Tomato irrigated with boron enriched fresh water: effects on leaves and berries

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Introduction. - Boron (B) is widely distributed throughout the environment. It is naturally present in many silicate minerals and there- fore can be found in many soils, but it is in the ocean that the most part of Earth's B occurs, being there the 10th most abundant element. High B concentration, mostly under the form of boric acid, $B(OH)_3$, and borate, $B(OH)^-_4$ (Argust, 1998; Ferreira, et al., 2006; World Health Organization, 2009), can also be found in other aquatic environments like surface and groundwater, occurring naturally or deriving from anthropogenic contamination.

In surface waters the presence of B can be influenced by factors as geochemical nature of the drainage area, proximity to marine coast and inputs from industrial and/or municipal effluents. With the exception of areas with particularly high natural B, its concentrations in surface waters are almost always less than about 0.5 mg/l (World Health Organization, 2009).

In the groundwater the presence of B is essentially due to the leaching of borates and borosilicates from rocks and soils, and its concentration can vary significantly throughout the world, reaching values lower than 0.3 mg/l and higher than 100 mg/l (World Health Organization, 2009). In Italy, the concentration of B in regional groundwater can reach values up to 8 mg/l (Polat et al., 2004). These high values of B in water can be very problematic since conventional water treatment does not remove B to any appreciable extent, and the World Health Organization (2008) defines B level of 0.5 mg/l as a guideline for drinking water. In fact, short and long-term oral exposures of rats, mice and dogs to boric acid or borax have demonstrated that the male reproductive tract is a consistent target of toxicity, giving origin to testicular lesions.

Even if B is an essential micronutrient for plants, signs of B toxicity like formation of necrotic patches on leaves and fruits, accelerated senescence, and ultimately plant death can be observed when plants are submit- ted to B concentrations higher than 0.5 mg/l (Nadav, 1999).

Tomato is a very versatile fruit that can be consumed fresh or in the form of processed products (Toor and Savage, 2005) but, as for other plant species, it is sensitive to high B concentrations (Kaya et al., 2009) since B affects both membrane functions and photosynthesis by increasing stomatal resistance (Eraslan et al., 2007).

Tomato has been associated with health protection and longevity (Gómez-Romero et al., 2007) due to its high nutritional value, influenced by the presence of antioxidant compounds such as vitamin C (ascorbic acid), vitamin E (tocopherol), carotenoids, flavonoids and phenolic acids (George et al., 2004; Sgherri et al., 2007). Indeed, these antioxidant compounds have the ability to neutralize reactive oxygen species (ROS) such as superoxide radicais (O_2^), hydroxyl radicais (OH"), and hydrogen peroxide (H_2O_2). In addition, they are worldwide recognised as beneficial for preventing widespread human diseases such as cancer and cardiovascuiar pathoiogies (Sgherri et al., 2007). Foiiowing abiotic stress conditions such as high B concentrations, antioxidant ieveis of tomato berries and ieaves couid change since piants react to oxidative damage activating antioxidative defence mechanisms. Depending on stress intensity, different organs of different piants can be endowed with higher ieveis of antioxidants and antioxidative enzymes to counteract the harmfui effects of ROS (Sgherri and Navari-Izzo, 1995) increasing nutraceuticai properties of edibie parts as observed in tomato fruits (Cervilla et al., 2007) and iettuce (Eraslan et al., 2007) foiiowing B stress.

Tomato is one of the most abundantiy produced and consumed fruit in the Mediterranean area, especially in many regions of Itaiy, iike Tuscany. This region suffers from B contamination of fresh water (Muti, 2006), thus we studied the effect of B excess on ieaves and berries of tomato piants with the aim to verify whether, together with an antioxidative response at ieaf ievei, this micronutrient can induce differences in the nutritionai vaiue of fruits.

Materiais and methods. - Plant Material. - Tomato (Lycopersicon esculentum L. cv. Caramba) piants were grown during the spring of 2009 in a giasshouse with a minimum night temperature and a daytime ventilation temperature of 16 and 28°C, respectively, being the mean value of daily radiation 9.2 MJ/m^2

with a maximum photon flux
density of 500-700 umol/m^2 s. A closed-loop rockwool culture was used. Two boron levels in the nutrient solutions were applied corresponding to 0.25 mg/l (control) and 2.0 mg/l (treatment) concentrations. The macronutrient composition (mol/m^3) of nutrient solution (Hoagland's 2) was the following: N-NO_3 11.5, P-H_2PO_4 1.2, K 7.0, Mg 0.8, Ca 4.0, added with micronutrients such as Fe, Cu, Zn, Mn, Mo at the concentrations of 20.0, 1.0, 5.0, 10.0 and 1.0 uM. respectively. B was added as boric acid (H_3BO_3). The recycling nutrient solutions were checked daily for EC and pH (5.5-6.0) and partially replaced every two weeks. Irrigation was controlled by a timer that opened the irrigation lines for two minutes up to 12 times per day, depending on growing stage and environmental conditions.

Two harvests of fruits and leaves were carried out with one month interval between them. Fruits were picked at red-ripe stage from the third and fifth truss of separate plants early in the morning. Fruits with more than 90% of the fruit surface red were considered to be at the red-ripe stage (Sgherri et al., 2008). Two leaves just below and above each chosen truss were harvested at the same time of the fruits. At harvest, fresh weights of fruits and leaves were taken and samples were frozen in liquid nitrogen and stored at -80°C. Some samples were dried in the oven till constant weight and the dry weight was determined.

Boron Determination. - Aliquots of fresh samples were wet ashed in a mixture of nitric-perchloric acids. The digestion was conducted for 2 h at 200-230°C within air- open teflon vessels. After digestion B was determined spectrophotometrically at 420 nm (Schimadzu UV-VIS 1204 spectrophotometer, Tokyo, Japan) by the Azomethine-H method (Lohse, 1982).

Leaf Extracts. - For ascorbate and glutathione determinations leaves were homogenized in ice-cold 5% (w/v) trichloroacetic acid containing 4% (w/v) polyclar AT. For glutathione reductase activity leaves were extracted with 1 mM potassium phosphate (pH 7.5), containing 0.4 mM Na_2EDTA, 9.9 mM isoascorbic acid and 2% (w/v) poly- clar AT. For ascorbate peroxidase activity determination leaves were homogenized with 50 mM potassium phosphate (pH 7) containing 1 mM ascorbate. Determinations were performed on the supernatant after centrifugation at 4°C for 15 min at 12100 g.

Fruit Extracts. - Lipid extracts from tomato berries were obtained by homogenizing them with a solution of methanol/chloroform (1:2 v/v) as previously reported by Sgherri et al. (2010). After filtration, extracts were washed with KCl 0.88% in order to eliminate salts. Chloroform phases were taken to dryness with a rotary evaporator. Some samples were re-suspended in chloroform/ethanol (1:5 v/v) and used for determination of antioxidant activity and tocopherol composition. Other samples were re-suspended in chloroform/hexane (1:5 v/v) for lycopene determination.

Aqueous extracts were obtained, as previously reported by Sgherri et al. (2010), using Milli Q water accurately degassed. After centrifugation at 12100 g for 15 min, the pellet was discarded and the supernatant was used for determination of antioxidant activity, ascorbate and total phenol concentrations.

Antioxidant Activity. - Antioxidant activity of berries was determined both for lipophilic and hydrophilic extracts using the ABTS assay. Radical cation ABTV was generated as described by Re et al. (1999). The radical solution was diluted with ethanol or water for lipophilic or hydrophilic extracts, respectively, in order to obtain an absorbance of 0.700 (±0.020) at 734 nm. After addition of the extract, the decrease in absorbance was monitored and compared to that of the Trolox standard. The results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC)/g DW of plant material.

Ascorbate and Dehydroascorbate Determinations. - Ascorbate (AsA) and total ascorbate (AsA + DHA) were determined as previously reported by Sgherri and Navari-Izzo (1995). Total ascorbate was determined by means of the reduction of dehydroascorbate (DHA) to ascorbate by 1 mM dithiotreitol. A standard curve covering a 0-25 nmol AsA range was used. DHA levels were estimated on the basis of the differ- ence between total ascorbate and AsA amounts.

Glutathione and Oxidized Glutathione Determinations. - The total (GSH + GSSG) and GSSG determinations were performed by the 5,5'-dithio-bis-nitro-benzoic acid- GSSG reductase recycling procedure as reported by Sgherri and Navari-Izzo (1995). GSSG was determined after GSH had been removed by derivatization with 2-vinylpyri- dine. Changes in absorbance of the reaction mixtures were detected at 412 nm at 25°C. GSH, expressed as GSSG equivalents, was obtained by the difference between total and oxidized glutathione contents. The total glutathione contents were calculated from a standard curve (1-10 nmol of GSH equivalents).

TBARS. - Leaves were extracted with 50 mM potassium phosphate buffer (pH 7.8) containing 2% (w/v) polyvinyl polypyrrolidone, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM cysteine. After centrifugation at 12100 g for 15 min the supernatant was used for the determination of TBARS, using the thiobarbituric acid method described by Pérez-López et al. (2009). The TBARS concentration

was determined by the extraction coefficient of 155 mM^{-1} cm^{-1} after subtracting non-specific absorbance at 600 nm and sugar absorbance at 440 nm, using the following formula:

$$[TBARS] = A - B$$

where $A = (A_{532+TBA} - A_{600+TBA}) - (A_{532-TBA} - A_{600-TBA})$
and $B = (A_{440+TBA} - A_{600+TBA}) \times 0.0571$.

GR and APX activity. - GR activity was determined in the supernatant of leaves according to the method reported in Navari-Izzo et al. (1997) by monitoring the increase in absorbance at 412 nm at 30°C. APX was assayed by measuring the oxidation of ascorbate at 219 nm at 25°C according to SGHRRRI and NAVARÍ-IZZO (1995). Corrections were made for the low, non-enzymatic oxidation of AsA by H_2O_2 and for the oxidation of AsA in the absence of H_2O_2 i.e. for the presence of intracellular H_2O_2 and the autoxidation of AsA.

Total Phenols. - Total phenols of berry aqueous extract were determined according to Sgherri et al. (2003), recording the absorbance at 280 nm before and after addition of PVP to the extract (1:10 w/v), to absorb the phenolic compounds. Calculations were performed using the absorbance obtained by the difference between the two readings, and by using a calibration curve for total phenolics prepared with gallic acid as standard.

Lycopene. - Lycopene determinations were performed in the lipophilic extracts from tomato berries re-suspended in chloroform/hexane (1:5 v/v) at 503 nm using hexane as blank. Calculations were performed according to the formula of JAvAnmArdi and KubotA (2006) as follows:

$$\text{Lycopene (mg/Kg)} = (X/Y) \times A_{503} \times 3{,}12$$

where X represents the volume of hexane, Y the dry weight of the fruit tissue, A_{503} the absorbance at 503 nm and 3.12 is the extinction coefficient.

Tocopherols. - Tocopherols were determined in the lipid extracts from tomato berries according to the method reported by SghErsi et al. (2008). a-, y- and S-tocopherols were determined by isocratic RP-HPLC using a Shimadzu apparatus (model LC-20AD) with an electrochemical detector (Metrohom model 791) equipped with a glassy carbon electrode and LC Solution software (Shimadzu) for peaks integration. We could not determine (S-tocopherol because the detector response for this isomer was under the limit of detection. Detection was performed according to Galatro et al. (2001) at +0.6 V and 25°C with a Nova Pak C-18 4 um column (3.9 mm x 150 mm). The extracts were eluted with 95% methanol containing 20 mM $LiClO_4$ at a flow rate of 1 ml/min. Standard mixtures of a-, y-, and S-tocopherol in the range of 25-75 ng in 20 ul of injection volume were injected to calculate the calibration curve.

Statistical Analysis. - The results are the means from three replicates of three independent experiments (n=9). The significance of differences among mean values was determined by one-way ANOVA. Comparisons among means were performed using the Duncan's Multiple Range test. Means in figures accompanied by different letters are significantly different at $P < 0.05$. When necessary an arc sin or angular transformation was applied before statistical analysis.

Results and discussion. - In plants B is well known to play an important role in structure of cell-walls and membrane functions (Brown et al., 2002). Unlike other plant nutrients, B, in the form of boric acid, can easily enter the plant passing directly across phospholipid bilayers (REid, 2010). Due to this facility in entering plant tissues, when external B concentration is increased also B content in plants tends to be higher. Indeed, in our experiment (Fig. 1A) B content in leaves increased by 17% following the treatment at both harvests. In agreement with these results, increases in leaf B content were observed, other than in tomato (CErvilla et al., 2007), in grapevine (GunEs et al., 2006), orange (KElEs et al., 2003) and chickpea (Ardic et al., 2008) as well. In contrast with leaves, tomato berries did not show increases in B content with the 2 mg/l B treatment either at the 1st or 2nd harvest (26 mg/l).

The higher B content in leaves at the 1st harvest could have induced an oxidative stress resulting in peroxidation (Fig. 1B) due to an increased production of ROS. Increases in peroxidation following B treatment were also observed in grapevine (GunEs et al., 2006), chickpea (Ardic et al., 2009) and in tomato leaves (CErvilla et al., 2007). However, the same increase in B content did not determine an oxidative damage in tomato leaves at the 2nd harvest (Figs. 1A and 1B) likely because leaves are

endowed with a higher enzymatic antioxidant bulk. Indeed, at the 2nd harvest control leaves showed higher GR and APX activities com- pared to the control ones at the 1st harvest (Figs. 2A and B). Moreover,

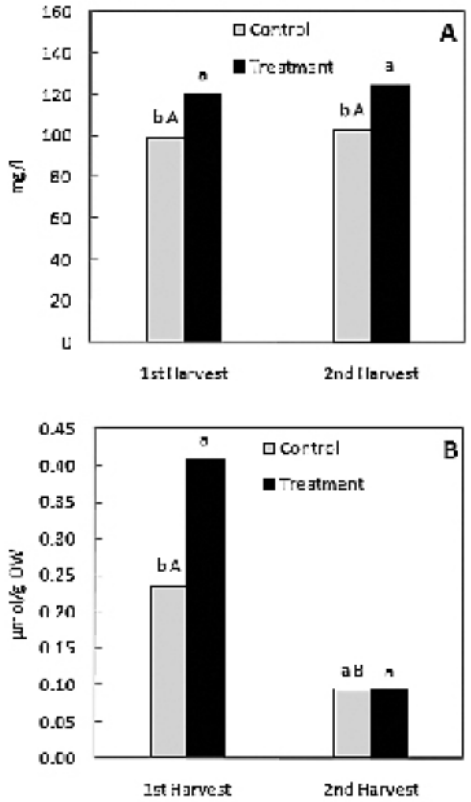

Fig. 1. - (A) Boron content and (B) thiobarbituric acid reactive substances (TBARS) in tomato leaves of control and boron treated plants. Means accompanied by different letters are significantly different at $P < 0.05$. Small letters refer to differences between samples subjected to different boron concentrations (0.25 and 2.0 mg/l) within the same harvest. Capital letters refer to differences between control samples harvested at different times (1st and 2nd harvest).

even if at the 2nd harvest GR and APX activities decreased respectively by 28 and 62% in comparison with the control, their activities showed values two- and three-fold higher (for GR and APX, respectively) than those detected at the 1st harvest (Fig. 2). The mechanism by which B affects antioxidative enzymes activity is unknown and there are contrasting reports on the response of these enzymes to B excess. In grapevine under B toxic conditions Gunes et al. (2006) observed a decrease in APX activity concluding that other enzymes such as CAT, that increased its activity with the treatment, could have a more important role than APX in H_2O_2 detoxification. In contrast, Cervilla et al. (2007) and Karabal et al. (2003) found increases in GR and APX activities in tomato and barley leaves, respectively, concluding that B can affect differently the enzymatic activity of APX under stress conditions.

Total glutathione (GSH + GSSG) and GSH amounts were also reduced in the treatment at the 1st harvest (Fig. 3A) supporting the hypothesis that at this time tomato leaves were more susceptible to oxidative damage. However, the fact that at both harvests nor GSH/ GSSG or AsA/DHA ratio changed with the treatment (Figs. 3A and 3B), indicates that the residual GR and APX activities were sufficient to make the glutathione/ascorbate cycle well working. This is particularly important since in that cycle APX detoxifies hydrogen peroxide and GR

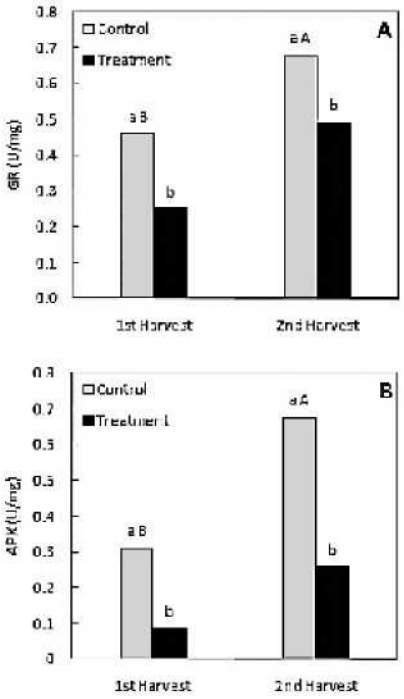

Fig. 2. - (A) Glutathione reductase (GR) and (B) ascorbate peroxidase (APX) in tomato leaves of control and boron treated plants. The other details are as in Figure 1.

reduces GSSG back into GSH, through NADPH (Secenji et al., 2008). As a consequence, the 2 mg/l B treatment was a subsymptomatic dose at the fruit level. Nor visible damage or increases in B concentrations were indeed observed in the treated tomato berries in which all substances tested remained unchanged com- pared to the control (Figs. 4 and 5).

Nutritional value of tomato fruits depends on the presence of non-enzymatic antioxidants such as ascorbic acid, phenols, lycopene and tocopherols. In particular, AsA is mostly associated with ROS scavenging and the regeneration of a-tocopherol (Eraslan et al., 2007). It is reported that B application elevates AsA content in potato (Mondy and Munshi,
1993), orange (Keles et al., 2004) and tomato (Cervilla et al., 2007). This increase can be due to the reduction of its consumption in growth processes, a-tocopherol regeneration, synthesis of cell wall glycoproteins, photosynthesis and glutathione metabolism following B toxicity. In the present experiment, as well as in that of Eraslan et al. (2007) in lettuce, the lack in AsA increase may be explained with the fact that other Authors (Cervilla et al., 2007) used B concentrations approximately 10-times higher which caused increases in total ascorbate by 75%. In the present work, although a reduction in AsA content of leaves following B treatment was detected (Fig. 3B) and in spite of the fact that a similar amount of total ascorbate was observed both in leaves and berries (Figs. 3B and 4B), DHA was found to be two times more concentrated in leaves. These observations suggest that the rate of ascorbate oxidation was higher in leaves than in berries,

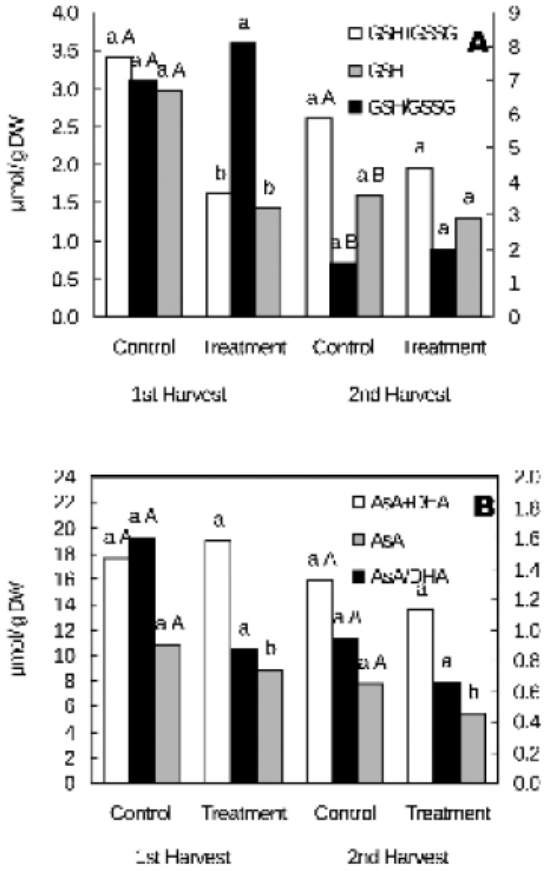

FIG. 3. – (A) Reduced glutathione (GSH, left axes), total glutathione (GSH + GSSG, left axes) and GSH/GSSG ratio (right axes) and (B) ascorbic acid (AsA, left axes), total ascorbate (AsA + DHA, left axes) and AsA/DHA ratio (right axes) in tomato leaves of control and boron treated plants. The other details are as in figure 1.

likely because B is transported by the transpiration flow in the leaf where it accumulates (Gunes et al., 2006), whereas no B is translocated to the fruit.

In agreement with the constant AsA content, also unchanged hydrophilic anti- oxidant activities and total phenols contents were observed in tomato berries (Fig. 4A, B and C). These results suggest that the antioxidant power of the aqueous fraction of tomato berries was not influenced by the 2 mg/l B treatment.

According to the observations of Sgherri et al. (2007) in tomato subjected to moderate salinity stress conditions, vitamin E was pre- dominantly represented by a-tocopherol and only trace amounts of ô-tocopherol were detected (Fig. 5C). It is known that AsA is involved
in a-tocopherol regeneration (Munné-Bosch et al., 2003) and, as AsA content (Fig. 4B) did not change with the treatment, the same occurred for a-tocopherol concentrations (Fig. 5C). Unchanged values following the treatment were observed in berries also for lycopene concentrations and lipophilic antioxidant activity (Fig. 5A), showing that also lipophylic antioxidants of the fruit were not influenced by B treatment.

In conclusion, when tomato plants are irrigated with fresh water containing up to 2 mg/l B, the toxic element can be translocated to the leaves giving rise to an oxidative burst, but not to the berries which maintain unaltered their nutritional value.

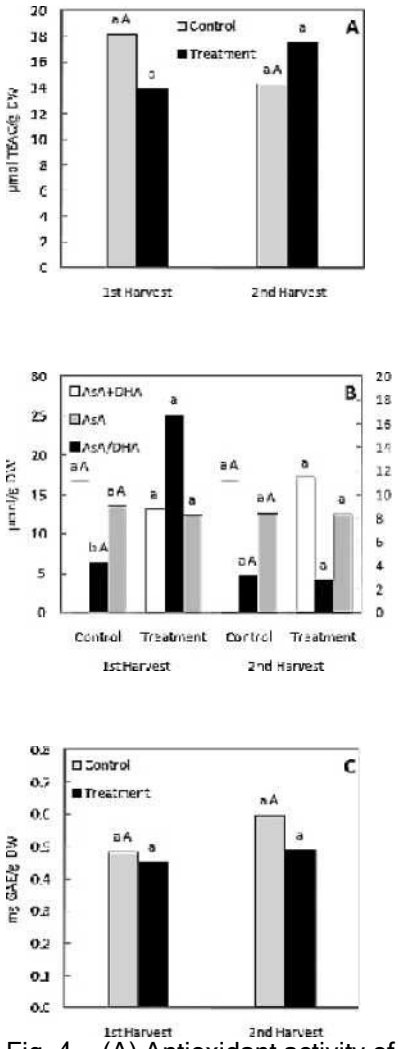

Fig. 4. - (A) Antioxidant activity of aqueous extract, (B) ascorbic acid (AsA, left axes), total ascorbate (AsA + DHA, left axes) and AsA/DHA ratio (right axes) and (C) total phenols in tomato berries of control and boron treated plants. The other details are as in figure 1.

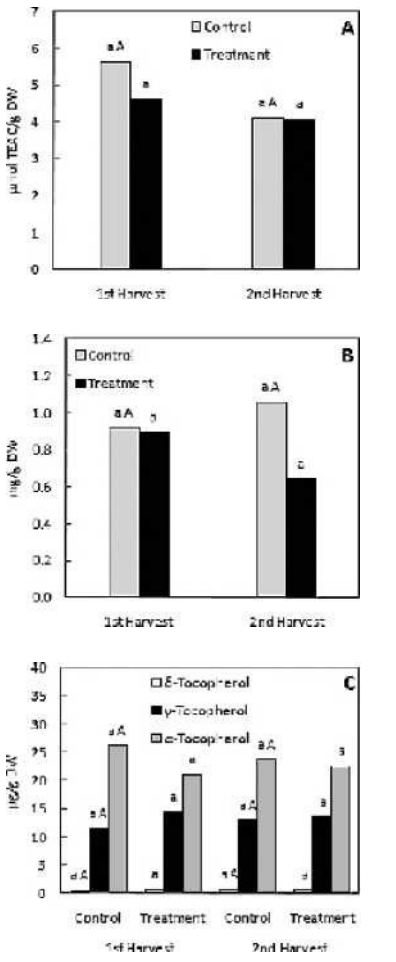

Fig. 5. (A) Antioxidant activity of lipid extract, (B) lycopene and (C) a-, γ- and ô-tocopherols in tomato berries of control and boron treated plants. The other details are as in figure 1.

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Summary. - The aim of this research was to study the effect of irrigation with B enriched fresh water (2 mg/l) on tomato plants. In particular, damage to leaves and the nutritional value of berries were investigated. Tomato berries were analysed for the levels of lipophilic and hydrophilic antioxidant activities, lycopene, tocopherols, ascorbic acid and phenols, whereas leaves were investigated for the contents of thiobarbituric acid reactive substances (TBARS), ascorbic acid, glutathione, glutathione reductase (GR) and ascorbate peroxidase (APX) activities. In comparison with the control, leaves showed higher amounts of B and TBARS and lower contents of glutathione as well as decreased GR and APX activities. Moreover, with time control leaves showed decreases in TBARS and reduced glutathione (GSH) contents and increases in oxidised glutathione (GSSG) amount and GR and APX activities. In contrast, nutritional value of tomato berries did not change with the treatment since both antioxidant activity and major antioxidants amounts remained constant.

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