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Nitrogen Nutrition Effect on Aeroponic Basil (*Ocimum basilicum* L.) Catalase and Lipid Peroxidation

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Abstract

Considering the physiological importance of nitrogen (N) for the plant growth and its controversial role in the plant oxidative status, the objective of this research was to investigate the effect of three different N nutrition solution concentrations (1.8, 3.6 and 11.5 mM) on leaf and root oxidative stress of aeroponically cultured basil (*Ocimum basilicum* L.) plants. Catalase (CAT) activity and lipid peroxidation (LP) were used as oxidative stress indexes at two different growth stages (10 and 15 week-old plants respectively). Leaf and root CAT activity was enhanced by the increment of N concentration at both growth stages of the plants. Especially in younger, high N nourished plants, 130 and 149% increments of the leaf and root CAT activities were observed respectively, in comparison with the low N nourished ones. Moreover, the root enzyme seems to be a bifunctional catalase-peroxidase considering its insensitivity to aminotriazole. On the other hand, root LP seems to be unaffected at this N concentration range whereas leaf LP was enhanced at high N levels, especially in younger plants. These results suggest that increased N nutrition induces oxidative stress mainly in the leaves of aeroponically grown basil plants while the increase of CAT activity probably represents a part of plant's antioxidative defense against potent cellular damage similar to membrane lipid peroxidation.

Keywords: ammonium, antioxidative defense, nitrate, peroxidase, oxidative stress

Abbreviation: CAT-catalase; N-nitrogen; LP-lipid peroxidation; ROS-Reactive Oxygen Species

Introduction

Sweet basil (*Ocimum basilicum* L.) is one of the most popular culinary herbs of the Lamiaceae family cultivated worldwide. It is also used for cosmetical and pharmaceutical preparations as it contains large amounts of essential oils (Sifola and Barbieri, 2006; Kiferle *et al.*, 2013) and antioxidant polyphenolic compounds with anticancer, antibacterial, antifungal and anti-inflammatory capabilities (Nguyen and Niemeyer, 2008; Nurzynska-Wierdak *et al.*, 2013). Basil is generally cultivated in open field (Kiferle *et al.*, 2013), but it is also commercially produced in greenhouses (Nguyen and Niemeyer, 2008). The major advantage of basil greenhouse hydroponic cultivation is the manipulation of growing conditions such as all-year round production, higher quality and ease of processing of harvested material on account of minimal contamination from pollutants, pests and pathogens (Nguyen and Niemeyer, 2008; Kiferle *et al.*, 2013).

Many biochemical compounds present in plant cells contain nitrogen. Besides, most natural and agricultural ecosystems show dramatic gains in productivity after fertilization with inorganic N, attesting to the importance of this element (Taiz and Zeiger, 2002). On the other hand, plant growth decreases under excessive N levels, as most plant species show smaller leaves and stunted root systems, and in severe cases plants can be lead to death (Chen *et al.*, 2009). Inorganic N is available to plants as nitrate (NO_3^-) and ammonium (NH_4^+) ions varying both temporally and spatially in open fields' soils. Nitrate is generally present in higher concentrations (1-5 mM) than ammonium (20-200 μM) in the soil solution of agricultural soils (Hawkesford *et al.*, 2012). In soilless culture, N is generally supplied as NO_3^- at concentration close or higher than 10 mM (Kiferle *et al.*, 2013). Although nitrate is more mobile in the soil than ammonium and therefore more available to plants (Hawkesford *et al.*, 2012) plant species can exhibit a differential capacity to take up particular forms of inorganic N. Many studies explore the plants' growth responses towards nutrition with varying NO_3^-:NH_4^+ ratios (Everett *et al.*, 2010).

Production of Reactive Oxygen Species (ROS) is inevitable even under optimum growth conditions. Under conditions of many environmental stresses, enhanced generation of ROS disturbs the normal redox environment of plant cells and damages the cellular components (Tewari *et al.*, 2007; Zhang L.-X. *et al.*, 2007). The role of N in the plant oxidative status is quite controversial. Some authors suggest that high N concentrations

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improve enzyme antioxidant defense (Štajner *et al.*, 1997; Zhang L.-X. *et al.*, 2007) and decrease LP (Zhang L.-X. *et al.*, 2007) while other studies indicate that different N levels have no significant effect on LP per se but under water- (Saneoka *et al.*, 2004) or cadmium-stress conditions (Zhang *et al.*, 2014) LP has been decreased with increasing N levels. Besides, it has been suggested that under N excess stress conditions LP was increased (Wei *et al.*, 2009) while N deficiency enhances both LP and enzyme antioxidant responses (Tewari *et al.*, 2007).

Aeroponic system is a successful cultivation method for several plant species, primarily because of the highly aerobic environment for the plant's roots. Besides, the aeroponic system makes it possible to examine completely intact roots without disturbing or damaging them, and to obtain clean samples without interference from mechanical substrates (Zobel *et al.*, 1976).

Ocimum basilicum L. is an economically interesting plant, field and hydroponically cultivated (Kiferle *et al.*, 2013). In the present study, we examined the effect of three different N nutrition concentrations on leaf and root oxidative status of aeroponically cultivated sweet basil plants at two different growth stages. The CAT activity and the LP were used as antioxidative defense and oxidative damage indexes, respectively.

Materials and Methods

Plant growth

This study was conducted during Autumn-Winter 2013 in an automated glasshouse located at the former Technological Educational Institute of Messolonghi at Western Greece. Sweet basil (*Ocimum basilicum* L.) seeds were sown in 80 pots with soil and irrigated with water. When the seedlings reached about 10 cm height, they were transplanted (after washing the roots in order to remove the soil) in the glasshouse at the established novel "Fully Automated Aeroponic Growing System". This closed recirculated aeroponic growing system consisted from three canals (one for each of the nitrogen levels) with three replications. The canals were made up of polystyrene rectangular sections with length 10 m, width 0.67 m and height 0.30 m, and internal thin plastic layer, to allow reuse of the nutrient solution. They were covered by polystyrene panels with holes on which plants were placed. A high pressure irrigation system of two parallel pipes with aeroponic sprayers was installed inside the structures in the bottom of the canals. The roots of the plants were sprayed by the nutrient solution for 25 s every 5 min. The system was fully electronically controlled. Plants were grown under natural temperature and daylight conditions. Root zone temperature into growing canals was automatically controlled to 20 °C.

Nutrient solutions

The irrigation solutions were prepared at three different N levels: i) 1.8 (low), ii) 3.6 (medium) and iii) 11.5 mM (high level, usually used in soilless culture) while all the other macro- and micronutrient concentrations remained constant. The solutions contained the following: a) macronutrient levels in mM: N (as nitrate plus ammonium, respectively), NO_3^-: 1.70, 3.40, 10.9 and NH_4^+: 0.1, 0.2, 0.6; K, 6.5; Ca, 3.0; Mg, 0.9; P, 1.6 and b) micronutrient levels in μM: Fe, 30.0; Mn, 5.0; Zn, 4.0; Cu, 0.75; B, 30.0; Mo, 0.53. The pH was adjusted to 5.6-6.0 and the electrical conductivity kept at 1.70 dS m^{-1}.

Experimental measurements

All the below measurements were conducted 5 and 10 weeks after the installation of the seedlings to their respective N nutrition treatment (called younger and older plants, respectively). The leaf samples were taken from healthy, completely expanded leaves of the 3rd node from the top of the shoot. The root samples were taken from lateral roots. For each experiment, the leaf and root sampling were conducted from three plants per N treatment. The results are the means of three independent experiments.

The samples were collected from the plants, wrapped in plastic bags and transferred immediately to the lab for LP, CAT activity, protein concentration and dry weight estimations. The samples were washed gently with deionised H_2O and blotted with paper. Similar samples were obtained and either dried to constant weight in an oven at 85 °C or homogenized.

All reagents were purchased from Sigma (St. Louis, MO, U.S.A.) and Merck (Darmstadt, Germany).

Lipid peroxidation

The leaf and root samples were ground at 4 °C under dim light (to prevent artificial LP) in a porcelain mortar with homogenization buffer containing 50 mM Na_2HPO_4 pH 7.2, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM butylated hydroxyanisole (BHA) and 0.15% ethanol. The homogenization was carried out using 5 mL buffer per g fresh sample weight. The homogenate was assayed for LP products by a modified thiobarbituric acid (TBA)-based method (Buege and Aust, 1978). Specifically, 0.5 mL homogenate was mixed with 0.5 mL TBA reagent containing 0.5% (w/v) TBA, 20% (w/v) trichloroacetic acid (TCA) and 0.33 N HCl. To the resulting mixture was added 5 μL 2% (w/v) of the lipid antioxidant BHA (in absolute ethanol) to prevent artificial LP during the assay. The mixture was incubated at 100 °C for 15 min and brought to room temperature (RT). To that was added 1 mL 1-butanol, mixed by vigorous vortexing, centrifuged at 15,000 *g* for 3 min, and the absorbance determined with a Shimadzu UV-1601 spectrophotometer (Shimadzu Corp., Japan) of the upper butanol layer was measured at 535 and 600 nm (subtracting the non-specific absorbance) against a sample blank (0.5 mL sample mixed with 0.5 mL 20% TCA in 0.33 N HCl and with 5 μL 2% w/v BHA) and a reagent blank (0.5 mL homogenization buffer mixed with 0.5 mL TBA reagent and 5 μL 2% w/v BHA). Absorbance difference $A_{(535-600)}$ was converted to malondialdehyde (MDA) equivalents with the molar extinction coefficient $1.56 \cdot 10^5 \cdot M^{-1} \cdot cm^{-1}$. LP was defined as TBA-reactive substances (TBARS) and was expressed in nmol MDA equivalents per g dry weight.

Catalase activity

The samples were ground at 4 °C in a porcelain mortar with homogenization buffer containing 100 mM K_2HPO_4 pH 7.0, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.3% ethanol. The homogenization was carried out using 5 mL buffer per g fresh sample weight. The homogenate was centrifuged at 3,300 *g* for 5 min, at 4 °C. The resulting supernatant was subsequently used for both CAT activity and protein concentration estimation. Catalase was assayed by its depletion of H_2O_2, followed at 240 nm (Blum and Fridovich, 1983).

The standard CAT assay consists of mixing (in a 30 °C water bath) 0.85 mL sample (proper dilution of the homogenate

supernatant), 0.05 mL of the homogenization buffer and 0.1 mL 0.09 M H_2O_2 stock solution (made fresh in homogenization buffer). The linear absorbance decrease (vs time) of the assay mixture was measured at 240 nm in a Shimadzu UV-1601 spectrophotometer. The absorbance decrease rate of consumed H_2O_2 was converted to CAT units (U) from a pure CAT standard curve.

The aminotriazole inhibition assay test of CAT consists of mixing 0.85 mL sample, 0.05 mL 0.4 M aminotriazole (made fresh in homogenization buffer) and 0.1 mL 0.09 M H_2O_2 stock solution. Specifically for the aminotriazole effect experiments, the H_2O_2 absorbance decrease of both standard and inhibition assay mixture was measured at a wavelength that the aminotriazole spectrum does not interfere significantly to the H_2O_2 one that is 253 nm.

Protein concentration

The protein concentration was determined by a modification of a Coomassie Brilliant Blue-based method (Assimakopoulos *et al.*, 2008). Specifically, 0.063 mL of various dilutions of the sample (homogenate supernatant) was mixed with 0.02 mL of 0.5% (v/v) Triton X-100 and 0.017 mL of 6 N HCl. The mixture was incubated at 100 °C for 10 min, brought to RT, mixed with 0.9 mL of 0.033% (w/v) Coomassie Brilliant Blue G-250 stock reagent (made in 0.5 N HCl, stirred for 30 min, filtered through Whatman #1 filter paper by water pump aspiration, and stored in the dark) and incubated for 5 min at room temperature. The absorbance at 620 nm (against appropriate sample and reagent blanks) of the mixture was converted to mg of protein from a Bovine Serum Albumin standard curve, using a Shimadzu UV-1601 spectrophotometer.

Data analysis

The results were obtained from three independent experiments and expressed as mean ± standard error of mean (SEM). The D'Agostino-Pearson normality test used in order to test the distribution of data. Statistical differences between the three different nitrogen levels in leaves and roots were calculated using ANOVA test with Tukey's multiple comparison test. The statistical significance level was set at a = 0.05. Data were plotted and analyzed using GraphPad Prism V5.0.

Results and Discussion

Catalase was primarily observed in tobacco leaf by Loew (1900) who characterized, named the enzyme and suggested that "there seems to exist no plant and no animal which is without that peculiar enzyme". Catalases and peroxidases are the two major systems for the enzymic removal of hydrogen peroxide (H_2O_2) in plants. Catalases are highly active enzymes that do not require cellular reductants as they primarily dismutate H_2O_2 to H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$) (Willekens *et al.*, 1995; Mhamdi *et al.*, 2010). The enzyme activity from both basil leaves and roots elevated during the increment of N nutrition concentrations, though the phenomenon is more intense in younger (Fig. 1A) than in older plants (Fig. 1B). In younger high N nourished plants, we observed 130 and 149% statistically significant increments of the leaf ($p < 0.0001$) and root ($p < 0.05$) CAT activities, respectively, in comparison with the low N nourished ones (Fig. 1A). However, the N nutrition effect on CAT activity is moderated in older plants since the increments are 44 and 55% for leaves and roots, respectively (Fig. 1B). The N induced leaf CAT activity increment has been also suggested for sugar beet (Štajner *et al.*, 1997), maize (Zhang L.-X. *et al.*, 2007) and poplar plants (Zhang *et al.*, 2014) but there are no data about the N nutrition effect on roots CAT activity. However, it has been suggested that the leaf CAT activity increases under N deficiency too (Tewari *et al.*, 2007).

Our results indicate that under all N concentrations, the younger basil plants' leaf CAT activity is 78–177% higher than root's activity ($p < 0.0001$) whereas in older plants this pattern is absent (p = ns) (Fig. 2A). Leaf CAT activity has been also referred to be significantly higher than the root one in maize (Redinbaugh *et al.*, 1990) and barley (Azevedo *et al.*, 1998). On the other hand, the enzyme activity of Citrus young roots has been reported to be higher than the leaves' one (Chouliaras *et al.*, 2004). We also found that in younger plants leaf CAT activity is equal or higher (at high N nutrition) than in older ones (Fig. 2B) indicating that the leaf enzyme activity may decreases during the plant ageing which is in accordance with findings in maize leaves (Zhang L.-X. *et al.*, 2007) while the opposite phenomenon appears with regard to basil root CAT enzyme (Fig. 2B).

Angiosperm species studied to date all contain three catalase genes. This includes monocots and dicots such as tobacco, Arabidopsis, maize, pumpkin, and rice (Mhamdi *et al.*, 2010). The CAT genes expression in different plants' tissues (photosynthetic, vascular, reproductive and seed) has been classified based on the naming of the tobacco genes i.e. Cat1, Cat2 and Cat3 (Willekens *et al.*, 1995; Mhamdi *et al.*, 2010). Our research shows a quite different activity profile between the leaf and root enzyme. Moreover, a basil CAT inhibition assay test with the presence of 20 mM aminotriazole revealed 67–77% decrease of the leaf enzyme activity while the root enzyme was decreased only 31–42% ($p < 0.05$) (Table 1) which corresponds to *in vivo* and *in vitro* findings in tobacco seedlings although both their aminotriazole sensitive and insensitive examined CAT forms were leaf ones (Havir, 1992). Thereby, it seems that in basil roots dominates a bifunctional catalase-peroxidase isoenzyme while in leaves dominates a typical monofunctional CAT considering that bifunctional catalases-peroxidases are insensitive to the classical CAT inhibitor aminotriazole (Willekens *et al.*, 1995; Mhamdi *et al.*, 2010, 2012).

ROS can seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Chen *et al.*, 2009). Therefore, LP can be used as a marker of the cellular damage caused by oxidative stress. Responding to different N nutrition concentrations, root LP seems to be unaffected in both younger and older basil plants (Fig. 3). On the other hand, leaf LP in younger plants shows an 41% increment which observed in the maximum N concentration in contrast with the low N ($p < 0.05$) (Fig. 3A) while in older plants we observed: i) a 44% increment in the medium concentration and ii) no effect in the maximum concentration, in comparison with the low N concentration (p = ns) (Fig. 3B). In bibliography, there are some controversial results about the effect of N nutrition to plant leaf LP while there are no results about the effect to root LP. In particular, N nutrition (with urea as N source) significantly decreased leaf MDA content (Zhang L.-X. *et al.*, 2007) while other studies indicate that different N levels (with ammonium nitrate as N source) have no significant effect on leaf LP per se but under water- (Saneoka *et al.*, 2004) or cadmium-stress conditions (Zhang *et al.*, 2014) LP has been

decreased with increasing N levels. Besides, it has been suggested that leaf LP was increased under both N deficiency (with KNO_3 and $Ca(NO_3)_2$ as N source for the control plants) (Tewari *et al.*, 2007) and N excess (with calcium nitrate) stress conditions (Wei *et al.*, 2009).

Table 1. Aminotriazole (20 mM) inhibition effect on leaf and root catalase activity

Nitrogen treatment (mM)	Plant organ	% inhibition
	Younger plants	
1.8	Leaves	72 ± 6 Aa
1.8	Roots	42 ± 1 Bb
3.6	Leaves	74 ± 8 Aa
3.6	Roots	35 ± 8 Bb
11.5	Leaves	77 ± 10 Aa
11.5	Roots	32 ± 10 Bb
	Older plants	
1.8	Leaves	67 ± 8 Aa
1.8	Roots	31 ± 7 Bb
3.6	Leaves	67 ± 7 Aa
3.6	Roots	31 ± 4 Bb
11.5	Leaves	75 ± 2 Aa
11.5	Roots	38 ± 9 Bb

Data are mean ± SE (n = 3). Means with the different letter are significantly different from each other (Tukey test, $p < 0.05$). Capital letters represent comparison between leaves and roots. Lower case letters represent comparison between nitrogen treatments.

Our study indicates that under all N concentrations, the younger basil plants' leaf LP is 35-95% higher than the root one but in older plants these increments tend to be annihilated with the exception of the medium N concentration treatment (Fig. 4A). Likewise, leaf LP has been reported to be higher than the root one in tobacco (Popov *et al.*, 2010) and sugar beet (Kiprovski *et al.*, 2014) while the opposite phenomenon has been reported in *Bruguiera gymnorrhiza* and *Kandelia candel* (Zhang F.-Q. *et al.*, 2007). Besides, a recent research on maize reported root LP being whether higher or similar with the leaf one, depending on the particular plant genotype (Tang *et al.*, 2010).

Our results also indicate that in younger plants leaf LP is significantly higher (especially at high N nutrition, likewise the leaf CAT activity) than in older ones (Fig. 4B). In contrast, leaf LP increased during the plant ageing in other plants (Zhang L.-X. *et al.*, 2007). Other researchers compare LP status between younger and older leaves of the same plant that is upper and lower leaves, respectively. Their findings usually suggest increased LP at the lower (older) leaves (Wang *et al.*, 2012) although it has been reported the opposite result too (Dias *et al.*, 2014). On the other hand, root LP seems to be unaffected during ageing of the pant (Fig. 4B). There are inadequate data about the root LP status during the plant ageing although it has been reported that during maize seedling maturation root LP is increased (Tang *et al.*, 2010).

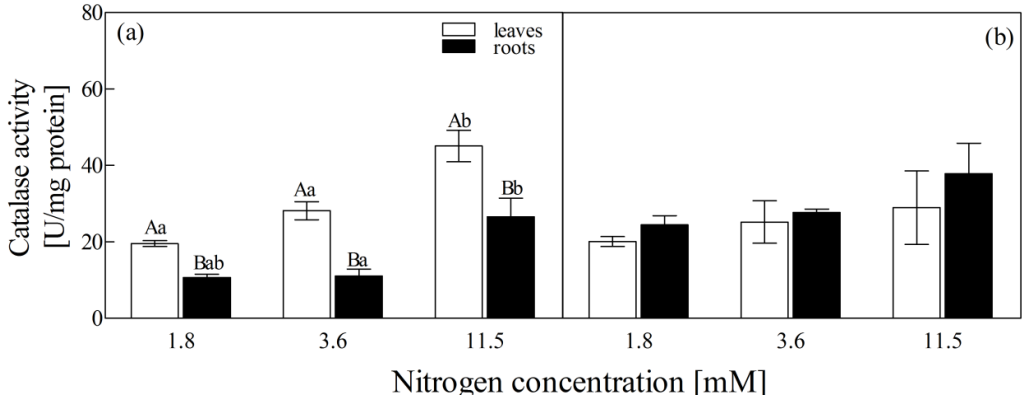

Fig. 1. Effect of nitrogen treatments on leaf and root catalase activity of younger (a) and older (b) basil plants

Vertical bars represent mean ± SE (n = 3). Means with the different letter are significantly different from each other (Tukey test, $p < 0.05$). Capital letters represent comparison between leaves and roots. Lower case letters represent comparison between nitrogen treatments

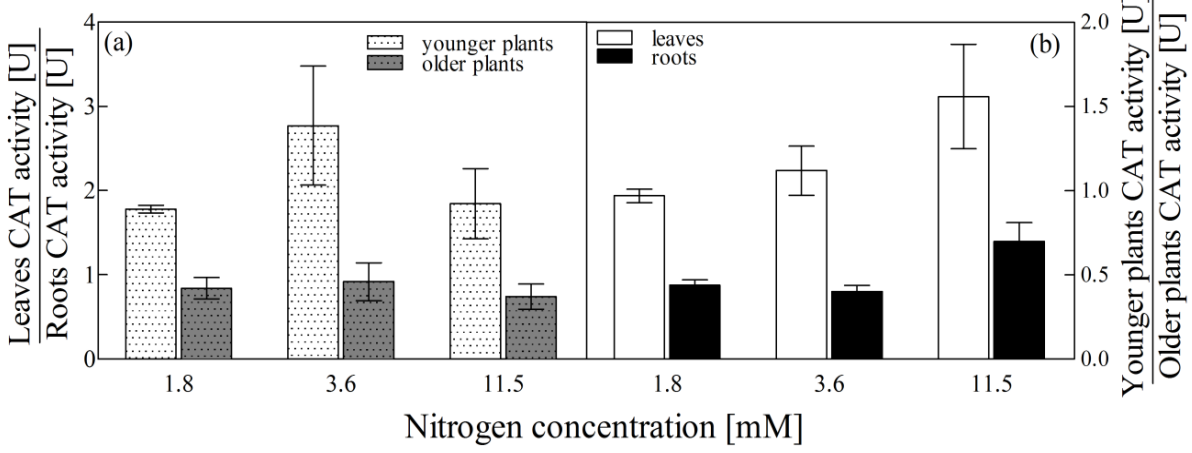

Fig. 2. Effect of nitrogen treatments on a) leaves to roots and b) younger to older plants catalase activity ratios

Vertical bars represent mean ± SE (n = 3)

Although N is an essential macronutrient element, some N compounds (ammonium, nitrite etc.) can be toxic for plants (Taiz and Zeiger, 2002; Guo *et al.*, 2007). Nitrate and ammonium are the main sources of N for crop plants (Kiferle *et al.*, 2013). In many plants, when the roots receive small amounts of nitrate, nitrate is reduced primarily in the roots. As the supply of nitrate increases, a greater proportion of the absorbed nitrate is translocated to the shoot and assimilated there (Taiz and Zeiger, 2002). The first step of the nitrate reduction into nitrite is catalyzed by nitrate reductase in the cytosol, and nitrite is further reduced to ammonium by nitrite reductase in the plastid/chloroplast. Finally, the ammonium assimilation into glutamate by glutamine synthetase/glutamate synthetase (GS/GOGAT) occurs. With the last reaction, plant cells (especially in roots) avoid ammonium toxicity by rapidly converting into amino acids either the absorbed ammonium or the ammonium generated from nitrate assimilation, photorespiration and protein degradation (Taiz and Zeiger, 2002; Guo *et al.*, 2007).

Recent studies investigate the effect of N deficiency (Tewari *et al.*, 2007), excess (Wei *et al.*, 2009) or form (Chen *et al.*, 2009) on the oxidative status of plants. It is considered that N plays an important role in the enzymic antioxidant defense and the lipid peroxidation metabolism of plants (Štajner *et al.*, 1997; Zhang L.-X. *et al.*, 2007) especially under different environmental stresses (Saneoka *et al.*, 2004; Zhang *et al.*, 2014). There are several methodological differences between the studies which explore the influence of N nutrition in basil plants, such as hydroponic (Kiferle *et al.*, 2013) or field culture (Sifola and Barbieri, 2006), winter (Nurzynska-Wierdak *et al.*, 2013), spring (Kiferle *et al.*, 2013), summer or fall seeding (Nguyen and Niemeyer, 2008) and a wide range of N treatment concentrations from 0.1 mM (Nguyen and Niemeyer, 2008) to 0.9 g dm^{-3} of growing medium (Nurzynska-Wierdak *et al.*, 2013) that is 64 mM.

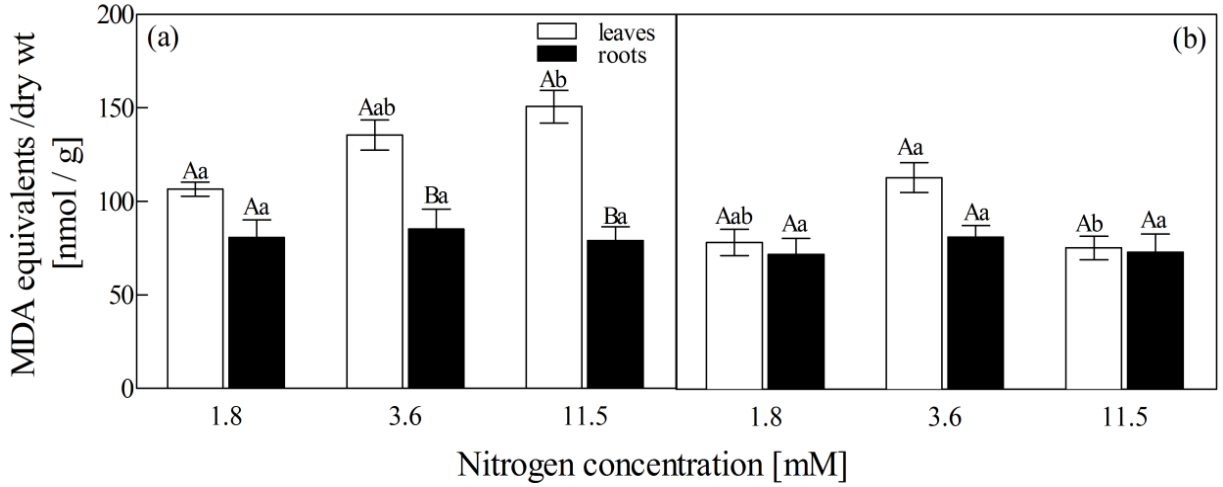

Fig. 3. Effect of nitrogen treatments on leaf and root lipid peroxidation of younger (a) and older (b) basil plants.
Vertical bars represent ± SE (n = 3). Means with the different letter are significantly different from each other (Tukey test, $p < 0.05$). Capital letters represent comparison between leaves and roots. Lower case letters represent comparison between nitrogen treatments

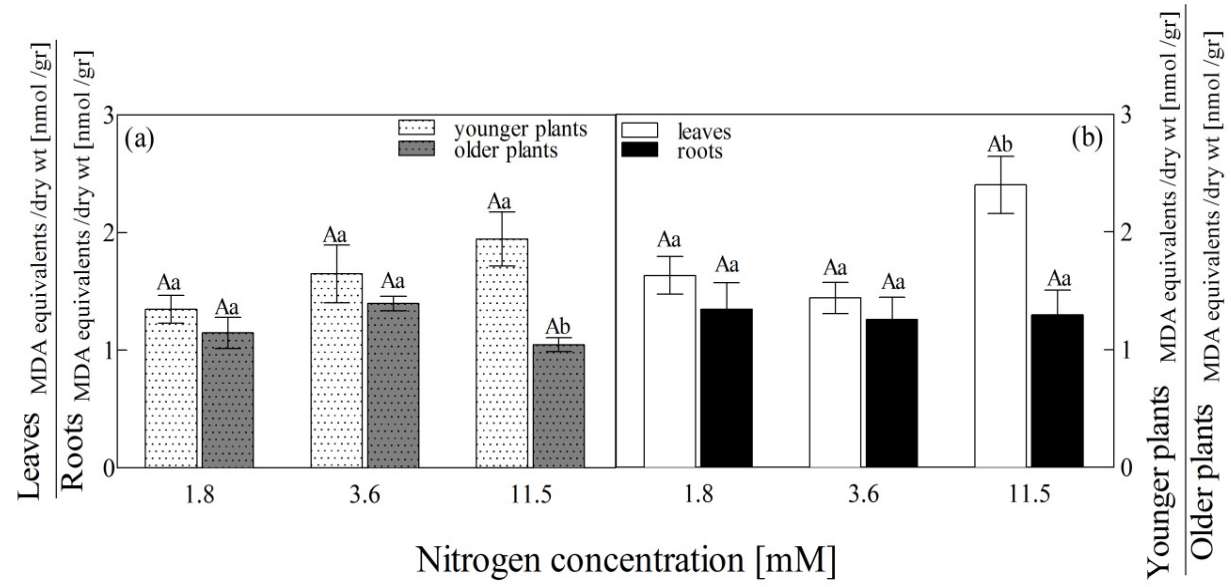

Fig. 4. Effect of nitrogen treatments on a) leaves to roots and b) younger to older plants lipid peroxidation ratios
Vertical bars represent ± SE (n = 3). Means with the different letter are significantly different from each other (Tukey test, $p < 0.05$). Capital letters represent comparison between i) younger and older plants (panel (a)) and ii) leaves and roots (panel (b)). Lower case letters represent comparison between nitrogen treatments

In spite of the above alterations, it is considered that in basil plants the increased N nutrition decreases both phenolics concentration and antioxidant activity (Nguyen and Niemeyer, 2008; Kiferle *et al*., 2013) while enhances the accumulation of essential oils (Sifola and Barbieri, 2006; Nurzynska-Wierdak *et al*., 2013) which also exhibit antioxidant activity (Amorati *et al*., 2013). These contradictious findings correspond to our results which indicate that aeroponically treated high N nutrition enhances both the enzymic antioxidative defense in leaves and roots and the leaf oxidative damages, implying that N nutrition induces oxidative stress while the plants develop adaptations of their enzymic and non-enzymic antioxidant defense. Therefore, it is quite ambiguous whether the increased N concentrations improve antioxidative defense as it has been proposed for other plants (Štajner *et al*., 1997; Zhang *et al*., 2014) or induce oxidative stress.

Conclusions

Basil oxidative stress responses (lipid oxidative damage and CAT antioxidative defense) against N nutrition appear to be more intense in leaves than in roots, especially in younger plants. Moreover, the strong CAT activity response in both leaves and roots in comparison with the relatively weak lipid damage (only in leaves) indicates a sufficient plant antioxidative defense adaptation. Further research, concerning other antioxidative defense or cellular oxidative damage indexes will clarify better the oxidative role of N nutrition especially at the conventional concentrations used for this crop production in soilless culture.

In point of the different basil CAT activity profiles between leaves and roots they may reflect the dominance of different isoenzymes as it is affirmed by their differential sensitivity against aminotriazole.

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