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Influence of photon flux density and high salinity on the level of some components of the antioxidative defence system in lettuce leaves

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Abstract. The amount of health-promoting substances biosynthesized in lettuce leaves is influenced by environmental stress factors such as photon flux density and salinity. As ubiquitous protective metabolites, carotenoid pigments and ascorbic acid (vitamin C) accumulate in higher concentrations when lettuce plants are exposed to high irradiance and salt stress, in relation with the oxidative damage caused by the environmental constraints. Accumulation of reactive oxygen species in photosynthetic cells induces an enhanced activity of the antioxidative protective system. This is reflected by changes in the catalytic activity of ascorbate peroxidase, responsible for the regulation of hydrogen

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peroxide level in different cell compartments, through a reaction which involves oxidation of ascorbate. High photon flux density increases the carotenoid content of young leaves and salt stress further enhances this increment, although by itself it does not cause the accumulation of these photoprotective pigments. Water-soluble sugars, which may play a role in osmoregulation and represent a source for the synthesis of ascorbate, are present in higher amount under intense light, but their concentration decreases when enhanced irradiation is associated with salt stress. High photon flux density and high salinity do not exhibit synergism concerning the increment of vitamin C content of lettuce leaves. A good molecular marker of antioxidative defence is the increased enzymatic activity of ascorbate peroxidase under high irradiance, this being further enhanced by the exposure of plants to 80 mM sodium chloride. In conclusion, the cultivation of lettuce under moderately high photon flux density (660 micromole photons m^{-2} s^{-1}) and under mild salt stress (80 mM NaCl) increases vitamin C and the carotenoid pigment content of its leaves, which is a beneficial property for consumers.

1 Introduction

Lettuce is a widespread vegetable that represents a valuable source of vitamins, inorganic nutrients and other health-promoting substances. The amount of different primary and secondary metabolites largely depends on the prevailing growth conditions. When environmental stress factors impair the steady state of different vital functions, acclimatization processes result in concerted metabolic changes that prevent or compensate damages caused by adverse developmental conditions. For example, it was demonstrated UV-B radiation, high light intensity, water stress, mild heat shock and chilling stress induce the biosynthesis of some phenolic compounds (e.g., quercetin-3-O-glucoside, chlorogenic acid, chicoric acid) with health-promoting qualities in the human diet (*Oh et al.*, 2009). An increased antioxidative capacity is conferred by activation of protective enzymes that reduce membrane damage (*Mahmoudi et al.*, 2010), as well as by accumulation of α-tocopherol (vitamin E) and glutathione, which also contribute to the nutritive value of lettuce leaves (*Rios et al.*, 2008). Specific phytochemicals produced through the shikimic acid pathway may accumulate due to a large increase in the expression of the gene encoding phenylalanine ammonia-lyase, the key enzyme for the biosynthesis of secondary phenolic compounds with protective properties (e.g., flavonoids). The transcription of this enzyme is especially enhanced by a series of abiotic stress factors (*Oh et al.*, 2009). The activities of the above-mentioned

key enzyme and of polyphenol oxidase are also responsible for the chemical browning, which reduces the visual quality of cut lettuce (*Altunkaya et al.*, 2008; *Roura et al.*, 2008). In red lettuce cultivars, environmental stress factors also enhance the production of anthocyanins (cyanidin-3-O-(6"-malonyl-β-glucopyranoside) and its methyl ester), which are absent from green lettuce plants and exhibit antioxidative properties, which results in a significantly reduced lipid peroxidation and cycloxigenase activity in red lettuce leaves (*Mulabagal et al.*, 2010). This is also the reason why red lettuce varieties were found to be better dietary sources of natural antioxidants, with higher levels of flavonols, which play a role in preventing cardiovascular diseases (*Llorach et al.*, 2008). Phenolic antioxidants generally exhibit a certain synergism with other reducing compounds, such as tocopherols, ascorbic acid, cysteine and carotenoids, which also contribute to the overall antioxidant capacity of leafy vegetables (*Altunkaya et al.*, 2009). For greenhouse-grown broccoli, it was shown that temperature increase under low light intensity affects the amount of phytochemicals such as antioxidative carotenoids, prooxidative chlorophylls and protective glucosinolates (*Schonhof et al.*, 2007).

The photon flux density of the incident light is a major environmental factor that determines the entire metabolic activity and vitality of plants, not only because it is the crucial energy source for the photosynthetic biomass production, but also because it may be the main source of photo-oxidative damage that triggers a network of concerted photoprotective mechanisms. While low light causes an imbalance in the energetic metabolism of plants that limits growth and development, excessively high photon flux densities may cause the photoinhibition of photosynthetic components and may lead to the overproduction of reactive oxygen species in chloroplasts. As a defence mechanism, overexpression of antioxidative enzymes and enhanced production of non-enzymatic antioxidants occur, and various repair processes take place in the thylakoid membranes, in metabolones and in the genetic material (*Pogány et al.*, 2006).

For greenhouse-grown and field-grown vegetables, such as lettuce, the salinity of irrigation water may represent a major environmental stress factor that limits growth and induces metabolic tolerance. Because lettuce cultivars are moderately salt-sensitive plants, high salinity is one of the most frequent limiting factors of growth and development in the case of lettuce. On a shorter time scale, salt stress inhibits growth of stem and leaves, mainly because of its osmotic effect (it causes difficulties in water supply, leading to the partial dehydration of leaves). This effect can be overcome by osmoregulation (accumulation of compatible solutes, such as proline, sucrose, trehalose, sugar

alcohols etc.) and by the downregulation of stomatal conductance, which has negative secondary effects on carbon assimilation and on the aquisition of mineral nutrients (*Ünlükara et al.*, 2008; *Younis et al.*, 2009). If salt stress lasts for longer periods (weeks and months), the chemical toxicity of excessive sodium ions becomes prevailing over the osmotic effect. Accumulation of sodium ions impairs the homeostasis of potassium and calcium in cell compartments, inhibits different enzymes and induces an oxidative stress by the generation of high amounts of reactive oxygen species. This chemical stress caused by high salinity is counteracted by the sequestration of sodium ions in the vacuole, by the enhancement of the antioxidative activity and by a series of secondary defence mechanisms that may vary among species and varieties, according to their degree of salt sensitivity, tolerance or resistance (*Ahmad et al.*, 2012; *Eraslan et al.*, 2007; *Kohler et al.*, 2009).

Many external adverse factors induce similar negative effects in living organisms, manifested as oxidative stress. This is why plants may develop cross-tolerance towards different environmental stressors by the activation of the antioxidative protective system. Radical scavengers and reducing agents (such as ascorbate, tocopherol, glutathione, simple phenolic compounds, flavonoids, carotenes and xanthophylls) represent the non-enzymatic component of the antioxidative system, while the enzymatic component includes the different isoenzymes of superoxide dismutase, catalase, peroxiredoxins, glutathione reductase, dehydroascorbate reductase, ascorbate peroxidase and other peroxidases (*Pogány et al.*, 2006; *Shigeoka et al.*, 2002; *Xu et al.*, 2008).

The aim of this research is to investigate the influence of moderately high (non-inhibitory) photon flux density and moderately high salinity, as well as of the interaction of these abiotic environmental factors on the concentration of some health-promoting metabolites, such as ascorbic acid and carotenoid pigments, as well as on a representative enzyme of the antioxidative system (i.e. ascorbate peroxidase) and on the water-soluble sugar content of fresh lettuce leaves, in order to improve nutritive value by modulating growth conditions.

2 Material and methods

Lettuce (*Lactuca sativa* L.) plantlets belonging to the May King cultivar (one of the most widespread varieties, cultivated all around Europe) were grown hydroponically in Hoagland's inorganic nutrient solution, in a growth chamber (Versatile Environmental Test Chamber, Sanyo) with a light intensity of 220 μM photons m^{-2} s^{-1} of photosynthetically active radiation, at 20 °C and a

constant atmospheric humidity of 70%. Four-week-old plants were separated into four experimental groups: the control group was grown for one more week under the above-mentioned conditions, a second group was illuminated with high light of 660 μM photons m^{-2} s^{-1}, a third one was put in Hoagland solution supplemented with 80 mM sodium chloride (p.a.), while the fourth group was subjected to the combined action of 660 μM photons m^{-2} s^{-1} and 80 mM NaCl. Measurements were performed on young but fully expanded leaves exposed for seven days to the above-mentioned conditions.

Carotenoid pigments were extracted with dimethylformamide and determined spectrophoto-metrically. 0.1 g fresh leaf material was immersed in 4 ml dimethylformamide and kept for 48 hrs in darkness for the complete extraction of photosynthetic pigments; then the extract was centrifuged for 5 min at 7000 g, and the absorbance of the supernatant was measured at 470 nm (*Bartha et al.*, 2010).

Water-soluble sugars were determined based on the absorbance of the green product resulting from the incubation of leaf extracts with anthrone solution. 0.5 g fresh leaves were homogenized in a final volume of 10 ml of 96% ethanol (v/v); the extract was centrifuged for 10 min at 5500 g, then 100 μL of supernatant was mixed with 3 μL freshly prepared anthrone solution in 72% H_2SO_4; the mixture was incubated for 10 min in boiling water and the absorbance of the green product was measured at 620 nm. Standard curve was obtained with glucose (*Bartha et al.*, 2010).

Ascorbic acid content was determined photometrically by the change in absorbance at 265 nm upon addition of 4 units (10 μL) of ascorbate oxidase. 1 g fresh leaf material was ground in a chilled mortar with a final volume of 4 ml 6% trichloroacetic acid; the mixture was centrifuged for 15 min at 4 °C and 15600 g, then 200 μL of supernatant was mixed with 790 μL of 0.1 mM phosphate buffer (pH 5.6). Upon the addition of 10 μL ascorbate oxidase solution, the absorption decrease at 265 nm was measured using an extinction coefficient of 14 mM^{-1} cm^{-1} (*Xu et al.*, 2008).

Ascorbate peroxidase activity was determined spectrophotometrically, based on the oxidation of ascorbic acid initiated by addition of hydrogen peroxide, and measured through decrease in the absorbance of the reaction mixture at 290 nm. 1 g leaf material was ground in a prechilled mortar with 5 ml extraction solution consisting of 50 mM phosphate buffer (pH 7.8), 1 mM Na_2-EDTA, 1 mM ascorbic acid and 2% water-soluble polyvinyl-pyrrolidone. The homogenate was centrifuged for 20 min at 15000 g. 50 μL of the supernatant (as plant enzyme extract) was mixed with 1.75 mL phosphate buffer (pH 7.8) containing 1 mM Na_2-EDTA and 100 μL of 10 mM ascorbic acid. The reaction

was started with the addition of 100 µL of 20 mM hydrogen peroxide, and after a lag period of 40 s the decrease of absorbance at 290 nm was registered for 3 min. A molar extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for the ascorbic acid and the reference mixture contained distilled water instead of hydrogen peroxide. Enzyme activity was expressed as consumed hydrogen peroxide per minute per mg protein, considering that 1 mole ascorbic acid reduces 1 mole of hydrogen peroxide. The protein content of leaf materials was assayed according to Bradford's method, with bovine serum albumine as a standard (*Bartha et al.*, 2009).

Every experimental setup had 4 repetitions. Statistical analyses of experimental data were performed in R environment (version 2.14.1), using the Shapiro-Wilk test for normality, Bartlett's test for homogeneity of variances and the post-hoc Tukey HSD test for the significance of differences between treatments (at $P < 0.05$).

3 Results

Environmental factors, such as light, temperature, water availability and mineral nutrient supply, have a determining influence on plant metabolism and development. Adverse external parameters are perceived as stress factors that induce changes in the main physiological functions like photosynthesis, mineral nutrition, water relations, secondary metabolism, growth and development. Metabolic plasticity enables plants to develop tolerance to stress factors, while physiological processes reach new steady state levels through a complex network of down- and upregulation mechanisms. Because of a considerable similarity between biochemical processes in the different living organisms, defence products that enable the survival of plants under changed environmental conditions may have benefic influence also on consumer organisms. For example, metabolites used by plants to overcome oxidative stress caused by reactive oxygen species have a demonstrated health-promoting action in humans upon consumption of plant products (*Mulabagal et al.*, 2010). In this context, lettuce is an important vegetable as a source of useful phytochemicals, especially when its fresh leaves are consumed in salads. A common group of metabolites synthesized by plants and related to photoprotection of chlorophylls in the photosynthetic apparatus of leaf cells is represented by the carotenoid pigments (carotenes and xanthophylls). Their role in preventing the generation of singlet oxygen in illuminated thylakoid membranes and in neutralizing the already produced singlet oxygen is well-established. Zeaxanthin and anther-

axanthin, as the two photoprotective pigments of the xanthophylls cycle, but also lutein, β-carotene, licopene and other carotenoids have an important antioxidative effect on all types of cells by protecting vital molecules (proteins, nucleic acids, unsaturated fatty acids in lipids, pigments) from oxidative damage (*Younis et al.*, 2009). These carotenoids cannot be produced by the human organism, so our carotenoid supply depends on plants. The present experiments showed that the carotenoid pigment content of lettuce leaves increases considerably if photon flux density is elevated from 220 to 660 μM photons m^{-2} s^{-1}. Even though mild salt stress exerted by 80 mM sodium chloride did not influence significantly the overall carotenoid content of the leaves, if salt stress was associated with increased light intensity, it acted synergistically with higher photon flux density and resulted in the further increment of the carotenoid content (Figure 1).

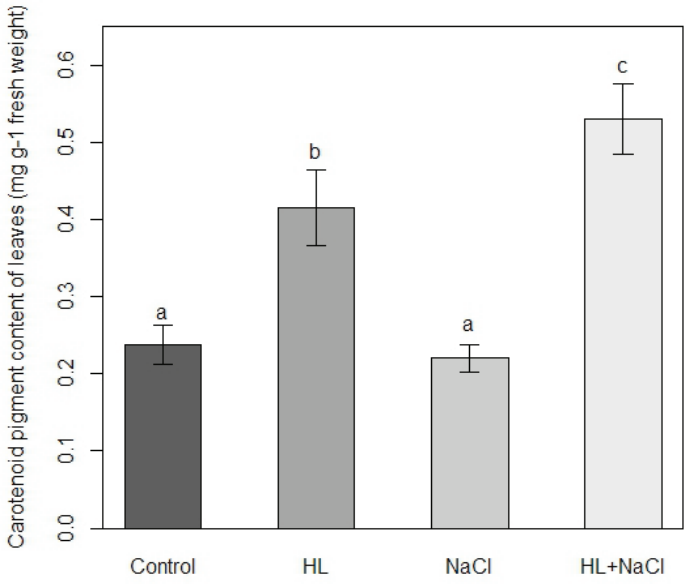

Figure 1: Influence of high light (HL) and salt stress – separately and in combination – on the carotenoid pigment content of lettuce leaves. For experimental conditions, see the *Material and methods* section. Bars represent means ± SE (n = 4). Different letters indicate significant differences at P < 0.05

Considering that carotenoid pigments are helth-promoting phytochemicals, their increased amount improves the nutritive value of lettuce grown under high light intensity and under mild salinity stress. *Oh et al.* (2009) have

demonstrated that chilling stress, heat shock, high light intensity (800 μM photons m^{-2} s^{-1} for one day) and other mild environmental stresses improve the health-promoting quality of lettuce with no significant adverse effects on its growth and fresh biomass yield. *Mahmoudi et al.* (2010) have found that treatment with 100 mM NaCl enhanced carotenoid biosynthesis in salt tolerant lettuce varieties. This enhancement was not observed in our experiments with the May King cultivar.

Water-soluble sugars are important products of photosynthetic carbon assimilation; furthermore, they represent intermediates for the biosynthesis of different defence products (e.g., biosynthesis of ascorbic acid involves D-mannose and L-galactose), and some simple sugars may play an important role in osmoregulation as compatible solutes of plant cells (e.g., sucrose, trehalose). This is the reason why we have investigated the influence of increased photon flux density and of high salinity on the water-soluble sugar content of lettuce leaves. While intense light caused a significant increase in the sugar content, salt stress had no effect on it, and it anihilated the effect of high light when they were applied in combination (Figure 2).

This may reflect that water-soluble sugars are not the main osmoregulators in lettuce leaves, thus their synthesis is not enhanced by the osmotic component of salt stress. While higher light energy input results in the accumulation of simple sugars in leaf cells (most probably as sucrose produced in the cytoplasm of mesophyll cells and loaded into the phloem vessels of leaf veins), salt stress disturbs carbohydrate metabolism and exerts an indirect inhibitory influence on water-soluble sugar accumulation. In other experiments, performed under generally similar conditions, it was found that different degrees of salt stress have no significant influence on the simple sugar content of several lettuce varieties (*Bartha et al.*, 2010). It is worth mentioning that salt stress did not cause any significant dehydration of leaves (their water content was around 94% in the control and around 91% under the influence of 80 mM NaCl – data not shown), so there is an efficient osmoregulation in lettuce, but this is probably not achieved with a significant contribution of water-soluble sugars.

Along carotenoids, which are lipophylic antioxidants occurring in biomembranes (thylakoid membranes) and in plastoglobuli as metabolic inclusions of the plastid stroma, ascorbic acid (vitamin C) is a major water-soluble antioxidant, being present in different compartments of the plant leaf cells in average concentrations of around 5 mM (with an average of 25 mM in the stroma of chloroplasts). It cannot be produced by the human organism, being supplied through diet. Because of its reducing property, ascorbic acid detoxifies

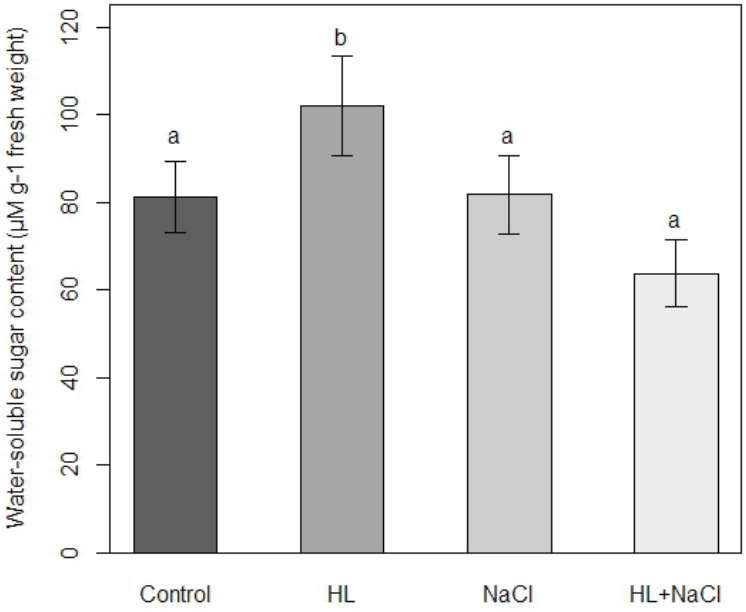

Figure 2: Influence of high light (HL) and salt stress (80 mM NaCl) on the water-soluble sugar content of lettuce leaves. Bars represent means ± SE (n = 4). Different letters indicate significant differences at P < 0.05, according to the post-hoc Tukey HSD test

hydrogen peroxide and it also contributes to the regeneration of vitamin E during the neutralization of hydroxyl radical, which is one of the most potent reactive oxygen species along hydrogen peroxide and the superoxide radical anion (*Pogány et al.*, 2006). In cell compartments where there is no catalase enzyme, the reduced form of vitamin C is used by ascorbate peroxidase to convert hydrogen peroxide into two molecules of harmless water. During this process, ascorbate is oxidized to dehydroascorbate, and the enzyme dehydroascorbate reductase ensures the regeneration of reduced vitamin C. For an efficient defence against oxidative stress generated by accumulation of hydrogen peroxide, a sufficiently high amount of vitamin C and a constantly high molar ratio between its reduced and oxidized form is needed. For this reason, the vitamin C content of plant organs is a molecular indicator of oxidative stress tolerance, and it also increases the health-promoting quality of food plants, considering that vitamin C has the same benefic action in the human organism. Under our experimental conditions, high photon flux density and salt stress both increased the ascorbic acid content of lettuce leaves, but no

synergistic or antagonistic interaction could be observed between the two environmental factors (Figure 3). Probably because the applied light intensity caused a significant photo-oxidative influence in leaf cells, it resulted in a higher vitamin C accumulation than salt stress exerted by 80 mM sodium chloride. The results are in agreement with several other findings, which demonstrates that various abiotic and biotic stress factors that induce oxidative damage increase the antioxidative capacity of plants through a higher ascorbic acid content (*Altunkaya and Gokmen*, 2008; *Kohler et al.*, 2009; *Rios et al.*, 2008; *Schonhof et al.*, 2007; *Xu et al.*, 2008).

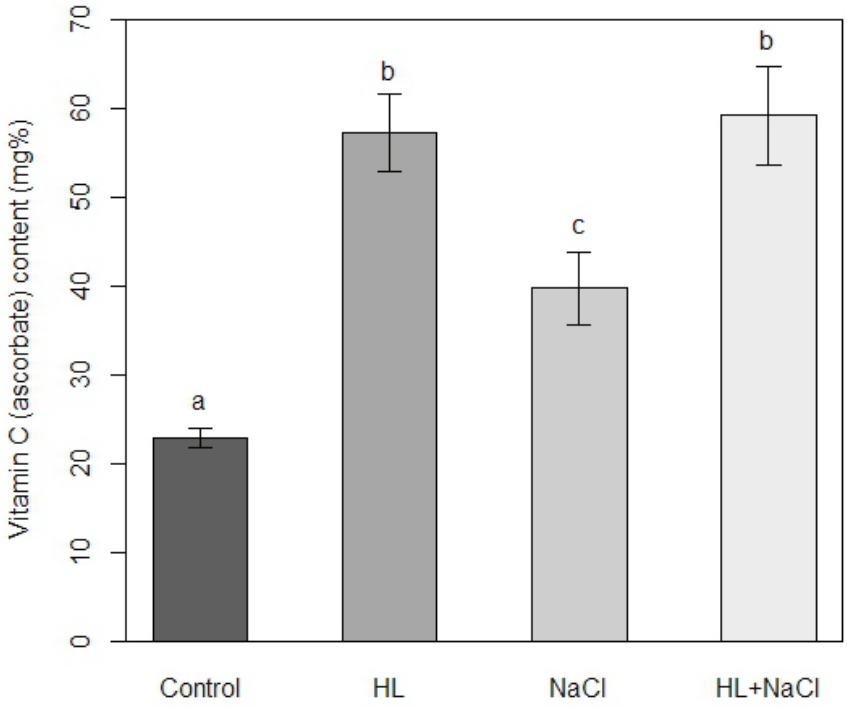

Figure 3: Influence of high light (HL) and salt stress on vitamin C content of fresh lettuce leaves. Bars represent means ± SE (n = 4). Different letters indicate significant differences at $P < 0.05$, according to the Tukey HSD test

The main antioxidative protective enzyme, which regulates the level of hydrogen peroxide in the micromolar concentration range by converting the excessive amount into water through reduction with ascorbic acid, is represented by the different isoforms of ascorbate peroxidase. It accumulates upon oxida-

tive stress in tolerant plants and its enzymatic activity decreases in sensitive species or intraspecific varieties; thus, it is considered a good biochemical marker of stress tolerance (*Pogány et al.*, 2006; *Shigeoka et al.*, 2002; *Younis et al.*, 2009). Its activity increased in lettuce leaves exposed to elevated light intensity; it was not influenced significantly by mild salt stress exerted by 80 mM sodium chloride, but salt stress enhanced the effect of high light when the combination of the two factors was applied (Figure 4). This suggests that high salinity acts synergistically with high light intensity in triggering this enzymatic component of the antioxidative defence system of plant cells, and the interaction of high irradiance with salt stress results in a higher catalytic activity of ascorbate peroxidase. This is in accordance with the elevated vitamin C level of leaves exposed to high photon flux density, vitamin C being the organic substrate which accomplishes the reduction of hydrogen peroxide.

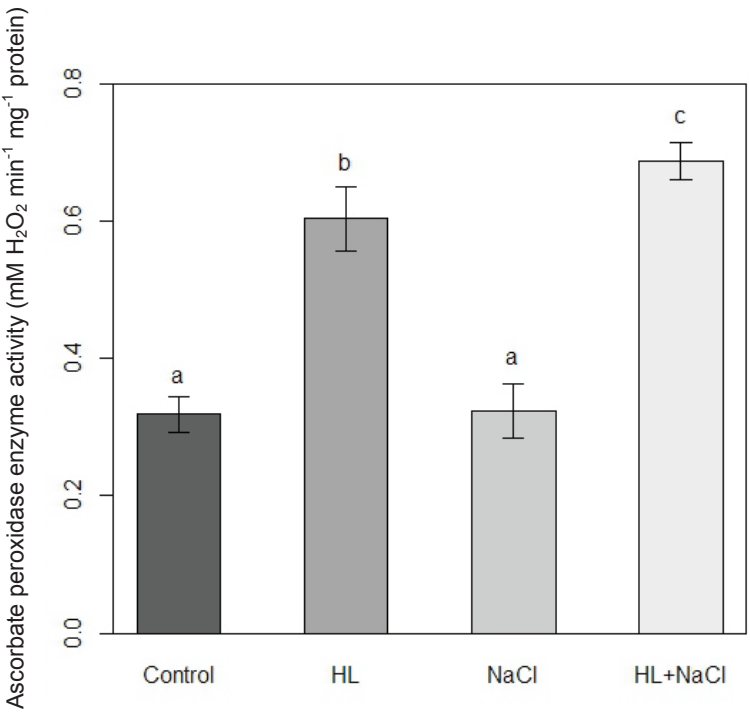

Figure 4: Influence of high light (HL) and salt stress on the enzymatic activity of ascorbate peroxidase in lettuce leaves. Bars represent means ± SE (n = 4). Different letters indicate significant differences at $P < 0.05$

4 Conclusions

Higher photon flux density increases carotenoid pigment content, water-soluble sugar concentration, vitamin C content and ascorbate peroxidase activity in lettuce leaves, thus conferring them a higher nutritive value related to enhanced antioxidant properties. Even though salt stress has no stimulating effect on carotenoid and simple sugar content of leaves, high salinity enhances the positive influence of high light on the accumulation of carotenoids and on the antioxidative activity of ascorbate peroxidase, and it reverses the action of elevated irradiance on water-soluble sugar content. Combination of moderately high photon flux density (660 μM photons $\mathrm{m}^{-2}\mathrm{s}^{-1}$) and mild salt stress (80 mM NaCl) improves the content of healthy phytochemicals in fresh lettuce leaves.

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