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EFFECTS OF CHLORIDE SALINITY ON NON-ENZYMATIC ANTIOXIDANT ACTIVITY, PROLINE AND MALONDIALDEHYDE CONTENT IN THREE FLUE-CURED CULTIVARS OF TOBACCO

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Abstract: High salinity causes ion poisoning and subsequent oxidative stress. During oxidative ion poisoning, non-enzymatic defense systems such as carotenoids, phenols and flavonoids are activated. In this study, the effects of various concentrations of chloride in the irrigation water (10, 20, 40 and 80 mg/l) on carotenoids, phenols, flavonoids and proline content in three varieties of tobacco plant (Virginia, Kentucky and Cooker) were investigated. Malondialdehyde content was also measured, as a lipid peroxidation index. The highest level of β-carotene was observed in Virginia at 10 mg/l chloride and the lowest quantity was seen in Kentucky at 20 mg/l chloride. Kentucky had the highest and lowest levels of flavonoids at 80 and 40 mg/l concentrations of chloride respectively. Highest phenol content was observed in the presence of 10 mg/l chloride in Virginia. Maximum and minimum MDA concentrations were observed respectively in 20 and 80 mg/l concentration of chloride in Cooker cultivar. Raising chloride concentrations in irrigation water caused a substantial decrease in proline content only in the Kentucky variety.

Keywords: Oxidative stress, β-carotene, chloride, flavonoids, tobacco, total phenol

Introduction

Chloride is generally abundant in the environment. It exists as a monovalent micronutrient in soil water. Chloride ion is extremely mobile and easily lost in soils, which have a leaching effect on the substance. Since chloride is present in different sources, such as irrigation water, soil, rain, fertilizers and air pollution, more attention is given to its poisoning effect rather than to its deficiency. On the other hand, chloride is one of the necessary components for plant growth. For instance, chloride is taken up very rapidly by tobacco plant [RHOADS, 1975]. Tobacco can accumulate chloride very rapidly in considerable amounts, equivalent to up to 100 g Cl per kg leaf dry weight [McCANTZ & WOLTZ, 1967]. While small quantities of chloride in nutrients has positive effects on tobacco yield and market value of the leaf, high concentrations in the soil cause abnormal growth and unfavorable properties in the cured leaf. Increasing quantities of chloride in cured leaves reduces the burning rate and causes undesirable effects, such as an increase in hygroscopy, dinginess or uneven colors and adverse odors. In recent years several researchers have focused on studying the role of chloride in creating such effects [PEELE & al. 1960, FLOWER, 1999].

High salt content has a decreasing effect on the osmotic potential of soil and causes water stress in plants. Interaction between salts and inorganic nutrients may result in

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an imbalance in feeding [McCUE & HANSON, 1992]. It decreases the regeneration of $NADP^+$ in Calvin cycle, which as a consequence reduces the photosynthetic electron transport chain dramatically and eventually generates super oxide radicals [SHUN-WU & KE-XUAN, 2004; LI & JIN, 2007]. In case of oxidative stress in plants, natural antioxidants, which prevent the adverse effects of oxidative stress, will increase. Some of the non-enzymatic antioxidants present in plants are polyphenols. These substances play key roles in the plant defense system and growth. In addition, these components contain the necessary information on the cell oxidation status and regulate gene expression related to optimizing defense via responses to biotic and abiotic stress [SHAO & al. 2008]. On the other hand, the amino acid proline is one of the most effective compounds for regulation of osmosis in plants under drought and salinity stress. Osmosis regulators are very soluble chemicals which increase resistance and tolerance of plants against stress by maintaining cell turgor and stability of the cytoplasmic enzymes. Despite the economic value of tobacco in the north of Iran, there has been no recent investigation on the effect of increasing soil chloride concentrations on tobacco growth responses. One of the reasons for the higher chloride concentrations in soil is that irrigation water at present contains higher levels of chloride than before, whereas in the past it was not considered a serious problem in tobacco growing areas. Recent measurements confirmed a rise in chloride concentration in soil and irrigation water in the north of Iran. This study was conducted with three main purposes:

1) To investigate the effects of different chloride concentrations on quantity and activity of some non-enzymatic antioxidants, such as total phenol and flavonoids.
2) To study the effects of different chloride concentrations on proline and MDA content in tobacco.
3) To determine the critical concentration of chloride in the three studied cultivars of tobacco and to establish an acceptable tolerance level for chloride in the water used for irrigating tobacco.

Materials and methods

***Sample preparation*:** Effects of four concentrations of chloride in irrigation water (10, 20, 40 and 80 mg/l) on proline content and some non-enzymatic antioxidants such as β-carotene, total phenol and flavonoids were investigated in three flue-cured cultivars of tobacco, namely Cooker 347, Virginia E1 and Kentucky 326, by spectrophotometry using factorial design (4×3) with 4 pot replications. The selected cultivars are the highest quality commercial cultivars in the north of Iran. Data were analyzed using standard analysis of variance (ANOVA), and means were separated using Duncan comparisons at $P \leq 0.05$.

***Plant culture and treatment application*:** An outdoor pot experiment was established during 2000 growing seasons at the Institute of Tobacco Research in Rasht in the northern part of Iran. Seedlings were prepared using the float system. In this study, plants in pots were grown according to normal practices used for tobacco production in Guilan. Plastic pots were filled with a mix consisting of 4 volume of soil and 1 volume of fine perlite. Defined basic fertilizer N-P-K was added to every pot upon soil analysis results before planting. Uniform seedlings (12-15 cm) were selected and transplanted to pots, one plant per pot. There are three vegetative growth phases in tobacco plants, including root development, fast growth phase and leaf ripening. Naturally, the fast growth phase is usually the most sensitive. Irrigation was therefore carried out twice a week with 2 liters of salt solution for each 6 liter pot. This amount of water did not provoke leaching and

chloride ions were added to water as $CaCl_2$. Electrical conductivity (EC) of the solutions was measured before irrigation. The analysis of all factors in this study was carried out using fresh and well developed leaves harvested from seedlings 60-65 days after transplanting.

***Extraction and assay of β-carotene*:** To estimate total β-carotene, leaf tissues (0.25 g) were first homogenized in a chilled (v/v) acetone-hexane mixture; the homogenates were centrifuged at 5000 rpm for 10 min at 4 ºC in the dark. The absorbance of the acetone-hexane extracts was measured at 663, 645, 505 and 453 nm. The amount of β-carotene was calculated according to NAGATA & YAMASHITA (1992).

***Non-enzymatic antioxidants extraction*:** For the preparation of tobacco extracts for non-enzymatic antioxidants, including phenols and flavonoids, the leaves of the three cultivars of tobacco were well ground separately in a mortar with liquid nitrogen. 0.25 g of the homogenized sample was weighed and transferred to falcon pips with label. 5 ml 80 % methanol was added to each falcon pip and stirred. Then the extract was centrifuged at 5000 rpm for 10 min at 0 ºC. The supernatants were collected and kept in a refrigerator at -20 ºC.

***Total flavonoid assay*:** The assessment of total flavonoid content in the obtained extracts was done using a colorimetric method [CHANG & al. 2006] with some modification. Sample absorbance was read at 510 nm. Different concentrations of catchin were used to draw the standard curve.

***Total phenol assay*: The method of SINGLETON & ROSSI** (1965) was used for total phenol assay using Folin-Ciocalteu solution. Total phenolic content (mg/g f.w.) was determined using the standard curve of gallic acid ($R^2 = 0.987$). The use of this method is mainly restricted due to the fact that it cannot distinguish between different types of phenols and only total phenol is measured.

***Proline measurement*:** Extraction of proline was carried out in accordance with BATES & al. (1973) and absorbance of solution color (included toluene and proline) was read at 520 nm. Pure proline (Merck) was employed as a standard reference and results were expressed as proline equivalents (ng of tobacco leaves on fresh weight basis).

***Extraction and measurement of malondialdehyde (MDA)*:** About 0.5 to 1.0 gr of tissue was homogenized in 5 ml of 5% (w/v) trichloroacetic acid and the homogenate was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was mixed with an equal volume of thiobarbitoric acid (0.5% in 20% (w/v) trichloroacetic acid), and the mixture was boiled for 25 minutes at 100 ºC followed by centrifugation for 5 minutes at 7,500 rpm to clarify the solution. Absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the A600. MDA content was calculated using an extinction coefficient of 155 M-1 cm^{-1}. Values of MDA content were taken from measurement of three independent samples and SEs of means were calculated [HEATH & PACKER, 1968].

Results

***β-Carotene assay*:** Changes of β-carotene content in mature leaves of tobacco cultivars Cooker 347, Virginia E1, and Kentucky 326 were investigated at different chloride concentrations (10, 20 40 and 80 mg/l). Studying mature leaves shows that the highest content (26.56 mg/g f.w.) of β-carotene is to be found in Virginia at 10 mg/l chloride and the lowest quantity (13.24 mg/g f.w.) in Kentucky at 20 mg/l chloride (Fig. 1).

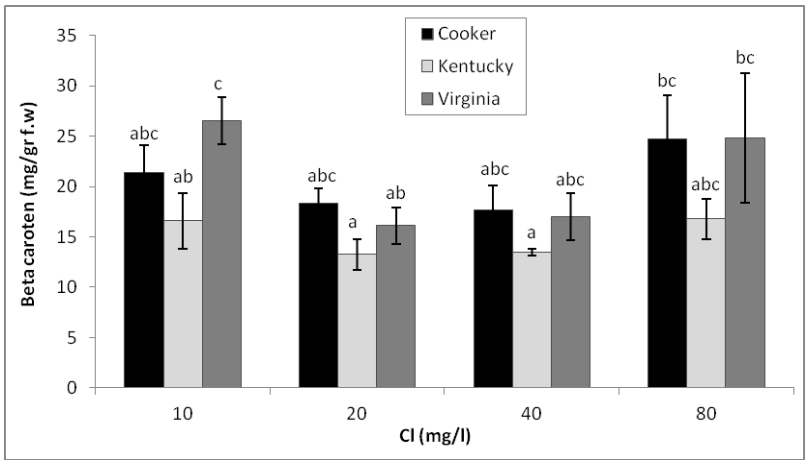

Fig. 1. β-carotene concentration in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively)

***Total flavonoid assay*:** Changes of the flavonoids were investigated in the tobacco cultivars under different concentrations of chloride. The total flavonoid content in samples varied from 0.16 to 0.45 mg/g f.w. with the lowest and the highest levels observed in 40 and 80 mg/l chloride concentration for Kentucky (Fig. 2).

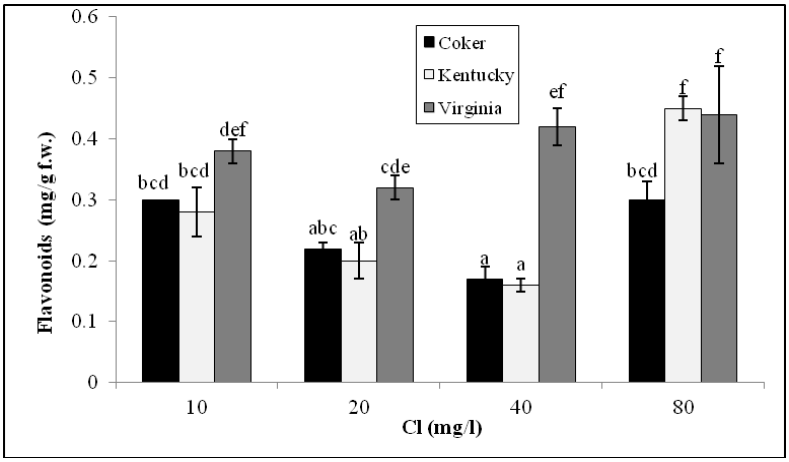

Fig. 2. Flavonoid content in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively). Different concentrations of catchin were used to draw the standard curve

***Total phenol assay*:** Results obtained show that total phenol varied from 56.64 mg/g f.w. in the Kentucky cultivar under 20 mg/l concentration of chloride to 79.44 mg/g in Virginia under 10 mg/l concentration of chloride (Fig. 3).

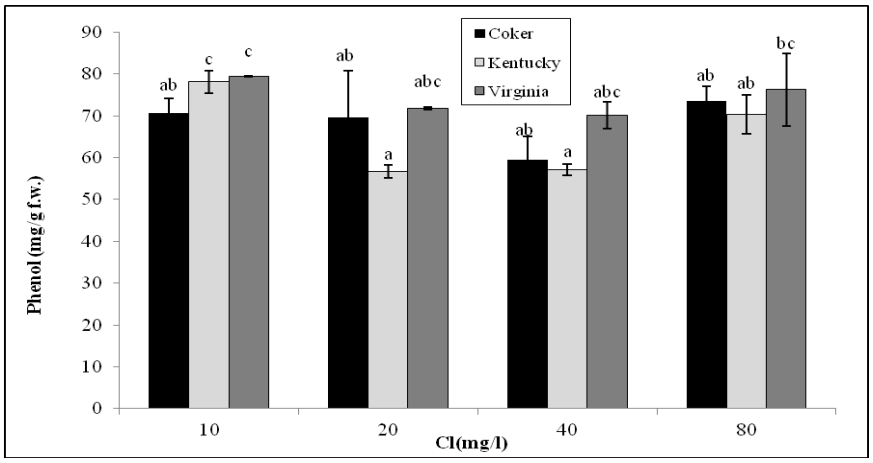

Fig. 3. Phenol concentration in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively). Total phenolic content was determined using the standard curve of gallic acid

***Measurement of Proline*:** The results showed that proline content in Kentucky cultivar varied from 437.8 to 2,169.4 mg/g f.w. The highest and the lowest proline content were observed at 10 mg/l and 20 mg/l chloride, respectively (Fig. 4).

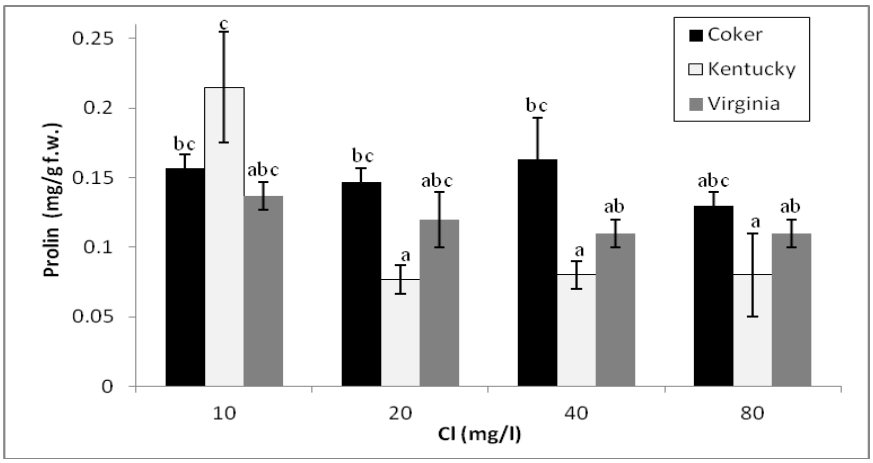

Fig. 4. Proline concentration (mg/g f.w.) in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively). Pure proline was employed to obtain the standard curve

***Lipid peroxidation*:** Lipid peroxidation is a process related to free radicals and its concentration is generally used as an index for oxidative stress and also as a marker for plant sensitivity to environmental stress. MDA assay in mature leaves of different cultivars showed different results. Maximum and minimum MDA concentrations were observed respectively in 20 and 80 mg/l concentration of chloride in Cooker cultivar (Fig. 5).

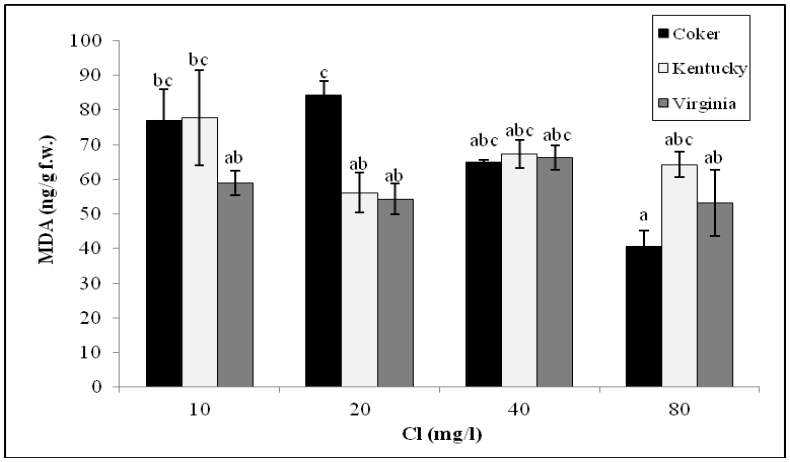

Fig. 5. MDA concentration in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively)

Discussion

As is shown in the results, non-enzymatic antioxidant responses to different concentrations of chloride in irrigation water are different, but the differences do not have the same statistical significance. Not only different concentrations, but also different cultivars have a significant impact on how the samples under treatment do react. In this study, MDA content was used primarily as a lipid peroxidation index and as a marker of oxidative stress.

***β-carotene*:** As seen in figure 1, with some exceptions, there are no meaningful differences between treatments and control; and this may be interpreted as absence of oxidative stress in the used range of chloride concentrations. Studies on other plants show a decrease in carotenoids content, especially β-carotene, in salt stress with chloride concentrations of up to 100 mM [STOEVA & KAYMAKANOVA, 2008; AYALA-ASTORGA & ALCARAZ-MELENDEZ, 2010]. In contrast, other studies seem to show an increase in other carotenoids during salt stress [RAO & al. 2007].

***Flavonoids*:** Figure 2 shows the differences between flavonoids content in the three cultivars. It emphasizes the results of interaction between two factors, namely chloride concentration and cultivar. Decreasing levels of flavonoids with chloride concentrations of 20 and 40 mg/l indicate that these concentrations are optimal and result in the least stress. Conversely, increasing chloride concentration to 80 mg/l results in higher levels of flavonoids and stress, except in the Kentucky variety, which exhibited more sensitivity and flavonoid levels increased from 40 mg/l chloride). The correlation between

the two antioxidant factors – β-carotene and flavonoids – in addition to changes in MDA, can lead us to an understanding of optimum chloride concentrations in the irrigation water.

***Total phenol*:** Investigation of the total phenols, as non-enzymatic antioxidants, shows that 10 mg/l concentration of chloride is a stressful concentration. Phenol content is maximal at 10 mg/l of chloride and with increasing chloride concentrations (20 and 40 mg/l) phenol values decrease; finally at 80 mg/l chloride, phenol content increase to its original level at 10 mg/l chloride concentration (Fig. 3). Interaction between concentrations and cultivars in the section indicates that the optimal response for the Kentucky cultivar is 80 mg/l chloride. This finding reinforces the results obtained from studies on flavonoids. Increasing phenol content during salt stress has been reported in several studies [MUTHUKUMARASAMY & al. 2000; AGASTIAN & al. 2002; NAVARRO & al. 2006].

***Proline*:** As mentioned in the introduction, proline has a significant role in salt and drought stress [HUA & al. 1997]. It is therefore desirable to measure proline in order to shed light on this question. Fig. 4 shows that in matured leaves we can see a significant decrease in proline content for Kentucky and a slight decrease in other cultivars together with increasing chloride concentrations. It is important to note that chloride ion probably causes more disorder in ion exchange in the rhizosphere than in osmosis. Given the situation we need to use a more multifaceted metabolism for a deeper explanation.

***Lipid peroxidation*:** Figure 5 shows that MDA content in mature leaves of Cooker cultivar increases with increasing chloride concentrations up to 20 mg/l, after which it decreases. Although variations are not meaningful in treatments which received chloride concentrations of less than 80 mg/l chloride, at 80 mg/l there was significantly reduced lipid peroxidation in the Cooker variety, as opposed to control. This means that concentrations below 80 mg/l chloride caused higher stress, and so led to higher concentrations of MDA. This result is not unexpected with regards to tobacco, as it easily absorbs chloride and needs it as a macroelement [DAVIS & NIELSEN, 1999]. It may therefore be concluded that chloride content of 10, 20 and 40 mg/l in irrigated water and soil could not supply sufficient chloride to provide the plants with the necessary minerals necessary for optimal growth. As a result, increasing chloride content to 80 mg/l created the conditions for a reduced lipid peroxidation. This situation was seen more or less in all three cultivars. Nonetheless, responses were not identical; in cultivars Virginia, Kentucky and Cooker lipid peroxidation decreases with the increasing chloride content, while the decrease is significant in the Cooker and not significant in the other two cultivars. The result obtained in our study show that high chloride concentrations in the soil result in increases in MDA levels; however this result needs to be verified in further studies. Investigations on other plant species show that stress due to increasing chloride content, especially where sodium ions are also present, results in lipid peroxidation, but does not significantly affect MDA content; these changes seem to be dependent on both chloride concentration and plant cultivar. So, in mean concentrations of chloride either no changes were reported [NOR'AINI & al. 1997] or even a decrease in lipid peroxidation occurred [KSOURI & al. 2007]. Conversely, in other species the same concentrations could induce an increase in lipid peroxidation meaningfully [KSOURI & al. 2007; ESFANDIARI & al. 2007].

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