

DOES OVERHEAD IRRIGATION WITH SALT AFFECT GROWTH, YIELD, AND PHENOLIC CONTENT OF LENTIL PLANTS?

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Abstract - Overhead irrigation of lentil plants with salt (100 mM NaCl) did not have any significant impact on plant growth, while chlorophyll content and chlorophyll fluorescence parameter F_v/F_m were affected. Under such poor irrigation water quality, the malondialdehyde content in leaves was increased due to the lipid peroxidation of membranes. In seeds, the total phenolic content (TPC) was correlated to their total antioxidant capacity (TAC). High performance liquid chromatography-mass spectrometry (HPLC-MS) detection showed that flavonoids (catechin, epicatechin, rutin, *p*-coumaric acid, quercetin, kaempferol, gallic acid and resveratrol) appear to be the compounds with the greatest influence on the TAC values. Catechin is the most abundant phenolic compound in lentil seeds. Overhead irrigation with salt reduced the concentration of almost all phenolic compounds analyzed from lentil seed extracts.

Keywords: Antioxidant activity, chlorophyll, *Lens culinaris* Medik, lipid peroxidation, overhead irrigation, phenolics

INTRODUCTION

Lentils are believed to have originated and been consumed since pre-historic times. They are one of the first crops to have been cultivated. Lentil seeds dating back 8,000 years have been found at archeological sites in the Middle East. The lentil is considered an “ancient crop for modern times” (Sandhu and Singh, 2007). In the modern and technologically advanced world, among other crops the annual legume lentil crop (*Lens culinaris* Medik.) is widely cultivated in more than 35 countries of 5 different continents, throughout Europe, Asia, and North Africa. This crop became very popular among the people of the third world countries as an important protein source. The plants are grown for their small lens-shaped edible seeds, which are rich in protein (35-40%) and carbohydrates, and are a good source of calcium, phospho-

rus, iron and B vitamins. Lentil seeds provide high levels of protein and, when consumed in combination with cereals, they provide adequate amounts of essential amino acids for the human diet. Lentil seeds are rich in polyphenols, which have antioxidant, antimutagen and free radical-scavenging abilities and their potential effects are beneficial to human health in the prevention of many chronic diseases, so the investigation of the polyphenolic content in lentil seeds is worthwhile (Dueñas et al., 2002, 2003). Their relatively short cooking time provides an additional advantage. Lentil production is equally beneficial for producers, as lentils have a high tolerance for extreme environmental conditions such as drought and hot temperatures, and can be grown in semiarid regions without irrigation. Although the lentil is drought tolerant in comparison with other temperate grain legumes, drought stress is the major restriction on lentil

yield in lentil growing regions, worldwide (Muehlbauer et al., 1995, 2006). Lentils are well adapted to dry land farming, but under severe water stress the crop responds favorably to irrigation.

It is well known that a growing crop has a basic demand for good water quality to produce the maximum yield. Given that water deficiency for crop production has become a common problem worldwide, new irrigation strategies must be established to use the limited water resources more efficiently. Overhead irrigation might partly mitigate the problem of water deficiency. Unlike surface irrigation systems, sprinklers are more efficient as the quantity of water to be applied can be adequately controlled. Regular watering of crop plants leads to the accumulation of salts in the soil and thereafter emerges as salt stress. High soluble salts can directly injure roots, interfering with water and nutrient uptake. Salts can accumulate in plant leaf margins, causing burning of the edges. The most common salt in irrigation water is sodium (Na^+). Drought and salt stress are the main threat to lentil growing. Modern agricultural management practices apply different strategies to alleviate the impact of these stresses on the quality and quantity of the harvested yield. Supplemental irrigation is a viable option that can be used by farmers in the Mediterranean region to increase lentil production under water shortage conditions. Also, agricultural scientists are trying to improve dry land lentil yields through breeding with the objective of increasing lentil adaptation to environmental stress. The key strategy used to combat drought has been to match crop development with the period of soil moisture availability.

Different physiological and biochemical methods are used for screening the effect of various biotic and abiotic stresses on crop production. Plant growth performance, chlorophyll content in leaves, chlorophyll fluorescence *in vivo*, malondialdehyde (MDA) content and antioxidant activity (total phenolic content and total antioxidant capacity) are tested in this study on lentil plants exposed to overhead irrigation with water supplemented with salt.

The aim of this study was to investigate if overhead irrigation with salt affects the growth, yield and phenolic content of lentil plants.

MATERIALS AND METHODS

Plant material

Lentil seeds (*Lens culinaris* Medik. cv. "Dimitra" - local Greek variety) were sown by hand individually and randomly in thirty-nine experimental plots. A field experiment was conducted from November to June in northern Greece. The experiment was established on a sandy loam (Xerofluvent) soil consisting of 644 g sand, 280 g silt, 76 g clay, 7 g organic C·kg⁻¹ with a pH of (1:2 H₂O) 7.6. The electrical conductivity (EC) of the saturated soil extract at 0-60 cm depth was 2 dS/m. Nitrogen and phosphorus were applied as ammonium sulpho-phosphate (20-10-0, F-TOP Ledra Ltd, Thessaloniki) at 100 and 50 kg·ha⁻¹, respectively, and incorporated into the soil prior to lentil planting. An additional 50 kg N·ha⁻¹ as ammonium nitrate (33.5-0-0, F-TOP Ledra Ltd, Thessaloniki) was applied in late March. A set of 21 plants from each plot was foliar-sprayed (to run-off) with a low-pressure hand-wand sprayer two times at 14-day intervals with 100 mM NaCl. The first application was made 7 weeks after germination. Control plants were treated with water and surfactant (Syngenta, Ontario, Canada).

Lipid peroxidation assay

The level of lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by thiobarbituric acid (TBA) reaction as described by (Health and Packer, 1968; Hodges et al., 1999). Leaf tissue was homogenized in TBA solution was comprised of 20.0% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene, at 4°C. Absorbances were read at 440 nm, 532 nm, and 600 nm. The concentration of MDA was calculated from the difference of absorbance at 400 nm, 532 nm and 600 nm using the extinction coefficient 157 mM⁻¹cm⁻¹ and expressed as nmol MDA·g⁻¹ fresh weight (FW).

Chlorophyll determination

Total chlorophyll concentration (Chl a+b) was calculated from the equations previously reported by Wintermans and Motts (1965). Samples (five discs each) were taken from plants of each treatment with ten fully expanded leaves. Leaf discs were extracted with 96% ethanol in a water bath at 80°C. Full extraction of chlorophyll was achieved when the sample was discolored. The absorption of the extracts was measured at 665 nm and 649 nm with an LKB Ultraspec II spectrophotometer expressed as mg·g⁻¹ FW.

Photosynthetic parameters

Chlorophyll fluorescence indices provide direct information on the functionality and effectiveness of photosynthesis (Lichtenthaler et al., 2005; Maxwell and Johnson, 2000). The chlorophyll fluorescence emission was measured from fully developed leaves *in vivo* with a pulse-amplitude modulation fluorometer (Plant Efficiency Analyzer-PEA, Hansatech 2.02, Walz, Effeltrich, Germany), as previously described by Kofidis et al. (2008). The minimum fluorescence yield (F_o) of the plants adapted to darkness for 20 min was determined under weak red modulated radiation. The mid-part of the front was held in the leaf clip of the fluorometer at a standard distance from the optic fiber probe and a weak 5s far-red (735 nm) pulse was sent to fully oxidize the electron transport chain. The maximum fluorescence yield (F_m) of the dark-adapted plants was reached by exposing the PSII to a saturating pulse (0.8s) of white light. The difference between F_m and F_o gave the variable fluorescence ($F_v = F_m - F_o$). The maximum photochemical efficiency of PSII was calculated as the ratio of variable fluorescence to maximal fluorescence (F_v/F_m), and represents the efficiency of open PSII in the dark-adapted state.

Determination of total phenolics content (TPC) and total antioxidant capacity (TAC)

For extraction of phenolics, the lentil seeds were homogenized in 80% methanol at a 1:5 FW:methanol),

using *Fast Prep-24 System* (M.P. Biomedicals, Irvine, California, USA) for sample homogenization. The homogenates were centrifuged at 10,000 x g for 20 min and supernatants were used for further analyses. Four extracts were prepared for each sample analyzed.

The amount of TPC in extracts was determined according to the Folin-Ciocalteu's spectrophotometric (2501 PC Shimadzu, Kyoto, Japan) procedure (Singleton and Rossi, 1965) using gallic acid (GA) as a standard for the calibration curve. Samples were mixed with 0.25 N Folin-Ciocalteu reagent, and after 3 min 0.2 M sodium carbonate solution was added and incubated for 60 min. Results were read at 724 nm and expressed as mg eq-GA g⁻¹ FW.

Determination of TAC was done following the ABTS method (Arnao et al., 1999). The reaction mixture contained 2 mM ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), 15 μM hydrogen peroxide and 0.25 μM horse radish peroxidase (HRP) in 50 mM phosphate buffer pH 7.5. The reactions were monitored at 730 nm (2501 PC Shimadzu, Kyoto, Japan) at 25°C until a stable absorbance was obtained due to ABTS radical formation. Afterwards, different concentrations (0.1-0.8 mM) of ascorbic acid (AsA) were added for a standard curve set-up. The adding of the methanol extracts of lentils to the reaction mixture resulted in absorbance decreasing because of ABTS radical depletion. Absorbance alterations were read from standard curve and results were expressed mg eq AsA·g⁻¹ FW.

HPLC-MS analysis of phenolic compounds

Quantification of individual phenolic compounds was done by reversed phase HPLC-MS analysis. Samples were injected in Waters HPLC system consisting of 1525 binary pumps, thermostat and 717+ autosampler connected to the Waters 2996 diode array and EMD 1000 Single quadrupole detector with ESI probe (Waters, Milford, USA). Separation of phenolics was performed on a XTerra MS C18 RP column 2.1 x 50 mm size with 3.5 μm particle diameter (Waters, Milford, USA) connected to the appro-

priate guard column. Two mobile phases, A (0.1% formic acid) and B (acetonitrile) were used at a flow of 0.4 mL min⁻¹ with the following gradient profile: the first 15 min from 10 to 30% B, followed by 15 min reverse to 10% B and with additional 5 min of equilibration time. For LC/MS analysis, signals for each compound were detected in negative ESI SIR Mode with the following parameters: capillary voltage 3.0 kV, cone voltage -35 V, extractor and RF lens voltages were 3.0 and 0.2 V respectively. Source and desolvation temperatures were 140°C and 400°C, respectively, with N₂ gas flow of 500 L·h⁻¹. The data acquisition and spectral evaluation for peak confirmation were carried out by the Waters Empower 2 Software (Waters, Milford, USA).

Statistical analyses

An analysis of variance (ANOVA) was performed for the lentil parameters (plant height, dry weight per plant, grain yield-seed weight, and 1000-seed weight) using the SPSS 11.0.1 for Windows statistical package (SPSS, Chicago, USA). For comparison of the means, Fisher's Protected LSD test at the 5% level was employed. Data for TPC, TAC and specific phenolic compounds were subjected to statistical analysis using Mann-Whitney U nonparametric test (Statistica 6.0; StatSoft, Inc., USA).

RESULTS

Growth measurements

Lentil plant growth performance was not affected by overhead irrigation with salt as compared to the control plots (Fig. 1A), while the dry weight per plant (Fig. 1B) and seed weight per plant (Fig. 1C) were affected after this treatment.

Photosynthetic parameters and MDA content

Although salt treatment reduced the chlorophyll content in the lentil leaves (Fig. 2A), the fluorescent parameters F_v/F_m and F_v/F_o ratio were increased, pointing to the absence of detrimental salinity effects on leaf photochemistry (Fig. 2C, D). The MDA con-

tent in lentil leaves was increased upon salt treatment (Fig. 2B).

Phenolic pattern of lentil seeds

Total phenolic content (TPC) and total antioxidant capacity of the phenolic compounds (TAC) were determined from lentil seeds treated with NaCl. TPC and TAC parameters followed the same pattern displaying reduced values upon treatment (Fig. 3A, B). The qualitative and quantitative profiles of the phenolic compounds were determined by HPLC (Table 1). HPLC analysis detected phenolic acids (gallic acid and *p*-coumaric acid) and flavonoids (epicatechin, catechin, resveratrol, kaempferol, quercetin and rutin). Among the detected flavonoids, catechin was dominant in all treatments. The content of specific phenolic compounds was decreased by salt treatment (Table 1).

DISCUSSION

Salt stress induces various biochemical and physiological responses in plants and affects almost all plant metabolic processes (Parida and Das, 2005) as alterations in the integrity of cell membranes (Bor et al., 2003), inhibition of different enzymatic activities and photosynthesis (Sairam and Tyagi, 2004; Moradi and Ismail, 2007). The general response of plants to salinity is reduction in growth (Mamo et al., 1996; Ghoulam and Fares, 2001). The data presented in this study indicated that overhead irrigation with 100 mM NaCl did not have any significant effect on plant height and dry weight per plant. The absence of salt effect on lentil growth attributes argues in favor of the salt tolerance of the local Greek lentil cultivar "Dimitra" used in this study. Above all the valuable information obtained in this study is that the lentil cultivar "Dimitra" could be cultivated in environments where the salinity of the soil is a frequent constraint, as previously reported by Sidari et al. (2007) for two lentil cultivars "Ustica" and "Pantelleria" found in a southern Italian semi-arid environment. The salt treatment performed in this study affected cell membrane integrity, recorded by the increased MDA content, which is in the

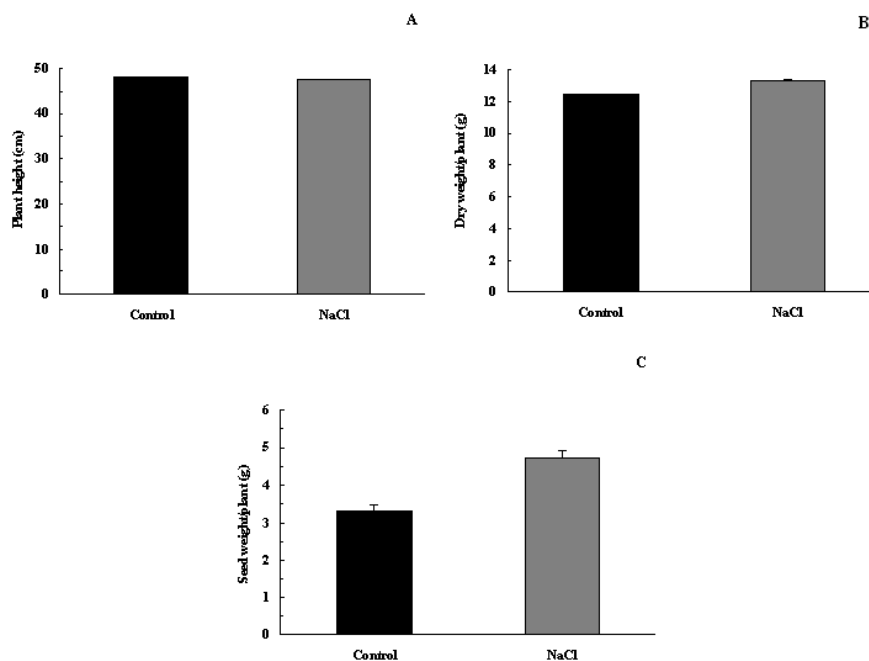


Fig. 1. Effects of overhead irrigation with NaCl (100 mM) on A-plant height (cm), B-dry weight/plant (g), C-seed weight/plant (g) of *Lens culinaris* Medik. plants. Vertical bars indicate S.E. (n=4).

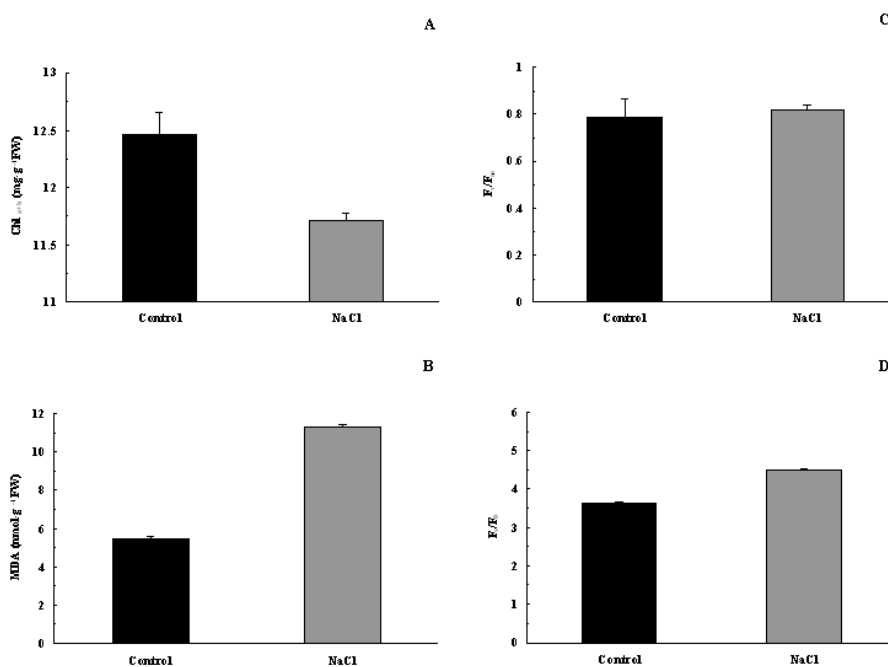


Fig. 2. Effects of overhead irrigation with NaCl (100 mM) on A-chlorophyll content chl a+b (mg·g⁻¹ FW), B-the level of lipid peroxidation determined as MDA content (nmol·g⁻¹ FW), and C, D-chlorophyll fluorescence parameters (F₀ – initial fluorescence, F_v – variable fluorescence, F_m – maximal fluorescence) of *Lens culinaris* Medik. plants. FW-fresh weight. Vertical bars indicate S.E. (n=4).

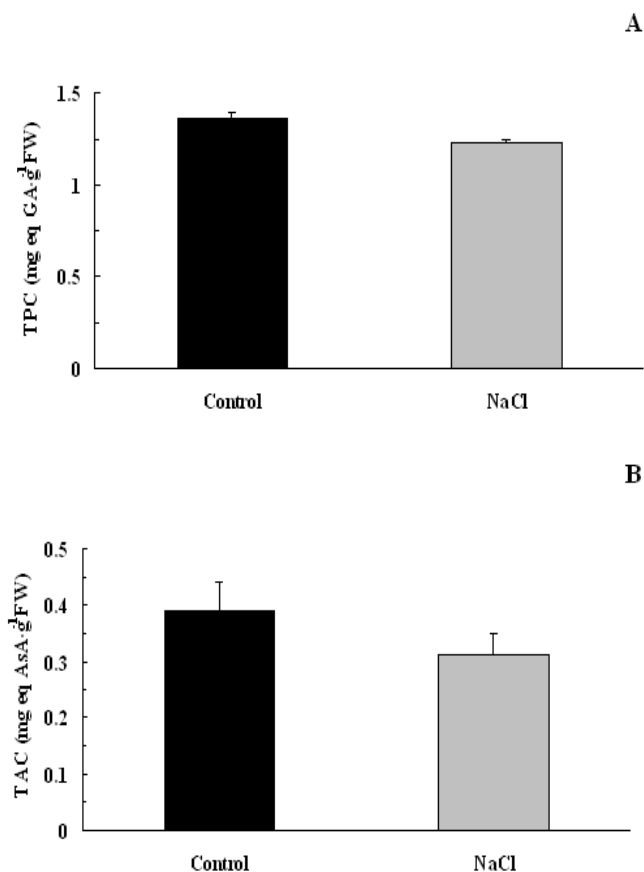


Fig. 3. Effects of overhead irrigation with NaCl (100 mM) on A-total phenolic content (TPC) in mg eq GA-g⁻¹ FW and B-total antioxidative capacity (TAC) in mg eq AsA-g⁻¹ FW in lentil seeds extracts. FW-fresh weight. Vertical bars indicate S.E. (n=4).

line with previously reported data (Özdemir et al., 2004, Jovanović et al., 2011). The chlorophyll content and fluorescence were also affected by salinity, which is in accordance with one group of data (Masojidek and Hall, 1992; Belkhodja et al., 1994), but opposite to other reports (Robinson et al. 1983; Morales et al. 1992). These conflicting data could be explained by “crosstalk” between different environmental stresses such as salt and high temperature (Alexieva et al., 2003). Moreover, the lentil plants screened for salt tolerance in this study were grown under natural field conditions of Mediterranean northern Greece and were probably exposed to different environmental constraints. The interaction between these stresses could be taken as a part of

the adaptive mechanisms of plants to survive under saline conditions and high temperatures.

Oxidative stress induced by salinity is closely associated with a diverse assortment of diseases and with depressed levels of antioxidant molecules, low levels of enzymes which form a part of the antioxidant defense system and increased levels of oxidation products (Fernandez-Orozco et al., 2003). Natural phenolics exert their beneficial health effects mainly through their antioxidant activities (Bravo, 1998; Dueñas et al., 2002, 2003). These compounds are capable of removing free radicals, chelating metal catalysts, activating antioxidant enzymes, and inhibiting oxidases (Heim et al., 2002). The

Table 1. Effects of overhead irrigation with NaCl on concentration of phenolic compounds ($\mu\text{g}\cdot\text{g}^{-1}$ FW) in lentil seeds detected by HPLC. FW-fresh weight. Values are means \pm S.E., $n=4$. Means marked with different letters are significantly differ at $p = 0.05$.

Phenolic compounds	Control	NaCl
Concentration	$\mu\text{g}\cdot\text{g}^{-1}$ FW	
Resveratrol	2.79 \pm 0.06 a	2.71 \pm 0.00 b
<i>p</i> -Coumaric acid	5.93 \pm 0.72 a	4.70 \pm 0.58 a
Kaempferol	0.59 \pm 0.01 a	0.56 \pm 0.01 b
Gallic acid	11.65 \pm 0.68 a	9.02 \pm 0.30 b
Quercetin	1.22 \pm 0.63 a	0.23 \pm 0.00 b
Catechin	66.86 \pm 1.56 a	23.17 \pm 5.18 b
Epicatechin	2.73 \pm 0.24 a	2.64 \pm 0.10 a
Rutin	3.20 \pm 0.10 a	4.33 \pm 0.12 b

contributions of total phenolics to the total TAC was reported previously (Fernandez-Orozco et al. 2003; Amarowicz et al., 2003; Han and Baik, 2008). Recent data (Amarowicz et al., 2009) demonstrated that phenolic acids from red lentil extract participated in TPC by about 20%, which is in line with our results. In our lentil extracts, the correlation coefficient between potent antioxidant capacities and high contents of phenolic substances in salinity treatments was $r^2 = 0.76$ (data not shown). These results indicated that different phenolic substances had different degrees of contribution to the overall antioxidant activities (Amarowicz et al., 2004). In addition, it is becoming increasingly apparent that phenolics can display various biological activities through a number of molecular mechanisms and that not all of these activities could be directly related to the molecular function as antioxidants. The data previously reported indicate that the content of phenolic compounds in plants can be changed by salt stress, which is critically dependent on the salt sensitivity of plants (Kim et al., 2007) and the level of salt stress (Yuan et al., 2010).

In conclusion, overhead irrigation of lentil plants should be conducted with care, keeping in mind the quality of the water used for this purpose. Although the concentration of salt applied in this work was relatively high, the growth of the lentil plants was not impaired by overhead irrigation. The absence of salt

effects on plant growth could be explained by a possible high salt tolerance of the lentil cultivar "Dimitra" grown in this Mediterranean area. Interestingly, seed yield was increased upon salt treatment. The phenolic content of lentil plants and seeds was reduced by overhead irrigation with salt. It is well recognized that saline water use through sprinklers may cause leaf burn due to the accumulation of toxic quantities of salt, which is very important for sensitive crops that exhibit high rates of foliar salt absorption during periods of high evaporative demand (Minhas, 1996). Overhead watering with salt performed in this study did not cause any apparent yellowing of the lentil leaves. These data implicate the use of overhead irrigation by sprinklers rather than by surface for growing this lentil cultivar in areas hit by salinity.

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