Biochemical response of *Olea europaea* cv. Gemlik to short-term salt stress

Mehmet Ali DEMİRAL¹, Deniz AKTAŞ UYGUN², Murat UYGUN², Erkan KASIRĞA¹,
Arife Alev KARAGÖZLER²

¹Department of Soil Science and Plant Nutrition, Faculty of Agriculture, Adnan Menderes University, 09100 Aydın - TURKEY
²Department of Chemistry, Faculty of Science and Arts, Adnan Menderes University, 09010 Aydın - TURKEY

Received: 30.12.2009

Abstract: One-year-old olive (*Olea europaea* L. cv. Gemlik) seedlings were exposed to increasing levels of salinity in pot culture. The experiment was laid out in a randomized block design with 6 replicates. Dry matter (DM) percentage, sodium (Na) and chloride (Cl) concentrations, 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity, reducing power (RP), total phenolic content (TPC), and proline (PRO) content were determined in the leaves of the plants. The results showed that the olive plant used physiological and biochemical mechanisms consecutively to alleviate the effects of salt stress. The type of mechanism used was related to the severity of the salt stress. Salinity-induced variation in DPPH scavenging activity correlated well with the PRO and TPC variations of the leaves. The results showed that DPPH radical scavenging activity may be evaluated as a reliable parameter to assess the ability of antioxidants to hinder salt stress in olive plants.

Key words: Chloride, dry matter, DPPH scavenging activity, reducing power, sodium, total phenolics

*Olea europaea* cv. Gemlik bitkisinin kısa süreli tuz stresine biyokimyasal tepkisi


Anahtar sözcükler: Klor, kuru madde, DPPH süpürme aktivitesi, indirgeme gücü, sodyum, toplam fenoller
Introduction

In the Mediterranean basin, the availability of fresh water is one of the major limitations for crop production. Therefore, the use of non-conventional water resources, such as saline water and reclaimed sewage effluent, has increased in recent years. The utilization of such water resources accelerates the salinization of the upper layer of the soil, where most root activity takes place, and generally decreases crop production (1,2). However, many plant species, such as the olive, in the Mediterranean basin have displayed an improved array of antioxidant defenses to resist salt stress.

Phenolic compounds are natural antioxidants (3) and the levels of these metabolites can be used in the assessment of the antioxidant defense system capacity of plants under stress. For this purpose, biochemical parameters such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and reducing power (RP) assays may be used. DPPH assay is one of the most easy, rapid, sensitive, and reliable ways to evaluate the ability of antioxidants to scavenge free radicals, which are known to be a major factor in the biological damage caused by oxidative stress (4,5). Additionally, RP is one mechanism for the action of antioxidants (6).

The exposure of plants to salinity commonly results in a water deficit in plant cells. According to Tester and Davenport (7), salt-stressed plants need to maintain internal water potential below that of the soil to maintain turgor and water uptake for growth. This requires an adjustment in osmotica, either by the uptake of soil solutes or by the synthesis of metabolically benign (compatible) solutes. This drought component of salinity poses a dilemma for plants: the major cheap solutes in saline soils are Na⁺ and Cl⁻, but these are toxic in the cytosol. Compatible solutes are non-toxic, but are energetically much more expensive. However, the use of compatible solutes in osmotic adjustment provides some important advantages for plants. First of all, these solutes can accumulate to high concentrations without interfering with the plant metabolism (8). Additionally, because of their specific hydrophilic structure they have osmoprotective functions. They can replace water on the surface of proteins, protein complexes and membranes (9).

The olive (Olea europaea L.) plant is considered as a moderately salt tolerant plant (2,10) and grows preferentially in semi-arid areas where irrigation is required to produce maximum yields. In these areas, most of the soil and water resources contain excess amounts of salt that can inhibit growth and reduce yield in the olive. However, previous findings have demonstrated that the response of the olive plant to salt stress varies depending on the specificity of the cultivar (10,11). The objective of this study was to determine the biochemical response of Olea europaea cv. Gemlik to salt stress. The biochemical findings were also corroborated by sodium (Na) and chloride (Cl) concentrations as well as the dry matter (DM) percentage of the leaves.

Materials and methods

Plant material and salt treatments

The Gemlik olive (Olea europaea L.) cultivar was used in the experiment. One-year-old, homogeneous, self-rooted plants produced through a mist-propagation system were planted in 18 L containers with a soil/coarse sand mixture (1/1.5, w/w). The containers were covered with aluminum foil to prevent evaporation that could cause the accumulation of salt on the surface. The experiment was conducted using irrigation water with 3 different salinity levels (4.0 dS m⁻¹, 8.0 dS m⁻¹, and 12.0 dS m⁻¹) (10). The salinity levels were adjusted by the addition of appropriate amounts of NaCl to half-strength Hoagland’s solution (12). Half-strength Hoagland’s solution was used as the control treatment. The experiment was laid out in a randomized block design with 6 replicates and 1 plant per pot. The seedlings were grown for 1 month using half-strength Hoagland’s solution before the application of saline solutions to the plants commenced. The salt concentration of the applied solutions was increased gradually in order to prevent any possible shock effect of salinity on the experimental plants. The experiment was carried out for 4 months after planting.

Chemical analysis

For chemical analysis, all the leaves of each plant were collected, weighed, and dried in a forced-air oven (Memmert UM 500, Schwabach, Germany) at 70 °C for 72 h. Dry samples were weighed and the DM percentage was calculated by using the fresh weights of the samples. The samples were then prepared for
analysis by grinding in a stainless steel mill (IKA A 11 Basic, Staufen, Germany). The ground samples were wet digested in a mixture of nitric/perchloric acid (HNO₃/HClO₄) (4/1, v/v) solution. The sodium contents in the digest were determined using flame photometry (Jenway PFP7, Staffordshire, UK) (13). Chloride was extracted from 0.1 g of ground sample with 10 ml of deionized water by shaking the mixture for 2 h. The chloride concentrations of the extracts were measured by chloridimeter (Jenway PCLM3, Staffordshire, UK) (14).

**Biochemical analysis**

**Proline analysis**

Proline (PRO) analysis was performed on dried leaf samples according to Bates (15). In brief, a 0.5 g ground leaf sample was homogenized in 10 mL of sulphasalicylic acid (3%, w/v) and filtered through filter paper. After this, 2 mL of the filtrate was mixed with 2 mL of acid ninhydrin solution (ninhydrin/glacial acetic acid/6 M H₃PO₄) (1.25/30/20, w/v/v) and 2 mL of glacial acetic acid and kept at 100 °C for 1 h. The reaction was stopped by transferring the mixture to an ice bath, and 4 mL of toluene was added to the mixture and it was vortexed for 15-20 s. The toluene phase was aspirated and absorbance at 520 nm was measured in a Shimadzu UV-1600A model spectrophotometer using pure toluene as a blank. A calibration curve was prepared with pure PRO. Results were expressed as μM PRO g⁻¹ DM.

**Preparation of plant extracts**

Two solvents (methanol and water) with distinct polarity (dielectric constants 33.0 and 78.5, respectively) were employed for the preparation of plant extracts. In brief, 0.25 g of ground leaf was mixed with 5 mL of solvent and the mixture was shaken for 2 h in a shaker (Heidolph Promax 2020, Schwabach, Germany). The filtrate was then used for analysis immediately.

**Determination of total phenolic content**

The total phenolic content of the extracts was assayed according to the Folin-Ciocalteu method (16). In brief, 300 μL of the filtrate was added to 45.9 mL of water in a 100 mL flask. Then 1 mL of undiluted Folin-Ciocalteu Reagent (FCR) was added to the mixture. After 3 min, 3 mL of Na₂CO₃ (2%, w/v) solution was added to each flask. The flasks were shaken (Heidolph Promax 2020, Schwabach, Germany) at 150 rpm for 2 h in the dark at room temperature. Absorption at 760 nm was measured in a Shimadzu UV-1600A model spectrophotometer. Gallic acid was employed as a standard and the results were expressed as mg gallic acid equivalents (GAE) g⁻¹ extract.

**Determination of reducing power**

The reducing power of the extracts was measured according to the method of Oyaizu (17), and 1 mL of the filtrate was mixed with 2.5 mL of phosphate buffer (0.2 M; pH 6.6) and 2.5 mL of K₃Fe(CN)₆ (1%, w/v). The mixture was incubated at 50 °C for 20 min and 2.5 mL of TCA (10%, w/v) was added. After centrifugation for 10 min at 1000 × g (Universal 32 R, Hettich, Tuttlingen, Germany), 2.5 mL of the supernatant was added to the tubes containing 2.5 mL of distilled water and 0.5 mL of FeCl₃.6H₂O (1%, w/v). The absorbance of resulting solution was measured at 700 nm using water as blank. A control was also prepared replacing water with plant extract. Ascorbic acid, which possesses relatively strong RP, was used as standard and RP was expressed as % ascorbic acid.

**DPPH radical scavenging assay**

The radical scavenging activity of the plant extracts against the DPPH radical was determined spectrophotometrically according to Brand-Williams et al. (18). The principle of the assay is based on the color change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant. In brief, 1 mL of 0.1 mM DPPH in methanol or water was mixed with 3 mL of extract solution at differing concentrations (5-250 μg mL⁻¹) and the mixture was vortexed. The samples were kept in the dark for 30 min at room temperature and then the decrease in absorbance at 517 nm was measured. Absorbance of DPPH solution in the absence of plant extract was measured as a control. DPPH radical scavenging activity was expressed using the formula: % DPPH radical scavenging activity = \[(A₀ - A₁)/A₀\] × 100, where A₀ was the absorbance of the control and A₁ was the absorbance of the sample. DPPH scavenging activity is best presented by IC₅₀ value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Therefore, extract concentrations providing 50% inhibition (IC₅₀) were calculated using the data.
Biochemical response of *Olea europaea* cv. Gemlik to short-term salt stress

**Statistical analysis**

Regression analyses were carried out according to Little and Hills (19) for the investigation of the relationships between salinity and the experimental data.

**Results and discussion**

**Dry matter accumulation**

One of the important approaches to assess the effects of salinity on plants is to measure growth parameters such as DM accumulation. The effects of salinity on leaf DM accumulation are given in Figure 1 and Table 1. As seen in Figure 1, salinity increased the leaf DM accumulation of the cultivar. The elevation of DM content started from 4 dS m\(^{-1}\) salinity.

**Sodium and chloride concentrations**

The effect of salinity on the Na and Cl content of the leaves is given in Figure 2 and Table 1. Salinity increased the Na content of the leaves significantly. The sodium accumulated by the plants was proportional to the applied salt concentrations. Chloride accumulation in the leaves increased up to 8 dS m\(^{-1}\) before a slight decrease was observed.

**Proline, total phenolic content, reducing power, and DPPH scavenging activity**

The effects of salinity on the PRO, TPC, DDPH scavenging activity, and RP of the leaves are given in Figures 3-6 and Table 2. Salinity increased the PRO content of the plants up to 8 dS m\(^{-1}\) before a decline was observed (Figure 3).

---

**Figure 1.** Variation in leaf DM accumulation of *Olea europaea* cv. Gemlik under salt stress.

**Figure 2.** Variation in leaf Na and Cl concentrations of *Olea europaea* cv. Gemlik under salt stress.

**Table 1.** Relationships between salinity and DM, Na, and Cl contents of *Olea europaea* cv. Gemlik leaves.

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Dependent variable</th>
<th>Regression equation</th>
<th>Determination coefficient ((R^2))</th>
<th>Correlation coefficient ((r))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity</td>
<td>DM (%)</td>
<td>(Y = 0.009X^2 - 0.007X + 41.67)</td>
<td>98.1</td>
<td>0.990**</td>
</tr>
<tr>
<td></td>
<td>Na (%)</td>
<td>(Y = 0.031X + 0.03)</td>
<td>95.4</td>
<td>0.976**</td>
</tr>
<tr>
<td></td>
<td>Cl (%)</td>
<td>(Y = -0.002X^2 + 0.062X + 0.175)</td>
<td>95.2</td>
<td>-0.975**</td>
</tr>
</tbody>
</table>

*, ** Significant at \(P \leq 0.05\) and 0.01, respectively

436
The TPC of water extracts decreased with salinity, whereas the TPC of methanol extracts decreased up to 8 dS m\(^{-1}\) salinity and then increased (Figure 4). The DPPH scavenging activities of water and methanolic extracts were increased up to 8 dS m\(^{-1}\) salinity. However, the response of water and methanol extracts to salinity after 8 dS m\(^{-1}\) was not similar (Figure 5).

Water extract exhibited higher DPPH scavenging activity than that of methanolic extract. The RP of both extracts decreased with salinity (Figure 6).

The relationships between antioxidant components and antioxidant parameters are presented in Table 3. Although salinity-induced PRO content showed a positive correlation with DPPH scavenging activity,
Biochemical response of *Olea europaea* cv. Gemlik to short-term salt stress

The correlation with RP was negative. TPC exhibited a positive correlation with all antioxidant properties measured in the study.

The DM accumulation of the leaves showed a positive and significant correlation with salinity (Table 1). It could be said that this might be accomplished by adjusting the osmotic potentials of the cells in the leaves through the accumulation of Na, Cl and PRO (Figures 2 and 3). The exposure of plants to salinity commonly results in a water deficit in plant cells, and maintaining osmotic homeostasis requires an adjustment in osmotica, either by the uptake of soil solutes or by the synthesis of metabolically compatible compounds (7). According to Verbruggen et al. (20), a neglected aspect in the PRO metabolism concerns its importance during the stress relief phase. Therefore, it may be concluded that Na, Cl, and PRO contributed significantly to the osmotic adjustment in the leaf cells up to the 4 dS m⁻¹ salinity level. Since the DM accumulation of the leaves remained constant up to 4 dS m⁻¹, this phase

---

**Table 3. Relationships between antioxidant components (PRO, TPC) and antioxidant properties (RP, DPPH scavenging activity) of *Olea europaea* cv. Gemlik leaves.**

<table>
<thead>
<tr>
<th>Antioxidant components</th>
<th>Antioxidant properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RP (%)</td>
</tr>
<tr>
<td></td>
<td>Aqueous extract</td>
</tr>
<tr>
<td>PRO (μM g⁻¹ DW)</td>
<td>−0.911**</td>
</tr>
<tr>
<td>TPC (mg GAE g⁻¹ DW)</td>
<td></td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>0.986**</td>
</tr>
<tr>
<td>Methanolic extract</td>
<td>--</td>
</tr>
</tbody>
</table>

*, ** Significant at P ≤ 0.05 and 0.01, respectively. ns: non-significant
can be accepted as the “stress relief phase” for the cultivar. It is known that the olive plant is moderately salt tolerant (2,10) and yield reduction is about 10% when the electrical conductivity of soil solution is 4-6 dS m\(^{-1}\). This value can be as high as 6-8 dS m\(^{-1}\) with soils containing high calcium levels (21).

However, as depicted in Figure 1, leaf DM accumulation increased sharply after the 4 dS m\(^{-1}\) salinity level. It is probable that the plants limited the uptake and translocation of water containing high amounts of dissolved salts to protect their photosynthetic organs. It is known that *Olea europaea* L. cultivars have an effective salt-exclusion mechanism operating in their thin roots. The control mechanism includes limiting salt translocation to the leaves rather than salt absorption by the roots (10). Additionally, the process is also supported by some other mechanisms such as stomata closure (22) and leaf tolerance to dehydration (23). It is most probable that the increase in DM content is a consequence of the decrease in the water content of the leaves. Similar results were also reported by researchers in an oilseed plant (*Matthiola tricuspidata*) (24), and in various olive (*Olea europaea* L.) (25) and barley (*Hordeum vulgare* L.) cultivars (26).

PRO content was increased significantly in relation to the severity of salt stress up to 8 dS m\(^{-1}\) salinity, and then a reduction was observed (Figure 3). It seems that the test plants used PRO synthesis to alleviate the effects of osmotic stress induced by salinity. This result was confirmed by earlier findings suggesting that PRO accumulation is related to the efficiency of the salt tolerance mechanism of the olive tree (27). It is probable that the length and rank of the synthesis process were related to the salt tolerance level and biochemical strategy of the cultivar. Therefore, PRO accumulation was reduced rapidly after 8 dS m\(^{-1}\) salinity, where the existing salinity tolerance mechanisms of the cultivar were hampered.

There are some inconsistent results about the probable reasons of PRO accumulation in plants under stress. According to Ramajulu and Sudhakar (28) PRO accumulation is one of the results of the adaptation of plants to salinity. Some authors did not observe any appreciable increase in PRO content (29,30), while others consider an enhanced PRO level merely as a stress effect (31). It has been reported that PRO accumulation appears to be a reaction to salt-stress damage (32) or a symptom of salt-susceptibility (33) and not a plant response associated with salt tolerance. Based on the results of this study, PRO accumulation can be used as a biochemical marker for elementary salt stress in *Olea europaea*.

The total phenolic content of the leaves exhibited significant correlations with salinity (Table 2). In this study, samples were extracted using two different solvents, i.e. water and methanol. Water was used to extract hydrophilic phenolics, whereas methanol was used to extract slightly hydrophobic phenolics. The TPC of the water extracts was decreased by the increased salinity. However, the TPC of the methanolic extracts decreased up to 8 dS m\(^{-1}\) and then increased (Figure 4). Additionally, the cultivar had relatively high TPC compared to other plants. Agastian et al. (34) reported that the TPC of different mulberry genotypes varied between 5.7 mg and 13.20 mg GAE g\(^{-1}\) under different salinity levels. Hanen et al. (35) reported a level of about 3.5 mg GAE g\(^{-1}\) DM in a medicinal plant, *Cynara Cardunculus* L., and Navarro et al. (36) found about 5 mg p-coumaric acid g\(^{-1}\) DM in pepper grown under salt stress.

Many researchers stated that the synthesis and accumulation of phenolic compounds is generally stimulated in response to biotic and abiotic stresses in plants (37,38). However, different findings have also been published by other researchers. For example, according to Ksouri et al. (39) salinity reduced the TPC of *Cakile maritima* (cv. Tabarka) leaves. The authors stated that relationship between salinity and the TPC of the leaves is possibly related to the salinity tolerance level of the plants. As reported by Agastian et al. (34), the TPC and PRO contents of different mulberry genotypes increased at low salinity levels (1-2 dS m\(^{-1}\)) and decreased at high salinity levels (8-12 dS m\(^{-1}\)). The results of the present study correlate well with the aforementioned reports.

DPPH scavenging activity and RP presented significant but various correlations with salinity (Table 2). It is known that the ranking of antioxidant activity is strongly dependent on the test system (40).
The results showed that DPPH scavenging activity expressed as IC$_{50}$ was greatly affected by salinity. IC$_{50}$ values obtained with 2 different extraction solvents (i.e. water and methanol) increased with the salinity level up to 8 dS m$^{-1}$. The DPPH scavenging activities of water extracts decreased at higher salinity levels. However, the DPPH scavenging activities of methanolic extracts stayed more or less constant after 8 dS m$^{-1}$ salinity. Water extract exhibited a more efficient DPPH scavenging activity than did the methanolic extract (Figures 5). On the other hand, the decrease in DPPH IC$_{50}$ values correlates well with the increase in TPC extracted with methanol (Figures 4 and 5, Table 3). It is known that a lower IC$_{50}$ value reflects improved DPPH radical scavenging activity (41). Phenolic compounds belong to the category of natural antioxidants and their abundance is positively and directly related to the antioxidant capacity of plants (3).

Increased salinity led to a general decrease in RP, and methanolic extracts demonstrated higher RP values than those of water extracts (Figures 6). Probably, in the initiation of the salt treatment the plants’ primary metabolite production mechanisms were damaged and the production of metabolites responsible for the reducing mechanism was decelerated. It may be speculated that at this point the plants’ defense mechanism took over and produced secondary metabolites, i.e. phenolic compounds (Figure 4) (42).

In conclusion, the results of this study demonstrate that the olive plant used physiological and biochemical mechanisms consecutively to alleviate the effects of salt stress. The types of mechanisms used were related to the severity of salt stress. It is probable that Na, Cl, and PRO contributed significantly to osmotic adjustment in the leaf cells at the 4 dS m$^{-1}$ salinity level. However, at higher salinity levels the cultivar decreased the Na and Cl concentrations of the leaves. It also supported this process by higher PRO synthesis. This phase continued up to the 8 dS m$^{-1}$ salinity level and then increased TPC synthesis was replaced by decreased PRO synthesis. Salinity-induced variation in DPPH scavenging activity correlated well with the PRO and TPC variations of the leaves and was evaluated as a reliable parameter to assess the ability of antioxidants to hinder salt stress in the olive plant. The results obtained in this investigation may be significant for a more complete understanding of the behavior of *Olea europaea* L. under salt stress. In addition, since the cultivar is widely planted in semi-arid areas, these findings may be valuable for practical applications.

**Acknowledgment**

This work was partly supported by the Adnan Menderes University Scientific Research Foundation, Project No. FBE-08019.

**Corresponding author:**

**Mehmet Ali DEMIRAL**  
*Department of Soil Science and Plant Nutrition, Faculty of Agriculture, Adnan Menderes University, 09100 Aydın - TURKEY*  
*E-mail: mademiral@yahoo.com*

**References**


Biochemical response of *Olea europaea* cv. Gemlik to short-term salt stress