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Determination of some physiological parameters in spinach (*Spinacia oleracea* L.) cultivated in Kosovo

Beobachtungen einiger physiologischer Parameter bei Spinat (*Spinacia oleracea* L.) angebaut im Kosova

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Summary

The objective of this work was to investigate the levels of photosynthetic pigments, antioxidant enzyme activities (superoxide dismutase [SOD], catalase [CAT], peroxidase [POX], ascorbate peroxidase [APX], glutathione reductase [GR], and MDA), total glutathione (GSH), total ascorbate (AsA), and lipid peroxidation of two spinach cultivars (Clipper and Matador) sampled in different locations in Kosovo (Prizren, Ferizaj, and Obiliq). The photosynthetic pigments, chlorophyll *a* and *b* as well as carotenoids, were extracted by 80% acetone, and concentrations were calculated based on the absorbance values at 663, 645, and 480 nm. There was a significant difference in SOD, APX, and GR activities between varieties at each location. The MDA and AsA contents in Obiliq were the highest, compared to other locations. From the increase in lipid peroxidation level, it can be assumed that the degradation in pigment content was caused by the decrease in total AsA content and APX and GR enzyme activities, which are the main enzymes of the AsA–GSH cycle in chloroplasts.

Keywords: Spinach, Lipid peroxidation, Antioxidant Enzyme Activities, Pigment Content

Zusammenfassung

Ziel der vorliegenden Untersuchung war es, den Einfluss von zwei Spinat-Genotypen (Clipper und Matador), angebaut an unterschiedlichen Standorten in Kosovo (Prizren, Ferizaj, Obiliq), auf die Gehalte an Photosynthesepigmenten, die Aktivität antioxidativer Enzyme (SOD, CAT, POX, APX, GR und MDA), Gesamt-Glutathion (GSH), Gesamt-Ascorbat (AsA) und Fett-Peroxidierung festzustellen. Die Photosynthesepigmente Chlorophyll a und b sowie Carotinoide wurden mit 80-%igem Aceton extrahiert, und die Konzentrationen wurden errechnet auf Basis der Absorption bei 663, 645 und 480 nm. Es gab nachweisbare Unterschiede zwischen den Sorten an allen Orten hinsichtlich der Aktivitäten von SOD, APX and GR. Die Gehalte an MDA und AsA waren am Standort Obiliq am höchsten verglichen mit den übrigen Standorten. Ein Anstieg der Fett-Peroxidierung lässt vermuten, dass der Abbau der Pigmentgehalte durch einen Abfall im Gesamt Asa und in den Aktivitäten von APX und GR verursacht wurde. Letztere sind die Hauptenzyme des AsA-GSH-Zyklus in Chloroplasten.

Schlagwörter: Spinat, Lipidperoxidation, Antioxidationsenzym-Aktivitäten, Pigmentgehalt

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1. Introduction

Researchers have given special importance to the presence of antioxidants in plants due to the increasing interest among consumers to improve the way of nutrition and prevent various chronic diseases (Moore et al., 2006). Spinach (*Spinacia oleracea* L.) is a leaf-producing vegetable and belongs to the Amaranthaceae family (Hassler, 2018). Spinach is consumed worldwide (Morelock and Correll, 2008). In 2016, the production of spinach in the world was 26.7 million tons (FAOSTAT, 2018), while in Kosovo, the production was 1348 tons in 2018 (KAS, 2018). Spinach is known for its low content of fats and calories and has a high content of vitamins, minerals, and fiber (Alvino and Barbieri, 2016). Spinach is also characterized by high antioxidant activity (Ismail et al., 2004; Ou et al., 2002). Various biotic and abiotic stresses inhibit plant growth and development, as well as reduce its yield and quality (Apel and Hirt, 2004). Photosynthetic pigments in plants, proline, glycine, nitrogen compounds such as proteins, sugars, phenolic compounds, flavonoids, ß-carotene and lycopene, and enzymatic antioxidants such as superoxide dismutase (SOD) degrade under environmental stress conditions (Koca et al., 2007). This is due to the excessive accumulation of reactive oxygen species (ROS) containing single oxygen, superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (Savvides et al., 2016). ROS molecules are highly reactive and cytotoxic, and they can damage DNA molecules, RNA, proteins, carbohydrates, lipids, and can result in cell death. The plant possesses enzymatic mechanisms which enable cleansing of the body from ROS. These enzymes include SOD, peroxidase (POX), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX), and ascorbate peroxidase (APX). SOD acts as the first line of defense against ROS, dismutating superoxide anion radical to H_2O_2. APX, GPX, POX, and CAT, subsequently decomposing H_2O_2 (Apel and Hirt, 2004). APX is a key component of the ascorbate glutathione cycle in chloroplasts. It also plays a role in ROS scavenging in cytosol, mitochondria, and peroxisomes (Miller et al., 2010). The POXs play a role in physiological processes such as auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, defense against pathogens, and cell elongation (Passardi, 2005). Likewise, POXs and CATs play an important role in the fine regulation of ROS concentration in the cell through moderation of H_2O_2 deactivation. The balance of SOD, POX, and CAT activities is crucial for suppressing toxic ROS levels in the cell (Apel and Hirt, 2004). SOD also contributes to the formation of H_2O_2 and dismutation of O_2. APX and GR are involved in the ascorbate (AsA)–glutamate (Glu) cycle that occurs in chloroplasts and are responsible for converting H_2O_2 to water (Apel and Hirt, 2004). The main function of POXs and CATs in peroxisomes is to remove H_2O_2 (Miller et al., 2010). In this paper, our research had two main targets, which are to investigate (i) the influence of different locations on the content of photosynthetic pigments (Chl *a*, Chl *b*, Chl total) and (ii) the influence of different locations on the activity of enzymatic antioxidants (SOD, APX, CAT, POX, GR), total glutathione (GSH), AsA, and the level of lipid peroxidation in two different cultivars of spinach.

2. Materials and methods

2.1. Experimental design

The research involved three locations of Kosovo: Ferizaj (longitude: 21°09'39", latitude: 42°22'15" at the elevation 580 m), Obiliq (longitude: 21°03'88", latitude: 42°41'31" at the elevation 540 m), and Prizren (longitude: 20°43'59", latitude: 42°16'49" at the elevation 356 m), and two spinach cultivars were studied with origin from the Netherlands: Clipper and Matador. The Prizren locality belongs to the Dukagjini region, which is characterized by a modified climatic condition due to the influence of Adriatic Sea through river Drini i bardhe (altitudes are from 350 to 450 m). The summer temperatures in this region sometimes reach 35°C–40°C. The localities of Ferizaj and Obiliq belong to the region of Kosovo (altitudes from 510 to 590 m), which is characterized by an average rainfall of 613.3 mm per year and the mean annual temperature is about 10.27°C (HMIK, 2020). Summer temperatures in this region sometimes exceed 35°C, resulting in high evapotranspiration. The experiment was planned according to the random split plot method with three replications in three locations. The general formula was as follows: three locations × two fields per location = six fields × three replications = 18 plots. Each plot had a size of 1 × 1 m^2. The basic fertilization with N:P:K (15:15:15) was applied before sowing of the spinach. The quantities of fertilizers were N (40 kg ha^{-1} or 4 g^2), P (60 kg ha^{-1} or 6 g^2), and K (60 kg ha^{-1} or 6 g^2). During the vegetation period, the Nitrogen (N) was applied as urea fertilizer (46% N) on quantity 30 kg ha^{-1} or 3 g^2) during the three to four leaves phase of the plants. The sowing of spinach seed on the experimental field was done in May 2018, and plants were harvested 120 days after sowing (August). Irrigation was done after the application of the fertilizer with Urea

46% (Nitrogen a white crystalline), irrigation treatment was done only once during the vegetation. The middle section of leaves was selected for analyses. The collection of the samples of leaves was done in the morning (about 10 o'clock) for spinach cultivars at all localities.

Soil and biochemical analyses were carried out in Plant Stress Physiology and Soil Laboratory of the Department of Agricultural Biotechnology at Tekirdağ Namik Kemal University (Turkey). The sensitive samples were transported in boxes cooled by dry ice. Texture analysis of the soil samples used in the experiment was made according to Tuncay (1994). The type of soil at the three locations was alluvium, but with some differences regarding lime content, pH, organic matter (OM), and texture (Table 1).

2.2. Determination of pigment contents

Plant samples (0.5 g leaf) were homogenized in 80% acetone at 4°C. The homogenate was filtered, and the supernatant was used for the analysis of pigments. Then, the supernatant was measured spectrophotometrically at 480, 645, and 663 nm. Pigment contents were calculated in mg g^{-1} leaf fresh weight (FW) by applying the absorption coefficient equations described by Lichtenthaler (1986) and Aliu et al. (2013, 2018).

Chl *a* = (12.7 × A663 – 2.69 × A645) × 10/mg FW

Chl *b* = (22.9 × A645 – 4.68 × A663) × 10/mg FW

Total Chl = (20.2 × A645 + 8.02 × A663) × 10/mg FW

Carotenoid = (A480 + A663 × 0.114 – A645 × 0.638)/112.5 × 10/mg FW

2.3. Determination of lipid peroxidation

Leaf samples (0.5 g) were placed in liquid nitrogen and homogenized in 2.5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 5 min at 4°C. After centrifugation, 1 mL of supernatant was mixed with 4 mL of 20% TCA. The solution contained 0.5% thiobarbituric acid. This mixture was heated at 95°C for 30 min and then immediately cooled in an ice bath. After cooling, it was centrifuged at 1000 rpm for 15 min at 4°C. The absorbance of the supernatant was measured at 532 and 600 nm. Thiobarbituric acid reactive substances (TBARS) were determined by its extinction using a coefficient of 155 mM^{-1}.

2.4. Determination of antioxidant enzyme activities

The leaf total protein content of the enzyme extract was assayed by Bradford method using BSA (Bovine serum albumin) as a standard (Bradford, 1976). The SOD activity was measured as the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich, 1971; Giannipolities and Ries, 1977). One unit of SOD was defined as the amount of enzyme that inhibits 50% of NBT photoreduction. The POX activity was measured according to Kanner and Kinsella (1983). The reaction mixture contained 50 mM Na-acetate buffer (pH 6.5), 90 mM H_2O_2, 100 mM pyrogallol, and 0.01 mL enzyme extract in a final assay volume of 1 mL. The POX activity was measured spectrophotometrically at 300 nm. The APX activity was measured according to Nakano and Asada (1981). The reaction mixture contained 0.05 M Na-phosphate buffer (pH 7), 0.5 mM ascorbate, 0.1 mM EDTA. Na_2 (Ethylenediaminetetraacetic acid), 1.2 mM H_2O_2, and 0.1 mL enzyme extract in a final assay volume of 1 mL. One unit of APX was defined as 1 mmol mL^{-1} ascorbate oxidized per minute. The GR activity was measured according to Foyer and Halliwell (1976). The reaction mixture contained 0.025 mM Na-phosphate buffer (pH 7.8), 0.5 mM GSSG (Glutathione disulfide), 0.12 mM NADPH Na_4, and 0.1 mL enzyme extract in a final assay volume of 1 mL. The CAT activity was measured by finding the initial rate of disappearance of H_2O_2 (Bergmeyer, 1970). The reaction mixture contained 3% H_2O_2 and 0.1 mM EDTA in 0.05 M Na-phosphate buffer (pH 7) and 70 µL enzyme extract in a final assay volume of 1 mL. The decrease in H_2O_2 was measured as a decline in optical density at 240 nm, and the activity was calculated as µmol H_2O_2 consumed per minute.

Table 1. Soil characteristics at different locations in Kosovo
Tabelle 1. Bodenmerkmale verschiedener Standorte im Kosovo

Location	Lime, %	pH	EC *× 10^6	OM*, %	Clay, %	Silt, %	Sand, %
Ferizaj	0.4	7.4	296	2.74	33.4	32.6	34.0
Prizren	1.8	5.9	186	2.56	22.7	39.4	37.9
Obiliq	0.4	6.8	432	1.82	35.5	37.9	27.3

*OM; organic matter, EC; Soil electrical Conductivity.

2.5. Determination of GSH and AsA levels

GSH amounts of plants were determined according to Queval and Noctor (2007). The total AsA content was determined by calculating the change in A_{265nm} as a result of addition of ascorbate oxidase according to the method of Foyer et al. (1983).

2.6. Statistical analyses

All parameters in the study were analyzed by analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) program (version 19). The Duncan multiple range test was used to compare treatment means.

3. Results

The results for SOD and GR activities showed that there was a statistically significant difference between the varieties at each location (Ferizaj, Prizren, and Obiliq). The highest SOD activity was found in Clipper cultivar grown in Prizren (382.60 U mg^{-1} protein), and the highest GR (225.60 U mg^{-1} protein) activity was found in Matador cultivar grown in the locality of Obiliq. The highest POX (3.92 U mg^{-1} protein) activity was found in Matador and Clipper (5.75 U mg^{-1} protein) cultivars in Obiliq, while the highest CAT activity was found in Clipper cultivar grown in Prizren locality. Results related to parameters are presented in Table 2.

The APX activities of the Matador cultivar grown in Obiliq and the Clipper cultivar grown in Prizren were significantly higher than in Ferizaj, respectively. The APX activity in Matador cultivar ranged between 1019.36 and 4673.81 U mg^{-1} protein and in Clipper cultivar ranged from 156.43 to 3095.50 U mg^{-1}protein. The CAT activity in our research gave different results; in Matador cultivar, it ranged between 1.16 and 4.27 mg g^{-1} protein and in Clipper, it was from 3.45 to 10.94 U mg^{-1} protein. The differences in CAT activities between cultivars were statistically significant at a level of probability of 0.05. The SOD activity in Matador cultivar was between 61.80 and 126.67 U mg^{-1} protein, while in Clipper, it ranged between 88.62 and 382.60 U mg^{-1} protein. The POX activity in Matador cultivar was between 1.25 and 3.92 U mg^{-1} protein, and in Clipper cultivar, it was from 0.58 to 5.75 U

Table 2. Antioxidant enzyme activities (SOD, APX, CAT, POX, and GR) in U mg^{-1} protein and the contents of MDA (µmol g FW^{-1}), GSH (µmol g^{-1} FW), and tot AsA (µmol g^{-1} FW) in spinach varieties collected from different locations
Tabelle 2. Antioxidative Enzymaktivitäten (SOD, APX, CAT, POX, and GR (U mg^{-1} Protein)) und Gehalte an MDA (µmol g FW^{-1}), GSH (µmol g^{-1} FW) und Gesamt-AsA (µmol g^{-1} FW) in Spinat-Genotypen von verschiedenen Standorten.

Enzyme	Genotype	Ferizaj	Prizren	Obiliq
SOD	Matador	126.67^{c*}	99.53^{d}	61.80^{e}
	Clipper	88.62^{d}	382.60^{a}	167.24^{b}
APX	Matador	1200.20^{c}	1019.36^{c}	4673.81^{a}
	Clipper	1094.41^{c}	3095.50^{b}	156.43^{d}
CAT	Matador	4.27^{b}	2.67^{d}	1.16^{e}
	Clipper	4.48^{b}	10.94^{a}	3.45^{c}
POX	Matador	$1.25^{c,d}$	2.04^{c}	3.92^{b}
	Clipper	$1.27^{c,d}$	0.58^{d}	5.75^{a}
GR	Matador	197.54^{b}	165.67^{c}	225.60^{a}
	Clipper	65.90^{e}	135.58^{d}	52.25^{c}
MDA	Matador	28.89^{b}	3.62^{d}	2.94^{d}
	Clipper	13.69^{c}	10.73^{c}	38.70^{a}
GSH	Matador	$1.04^{a,b}$	$1.79^{b,c,d}$	0.44^{a}
	Clipper	1.29^{bc}	2.12^{c}	$1.91^{c,d}$
Tot AsA	Matador	$9.29^{a,b}$	$10.76^{a,b}$	11.85^{a}
	Clipper	$9.50^{a,b}$	9.95^{b}	7.47^{b}

* Means with different superscript letters within a column are significantly different at 005 probability level.
FW, fresh weight; SOD, superoxide dismutase; CAT, catalase; AsA, ascorbate; GSH, glutathione; GR, glutathione reductase; MDA; Methylenedioxyamphetamine, POX, peroxidase; APX, ascorbate peroxidase.

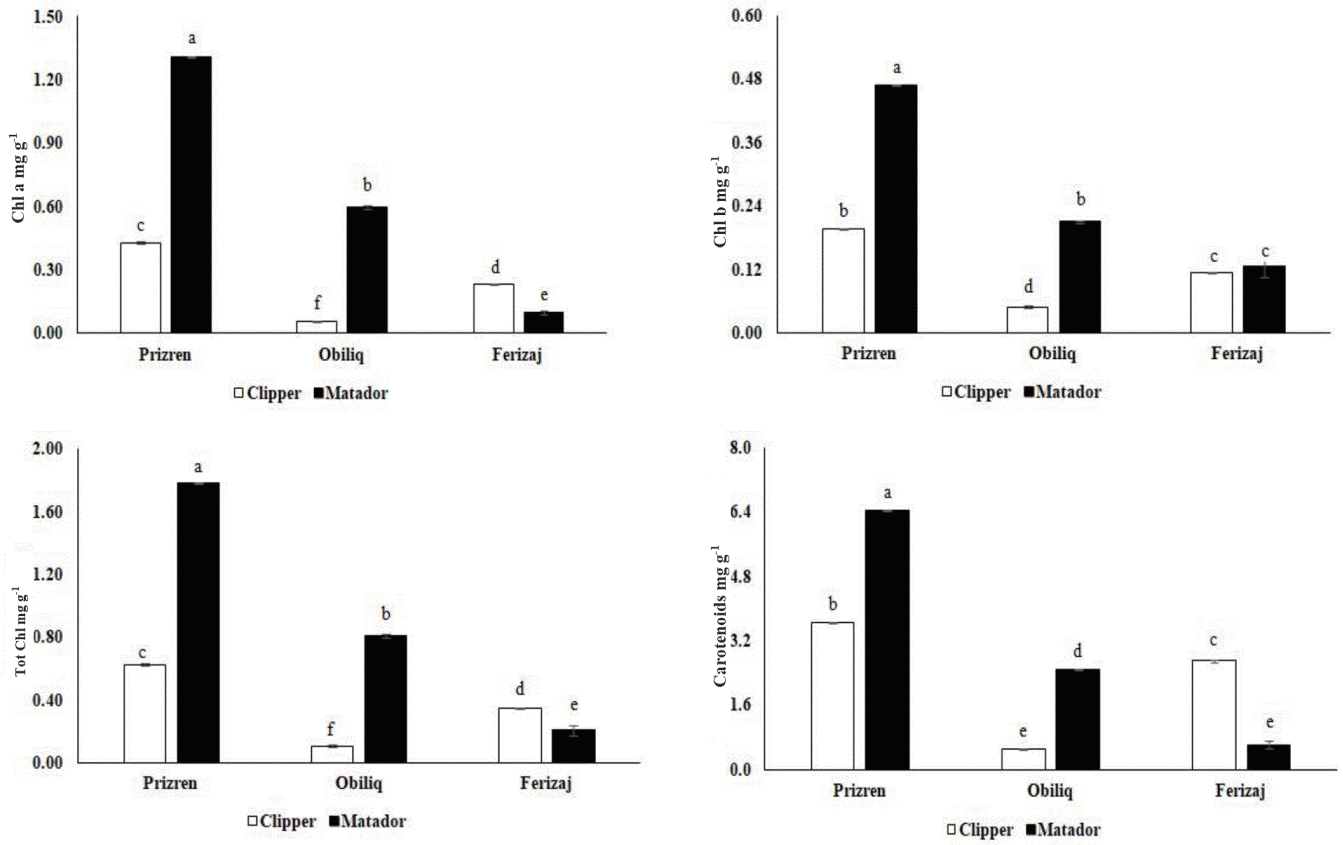

Figure 1. Pigment contents of spinach (Chl *a*, Chl *b*, tot Chl *a* + *b*, carotenoids; mg g^{-1}) in different locations and cultivars. Different letters (a–f) indicate significant differences.
Abbildung 1. Pigmentgehalte von Spinat (Chl *a*, Chl *b*, tot Chl *a*+*b*, Carotenoids; mg g^{-1}) in Abhängigkeit von Standort und Sorte. Unterschiedliche Buchstaben (a-f) zeigen nachweisbare Mittelwertdifferenzen.

mg^{-1} protein. The GR activity ranged between 165.67 and 225.60 U mg^{-1} protein in Matador cultivar and from 52.25 to 135.58 U mg^{-1} protein in Clipper. Also, the MDA activity of spinach was between 2.94 and 28.89 mol g^{-1} FW in Matador cultivar and between 10.73 and 38.70 mol g^{-1} FW in Clipper cultivar. The highest value of MDA, the final product of lipid peroxidation, was in Clipper variety cultivated in Obiliq. No significant difference was found in the GSH content of Clipper cultivar at different locations, but there were significant differences in Matador cultivar between Obiliq and Prizren locations. The total GSH content of spinach was between 0.44 and 1.79 µmol g^{-1} FW in Matador cultivar and from 1.29 to 2.12 µmol g^{-1} FW in Clipper cultivar. There was no significant effect on the total AsA content for the two spinach cultivars at different locations, but there were significant differences between the AsA contents in the two spinach cultivars grown in Obiliq. The total AsA content of spinach was between 7.47 and 9.95 µmol g^{-1} FW in Matador cultivar and from 9.29 to 11.85 µmol g^{-1} FW in Clipper cultivar. There was a significant difference in the total AsA content in Obiliq. We found a significant difference in the content of some pigments (total Chl *a* + *b*, Chl *a*, Chl *b*, and carotenoids) between cultivars at each location. The highest pigment content was found in Matador cultivar grown in Prizren (1.31, 0.47, 1.77, and 6.44 mg g^{-1}, respectively) and the lowest pigment content was found in Clipper cultivar grown in Obiliq (0.05, 0.04, 0.11, and 0.51 mg g^{-1}, respectively). The content of Chl *a* was between 0.10 and 1.31 mg g^{-1} in Matador and between 0.05 and 0.43 mg g^{-1} in Clipper. There was a significant difference in the pigment content of Chl *a* between cultivars at each location. For the cultivar Matador, the content of Chl *b* was between 0.12 and 0.47 mg g^{-1}, while for Clipper, it was from 0.04 to 0.19 mg g^{-1}. The total Chl *a* + *b* was found between 0.20 and 1.77 mg g^{-1} in Matador and between 0.11 and 0.62 mg g^{-1} in Clipper. The pattern of carotenoid content was similar to that of total Chl *a* + *b*. Results are presented in Figure 1.

4. Discussion

Various biotic and abiotic stresses cause the production of ROS and lead to the appearance of oxidative stress in plants (Foyer and Noctor, 2000). Plants primarily deal with oxidative stress via an endogenous defensive mechanism consisting of different enzymatic (SOD, CAT, APX, GR) and nonenzymatic (AsA, GSH) reactions (Hasanuzzaman et al., 2020). It has been reported that the activity of enzymes that enable the purification of ROS is higher in plant genotypes that are tolerant, while in sensitive plant genotypes, lower level of enzymes is observed. Therefore, different plant genotypes react differently to biotic and abiotic stresses due to variations in the antioxidant system (Nawaz and Ashraf, 2007). Based on the results of this study on the activities of enzymes POX, APX, SOD, and CAT, as well as the content of MDA, we found that oxidative stress plays an important role as an indicator of the state of stress in the spinach plant. H_2O_2 and O_2 are highly reactive molecules and can cause serious damage to cells such as DNA molecules, membranes, and proteins. SODs are antioxidant enzymes that enable the breakdown of superoxide into H_2O_2 and molecular oxygen. McCord and Fridovich (1969) first reported this phenomenon through a cupro-zinc protein (erythrocyte) derived from bovine erythrocytes. The content of MDA, indicative of the level of lipid peroxidation, was highest in Obiliq. Due to the increase in the level of lipid peroxidation, we assume degradation in the pigment content caused by the decrease in the total AsA content and the activities of the APX and GR enzymes, which are the main enzymes of the AsA–GSH cycle in chloroplasts. These results were obtained by Fatima and Ahmad (2005) with culinary onions and Vitoria et al. (2001) with radish seedlings. Shah et al. (2001) reported similar results from a study in which they applied heavy metals to paddy plants. The soil of Obiliq region showed some differences in pH and the content of EC and OM, and may be, this was connected to physiological and biochemical parameters. Enzymatic antioxidants include SOD, APX, GPX, and CAT. These enzymes are present in almost all subcellular divisions. Usually, cellular organelles contain more than one antioxidant enzyme, which enable the clearance of ROS (Scandalios, 2005). Based on previously published results, we can say that the level of APX activity in general increases along with the activities of other enzymes, such as SOD, CAT, and GSH, at the time of environmental stress (Shigeokaet et al., 2002).

5. Conclusions

During the examination of the physiological and biochemical parameters of the Clipper cultivar of spinach plants grown in Obiliq, it was found that the content of MDA, which is an indicator of the level of lipid peroxidation, was at the highest level in this cultivar. The increase in the level of lipid peroxidation that occurred in chloroplasts was due to the increase in the level of activity of the enzymes APX and GR, which are the main enzymes of the AsA–GSH cycle. However, one reason for this result may be that Obiliq is the most polluted site in Kosovo. In general, the results of the present study clearly indicated that different levels of soil texture, that is, pH, EC, and OM, had significant effects on growth, chlorophyll, and physiological attributes of spinach plants.

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