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Comparative analysis of the relationship between morphological, physiological, and biochemical properties in spinach (*Spinacea oleracea* L.) under deficit irrigation conditions

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Abstract: Drought is an abiotic stress factor that negatively affects plant development and causes economic losses in plant production, and its effect is getting significant every day. Spinach contains a high amount of water and drought stress affects its development as well as causing changes in its biochemical contents. In this study, it is aimed to determine the changes in the morphological, physiological, and biochemical contents of spinach grown under deficit irrigation conditions in different stages of development. For this purpose, by considering five different irrigation levels (I_{100}, I_{80}, I_{60}, I_{40}, and I_{20}), and 4 harvest times determined according to the development periods of spinach, a total of 20 experiment units were designed with 3 replications in a completely randomized experimental design. Growth parameters were negatively affected by severe deficit irrigations such as I_{20} and I_{40}. While the deficit irrigation caused a decrease in the relative water content (RWC), protein, and chlorophylls a and b, it increased membrane damage and carotenoid content. Furthermore, deficit irrigations have shown significant increases in proline, hydrogen peroxide, malondialdehyde, superoxide dismutase, catalase, and peroxidase. When different irrigation levels and different harvest periods were evaluated together, water use efficiency (WUE) values ranged between 2.31 and 5.02 g/L, and the highest WUE value was obtained at the I_{40} irrigation level and harvest III. As a result of the principal component analysis conducted to evaluate all the data obtained from the trial subjects together, the most important parameters explaining the effect of harvest time and deficit irrigation together were underground fresh weight, number of leaves, hydrogen peroxide, and peroxidase. Consequently, considering the growth parameters, the I_{100} irrigation level without deficit irrigation and the I_{80} irrigation level with mild water stress resulted in considerable outputs, especially in the harvests III and IV.

Key words: Antioxidant enzyme, deficit irrigation, growth, physiological, spinach

1. Introduction

Drought is one of the most important abiotic stress factors that limit agricultural production in many regions of the world, especially in arid and semiarid areas. As a result of global climate change, many regions are faced with water scarcity, (Bacon, 2004; Pachauri et al., 2014) and accordingly, 45% of agricultural areas have drought stress (Ashraf and Foolad, 2007). Approximately 70% of usable water in arid and semiarid regions is used in agricultural irrigation (Wisser et al., 2008). In agriculture, the most basic approach in irrigation water management is not to use excessive water in conditions where the water supply is sufficient, whereas, in conditions where the water supply is insufficient, it is to obtain the highest amount and quality product with the available water. In this context, it is aimed to determine the responses of plants to water restriction especially in arid and semiarid areas for the efficient and sustainable use of water resources (Yavuz et al., 2015; Gavili et al., 2019).

Drought stress in plants is an important factor affecting some biochemical and physiological contents as well as limiting growth and development (Srivastava and Srivastava, 2014). Drought causes disruption of water balance in the plant (Silva et al., 2009) and causes great damage due to the formation of reactive oxygen species (ROS) (Zgallaï et al., 2006). On the other hand, drought can also be detrimental by triggering lipid peroxidation and protein breakdown in plant tissues (Sairam and Saxena, 2000). Plants show some reactions to avoid the negative effects of drought. Development may be limited in a plant exposed to water stress and changes may occur in some of the plant's morphological appearances. Plants protect themselves from water stress by regulating their osmotic balance. When drought stress is observed, the plant tries to survive by synthesizing osmoprotectants such as proline (Liu et al., 2011). Plants have antioxidant enzyme systems, such as catalase (CAT), superoxide dismutase (SOD),

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peroxidase (POD), ascorbate peroxidase (APX), and nonenzymatic (tocopherol, ascorbate, and glutathione) antioxidants (Lehtimäki et al., 2010).

Spinach (*Spinacea oleracea* L.) is one of the vegetables that is grown and consumed in many parts of the world, except for the hot summer seasons. World spinach production is approximately 26.2 million tons. China is the first with 23.8 million tons, America is the second with 0.34 million tons (USDA), and then Turkey with 0.23 million tons[1]. Spinach, which is consumed fresh, is an important vegetable with a high content of minerals and vitamins, in particular vitamin C, which are beneficial to human health (Ekinci et al., 2015). Moreover, it is an important dietary product and is rich in derivatives of glucuronic acid and p-coumaric acid with strong antioxidant activity (Xu and Leskovar, 2015).

Since spinach has a high water content, irrigation is necessary for increasing yield and quality in many regions. On the other hand, suitable irrigation programs should be determined in arid and semiarid areas, under limited irrigation conditions (Leskovar et al., 2012; Xu and Leskovar, 2015; Ekinci et al., 2015; Jabeen et al., 2019). As in many vegetables, it is obvious that there will be changes in morphological, physiological, and biochemical contents as a result of water stress in spinach. Although there are many studies on deficit irrigation in spinach, there is no comprehensive study examining the effect of drought stress on different harvest periods. Thus, changes in the morphological, physiological, and biochemical contents of spinach harvested in different vegetation periods under deficit irrigation conditions were determined.

2. Materials and methods

2.1. Experimental area

The present study was conducted between 1 Nov 2019 and 2 Jan 2020, in glass greenhouses at the Faculty of Agriculture, Selçuk University, Konya/Turkey (located at 38°01’49”N and 32°31’32”E). With the automatic climate station placed in the greenhouse, some climatic parameters such as temperature and relative humidity were measured and recorded (Figure 1). When the climate data received were analyzed, the relative humidity in the greenhouse ranged between 39.1 and 69.5%. The highest temperature was measured as 40.7 °C and the lowest temperature as 7.7 °C and the average temperature varied between 11.4 and 22.9 °C during the vegetation period. As can be seen from the climate data, no climate factor restricting spinach cultivation has been encountered. In the research, a certain amount (1:2 respectively) of maturated cattle manure mixed soil was used as a growing medium. As a result of the soil analysis, the lime content was determined as 7.8% and the structure was clay-loam, the organic matter was 3.05, the pH value was 7.98, and the electrical conductivity (EC) was 1278 µS / cm. In terms of weight percent of the trial soil, the field capacity (FC) and wilting point (WP) values were 27.4% and 14.8%, respectively.

2.2. Plantation and irrigation

The study was carried out in plastic pots with a bottom diameter of 19 cm, a top diameter of 29 cm, a height of 24 cm, and containing 13.3 kg of seedling mixture. Red Kitten F1 cultivar was used as plant material. In the study, which was established according to the completely randomized experimental design, 5 irrigation levels (I_{100}, I_{80}, I_{60}, I_{40}, and I_{20}) were used and 4 harvests were carried out from the 3–4 leaf stage to harvest at 7 day intervals during the development period of the spinach. The experiment was established with 3 replicates and 3 pots per repeat, 8 seeds were planted in each pot and after germination, thinning was performed so that 4 plants remained in each pot.

The amount of irrigation water applied to the pots was determined by the gravimetric soil moisture measurement method. For this purpose, when the useful water capacity in control I_{100} subject decreased to 30%–35%, irrigation was carried out and soil moisture was irrigated to near the field capacity each time. Immediately after seed sowing (November 1), 1.48 L (approximately 31.4 mm) of irrigation water were applied to each pot, and soil moisture was increased to FC level. Scheduled irrigation was started approximately 7 days after the sowing seeds and irrigation water was applied to the trial pots a total of 10 times. The irrigation levels were 100% or full irrigation (I_{100}: nonstressed), 80% of full irrigation (I_{80}), 60% of full irrigation (I_{60}), 40% of full irrigation (I_{40}), and 20% of full irrigation (I_{20}). According to the experimental design, 5 different irrigation treatments were applied. Irrigation level treatments were based on the application of the amount of water at full irrigation treatments depending on irrigation intervals. In every application, the amount of irrigation water in I_{100} treatments was calculated from the difference of the field capacity and measured soil content according to irrigation intervals. The amount of irrigation water applied to the pots ranged from 53.0 mm (I_{20}) to 139.2 mm (I_{100}). The first harvest was made 42 days (Dec 12, 2019) after the start of scheduled irrigations, other harvests were realized at 7 day intervals (Dec 19 and 26, and Jan 2) for a total of 4 times. The morphological, physiological, and biochemical parameters given below were determined after each harvest.

2.3. Growth parameters

Leaf numbers and leaf area (winfolia computer program) were determined in the samples of plants and leaves taken during harvests (Ipek et al., 2014). Then, each pot was

[1] FAO (2018). Food and Agriculture Organization of the United Nations. FAOSTAT [online]. Website http://www.fao.org/faostat/ en/ [accessed 18.03.2020].

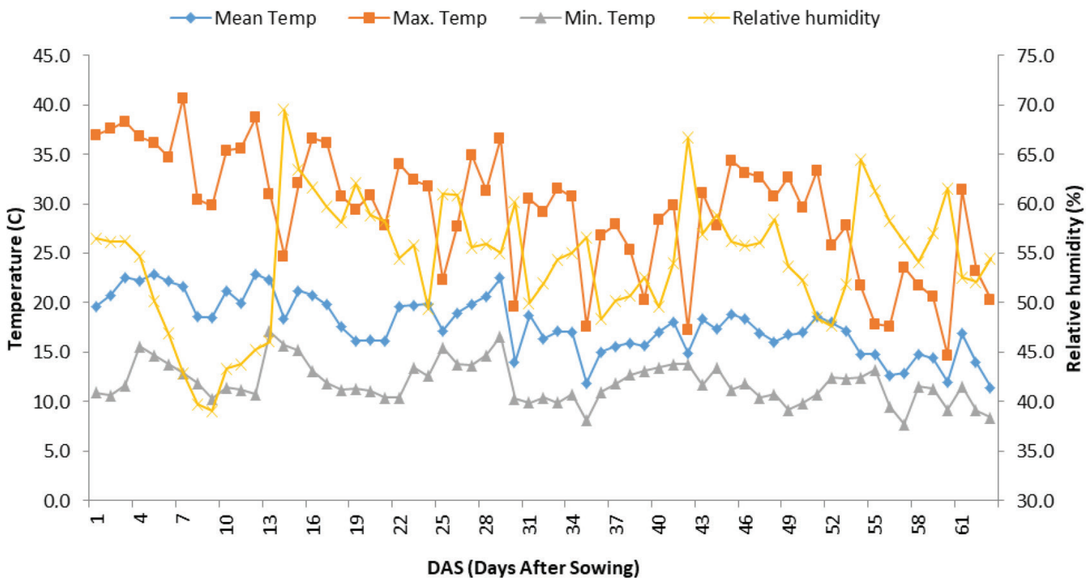

Figure 1. Temperature and humidity change graph at 63 days after sowing (DAS) in greenhouse condition. Statistically significant according to $P < 0.05$.

harvested separately, and the above and below ground parts of the plants were harvested, separated, cleaned, and weighed to determine the aboveground fresh weight (g) and underground fresh weight (g) per plant. The samples taken from these materials were dried in a 72 °C oven until they reached a constant weight and the dry weight of aboveground (g) and underground (g) parts were determined.

2.4. Physiological parameters

The fresh weight of the discs taken from the leaf samples was taken and their weights in turgor status were recorded by saturating with distilled water. After that, the samples were dried in an 80 °C oven for 48 h, then their dry weights were determined again. Relative water content (%) was determined according to Kaya et al. (2003). Membrane damage (%) was determined in leaf discs taken from applications according to Lutts et al. (1996).

2.5. Biochemical parameters

To determine the total carotenoids, chlorophyll a and b content in leaf samples, the samples ground in 10 mL acetone were centrifuged and read spectrophotometrically at 470, 652, and 663 nm, respectively. Chlorophyll a and b content has been determined according to Lichtenthaler and Buschmann (2001); the carotenoid amount has been calculated according to the Jaspars formula (Witham et al., 1971), and Bradford (1976) method was used for determination of protein contents. The samples were read at 520 nm and proline contents were determined according to Bates et al. (1973). After the plant sample crushed in 10 mL of 3% sulfosalicylic acid was centrifuged for 10 min at 15,000 rpm, 2 mL was taken from this sample, and then 2 mL enzyme, 2 mL glacial acetic acid, and 2 mL acid-ninhydrin were added and kept in an oven at 100 °C. Then, the samples were cooled and 4 mL of toluene was added and mixed for 30 s in the vortex. Hydrogen peroxide (H_2O_2) content was determined by the spectrophotometric method at 390 nm according to Pick and Mizel (1981), and malondialdehyde content (MDA) with measurements of 532 and 600 nm as described by Cakmak and Horst (1991).

While preparing enzyme extracts, 1 g of plant sample was ground and 5 mL of cold homogenate buffer (1% PVP and 0.1 M KH_2PO_4 containing 1 mM EDTA, pH: 7) was added and centrifuged at 15,000 rpm and + 4 °C for 15 min. The preparations obtained were used as a source for the measurements of antioxidant enzyme activity (Angelini and Federico, 1989). To determine SOD activity, samples prepared according to Agarwal and Pandey (2004) were read at 560 nm, and the content of enzymes causing inhibition were determined and calculated. The change in absorbance was observed at 240 nm for 3 min, and 0.01 A_{240} unit per min was considered to be equivalent to 1 unit of CAT activity (Chance and Maehly, 1955). Peroxidase (POD) content was determined based on the method described by Chance and Maehly (1955). The change in absorbance was noted every 30 s at 470 nm, and 1 unit of enzyme activity was considered to be equal to a change of 1.0 A_{470} unit per min.

2.6. Water use efficiency (WUE)

The WUE values were calculated by proportioning the above-ground wet spinach weights (g) obtained from the experimental subjects to the plant water consumption amounts (liter) calculated for each experiment.

$$WUE = \frac{AFW}{ET}$$

WUE = water use efficiency (g / L); E_y = above-ground wet spinach weights (g); ET = seasonal evapotranspiration (liter).

2.7. Statistical analysis of the data

Morphological, physiological, and biochemical changes obtained in spinach at different irrigation levels and different harvest times were analyzed in the JMP-14 package program according to 5% levels. Fairly close correlations between parameters were revealed by making correlation analysis in the same package program according to 5% levels. The data were also subjected to principal component analysis (PCA) to identify parameters that reveal significant changes. Loading plot graphs were prepared to show the relationship between the parameters from PC1 and PC2, which are formed as a result of the analysis. Furthermore, score plot graphs were drawn to determine the effects of harvest time and irrigation levels.

3. Results and discussion

3.1. Effect of deficit irrigation on the growth parameters

As a result of the statistical analysis, it was observed that harvest time and irrigation levels had important effects on growth parameters. Although no significant effect of irrigation levels was observed, except for the effect of I_{20} on the above-ground wet weight, the application of water restrictions in the subsequent harvests led to serious reductions in above-ground wet weight. However, fresh weights obtained from the I_{100} and I_{80} irrigations in the last harvest were statistically in the same group. Aboveground dry weight also produced similar results with fresh weight, and dry weights obtained from the I_{100} and I_{80} irrigation levels in the last harvest were in the same group, statistically (Figures 2a and 2b). Leskovar and Piccinni (2005) reported that 50% of water restriction caused significant losses in the fresh weight of three spinach cultivars. In another study conducted in spinach, 50% of water restriction was caused by significant losses in fresh and dry weight aboveground (Xu and Leskovar, 2015). When underground fresh and dry weights were examined, both parameters gave almost similar results, while the effects of irrigation levels were not detected in the first harvest, significant differences occurred in subsequent harvests. The highest underground fresh weights were obtained from the I_{100} irrigation level during the III and IV harvest periods, while the highest underground dry weight was obtained from the I_{100} and I_{80} irrigation levels in the same statistical group in the last harvest (Figures 2c and 2d). Mozafari et al. (2019) reported that drought applied to strawberries caused significant weight loss in root and green parts. Although the number of leaves is not affected by the irrigation restriction in the first harvest, serious decreases were observed with the increase in the number of leaves in the subsequent harvest periods (Figure 2e). In the leaf area, there were statistically significant differences between irrigation levels at the first harvest, and significant reductions were observed in the I_{20} and I_{40} irrigation levels where severe water constraints were applied. Another interesting point in the figure is that there is no statistically significant difference between the I_{100} and I_{80} irrigation levels, where the highest leaf area is obtained, in all other harvests except for the first harvest (Figure 2f). In spinach, 50% drought stress significantly reduced leaf area and leaf number (Xu and Leskovar, 2015). Drought stress had negative effects on the fresh and dry weight of underground and aboveground, as well as the number of leaves and leaf area (Ors and Suarez, 2017). When a general evaluation is made in terms of growth parameters among the irrigation levels, except for the I_{20} level during the first harvest period, the effects on vegetative growth, except the leaf area, were not found to be statistically significant. Therefore, until the first harvest period, cell division occurred, but the required water for the growth of the formed organs (shoots and leaves) was not sufficient at I_{20} and I_{40} levels. However, from the second harvest, the amount of water in the soil at the levels with limited irrigation has fallen below the required amount for cell division and consequently caused significant differences in the number of leaves.

3.2. Effect of deficit irrigation on physiological parameters

As a result of the statistical analysis, it has been determined that irrigation levels have important effects on relative water content (RWC) and membrane damage. The highest RWC values were obtained in the first harvest period, RWC were also lower in other harvests (Figure 3a). RWC is a widely used index to display the water stress of plants (Siddique et al., 2000). RWC is high in plants, there is more water nutrient intake from the soil and, in stress conditions, water deficiency appears. When membrane damage is examined, although low values were obtained in the first two harvest periods and irrigation level had no effect, high values were obtained in all irrigation levels in the last two harvests, in particular in the last harvest, irrigation restriction significantly increased membrane damage (Figure 3b). Deveci and Tuğrul (2017) stated that tissue membrane damage is an expression of their ability to maintain membrane integrity under stress in plants. They reported that, as a result of damage to the cell membranes of plants exposed to stress, the water-soluble substances in the cell flow into the intercellular spaces, which increases tissue electrical conductivity. According to these results, plants were not affected by water stress in the first two harvests. After the second harvest, the water stress started to affect the plant development negatively and thus, membrane damage occurred. Water deficit

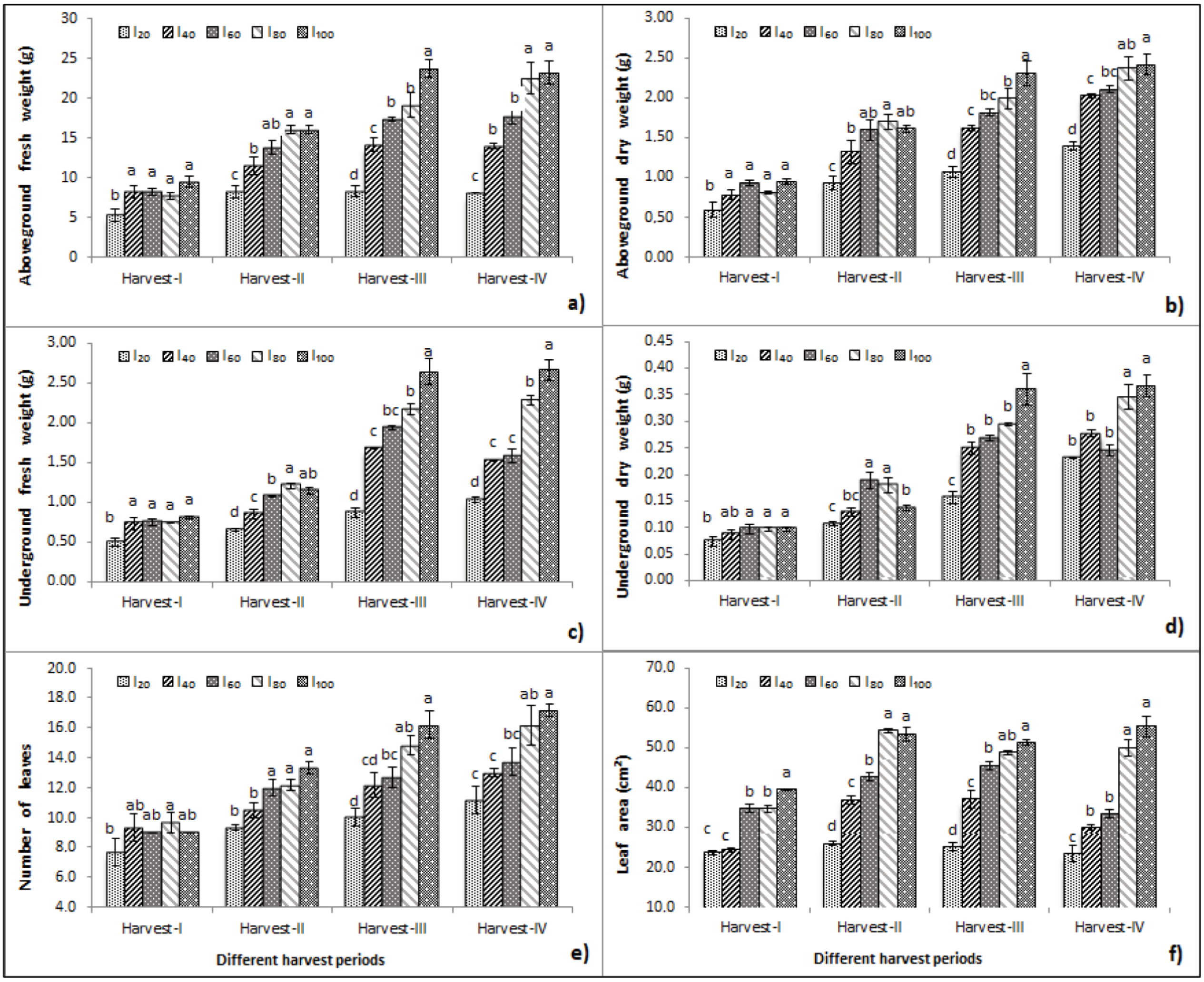

Figure 2. Growth characteristics of spinach under deficit irrigation conditions and different harvest time.

according to the control (full irrigation = I_{100}) caused the membrane damage to increase and RWC to decrease. Arıkan et al. (2018) stated that abiotic stresses increase membrane damage in blackberries. Likewise, limited irrigation has negative effects on RWC and membrane damage in blackberries (Ipek et al., 2018). On the other hand, Mozafari et al. (2019) reported that drought reduced RWC in strawberries. Similarly, drought stress negatively affected the RWC content in spinach (Xu and Leskovar, 2015).

3.3. Effect of deficit irrigation on biochemical parameters

As a result of the statistical analysis, it has been observed that limited irrigation applications have an important effect on biochemical parameters. While the chlorophyll value decreased only in the applications of high-water restrictions in the first two harvests, the I_{100} and I_{80} irrigation levels were statistically in the same group and showed less reduction than other irrigation levels in the other two harvests (Figure 3c). When chlorophyll b values were analyzed, although excessive and moderate water restrictions in the first three harvests lead to significant reductions, only the I_{20} and I_{40} irrigation levels decreased in the last harvest (Figure 3d). It is reported that change of the chlorophyll a and b content occur due to inhibition of chlorophyll biosynthesis and enzymes, degradation of pigments caused by disruption of chloroplast membrane integrity and interruption of other metabolic activities (Foyer and Shigeoka, 2011). Considering the results we obtained, it is thought that the changes occur due to chlorophyll biosynthesis and inhibition of the enzymes responsible for synthesis and the disruption of metabolic events. Moreover, the fact that the study was carried out in the winter period and the air condition? was turned off affected the light intensity, and changes occurred between the periods. In a water constraint study in spinach, it has been reported that chlorophyll a and b content decreases

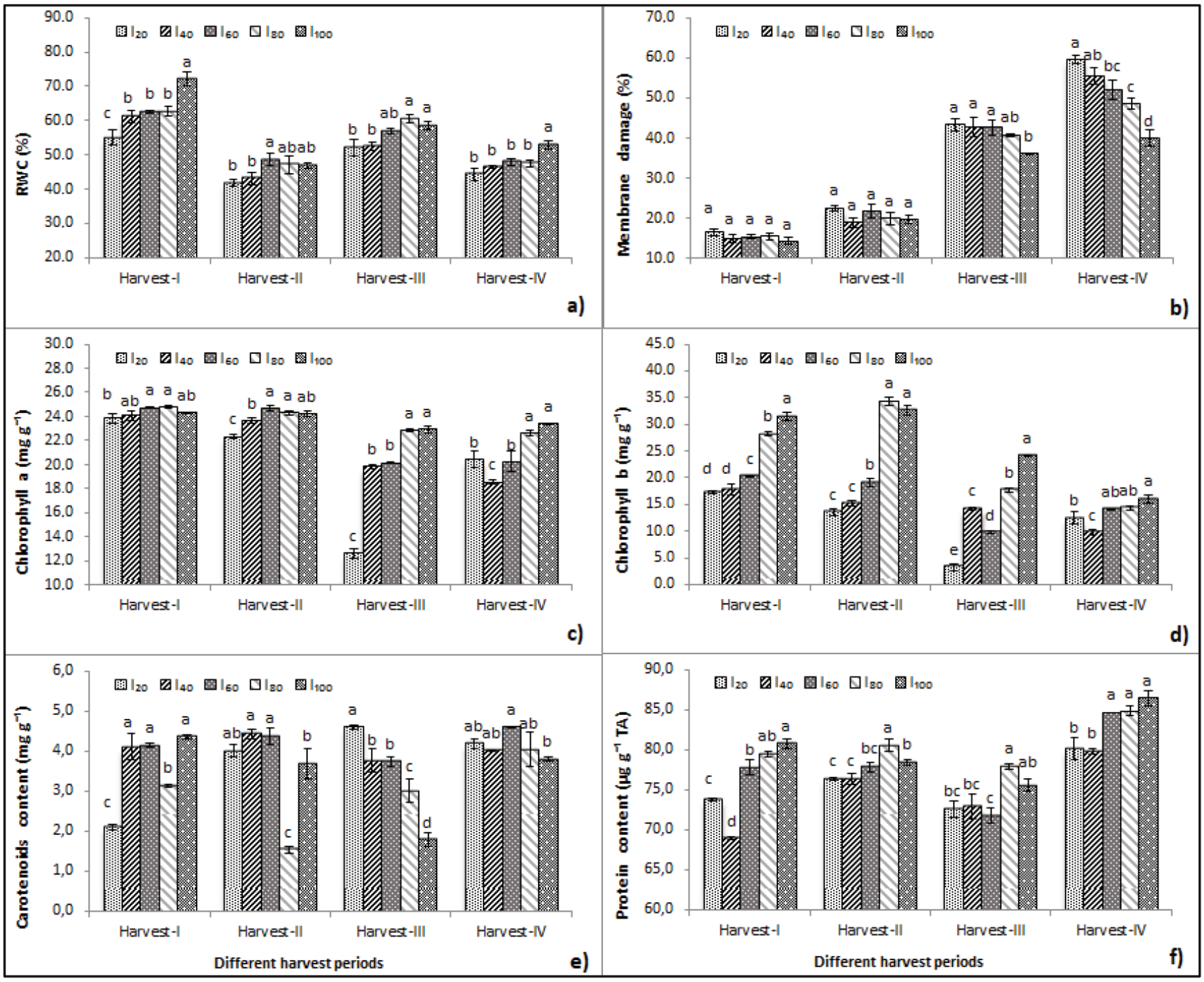

Figure 3. Physiological and some biochemical characteristics of spinach under deficit irrigation conditions and different harvest time.

as drought severity increases (Jabeen et al., 2019). In previous studies, similar results were observed by Akram et al. (2016) in radish, Bandian et al. (2016) and Yasmeen et al. (2013) in wheat, and Zhang et al. (2018) in spinach. In terms of carotenoid content, the effects of irrigation levels changed during the harvest periods, and although there were significant differences between the pots in the first three harvests, there was no significant difference between the pots in the final harvest (Figure 3e). Carotenoids are molecules that are located together with the chlorophyll in the leaves, work indirectly in photosynthesis, and have antioxidant properties (Keyvan, 2010; Smirnoff, 2005). Similarly, an increase in carotenoid content was observed as a result of the drought stress in spinach (Xu and Leskovar, 2015). The change in the carotenoid content in the last harvest was thought to be related to light and it was concluded that cloudy weather affects the carotenoid content in this period.

When the protein content was analyzed, a lower protein ratio was obtained in severe restriction applications compared to other irrigation levels in all harvest periods (Figure 3f). Although there was not much change in the proline content of the first two harvests, proline content increased significantly in the I_{20} and I_{40} irrigation levels, particularly in the final harvest, and they were included in the same statistical group (Figure 4a). It is well known that plants synthesize proline when encountering any stress factor and the proline content increases as the severity of stress rises. It is reported that proline plays an important role in biological events, such as ensuring osmotic balance, eliminating radicals, membrane integrity, protecting protein and DNA structure, and usable nitrogen accumulation (Keyvan, 2010). Indeed, with the increase of drought stress in spinach, the content of proline has increased (Jabeen et al., 2019). In previous studies, similar results were achieved by Akram et al. (2016) in radish and wheat. In our results, the deterioration of osmotic balance in severe water constraints and restriction of metabolites consequently in the last harvest period caused a significant increase in proline.

In all harvests, irrigation levels affected H_2O_2 content, which increased with increasing stress severity (Figure 4b). The high content of H_2O_2 causes oxidative stress and thus, ROS formation (Cruz et al., 2013). H_2O_2, which is one of the important ROS compounds formed as a result of oxidative stress, is used as an important index for measuring and comparing the damaging effects of stresses on plants (Hossain et al., 2015). As a result of the increased water deficit in spinach, H_2O_2 increased and became an important criterion for measuring the effect of stress. It has been reported that the content of H_2O_2 increases with increasing drought stress in spinach (Jabeen et al., 2019). MDA content was found high in all harvests and severe and moderate water constraints. On the other hand, I_{100} and I_{80} irrigation levels were determined as applications that accumulate the least amount of MDA (Figure 4c). A high amount of MDA content indicates an increase in lipid peroxidation accumulation due to stress (Türkan et al., 2004). According to the results obtained, water stress causes lipid peroxidation accumulation and prevents cellular transmission under severe restrictions. Similarly, in previous studies, Yadegari et al. (2014) and Hameed and Iqbal (2014) reported that drought stress increased the MDA activity with respect to tomato and wheat, respectively. Although SOD activity was found high at the first harvest at all irrigation levels, the other harvests produced lower SOD values. When all harvest periods are examined together, low SOD values were observed in the I_{100} irrigation level (without water stress) and I_{80} irrigation level (with mild water stress) (Figure 4d). The highest CAT values were determined in the third harvest period and irrigation applications with severe stress. However, when CAT activity was evaluated in the last harvest, all irrigation levels gave relatively similar values and were statistically in the same group (Figure 4e). Although irrigation levels did not have much effect on POD activity in the first two harvests, there were important differences in the last two harvests. When the figure is analyzed, it is seen that in the last two harvests, as the severity of stress increases, there are significant increases in POD activity (Figure 4f). Increasing antioxidant enzymes such as SOD, CAT, and POD is an important way to neutralize ROS produced in plants under stress conditions and is a method used to determine the effect of stress. The high secretion of these enzymes reveals the severity of stress and the ability of plants to cope with stress (Mozafari et al., 2019). According to the results obtained in spinach, irrigation deficit has been shown to increase enzyme activities. Aghaie et al. (2018) reported that drought stress caused 18% increase in CAT activity and 22% increase in SOD activity in some genotypes. Yuan et al. (2016) reported that drought stress increased SOD, CAT, and POD activity in their study. Previous studies reported that drought stress increased SOD, CAT, and POD activities in spinach (Jabeen et al., 2019), maize (Moussa and Abdel-Aziz, 2008), radish (Akram et al. 2016), and myrobalan 29C rootstock (Ipek and Pırlak, 2018).

3.4. PC and correlation analysis in morphological, physiological, and biochemical parameters

Morphological, physiological, and biochemical parameters of spinach obtained from different irrigation levels and different harvest periods were subjected to PCA analyses (Table 1). As a result of PCA, the study explained a high rate of 73.86% in two components. In studies conducted, it is reported that the first two components should explain more than 25% of the study to use PC analysis (Mohammadi and Prasanna, 2003; Mozafari et al., 2019; Seymen et al., 2019). A strong explanation of PCA will give important results about the suitability of this analysis and the parameters being considered. PCA is a widely used method in defining and evaluating stress tolerance in drought studies (Meng et al., 2009; Xiu et al., 2018). As a result of PCA, the first component (PC1) explained 40.7% of the variation, ADW, UFW, UDW, NL, MD, H_2O_2, MDA, CAT, and POD parameters were the highest described parameters. The second component (PC2) explained 33.15% of the variation, while AFW, UFW, NL, and LA were the parameters described in high positivity, while PL, H_2O_2, and POD were the parameters explained in the negative direction. UFW, NL, H_2O_2, and POD parameters, which were explained high in both components, revealed the importance of both the water constraint and harvest time of the study. Pouri et al. (2019) determined important parameters with the same method in their drought study in corn.

Using the PC1 and PC2 components, a loading plot chart was created to examine the interrelationship between morphological, physiological, and biochemical parameters (Figure 5). It is reported that if the angle between the vectors in the figure is < 90°, there is a positive relationship, if > 90°, there is a negative relationship, and if the angle between the vectors is 90°, there is no significant relationship (Yan and Kang, 2003). When the figure is examined, it is seen that there is a high positive correlation between Ca and Cb, and they have a high negative correlation with MDA, POD, CAT, H_2O_2, and PL. Besides, it is seen that the strongest negative correlations are between SOD and growth parameters. To interpret similar relationships, the data were subjected to correlation analysis and significant correlations ($P < 0.01$) were revealed (Table 2). The important relationships shown in the figure are also seen in the correlation table, while there was a high positive

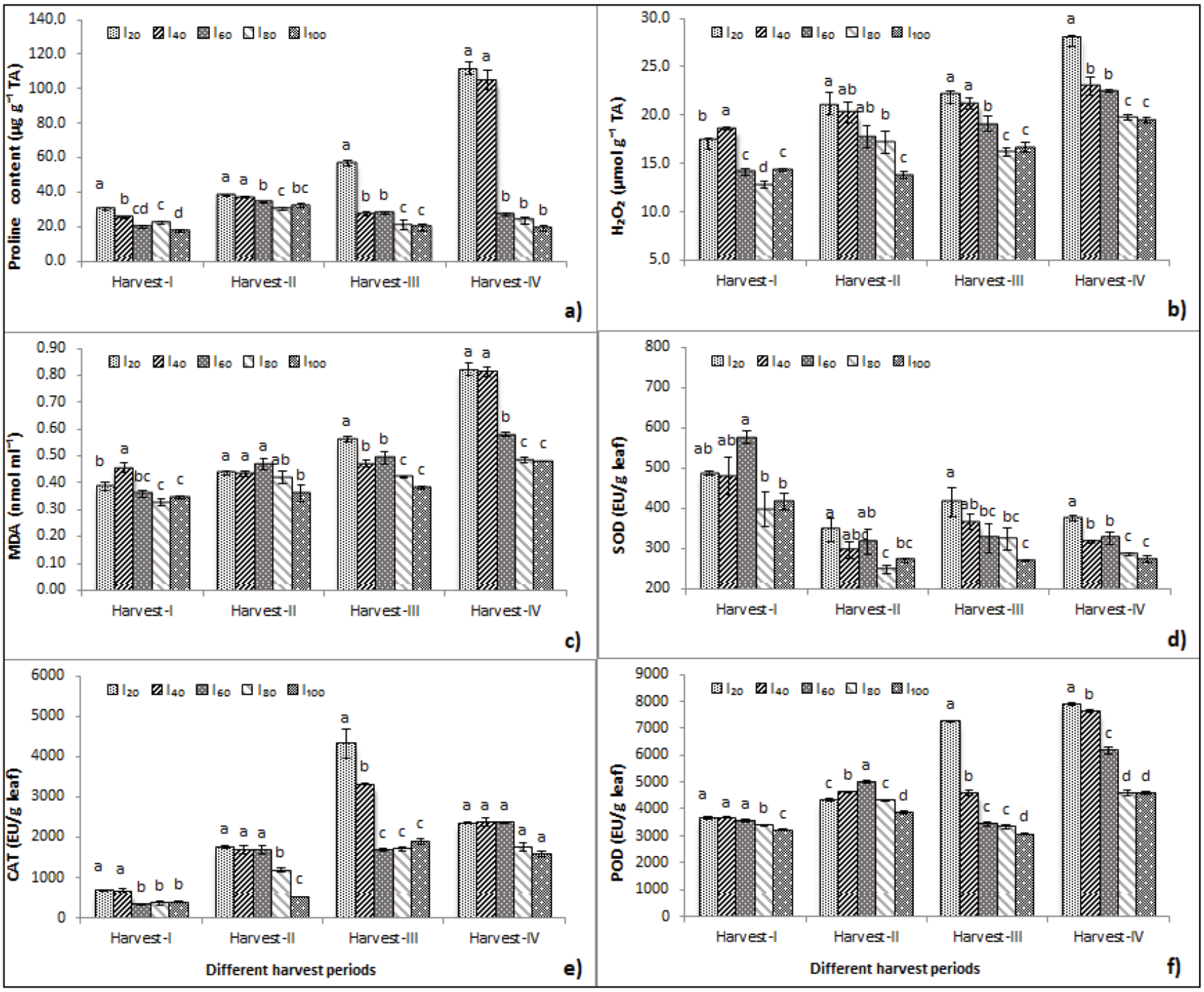

Figure 4. Biochemical characteristics of spinach under deficit irrigation conditions and different harvest time.

correlation between chlorophyll b and a (r = 0.735), the parameters expressed above showed a high negative correlation. The negative relationship of SODs with growth parameters was also seen in the correlation table. When examining the correlations, another important point is that all of the growth parameters (AFW, ADW, UFW, UDW, NL, and LA) created high positive correlations among themselves. It has been determined that there is a high positive correlation between MDA, CAT, and POD and with H_2O_2. Xiu et al. (2018) reported that correlation analysis is an important method for evaluating parameters and antioxidant enzyme contents show a high correlation with each other in drought stress. Mozofari et al. (2019) determined the relationship between parameters with a similar method in the drought stress study conducted in strawberries and reported a positive correlation between H_2O_2 and MDA and a negative correlation in others. Besides, there was a high positive correlation between SOD, POD, proline, and growth parameters. Similarly, in Achillea species a high positive correlation between H_2O_2 and MDA was reported (Gharibi et al., 2016).

Using the PC1 and PC2 components, a score plot chart was created to explain the harvest periods and irrigation levels (Figure 6). When evaluating the projection of PC1 in the figure, the harvest times are listed in a row. However, when PC2 was examined, severe irrigation levels were in the negative region, while moderate irrigation stress and full irrigation subjects were in the positive region. In this case, while the PC1 component was the best component to explain the harvest time, irrigation constraint is best explained with the PC2 component. When the parameters are summarized, UFW, NL, H_2O_2, and POD parameters are important both at harvest time and irrigation levels. The most important parameters explaining the harvest time are ADW, UDW, MD, MDA, and CAT, while the most important parameters explaining the water constraint are

Table 1. PCA results regarding morphological, physiological, and biochemical characteristics of spinach under deficit irrigation conditions and different harvest time.

Items	PC1	PC2
Eigenvalue	7.32	5.96
Percentage of variance	40.70	33.15
Cumulative variance	40.70	73.86
Eigenvectors		
AFW	0.222	**0.318**
ADW	**0.294**	0.238
UFW	**0.248**	**0.270**
UDW	**0.308**	0.193
NL	**0.266**	**0.274**
LA	0.068	**0.378**
RWC	-0.201	0.097
MD	**0.343**	-0.063
Ca	-0.217	0.227
Cb	-0.207	0.238
CT	0.067	-0.201
PT	0.132	0.159
PL	0.179	**-0.269**
HO	**0.265**	**-0.242**
MDA	**0.271**	-0.224
SOD	-0.222	-0.215
CAT	**0.271**	-0.160
POD	**0.244**	**-0.265**

Principle component (PC), Aboveground fresh weight (AFW), Aboveground dry weight (ADW), Underground fresh weight (UFW), Underground dry weight (UDW), Number of leaves (NL), Leaf area (LA), Relative water content (RWC), Membrane damage (MD), Chlorophyll *a* (Ca), Chlorophyll *b* (Cb), Carotenoids content (CT), Protein content (PT), Proline content (PL), H_2O_2 content (HO), Malondialdehyde content (MDA), Superoxide dismutase content (SOD), Catalase content (CAT), Peroxidase content (POD).

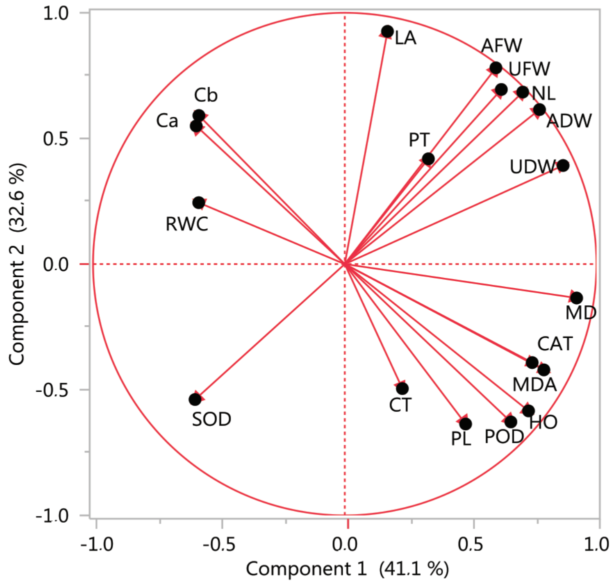

Figure 5. Loading plot based on PC 1 and 2 obtained from PCA using morphological, physiological, and biochemical characteristics of spinach under deficit irrigation conditions and different harvest time.

AFW, LA, and PL. When the positive intersection region of PC1 and PC2 is examined, this region has been the area where growth parameters are best explained, and H3-I_{100}, H4-I_{100}, H3-$I_{80,}$ and H4-I_{80} applications had the best results in this region. Finally, it has been revealed that 20% of water savings will be provided with the I_{80} water constraint in achieving economic yield.

3.5. Water use efficiency

The results regarding the WUE, which is an important criterion in the evaluation of irrigation programs and expressed as the unit water utilization rate, are presented in Figure 7. When different irrigation levels and different harvest periods are evaluated together, WUE values changed between 2.31 and 5.02 g/L, and the highest WUE value was obtained at the I_{40} irrigation level and harvest III. When irrigation levels are examined according to different harvest periods, the highest WUE values in irrigation levels of I_{100}, I_{60}, and I_{40} were obtained in the harvest III period. On the other hand, WUE values increased in parallel with the harvest period at the level of I_{80} irrigation, where light water stress was applied, and the highest WUE value was reached in harvest IV. In the I_{20} trial pot exposed to excessive water stress, the highest WUE value was obtained in the harvest II period, and in the harvests made after this period, there was a decrease in WUE values. It is aimed to provide the highest benefit from unit water in arid and semiarid areas. In this context, it is clearly understood from the study results that water can be saved considerably by irrigating at the I_{40} irrigation level of the spinach grown in the greenhouses in the autumn-winter period and dry regions with insufficient water resources. Similar results were also reported by Nishihara et al. (2001), Leskovar and Piccinni (2005), Ors and Suarez (2017) and Gavili et al. (2018) in spinach.

4. Conclusion

It has been shown that significant changes occur in morphological, physiological, and biochemical parameters of spinach, both in different harvest periods and in deficit irrigation levels. While severe water constraints negatively

Table 2. Correlation coefficients between morphological, physiological, and biochemical characteristics of spinach under deficit irrigation conditions and different harvest time.

	AFW	ADW	UFW	UDW	NL	LA	RWC	MD	Ca	Cb	CT	PT	PL	HO	MDA	SOD	CAT	POD
AFW	1.00																	
ADW	**0.94**	1.00																
UFW	**0.94**	**0.92**	1.00															
UDW	**0.87**	**0.94**	**0.95**	1.00														
NL	**0.96**	**0.96**	**0.94**	**0.92**	1.00													
LA	**0.82**	**0.68**	**0.68**	0.54	**0.73**	1.00												
RWC	-0.11	-0.28	-0.01	-0.18	-0.20	0.05	1.00											
MD	0.43	**0.65**	0.58	**0.75**	0.57	-0.01	-0.37	1.00										
Ca	0.04	-0.12	-0.09	-0.24	-0.04	0.36	0.25	**-0.66**	1.00									
Cb	0.05	-0.11	-0.12	-0.27	-0.03	0.48	0.33	**-0.63**	**0.73**	1.00								
CT	-0.22	-0.09	-0.21	-0.12	-0.14	-0.36	-0.13	0.21	-0.33	-0.48	1.00							
PT	0.44	0.52	0.36	0.40	0.50	0.41	-0.19	0.27	0.21	0.22	0.08	1.00						
PL	-0.27	0.00	–0.17	0.07	–0.09	–0.48	–0.49	0.54	–0.50	–0.43	0.24	0.02	1.00					
HO	–0.03	0.21	0.08	0.32	0.10	–0.44	**–0.62**	**0.74**	**–0.63**	**–0.73**	0.36	0.03	**0.72**	1.00				
MDA	–0.00	0.28	0.11	0.36	0.16	–0.38	–0.49	**0.79**	**–0.60**	**–0.61**	0.35	0.151	**0.89**	**0.87**	1.00			
SOD	**–0.74**	**–0.76**	**–0.61**	**–0.62**	**–0.74**	**–0.66**	0.51	–0.35	0.01	–0.12	0.20	–0.41	–0.04	–0.15	–0.14	1.00		
CAT	0.16	0.33	0.27	0.43	0.25	–0.20	–0.43	**0.71**	**–0.88**	**–0.71**	0.26	–0.11	0.43	**0.72**	0.58	–0.25	1.00	
POD	–0.13	0.15	–0.07	0.18	0.04	–0.43	–0.58	**0.67**	**–0.69**	–0.58	0.44	0.20	**0.85**	**0.83**	**0.90**	–0.10	**0.68**	1.00

Aboveground fresh weight (AFW), Aboveground dry weight (ADW), Underground fresh weight (UFW), Underground dry weight (UDW), Number of leaves (NL), Leaf area (LA), Relative water content (RWC), Membrane damage (MD), Chlorophyll *a* (Ca), Chlorophyll *b* (Cb), Carotenoids content (CT), Protein content (PT), Proline content (PL), H_2O_2 content (HO), Malondialdehyde content (MDA), Superoxide dismutase content (SOD), Catalase content (CAT), Peroxidase content (POD), values in bold = statistically significant according to P < 0.05.

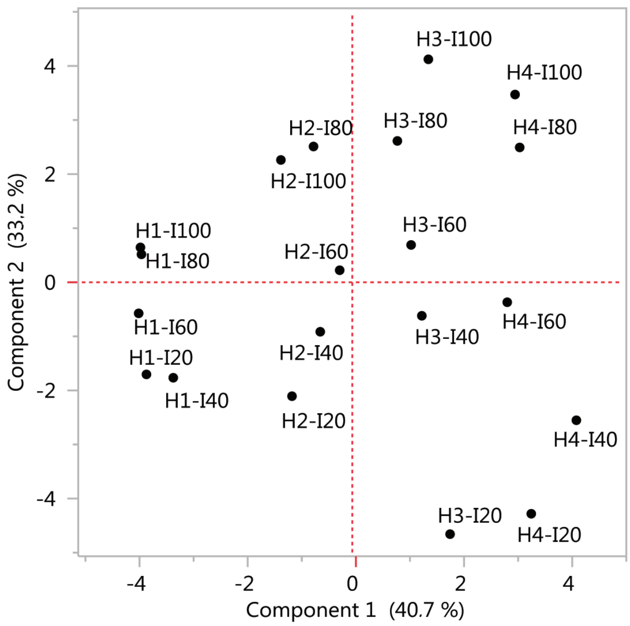

Figure 6. Score plot based on components 1 and 2 obtained from PCA using morphological, physiological, and biochemical characteristics of spinach under deficit irrigation conditions and different harvest time.

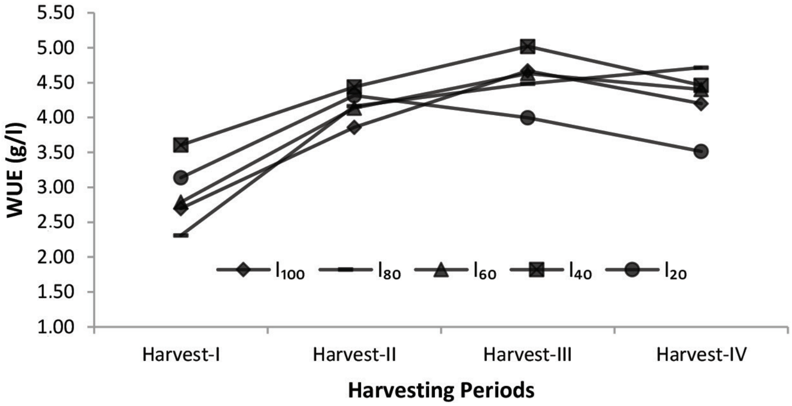

Figure 7. Water use efficiency of spinach under deficit irrigation conditions and different harvest time.

affect growth parameters, it caused a decrease in RWC, protein, chlorophyll a and b, and increased membrane damage and carotenoid content. Also, water constraints have shown significant increases in proline, H_2O_2, MDA, SOD, CAT, and POD. According to the PCA, while aboveground and underground dry weight, membrane damage, MDA, and CAT parameters produced the most important differences at different harvest times, aboveground fresh weight, leaf area, and proline parameters were in the water constraints. The parameters explaining the water constraint and harvest times were underground fresh weight, number of leaves, H_2O_2, and POD. H3-I_{100}, H4-I_{100}, H3-I_{80}, and H4-I_{80} applications in the positive region of PC1 and PC2 gave the best results in growth parameters. As a result, it has been determined that economic yields have been reached from the harvests at 56 (harvest III) days after seed sowing, and to obtain high yield and quality in areas with sufficient water resources, it has been recommended to either fully water or make 20% restriction from irrigation water of the spinach. On the other hand, according to the WUE results, it is revealed by the results of the research that the water level can be saved significantly by applying the I_{40} irrigation level in spinach in arid and semiarid regions.

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