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Short-Time Hypoxia and Post Aeration Alter Relative Membrane Permeability and Total Protein in Root and Leave of Sunflower Cultivars

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Authors' contributions

This work was carried out in collaboration between all authors. Author AM proposed the idea, correct and edits the manuscript and author SS managed the experiments and put the data together as a paper and author MM helped to finalize the first draft of the manicurist. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: There are varieties of factors that results into hypoxia in plant's root zone. Hypoxia causes oxidative stress and lipid peroxidation, which seriously affect plant growth. In this experiment, effects of hypoxia and post aeration on root and shoot dry weight, relative membrane permeability (RMP) and total protein (TP) of roots and leaves were evaluated.
Study Design: Department of Biology (Plant Science Division), University of Isfahan and University of Arak in Iran, between September 2010 and April 2012.
Methodology: Seventeen days old seedlings of four sunflower cultivars (Lacomca, Record, Progress and Hysun33) were subjected to four days of hypoxia and then re-aerated for four days. Plants were sampled daily during hypoxia and post aeration to measure root and shoot dry weight, RMP and TP of roots and leaves. RMP was assayed by level of electrolyte leakage from plasma membrane with electrical conductivity meter and TP was measured according to Bradford's method.
Results: Hypoxia caused reduction of plant dry weight differently in different cultivars. Lacomca and Progress cultivars in comparison to Record and Hysun33 cultivars showed less reduction in root dry weight (almost 18% versus 49.4% and 36.1%, respectively). The

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highest and the lowest reduction in shoot dry weight was observed in Hysun33 (24.5%) and Lacomca (9.7%) cultivars; these reductions were correlated with the amounts of increase in leave RMP in the selfsame cultivars (14.5% and 5.0%, respectively). The highest increase in root and leave TP was obtained for Record cultivar (100.8% and 490.1%, respectively), nevertheless, the lowest increase was measured in root (26.6%) and leave (94.9%) of Lacomca cultivar. Re-aeration for 4 days after hypoxia showed continuous reduction in roots and shoots dry weight and also optimized the amount of RMP and TP of roots and leaves.

Conclusion: Hypoxic condition caused plant dry weight reduction; however, root dry weight reduction was higher than shoot. Hypoxia also increased root and leave RMP and TP. The amount of leave RMP was higher than that of root. The addition of leave RMP was correlated with dry weight reduction; however, this trend was not the same in root.

Keywords: Helianthus annuus L.*; hypoxia; post-aeration; relative membrane permeability; total protein.*

ABBREVIATION

PUFA - polyunsaturated fatty acid; ROS - reactive oxygen species; RMP - relative membrane permeability; TP - total protein.

1. INTRODUCTION

Oxygen is an essential element for plant growth and metabolism [1]. Some conditions such as over irrigation, soil compaction, flooding and over rainfall and poor drainage [2,3,4,5] results into hypoxia which often happens in rhizosphere. One of the primary stresses in flooded soil is oxygen deprivation [6] simply call hypoxia. Hypoxia leads to block electron transport chain [7] and consequently produces reactive oxygen species (ROS) and oxidative stress in cell [1,3]. ROS included radical and non-radical forms of oxygen related chemicals [8] that cause severe damage to lipid, DNA, protein and carbohydrate of plant cells [9,10]. These negative effects can alter cellular metabolism and dramatically reduce root and shoot dry weight and eventually crop productivity [2,11]. Drew (1997) reported that under oxygen deficiency, accumulation of toxic products of fermentation, lower energy production and lack of substrate for respiration result into root injury [2]. Main sources of ROS production in plant are mitochondria, chloroplast and peroxisome [8,12]; however, in hypoxic condition mitochondria is more important than others [13,14,15]. According to Vartapetian and Jackson [16], when plants subject to hypoxic or anoxic condition the oxygen-dependent pathways, especially the energy-generating system, are suppressed, the functional relationships between roots and shoots are disturbed, and both carbon assimilation and photosynthetic utilization are affected.

Numerous studies have shown that hypoxia triggers the formation of ROS and induce oxidative stress in plants [1,3]. Membrane lipid peroxidation is one of the injurious results of ROS [17]. Polyunsaturated fatty acids (PUFA) are suitable substrates for membrane lipid peroxidation [14,17]. ROS, especially hydroxyl radicals and singlet oxygen attract to methylene group of PUFA and producing conjugated dienes, lipid peroxy radicals and hydroperoxides. Although, malondialdehyde resulted from decomposition products of hydroperoxides [18], these products are very reactive and lead to injury of membrane integrity and reduce relative membrane permeability (RMP) [19].

To control destructive effects of ROS and protect plant cell from its effects, antioxidant system, that includes enzymatic antioxidant such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POD) and glutathione reductase (GR) [8,12] and non-enzymatic antioxidant such as ascorbate [20], glutathione [21], phenolic compounds [22] and tocopherols [23] is activated. Therefore, the level of these components defines the rate of plant tolerance to hypoxia. One of the indexes that could be affected under oxidative stress is the amount of total protein, and protein compounds included different enzymes which changes under hypoxic condition [24].

Previous studies have shown that different plants have different ability to tolerate hypoxic condition even cultivars belong to same species [25,26,27]. Results of different studies indicate that hypoxia leads to increase in relative membrane permeability in leaves of *Zea mays* [28]*; Capsicum annum* L. [29], *Malus hupehensis* and *Malus toringoides* [30]. However, Bai et al. [30] showed that growth of both species of *Malus* was inhibited by hypoxia.

More than one third of irrigated lands are affected by hypoxic condition due to improper drainage, soil compaction and non-uniform rainfall especially at early stage of growth. Therefore, reduction of growth and consequently yield due to hypoxia for different crops is a frequent condition [31,32]. Unsuitable condition for growth during and after hypoxia, even in short period of time, has been reported for different crops such as wheat [33], cotton and rice [34], especially at early stage of growth [35,36], in different regions; for instance, South East Asia, Australia, USA as well as in Iran.

Sunflower (*Helianthus annuus* L.) with almost 40-50% valuable edible oil is one of the most important oilseed crops in the world. Sunflower, which mostly planted in dry land and Northern provinces of Iran, almost accounts for one third of the oilseed crops cultivated in Iran. Non-uniform rainfall and heavy soil in dry lands of Iran cause temporary hypoxic condition especially in early stage of growth which coincided with spring rains. This condition damages root growth via ROS formation and membrane degradation. Therefore this research was conducted to evaluate the effect of short period of hypoxia and re-aeration on growth (shoot and root dry weight), membrane permeability and total protein of root and leave of different sunflower cultivars.

2. MATERIALS AND METHODS

2.1 Plant Material and Treatments

Seeds of sunflower (*Helianthus annuus* L.) cultivars (Lacomca, Record, Progress and Hysun33) were obtained from Oil Seeds Research Center of Isfahan, Iran. Sterilized seeds were germinated in dark for three days and then transferred into pots containing perlite for four days. Seven days old seedlings were moved into aerated hydroponic containers containing full strength Hoagland's solution [37]. Seedlings were grown in a growth chamber (27/20ºC day/night, with a 16/8 h day/night photoperiod, irradiance of 1500 lux) for ten days for environmental adjustment. Then seventeen days old seedlings were subjected to hypoxic condition for four days using N_2 (99.99%) bubbling procedure to remove O_2 from medium [38]. In contrast, the control plants were continuously aerated with regular air. Plants pre-exposed to hypoxia for four days, were aerated for four more days to assay the rate of recovery. Simultaneously, control plants were aerated continuously. Four days of hypoxia was selected as the period of low oxygen availability which usually is predominant in dry land regions of Northern and Western provinces of Iran. The amounts of dissolved oxygen in aerated and hypoxic condition were 5.92 and 1.94 mg L^{-1}, respectively. Plants were sampled daily during hypoxia and two days interval during post aeration. Plant samples were split into root and shoot and then each plant part was divided into two subsamples; one subsample was weighted and oven dried (70ºC) and the other used as fresh for RMP analysis or immediately frozen (-80ºC) in liquid nitrogen for protein analysis.

2.2 Assay of Relative Membrane Permeability

Relative membrane permeability (RMP) was assayed by level of electrolyte leakage from plasma membrane with electrical conductivity meter using the method described by Dionisio-Sese and Tobita [39]. Equal size of fresh plant sample (root and leave) separately was placed in test tubes containing 10 mL distilled water. The tubes were shaked for 4 h at room temperature and the initial electrical conductivity of the medium (L_1) was analyzed using an electrical conductivity meter (Consort C535 Multi-parameter Analyzer). The samples were autoclaved at 100ºC for 5 min to release all electrolytes; cooled to 25ºC, and then the final electrical conductivity (L_2) was measured. RMP was calculated using $RMP = L_1/L_2 \times 100$.

2.3 Assay of Total Protein

Total protein (TP) was measured according to Bradford [40]. 0.5 g sample was homogenized with one ml cold Tris-HCl buffer 50 mmol L^{-1} (pH = 8.4). The homogenate was centrifuged at 10,000 *g* for 25 min at 4ºC and the supernatants were mixed with Bradford reagent and then absorbance was read at 595 nm. Standard curve of Bovine Serum Albumin (BSA) was used to calculate the amount of protein.

2.4 Statistical Analysis

The experiment was conducted according to a factorial test in a completely randomized design with three replicates. Statistical analysis was carried out using Sigma Stat for ANOVA as well as Duncan's multiple-range test or t-test to compare the means values.

3. RESULTS AND DISCUSSION

3.1 Root and Shoot Dry Weight

Shoot and root dry weights of aerated sunflower cultivars (Lacomca, Record, Progress and Hysun33) were increased differently with time. The highest and the lowest amounts of root dry weight were observed in Lacomca (664.0 mg $plant^{-1}$) and Progress (299.7 mg $plant^{-1}$) cultivars, respectively, when seedlings were 25 days old. Simultaneously, the highest and the lowest shoot dry weights were 2721.5 and 943.7 mg $plant^{-1}$ in the same cultivars, respectively. These numbers indicated that Lacomca and Progress had the highest and the lowest root growth rates (26.56 and 11.99 mg day^{-1} $plant^{1}$, respectively), and shoot growth rate in the same cultivars were 108.86 and 37.75 mg day^{-1} $plant^{-1}$, respectively.

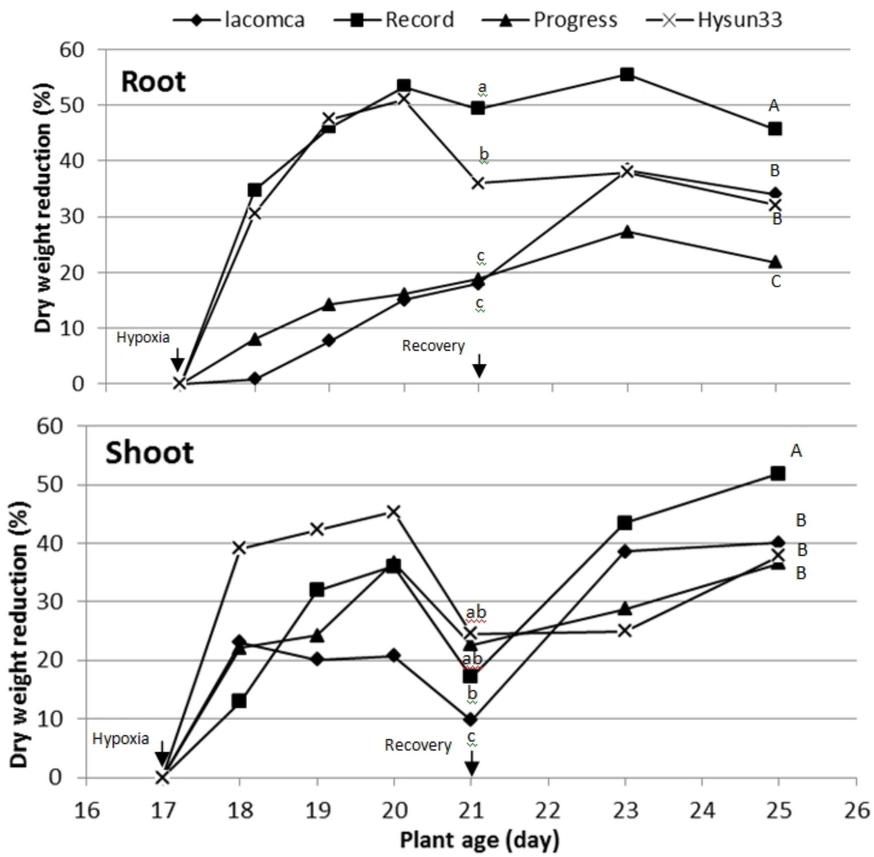

Fig. 1. Mean values (n= 6) of root and shoot dry weight reduction (%, mg plant $^{-1}$) of four sunflower cultivars under hypoxia and post-aeration. The reduction was calculated on daily basis for treated plant compared to its control at the same day. Difference in lower and upper case letters indicated significant difference between mean values (Duncan's rang test, P < 0.05) at the end of hypoxia and post-aeration, respectively

Hypoxic condition caused root and shoot dry weight reduction differently in different sunflower cultivars. The reductions were calculated on daily basis for each hypoxic condition compared to its control plant at the same day. The results (Fig. 1) showed that root dry weigh reductions of Record (49.4%) and Hyssun33 (36.1%) cultivars were higher and non-linear during 4 days of hypoxia but lower and linear in Lacomca (17.9%) and Progress (18.8%). Simultaneously, after four days of hypoxia the highest and the lowest shoot dry weight reductions were found in Hysun33 (24.5%) and Lacomca (9.7%) cultivars, respectively. In general, root dry weight reductions were higher than shoot and also the compatibility of shoot to hypoxia is much faster than root. Therefore shoots were adjusted much better than roots and almost reached to their control values at the end of 4 days of hypoxia.

Aerated condition after hypoxia did not improve the status of plant’s growth and consequently, root and shoot dry weight reductions were observed continuously in different cultivars (Fig. 1). The root dry weight reductions were almost constant and higher in Record but less in progress cultivars up to the end of re-aeration (25^{th} day of growing). In contrast, shoot dry weight reductions were continued linearly during re-aeration in all cultivars (Fig. 1). Therefore at the end of four days of post aeration the highest root and shoot dry weight reductions were occurred in Record cultivar (45.6% and 51.9%, respectively), whereas the lowest reductions were observed in Progress cultivar (21.8% and 36.6%, respectively). It should be mentioned that the total reduction of dry weight of plant parts at the end of the experiment come from the effects of hypoxia as well as re-aerated condition.

3.2 Root and Leave Relative Membrane Permeability

Relative membrane permeability (RMP) in aerated plants was increased with time especially at the early stage of growth (up to 21^{st} day) and afterward increased smoothly in all cultivars (Figs. 2 and 3). At the beginning of the experiment (17 old days' seedlings) the lowest and the highest amounts of root RMP were observed in Lacomca (72.1%) and Record (83.9%), respectively ($P < 0.05$). However, for leave, their amounts were observed in Hysun33 (71.0%) and Lacomca (79.0%) cultivars, respectively, ($P < 0.05$). The results indicated that the amounts of RMP are age- dependent and increases as plant get older.

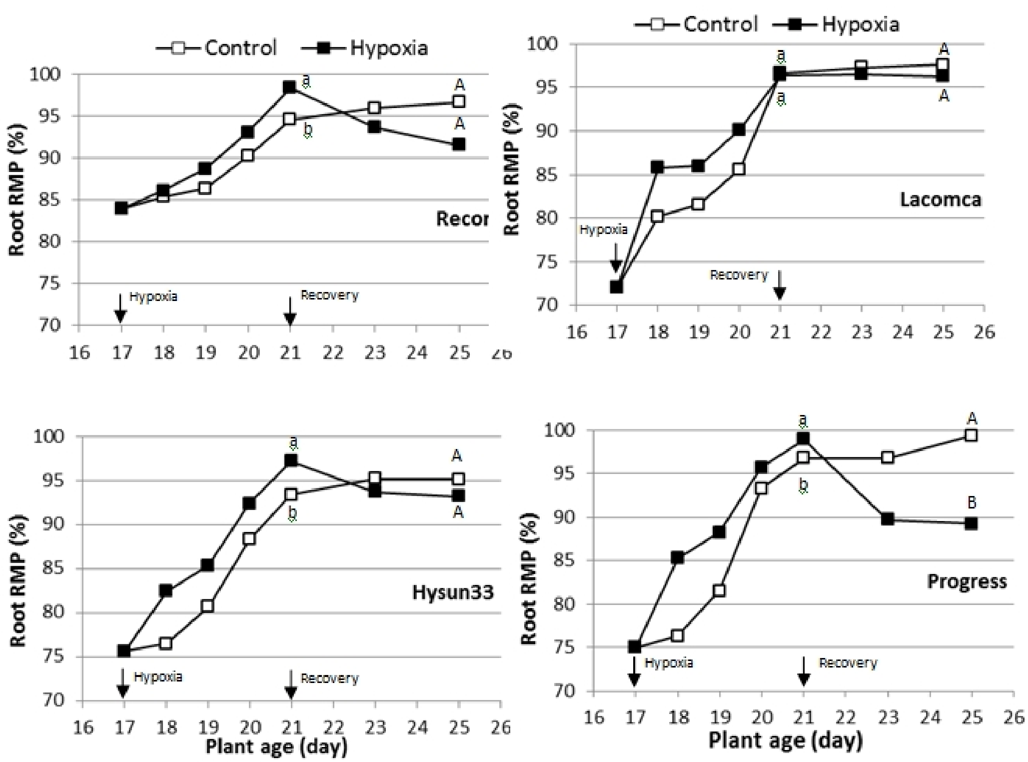

Fig. 2. Mean values (n= 6) of root's relative membrane permeability (RMP; %, µS cm^{-1}) of four sunflower cultivars under hypoxia and post-aeration. Difference in lower and upper case letters indicated significant difference between mean values (Using t-test, α < 0.05) at the end of hypoxia and post-aeration, respectively

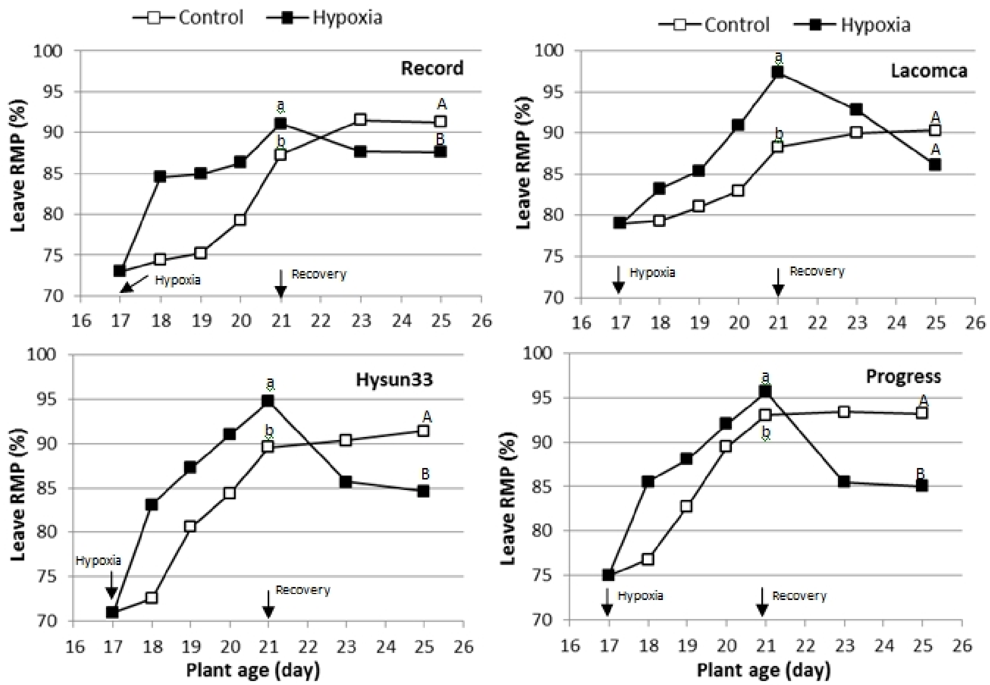

Fig. 3. Mean values (n= 6) of leave's relative membrane permeability (RMP; %, µS cm^{-1}) of four sunflower cultivars under hypoxia and post-aeration. Differences in lower and upper case letters indicated significant difference between mean values (Using t-test, α < 0.05) at the end of hypoxia and post-aeration, respectively

Hypoxic condition leads to increase root and leave RMP in four cultivars (Figs. 2 and 3); however, the addition of RMP formation due to hypoxic condition was higher in leave than in root. Higher differences of root's RMP were observed at the first day of hypoxia and afterward the differences were less except in Record cultivar. The highest and the lowest additions of root's RMP were obtained in Progress (11.7%) and Record (0.9%) cultivars, respectively. At the same time, the highest and the lowest additions of leave's RMP were observed in Hysun33 (14.5%) and Lacomca (5.0%) cultivars (Fig. 3).

During 4 days of re-aeration after hypoxia (Figs. 2 and 3), root and leave RMP were decreased smoothly and finally at the end of post aeration, their amounts were lower than their control values. These values might be an indication of membrane recovery.

3.3 Root and Leave Total Protein

In aerated plants, total protein (TP) of whole plant (root and leave) was increased with time. However, the average amounts of TP in leaves (2134.90 µg g^{-1} FW) of control plants were much higher than roots (218.72 µg g^{-1} FW); hence, leaves protein was associated much more than root in TP of whole plant (Figs. 4 and 5). Although in control plants, root and leaf TPs were varied at early days of experiment, the differences due to the age and cultivar were also statistically significant (*P < 0.05*). Hence, the differences in TPs of root and leave of control plants at the end of experiment were statistically significant.

Under hypoxic condition, additions of root and leave TP were observed in different cultivars in comparison to control plants. After four days of hypoxia, the highest and the lowest additions of root TP were obtained in Record (100.8%) and Lacomca (26.6%) cultivars, respectively (Fig. 4). At the same time, the highest and the lowest leave TPs were measured for the same cultivar (490.1% and 94.9%, respectively) (Fig. 5).

In re-aerated plants which pre-exposed to hypoxia, root total protein was decreased sharply at early days of re-aeration and afterward reached to lower than their control values (Fig. 4). Simultaneously, leave TP follows same trend at early stage of re-aeration and their values were decreased sharply. However, during 4 days of re-aeration, reduction of leave TP was more in Record and Hysun33 and less in Progress and Lacomca cultivars (Fig. 5); consequently, after 4 days of re-aerated condition, Progress cultivar had higher leave TP in compare to its control plant.

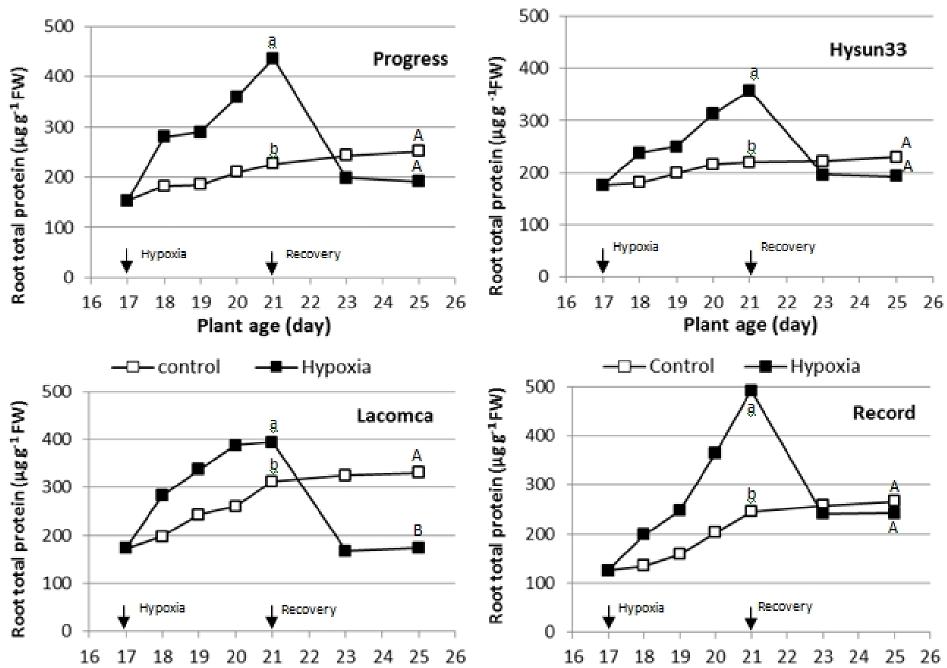

Fig. 4. Mean values (n= 6) of root's total protein (µg g^{-1} FW) of four sunflower cultivars under hypoxia and post-aeration. Differences in lower and upper case letters indicated significant difference between mean values (Using t-test, α < 0.05) at the end of hypoxia and post-aeration, respectively

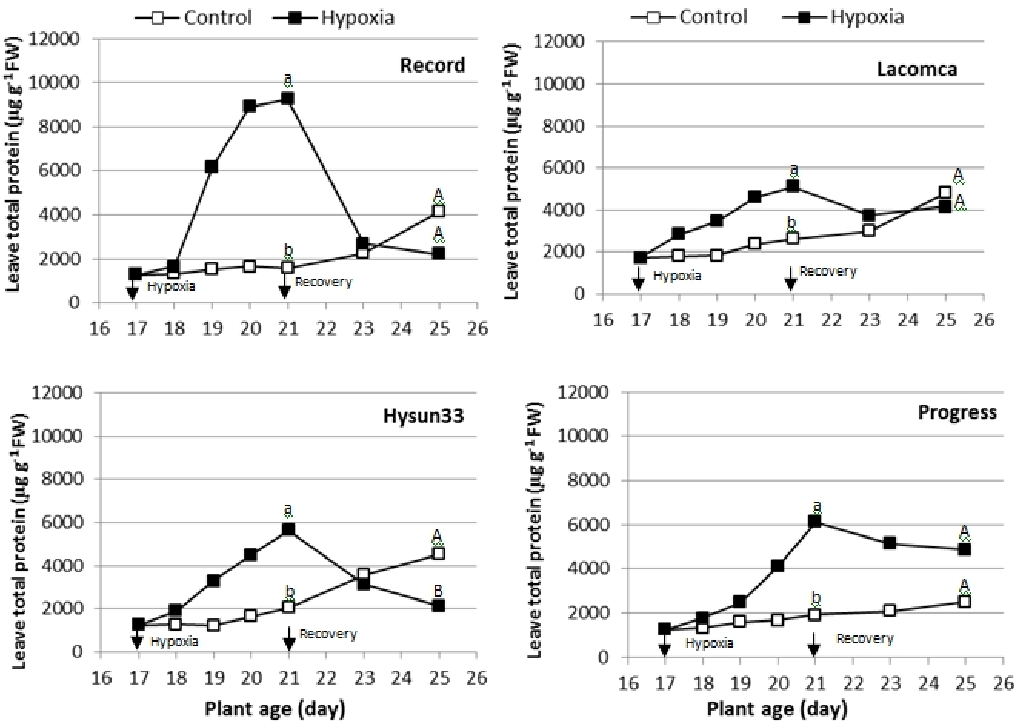

Fig. 5. Mean values (n= 6) of leave's total protein(µg g^{-1} FW) of four sunflower cultivars under hypoxia and post-aeration. Differences in lower and upper case letters indicated significant difference between mean values (Using t-test, α < 0.05) at the end of hypoxia and post-aeration, respectively

3.4 Discussion

In general, root and shoot dry weight of plants increase non-linearly with time and their growth rates are different in different plant as well as different condition [41]. In this study, growth rate of aerated plants was non-linear as expected; however, different cultivars had different growth rates. When plants were 25 days old, the highest and the lowest root and shoot dry weights were observed in Lacomca and Progress cultivars, respectively. The differences in growth rates of different cultivars and consequently, difference in their dry weights could affect their tolerance to environmental stresses; even in plants with the same age. Considering dry weight, the result was different in each cultivar; Lacomca and Record cultivars had higher plant dry weight in aerated condition in comparison to other cultivars, whereas under hypoxic condition, Lacomca and Record cultivars showed the lowest and the highest root dry weight reduction, respectively. In contrast, Progress and Hysun33 cultivars had shown lower root and shoot dry weights in aerated condition in comparison to other cultivars, but root dry weight reduction at hypoxic condition was low for Progress and high for Hysun33.

Hypoxia dramatically reduces root and shoot dry weights and consequently crop productivity [2,11,16]. In this experiment, four days hypoxic condition caused higher and non linear root dry weight reductions in Record and Hysun33 cultivars and lower and liner in Lacomca and

Progress. Simultaneously, the highest and the lowest shoot dry weight reductions were found in Hysun33 and Lacomca cultivars, respectively. The results of other experiments show similar trend. For example, Oxygen deficiency leads to alteration in cellular metabolism and reduction in growth of plants [42] and plant survival capacity [43]. Fakhri-Filsouf (2011) observed that 2 and 4 days of hypoxic condition in sunflower resulted into reduction of dry weights in roots (15.0% and 29.5%, respectively) and shoots (8.0% and 16.0%, respectively); these reductions were higher in root than in shoot [44]. In another experiment, Bai et al. [2010] observed that hypoxic condition of 4 to 20 days in two *Malus* species (*M. hupehensis* and *M. toringoides*) inhibited the growth of both species differently. *M. hupehensis* was more tolerant to hypoxia (root and shoot dry weight reduction, 14.2% and 22.9%, respectively) due to less oxidative damage compared to *M. toringoide*s (21.4% and 42.9%, respectively). Grassini et al. [2007] showed that waterlogging causes 5-60% reduction in sunflower production [36]. The reduction in sunflower yield is one of the consequent of reduction in vegetative growth during waterlogging. Bennicelli et al. (1998) observed that deficient soil aeration at 12 h causes reduction of root and shoot dry weight of KLG2210 cultivar of *Zea mays* [45].

In this experiment, the reductions of shoot dry weight under hypoxic condition were higher for Lacomca and Record and lower for Progress and Hysun33 cultivars in comparison to their control plants. This means that under hypoxic condition Lacomca and Progress cultivars were more tolerant to hypoxia in compare to others. This trend should be due to the higher growth rates of Lacomca and Record at aerated condition or the different behavior of sunflower cultivars in hypoxic condition such as ROS formation and lipid peroxidation [14,15]. Although alcohol and lactate dehyrogenases and pyruvic dehydrogenase complex activities, the key enzymes in fermentation process for generation of NAD^+, are important [46]. Plants decrease their oxygen consumption in response to low oxygen concentration by decrease in ATP consumption and inhibition of respiration via change in energy-conserving pathway of sucrose degradation [1]. All of mentioned processes as well as noticeable decreases in photosynthesis and RuBPC activity after flooding [47] are the reasons for reduction of plant growth.

Oxidative stress triggers membrane lipid peroxidation in the cells, consequently reduces membrane integrity and fluidity [28]. In such cases, increase in leakage of plasma membrane and reduction of RMP would be expected. Therefore, root and shoot dry weight reduction is most likely phenomena as a consequent of membrane lipid peroxidation. Shokrollahi et al. (2012) reported the amount of MDA (as an index of membrane lipid peroxidation) of root and leave for the same cultivars. They stated that four days of hypoxia increased MDA production in root and leave of sunflowers cultivars differently. The additions of root's MDA in Record (170.6%) and Hysun33 (115.4%) were higher than in Progress (51.7%) and Lacomca (42.3%) cultivars. Simultaneously, Lacomca has the highest addition of leave's MDA (151.1%), and Hysun33 cultivar had the lowest amounts of leave's MDA (9.6%) in comparison to their control [48]. Their results indicated that the variation in lipid preoxidation of different cultivars might be one of the reasons for different dry weights in different cultivars.

In this experiment, root and leave relative membrane permeability (RMP) in aerated plant were increased with plant age. The rise of RMP has happened after transferring plant to hydroponic condition. Therefore increase in RMP might be due to transferring plant to new medium and/or increasing in the age of plant (Figs. 2 and 3). In addition, hypoxic condition leads to increase root and leave RMP in four cultivars and RMP production increased daily similar to control plants. Other studies also show that hypoxic condition raises the amount of

RMP in plant parts. For instance, Jamei et al. [2009] reported that 192 h hypoxia increased RMP in leaves of *Zea mays* by 148% in comparison to control plant. Malekahmadi et al. [2005] shown that 3, 5 and 7 days of waterlogging lead to a significant raises of electrical leakage in leaves of *Capsicum annum* [29]. Hypoxic condition in two *Malus* species (*M. hupehensis* and *M. toringoides*) also led to increase RMP [30].

The relation between the reduction of plant parts and the addition of RMP is an important subject in hypoxic condition. According to our results, Progress cultivar had the lowest reduction of root dry weight and the highest addition of root RMP; however, the highest reduction of root dry weight and the lowest addition of root RMP were observed in Record cultivar. The correlation between shoot dry weight reduction and the additions of leave RMP was different in compare to root's reaction. The highest reduction of shoot dry weight was correlated with the highest addition of RMP in Hysun33; simultaneously the lowest reduction of shoot dry weight and the lowest addition of RMP has happened in Lacomca cultivar. Therefore it can be concluded that leave's RMP was more correlated with shoot dry weight reduction than root.

In aerated plants, firstly, plant total protein was smoothly increased with the age of plant and secondly, the amount of total protein was much higher in leaves than roots; hence, the trend of plant total protein mostly determined by the leave protein. Under hypoxic condition, addition of root and leave total protein was observed in treated plants in comparison to their controls (Figs. 4 and 5). Additional of total plant protein in Record cultivar was significantly different ($P < 0.05$) in comparison to other cultivars. However, after four days of hypoxia, the highest addition of root and leave total protein was observed in Record and the lowest was measured in Lacomca cultivars. Simultaneously, the same cultivars showed the highest and the lowest plant dry weight reduction, respectively. Therefore, it could be mentioned that addition of proteins such as antioxidant enzymes in whole plant which synthesized under hypoxic condition had affected plant tolerance.

Re-aeration after hypoxia didn't improve the status of plant and caused root and shoot dry weight reduction in all cultivars (Fig. 1). Therefore, the adverse effects of hypoxia could be seen continuously; even when plants settled in aerated condition. Our result indicated that after four days of post aeration the highest reduction of root and shoot dry weights were occurred in Record cultivar, whereas the lowest reduction were observed in Progress cultivar. Similar results were reported for different crops in different experiments. For example Garnczarska et al. [2004], reported that exposure of lupine roots to increased oxygen availability after plant imposed to hypoxia caused a two fold increase in concentration of free radicals [49]. Drew [1997] suggested that perturbation of the cell structure and function during post-anoxia can be far more severe than during the period of uninterrupted anaerobiosis [2]. In Skutnik and Rychter [2009] study on *Barley* leave, it has been reported that production of ROS increased during post-aeration for 1 h in plant hypoxically pretreated for 4 h [50]. Our result indicated that during re-aeration the amount of root and leave RMP (Figs. 2 and 3) and leave total protein (Figs. 4 and 5) were dropped in all cultivars and finally approached to their control values.

4. CONCLUSION

Hypoxic condition causes plant dry weight reduction in four cultivars. The dry weight reduction was higher in root than shoot. However, Lacomca and Progress cultivars with less reduction of shoot and root dry weight showed more tolerance to hypoxia than Record and Hysun33 cultivars. Re-aeration after 4 days of hypoxia leads to continued reduction of root and shoot dry weight. Under hypoxic condition, addition of root and leave RMP and total protein were observed in hypoxic condition. Increase in leave RMP was more correlated with shoot dry weight reduction; however, this trend was not the same in root.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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