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The effects of seasonal changes on biochemical variations of some Citrus species

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Abstract

Seasonal changes cause an array of physiological, biochemical and morphological changes in plants, thereby affecting plant growth and development. The measurement of some biochemical indexes is a method for the evaluation of cultivars and the explanation of their behavioral patterns from normal to severe environmental changes. This study was carried out to investigate the effects of seasonal changes on the changes of antioxidant capacity, total protein, peroxidase (POD) and superoxide dismutase (SOD) in five citrus species (the Valencia orange, Kinow tangerine, Mexican lime, Persian lime and Darab native orange) during the four seasons of the year with three replications in the statistical design split plot. The leaves of these species were sampled in May, August, November and February in the range of the average temperatures 21-25, 31-35, 15-20 and 10-15 ° C, respectively. Results show that amount of protein, total antioxidant capacity and POD in the spring (the time of fruit growth and development) was great. The comparison between the data averages demonstrated that the protein and the POD enzyme have the minimum activity in the summer, whereas the SOD had the maximum activity in the summer (as the hottest season of the year).The amount of POD enzyme in Persian lime and Mexican lime was considerably greater than that of the other investigated cultivars. These two cultivars can be a source of producing the commercial POD. The greatest amount of protein was observed for the Darab native orange and Persian lime and the smallest amount was observed in the Valencia orange in the summer.

Key words : citrus, antioxidant enzyme, environment, stress, superoxide dismutase

INTRODUCTION

The environmental stress results in the oxidative stress through producing and accumulating reactive oxygen species (ROS). Oxygen radicals could be controlled via antioxidant compounds. Still, a risk for severe cellular damage might take place when ROS are produced excessively under stress circumstances [1]. Seasonal alterations can be represented by variations in plant metabolism and have influences upon biosynthetic pathways [2] Plants have evolved a countless number of defense systems to survive continuous biotic attacks perpetually making changes to weather and other environmental conditions [3]. However, the plant antioxidant response varies with such exogenous factors as plant development environments, contributing to resistance or sensitivity [4]. Moreover, the lipid membrane free radical-induced peroxidation is not only a reflection but also a measure of stress-induced cellular damage [5]. In order to deal with the oxidative damage under considerably adverse circumstances, plants have produced antioxidant defense systems like the antioxidant enzymes SOD, APX, POD, and CAT [6]. The antioxidant enzymes levels are higher in tolerant species than those in sensitive species under miscellaneous environmental stresses [5]. The antioxidant activity is principally pertinent to the producing of chemical agents utilized for the plant protection. The plant free radical scavenging action observation can function as a monitor for the stressor conditions (e.g. seasonal and climatic variations) which generate these antioxidant compounds. Alternatively, changes in antioxidant compounds and enzyme activities reveal the effects of environmental stresses upon plant metabolism and have impacts on the cellular antioxidant defense capacity. As mentioned above, the antioxidant activity varies with the physiological and biochemical parameters [7]. Accordingly, enzyme activities are regarded as a significant indicator in plant response and behavior toward seasonal variations. A change in both time and location makes the seasonal environment complicated to foresee the plant responses to the varying conditions of the environment [8]. The equilibrium between ROS production and activities of antioxidative enzyme specifies whether oxidative signaling or damage will take place [9]. POD is a crucial multifunctional enzyme and comprises roughly 15% of the entire cellular proteins [10]. POD participates in certain essential physiological processes and is omnipresent in plants [11]. PODs adjust the cellular H2O2 levels and ROS production and catalyze the H2O2 reduction by applying a variety of substrates [12]. The DPPH radicals scavenging activity tends to be utilized as a basic screening method for testing the antiradical activity of a great number of compounds. Lester, 2008 [13] proved that since antioxidants can scavenge/neutralize ROS, the tissue with high antioxidant capacities would show further resistance to oxidative stress than that with lower antioxidant potentials.

The enzyme superoxide dismutase (SOD), a radical superoxide scavenger, is a vital instrument to protect plants against the oxidative stress and makes O2- into H2O2, which is, then, converted to H2O by ascorbate peroxidase (APX) through the oxidation of AA or by catalases (CAT) [14]. Under thermal stress conditions, namely heat or chilling temperatures, metabolic and physiologic plant processes are disrupted. As a result, there will occurs a protein aggregation and denaturalization in chloroplasts and mitochondria, destruction in membrane lipids, and a production of toxic compounds, thereby leading to the ROS overproduction [15]. Plants are less vulnerable to climatic variations, probably due to their natural properties and great adaptability [8].

This occurs due to the differential efficiency of antioxidants in neutralizing the noxious effects of ROS on the cells. The purpose of the present study is to contribute to a better understanding of the physiological responses of the Valencia orange, Kinow tangerine, Mexican lime, Persian lime and Darab native orange plants to the seasonal changes. In fact, the influence of the four seasons on the contents of proteins, peroxidase (POD), antioxidant capacity, and superoxide dismutase (SOD) will be investigated in different citrus species.

MATERIAL AND METHODS

Four experiments (each in one season) were carried out. The summer, fall, winter and spring tests were done in February/March, May/June, August/September and November/December, respectively. The cultivars in the test were 7 years old trees of five citrus species (the Valencia orange, Kinow tangerine, Mexican lime, Persian lime and Darab native orange) and the leaves were used and they were selected randomly from the mature and immature leaves. The temperature range of the sampling days was chosen based on the ten-year average temperatures of each season, which had been recorded at the synoptic station in the sampling location.

Enzyme extracts were obtained at 4ºC. For enzyme extracts, 0.5 g of the leaf specimens was homogenized with 50 mM potassium phosphate buffer (pH 7), containing 0.5 mM EDTA at 2ºC (w/v) polyvinylpolypyrrolidone (PVPP). The samples were centrifuged at 14,000 rpm for 15 min, and supernatants were applied to measure the enzyme activity. The whole spectrophotometric analyses were evaluated by Shimadzu (UV 1800) [16]. Superoxide dismutase (SOD) activity was estimated by its capability to prevent the photochemical decrease of nitro blue tetrazolium (NBT) at 560 nm. The reaction mixture (1 mL) was composed of 75 µM NBT, 13 mM L-methionine, 0.1 mM EDTA and 2 µM riboflavin in 50 mM potassium phosphate buffer (pH 7). The reaction and control mixture were laid for 15 min in 300 µmol m^{-1} s^{-1} irradiance at 25ºC. A non-irradiated reaction blend was employed as blank.

One unit of the SOD activity was regarded as the amount of SOD needed to create a 50ºC inhibition of the NBT photochemical reduction. The specific enzyme activity was considered as units per mg of the fresh wet leaf [17]. The Peroxidase (POD) activity was measured based on Ballester et al, 2006 [16]. The reaction blend constituted 475 µL H2O2 100 mM , 475 µL guaiacol 45 mM, and 100 µL enzyme extract in a final volume of 1000 µL. The reaction was launched by adding the enzyme extract. The tetraguaiacol formation was estimated at 470 nm. One unit of the enzymes was defined as the quantity of enzyme to disintegrate 1 µM of H2O2 per min at 25□C and the enzyme activity was viewed as units per g of the fresh wet leaf. The antioxidant capacity of the leaves was assessed in accordance with the method of Abd-Ghafar et al, 2010 [18].

Measurement of total protein:

The water-soluble proteins concentration in the supernatants of each leaf extract was measured by the procedure of Bradford, 1976 [19] utilizing the bovine serum albumin (BSA) as standard. Each supernatant was tested three times for both protein concentration and enzyme activities.

Statistical analyses:

The experiments were conducted using a completely randomized design with three replications. A preliminary test was run prior to the main experiment reported here. Data were analyzed as a 2-factor linear model via PROC GLM procedure by SAS software (ver. 9.1 2002-2003), where treatments and storage time were the factors.

RESULTS

The effects of seasonal environments on total protein changes:

The effects of the cultivar, various harvesting times and their interaction on Protein were significant ($P < 0.01$). The comparison between the data averages indicated that within species had a great effect on the amount of leaf protein. In autumn, due to the ripening of fruits, the amount of protein increases once again. In comparison with the species, the Darab native orange showed the highest protein activities (15 mg/swgr) and Kinow tangerine, (12.3 mg/swgr, respectively) in the autumn. Persian lime showed exceptional activities (23 activity/g FW) in the spring it had the second lowest activity, as compared to the other cultivars (Fig 3).

The effects of seasonal environments on total antioxidant capacity changes:

The effects of the cultivar, various harvesting times and their interaction on antioxidant capacity were significant ($P< 0.01$). It was indicated that the content of total antioxidant capacity was greater in the leaves in the spring (58%) and is the lowest in the leaves in summer (13%) and autumn (11%) and after that it increased in the winter (52%) (Fig 4).The highest antioxidant capacity in the winter was observed in Kinow tangerine (56%) and Persian lime (59%) (Fig 6).

The effects of seasonal environments on POD changes:

The effects of species, various harvesting times and their

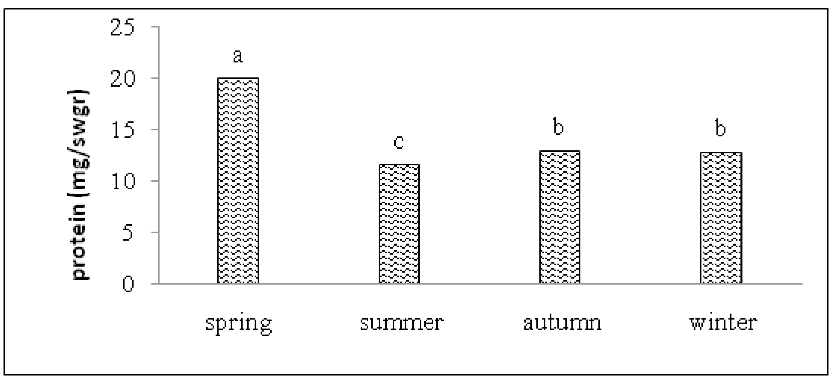

Figure 1 : The simple effect of seasons on the total protein content.

interaction on POD were significant ($P < 0.01$). As can be observed in the present study, POD had a strong activity in the spring (275 U/mg FW min), while it showed the lowest activity in the warmest time in the summer(175 U/mg FW min) after that observed a re-increasing of POD in the autumnal leaves(200 U/mg FW min) (Fig 7). As compared to the genotypes, Mexican lime and Persian lime showed the highest POD activities in the 4 seasons (400 U/mg FW min) and Valencia had the lowest activity (50 U/mg FW min) (Fig 8).

The effects of seasonal environments on SOD changes:

The results indicated that the effects of the cultivar and various harvesting times on the SOD enzyme activity are significant ($P< 0.01$) and interaction between cultivar and various harvesting time factors in regard to the SOD activity is not considerable. As can be seen in figure10 the high temperatures increased the SOD enzyme activity. In this study, the temperature rise led to a remarkable increase in the SOD activity.SOD was more important in kinow tangerine and did not show any significant difference, as compared with the persian lime (1.7 U/mg /FW) (Fig 11).

DISCUSSION

It was revealed that the amount of total protein is higher in the young and mature leaves in the spring and is the lowest in the summer leaves. Shao[20] showed that in the spring the plant makes

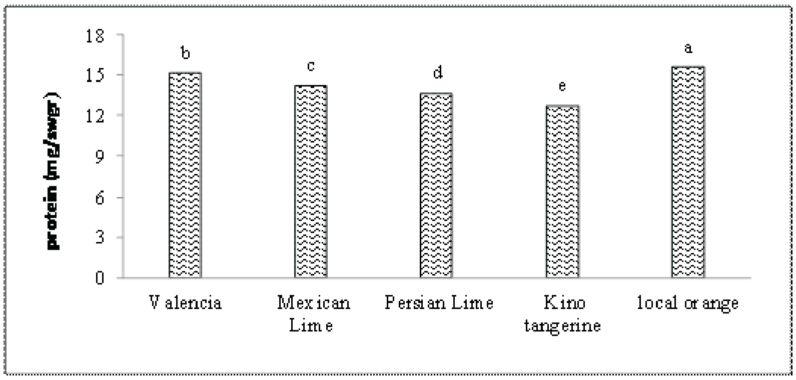

Figure 2 : The simple effect of species on the total protein content

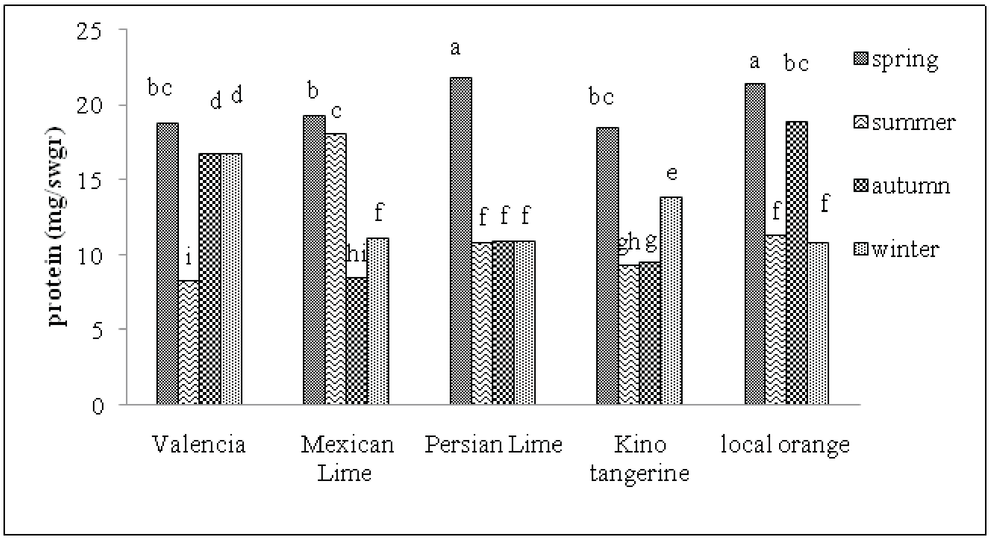

Figure 3 : The interaction of season and species effects on the total protein content

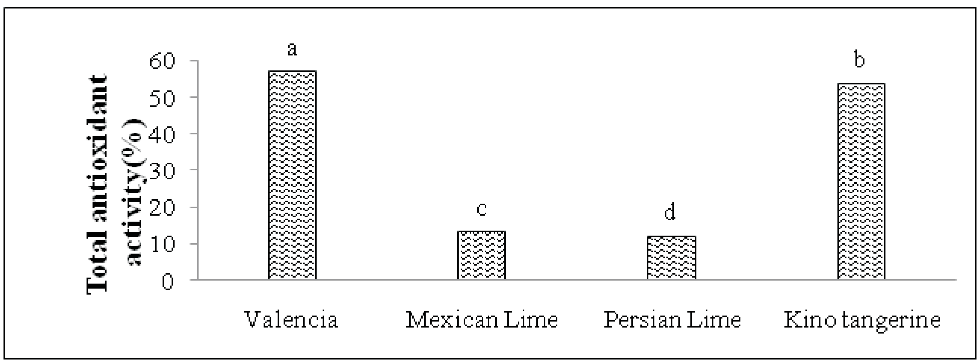

Figure 4 : The simple effect of seasons on the total antioxidant capacity

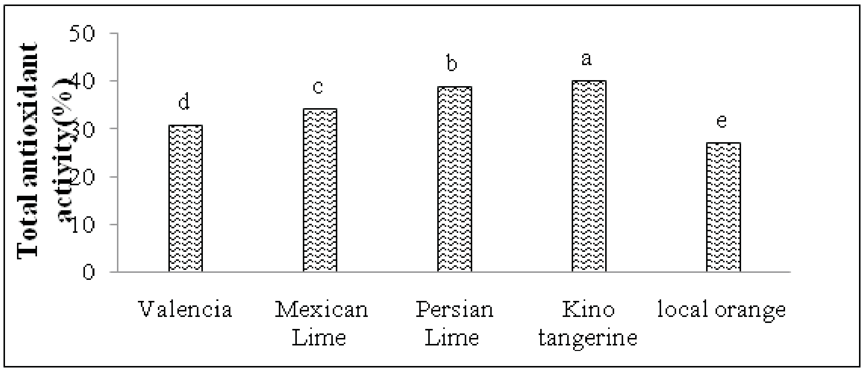

Figure 5 **:** The simple effect of species on the total antioxidant capacity

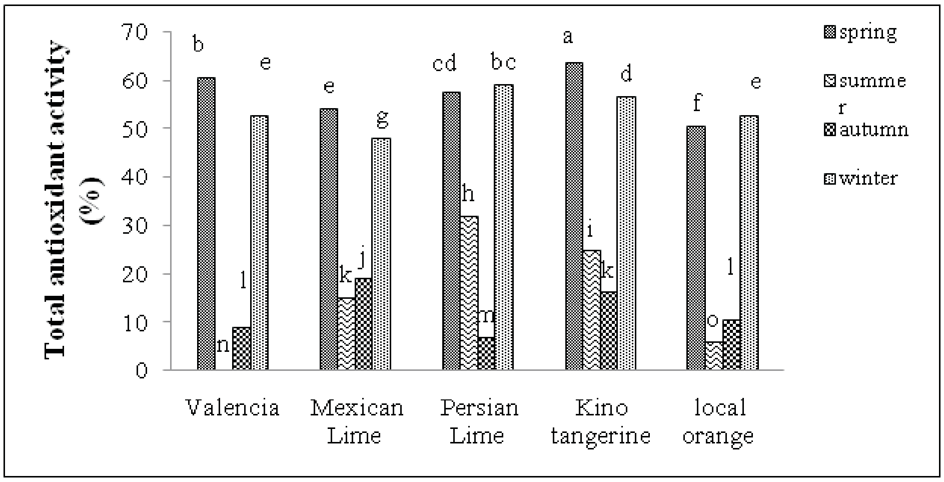

Figure 6 : The interaction of seasons and species effects on the total antioxidant capacity

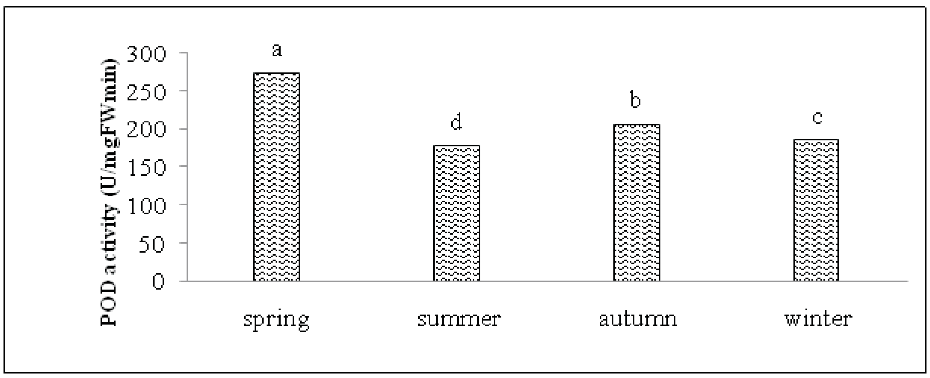

Figure 7 : The simple effect of seasons on POD enzyme activity

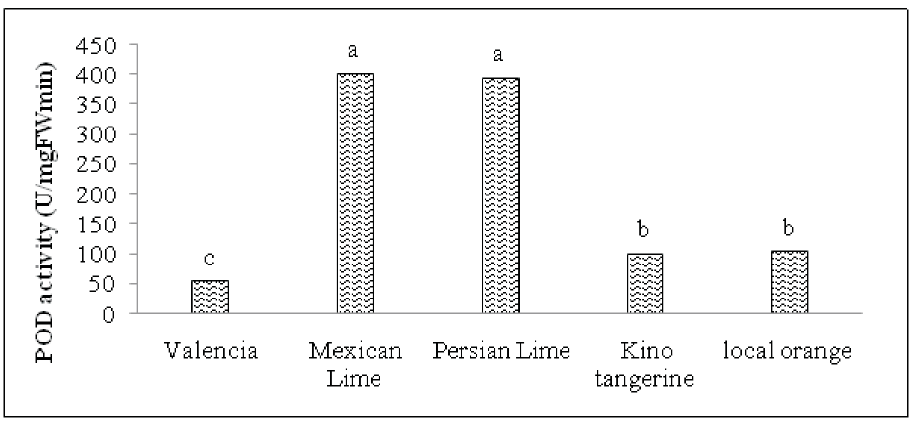

Figure 8 : The simple effect of species on POD enzyme activity

use of its whole capacity for survival. A decline in the protein content may be in view of inhibiting protein synthesis or protein degradation [15]. Changes in the levels of antioxidants may also take place during the aging of the plant, which occurs concomitantly with higher protein degradation, and loss of chlorophyll [6]. The high temperature in the summer (the ten-year average temperature 38ºC) can result from a reduction in the amount of protein in this season. In a similar investigation, [15] was reported that a drop in the total protein is an indirect damage arising from high temperatures. The main reason for this phenomenon in the summer could be the transmission of the nutrients from leaves to fruits. Moreover, leaf aging may play a part in this case [21]. Another reason is the plant dormancy under unfavorable environmental conditions. During unfavorable environmental circumstances, the dormancy takes place and the plant activities are interrupted [22]. The rapid accumulation of Heat Shock Proteins (HSPs) in the sensitive organs can play a major role in the protection of the metabolic apparatus of the cell, thereby acting as a key factor for plant conformity and survival under heat stress [23]. It appears that the spring and fall temperatures were the desirable growth ones for most of the cultivars, resulting from higher protein levels. The climate had a conspicuous influence on the plant and the enzyme indexes. The cool spring weather leads to an increase in cell reproduction, thereby producing larger fruits at the end of the season. Thus, the fruits growing in the warm climate are smaller than those growing in the cooler climate[24].

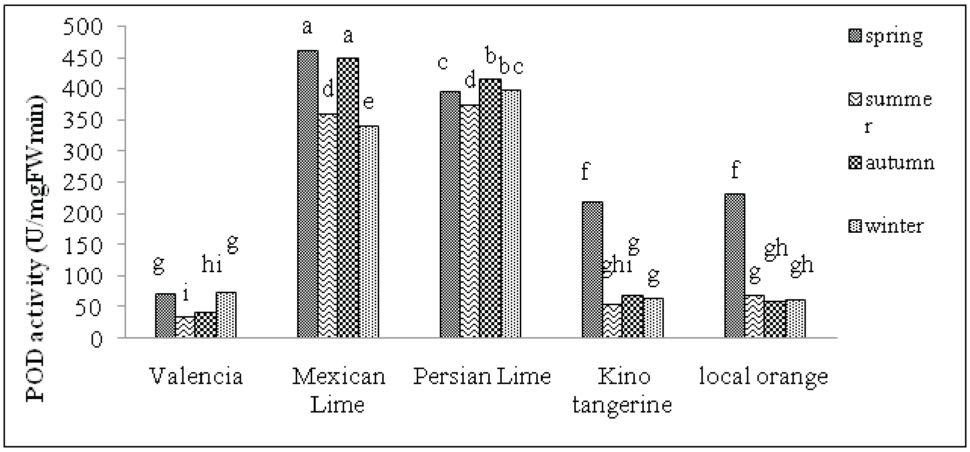

Figure 9 : The interaction of seasons and species effects on POD enzyme activity

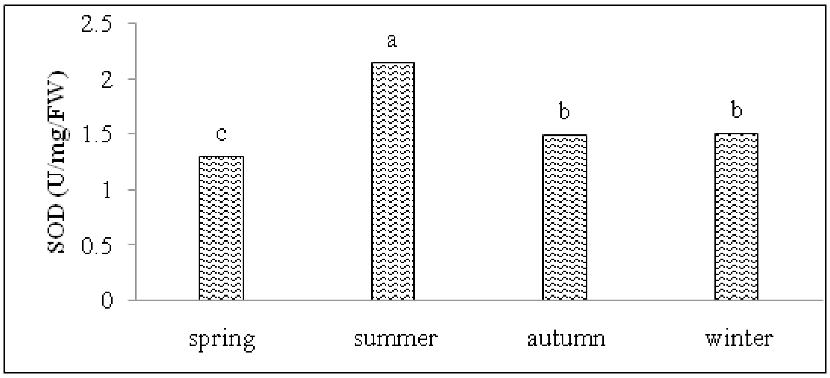

Figure 10 : The simple effect of seasons on SOD enzyme activity

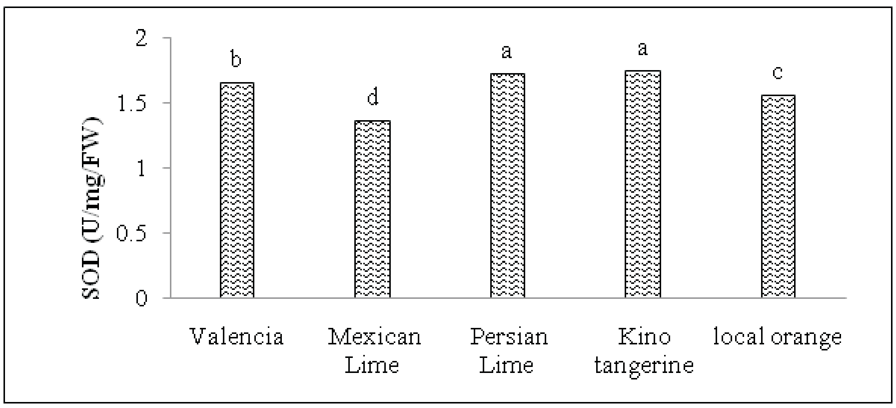

Figure 11 : The simple effect of species on SOD enzyme activity

Antioxidants have to play a direct role in protecting plants in an inappropriate environment [25]. Additionally, it has been indicated that the content of total antioxidant capacity was greater in the leaves in the spring and was the lowest in the leaves in the summer and fall and then increased in the winter (Fig 4). It is already known that the alleviation of oxidative damage tends to be correlated with an efficient antioxidant capacity under low temperature stress [26]. In this experiment, the highest antioxidant capacity in the winter was observed in Kinow tangerine and Persian lime (Fig 6). This result discloses that these species can greatly ameliorate their antioxidant capacity under low temperature. This result was in consistency with the previous finding by Rapisarda et al, [27] who showed the increase of the antioxidant capacity during low-temperature stress in the Citrus fruit. Increases in the activity of leaf antioxidant systems have been proved to be indispensible in the acclimation of plants to winter conditions [28].

The formation and level of such antioxidants may be seasonality marked in response to changes in the environmental conditions, even in the absence of anthropic interferences. This is plausible because the seasonality in solar irradiation, photoperiod, temperature and relative humidity, among other meteorological factors, as well as influencing the stomata aperture, regulates the photosynthesis and respiration processes on chloroplasts and mitochondria and thus the natural production of ROS in the cells. Variations in the levels of antioxidants may also occur during the aging of the plant, which occurs concomitantly with higher protein degradation, and loss of chlorophyll [6]. The key role of the POD enzyme activity has been proved for plant development processes, which scavenge the H2O2 in cell organelles [29]. As can be observed in the present study, POD had a strong activity in the spring, while it showed the lowest activity in the warmest time in the summer after that observed a re-increasing of POD in the autumnal leaves (Fig 7).

The re-increasing of POD in the autumnal leaves can be a result of approaching the induction time and beginning the cold days in view of the specific fruit ripening time in certain species. It has been observed that POD includes the auxin catabolism. For example, a research on strawberry prior to ripening revealed a reduction in the amount of auxin and subsequently the greatest POD enzyme activity [30]. PODs are involved not only in scavenging H2O2 but also in plant growth, development, lignification, suberization, and cross-linking of cell wall compounds [12]. As compared to the genotypes, Mexican lime and Persian lime showed the highest POD activities in the four seasons. Tolerant plants often have higher POD activity than sensitive plants under stress conditions; this is true for salt-tolerant tomato [31].

Recently, there has been an increasing interest in the production of enzymes from the plant tissue culture. The studies in the literature have shown that call from plant sources like cowpea, green pea and radish are the possible sources of the commercial production of peroxidase [32]. At present, peroxidase is commercially produced only from the horseradish roots. In addition, other studies revealed that there is another potential peroxidase source from *Citrus aurantifolia* (lime) flavedo callus [33].

Superoxide dismutase makes superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2), POD reduces H_2O_2 to water utilizing a miscellany of substrates as electron donors. Ascorbate peroxidase makes use of ascorbate as an electron donor to decrease H_2O_2 to water, and CAT turns H_2O_2 into water and oxygen. In the presence of O_2^- and H2O2, the trace amounts of transition metals can give rise to the highly toxic hydroxyl radical (OH^-). The rapid detoxification of both O_2^- and H_2O_2 is, therefore, necessary to prevent the oxidative damage. Numerous studies indicate that the activity of antioxidant enzymes is correlated with plant tolerance abiotic stresses [5].

As can be seen in Figure10, the high temperatures increased the SOD enzyme activity. In this study, the temperature rise led to a remarkable increase in the SOD activity. The enhancement of the SOD activity in summer indicated that it has the highest concentration of the superoxide radicals at this temperature, as compared to the other temperatures. Superoxide dismutase is the first line to protect the antioxidant enzyme against the oxygen radical damages [34].

During the hottest seasons of the year, the respiratory rates of the plants are higher and can also induce an increasing antioxidant response due to a consequent higher ROS production in mitochondria. This respiratory increase is associated with an increase in the NADH synthesis [35]. which is related to the production of enzymes such as SOD. SOD was more important in Darab local orange and did not show any significant difference, as compared with the Valencia orange. Zhang et al[36] found a significant copper-zinc SOD increase in *Elsholtzia haichowensis* during the enhancement of respiration rates induced by some stress factors. Therefore, plants have evolved various mechanisms to cope with the stresses imposed by naturally fluctuating environmental conditions, generally modulated by the gene expression and synthesis of compounds that may result in higher stress tolerance.

CONCLUSION

Overall, it has been concluded from this study that the quantity of protein, entire antioxidant capacity and POD in the spring (the fruit growth and development time) is remarkable. Through comparing the data averages, it has been revealed that the protein and the POD enzyme have the least activity in the summer, while the SOD has the greatest activity in the summer (as the hottest season). Also, the proportion of POD enzyme in Persian lime and Mexican lime is significantly more than that of the other studied cultivars. These two cultivars can produce the commercial POD. The highest amount of protein has been obtained for the Darab native orange and Persian lime and the lowest amount has been attained in the Valencia orange in the summer.

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