

Quantitative and qualitative comparison of antioxidant activity in the flavedo tissue of three cultivars of citrus fruit under cold stress

Mansour Afshar Mohammadian, Zahra Khosravi Largani, Reza Hasan Sajedi

Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran

*Corresponding author: afshar@guilan.ac.ir

Abstract

Low temperatures constitute a major risk for the production of citrus fruit because citrus plants are sensitive to low temperatures and harvesting coincides with winter. The production of reactive oxygen species (ROS) is a biochemical change in plants facing cold stress. Plants may increase levels of antioxidants as an adaptation for preventing oxidative stress. In this study, three citrus cultivars (*Citrus unshiu*, *Citrus sinensis* and *Citrus lemon*) were examined to compare the activities of three antioxidants: ascorbate peroxidase (APX), peroxidase (POD) and superoxide dismutase (SOD), in the flavedo tissue of unripe and ripened fruits. Enzyme activity levels were examined under various cold temperature treatments (range: 3, 0, -3 and -6°C) and a control (15°C). The results indicate that SOD activity was highest in all three cultivars under cold treatments. Unripe and ripened *Citrus unshiu* fruits had the maximum level of APX enzyme activity in the cold treatments. Similarly, the activity of POD enzyme in *Citrus unshiu* fruits significantly increased during temperature reductions. Overall, *Citrus unshiu* appears the most resistant to cold treatments compared with the other two cultivars.

Keywords: Citrus fruit, Cold stress, ROS, APX, POD, SOD.

Abbreviations: ROS (Reactive oxygen species), APX (Ascorbate peroxidase), POD (Peroxidase), SOD (Superoxide dismutase).

Introduction

Chilling, as a result of low, non-freezing temperatures, is one of the major environmental factors influencing the growth, development, survival and geographical distribution of plants (Levitt, 1972). While citrus are grown across a variety of climatic conditions, ranging from hot-humid equatorial conditions through to warm-subtropical climates (Bowman, 1956); citrus distribution is often limited by sensitivity to low temperatures (Guo et al., 1998; Guo et al., 2000). Thus, optimum temperatures for the growth of citrus species range between 22 - 30°C (Sun and Ma, 1999). Furthermore, citrus fruits have a long life on the tree; therefore, cell damage may occur in the flavedo tissue (i.e. the outer pigmented layer of the peel) when fruit are exposed to temperatures less than 12°C (Schirra and Mulas, 1995; Martinez and Lafuente, 1997; Kietsuda and Diane, 2010). A common consequence of exposure to low temperatures is the increased production of reactive oxygen species (ROS), namely: hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\cdot) and superoxide (O_2^-) (Asada, 2006). Environmental stresses like cold temperatures can inhibit the growth and photosynthetic capacity of plants when production of ROS is greater than the level of antioxidant defense (Zhang et al., 2001). Thus the accumulation of ROS induces oxidative stress to proteins, membrane lipids and other cellular components (Iturbe et al., 1998). The antioxidant defense system in the plant cell therefore includes both enzymatic (e.g. ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), etc.) and nonenzymatic (Sudhakar et al., 2001; Peng et al., 2011). The enzyme SOD is a metalloprotein that catalyzes the initial step in the water-water

cycle in chloroplasts and the dismutation of O_2^- to H_2O_2 and molecular oxygen (Scandalios 1993; Garcia et al., 2001; Molassiotis et al., 2006; Cervilla et al., 2007). The role of SOD during environmental stress has received much attention because ROS is produced in plants under a range of adverse conditions (Bowler et al., 1992). SOD is the only plant enzyme capable of scavenging O_2^- , whereas H_2O_2 can be catabolized directly by catalases or with the help of various reductants (e.g. APX and the heterogenous group of guaiacol peroxidases) (Dat et al., 2000). For instance, a concentration of 10 μM H_2O_2 caused a 50% inhibition of CO_2 assimilation in isolated chloroplasts (Kaiser, 1976). The ROS H_2O_2 , is a less reactive oxidant when compared to other ROS; nevertheless, H_2O_2 may be particularly harmful because it is relatively stable and may spread within or among cells by diffusion. Furthermore, APX is a heme peroxidase, and uses two molecules of ascorbate to reduce H_2O_2 to water thereby generating two molecules of monodehydroascorbate (MDHA) (Miyake and Asada 1992). Substrate affinity is also an important parameter in assessing the relative contribution of the different enzymes to H_2O_2 detoxification, and APX has a very high affinity to H_2O_2 (Mittler 2002). The enzyme POD is widely found in animals, plants and microbes and like most peroxidases use various phenolic substrates for H_2O_2 elimination, thus making them very useful as general indicators for oxidative stress (Gaspar et al., 1991; Hiraga et al., 2001). Protection of cells by antioxidants is believed to be a major mechanism for resistance to oxidative injury (Kraus and Fletcher, 1994; Manish et al., 2011), hence the efficacy of resistance is dependent upon the

level of antioxidant enzyme activity (Lee and Lee, 2000). Therefore, cold tolerance in plants is primarily related to the activity of antioxidants, such as ROS scavengers to combat oxidative stress. So far, there have only been a few studies comparing the activities of antioxidant enzymes of various citrus cultivars exposed to chilling stress. In addition, since the flavedo tissue of the citrus fruit is the first protective layer facing environmental stress, the presence of antioxidants in the flavedo tissue (usually discarded as waste), may prove a rich source of antioxidants. The aim of this study was therefore to compare the activities of selected antioxidant enzymes in the flavedo tissue of unripe and ripened fruits of three citrus cultivars (*Citrus unshiu*, *Citrus sinensis* and *Citrus lemon*) under various cold temperature treatments.

Results

Changes in the activities of antioxidant enzymes of three citrus cultivars

Enzyme activity in the flavedo tissue of three citrus fruit cultivars showed *Citrus unshiu* had maximum APX activity, while *Citrus sinensis* had minimum APX activity in both unripe and ripened fruit. However, the highest level of APX activity in *Citrus unshiu* unripe fruits was at 3 °C, and 0 °C for ripened fruits (Fig. 1A). *Citrus lemon* had the highest activity of POD enzyme in unripe and ripened fruit at 0°C, while unripe and ripened *Citrus unshiu* fruit had the lowest POD activity (Fig. 1B). Similarly, *Citrus lemon* had the highest SOD activity and *Citrus sinensis* the lowest SOD activity in ripened fruit for all cold temperature treatments. However, overall there were no significant differences ($P < 0.05$) in the SOD activity of *Citrus lemon* during the treatments. *Citrus unshiu* showed the highest and lowest levels of SOD activity at -6°C and 15°C, respectively (Fig. 1C).

Analysis of enzymatic activities using zymogram

The results of the enzyme activities of unripe fruits of the three citrus cultivars using a zymogram indicated that POD enzyme activity reached maximum levels for *Citrus lemon* and minimum levels in *Citrus sinensis* in all treatments (Fig. 2). In the zymogram gel for SOD activity, two separate bands were observed in the flavedo tissue of unripe fruit, indicating at least two isoenzymes in three cultivars (Fig. 3). Meanwhile, the thickness of bands in the -6 °C treatment compared to the bands observed in the 15 and 0 °C treatments were highest in *Citrus lemon*, which correlates with the results previously found using a spectrophotometer.

Discussion

Environmental stresses increase the formation of ROS that then oxidize photosynthetic pigments, membrane lipids, proteins and nucleic acids (Smirnoff, 1993; Egert and Tevini, 2002). Several lines of evidence suggest that low temperatures alter cellular homeostasis by increasing the level of ROS at the transcript, protein and activity levels (Suzuki and Mittler, 2006). In addition, the induction of oxidative stress by low temperatures is likely to be the main factor contributing to frost injury in different fruits (Sala, 1998). For instance, Sala (1998) observed that chilling injuries were reduced in cold-tolerant mandarin cultivars that had a more efficient antioxidant system. Plants with high levels of either constitutive or induced antioxidants were reported to have greater resistance to oxidative damage (Sudhakar et al., 2001), so it can therefore be hypothesized that differences in stress tolerance among plant species and intraspecific genotypes are intrinsically associated with the

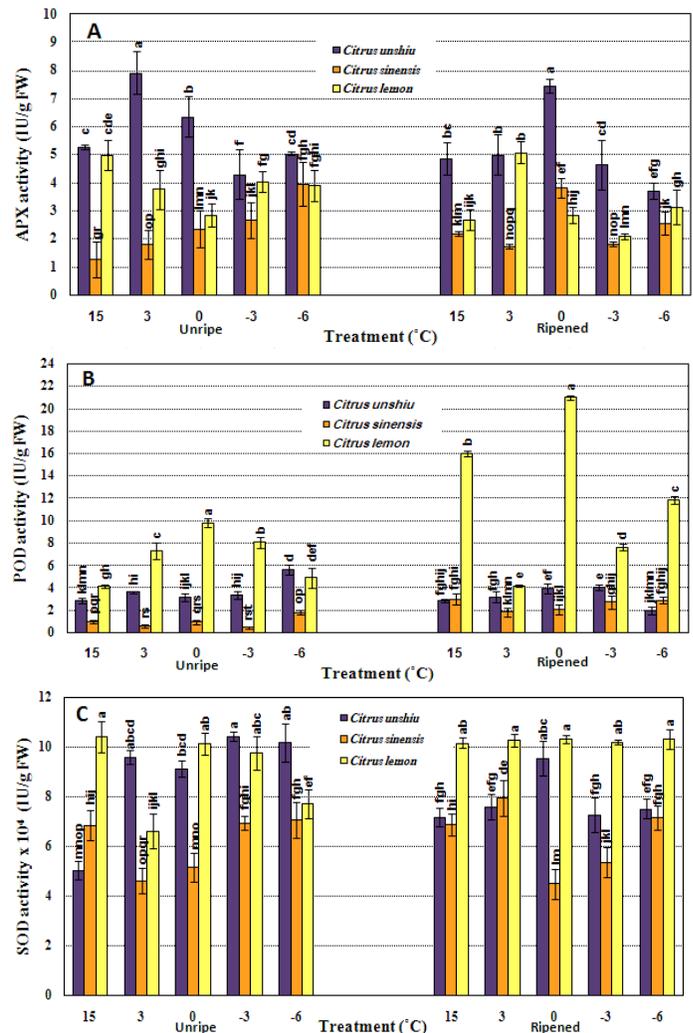


Fig 1. Changes in the activity of (A) ascorbate peroxidase (APX); (B) peroxidase (POD) and (C) superoxide dismutase (SOD) in the unripe and ripened fruit flavedo tissue of three citrus cultivars (*Citrus unshiu*; *Citrus sinensis* and *Citrus lemon*) under various cold temperature treatments: 3, 0, -3 and -6 °C. Control temperature 15°C. Results are mean of 3 determinations \pm SD. Different letters in the same column show significant differences at $P < 0.05$.

development of antioxidant systems under stress (Mittler, 2002). Dismutation of the superoxide radical by SOD may be the primary step in the defense against chilling treatment, thus playing a central role in the plant antioxidant system. The activity of SOD is inversely correlated with the rate of superoxide radical generation in chilling-sensitive plants (Lukatkin, 2002; Xihong et al., 2011). The results of this study indicated that the level of SOD activity in the flavedo tissue of ripened *Citrus lemon* fruit were significantly higher compared to unripe fruit ($P < 0.05$). However, no significant difference was found ($P < 0.05$) in the activity level of SOD in ripened *Citrus lemon* fruit across all temperature treatments, possibly indicating the active action of the SOD enzyme for scavenging superoxide radicals (Fig.1C). The flavedo tissue of unripe *Citrus unshiu* fruit showed a significant increase in SOD activity with decreasing temperature ($P < 0.05$), while this pattern was the same for ripened fruit from 15 to 0°C. Varying levels of SOD activity have been reported in a

suite of plants facing low temperatures (Scandalios, 1993; Foyer et al., 1994; Allen, 1995; Jevremović et al., 2010), which is attributed to the complexity of the detoxification system of ROS, since changing one enzyme may not change the capacity of the whole pathway. Recently, APX was proposed to be very important for adaptation to low temperatures (Peltzer et al., 2002; Morad et al., 2011). In this study, the APX activity was different under various low temperature treatments for different cultivars. For instance, as temperature decreased, the level of APX enzyme activity in the flavedo tissue of unripe *Citrus sinensis* fruit significantly increased ($P < 0.05$) (Fig. 1A), indicating the ability of APX enzyme for scavenging free radicals during temperature reductions. For unripe *Citrus lemon* fruits, the activity of APX enzyme significantly decreased in the 15 to 0°C temperature treatments ($P < 0.05$), indicating a higher sensitivity to low temperatures in comparison with *Citrus sinensis*. However, APX activity of *Citrus lemon* significantly increased after a 42 h chilling period ($P < 0.05$), suggesting that more time or lower temperature is required to increase its activity. The enzyme APX, which is localized in chloroplasts, mitochondria, microbodies, and cytosol, is essential to the decomposition of hydrogen peroxide in plants. Allen et al., (1997) and Mittler (2002) reported that transgenic tobacco plants expressing gene constructs for either cytosolic APX, or chimeric chloroplast-targeted cytosolic APX from pea plants, have increased protection against Methyl Viologen mediated membrane damage compared to untransformed control plants. These transgenic plants had increased protection from photooxidative stress (exposure to high light intensity and chilling temperature for 4 h). These results seem to indicate that increased scavenging of H_2O_2 in either the chloroplasts or cytosol can reduce oxidative stress in chloroplasts. The simultaneous expression of SOD and APX provided much better protection from oxidative stress than single expression of SOD or APX, showing the cumulative effect of two enzymes in ROS scavenging activity (Mittler, 2002). Peroxidase (POD) activity plays an important role in the oxidative degradation of phenolic compounds, which can lead to the production of brown polymers (Tomás-Barberán and Espín, 2001). Some authors mentioned a relationship between POD activity and physiological disorders in vegetables (Robinson, 1991); therefore POD activity in fruit peel may be linked to the higher cell damage as a response to stress (Li, 2003). The results of this research indicated that POD enzyme activity in the flavedo tissue of unripe *Citrus lemon* and *Citrus unshiu* fruit significantly increased during temperature reductions ($P < 0.05$) (Fig. 1C), which was further confirmed in zymogram gel observations (Fig. 2). The increasing activity of POD enzyme activity in the flavedo tissue of ripened *Citrus unshiu* fruit had the same pattern as unripe fruit during temperature reduction, possibly indicating the higher tolerance of *Citrus unshiu* compared to the other two cultivars. In general, however, the balance of SOD, POD and APX enzyme activities appears critical to cell survival during chilling stress. In conclusion, the results of the current study indicate that the SOD enzyme had the highest level of activity in the flavedo fruit tissue of all three cultivars under various cold treatments. In particular, the unripe and ripened fruits of *Citrus unshiu* showed maximum APX enzyme activity and *Citrus sinensis* showed minimum activity during temperature reductions. Also, the activity of POD enzyme in *Citrus unshiu* fruits increased significantly during temperature reductions. Therefore, *Citrus unshiu* seems to be more resistant to low temperature treatments when compared to *Citrus sinensis* and *Citrus lemon*.

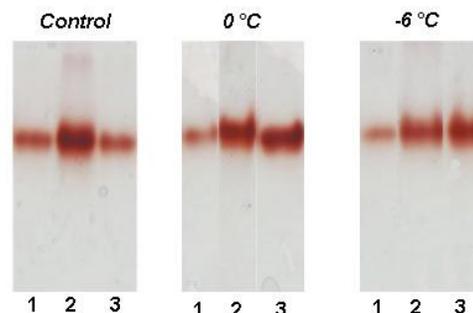


Fig 2. Native-PAGE of peroxidase (POD) in the unripe fruit flavedo tissue of three citrus cultivars (1 = *Citrus unshiu*, 2 = *Citrus sinensis* and 3 = *Citrus lemon*), indicating the maximum POD enzyme activity levels for *Citrus lemon* and minimum levels in *Citrus unshiu* under cold temperature treatments: 0 and -6 °C. Control temperature 15°C.

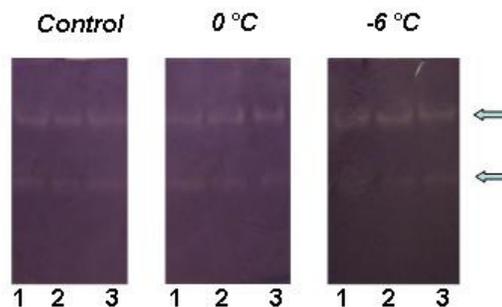


Fig 3. Native-PAGE of superoxide dismutase (SOD) showing two separate bands in the flavedo tissue of unripe fruits in three cultivars (1 = *Citrus unshiu*, 2 = *Citrus sinensis* and 3 = *Citrus lemon*) under cold temperature treatments: 0 and -6 °C. Control temperature 15°C.

Materials and methods

Plant materials and treatments

The fruits of three cultivars (*Citrus unshiu*, *Citrus lemon* and *Citrus sinensis*) were harvested from the garden of the Citrus Institute (Tonekabon city, Iran) in December 2009. According to skin color, fruit were either harvested before ripening (unripe) and after commercially matured ripening (ripened). Samples of the cultivar fruits were then stored for 10 h across a variety of cold temperature treatments (range: 3, 0, -3 and -6°C) and a control temperature (15°C). The flavedo tissues samples of unripe and ripened fruit for each cultivar were manually separated, frozen in liquid nitrogen and then stored at -20 °C prior to analysis.

Enzyme extractions

Samples of flavedo tissue (0.5 g) were homogenized in 1 ml of ice cold solution. For SOD and POD analysis, the solution contained 50 mM potassium phosphate buffer (pH 7), and 0.5

mM EDTA. For APX analysis the solution contained 1 ml of 50 mM cool potassium phosphate buffer (pH 7) containing 2% (w/v) vinylpyrrolidone (PVP) and 1 mM EDTA. The homogenates were centrifuged at 14000 rpm for 15 min, at 4°C and stored at -70°C for further experiments.

Determination of antioxidant enzyme activities

The activity of SOD was determined using the methods outlined by Giannoplitis and Ries (1977). Samples were placed into 1 ml of reaction solution (containing 13 mM methionine, 75 μ M nitro blue tetrazolium chloride (NBT), 0.21 mM riboflavin, 0.1 mM EDTA, 50 mM potassium phosphate buffer (pH 7) and enzyme extract) were dark adapted then briefly exposed to fluorescent light (15 min). The initial rate of reaction, measured by the difference in an increase in absorbance at 560 nm, is proportional to the amount of enzyme; therefore, one unit of SOD activity was defined as the amount of enzyme required for the inhibition of photochemical reduction of NBT by 50%. Similarly, POD activity was determined by measuring an increase in absorption at 470 nm, according to Kalir et al. (1984) where POD activity was determined by monitoring the increase in absorbance at 470 nm during the oxidation of guaiacol. One unit of peroxidase was defined as the amount of enzyme that caused the formation of 1 mM of tetraguaiacol per minute. To determine APX activity, a spectrophotometer was used to measure the rate of ascorbate oxidation at 290 nm for 2 min. The reaction solution used for this analysis contained 50 mM potassium phosphate buffer (pH 7), 0.1 mM EDTA, 0.5 mM ascorbic acid (AsA), 0.1 mM H₂O₂ and enzyme extract. APX activity was calculated by following the decrease in absorbance of AsA (extinction coefficient 2.8 mM⁻¹ cm⁻¹) according to Biyan Zhou et al., (2005).

Zymogram analysis for SOD and POD

Changes in the enzyme activities of unripe fruits of the three citrus cultivars were investigated using a zymogram for two cold treatments (0 and -6°C) and a control (15°C). Non-denaturing gel electrophoresis was performed according to Davis (1964) using 7.5% separating gel and 5% stacking gel at 4°C. Protein bands with SOD activity were visualised by activity staining, according to Beauchamp and Fridovich (1971). After 20–25 min incubation in a staining solution (contained: 50 mM potassium phosphate buffer (pH 7) containing 28 mM TEMED, 120 μ M riboflavin and 2.5 mM NBT) at ambient room temperature, the gels were placed into distilled water and exposed in a light box until the SOD activity bands became visible. For POD staining, gels were incubated in a solution of 25 mM potassium buffer (pH 7) for 15 min, then submerged again in a freshly prepared solution containing 18 mM guaiacol and 25 mM H₂O₂ in 25 mM K-phosphate buffer (pH, 7) until the POD activity-containing bands were visualized (Fielding and Hall, 1978).

Statistical analysis

Mean values (n = 3) of enzyme activity level were compared using a one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test (P < 0.05) using SAS 8.3 software (SAS Ins. Inc., Cary, USA).

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