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Transcript levels of antioxidative genes and oxygen radical scavenging enzyme activities in chilled zucchini squash in response to superatmospheric oxygen

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Abstract

The transcript levels of antioxidative genes including Mn-superoxide dismutase (Mn-SOD), Cu/Zn SOD, ascorbate peroxidise (APX), and catalase (CAT) were relatively constant during storage at 5 °C with high oxygen treatment in freshly harvested zucchini squash (*Cucurbita pepo* L. cv. Elite). However, the expressions of alternative oxidase (AOX) were induced slightly in squash treated with 60% and 100% oxygen for 3 days when compared with control squash. These increases in AOX transcript levels were correlated with the increased chilling resistance in the treated squash. The corresponding oxygen radical scavenging enzyme activities including SOD, APX, CAT, and peroxidise (POD) in treated samples were also higher than those in the control for the first 3 days at 5 °C. Transcript levels of AOX increased substantially between 3 and 6 days in all treatments suggesting the involvement of alternative respiratory pathway during chilling stress. All of the enzyme activities in 100% oxygen treated squash started to decline after 6 or 9 days of cold storage to a level comparable or lower than those of the control. These declines were correlated to the loss of chilling resistance in the 100% oxygen treated tissue as indicated in the chilling injury index. However, squash treated with 60% oxygen maintained elevated levels of all enzyme activities except POD and sustained the least chilling injury throughout the 15 days of storage at 5 °C. The oxygen radical absorbance capacity (ORAC) values and total phenol content remained high in squash treated with 60% and 100% oxygen for the first 9 days, then their levels in the 100% oxygen treated samples declined sharply while those in the 60% oxygen treated samples maintained elevated, indicating that both ORAC activity and phenolic content may also contribute to the resistance of tissue against chilling injury. The 100% oxygen treated squash showed the lowest respiration rate and 60% oxygen treated samples had the lowest ethylene production. These data may also be an indication of the low chilling injury in the high oxygen treated squash.

Keywords: Zucchini squash; Alternative oxidase; Scavenging enzymes; High oxygen; Chilling injury

1. Introduction

Symptoms of chilling injury can be the consequence of oxidative stress resulted from excess reactive oxygen species (ROS) that induce peroxidation and breakdown of fatty acids in membrane lipids (Lyons and Raison, 1970; Lyons, 1973). ROS scavengers, on the other hand, increase the degree of unsaturation of 18-carbon fatty acids in the polar lipids and act as antioxidant to reduce the severity of chilling injury (Wang and Baker, 1979; Purvis, 2002). Low temperature causes an increase in ROS levels and induces oxidative stress in plants (Karpinski et al., 2002). Defense against low temperature oxidative stress in plants relies on the tight balance of ROS levels by multiple mechanisms includes lipid peroxidation (O'Kane et al., 1996); the enzymatic ROS scavenging systems as described in Møller (2001) and Mittler (2002) with superoxide dismutase (SOD), catalase (CAT), the glutathione peroxidase system and thioredoxin system and ascorbate–glutathione cycle (Karpinski et al., 1993; Walker and McKersie, 1993; Dipierro and Leonardis, 1997). On the other hand, the presence of ROS avoidance genes includes alternative oxidase (AOX) in thylakoids (immutans)

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reduces the production of peroxides and prevents oxidative damage in mitochondria (reviewed in Mittler, 2002). This concept of AOX acting as an antioxidant enzyme has been shown in isolated bell pepper mitochondria (Purvis, 1997) and in intact tobacco cells (Maxwell et al., 1999). We previously investigated the correlation of this ROS avoidance gene with chilling injury and showed that AOX at the RNA transcript level and enzymatic level were both induced during the low temperature storage period and were correlated with chilling resistance (Fung et al., 2004; Tian et al., 2004). Another major part of plant antioxidants system is the involvement of non-enzymatic compounds, includes reduced glutathione, vitamin E, various types of secondary metabolites mostly consisting of total phenolic compounds like flavones, flavonols, proanthocyanidins. The behaviour of these non-enzymatic compounds has also been linked to function as a ROS scavenger (Tappel, 1972; Jiménez-Escrig et al., 2001; Siddhuraju et al., 2002). The phenolic compound is induced in petunia during the 3 weeks of cold acclimation while its antioxidant capacity is moderately related to its chilling tolerance (Pennycooke et al., 2005).

In zucchini squash, oxidative stress and lipid peroxidation was demonstrated during chilling in the peel tissue (Hariyadi and Parkin, 1991). Increase in squash chilling resistance by temperature preconditioning was associated with the protection against lipid membrane breakdown (Kramer and Wang, 1989; Wang et al., 1992) possibly with the increased activities of catalase, peroxidise (POD), superoxidase dismutase, and ascorbate peroxidise (APX) antioxidant system (Wang, 1995). A number of postharvest treatments focused on improving quality of cold-stored squash; e.g. MeJA treatment, heat shock, low temperature conditioning, intermittent warming, dipping in free radical scavengers, and controlled atmosphere (CA) with low oxygen (Wang and Baker, 1979; Mencarelli et al., 1983; Wang, 1994; Wang and Qi, 1997; Wang and Buta, 1999). More recently, elevated oxygen modified atmosphere has been shown to prolong shelf life of various produce (Kader and Ben-Yehoshua, 2000; Lu and Toivonen, 2000; Van der Steen et al., 2002; Wszelaki and Mitcham, 2000; Zheng et al., 2003) and minimally processed fresh-cut produce (Amanatidou et al., 2000; Lu and Toivonen, 2000; Allende et al., 2004). It was suggested that high oxygen resulted in suppression of microbial growth and therefore resulted in the control of decay (Amanatidou et al., 1999, 2000). However, little is known about the mechanisms on how the high level of atmospheric oxygen maintains quality of fresh produce.

Transcript profiles of scavenging genes were found to be relatively constant during the early cold-storage period in peppers when transcript profiles of AOX genes were dramatically induced (Fung et al., 2004). In the present study, we investigated the relation of transcript and enzymatic levels of ROS scavenging genes (SOD, CAT, APX) with chilling resistance in zucchini squash exocarp tissue. We further determined the overall antioxidant activity of zucchini under high oxygen condition by measuring two related parameters, the ORAC and total phenolic compounds. The respiration rate and ethylene production of zucchini squash in response to high oxygen treatment were also measured.

2. Materials and methods

2.1. Plant materials and postharvest treatments

Zucchini squash fruit (*Cucurbita pepo* L. cv. Elite) were freshly harvested from local farm in Maryland during the summer season of 2001. Fruit were selected for their uniformity in size ranging 16–22 cm in length. Three sets of 45 fruit were placed in each 18-L airtight glass jars for each treatment. The jars were placed at 5 °C and connected to a continuous flow (2 mL/s) of humidified air (control) and 60% or 100% O_2 (balance N_2 in all high O_2 treatments). The gases were checked regularly with an O_2/CO_2 analyzer (AMETEK, Pittsburgh, PA) and maintained at ±2% during the duration of the experiment. Samples were taken initially and at 3-day intervals during storage. At each time point, exocarp tissues were taken from each fruit, frozen in liquid nitrogen and stored at −80 °C until analyzed.

2.2. Evaluation of chilling and ripening characteristics

Three squash were chosen at random from each replicate at each sampling time and examined for chilling injury. The degree of chilling injury, as judged by the extent of surface pitting, was evaluated 1 day after being transferred to air at room temperature (20 °C) by rating on a scale of 1–5, with 1 = no abnormality; 2 = trace; 3 = slight; 4 = moderate; and 5 = severe chilling injury.

2.3. CO_2 and C_2H_4 determinations

Three squashes from each of three replications at each time point were enclosed in 3.8 L glass jars at 5 °C. Ten millilitres of headspace gas was taken from each jar at the end of 1 h enclosure. Ethylene production was determined by gas chromatography using flame ionization detection (Carle Instruments, Inc., Fullerton, CA), and CO_2 was measured with an infared gas analyzer (Model CD-3A, AMETEK Applied Electrochemistry, Pittsburgh, PA).

2.4. Preparation of gene specific probes and family specific probes

Complementary DNA fragment corresponding to conserved region of AOX gene were obtained from zucchini fruit and that of seven genes encoding antioxidative enzymes were obtained from peppers according to Fung et al. (2004). Genes encoding antioxidative enzymes selected for analysis included cytosolic ascorbate peroxidase, ascorbate peroxidase, thioredoxin peroxidase, catalase, peroxidase, Mn-superoxide dismutase and Cu/Zn-superoxide dismutase (see Table 1 in Fung et al., 2004 for primer sequence). Complementary DNA was prepared with SuperScript™ reverse transcriptase (Invitrogen™) using odtRACE1 primer and total RNA extracted from zucchini fruit exocarp (skin peel) as template. PCR conditions were as outlined in Fung et al., 2004. PCR products for AOX were obtained using MST1 and MST2 degenerative primer as described in Tian et al. (2004), cloned into pGEM-T vectors (Promega) and confirmed by sequencing. PCR products for other antioxidative genes were

gel-purified and sequenced to confirm their identity before being used as probes for Northern analysis.

2.5. RNA gel blot analysis

Total RNA was extracted from zucchini fruit tissues by the procedure described previously (Verwoerd et al., 1989). Electrophoresed RNA was transferred to Hybond N^+ membrane using 20× SSC, according to manufacturer's instructions. RNA gel blot hybridization was performed according to Virca et al. (1990). Probes were labelled with [α-^{32}P]-dCTP by DNA random prime labelling RTS system (Invitrogen). After an overnight hybridization at 65 °C, unbound probe was removed by washing the membrane in 0.2× SSC at 65 °C. Membranes were sealed in plastic bags and exposed to X-ray film. Membranes were then stripped and rehybridized with an 18S ribosomal DNA probe from apple (*Malus domestica*) (Pechous, personal communication). Hybridization signal of 18S rRNA was used as RNA loading control.

2.6. Enzyme extractions

Two grams of exocarp tissues was ground with a chilled mortar and pestle in 10 mL of pH 7.0, 50 mM phosphate buffer or pH 7.5, Tris–HCl buffer (containing 3 mM $MgCl_2$, 0.1 mM EDTA, and 1% (w/v) insoluble PVP), and a small amount of washed sand at 0–4 °C. The homogenate was then centrifuged at 20,000 × *g* for 20 min at 0–4 °C and the supernatant was used for enzyme activity assay.

2.7. Enzyme assays

2.7.1. Superoxide dismutase (SOD) activity

SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The assay mixture (3 mL) contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine (MET), 75 μM NBT, 4 μM riboflavin, 0.1 mM EDTA, and 100 μL of enzyme extract. Three millilitres of the assay mixture in uniform, transparent tubes was shaken and placed 50 cm below a lightbank consisting of four 30-W fluorescent lamps. The reaction was started by switching on the light, after 10 min the light was turned off, and the absorbance by the assay mixture at 560 nm was recorded. A similar assay mixture covered with aluminium foil served as a control. One unit of SOD activity is defined as the amount of enzyme that inhibits the NBT photoreduction by 50% under the condition of the assay.

2.7.2. Catalase (CAT) activity

Catalase activity was assayed according to the method of Kar and Mishra (1976). One unit of CAT activity is defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per 60 s per gram fresh weight at 25 °C.

2.7.3. Peroxidase (POD) activity

POD activity was measured using guaiacol as a donor and H_2O_2 as a substrate. Two millilitres of assay mixture [50 mM PBS (pH 6.5), containing 6 mM guaiacol and 4.5 mM H_2O_2] was added to 1 mL of crude enzyme extract. Increase in absorbance at 420 nm at intervals of 30 s up to 300s (at 25 °C) was recorded spectrophotometrically. One unit of POD activity is defined as the amount of enzyme that catalyzes the peroxidation of 1 mmol of guaiacol per 60 s per gram fresh weight.

2.7.4. Ascorbate peroxidase (APX) activity

APX activity was assayed according to the method of Nakano and Asada (1981) by measuring the oxidation of ascorbate at 290 nm. The assay mixture (1 mL) contained 50 mM phosphate buffer (pH 7.8), 15 mM H_2O_2, 5 mM ascorbate, 1 mM EDTA, and 100 μL of enzyme extract. The reaction was started by the addition of H_2O_2, the decrease in OD_{290} was recorded at 30 s up to 300 s. One unit of APX activity is defined as the amount of enzyme that oxidizes 1 mmol of ascorbate per 60 s per gram fresh weight.

2.8. Total phenolic content

Zucchini squash were extracted with 80% acetone containing 0.2% formic acid. Total phenolic content was determined with Folin–Ciocalteu reagent by the method of Slinkard and Singleton (1977) using gallic acid as a standard.

2.9. Oxygen radical absorbance capacity (ORAC) assay

The automated sample preparation was performed using a Precision 2000 instrument. The ORAC assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a microplate fluorescence reader (Huang et al., 2002).

2.10. Statistical analysis

Experiments were performed using a completely randomized design. Data were subject to statistical analysis by ANOVA. In case of a significant *F*-value, the means were compared by the least significant difference (LSD) test or Duncan's multiple range test at a significance level of $P = 0.05$.

3. Results

3.1. Effect of high oxygen on chilling injury

Summer squash are chilling sensitive and highly perishable (Hardenburg et al., 1986). It has been demonstrated previously that controlled atmospheres of low oxygen extended storage life of zucchini squash (Mencarelli et al., 1983). Storage at high levels of oxygen, on the other hand, received little attention. To test whether cold storage with high oxygen was effective in increasing chilling resistance in zucchini squash, we harvested fruit from a local farm and stored them at control atmosphere at 5 °C with air, high oxygen levels of 60% or 100%. Chilling injury severity was evaluated for each treatment (Fig. 1). Chilling injury symptoms were visible as pitting within 6 days of storage at 5 °C and progressed rapidly to severe level within 9

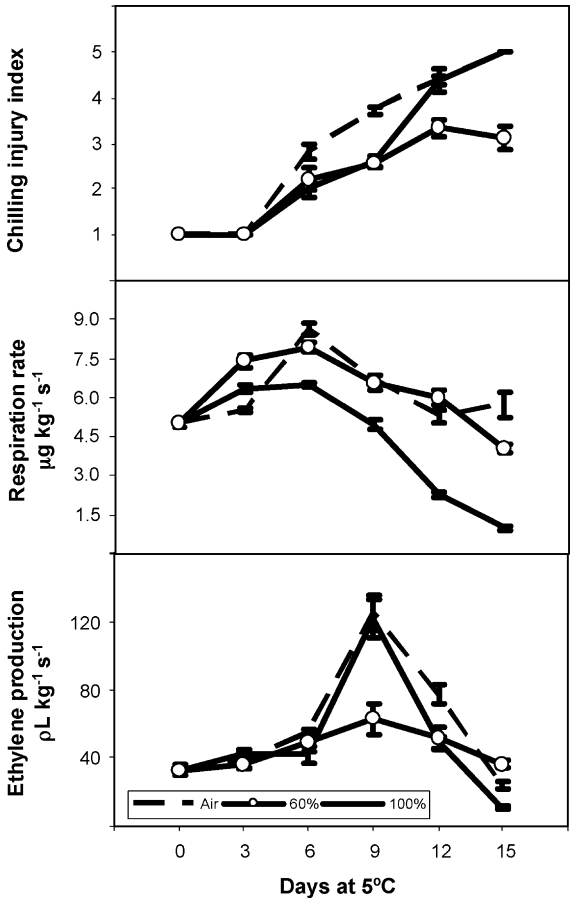

Fig. 1. Effect of superatmospheric oxygen on chilling injury (top panel) of zucchini squash stored at 5 °C. Fruit were harvested manually from a local farm and treated at 5 °C over a 2-week period with 60% or 100% oxygen or with air as a control. Chilling injury evaluation was performed based on the percentage of fruit surface area covered by pitting. Vertical bars represent S.E.; $n = 30$.

days of low temperature storage in air (Fig. 1, top panel). High O_2 treatments at 60% and 100% reduced the severity of chilling injury during the first 9 days at 5 °C. By day 12, however, the percentage of surface pitting increased dramatically in fruit treated with 100% oxygen, to level similar to that of untreated control. Chilling injury level of the 60% oxygen treated fruit stayed at lower moderate severity level till the end of experiment. High oxygen level of 60% was effective in suppressing surface pitting in zucchini squash fruit stored at 5 °C.

3.2. Effect of high oxygen on respiratory rate

The respiratory rate measured as CO_2 evolution of zucchini squash at harvest was at moderate level of around 5 $\mu g\,kg^{-1}\,s^{-1}$ (Fig. 1). During the 5 °C storage period, respiration rate for both treated and control fruit increased and peaked at day 6. Respiration rate for high O_2 treated fruit was higher than that of the control fruit initially at day 3 but levelled off at day 6 to a level lower than control. The sudden increased in respiration rate in control fruit at day 6 coincided with the higher chilling injury index described above and is consistent with the idea that defense mechanisms were put forth by the fruit to detoxify metabolic intermediates (e.g. ROS) resulted from cellular damage during chilling. After day 6, respiration rate of the 60% O_2 treated fruit and that of control fruit declines slowly to levels similar to that at harvest. Storage condition of 100% oxygen was the most efficient in reducing respiration throughout the storage period. By the end of the storage period, respiration rate of 100% O_2 treated fruit declined substantially to very low level of 1.5 $\mu g\,kg^{-1}\,s^{-1}$. The lower respiration rate for fruit at 100% O_2 before day 9 was possibly a contributing factor to its extended shelf life. However, further decreased in respiration rate at days 12 and 15 to lower than its harvest (day 0) seems to be detrimental to zucchini squash as it failed to maintain its chilling resistance.

3.3. Effect of high oxygen on ethylene production

Increased in ethylene production generally indicated the presence of biotic and abiotic stress in the non-climacteric commodities. Ethylene production of all treated and control fruit were similar during early storage period until day 6 (Fig. 1). At day 9, ethylene production increased dramatically in control and in 100% O_2 treated fruit to levels up to 2-fold higher than that of 60% O_2 treated fruit. After the peak ethylene production at day 9, the ethylene level of 100% O_2 treated fruit and control decreased to below harvest level by day 14. On the other hand, ethylene level of 60% O_2 treated fruit was relatively low and stable throughout the course of the cold storage experiment and the fruit in this treatment showed the least chilling injury symptoms.

Storage at 100% O_2 atmosphere was effective in suppressing respiration, but failed to reduce ethylene production after 9 days at chilling temperature. The dramatic inductions of ethylene at day 9 in control and 100% O_2 treated squash were correlated with the adverse changes in CI index in these treatments in the later stage of the experiment. Zucchini squash stored with 60% O_2 showed the least change in ethylene production and is consistent with our previous results when cucumbers were stored at a low but non-chilling temperature (13 °C) (Wang and Adams, 1982).

3.4. Effect of high oxygen on transcript levels of genes encoding antioxidative enzymes

We previously investigated bell peppers, another non-climacteric commodity, and demonstrated that transcript levels of several genes encoding antioxidative enzymes varied slightly in response to MeJA or MeSA treatment or to low temperature and showed little correlation with the degree of chilling resistance (Fung et al., 2004). However, levels of AOX transcripts increased significantly when peppers were pretreated with MeJA or MeSA and stored at low temperature. The change in AOX transcript levels is correlated with increased chilling resistance. We therefore set out to investigate if transcript levels of these genes follow the same expression pattern in zucchini squash stored in high oxygen condition.

Total RNA was extracted from exocarp tissues of zucchini squash freshly harvested from the local farm (day 0). Transcripts of several of these antioxidative genes were detected at harvest (Fig. 2). During the cold-storage period, transcript levels of antioxidative genes including MnSOD, Cu/Zn SOD, catalase,

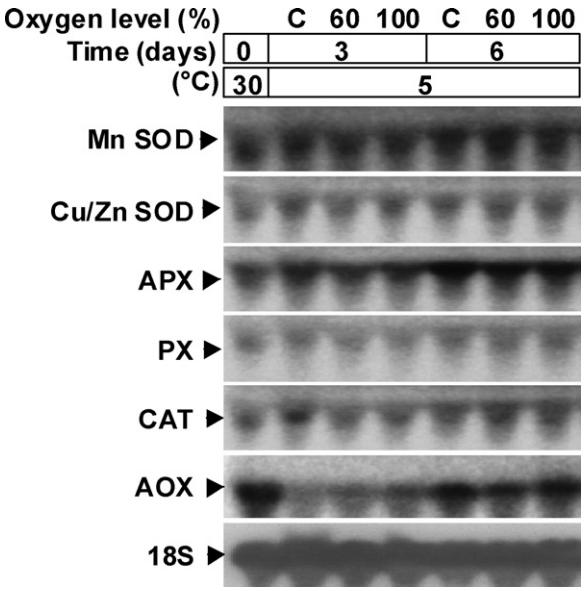

Fig. 2. Expression profiles of five different genes of antioxidative enzymes including Mn-superoxide dismutase (Mn-SOD), Cu/Zn SOD, ascorbate peroxidise (APX), peroxidise (PX), catalase (CAT) and alternative oxidase (AOX) gene in response to superatmospheric oxygen level in chilled zucchini squash fruits. Untreated control fruit is represented with (C). Zucchini squash were harvested from a local farm and treated as described in Fig. 1. Fruit from the first 6 days of experiments were used for Northern analysis. RNA gel blot analysis was performed as described in Section 2. 18S ribosomal fragment was used as probe for loading control and a typical blot is shown.

and the two different types of peroxidase were relatively constant in response to high oxygen CA treatment. The AOX transcript levels were slightly higher in squash treated with 60% and 100% oxygen for 3 days then in the control samples (Fig. 2). These higher AOX transcript levels were correlated with the greater chilling resistance in the treated squash (Fig. 1). Between 3 and 6 days, transcript levels of AOX increased substantially in both treated and control squash. The constant transcript level detected for the antioxidative genes in this study can be attributed to various factors ranged from timing and duration of transcript induction.

3.5. Effect of high oxygen on oxygen radical scavenging enzyme activities

Enzyme activity and transcript expression assay were performed using exocarp tissue (skin peel) since it is the location where chilling injury symptom first appeared. Superoxide dismutase (SOD) activity declined slightly in control fruit over the 2 weeks cold-storage period but its activity increased 1.5-fold in fruit stored in high oxygen CA (60% and 100%) within 3 days (Fig. 3). The enhanced SOD activity was then decreased slightly in the 60% oxygen CA fruit but drop drastically in the 100% oxygen treated fruit after day 9, to levels below that of control squash. Catalase (CAT) activity in control squash decrease dramatically by 2.5-fold within 3 days followed by a much slower decrease till the end of the 2 weeks cold-storage period. For squash stored at higher oxygen CA level, catalase activity declined to a lesser extent and maintained at higher level than control. However, after day 9, catalase activity in fruit stored at

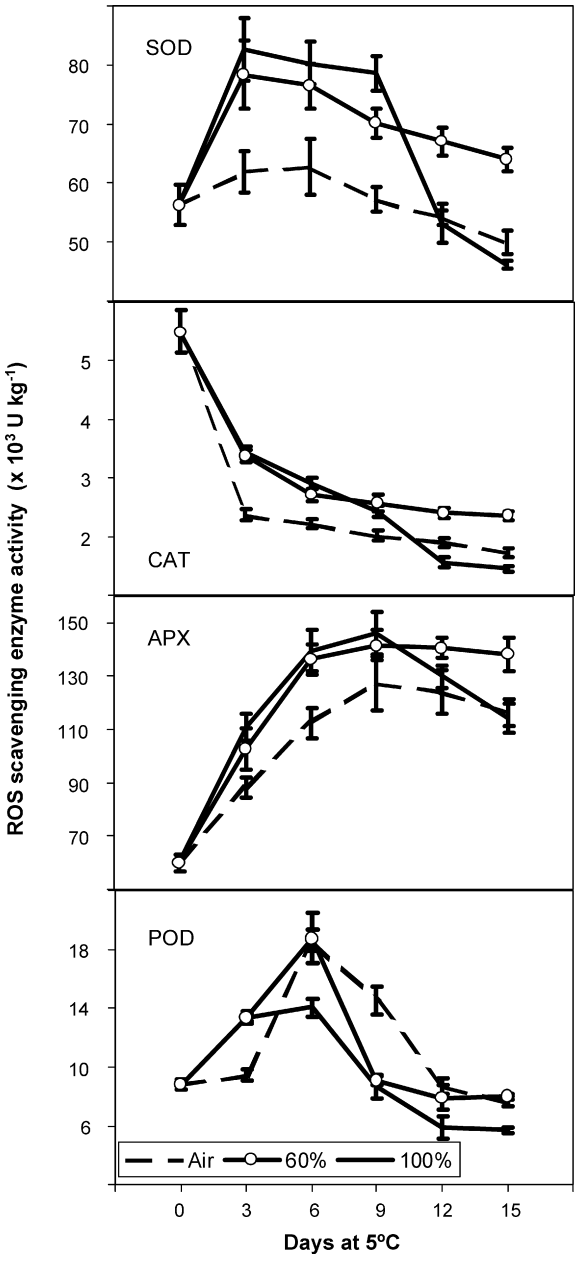

Fig. 3. Enzymatic activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidise (APX), and peroxidase (POD) (top to bottom) in response to superatmospheric oxygen at 60% or 100% were compared with those in air control. Vertical bars represent S.E.; $n = 3$.

100% oxygen dramatically reduced to levels lower than control. Ascorbic peroxidase (APX) activity in control squash increased 2-fold to maximum at day 9 and stays unchanged till the end of the storage period. Fruit stored at higher oxygen CA levels was able to maintain an even higher ascorbic peroxidase activity throughout the whole storage period. Again, after day 9, ascorbic peroxidase activity in 100% oxygen treated fruit reduced to level similar to that of control fruit. The peroxidise activity increased in high oxygen treated squash transiently and peaked at around days 3–6 before it return to harvest level or even lower for 100% oxygen CA squash. The POD activity increased substantially at day 6 in control samples and maintained at levels

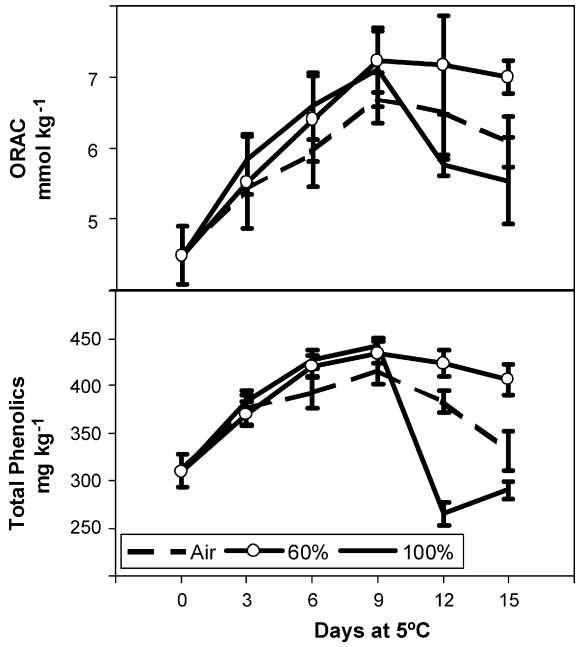

Fig. 4. Effect of different concentrations of oxygen on oxygen radical absorbance capacity (ORAC) and phenolic compound at 5 °C. Fruit were obtained and treated as in Fig. 1. Vertical bars represent S.E.; $n = 3$.

higher than 100% oxygen treated squash (Fig. 3). POD is ubiquitous enzymes that have diverse biochemical functions in higher plants (Gaspar et al., 1981). The activity of POD has been found to increase during storage at chilling temperatures in mangoes and zucchini squash (Zauberman et al., 1988; Wang, 1995). This increase was suggested as part of the chilling injury syndrome. The increase in POD activity accompanied by the decrease in CAT and APX activity could result in slow removal of H_2O_2 which leads to its accumulation in the tissue. Excessive amount of H_2O_2 could aggravate oxidative damage and intensify chilling injury.

3.6. *Effect of high oxygen on antioxidative capacity and total phenolic levels*

As we described in Section 1, ROS was the prominent factor that resulted in chilling injury. The overall antioxidative capacity and the total phenolic levels are generally used as an indicator of the ability of the commodity in reducing oxidative stress. Both ORAC and total phenolic levels increased steadily during cold-storage period and peaked at day 9 (up to 30% in control) (Fig. 4). Treatment with 60% oxygen level led to higher ORAC and total phenolic levels (by up to 50% compared with that at harvest) especially by the end of the storage period. For fruit stored at 100% oxygen, both the ORAC and total phenolic levels are similar to those in 60% oxygen treated fruit till day 9. At days 12 and 15, the ORAC and phenolic levels in 100% oxygen treated fruit decreased dramatically to levels lower than control squash. This decline indicate possible accumulation of toxic effect imposed by high oxygen level on squash tissues to a level when the tissue stop functioning as indicated by the sudden drop in respiration rate and ethylene production from their peak level.

4. Discussion

The changes in ROS scavenging enzyme activities and antioxidative capacity index (ORAC and phenolic levels) in response to superatmospheric oxygen levels in zucchini were investigated in this study. We established the relationship among respiration rate, ethylene production levels, fruit chilling injury index, and the antioxidative gene expression (enzymatic activities and RNA transcript levels). Chilling injury provoked a similar response to all treatments as evidence by the activities of the antioxidant enzymes, the ORAC and total phenolic assays. The dramatic change in antioxidative enzyme activity in response to cold storage and superatmospheric oxygen level, however, does not necessarily reflect change in their corresponding gene transcript levels (see below for details). More importantly, we employed two parameters (ORAC and total phenolic levels) that have been routinely used to measure the antioxidative status of targeted samples. Our results show that the ORAC and total phenolic levels in zucchini skin peels were both induced by cold storage and further enhanced by 60% oxygen storage. While the causal link of chilling injury is unknown, the enhanced antioxidative enzyme activities and the overall antioxidative capacity (ORAC and phenolic levels) seem to correlate with the reduced chilling injury.

The ROS antioxidative defense system includes ROS avoidance gene (AOX) and ROS scavenging gene of the ascorbate–glutathione antioxidative system (Møller, 2001). The transcript profile of selected ROS avoidance and scavenging genes in cold-stored zucchini is consistent with that observed previously in peppers during the early chilling period (Fung et al., 2004). In peppers, treatment with MeJA and MeSA reduced chilling injury and induced preferentially the transcript level of AOX gene while no changes in transcript levels of ROS scavenging genes were detected during the early cold storage at 0 °C (Fung et al., 2004). Here in this study, elevated level of oxygen was effective in reducing chilling injury in zucchini. Coincidentally, we observed slight induction of only AOX gene transcript during early chilling period at day 3 when no difference in chilling injury symptom was visible among treated and control squash. The detectable differences in AOX transcript levels among treated and control zucchini and peppers (Fung et al., 2004) suggested possible correlation between AOX gene expression level and chilling injury. During the rest of the cold-storage period, no observable differences in transcript levels for either ROS avoidance or ROS scavenging genes among high O_2 treated and control (day 6, Fig. 3; days 9–15, data not shown). However, the enzymatic activities of ROS scavenging genes were dramatically induced in the treated zucchini and the chilling injury was also reduced. The discrepancy in transcript expression profile (no change) and enzymatic expression profile (dramatic induction) can be explained as follows. First, ROS scavenging genes are likely belonging to a multi-gene family. Second, the use of heterologous DNA probe obtained from peppers may not detect transcript profile of all its respective zucchini gene members that may be responsible for the change in enzyme activity. Furthermore, ROS antioxidative enzyme might be subjected to regulation posttranslationally and resulted in induction

of enzymatic activity during cold storage. On the other hand, we observed unexpectedly high level of zucchini AOX transcript at harvest (day 0). Possible reason for this observation points to either pathogen attack or other environmental stresses before harvest. Higher initial AOX transcript level was also observed previously when peppers were preconditioned in cold before treatment (Fung et al., 2004).

The assay of ORAC and total phenolic levels measures the overall antioxidative capacity of zucchini against reactive oxygen species. Enzymatic activities of ROS scavenging gene were enhanced by elevated oxygen level during this early chilling period. Interestingly, the antioxidative capacity of the zucchini skin peel was also enhanced as indicated by the ORAC and total phenolic level measurements. All these results indicated that elevated oxygen level increased the ability of antioxidative defense mechanism in cold-stored zucchini presumably to control the ROS level and eventually the chilling injury severity. Storage at 60% and 100% oxygen levels seems to be just as effective in enhancing enzyme activity, ORAC and total phenolic levels in zucchini until after day 9 where they all decreased in zucchini treated with 100% oxygen. These drops possibly lead to increase in chilling injury. The high respiration rate and ethylene production of the 100% oxygen treated squash seems to predict the failure of this treatment. Ethylene production was effectively reduced only by 60% oxygen but not by 100% oxygen. This is consistent with our previous finding that skin tissue contained higher levels of ACC and was more sensitive to chilling than was the cortex tissue (Wang and Adams, 1982).

Taken together, chilling injury of zucchini squash was reduced initially by both 60% and 100% O_2 treatment. This reduction was correlated with the increases of AOX transcript accumulation, oxygen radical scavenging enzyme activities, ORAC values, and total phenolic content. However, after 9 days at 5 °C, squash exposed to 100% O_2 started to lose their resistance to chilling injury as reflected by the drastic decrease in all the parameter measured. Squash treated with 60% O_2 consistently maintained the lowest amount of chilling injury and the antioxidative enzyme activities in this treatment remained elevated throughout the cold storage. These data indicate that both the expression and activities of antioxidative enzymes may be one of the contributing factors in defending plant tissues against chilling injury, perhaps through avoiding (AOX) or scavenging (SOD, APX, CAT) the harmful ROS that are generated during chilling stress. Although we are yet to identify ROS scavenging gene transcripts that are corresponding to the changes in its activity, examination of the ORAC values and the metabolic level via total phenolics demonstrate a similar response with the enzyme activities of SOD, APX, and CAT in the treated and control squash.

Our data is consistent with the idea that global reprogramming of metabolism occurs during low temperature stress (Sung et al., 2003; Graya and Heath, 2005; Hannah et al., 2005). We observed that cold storage resulted in inhibition of primary metabolic pathways of respiration and induction of AOX transcript of alternative respiratory chain. This is also accompanied with the antioxidant system as demonstrated by their enzyme activities and by the ORAC assay that presumably function to provide immediate relieve to the adverse redox status. On the other hand, the plant system quickly remobilize its primary metabolites into various secondary metabolite biosynthetic pathway (Kaplan et al., 2004; Renaut et al., 2005) as shown also by our data from the total phenolic compound measurements (increased of 30% during cold storage). All these efforts developed in the zucchini squash exocarp tissues in response to cold storage, was enhanced by the superatmospheric oxygen treatment.

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