Production of Reactive Oxygen Species and Antioxidant Metabolism about Strawberry Leaves to Low Temperatures

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Abstract

This study examined the influence of low temperature on the production of reactive oxygen species and antioxidant metabolism in two strawberry (*Fragaria ananassa* Duch.) cultivars (cv. Zoji and Toyonaka). Low temperature treatment was imposed by maintaining the plants at 0℃ for 2, 4, 6, 8, 12, 24, 48 and 72 h in an artificial intelligent growth chamber. During the period of low temperature treatments, the activities of peroxidative (POD), superoxide disumutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate (DHAR), glutathione reductase (GR), the production of O_2^- and H_2O_2, as well as the contents of antioxidant such as dehydroascorbate and reduced glutathione were up-regulated exception of the contents of ascorabte and chlorophyll in compared with the control. Meanwhile, cv. Toyonaka showed more tolerant to low temperatures than cv. Zoji since it showed higher activities of antioxidative enzymes, more contents of osmolytes (proline and soluble sugar) and less lipid peroxidation during the low temperature treatments. The present study suggested that plant had signal molecules sensing the environmental stress, following erecting its defensive system protective them against the damage caused by stress environment. However, if the stress were too powerful, plants could not response to acclimate that stress, the plants would be injured or even be dead finally. In the two cultivars examined, cv. Toyonaka has a more efficient antioxidant system against low temperature than cv. Zoji.

Keywords: Antioxidant enzymes, Strawberry, Reactive oxygen species, Antioxidant, Low temperature stress, Lipid peroxidatiom

1. Introduction

Plant growth is affected adversely by different environmental abiotic stresses such as cold, salinity, drought and fluctuations in incident light (Berry and Bjorkman, 1982; Andrea *et al*., 2003). Low temperature is one of the major environmental factors affecting plant growth and development, survival and distribution (Delauney and Verma, 1993; Cao, *et al*., 2002). Therefore, it is important to understand how plants respond and adapt to such stress. The mechanism of the plant responding and adapting to low temperature stress including adjusting their cellular metabolism and invoking various defensive mechanism may provide a strategy to protect plant from low temperature stress or to select stress tolerant cultivars.

Under the low temperature stress conditions, oxidative stress was induced resulting from the production and accumulation of reactive oxygen species (ROS) (Yong *et al*., 2003), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxide radicals (OH^-), and singlet oxygen ($^1O_2$). The active oxygen species produced during stress susceptible to damage cellular components including lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrate and nuclei acid (Blokhina *et al*., 2003). Oxidative stress can lead to inhibition of the photosynthesis and respiration processes and, thus plant growth (Jiang and Huang, 2001). Plant has non-enzymatic and enzymatic system to protect cellular membranes and organelles from damaging effects of ROS. The non-enzymatic compounds mainly are the low molecular mass antioxidants, such as ascorbate, glutathione, β-carotenen andα-tocopherol while the enzymatic antioxidant system include the enzymes

regenerating the reduced forms of antioxidants, such as ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.6.4.2), and ROS-interacting enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) (Blokhina and Virolainen, 2003). The non-enzymatic and enzymatic systems in plant together keep the ROS in low levels and not to be injured by accumulation of ROS in normal conditions.

There are some reports about other species detailing changes in the activities of enzymes and the contents of antioxidants involved in antioxidant metabolism in response to low temperature stress (Li et al., 2000; Wu et al., 2004; Zhang, 2004), and results suggested that those plants grown under low temperature could induce the production of ROS and trigger the changes of defense systems. However, there is not a clear picture about the relationship between the antioxdative system and low temperature stress. Little is known about the effect of low temperature stress on the ascorbate-glutathione cycle (ASA-GSH cycle) in plants, and there is not enough information about the changes of the osmolytes and chlorophyll contents.

Strawberry is one of nutritious fruits in the world. Low temperature stress in winter is one of the serious problems in strawberry production. Tolerance to the abiotic and biotic stress is one of the major strawberry breeding objectives as well as high production, good quality and insect resistance. In this work, we report a more detail study of the effect of short-term low temperature on the production of ROS, the activities of SOD, CAT, POD and the enzymes involved in the ASA-GSH cycle (APX, MDHAR, DHAR and GR), as well as the contents of ASA, DHA, GSH, proline, soluble sugars and chlorophylls. Our major aims were to observe the two strawberry cultivars response to short-term low temperature stress, characterize the sensitivity of antioxidative systems to oxidative stress and discuss the role of ASA-GSH cycle in the alleviation of low temperature induced oxidative stress. Meanwhile, our working hypothesis was that the more tolerant to low temperature was associated with the higher levels antioxidative system.

2. Materials and methods

2.1 Plant materials and treatments

The leaves of strawberry (*Fragaria ananassa* Duch) cultivars (cv. Zoji and Toyonaka) were detached from 4-week-old in vitro cultures. The cultures were established from the shoot-tips of 'Zoji' and 'Toyonka' in the spring of 2007 and maintained on MS medium supplied with 0.5mg/L BA and 0.1mg/ L IBA with a 4-week interval in a growth chamber at 24±1℃ and a light intensity of 4800lx provided by cool-white fluorescent lamps. For low temperature treatment (0℃), the cultures were transferred to the artificial intelligent growth chamber. The leaves were detached as materials regularly after 0, 2, 4, 6, 12, 24, 48 and 72 h treatment.

2.2 Determinations of H_2O_2, O_2^-, lipid peroxidation and GSH

The detections of H_2O_2, O_2^- and GSH were measured by using the H_2O_2, O_2^- and GSH determination kit (Nanjing Jiancheng Bioengineering Institute NJBI). Lipid peroxidation was estimated through the level of malondialdehyde (MDA) production. The lipid peroxidation was also measured by using the MDA determination kit (Nanjing Jiancheng Bioengineering Institute NJBI).

2.3 Determination of proline and soluble sugar content

Proline and soluble sugar were determined according to the colorimetric method of acor ninhydrin and anthrone respectively (Xiong, 2003).

2.4 Chlorophyll quantification

The contents of chlorophylls were measured using spectrophotometer and determined (Xiong, 2003).

2.5 Determination of ASA and DHA

The determination of ASA was measured by using the method of dinitrophenylhydrazine. Reduced ascorbate was determined by the method of 2, 6-dichloroindophenol. DHA was calculated by subtracting reduced ascorabte from total levels (Takahama and Oniki, 1992).

2.6 Assay of the antioxidative enzymes activity SOD, CAT and POD

2.6.1 Assay of the activity SOD, CAT and POD

The SOD and CAT activities were assayed with the SOD and CAT determination kit (Nanjing Jiancheng Bioengineering Institute). One unit of SOD activity was defined as the amount of enzyme required for 1 mg tissue proteins in 1 ml of a reaction mixture SOD inhibition rates to 50% as monitored at 550nm. One unit of CAT activity was defined as 1 mg tissue proteins consumed 1 μmol H_2O_2 at 405nm for 1 second.

The POD activity was assayed according to the method of guaiacol (Xiong, 2003). One unit of POD activity was defined as 1 mg tissue proteins catalysised 0.01μmol H_2O_2 for 1 min.

2.6.2 APX, DHAR and GR enzyme extraction

According to the methods of Jin (Jin *et al*., 2003), with some modification, leaves of 0.1g were ground in a mortar in liquid nitrogen. The crude enzymes were extracted with 1.0mL phosphate buffer containing 2.0% polyvinylpyrrolidone (PVP), EDTA 1.0mmol/L and ascorbate (freshly prepared) 1.0mmol/L and 0.25% Triton x-100 at pH7.6 and then centrifuged with 16000g for 20 min at 4℃ right after. The supernatant was transferred into 1mL clean centrifugal tube and then kept in refrigerator at 4℃ ready for use.

2.6.3 Enzyme activity assay

Ascorbate peroxidase (APX, EC 1.11.1.11) was assayed by following the decrease in ascorbate at 290 nm (absorption coefficient 2.8 $nM^{-1}cm^{-1}$) for 1 min in 2mL of a reaction mixture containing 1.66mL of 0.5mmol/L ascorbate in phosphate buffer (pH 7.0), 0.26mL of 2mmol/L H_2O_2, both of which were freshly prepared, and 0.08mL of enzyme extract (Nakano and Asada, 1981). One unit of APX activity was defined as 1 mg tissue proteins catalysised 1μmol ascorbate at 290nm for 1 min.

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was assayed directly by following the regeneration of ASA at 265 nm for 1 min in 2mL of a reaction mixture containing 1.4mL phosphate buffer (pH 7.0), 0.2mL of 20mmol/L reduced glutathione (GSH) in phosphate buffer (pH 7.0), 0.2mL of 2mmol/L DHA and 0.2mL enzyme extract (Hossain and Asada, 1984). DHA was prepared freshly and kept at 4℃ to avoid oxidation. One unit of DHAR activity was defined as 1 mg tissue proteins catalysised 1μmol GSH at 265nm for 1 min.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined by following the rate of NADPH oxidation as measured decrease in absorbance at 340 nm. The determination of GR activity was determined with the assays kit (Nanjing Jiancheng Bioengineering Institute). One unit of GR activity was defined as 1 g tissue proteins consumed 1mmol NADPH at 340nm for 1 min.

2.7 Statistical analysis

The experiments were repeated at least 3 times with 3 replicates for each. All the data in this study were expressed as Means±SD. The data were analysed using one-way analysis of variance and Duncan's multiple range test at the 5% level of significance from the DPS 3.01 package for windows 2003.

3. Results and Discussions

3.1 Effects of low temperatures on H_2O_2 and O_2^-

Low temperature stress led to a continuous increase in the generation of O_2^- during the low temperature treatment (Fig. 1A). A significant increase in the production of O_2^- occurred within the first 6 h of treatment. After 24 h and 48 h of treatment, the O_2^- production of cv. Zoji and cv. Toyonoka reached the maximum values, and increased by 78% and 1500% respectively compared with the control. After that, the production of O_2^- decreased slowly. However, the generation of O_2^- in 'Zoji' in response to low temperature was relatively higher than that of 'Toyonoka'.

As the production increasing of O_2^-, the contents of H_2O_2 increased sharply right after transferring them to low temperature (Fig. 1B). The product of H_2O_2 kept in a high state to 8 h and 48 h respectively for 'Zoji' and 'Toyonoka'. Although it declined a little by the end of the treatment, it was 1.4 to 1.6 times higher than that of control. The fast increasing and keeping at a high level of production of O_2^- and H_2O_2 in both strawberry leaves treated with low temperature suggested that O_2^- and H_2O_2 might serve as signal molecules.

3.2 Effects of low temperatures on proline and soluble sugar contents

Both of proline and soluble sugars increased sharply right after transferring the cultures of strawberry to the low temperature (Fig. 2). After 24 h of low temperature stress, the proline in cv. Zoji reached the maximum, which were 11 times higher than that of the control, while the proline in cv. Toyonoka kept increasing to the end of treatment (Fig. 2A). The contents of soluble sugars reached the maximum after 8h and 24h of treatment respectively for cv. Zoji and cv. Toyonoka and then declined to a low level. As the treatment continuing, the soluble sugars increased again (Fig. 2B). Accumulation of proline and soluble sugars was reported in many plant species under diverse abiotic stress conditions (Yan et al., 2002) which might indicate that osmolyte could increase the tolerance of plants to stress conditions in some degrees (Xiong and Zhu, 2002).

3.3 Effects of low temperatures on chlorophyll and lipid peroxidation

Chlorophyll contents in live plants are an important factor in determining photosynthetic capacity (Jiang and

Huang, 2001). Environment stress such as salt and osmosis may lead to the decrease of the Chl level (Wu et al., 2006; Jiang et al., 1991). In our experiment, leaf Chl contents decreased gradually during low temperature stress and the Chl contents in cv. Zoji decreased much more than those in cv. Toyonaka (Fig. 3A). Jiang proved that Chl degradation was associated with the oxidative damage caused by ROS (Jiang et al., 1994).

The accumulation of MDA is often used as an indicator of lipid peroxidation (Wu et al., 2006). In our experiment, the contents of MDA were 1.3 to 2.6 times higher respectively than those of the control value, which indicated that the accumulation of ROS caused the membrane damage. In rice and cool-season turfgrasses plants, increase of lipid peroxidation was observed under stressed conditions (Yong et al., 2003; Jiang and Huang, 2001).

3.4 Effects of low temperatures on antioxidative enzymes activity and antioxidant contents

SOD constitutes the first line of defense against ROS that converts O_2^- into H_2O_2 and O_2 (Ruth et al., 2002), and play an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular compartments (Luis et al., 2002). The two cultivars treated at 0℃ showed that the SOD activity increased gradually and then decreased slowly (Fig. 4A). Meanwhile, SOD activity in the cv. Toyonaka responding to low temperature was relatively high compared to that in cv. Zoji, which suggested the cultivar 'Toyonaka' had stronger capacity to convert O_2^- into H_2O_2 and O_2 and lessen the oxidative stress and cv. Toyonaka was more tolerant to low temperature stress than cv. Zoji.

CAT, POD and APX that localized in peroxisomes, cytosol and chloroplasts also play an important protective role in controlling the levels of H_2O_2 generated primarily through SOD action. With the exception of POD activity in 'Zoji', the activity of CAT, POD and APX increased and decreased almost coordinately with SOD activity in two cultivars (Fig. 4A-D). Elevated CAT, POD and APX would lower H_2O_2 levels, which reduced the lipid peroxidation degree and lessen membrane damage. GR also may remove H_2O_2 within chloroplasts by maintaining more favourable levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) (Jiang and Huang, 2001). Meanwhile, DHAR use the GSH as the reducing to reduce DHA to ASA. The activity of GR and DHAR changed fluctuantly and also had the similar changing tendency with the SOD. The two enzymes activity had relatively higher in 'Toyonaka' except DHAR in 'Zoji' (Fig. 4EF). Increased GR and DHAR activity may maintain the pool of glutathione and ascorbate in the reduced state and lessen the oxidation by the H_2O_2.

Ascorbate and glutathione have been shown to act as antioxidants in the non-enzymatic detoxification of ROS (Ruth, 2004). Ascorbate plays an important role in the removal of H_2O_2 and GSH together with its oxidized form (GSSG), which maintains a redox balance in the cellular compartments (Ruth, 2002). Our data showed that the contents of ASA had a little decrease, but the contents of DHA (Fig. 5) and GSH increased (Fig. 6) when transferred to low temperature. These results confirmed that the enzymes activity changes of GR, APX and DHAR together with the antioxidant protected strawberry from the damages caused by the ROS.

In summary, our results clearly demonstrated that low temperature stress would trigger an increased production of ROS that mediated the production of oxidative stress and induced the changes of the plant defensive system. ROS has the dual function that one exacerbates damages and the other signals the activation of defensive systems (Dat et al., 2000). Plant itself could induced its defensive system to protect against free radical when suffered abiotic or biotic stresses, as shown by the transient increases of SOD, POD, CAT and APX activities and the contents of GSH and DHA in the initial periods of low temperature stress. The production and removal of ROS were in homeostasis in natural conditions. However, the longer the stress time was, the more ROS produced, so that antioxidative enzymes activity was impaired and the ROS could not be removed effectively and the lipid peroxidation level increased, which would result in plant injured or dead at last. The degree of injuring to plants relies on both the capacity of plant tolerance to the stress and the strength of the stress and its lasting times. Although the two strawberry cultivars examined in this study showed a similar way responding to low temperature stress, cv. Toyonaka exhibited more capacity to remove the ROS than cv. Zoji under low temperature. Such studies might be used in strawberry resistance breeding programme and the more tolerance variety selection.

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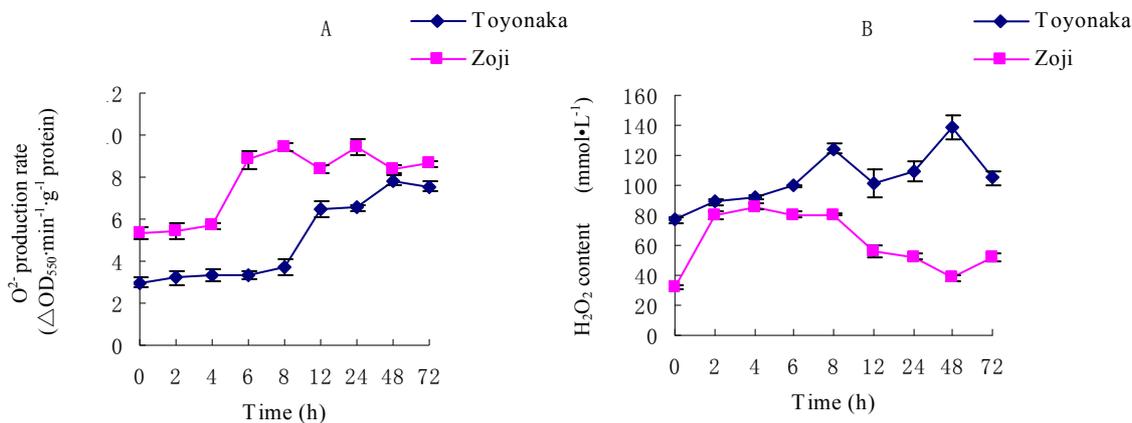
Figure 1. Time-course of changes in the production of O_2^- (A) and H_2O_2 (B) in leaves of two strawberry cultivars ('Zoji' and 'Toyonaka') treated with low temperatures
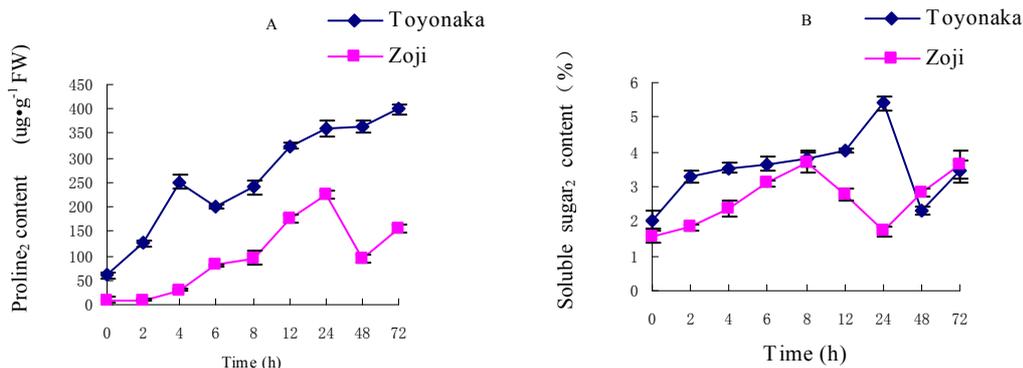
All values are means ± SD of three replicates

Figure 2. Time-course of changes in the contents of Proline (A) and soluble sugars (B) in leaves of two strawberry cultivars ('Zoji' and 'Toyonaka') treated with low temperatures
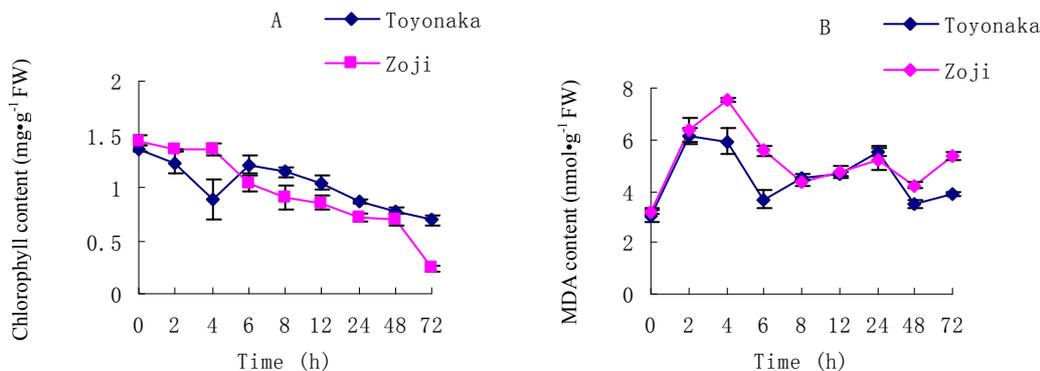
All values are means ± SD of three replicates

Figure 3. Changes of the chlorophyll (A) and MDA (B) contents in leaves of strawberry cultivars ('Zoji' and 'Toyonaka') treated with low temperatures. All values are means ± SD of three replicates
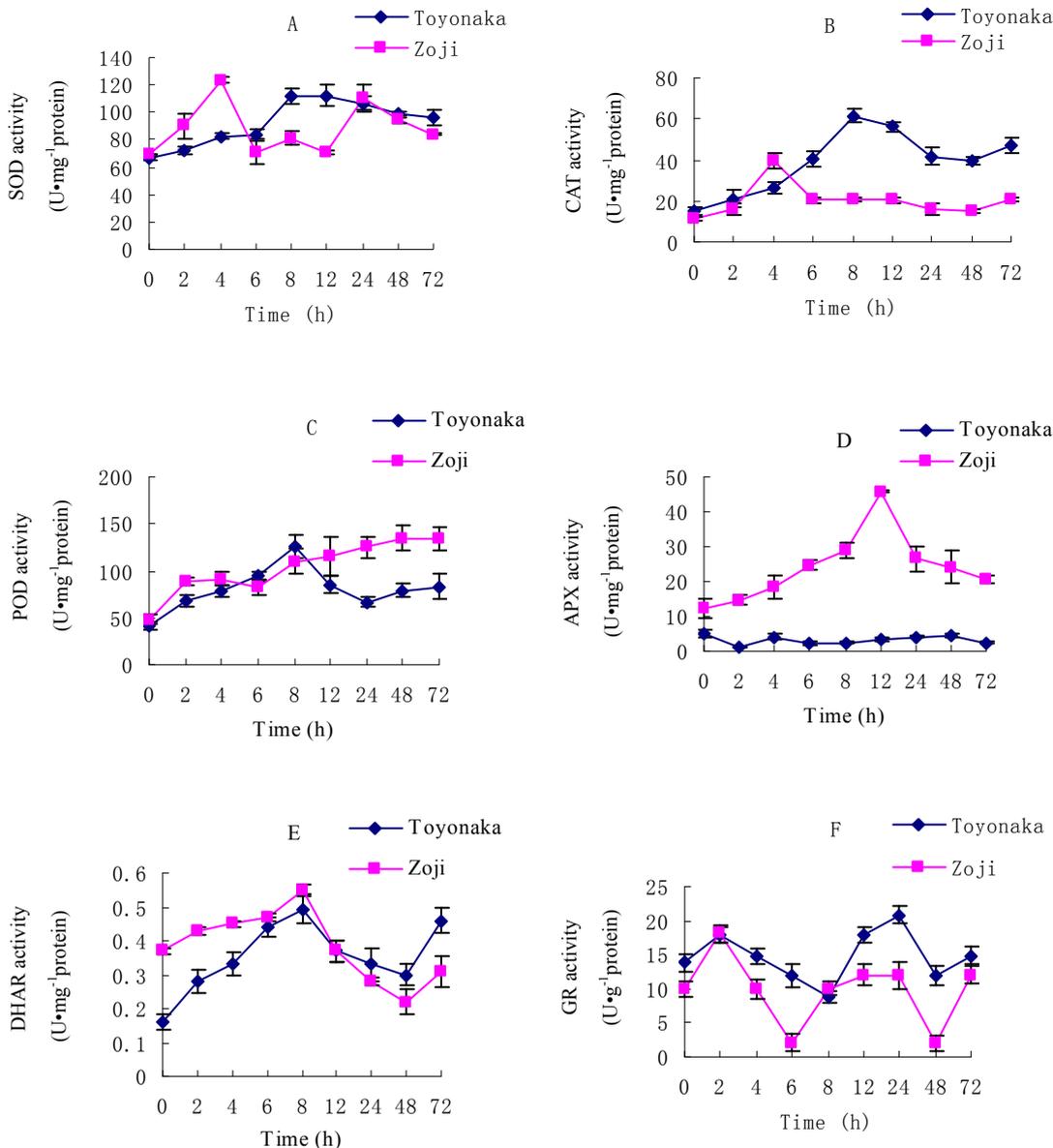
Figure 4. Changes of the antioxidative enzymes activities in leaves of two strawberry cultivars ('Zoji' and 'Toyonaka') treated with low temperatures. All values are means ± SD of three replicates
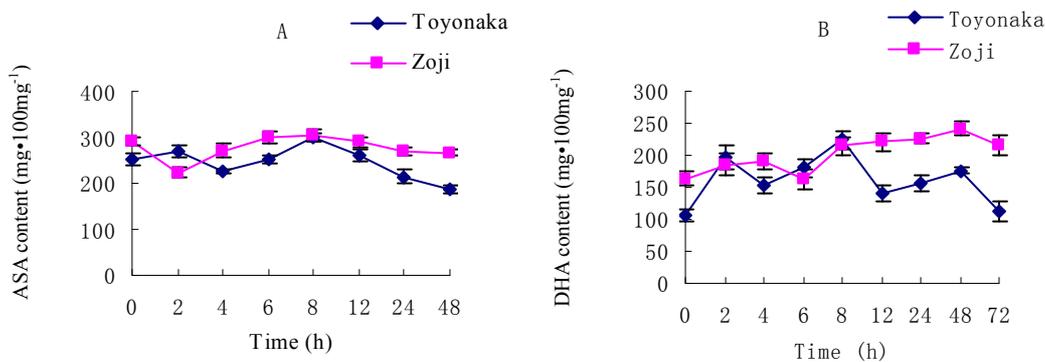

Figure 5. Changes of the ASA (A) and DHA (B) contents in leaves of two strawberry cultivars ('Zoji' and 'Toyonaka') treated with low temperatures. All values are means ± SD of three replicates
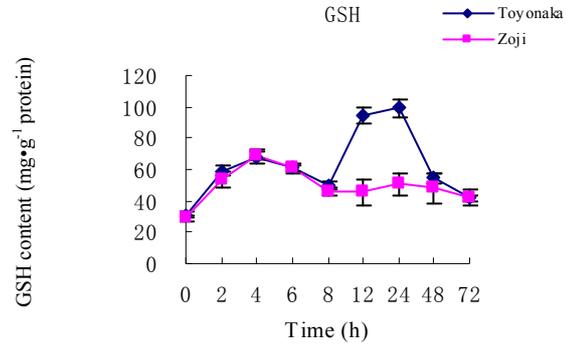
Figure 6. Changes of the GSH contents in leaves of two strawberry cultivars ('Zoji' and 'Toyonaka') treated with low temperatures. All values are means ± SD of three replicates