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New insights into the chilling injury of postharvest white mushroom (*Agaricus bisporus*) related to mitochondria and electron transport pathway under high O_2/CO_2 controlled atmospheres

Ling Li[a], Hiroaki Kitazawa[b], Rongfei Zhang[a], Xiangyou Wang[a,*], Liming Zhang[a], Shaoxuan Yu[a], Yanjie Li[a]

[a] School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, Shandong, 255049, China
[b] Food Research Institute, National Agriculture and Food Research Organization, Ibaraki 305-8642, Japan

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ABSTRACT

Mitochondria and their electron transport pathway (ETP) might have a potential role in response to chilling stress. Notwithstanding, it is a novel area and the relative research is still in the early stages. In this study, we attempt to explore the explicit mechanisms of the 80% O_2 + 20% CO_2 treatment on alleviating chilling injury (CI) to white mushrooms based on the ETP and mitochondria, with natural air as the control.

CI incidence, proportions of cytochrome pathway (CCP) and alternative pathway (AP), activities of key enzymes of CCP and AP, the relative gene expressions, mitochondrial structure and functions were investigated at 1 °C. The results reported that 80% O_2 + 20% CO_2 treatment prevented the occurrence of CI and retained the ATP yield. With regard to the ETP, the 80% O_2 + 20% CO_2 treatment restrained the decline in the proportion of CCP and boosted the proportion of AP through impacting the key enzymes and relative genes expressions. It also retarded mitochondria swelling and maintained higher levels of membrane fluidity and cytochrome c (Cyt c). Our results illustrated that 80% O_2 + 20% CO_2 dramatically alleviated the development of CI in white mushrooms, which could possibly be ascribed to the adjustment of ETP while retaining the structure and functions of the mitochondrion and the switching of its two conformations.

1. Introduction

As the most productive edible fungus in the world, white mushroom (*Agaricus bisporus*) has abundant nutritional and health value and is considered to be essential for human beings in their daily lives (Singh et al., 2010). Nevertheless, white mushrooms have their own particular structure and metabolic mechanisms. Additionally, they have no protective structure on their surface like higher plants, which brings about its very short shelf life of 1–2 days at ambient temperature (Yu et al., 2009). Consequently, cold storage is utilized as one of the main methods to preserve them.

When fruit or vegetables that are sensitive to low temperatures are stored at low but non-freezing temperatures, a post-harvest physiological disorder will appear. In general, the major symptoms of chilling injury (CI) are: discoloration, emergence of epidermis pitting, water-soaked and off-flavor, reduction of firmness, and the acceleration of rotting and decay (Min et al., 2018; Pan et al., 2017). Although there have been a sequence of studies analyzing the relationship between CI and cell membrane damage (Jin et al., 2014), phase transition of membrane lipids (Lyons, 1973), and reactive oxygen species (ROS) attack (Koushesh saba et al., 2012; Yang et al., 2012), deficient energy is arousing wide attention of researchers at present (Pan et al., 2017; Jin et al., 2015; Song et al., 2016). It is interesting to note that the abundant energy and enhanced activity of the related enzymes of energy generation suppressed the process of CI in postharvest papaya, peach, and banana fruit (Pan et al., 2017; Li et al., 2016a,b; Jin et al., 2015), nevertheless, the underlying mechanism has not been adequately elaborated upon. The electron transport pathway (ETP) of the

Abbreviations: CI, chilling injury; ROS, reactive oxygen species; ETP, electron transport pathway; CCP, cytochrome pathway; AP, alternative pathway; ATP, adenosine triphosphate; CA, controlled atmospheres; RH, relative humidity; RCR, respiration control rate; OPR, oxidative phosphorylation rate; ADP, adenosine diphosphate; Cyt c/a, cytochrome c/a; MPTP, permeability transition pore; COX, cytochrome c oxidase; AOX, alternative oxidase; ETC, electron transport chain; NO, nitric oxide

* Corresponding author.
E-mail address: wxy@sdut.edu.cn (X. Wang).

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mitochondria, which mainly includes the cytochrome pathway (CCP) and the alternative pathway (AP), is crucial and essential for providing adenosine triphosphate (ATP) for higher plants and enhancing the capability of resistance to cold, but inevitably produces ROS (Li et al., 2017; Yoshida et al., 2011). Since deficient energy and excess ROS have a close relationship with CI, it is time to access whether the ETP and mitochondria are relevant to CI. Much less is known about the impact of chilling stress on the ETP and the mitochondrial ultrastructure and functions in postharvest fruit and vegetables, but increasing interest over the years has begun to provide greater insight. For example, the mitochondria of some plants were assumed to be signaling organelles, orchestrating defense responses to biotic stress (Cvetkovska et al., 2013). Song et al. (2016) demonstrated that the symptoms of CI in peach fruit had been alleviated through maintaining the mitochondrial function in the hypobaric treatment. Yang et al. (1981) established that the oxidative phosphorylation rate of corn seedlings decreased and the AP changed under cold treatment of 4 °C, but different varieties had different responses to the cold. It would be interesting to ascertain whether a similar situation is responsible for the postharvest white mushrooms with special structure under chilling stress, which indefinitely was associated with the changes in ETP and mitochondria.

Controlled atmosphere (CA) is an effective and acknowledged method of preserving fruit and vegetables all over the world. As discussed earlier, CA also could alleviate the CI of fruits such as guavas (Murmu and Mishra, 2018), tomatoes (Park et al., 2018), or persimmon (Besada et al., 2015). In our previous study, appropriately high ratios of O_2 combining CO_2, such as an 80% O_2 + 20% CO_2 treatment, could sustain the good organoleptic and physiological qualities of postharvest white mushrooms during storage (Li et al., 2017). This treatment remarkably effective in delaying senescence and extending the storage period much longer in comparison to other preservation methods (Li et al., 2017), but its effect on CI requires further exploration.

In our pre-experiment, white mushrooms were susceptible to CI below 2 °C. Consequently, in this study, we focused on the CI, changes of energy metabolism, CCP, AP, and the mitochondrial ultrastructure and functions of white mushrooms under 80% O_2 + 20% CO_2 treatment at 1 °C. The aim of this work is to unearth the underlying mechanism of the relationship between the mitochondria, ETP and CI under high O_2 /CO_2.

2. Materials and methods

2.1. Mushroom materials and treatments

White mushrooms (*Agaricus bisporus*) were freshly harvested from Zibo City in Shandong Province, China. The experimental mushrooms were treated following the methods in our previous research (Li et al., 2017). They were randomly divided into two groups with 450 mushrooms per group: one was the 80% O_2+20% CO_2 treatment and the other group acted as the control. Three subgroups per group were utilized for replication and every subgroup included 150 mushrooms. Subsequently, the white mushrooms were stored at 1 °C and 90–95 % relative humidity (RH). ATP, CCP, AP, activities of enzymes, gene expression, mitochondrial respiration and functions were analyzed employing 30 mushrooms from each group every four days. Fresh weight of white mushroom was used for measuring parameters.

2.2. CI degree evaluation

The CI index was measured employing the method of Li et al. (2016a) with some modifications. The CI scale (P) was calculated with a rating scale ranging from 0 to 5 according to the percentage of CI area of common visual symptoms with epidermis pitting and water-soaked appearance; 0: P = 0; 1: P ≤ 20%; 2: 20% < P ≤ 40%; 3: 40% < P ≤ 60%; 4: 60% < P ≤ 80%; 5: P > 80%. CI index = (ΣCI scale × the number of the mushrooms affected)/(5 × the total mushrooms).

The CI incidence (%) was calculated by the following formula: The CI incidence = the number of CI mushrooms /the total number of mushrooms × 100%

2.3. ATP and energy charge measurement

Briefly, 2.5 g of mushrooms were ground with 15 mL of 0.6 mol L^{-1} perchloric acid, and then centrifuged at 15,000 × g for 15 min at 4 °C. ATP content was determined using high-performance liquid chromatography following the method described in our previous study (Li et al., 2017). Energy charge was calculated by [ATP + 1/2 ADP]/[ATP + ADP + AMP].

2.4. Determination of CCP and AP, the key enzymes activity and their genes expression

The proportions of CCP and AP were determined employing a liquid-phase oxygen measurement system (Chlorolab-2, Hansatech Company, UK) according to the method outlined in our previous research (Li et al., 2017, 2016b). In brief, 1.5 g tissue of mushrooms were cut into small pieces and put into reaction cup, then the total decreasing rate of respiration was tested, the value marked as Rt. Thereafter, 0.1 mL of 0.1 mol L^{-1} salicylhydroxamic acid (SHAM) was added to the reaction cup for AP measurement, and 0.1 mL of 0.1 mol L^{-1} NaN_3 was added for CCP measurement. The CCP and AP values were marked as Vc and Va, respectively. The proportions of CCP and AP were expressed as Pc and Pa, and their proportions were calculated as follows, Pc = (Rt-Vc) /Rt × 100% and Pa = (Rt-Va) /Rt × 100%.

The activities of COX and AOX and their gene expressions were measured according to the method described in our previous research (Li et al., 2017, 2016b).

2.5. Respiration control rate (RCR) and oxidative phosphorylation rate (OPR) of mitochondria measurement

Mitochondria were isolated and purified according to the methods described by Yang et al. (2014) and Qi et al. (2013) with some modifications. In brief, 10 g of mushrooms were ground in 50 ml of precooling extraction buffer (including 50 m mol L^{-1} Tris-HCl, 1 m mol L^{-1} EDTA, 0.5% polyvinylpyrrolidone, 50 m mol L^{-1} sucrose, 5 m mol L^{-1} cysteine, 0.1% bovine serum albumin, and 0.3% β-mercaptoethanol). After being filtered through four layers of cheesecloth, the filtrate was centrifuged at 1500 × g for 12 min. The supernatants were centrifuged at 18,000 × g for 15 min. The pellets were re-suspended twice in 10 mL of washing buffer containing 250 m mol L^{-1} sucrose, 300 m mol L^{-1} mannitol, 1 m mol L^{-1} EDTA, 0.1% bovine serum albumin and 10 m mol L^{-1} Tris-HCl (pH 7.2). Sucrose solutions of 8%, 20% and 45% were prepared with 10 m mol L^{-1} glycine, 1 m mol L^{-1} EDTA and 0.1% bovine serum albumin. The suspension was separated on a step Percoll gradient and centrifuged at 40,000 × g for 1 h. The mitochondria layer (during the interface of the 20% and 45% Percoll gradient) was centrifuged at 15,000× g for 20 min, and the wash was repeated once again. The pellets were re-suspended in 10 ml of wash medium.

The assay of respiration control rate (RCR) of mitochondria was conducted with a liquid-phase oxygen measurement system following the method of Pietro (1981) with some modifications. In brief, 0.25 mL mitochondrial suspensions was added to the 2.0 mL reaction mixture (including 10 m mol L^{-1} EDTA, 250 m mol L^{-1} sucrose, 10 m mol L^{-1} Tris-Mops, 5 m mol L^{-1} pH 7.4 KH_2PO_4, 0.5 mol L^{-1} succinic acid, and 0.1% bovine serum albumin). After recording the base line for 90 s, the 25 μL of 0.2 mol L^{-1} sodium succinate was added, and the respiration of IV was recorded as R_2. Similarly, 10 μL of 50 m mol L^{-1} adenosine diphosphate (ADP) was added 2 min later, and the respiration of III was recorded as R_1. When the ADP was fully consumed, the respiration of IV

reappeared. RCR was calculated by R_1/R_2, oxidative phosphorylation rate (OPR) was calculated by $ADP/(R_1-R_2)\times R_1$ (Ni, 2007).

2.6. Determination of mitochondrial membrane fluidity and cytochrome c/a (Cyt c/a)

The methods of Li et al. (2012) and Shi et al. (2013) were employed to measure the mitochondrial membrane fluidity with slight modifications using the F-4500 spectrofluorometer (Hitachi, Japan) at wavelengths of 400 nm for excitation and 480 nm for emission. Briefly, 0.4 mL mitochondrial suspensions was added to the 0.05 mL of 5 m mol L^{-1} 8-anilino-1-naphthalenesulfonic acid (ANS) and 5.5 mL of 0.3 mol L^{-1} mannitol solutions. After incubating at 25 °C for 10 min, the fluorescence intensities (F) was determined according to the following equation: $F = I_{II} + 2I_I$, where I_I and I_{II} were the fluorescence intensities observed with the excitation polarizer vertically oriented and the emission polarizer horizontally oriented, respectively. F is inversely correlated with membrane fluidity.

Cytochrome c/a (Cyt c/a) was determined according to the Tonshin et al. (2003) method with slight modifications. 0.4 mL of mitochondrial suspensions was added to 2.5 mL of 0.2% bovine serum albumin. The absorbances were immediately recorded at 550 nm and 630 nm.

2.7. Mitochondrial membrane potential ($\Delta\psi_{mit}$) and permeability transition pore (MPTP) measurement

Mitochondrial membrane potential ($\Delta\psi_{mit}$) was determined following the method of Braidot et al. (1998) with slight modifications. 0.4 mL of mitochondrial suspensions was added to 4 mL reaction mixture (2 m mol L^{-1} Hepes, 5 m mol L^{-1} $MgCl_2$, 5 m mol L^{-1} KH_2PO_4, 4.2 m mol L^{-1} sodium succinate, and 250 m mol L^{-1} sucrose, pH 7.4). Finally 0.1 mL of 1 mg L^{-1} rhodamin 123 was added to the mixture. After incubating the reaction mixture at 25 °C for 25 min, it was centrifuged at 8000 × g for 10 min at 4 °C. The pellets were suspended in 4 ml of reaction mixture, and the changes of fluorescence intensity within 3 min were determined using the F-4500 spectrofluorometer at wavelengths of 505 nm for excitation and 575 nm for emission.

Permeability transition pore (MPTP) assay was implemented by applying the method of Marchi et al. (2004) and Yang et al. (2014). In brief, 0.4 mL of mitochondrial suspensions was centrifuged at 8000 × g for 10 min at 4 °C. The pellets were suspended in 4 mL of the reaction mixture (5 m mol L^{-1} Hepes, 70 m mol L^{-1} sucrose, 220 m mol L^{-1} mannitol, and 5 m mol L^{-1} sodium succinate, pH 7.2). The absorbance was immediately recorded at 540 nm after added to 10 μL of 30% H_2O_2.

2.8. Assays of mitochondrial ultrastructure

Mitochondrial ultrastructure was analyzed employing the method of Vigani et al. (2016) with slight modifications. Small pieces of sample mushrooms were fixed in a mixture of 3% glutaraldehyde for 24 h and then rinsed for 4 times in 0.1 mol L^{-1} phosphate buffer (pH 7.0). They were subsequently fixed with osmic acid (1%) for 1.5 h and dehydrated in a graded ethanol series. After being embedded in spurr resin, ultrathin sections were cut and contrasted with uranyl acetate and lead citrate for 20 min, and examined with transmission electron microscopy (JEM-2100 F TEM, Japan) at 80 kV.

2.9. Statistical analyses

Reported values are averages of three replicates. Experiments were performed randomly, and data expressed as mean standard deviation. The *t*-test was carried out for pair-wise comparison. Significant differences are indicated by * and ** at $P < 0.05$ and $P < 0.01$, respectively. NS = not significant.

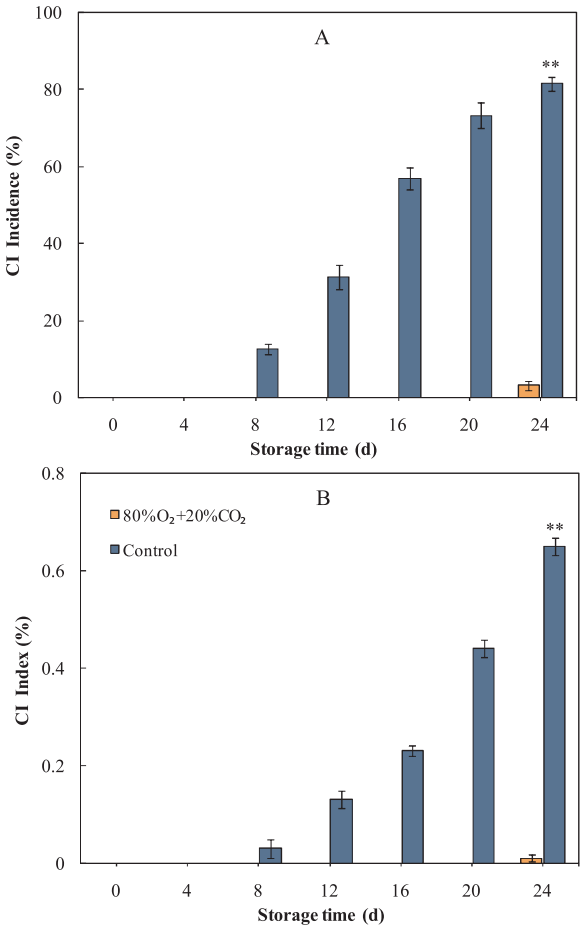

Fig. 1. CI Incidence (A) and CI Index (B) of white mushroom treated with high O_2/CO_2 at 1 °C. Air treatment was used as control. The vertical bars indicate standard deviation (n = 3). Significant differences are shown by ** ($P < 0.01$).

3. Results and discussion

3.1. Effects of high O_2 /CO_2 on the CI incidence and CI index

The symptoms of CI in the white mushrooms presented as a reduction in whiteness and firmness, epidermis pitting, water-soaked, and acceleration of rotting and decay. The CI incidence and CI index values of the control increased during the storage periods, while the mushrooms stored at 80% O_2 + 20% CO_2 exhibited almost no CI symptoms (Fig. 1A, B). The CI of the control was first observed on the 8th day with 12.5% of CI incidence and 0.03 of CI index, and increased up to 81.4% and 0.65 respectively at the end of storage. The finding illustrated that proper O_2 /CO_2 could significantly prevent the occurrence of CI in white mushrooms. Besada et al. (2015) also found that high CO_2 treatment inhibited CI development and fruit darkening in persimmon. Similar results were reported by Murmu and Mishra (2018) and Park et al. (2018) with high O_2 or CO_2 in alleviating CI symptoms in tomatoes and guavas.

3.2. Effects of high O_2 /CO_2 on the ATP and energy charge

Currently, several studies report that the development of CI in several kinds of products might partly be due to energy deficiency (Pan et al., 2017; Li et al., 2016a,b; Jin et al., 2014). Collectively, the ATP content and energy charge of 80% O_2 + 20% CO_2 treatment presented a gradual decrease trend and showed a higher level than the control (Fig. 2A, B). The changes of energy metabolism were in accordance with those of the development of CI during storage. As for the control,

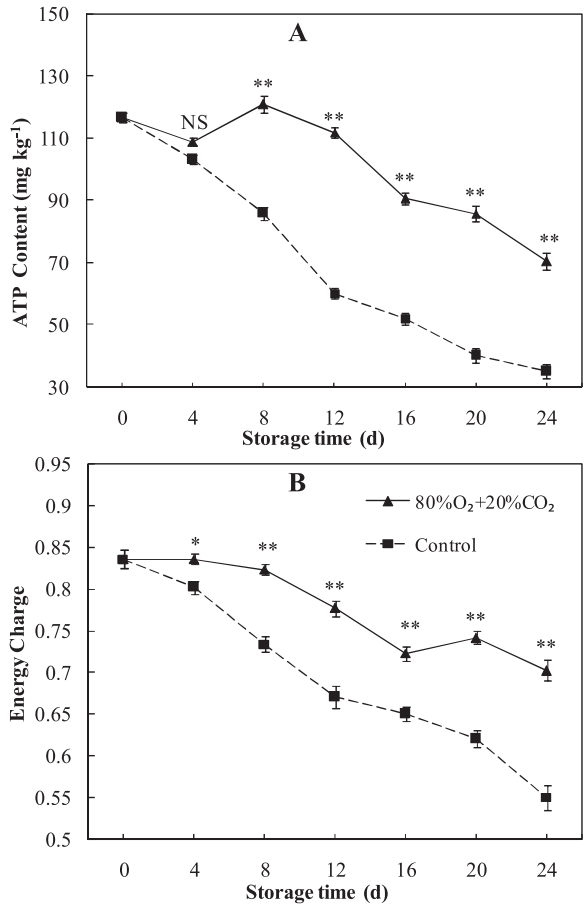

Fig. 2. ATP (A) and EC (B) of white mushroom treated with high O_2/CO_2 at 1 °C. Air treatment was used as control. Energy charge was calculated by [ATP + 1/2 ADP]/ [ATP + ADP + AMP]. The vertical bars indicate standard deviation (n = 3). Significant differences are shown by * ($P < 0.05$) and ** ($P < 0.01$). NS = not significant.

the ATP content and energy charge decreased sharply after the fourth day, and the CI developed on the eighth day respectively.

3.3. Effects of high O_2 /CO_2 on the electron transport pathway (ETP) of mitochondria: CCP, the key enzymes activity and the relative gene expression

Temperature is one of the primary factors affecting the respiration of plants, which is attributed to some respiratory enzymes being extremely sensitive to temperature (Atkin and Tjoelker, 2003). The changes in the proportion of CCP and cytochrome c oxidase (COX) activity of mushrooms under the 80% O_2 + 20% CO_2 and in the control displayed a general falling-off during storage (Fig. 3A, B). Compared to the control, the 80% O_2 + 20% CO_2 dramatically prevented the reduction of the proportion of CCP and COX activity ($P < 0.05$); the proportion of CCP of the control decreased by 7.7 times in comparison to the 80% O_2 + 20% CO_2 from the 0–12 t h days during storage. Mushrooms undergoing the 80% O_2 + 20% CO_2 treatment displayed a significantly higher level of *AbCOX2* transcript ($P < 0.05$) than the control during the down-regulation process (Fig. 3C). This paralleled the change of the proportion of CCP and COX activity. Low level of ATP appeared to block electron transferring at the terminal of respiratory electron transport chain (ETC) (Partridge et al., 1994), and COX is one of the complexes of ETC. In our study, the 80% O_2 + 20% CO_2 treatment increased the COX activity and ATP production, and further impacted on the CCP.

As discussed earlier, the proportion of CCP of some plants

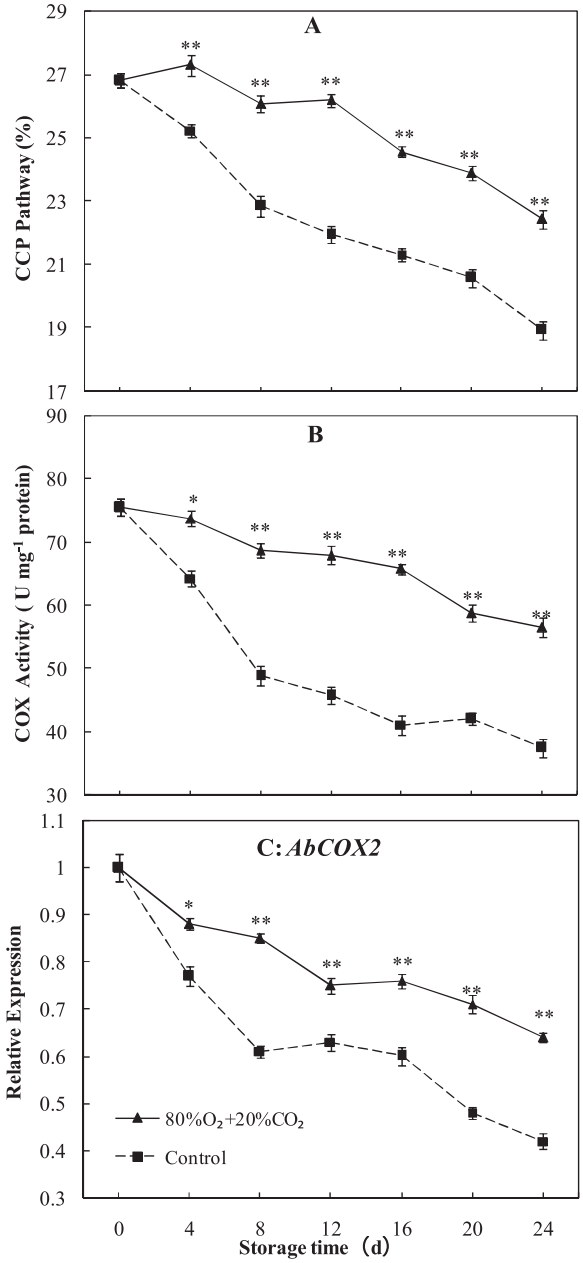

Fig. 3. Mitochondrial ETP: CCP (A), COX activity (B) and relative expression of *AbCOX2* (C) of white mushroom treated with high O_2/CO_2 at 1 °C. Air treatment was used as control. The vertical bars indicate standard deviation (n = 3). Significant differences are shown by * ($P < 0.05$) and ** ($P < 0.01$).

diminished under cold stress (Purvis and Shewfelt, 1993; Wagner, 1995), which might be owing to the deficient ADP in mitochondria and the COX being more sensitive to low temperatures (Stewart et al., 1990). Zhou et al. (2000) pointed out that cold stress could trigger alteration of the protein conformation of COX. In our study, the 80% O_2 + 20% CO_2 treatment prominently prevented the decline of COX activity and down-regulation of *AbCOX2*, which could delay the decrease in the proportion of CCP and further alleviate the CI of the white mushrooms.

3.4. Effects of high O_2 /CO_2 on the electron transport pathway (ETP) of mitochondria: AP, the key enzymes activity and the relative genes expression

The plant mitochondrial ETP includes a non-energy conserving AP, which dampens ROS production by uncoupling it from ATP turnover

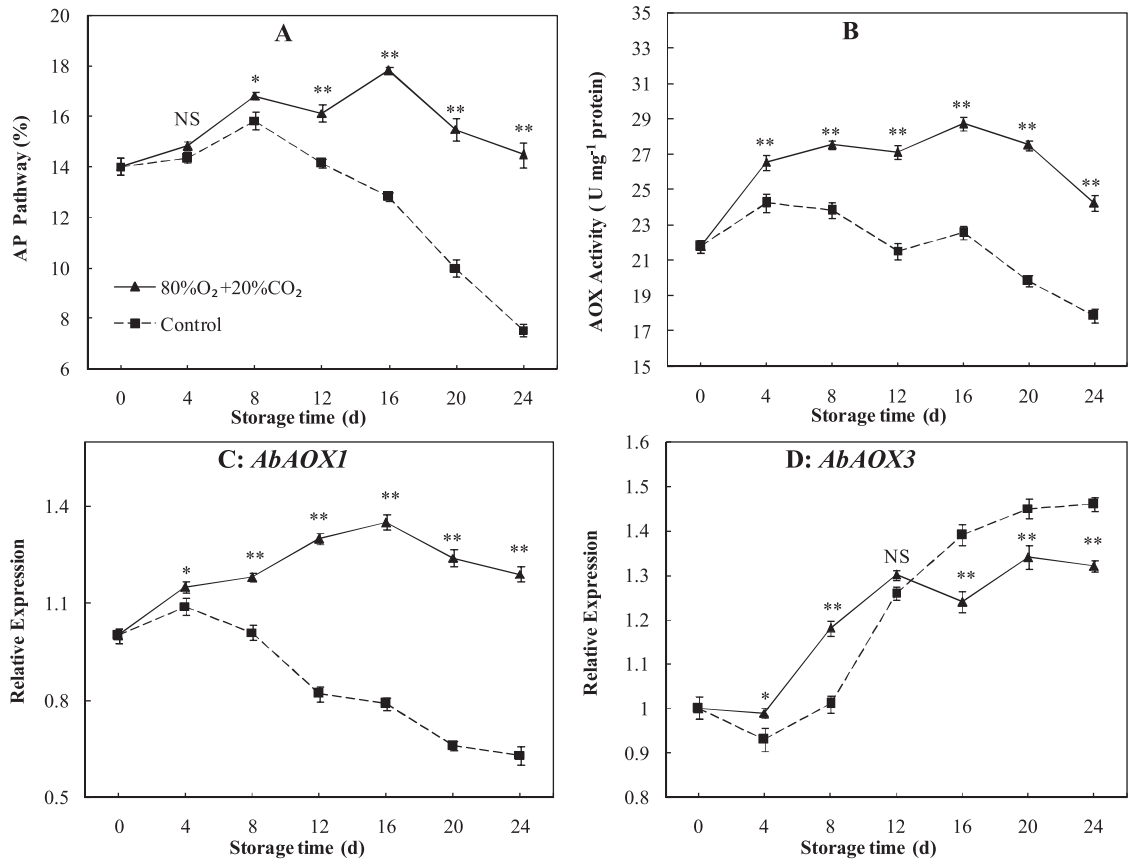

Fig. 4. Mitochondrial ETP: AP (A), AOX activity (B) and relative expressions of *AbAOX1* (C) and *AbAOX3* (D) of white mushroom treated with high O_2/CO_2 at 1 °C. Air treatment was used as control. The vertical bars indicate standard deviation (n = 3). Significant differences are shown by * ($P < 0.05$) and ** ($P < 0.01$). NS = not significant.

and enhance the endurance capability particularly under abiotic stress for some plants (Wang et al., 2011). Moreover, numerous studies have illustrated that the expression of the alternative oxidase (AOX) gene and AP could be induced by abiotic stresses such as chilling or freezing injuries (Wang et al., 2011). For example, studies in several plant species exhibited an increase in AOX transcript to cold stress (Atkin and Tjoelker, 2003; Vanlerberghe and McIntosh, 1992), though other studies did not find the same results (Gonzales-Meler et al., 1999; Svensson et al., 2002). Hence, our results explained whether the hypothesis also holds true for white mushrooms undergoing the chilling stress and the way that the high O_2 /CO_2 can moderate the CI by altering AP and AOX expressions. As shown in Fig. 4A and B, the changes in the proportion of AP and AOX activity increased at the beginning and then decreased with some fluctuations. The increase of the proportion of AP and AOX activity at the previous stage of storage might represent a crucial acclimation response to cold storage. The proportion of AP in the control group declined rapidly by 52.4% during the 8–24 t h days. Whereas, the proportion of AP undergoing the 80% O_2 + 20% CO_2 diminished only by 18.8% between the 0–16 t h days, indicating that the proper high O_2 /CO_2 could enhance tolerance to the chilling stress. Basically, the change of the proportion of AP was agreement with the development of CI during storage. The variation in expressions of *AbAOX1* were similar to the proportion of AP, and the expression of 80% O_2 + 20% CO_2 treatment was significantly higher than the control ($P < 0.05$) (Fig. 4A, C). In relation to the *AbAOX3* expression, the control increased swiftly and showed a dramatically higher level than the 80% O_2 + 20% CO_2 treatment during the later stage of storage ($P < 0.05$) (Fig. 4D). Our results demonstrated that the *AbAOX1* of the white mushrooms might have a close relationship with the cold stress, whereas the *AbAOX3* may be related to the dampening of ATP yield by the uncoupling mechanism, which was consistent with the results of Borecky and Vercesi (2005). Accordingly, the 80% O_2 + 20% CO_2 treatment could restrain the CI by up-regulating the *AbAOX1* expression, improving the AOX activity, and further enhancing the proportion of AP. Notably, the change of AP caused by cold stress was assumed to be the result of the close relationship with the variation of mitochondrial ultrastructure. Several studies have begun to focus on the potential role of AOX as a vital mitochondrial component of preharvest products under stress (Vanlerberghe, 2013), which is clearly an area that needs further confirmation, especially for postharvest fruit and vegetables.

3.5. Effects of high O_2 /CO_2 on the respiration control rate (RCR) and oxidative phosphorylation rate (OPR) of mitochondria

Under cold stress, the changes of mitochondrial function of some plants manifested across in oxidative phosphorylation uncoupling, reducing its activity and ATP generation (Jian and Wang, 2008). The disturbance of mitochondrial oxidative respiration prevailingly reduced the mitochondrial function and the energy yield; the oxidative phosphorylation uncoupling presented as the primary characteristic of these changes (Jian and Wang, 2008). Furthermore, RCR and the OPR were critical attributes reflecting the functions of oxidative phosphorylation. Specifically, RCR was generally utilized to estimate the mitochondrial structural integrity and the degree of oxidative phosphorylation uncoupling, and the OPR was deemed to be associated with the generation efficiency of energy (Ni, 2007). In our study, the RCR of the 80% O_2 + 20% CO_2 treatment decreased by 26.1% from the first to 16th days during storage, whilst those in the control group decreased 69.6%, respectively (Table 1). These findings illustrated that 80% O_2 + 20% CO_2 could prevent a notable decrease in RCR, and further retain well the

Table 1
Mitochondrial RCR and OPR of white mushroom treated with high O_2/CO_2 at 1 °C.

Treatment	R (mmol O_2 min^{-1} kg^{-1})	Storage time (d)			
		0	8	16	24
High O_2 /CO_2	R_1		33.23 ± 0.38*	31.45 ± 0.43**	27.28 ± 0.50**
	R_2		8.52 ± 0.13*	9.65 ± 0.12*	11.02 ± 0.16*
	RCR		3.90 ± 0.01*	3.26 ± 0.02**	2.48 ± 0.02**
	OPR		45.86 ± 0.56*	43.40 ± 0.43**	37.65 ± 0.29**
Control	R_1	35.32 ± 0.46	25.88 ± 0.58	18.43 ± 0.62	15.09 ± 0.57
	R_2	8.01 ± 0.15	12.41 ± 0.18	13.74 ± 0.09	14.46 ± 0.23
	RCR	4.41 ± 0.03	2.14 ± 0.02	1.34 ± 0.02	1.04 ± 0.03
	OPR	48.74 ± 0.36	35.71 ± 0.26	25.43 ± 0.22	20.82 ± 0.31

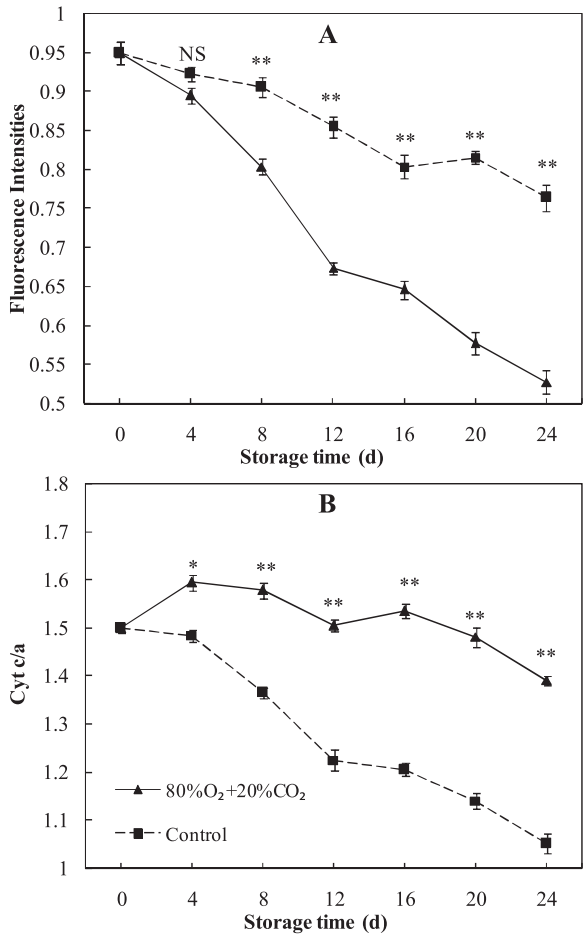

Fig. 5. Mitochondrial membrane fluidity (A) and Cyt c/a (B) of white mushroom treated with high O_2/CO_2 at 1 °C. Air treatment was used as control. The vertical bars indicate standard deviation (n = 3). Significant differences are shown by * ($P < 0.05$) and ** ($P < 0.01$). NS = not significant.

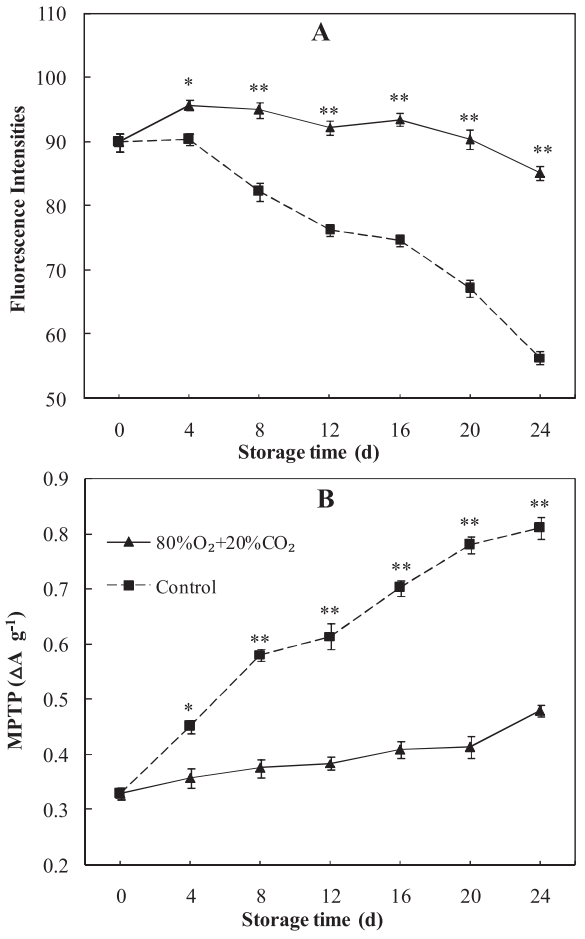

Fig. 6. Mitochondrial membrane potential (A) and MPTP (B) of white mushroom treated with high O_2/CO_2 at 1 °C. Air treatment was used as control. The vertical bars indicate standard deviation (n = 3). Significant differences are shown by * ($P < 0.05$) and ** ($P < 0.01$).

function of mitochondrial oxidative respiration, which might be attributed to the restraining cytochrome c dissociation from mitochondrial membrane and mitochondrial structure change.

3.6. Effects of high O_2 /CO_2 on the mitochondrial membrane fluidity and cytochrome c/a (Cyt c/a)

In the mitochondria, the overwhelming majority of the physiological and biochemical reactions were dependent upon their integrity and membrane fluidity. When the mitochondrial membrane fluidity dropped to a certain level, physiological metabolic disorders appeared, which caused the diminished function of cells and hence the senescence and death of the organisms (Blokhina and Fagerstedt, 2010). In the study, the fluorescence intensities (F) (reflecting the mitochondrial membrane fluidity) of the control underwent a sharp decline after the 4th day under the chilling stress (Fig. 5A), which was consistent with the research on rice and cucumber seedlings (Jian and Wang, 2008). It is interesting to note that the reduction of F treated with the 80% O_2 + 20% CO_2 was significantly prevented ($P < 0.05$), i.e., 19.6%, while that of the control was 44.5% during the whole storage. The results indicated that the 80% O_2 + 20% CO_2 treatment could conspicuously inhibit the decline of mitochondrial membrane fluidity.

Cyt c is a crucial component of ETC, whose reduction or deficiency could block the ETC, diminish ATP yield and boost the generation of ROS (Azad et al., 2008). Cyt c is ordinarily located in the mitochondrion, and its release to the cytoplasm signifies cell apoptosis (Robson

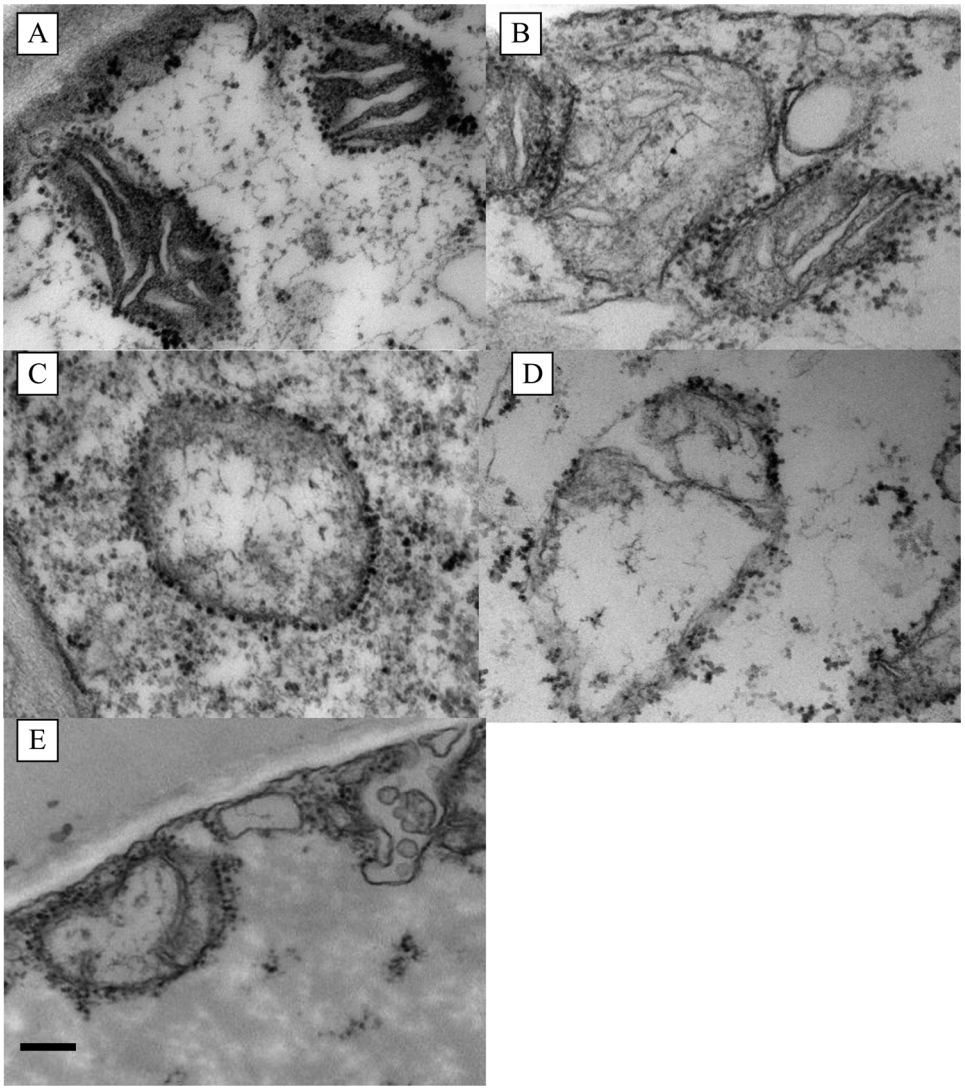

Fig. 7. Mitochondrial ultrastructure of the white mushroom treated with high O_2/CO_2 at 1 °C (A–E): the fresh white mushroom on the 0 day (A), treated with 80% O_2 + 20% CO_2 on the 16th day (B), the control group on the 16th day (C), treated with 80% O_2 + 20% CO_2 on the 24th day (D), the control group on the 24th day (E). All scale bars for A–E = 200 nm.

and Vanlerberghe, 2002). The variation tendency of Cyt c/a was roughly similar to the changes of mitochondrial membrane fluidity during the whole storage (Fig. 5A, B). It manifested as a decrease of 7.3% and 30.0% in the 80% O_2 + 20% CO_2 and the control, respectively. Our results indicated that high O_2/CO_2 could restrain the Cyt c release, and further retain the mitochondrial structure and functions.

3.7. Effects of high O_2 /CO_2 on the mitochondrial membrane potential ($\Delta\psi_{mit}$) and permeability transition pore (MPTP)

The function of the mitochondrial membrane potential is the hub of controlling the respiration and ATP yield, so it is crucial to confirm its impact of chilling stress. Therefore, it is also worth exploring how the mushrooms stored at 80% O_2 + 20% CO_2 respond to the stress and maintain the mitochondrial function. Under chilling stress, the mitochondrial membrane potential of the control displayed a sharp decrease of 37.2% during storage. Notably, the 80% O_2 + 20% CO_2 treatment displayed a reduction of a mere 5.3% (Fig. 6A). Loss of mitochondrial membrane potential is sometimes attributed to the opening of an MPTP, which in turn often results in the release of cyt c to the cytosol (Vanlerberghe, 2013).

In general, when the organism is healthy, the MPTP is closed, which could maintain chemical equilibrium in the mitochondria (Tiwari et al., 2002). Under oxidative stress or matrix (Ca^{2+}) increase, the MPTP opens and the swelling of the mitochondria occurs, inducing different types of cell death (Tiwari et al., 2002). Significant progress is imminently needed to be made currently to ascertain the response of the mitochondria to the chilling stress. The manner in which high O_2/CO_2 alleviates mitochondrial injury is worthy of more investigation. Song et al. (2016) pointed out that the mitochondrial membrane fluidity subsided and MPTP intensified continuously during the CI process in peach fruits. In our study, white mushrooms undergoing the 80% O_2 + 20% CO_2 exhibited a prominently lower MPTP than the control ($P < 0.05$), with 0.41 and 0.78 respectively on the 20th day (Fig. 6B). The results indicated that high O_2/CO_2 could retard the degree of MPTP opening, slow down the mitochondrial swelling speed, and further retain mitochondrial integrity.

3.8. Effects of high O_2 /CO_2 on the mitochondrial ultrastructure

The vital function of mitochondria under cold storage remains largely unknown.

As shown in Fig. 7A, the mitochondria of fresh mushrooms had an integrated structure and distinct cristae, along with an abundant

number of cristae and a wide intracristae surface area. The mushrooms that underwent the 80% O_2 + 20% CO_2 treatment still maintained mitochondrial structure integrity on the 16th day. The cristae were relatively clear and intracristae spaces were enlarged (Fig. 7B). Notwithstanding, the mitochondria of the control showed intact outer membranes, matrix volume dilation, cristae partially degraded and intracristae space was compressed (Fig. 7C). At the end of storage (on the 24th day), the mitochondria swelled, a large electron-transparent area appeared, outer membranes partially ruptured and the mitochondrial matrix with cristae became thin in mushrooms that underwent the 80% O_2 + 20% CO_2 treatment (Fig. 7D). In contrast, the mitochondria of the control mushrooms presented a significant swell, extensive rupture of the outer membrane, and almost complete disappearance of the cristae (Fig. 7E). Our results were similar to the findings of Chen (2012), who described that the mitochondrial structure was obscured along with the broken or disappeared membrane, destruction and reduction of the cristae and disintegration of the mitochondria when the root of sugarcane seedlings were under chilling stress. There are two types of mitochondrial conformations: orthodox with an expanded matrix volume and a compressed intracristae space, condensed conformation with a partial matrix contraction and dilated intracristae spaces, but the explicit mechanism requires further investigation (Vigani et al., 2016). The switch between these two conformations might suggest a dynamic process, which lies on the metabolic status of the cell such as ETP and mitochondrial respiration (Vigani et al., 2016). Interestingly, the mitochondria in cells from mushrooms that underwent the 80% O_2 + 20% CO_2 treatment manifested in the condensed conformation, while the control had orthodox conformation on the 16th day under chilling stress (Fig. 7B, C), which revealed that high O_2 /CO_2 exposure might impact on the ETP, mitochondrial respiration and functions by activating the conversion of the two mitochondrial conformations, and further alleviate the CI of white mushrooms.

As shown in earlier studies, 80% O_2+ 20% CO_2 suppressed the ROS and nitric oxide (NO) generation in comparison to other treatments and the control (Li et al., 2017). As for key enzymes of ETP, COX was significantly inhibited by NO, whereas AOX was NO-resistant (Cvetkovska et al., 2013). The present work reinforces the link between ETP and mitochondrial structure and functions. It provides a potential mechanism and theoretical analysis employing high O_2/CO_2 treatment to alleviate CI, including ETP, its key enzymes and mitochondrial function, ultrastructure, and the switching of mitochondrial two conformations in comprehensive and new ways for postharvest white mushrooms.

4. Conclusions

In conclusion, chilling stress can perturb the metabolism of white mushrooms through the disruption of the ETP metabolism and mitochondrial ultrastructure and functions. The 80% O_2+ 20% CO_2 treatment could prominently inhibit the occurrence of CI and the CI index of mushrooms and maintain higher energy level. Meanwhile, it prevented the proportion of CCP reduction and enhanced the proportion of AP by affecting their key enzymes and relative gene expressions. Furthermore, the mitochondrial functions and structures were satisfactorily retained under the 80% O_2+ 20% CO_2 treatment, displaying higher mitochondrial membrane fluidity and Cyt c, lower mitochondrial membrane potential and MPTP opening, and a relatively intact structure of mitochondria. The above results provide novel findings; collectively, they clearly illustrate that the ETP metabolism and mitochondrial ultrastructure and functions may be more prevalent under chilling stress, especially in postharvest fruit and vegetables.

Notes

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