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Research Article

Responses of Phospholipase D and Antioxidant System to Mechanical Wounding in Postharvest Banana Fruits

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Banana fruits are susceptible to mechanical damage. The present study was to investigate the responses of phospholipase D (PLD) and antioxidant system to mechanical wounding in postharvest banana fruits. During 16 d storage at 25°C and 90% relative humidity, PLD activity in wounded fruits was significantly higher than that in control (without artificial wounding fruits). The higher value of *PLD* mRNA was found in wounded fruits than in control. *PLD* mRNA expression reached the highest peak on day 4 in both groups, but it was 2.67 times in wounded fruits compared to control at that time, indicating that *PLD* gene expression was activated in response to wounding stress. In response to wounding stress, the higher lipoxygenase (LOX) activity was observed and malondialdehyde (MDA) production was accelerated. The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) in wounded fruits were significantly higher than those in control. The concentrations of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) in fruits increased under mechanical wounding. The above results provided a basis for further investigating the mechanism of postharvest banana fruits adapting to environmental stress.

1. Introduction

Mechanical damage is the main cause for losses in postharvest horticultural products [1, 2]. Wounds represent vulnerable points that may lead to severe damage and compromise organ survival rate. Membrane deterioration is an early and characteristic feature in plant cells undergoing mechanical injuries [3]. Membrane integrity loss is often associated with lipid peroxidation or phospholipid degradation. The increased lipid peroxidation, mediated and sustained by phospholipid-degrading enzymes such as phospholipase D (PLD) and lipoxygenase (LOX), results in membrane integrity loss, which has been noted in senescing petal tissues [4]. PLD and LOX in plants play important roles in phospholipid catabolism, initiating lipolytic cascade in membrane deterioration during senescence and stress [5, 6]. LOX, encoding a lipoxygenase involved in jasmonate (wounding-induced signaling molecule) synthesis, is transcriptionally upregulated in response to wounding in *Arabidopsis* [7]. It has been proposed that the liberated polyunsaturated fatty acids serve as substrates for LOX that produces activated oxygen and lipid peroxides leading to membrane damage [8]. PLD has been proposed to play several roles in wounding response. The increased PLD-mediated hydrolysis occurs in response to various stress conditions such as frost, senescence, and wounding [9]. Plants accumulate phosphatidic acid (PA) and unesterified fatty acids that are released from lipids, presumably by the action of wound-inducible phospholipases of types D after wounding [7, 10]. The wounding-induced activation of PLD may result from translocation of PLD to the membrane, which is mediated by an increase in cytoplasmic calcium and stimulated by low micromolar calcium levels

[11, 12]. In castor bean leaves, it has been reported that free fatty acid quantity and PLD activity increase with wounding. Increases in membrane-associated PLD and LOX have been observed in response to mechanical wounding in postharvest cucumber fruits [8].

A wound signal originates at injury site and propagates into adjacent tissue where it induces a number of physiological responses, including lipid degradation, peroxidation compound synthesis and accumulation, and subsequent tissue browning [13]. The oxylipin pathway is involved in wound responses in a number of plants. Antioxidant enzymes are critical in inhibiting oxidative stress. When reactive oxygen species (ROS) increases, chain reactions start in which superoxide dismutase (SOD) catalyzes the dismutation of superoxide radical ($O_2^{\bullet-}$) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). H_2O_2 is then detoxified by catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) [14]. CAT reduces H_2O_2 into water and O_2, whereas POD decomposes H_2O_2 by oxidation of cosubstrate such as phenolic compounds [15].

Banana (*Musa acuminate* L.) is one of the major commercial fruit crops grown in tropics and subtropics. It is very important to the economy of developing countries [16]. Banana fruits are very susceptible to mechanical damage during harvesting, handling, packaging, and transportation, which results in a substantial reduction in quality. Mechanical wounding significantly reduces the commercial value of banana fruits [17]. Lipid degradation and peroxidation activities are directly involved in natural and induced senescence and mechanical wounding. With regard to harvested banana fruits, it is important to discern potential roles of lipid degradation and antioxidant enzymes in association with mechanical wounding. The aim of this research was to explore the role of PLD, LOX, and oxidative stress in banana fruits subjected to artificial wounding and senescence processes. Molecular characterization of PLD in response to wounds and senescence was also analyzed. These innovative results will provide a scientific basis for further investigating the mechanism of postharvest banana adapting to environmental stress.

2. Materials and Methods

2.1. Plant Materials and Postharvest Treatments. Banana fruits were collected from a commercial orchard in Nanning of Guangxi province during July 2016 and transported into a laboratory in Guangxi Academy of Agricultural Sciences immediately. The fruits without infection and physical injury were chosen as plant materials at similar size and same mature period. They were randomly divided into wounded and control groups (100 fruits in each group). In wounded group, banana fruits were punctured at 3 cm intervals from the calyx to the stalk end by a 10 mm diameter stainless steel puncher. The fruits which were not punctured were used as control group. Both groups were packed into polyethylene bag (0.03 mm thick) and stored at 25°C and 90% relative humidity (RH). Every 6 fruits were taken for the determination every 2 d after wounding.

2.2. PLD Activity. PLD activity was assayed by means of highly specific and sensitive sandwich enzyme immunoassay technique (ELISA) [18]. The 96-well ELISA plates (Nunc, Wiesbaden, Germany) were coated with the purified plant PLD antibody (100 μL per well, 2 mg/mL diluted 1 : 1000 in PBS, 4°C, 8 h, pH 7.2–7.4). Plates were then blocked with 1% BSA in PBS for 16 h at 37°C. The wells were then washed twice with 200 μL of wash buffer. A series of PLD standards were prepared in range 10–120 U/L in PBS/0.1% BSA. Samples were also prepared in a series of dilutions from 1/5 to 1/50 in PBS. A total of 50 μL of standards and samples were added and incubated for 2 h at 37°C. The wells were then washed twice with 100 μL of wash buffer. Conjugate binding was performed by adding a biotin-conjugated antibody specific for PLD (100 μL of 0.1% conjugate antibody in 0.1% BSA/PBS). After being incubated for 60 min at 37°C, the plates were washed three times with wash buffer and three times with 100 μL of carbonate buffer. Horseradish Peroxidase (HRP) (100 μL of 15 mg HRP in 15 mL of carbonate buffer) was added and incubated for 10 min at 37°C. The absorbance of the plates was then read at 450 nm on an ultraviolet (UV) microplate reader (Bio-Rad Laboratories, Hercules, California, USA).

2.3. Semiquantitative RT-PCR Analysis. Total RNAs were extracted from 3 g of fresh banana pericarp tissues using CTAB method [19]. Total RNA samples were then dissolved in 50 μL of RNase free H_2O and stored at 80°C prior to RT-PCR. Total RNA (5 μg) was used for the cDNA synthesis with the Reverse Transcription System A3500 (Promega, Madison, WI, USA) according to the protocol provided by the manufacturer. The fragment of *PLD* cDNA was cloned by RT-PCR. PCR was carried out with an initial heat action step at 94°C for 6 min, and amplifications were achieved through 35 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 90 s. A final extension reaction was carried out for 6 min at 72°C. The resulting PCR product was isolated, cloned, and sequenced (Invitrogen, Shanghai, China).

The mRNA expression patterns of *PLD* gene were examined by semiquantitative RT-PCR. The house-keeping gene *Actin* (GenBank accession number AB046952) was used as an internal control. Protocols for total RNA extraction and synthesis of cDNA were described as above. Gene specific primers for *PLD* (*PLD*-S3: 5′-GAAATCGGGAGGTCAAGAAGAG-3′; *PLD*-A3: 5′-CTAAGTTGTGAGGATTGGAGG-3′) and *Actin* (forward: 5′-GATTCTGGTGATGGTGTGAGT-3′; reverse: 5′-GACAATTTCCCTTAGCAG-3′) were used in RT-PCR. PCR was carried out with an initial heat action step at 94°C for 5 min, and amplifications were achieved through 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified products (10 μL) were analyzed by 1% agarose gel electrophoresis [19].

2.4. LOX Activity. The frozen banana pericarp tissues (5 g) were ground finely in liquid nitrogen and then homogenized in 15 mL of 50 mM phosphate buffer (pH 7). After centrifugation at 10,000 ×g for 20 min at 4°C, the supernatant was collected and then used as crude enzyme extract. LOX

activity was assayed by monitoring the formation of conjugated dienes from linoleic acid at 25°C and 234 nm [20]. A total of 3 mL of reaction mixture contained 50 mM sodium phosphate buffer (2.8 mL, pH 7), 10 mM sodium linoleic acid solution (0.1 mL), and crude enzyme solution (0.1 mL). One unit of LOX activity was defined as a change of 0.01 in absorbance per minute at 25°C.

2.5. Activities of Antioxidative Enzymes. CAT and POD were extracted and assayed according to the modified methods of Oracz et al. [21]. Banana pericarp tissues (0.5 g) were extracted for 10 min with 2.5 mL of 0.1 M sodium phosphate buffer (pH 7) at 4°C. The extract solution was centrifuged for 15 min at 12,000 ×g. The supernatant was collected as enzyme solution for the determinations of enzymatic activities. The assay mixture (3 mL) for determining CAT activity consisted of 2.8 mL of 15 mM H_2O_2 prepared by 0.05 M sodium phosphate buffer (pH 7.8) and 0.2 mL of enzyme solution. The increase in absorbance at 240 nm was recorded for 3 min at 25°C. The assay mixture (3 mL) for determining POD activity consisted of 2.5 mL of 0.1 M sodium phosphate buffer (pH 7), 0.2 mL of 0.46% (v/v) H_2O_2, 0.2 mL of 4% (v/v) guaiacol, and 0.1 mL of enzyme solution. The increase in absorbance at 470 nm was recorded for 3 min at 25°C.

SOD was extracted and assayed according to the modified methods of Sun et al. [22]. Banana pulp tissues (0.5 g) were extracted for 10 min at 4°C with 2.5 mL of 0.05 M sodium phosphate buffer (pH 7.8) containing 0.1% (w/v) polyvinyl pyrrolidone. The extract solution was centrifuged for 20 min at 12,000 ×g. The supernatant was collected for analyzing SOD activity. SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). A total of 0.05 mL of enzyme solution was added to 3 mL of assay reagent consisting of 13 mM methionine, 63 μM NBT, 100 μM EDTA, and 1.3 μM riboflavin in 0.05 M sodium phosphate buffer (pH 7.8). The reaction solutions were incubated for 10 min under 4,000 lux illumination. The absorbance of sample was measured at 560 nm and 0.05 M sodium phosphate buffer (pH 7.8) was used as control.

The assay for APX activity was determined according to the modified method of Nakano and Asada [23]. APX activity consisted of 0.1 mM EDTA-Na_2, 0.5 mM ascorbic acid, and 0.1 mM H_2O_2 (above-mentioned reagents were prepared in 0.05 M sodium phosphate buffer, pH 7) and 0.1 mL of enzyme solution. The activity was recorded as the decrease in absorbance at 290 nm. One enzyme unit was defined as 1 mol of ascorbic acid oxidized per minute at 290 nm.

2.6. MDA Content. Malondialdehyde (MDA) content was measured according to the reported method of Sun et al. with a slight modification. Banana pulp tissues (3 g) were homogenized with 15 mL of 10% trichloroacetic acid and centrifuged at 15,000 ×g for 20 min [22]. One milliliter of supernatant was mixed with 3 mL of 0.5% 2-thiobarbituric acid, heated at 95°C for 20 min, and then immediately cooled in an ice-water bath. The absorbance was measured at 532 nm after centrifugation at 3,000 ×g for 10 min and the value for nonspecific absorbance 600 nm was subtracted. The amount of MDA was estimated as follows: (μM/g FW) = [6.45 (OD_{532} − OD_{600}) − 0.56 OD_{450}] × 5 mL/0.25 g.

2.7. Superoxide Anion Content. Superoxide radical ($O_2^{\bullet-}$) was determined by the conversion of nitro blue tetrazolium (NBT) staining according to Dunand et al. [24]. The banana powder (1 g) was vacuum-infiltrated with 10 mM NaN_3 in 10 mM potassium phosphate buffer (pH 7.8) for 1 min and incubated in 1 mg/mL NBT (in 10 mM potassium phosphate buffer, pH 7.8) for 20 min in the dark at room temperature.

2.8. H_2O_2 Content. H_2O_2 levels were determined according to the modified method of Velikova et al. [25]. The banana powder (0.5 g) was homogenized for 10 min in 2.5 mL of cold acetone. The homogenate was centrifuged for 15 min at 12,000 ×g. A total of 1 mL of supernatant was added to 0.1 mL of 20% (v/v) titanium tetrachloride ($TiCl_4$) and 0.2 mL of ammonia water. After centrifuging for 15 min at 12,000 ×g, the precipitate was collected and dissolved in 3 mL of 1 M H_2SO_4 and centrifuged for 5 min at 6,000 ×g. The absorbance of the supernatant was measured at 412 nm. The content of H_2O_2 was calculated from a standard curve. The increasing rate of H_2O_2 content (%) = $[(C_i - C_0)/C_0] \times 100$, where C_i indicated H_2O_2 content in banana fruits storing for different times, while C_0 was H_2O_2 content in banana fruits storing for 0 d.

2.9. Statistical Analysis. Experiments were arranged in a completely randomized design. All statistical analyses were performed by variance (ANOVA) analysis using Statistix version 8.0 (Analytical Software, Tallahassee, FL, USA). Treatment means were compared by Fisher's Least Significant Difference (LSD) test at a significance level of $P < 0.05$.

3. Results

3.1. Phospholipase D Activation and PLD Gene Expression in Postharvest Banana Fruits under Mechanical Wounding. PLD activity was correlated with mechanical wounding. From Figure 1, at 25°C and 90% RH, banana PLD activity demonstrated a rapid increase tendency in both control and wounded fruits with extended storage time and attained maximum on day 2 and day 4, respectively. PLD activity in wounded fruits was significantly higher ($P < 0.05$) than that in control throughout the entire storage period. After day 4, PLD activity increased 2-fold in wounded fruits while increasing only 1.6-fold in control. The accumulation of banana *PLD* mRNA in response to mechanical wounding was further investigated (Figure 2). Similar to PLD activity profile, *PLD* expression was found to be upregulated with the extended storage and reached the highest expression peak on day 4 in control (1.48 times the control on day 0) and then decreased on day 6. In wounded fruits, *PLD* expression also attained a maximum on day 4 (3.14 times to control on day 0). The higher value (3.95) of *PLD* mRNA was found in wounded fruits than that in control, and *PLD* mRNA expression in wounded fruits was 2.67 times that in control on day 4, indicating that *PLD* gene expression was

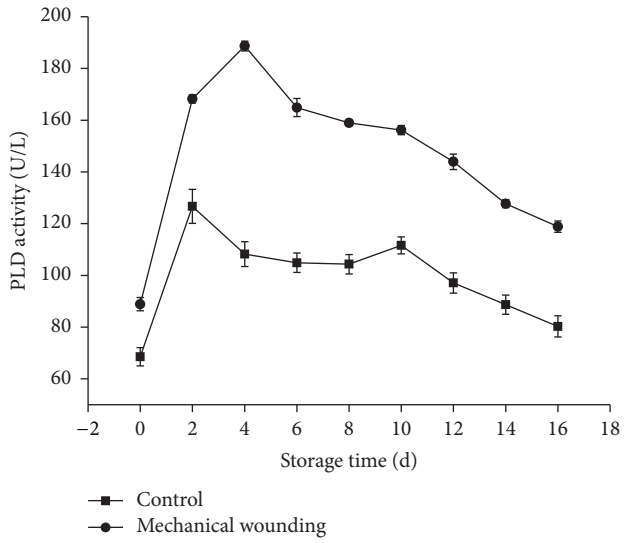

Figure 1: PLD activity in banana fruits dealing with mechanical wounding.

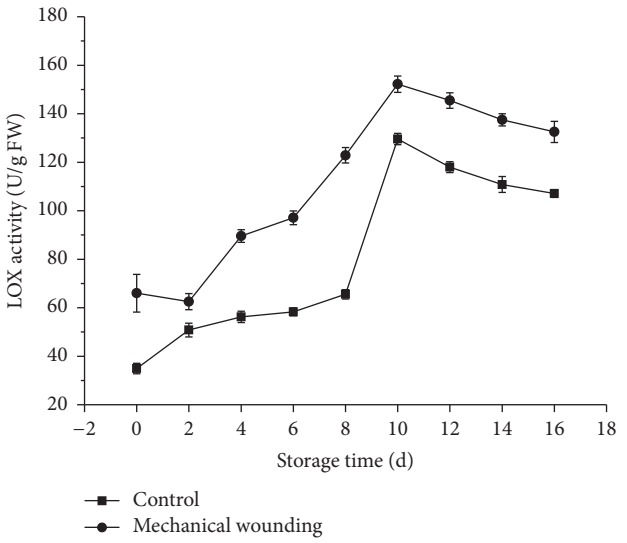

Figure 3: LOX activity in banana fruits dealing with mechanical wounding.

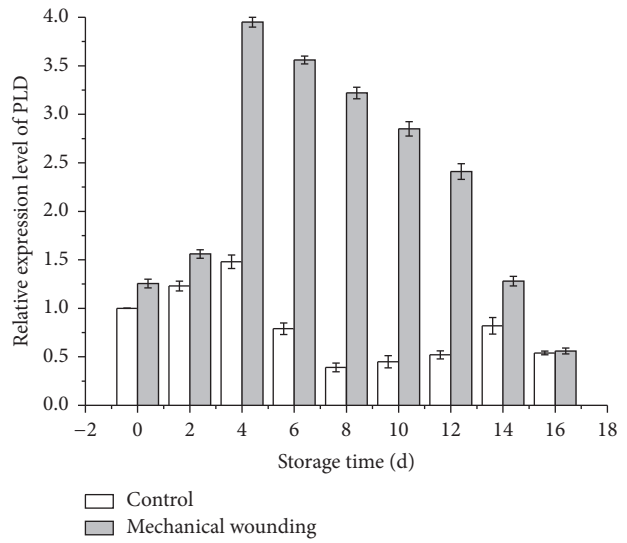

Figure 2: Expression analysis of *MaPLD* gene in banana fruits dealing with mechanical wounding.

activated by wounding. This was similar to the expression pattern found in lettuce [13]. These results suggested that PLD could be involved in producing wound signal and PLD activity in wounded fruits that were associated with the mRNA expression. PLD induced by wounding might stimulate corresponding physiological reactions related to fruit deterioration and senescence through increasing gene expression.

3.2. LOX Activity in Postharvest Banana Fruits under Mechanical Wounding. LOX is a key enzyme in phospholipid catabolism of plants, initiating a lipolytic cascade in membrane deterioration during senescence and stress. From Figure 3, at 25°C and 90% RH, LOX activity from banana fruits increased first and then decreased during 16 d storage and attained maximum on day 10 in both control and wounded fruits. Much higher LOX activity was observed in wounded fruits than that in control throughout the entire storage period. Similar results were obtained by Zhao et al. who reported that LOX might be the main hydrolytic enzymes of phospholipids in response to mechanical wounding in postharvest cucumber fruits [8].

3.3. Activities of Antioxidative Enzymes in Postharvest Banana Fruits under Mechanical Wounding. The activities of antioxidative enzymes SOD, CAT, POD, and APX in postharvest banana fruits were shown in Figure 4. From Figure 4(a), at 25°C and 90% RH, SOD activity rose rapidly during 16 d storage. It was higher in wounded fruits than that in control, but there was no statistically significant difference ($P > 0.05$) on day 4. CAT activity in control showed a peak on day 14 and then declined (Figure 4(b)). However, CAT activity in wounded fruits demonstrated a rapid increasing tendency throughout the entire storage period and was significantly higher ($P < 0.05$) than that in control on days 2, 4, 6, 8, and 16. POD activity exhibited increasing trends in control and wounded fruits (Figure 4(c)). POD activity in wounded fruits was significantly higher ($P < 0.05$) than that in control during 16 d storage. APX activity was higher in wounded fruits than that in control during 16 d storage. This enzymatic activity presented a peak on day 12 in control. However, it still increased and significantly higher values were found in wounded fruits on days 14 and 16.

3.4. MDA Content in Postharvest Banana Fruits under Mechanical Wounding. MDA is an indicator to assess fruit

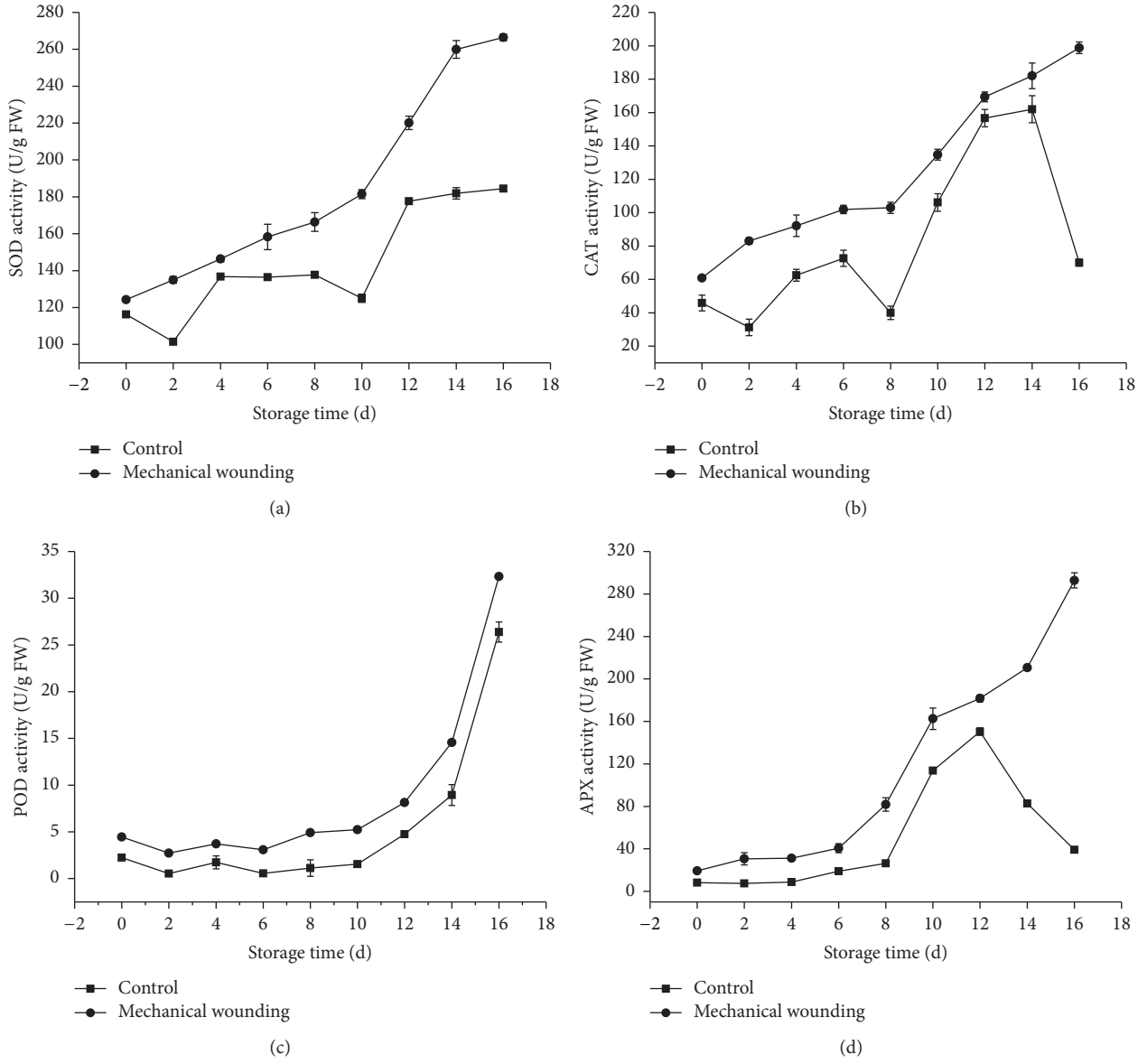

Figure 4: Activities of antioxidative enzymes SOD (a), CAT (b), POD (c), and APX (d) in banana fruits dealing with mechanical wounding.

damage progress, which represents the level of lipid peroxides and the structural integrity of cell membranes [26]. MDA level change is considered to be a marker for membrane lipid peroxidation of plants subjected to senescence or stress. From Figure 5, at 25°C and 90% RH, a continuous increase of MDA content was observed in both control and wounded fruits with extended storage time. MDA in wounded fruits was significantly higher ($P < 0.05$) than that in control during 16 d storage, indicating that mechanical wounding accelerated MDA accumulation in postharvest banana fruits.

3.5. Changes of $O_2^{\bullet-}$ and H_2O_2 Content in Postharvest Banana Fruits under Mechanical Wounding. Superoxide radical and H_2O_2 production in postharvest banana fruits response to mechanical wounding were investigated. Generally speaking, the contents of $O_2^{\bullet-}$ and H_2O_2 in control and wounded fruits exhibited ascending first and then descending trends (Figure 6) when storing fruits for 16 days at 25°C and 90% RH. From Figure 6(a), $O_2^{\bullet-}$ content in wounded fruits was significantly higher ($P < 0.05$) than that in control. It reached the highest peak on day 4 in control but continually rose to maximums on day 6 in wounded fruits. $O_2^{\bullet-}$ level in wounded fruits was about 5-fold higher than that in control on day 16. From Figure 6(b), H_2O_2 content in wounded fruits showed significantly higher ($P < 0.05$) level than that in control throughout the entire storage period. It reached the highest peak on day 4 in both groups. The above results suggested that H_2O_2 could significantly increase the production rate of $O_2^{\bullet-}$ in response to wounding stress.

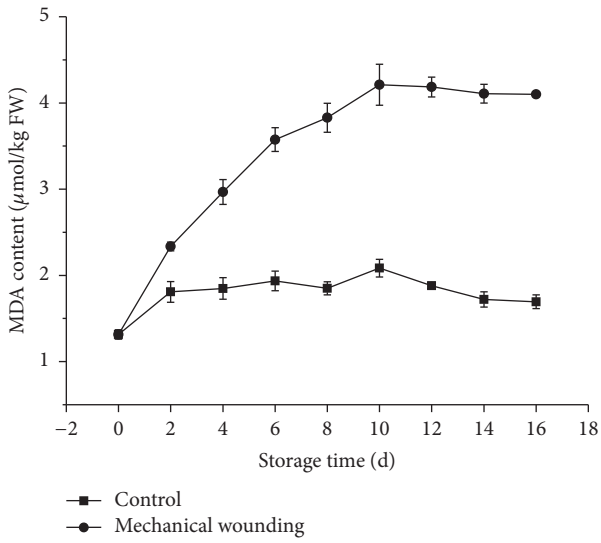

Figure 5: MDA content in banana fruits dealing with mechanical wounding.

4. Discussion

Lipase and PLD are very important enzymes involved in membrane phospholipid hydrolysis [27–29]. The activities of PLD and LOX not only have impacts on cellular membrane structure and stability but also play pivotal roles in regulating many critical cellular functions, including cellular signal transduction, vesicle trafficking, cell proliferation, hormone action, cellular backbone formation, seed germination, senescence, and defense responses [9, 30]. A direct correlation was found between lipid peroxidation and phospholipid hydrolysis [3]. PLD and LOX are proposed to initiate lipolytic cascades in membrane deterioration in response to senescence and environmental stress [11]. It is proposed that PLD initiates a lipolytic cascade in membrane deterioration [27]. The resultant phospholipid degradation products could be further catalyzed by LOX to produce activated oxygen and lipid peroxides, which lead to cellular compartmentalization and membrane damage [31]. In this study, the activities of PLD and LOX in wounded banana fruits were significantly higher ($P < 0.05$) than those in control throughout the whole storage period (Figures 1 and 3). The higher *PLD* gene expression accompanying higher PLD activity was observed in wounded fruits (Figure 2), and *PLD* gene is involved in the defense and repair process in the early damage and its effect reduced or is replaced after 4 days. Similar results were reported by Sang et al. [32], who found wounding increased the level of expression of the *PLDα* gene after stress in cucumbers. PLD and LOX induced by wounding might stimulate corresponding physiological reactions related to deterioration and senescence through increasing gene expression. Some evidences reported that the wounding-induced activation of PLD could result from PLD translocation to membrane, which is mediated by an increase in cytoplasmic calcium and stimulated by low micromolar calcium levels [10, 12]. In addition, PLD and PA promote the production of superoxide [33, 34], a reactive oxygen species that is involved in various defense responses. PLD and its catalyzed products, phosphatidic acid and linolenic acid, initiate the oxylipin pathway and cellular signal transduction, which may be involved in producing the wound signal responsible for increased wound-induced activity, accumulation of phenolics, and enzymatic browning in plant tissues [6].

Membrane lipid degradation is an essential feature of signal transduction pathways that occur in response to wounding stress. Antioxidant defense systems can reduce membrane lipid peroxidation and protect plants from ROS damage. Among antioxidant systems, SOD can scavenge $O_2^{\bullet-}$ radicals into H_2O_2, while H_2O_2 is further converted into water by CAT and APX [15]. In this study, the increasing rate of H_2O_2 and $O_2^{\bullet-}$ (major ROS) contents in wounded banana fruits exhibited ascending trend before day 4 (Figure 6), which suggested that the equilibrium between production and scavenging of H_2O_2 and $O_2^{\bullet-}$ was disturbed, and H_2O_2 and $O_2^{\bullet-}$ in banana fruits were largely produced during 4 d storage. Lipid peroxidation product MDA resulting from ROS activity was significantly higher ($P < 0.05$) than that in control (Figure 5). Although overproduced H_2O_2 was synchronously scavenged through chain reactions catalyzed by a series of antioxidant enzymes (such as SOD, CAT, POD, and APX) (Figure 4), H_2O_2 accumulation was more than its breakdown before 4 d storage. After day 4, the accumulation of H_2O_2 and $O_2^{\bullet-}$ slowed down because of the enhanced scavenging effects of antioxidant enzymes (Figure 6). The results indicated that antioxidant enzymes could scavenge overproduced ROS in banana fruits, retard peroxidation of membrane lipids, inhibit the loss of membrane function, and therefore control senescence of the fruits during storage. The overproduced ROS during storage of wounded banana fruits caused lipid peroxidation, induced membrane injury, destroyed membrane integrity, and resulted in cell senescence. The equilibrium between production and scavenging of ROS in banana fruits may be disturbed by mechanical wounding during storage and thereby it incurs fruit senescence.

Considering the above analysis, the biochemical characters (i.e., membrane-associated lipolytic enzymes such as PLD and LOX, ROS, and antioxidant enzymes) are related to the wounding stress. PLD plays a role in elicitor-induced production of ROS and is involved in signaling pathways in response to wounding stresses [35, 36]. PLD associated with membrane and soluble fractions had opposite behaviors in wound response. Wounding studies performed on *Arabidopsis* leaves showed that PLD was differently affected by wounding and transcript changes were observed after a few hours [37]. Further study is needed to genetically and physiologically characterize different PLD in banana fruits and the signaling pathway of the PLD so as to gain a better understanding on their function under environmental stress.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

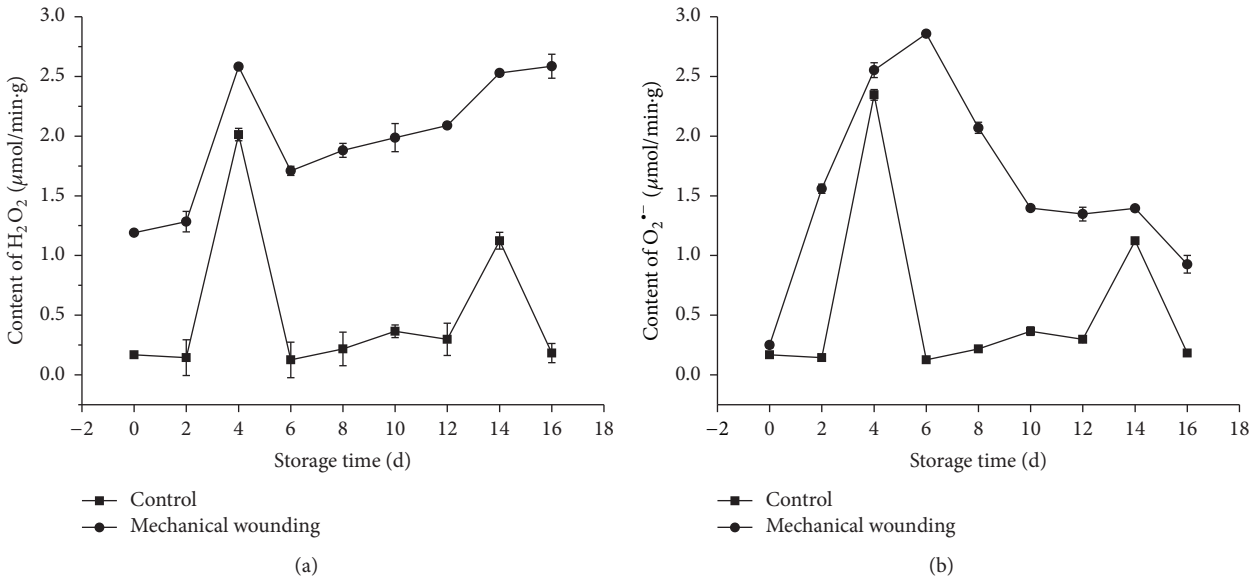

Figure 6: Changes of H_2O_2 (a) and $O_2^{\bullet-}$ (b) content in banana fruits dealing with mechanical wounding.

Acknowledgments

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