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Changes in Hydrogen Peroxide Content in Flower Buds of Japanese Pear (*Pyrus pyrifolia* Nakai) in Relation to Breaking of Endodormancy

Haruyuki Kuroda*, Toshihiko Sugiura and Daiyu Ito**

National Institute of Fruit Tree Science, NARO, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605

Summary

Hydrogen peroxide (H_2O_2) content was determined in flower buds of Japanese pear (*Pyrus pyrifolia* Nakai cv. Kosui) trees under natural conditions during the development and their release from endodormancy. H_2O_2 content increased gradually as the developmental stage index (DVI) accumulated, peaking in late December when endodormancy was broken; it then decreased during the ecodormancy stage. In unchilled flower buds from November 1 throughout the winter, H_2O_2 content did not change significantly and remained low. No budbreak was observed at the end of March which indicate that the increase in the level of H_2O_2 during chilling is closely associated with the breaking of endodormancy. H_2O_2 levels induced by applying hydrogen cyanamide to flower buds of cuttings throughout the endodormancy period promoted budbreak. This was accompanied by a depletion of H_2O_2 within one day, followed by a large increase 3-6 days later. However, cyanamide treatments at concentrations that had no promoting effect on budbreak resulted in a decrease in H_2O_2 content during the experimental period. The cumulative budbreak 22 days after cyanamide treatment was positively correlated with H_2O_2 content six days after the treatment, indicating that the endodormancy-breaking effect of cyanamide involves the elevation of H_2O_2 similar to that of chilling temperatures. These results suggest that H_2O_2 can substitute for the chilling requirement, and that the increase in H_2O_2 levels may trigger the sequence of reactions involved in the breaking of endodormancy.

Key Words: breaking of endodormancy, chilling requirement, developmental stage index, hydrogen peroxide, Japanese pear

Introduction

In temperate zone deciduous fruit trees, the endodormancy period is overcome by exposure to low temperatures. Although chilling requirements have been well established for most deciduous fruit trees, little is known about the specific physiological or biochemical events that are involved in the breaking of endodormancy.

There are numerous chemical and physical treatments that can overcome endodormancy; i.e. mineral oils (Chandler et al., 1937), calcium cyanamide (Iwasaki, 1980; Kuroi et al., 1963; Shulman et al., 1983), hydrogen cyanamide (H_2CN_2) (Nir and Lavee, 1993; Shulman et al., 1983; Siller-Cepeda et al., 1992), high temperatures (Orffer and Goussard, 1980; Tamura et al., 1993), freezing (Olmsted, 1951; Sparks et al., 1976), mechanical injury (Iwasaki, 1980; Paiva and Robitaille, 1978; Shulman et al., 1983), electric current (Kurooka et al., 1990) and anaerobic conditions (Erez et al., 1980; Tamura et al., 1993). The majority of these are environmental stress factors that Chandler et al. (1937) reported have a rest-breaking influence on deciduous fruit trees.

Environmental stresses increase the production of active oxygen species, such as superoxide radicals, hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot$OH) in plants (Elstner, 1982). Levels of intracellular H_2O_2 increase in plants in response to stress conditions, such as low temperature (Okuda et al., 1991 Prasad et al., 1994) and H_2CN_2 (Nir and Lavee, 1993). As stress conditions overcome endodormancy, stress-induced H_2O_2 seems to have a triggering effect on the breaking of endodormancy. However, little is known about the association of endogenous H_2O_2 with the breaking of endodormancy.

The purpose of this study was to determine the relationship between the breaking of endodormancy and H_2O_2 contents in flower buds of Japanese pear trees. Therefore, the changes in H_2O_2 content under natural conditions as well as the lack of chilling during the development and release from endodormancy were investigated. Furthermore, the effects of the rest-breaking

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* Corresponding author.

** Present address: Teaching and Research Center for Bio-coexistence, Faculty of Agriculture and Life Sciences, Hirosaki University, 7-1, Shimobukuro, Fujisaki, Aomori, 038-3802.

compound H_2CN_2 on H_2O_2 level on endodormant flower buds were also studied.

Materials and Methods

Developmental stage index (DVI) and H_2O_2 contents in flower buds under natural conditions

'Kosui' Japanese pear flower buds on two-year-old branches were collected weekly during the autumn-winter of 1998-1999 from mature trees trained to a trellis system at the National Institute of Tree Fruit Science (Tsukuba). The analysis of H_2O_2 in excised flower buds was duplicated on each sampling date. Hourly air temperatures were recorded in the orchard; DVI of the flower buds was calculated according to the chilling accumulation model for 'Kosui' as proposed by Sugiura and Honjo (1997). The model equates the relationship between air temperature (T [℃]) and developmental rate (DVR [hour^{-1}]) during endodormancy; i.e. the DVR value increases from -6 to 0℃ to a maximum between 0 to 6℃; DVR then decreases to zero by 12℃. Chilling is satisfied when DVI, cumulative value of DVR, reaches 1.0. A similar study was conducted during development and release of endodormancy in 1999-2000.

H_2O_2 contents in chilled and unchilled flower buds

Four-year-old 'Kosui' Japanese pear trees, grown in pots, were divided into two groups. One group was kept outdoors throughout the autumn-winter of 1999-2000 and exposed to ambient temperatures in Tsukuba. The other group was moved into a temperature-controlled sunlit chamber (phytotron) kept at 25 ± 1.0℃ on November 1, 1999. Each group consisted of four trees. To determine H_2O_2 content, flower buds on two-year-old branches were collected on each sampling date and assayed in triplicate.

Budbreak and H_2O_2 contents in H_2CN_2-treated flower buds

Two-year-old branches with flower buds, 30 cm in length, were prepared on November 27 and December 12, 2000 from mature 'Kosui' Japanese pear trees trained to a trellis system. On each date, the cuttings were sprayed to run-off with distilled water (control) or at 0.25, 0.5, or 1.0 M H_2CN_2. These concentrations were previously found to be effective on grapevines and deciduous fruit trees (Nir and Lavee, 1993; Shulman, 1983; Siller-Cepeda et al., 1992). After applying H_2CN_2, the cut ends were immersed in water in a phytotron kept at 25 ± 1.0℃ and the levels of H_2O_2 were determined 0, 1, 3, and 6 days later. Each assay was replicated twice. Percent budbreak was evaluated at 2-day intervals by measuring approximately 20 flower buds on three cuttings. Budbreak was defined when flower buds became swollen and the basal part of the bud scales appeared green. Phytotoxicity was evaluated visually from browning after 22 days of forcing and expressed as percentage dead flower buds.

Measurements of H_2O_2

H_2O_2 was assayed by the method of Okuda et al. (1991). Flower buds without bud scales (0.1 to 0.15 g FW) were ground with a mortar and pestle at 0℃ in the presence of 2.0 ml of 0.2 M $HClO_4$ and 0.15 g of sea sand. The slurry, kept at 4℃, was centrifuged at 20,000 g for 5 min. To remove $HClO_4$, the supernatant was neutralized to pH 7.5 with 4 N KOH, and the solution re-centrifuged at 1,000 × g for 1 min. An aliquot (0.2 ml) of the supernatant was applied to a 1-ml column of AG 1-X8 (0.8 cm i.d. × 2 cm; Bio-Rad) and the column was washed with 0.8 ml of distilled water. The eluate was assayed for H_2O_2 in a reaction mixture containing 1.0 ml of the eluate, 0.4 ml of 12.5 mM 3-(dimethylamino) benzoic acid in 0.375 M sodium phosphate buffer (pH 6.5), 0.08 ml of 1.4 mM 3-methyl-2-benzothiazoline hydrazone, and 0.02 ml (0.25 unit) of peroxidase in a total volume of 1.5 ml. The reactions were initiated by the addition of peroxidase at 25℃ and the increase in absorbance at 590 nm was recorded continuously. The data were compared with increases elicited by standard samples of H_2O_2, and the contents were expressed as $\mu mol \cdot g^{-1}DW$. The dry weight was calculated by drying the flower buds without bud scales for 24 hr at 90℃.

Results

DVI and H_2O_2 contents in flower buds under natural conditions

In flower buds of 'Kosui' Japanese pear under natural conditions, DVI started to accumulate on October 26 in 1998 and October 28 in 1999. It accumulated gradually as the air temperature decreased, and reached 1.0 on December 26 in 1998 and on December 27 in 1999 (Fig. 1). In both seasons, the chilling requirement of flower buds of 'Kosui' appeared to be satisfied by late December.

In 1998-1999, H_2O_2 content in flower buds increased gradually, accompanied by an accumulation of DVI, peaking at 2.28 $\mu mol \cdot g^{-1}DW$ on December 29 immediately after the breaking of endodormancy (December 26). Subsequently, H_2O_2 content decreased during the ecodormancy period (January 13). A similar trend was observed in flower buds during autumn-winter in 1999-2000. H_2O_2 content increased as the DVI accumulated, reaching a peak value 2.46 $\mu mol \cdot g^{-1}DW$ on December 29 near the date of completion of endodormancy (December 27), and decreased during the ecodormancy period (January 19)(Fig. 1).

H_2O_2 contents in chilled and unchilled flower buds

In flower buds of 'Kosui' Japanese pear kept outdoors during the autumn-winter of 1999-2000, DVI became

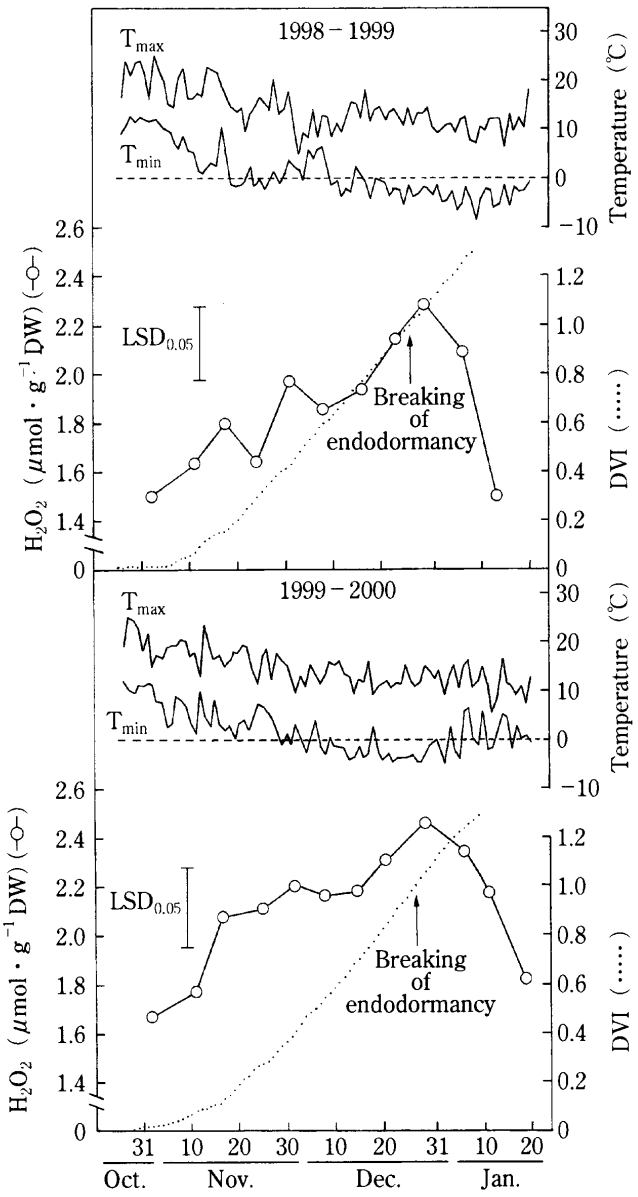

Fig. 1. Changes in H_2O_2 content (○) and developmental stage index (DVI, dotted line) in flower buds of 'Kosui' Japanese pear grown outdoors during endodormancy and its release in 1998－1999 and 1999－2000. T, Air temperature. Values represent means of two replications.

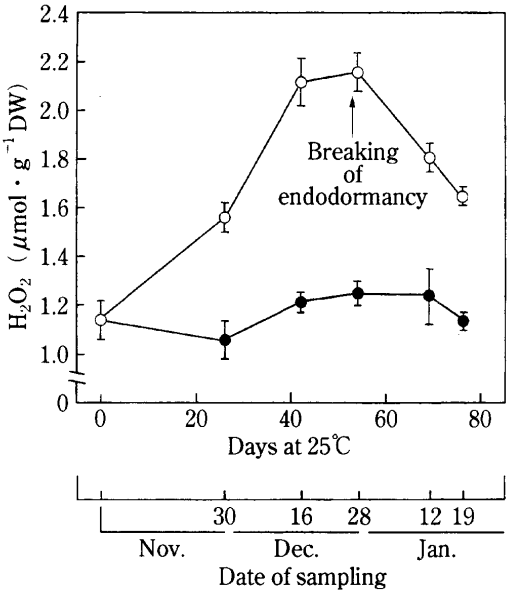

Fig. 2. Changes in H_2O_2 content in flower buds of 'Kosui' Japanese pear kept outdoors (○) and in a temperature-controlled sunlit chamber at 25 ℃ (●) during endodormancy and its release in 1999－2000. Values represent means ± SE of three replications.

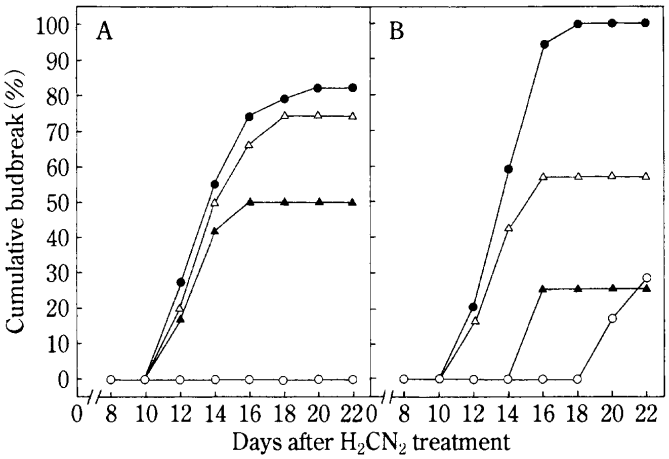

Fig. 3. Changes in cumulative percent budbreak in flower buds of 'Kosui' Japanese pear cuttings treated with hydrogen cyanamide. A, Treated on November 27, 2000; B, Treated on December 12, 2000. ○, Control; ●, 0.25 M; △, 0.5 M; ▲, 1.0 M. Values represent means of two replications.

1.0 on December 27, 1999, indicating that chilling was satisfied (Fig. 1), whereas flower buds kept in the phytotron from November 1, 1999, through the winter did not sprout, even at the end of March, 2000.

In flower buds kept outdoors, H_2O_2 content was almost the same as that in Fig. 1. That is, H_2O_2 content increased gradually with accumulating DVI, reaching a maximum level (2.16 μmol・g^{-1}DW) on December 28 immediately after the breaking of endodormancy (December 27). Subsequently, H_2O_2 content decreased during this period. However, in unchilled flower buds held in the phytotron, H_2O_2 content did not change significantly, remaining low and nearly constant during the experimental period (Fig. 2).

Budbreak and H_2O_2 contents in H_2CN_2-treated flower buds

In flower buds of 'Kosui' Japanese pear collected on November 27, 2000, no budbreak was observed after 22 days of forcing, whereas in the H_2CN_2-treatment, budbreak occurred 12 days after treatment at all concentrations; the cumulative budbreak after application increased for 22 days as H_2CN_2 concentration decreased (Fig. 3A). On cuttings collected on December 12, 2000, percent budbreak after 22 days of forcing was 34%. Treatment with 0.25 M resulted in 100% budbreak 18

days after application, whereas treatment at 0.5 or 1.0 M was less effective than that on November 27 (Fig. 3B).

H_2CN_2 treatment on flower buds collected on November 27, 2000, resulted in a rapid decrease in H_2O_2 content within one day of application. Three days after application, a significant increase in H_2O_2 content occurred and continued for three more days. The magnitude of H_2O_2 change was inversely proportional to the H_2CN_2 concentration (Fig. 4A). The 0.25 M H_2CN_2 treatment on flower buds collected on December 12, 2000, showed a similar change in H_2O_2 content (Fig. 4A) and induced the highest H_2O_2 content six days after application. However, in those treated with 0.5 or 1.0 M H_2CN_2, H_2O_2 content continued to decrease during the experimental period. H_2O_2 content in flower buds treated with 1.0 M H_2CN_2 decreased significantly (Fig. 4B), whereas in untreated flower buds, it remained relatively constant during the experimental period for both application dates (Fig. 4).

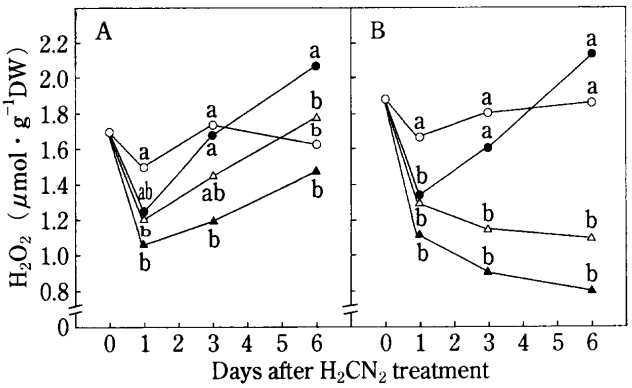

Fig. 4. Changes in H_2O_2 content in flower buds of 'Kosui' Japanese pear cuttings treated with hydrogen cyanamide. A, Treated on November 27, 2000; B, Treated on December 12, 2000. ○, Control; ●, 0.25 M; △, 0.5 M; ▲, 1.0 M. Values represent means of two replications. Different letters within each day represent significant differences at the 5% level in Duncan's multiple range test.

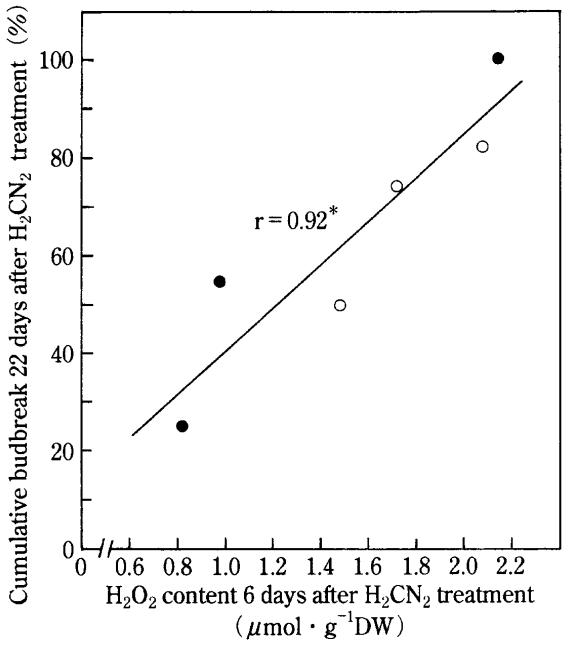

Fig. 5. Relationship between cumulative percent budbreak 22 days after hydrogen cyanamide treatment and H_2O_2 content 6 days after hydrogen cyanamide treatment in flower buds of 'Kosui' Japanese pear cuttings. ○, Treated on November 27; 2000; ●, Treated on December 12, 2000. *Significant at the 5% level.

The relationship between cumulative budbreak 22 days after H_2CN_2 treatment and H_2O_2 content six days after H_2CN_2 treatment (Fig. 5), indicate that the cumulative budbreak induced by H_2CN_2 treatment is correlated positively with the H_2O_2 content regardless of application date.

H_2CN_2 was phytotoxic, depending on the concentration and timing of application which affected percent budbreak. On December 12, close to the completion of the chilling requirement, 1.0 M H_2CN_2 was most phytotoxic, killing 75% of the buds, whereas at 0.25 M H_2CN_2, 17.6% of the buds died after budbreak. It was noted that at 0.5 and 1.0 M H_2CN_2, toxic symptoms appeared at budbreak (Table 1).

Discussion

Increased levels of intracellular H_2O_2 were induced by low temperatures (Okuda et al., 1991; Prasad et al., 1994) but they did not associated it with the breaking of endodormancy of deciduous fruit trees. In this study, we found that the H_2O_2 content in flower buds of Japanese pear increased gradually with the accumulation of DVI, peaking in late December when endodormancy was broken. Subsequently, it decreased during ecodormancy. In unchilled flower buds held in a phytotron from November 1 throughout the winter, H_2O_2 content did not change significantly, remaining at low levels throughout and no budbreak resulted even at the end of March. These results suggest that the increase in H_2O_2 level during chilling is closely related to the breaking of endodormancy, and that H_2O_2 can substitute for the chilling requirement.

Table 1. Phytotoxicity induced by hydrogen cyanamide in flower buds of 'Kosui' Japanese pear cuttings[z].

Concentration (M)	Phytotoxicity(%)	
	Treated on November 27	Treated on December 12
0 (Control)	0	0
0.25	0	17.6 (17.6)
0.5	26.7 (6.7)[y]	50.0 (7.1)
1.0	41.7 (16.7)	75.0 (0)

[z] Phytotoxicity was evaluated 22 days after H_2CN_2 treatment and expressed as percentage of dead flower buds.

[y] Values in parentheses are percentages of buds that died after budbreak.

As H_2O_2 is a potential source of the highly reactive ·OH radical, the removal of H_2O_2 is necessary if plants are to maintain metabolic integrity (Elstner, 1982). Indeed, appreciable levels of the metabolic activities of H_2O_2-scavenging systems that require glutathione (GSH) and ascorbate are known to exist in the cells of wintering apple trees (Kuroda et al., 1990), and such scavenging systems are related to the enhancement of metabolic organization in a cold environment via the maintenance of cellular homeostasis by compensation of oxidative stresses. However, our study showed that levels of H_2O_2 increased during endodormancy, even in the presence of such scavenging systems. Although the mechanism of elevated H_2O_2 as a result of chilling is unclear, the increase is a normal event for plants that require chilling temperatures for breaking of endodormancy. Thus, the increase in H_2O_2 content during chilling may be one of the biochemical events that mark fulfilment of the chilling requirement. Evidence to support this contention is given in Fig. 1 and 2, which show that the peak of H_2O_2 content coincided with the end of endodormancy.

GSH synthesis has been shown to respond either directly or indirectly to H_2O_2 (Smith et al., 1984). Fuchigami and Nee (1987) speculated that glutathione thiol-disulfides are involved in overcoming rest. Siller-Cepeda et al. (1992) also reported that an increase in GSH level is closely associated with the breaking of rest in peach buds. Although the physiological role of GSH in breaking of rest remains unclear, the increase in GSH levels during endodormancy might account for the increase in intracellular H_2O_2 concentration caused by low temperature.

H_2CN_2 is one of the most effective rest-breaking agents (Nir and Lavee, 1993; Shulman et al., 1983; Siller-Cepeda et al., 1992). However, how H_2CN_2 induces bud release in resting deciduous fruit trees is not yet known. Our study showed that the cumulative percent budbreak 22 days after H_2CN_2 treatment was positively correlated with H_2O_2 content six days after H_2CN_2 treatment. That is, H_2CN_2 treatment at concentrations that promoted budbreak resulted in a decrease in H_2O_2 content within one day followed by a large increase 3-6 days after application. In contrast, H_2CN_2 treatment at concentrations that had no promoting effect on budbreak resulted in a decrease in H_2O_2 content during this same period. These results indicate that the endodormancy breaking effect of H_2CN_2 involves the elevation of H_2O_2 levels similar to low temperatures.

H_2CN_2 treatment inhibits catalase activity, which is thought to increase H_2O_2 level (Amberger, 1984; Nir et al., 1986). In this study, however, H_2O_2 content decreased one day after H_2CN_2 treatment. Inoue et al. (1970) reported that cyanamide inhibits the activity of cytochrome *c* oxidase, an enzyme involved in the electron-transport chain. The electron-transport chain serves as a univalent pathway for the reduction of oxygen and production of active oxygen (Azzi et al., 1975; Cadenas et al., 1977). Thus, the decrease in H_2O_2 immediately after H_2CN_2 treatment may be responsible for inhibiting the electron-transport chain.

That an increase in H_2O_2 5-10 days after H_2CN_2 treatment promoted budbreak reported previously in grapevine buds (Nir and Lavee, 1993), agrees with our finding in that a significant increase in H_2O_2 content was observed 3-6 days after H_2CN_2 treatment that promoted budbreak. H_2CN_2 binds nonenzymatically to the thiol of GSH (Fuchigami and Nee, 1987) resulting in the detoxification of H_2CN_2. This finding is supported by Siller-Cepeda et al. (1992) who found a rapid depletion of GSH levels within 12 hr of H_2CN_2 application. Therefore, detoxification of H_2CN_2 by GSH may be responsible for the increase in H_2O_2 level observed three days after H_2CN_2 application. However, as the capacity of enzyme systems involved in the regeneration of GSH in the cell is limited, when the dosage of H_2CN_2 exceeds this capacity, GSH disappears, leading to cell damage. The levels of H_2O_2 produced following treatment with 0.5 and 1.0 M H_2CN_2 on December 12 declined probably because of its toxic effects that occurred before budbreak.

Our results indicate that the increase in H_2O_2 level is an integral part of breaking endodormancy. We propose that the elevation of H_2O_2 caused by stress conditions such as low temperature and H_2CN_2 triggers the sequence of reactions to overcome the endodormancy. Matsuda et al. (1994) reported that treatment with H_2O_2 alters the rate of synthesis of certain proteins in the crown of winter wheat which supports the idea that breaking of rest induced by low temperature is under the control of a genetic system (Lang, 1994). H_2O_2 seems to be one of many factors involved in the modification of gene expression concerned with the breaking of endodormancy. However, the mechanism on the production of H_2O_2 as a result of exposure to chilling remains to be determined.

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ニホンナシ花芽における自発休眠覚醒と過酸化水素の関係

黒田治之・杉浦俊彦・伊藤大雄 *

農業技術研究機構果樹研究所　305-8605　つくば市藤本 2-1

摘　要

自発休眠覚醒と過酸化水素 (H_2O_2) の関係を検討するために，ニホンナシ‘幸水’花芽における H_2O_2 含量を自発休眠期から他発休眠期にかけて調査した．H_2O_2 含量は自発休眠期の発育ステージ (DVI) の高まりに伴って増加し，自発休眠が覚醒した12月下旬にピークに達し，その後他発休眠期に減少した．一方，11月1日から冬を通して温暖処理 (25 ℃) をした花芽では，H_2O_2 含量は低い値で推移し，また萌芽は3月末に至っても観察できなかった．このようなことから，自発休眠期を通しての H_2O_2 含量の増加は自発休眠覚醒と密接に関係していることが示された．また，切り枝の花芽を用いて，休眠打破剤シアナミド (0, 0.25, 0.5, 1.0 M) を11月下旬と12月中旬に処理し，萌芽率と H_2O_2 含量の変化を調査した．自発休眠覚醒を促進したシアナミド処理は，処理後1日目には H_2O_2 含量を減少させたが，処理後3日以降には増加をもたらした．しかし，自発休眠覚醒を促進しなかったシアナミド処理では，H_2O_2 含量は減少し続けた．シアナミド処理後22日目の積算萌芽率と処理後6日目の H_2O_2 含量との間には正の相関が認められ，シアナミドの自発休眠打破効果は低温条件と同様に，H_2O_2 含量の増加と密接に関係していることが示された．以上の結果から，H_2O_2 は低温要求に代替するものであり，また H_2O_2 レベルの高まりは自発休眠覚醒に関連する一連の反応を始動させる要因であると考えられた．

* 現在：弘前大学農学生命科学部生物共生教育研究センター　038-3802　青森県藤崎町下袋7-1