Studies on Development of Cultivation Techniques for the Sustainable Production of Japanese Pear

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Studies on Development of Cultivation Techniques for the Sustainable Production of Japanese Pear

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Contents

Summary

By employing a well-organized traditional cultivation system that includes training trees on a flat trellis, farmers continue to produce stable harvests of Japanese pear (*Pyrus pyrifolia*); however, new serious problems that threaten stable production are on the horizon. Thus, new cultivation systems will be required for the future sustainable production of Japanese pear. The first problem is a consequence of global warming. Delayed flowering and dead flower buds have been frequently observed on the Japanese pear cultivar 'Kosui' when grown in heated plastic-houses and open fields in the southwestern regions of Japan, possibly due to global warming. In addition, high temperatures during the summer possibly inhibit flower bud formation because flower bud differentiation normally takes place after the cessation of shoot elongation. When summer temperatures are high, the current season's shoots (CSSs) continue to elongate without stopping and fail to produce flowers. Secondly, a declining workforce population is now apparent, thereby causing another hindrance to efficient farm management for such labor-intensive tasks as thinning, harvesting, and pruning. As aforementioned, the flat trellis training system is highly suitable for managing Japanese pear cultivation, but the shortage of workers due to the aging of farmers has progressed more quickly than expected and negatively affects the efficiency of Japanese pear cultivation. Therefore, new laborsaving management methods are certainly required. Artificial pollination is an important management technique used in Japanese pear cultivation because most Japanese pear cultivars are self-incompatible and require artificial pollination by hand and/or by insects. In addition, the effective period for artificial pollination is limited during the flowering period. Thus, reducing the labor required for the pollination work

has become an urgent problem for Japanese pear production. Considering these recent issues, the objective of this study was to maintain the sustainable production of Japanese pear by solving some of the following problems: i) abnormal flowering in Japanese pear such as delayed flowering and dead flower buds, ii) inhibition of flower bud formation during high temperature conditions in the summer, and iii) labor-intensive pollination. As for the abnormal flowering such as delayed flowering and dead flower buds, we proposed the following two possible reasons: i) shortage of sufficient chilling accumulation for endodormancy breaking in winter under heated plastic-house conditions and/or ii) failure to acquire cold hardiness in the fall under open-field conditions. Considering the former case, application of an endodormancy breaking agent such as α-ketol-octadecadienoic acid (KODA) was proposed and found to be a useful approach. Although not as effective as hydrogen cyanamide (HC), KODA may be preferable during the late endodormancy stages because this chemical has no apparent phytotoxicity for either plants or humans. In the latter case, compost application during the fall-winter season can adversely affect freezing tolerance through an increase in the nitrogen (N) content, thus promoting dead flower buds. Therefore, we suggested that the timing of N fertilization should be shifted to the spring season instead of fall and winter. In terms of inhibiting flower bud formation during the high temperatures of summer, we observed that application of KODA in July increased the number of lateral flower bud primordia on Japanese pear spurs. This result suggests that application of KODA in July could be a useful technique for decreasing the number of blind buds and promoting stable fruit production. Lastly, to reduce laborious hand pollination, we found that the level of fruit set after spray pollination using media containing 0.3% (w/v) pollen grains, 0.1% (w/v) agar and 10% (w/v) sucrose was almost the same as the level after hand

pollination in the ‘Kosui’ cultivar that has partial parthenocarpic properties. Moreover, addition of forchlorfenuron (CPPU) to the spray pollination media that was used for ‘Kosui’ increased the fruit set of ‘Hosui’. We hypothesized that the higher levels of fruit set by CPPU might be due to the induction of partial parthenocarpic properties in ‘Hosui’.

Abbreviations

ANOVA	analysis of variance
CPPU	forchlorfenuron [N-(2-Chloro-4-pyridyl)-N'-phenylurea]
CSSs	current season's shoots
FT	*FLOWERING LOCUS T*
HC	hydrogen cyanamide
JA	jasmonic acid
KODA	α-ketol-octadecadienoic acid
LSD	least significant difference
LT_{50}	lethal temperature 50
N	nitrogen
NIFTS	Institute of Fruit Tree and Tea Science, NARO
PG	polygalacturonase
PME	pectin methylesterase
TFL1	*TERMINAL FLOWER 1*
XG	xanthan gum

Chapter 1

General introduction

Pyrus spp. (pear) is a member of the Rosaceae family along with apple. The Japanese pear (*P. pyrifolia*) is the main cultivated species in Japan and accounts for 87.3% of the total planted area allocated to pears (Saito, 2016). According to a Food and Agriculture Organization of the United Nations (FAO) report (2013), Japan ranked eleventh in the world following China, the United States of America, Italy, Argentina, Turkey, Spain, South Africa, India, Netherland, and Belgium and accounts for 1.2% (approximately 294 thousand tons) of the total global production (Table 1). Japanese pears are widely cultivated from the northern (Hokkaido region) to the southern (Kyushu region) regions of Japan. The total area in Japan for Japanese pear cultivation was 12,400 ha in 2015, a decline of 3,500 ha since 2005 (MAFF, 2016). Moreover, the commercial value of Japanese pear production was estimated at more than 70 billion yen, which ranks fourth after citrus, apple, and grape production (Saito, 2016).

Pear has been a traditional tree fruit in Japan since A.D. 700, but the major cultivars that were first commercially produced in 1895 were ‘Nijisseiki’ and ‘Chojuro’ (White, 1990). The Horticultural Division of the Agricultural Research Station, affiliated with the Ministry of Agriculture and Commerce (now the Institute of Fruit Tree and Tea Science, NARO: NIFTS) in Japan began systematic breeding of Japanese pear in 1935. Up to the present, more than 20 *P. pyrifolia* cultivars have been developed. According to a Ministry of Agriculture, Forestry and Fisheries (MAFF) report (2016), the varietal share of the

cultivated areas in 2013 among leading cultivars and others was as follows: ‘Kosui’ (39.9%), ‘Hosui’ (26.5%), ‘Niitaka’ (9.3%), ‘Nijisseiki’ (7.6%), ‘Akizuki’ (3.4%), ‘Nansui’ (2.4%), ‘Shinko’ (2.3%), and ‘Gold Nijisseiki’ (1.7%) (Fig. 1). Two cultivars, ‘Kosui’ and ‘Hosui’, occupied approximately 70% of the total planted area.

Many fruit trees tend to continue the elongation of the CSSs under the warm and high humidity conditions commonly found in Japan. In addition, fruit growers are more frequently troubled by typhoons during the harvest period. To overcome these conditions, a flat trellis training system, which is 1.8-2.0 m high, has been traditionally used to cultivate Japanese pear in our country. Also, the main cultivar ‘Kosui’ was first planted in the 1970s, but presently these trees are becoming less productive. Accordingly, the replacement of these trees is necessary; however, tree replanting is not proceeding smoothly. One of the reasons for making less progress in replanting is that it takes a long period for orchards to mature when grown under the flat trellis training system. Shibata et al. (2008) suggested that the joint tree training system in which the apical tips of each tree are grafted into the bent part of the main stem of the adjacent tree under a flat trellis enabled high yields to be achieved more quickly. By using the joint tree training system, about 3 fewer years are required to mature an orchard compared to the flat trellis training system. In this way, several technical developments have been accomplished in line with climate and natural features of our country toward the sustainable production of Japanese pear. Recently, there have been several new problems to hinder the stable production of Japanese pear. Namely, we face two major concerns, global warming and the decline in the workforce population. Therefore, in this study, our goal was to develop a new cultivation system that would minimize the several problems caused by these two major

concerns.

According to the Intergovernmental Panel on Climate Change (IPCC) fifth Assessment Report (2013), each of the last three decades has been successively warmer at the Earth's surface than any preceding decade since 1850. In the Northern Hemisphere, 1983–2012 was likely the warmest 30-year period of the last 1400 years.

Japanese pear is widely cultivated throughout Japan. Especially in the southwestern regions, Japanese pear enjoys potentially high profitability in that harvest time is advanced by forcing inside plastic-houses. However, low temperature requirements for the development of complete endodormancy in Japanese pear are often not met now in regions where forcing occurs in heated plastic-houses. Thus, dysfunctional endodormancy breaking has become an obstacle to the stable production of Japanese pear (Kuroki et al., 2013). In addition, delayed flowering and dead flower buds have been frequently observed in the 'Kosui' cultivar grown in open field conditions, especially in the southwestern regions of Japan (Matsumoto et al., 2010) (Fig. 2). It was apparent from past records that this abnormal phenomenon became prominent during especially warm seasons in the fall and winter (Fujikawa et al., 2012).

Flower bud formation is influenced by environmental factors, and the degree of flower bud formation fluctuates year-to-year. Therefore, techniques to regulate flower formation have been desired. In Japanese pear, flower buds usually are formed on the apices of spurs and CSSs and on axillary buds of CSSs. Flower bud induction and development occurs after the cessation of shoot elongation (Ito et al., 2014). Flower buds differentiate in early

summer (from mid-June to mid-July in Japan), although the time differs with cultivars and districts (Banno et al., 1986). The flower bud type of Japanese pear is mixed, which means that flower buds contain both floral and vegetative primordia. Typical flower buds of Japanese pear are composed of twelve scales (Banno et al, 1985), a terminal inflorescence, and one or two lateral primordia that are growing points. In apple, Osanai et al. (1990) reported that the formation of flower buds tended to decrease as temperatures in July increased. There is a possibility that high temperatures during summer can inhibit flower bud formation because elongation of the CSSs continue in Japanese pear (Ito, 2015). In fact, the number of lateral primordia tend to decrease as the climate warms (Appendix 1).

Considering the aging of the domestic agricultural work force, the shortage of successors, and the low profitability of fruit production, the future sustainability of this sector is a major concern. In particular, farmers have faced an advancing age crisis for the last twenty years (Fig. 3). The types of work required for Japanese pear production in 2005 (MAFF, 2008) were as follows: training and pruning (31.9%), pollinating and thinning (26.1%), harvesting and grading (19.3%), weeding and agrochemical spraying (9.6%), and other tasks (13.1%) (Fig. 4). Koizumi et al. (2007) reported that the time needed for pruning trees that were using the joint tree training system was almost one-half that required by the flat trellis training system. Most cultivars of Japanese pear are self-incompatible. In general, artificial pollination by hand using compatible pollen and a conventional feathered stick is used for commercial production in Japan; however, the effective period for artificial pollination is limited (3-5 d) during the flowering period (Kagami et al., 1997). In addition, hand pollination is a labor-intensive process resulting in high labor

costs. As an alternative, a spray pollination technique using aqueous pollen grain solutions might reduce labor and other costs associated with fruit tree cultivation. A practical technique was successfully established for kiwifruit through the development of a pollen grain suspension medium (Hopping and Simpson, 1982). More recently, a liquid pollen grain suspension medium thickened with agar was also developed for kiwifruit (Yano et al., 2007). To our knowledge, the use of spray pollination with other fruits is quite limited and has not previously been examined in Japanese pear.

The objectives of this study were 1) to develop a countermeasure technique to prevent delayed flowering and dead flower bud formation in Japanese pear, 2) to develop a tool for regulating flower bud formation in Japanese pear, and 3) to develop spray pollination as a labor-saving pollination system in Japanese pear. This study will provide effective and scientific strategies to sustain the production of Japanese pear.

Table 1. Ranking of countries for pear production based on production statistics published by the FAO (2013).

Rank	Country	Production (thousand tons)
1	China	17,300
2	United States of America	795
3	Italy	743
4	Argentina	722
5	Turkey	461
6	Spain	425
7	South Africa	343
8	India	340
9	Netherland	327
10	Belgium	305
11	Japan	294

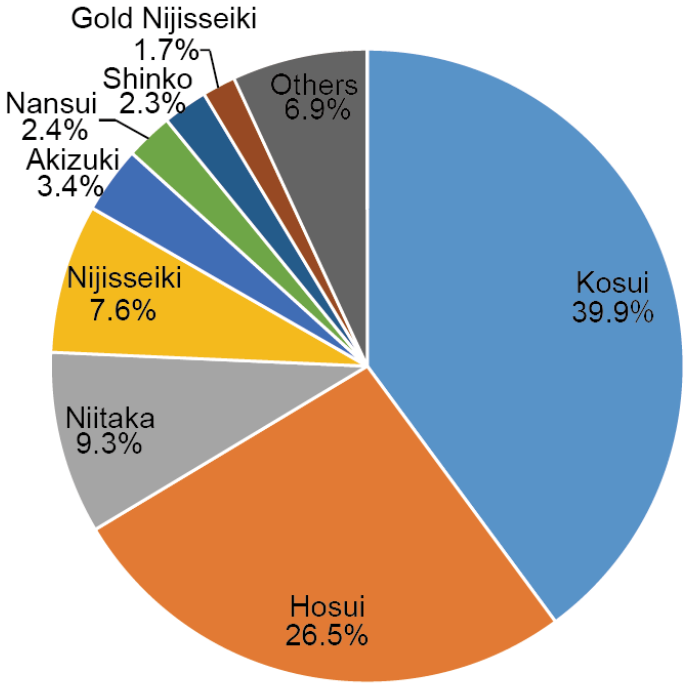

Fig. 1. Varietal shares of Japanese pear production in Japan (2013) based on statistics published by the MAFF (2016).

Fig. 2. Abnormal spring-season phenotype in Japanese pear ‘Kosui’. The tree in the foreground has dead flower buds and is delayed in flowering, whereas the tree in the background is normal.

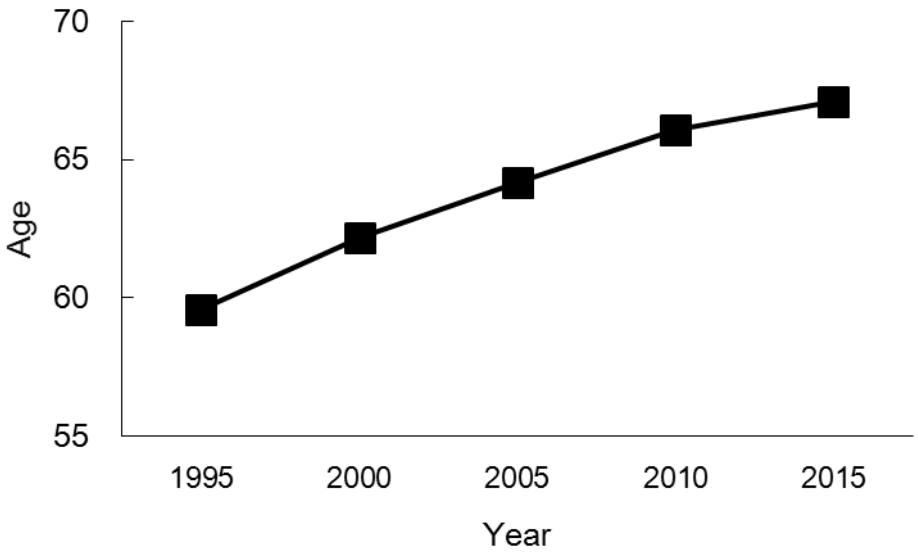

Fig. 3. Changes in the average age of core farmers in Japan (MAFF, 2016).

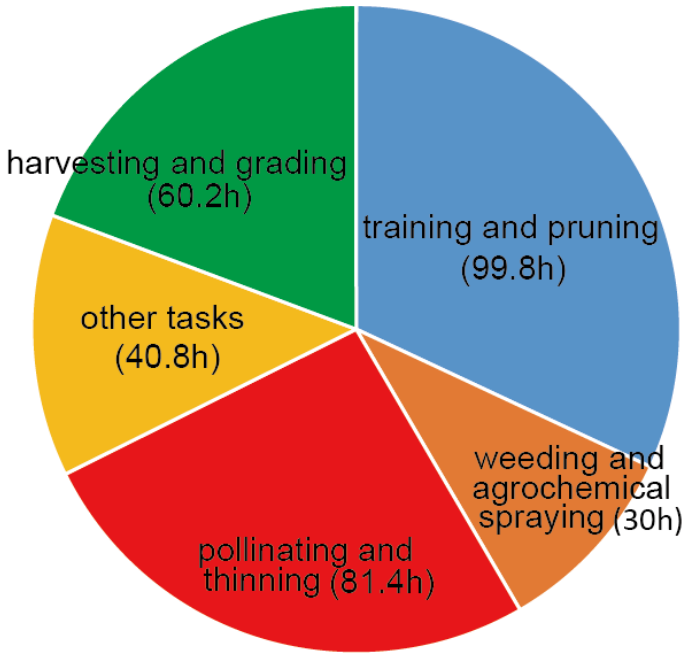

Fig. 4. The varietal share of types of work (time per 10a) required for Japanese pear production in 2005 (MAFF, 2008).

Chapter 2

An effective strategy for eliminating delayed flowering and dead flower buds in Japanese pear

Section 1 The use of KODA as an endodormancy breaking agent for flower buds of Japanese pear that has limited toxicity for plants and humans

Introduction

In woody plants, the ability of a plant or plant tissue to enter the quiescent physiological state known as endodormancy is an important adaptive strategy for surviving severe winter freezes (Lang, 1987). However, recent winter temperature aberrations which have been associated with global warming conditions have given new impetus to the study of endodormancy breaking, and particularly to dysfunctional breaking which may be due to changes in climate. Low temperature requirements for the development of complete endodormancy in Japanese pear are now often not met in southwestern regions of Japan, especially where forcing culture under heated plastic-house conditions is used. Thus, dysfunctional endodormancy breaking has become an obstacle to stable economic production of Japanese pear. In these regions, artificial cooling to compensate for insufficient natural chilling (Shulman et al., 1983; Erez, 1995) is now required to maintain commercial Japanese pear production.

A number of chemical and physical treatments can be used to overcome endodormancy,

including mineral oils (Chandler et al., 1937), calcium cyanamide (Iwasaki, 1980; Kuroi et al., 1963; Shulman et al., 1983), HC (Nir and Lavee, 1993; Shulman et al., 1983; Siller-Cepeda et al., 1992), thiourea (Shulman et al., 1983), diallyl sulfides in garlic (Kubota et al., 1999), high temperatures (Orffer and Goussard, 1980; Tamura et al., 1993), and hydrogen peroxide (Kuroda et al., 2005). However, some treatments, such as mineral oils or cyanamide, can sometimes cause negative effects not only to fruit trees, but also to humans. Other chemicals, which may have some endodormancy breaking effect, are not potent enough to compensate for the lack of chilling. Therefore, the identification of effective alternative chemicals or treatments that have robust and reproducible effects on endodormancy breaking with less toxicity for plants and humans, would be a great benefit to the fruit industry.

KODA is a signal compound expressed in *Lemna paucicostata* (Duckweed) after exposure to drought, heat or osmotic stresses (Yokoyama et al., 2000; Yamaguchi et al., 2001). KODA is an oxylipin, a common compound in green plants (Vick and Zimmerman, 1987). Oxylipins are bioactive lipids derived by oxygenation of polyunsaturated fatty acids (Lee et al., 2008). KODA is synthesized from linolenic acid (C18:3) by a 9-specific lipoxygenase (Howe and Schilmiller, 2002) (Fig. 5). In addition, applications of 10 or 100 μM KODA during paradormancy promote bud breaking in strawberry flower buds (Yokoyama, personal commun.), suggesting that KODA may also break endodormancy in deciduous fruit trees, including Japanese pear. In this study, we investigated the effect of KODA on breaking of endodormancy in flower buds of Japanese pear.

Materials and Methods

Plant materials

Mature Japanese pear trees grown at the NIFTS (Tsukuba, Ibaraki Prefecture, Japan), located at 36º 3'N and 140º 8'E, were used for all experiments. We selected 3-5 CSSs with 6-9 flower buds on shoots between 60-80 cm in length per treatment. All mature Japanese pear trees were managed according to the ordinary cultural practices used in Tsukuba.

Effect of KODA on endodormancy breaking in 'Kosui' flower buds

CSSs with flower buds in endodormant stage (Dec., 2006) were cut from mature Japanese pear 'Kosui' at the NIFTS. CSSs were sprayed to run-off with distilled water (control), 10, 100 or 1000 μM KODA, which was provided by Shiseido Co. Ltd., Tokyo, Japan. After treatment, cut ends of the CSSs were placed in distilled water in a phytotron at 25 ± 1.0 ºC. Water in the 500 mL vials was changed at 2-3 d intervals. The percentage of flower bud breaking, which is indicative of endodormancy breaking, was determined at 2-4 d intervals. Flower bud breaking was defined as the stage at which green tissue is visible. The bud breaking percentage was first calculated on each CSS with 6-9 flower buds. Then, summed up percentage from each CSS divided by the number of CSS (3-5 CSSs) gave the average bud breaking percentage. Seasonal effects on endodormancy breaking were determined by treating mid to late endodormancy. CSSs with flower buds cut and sprayed on 7 Dec., 2006, 15 Oct., 30 Oct., 15 Nov., 22 Nov., 29 Nov. and 6 Dec., 2007, and treated as above, but without a 1000 μM dose.

Comparison of KODA on three Japanese pear cultivars

CSSs from 'Hosui' and 'Natsushizuku' mature trees were cut and treated as above with distilled water or 100 μM KODA on 29 Oct., 11 Nov., 26 Nov., and 3 Dec., 2008 during endodormancy. 'Kosui', which was sampled on the same date served as a longitudinal control to confirm the reproducibility of the experiments conducted in 2006 and 2007.

Comparison of KODA and HC on endodormancy breaking of flower buds

KODA was compared to HC, which is registered in Japan as a dormancy breaking agent of Japanese pear. CSSs from 'Kosui' trees were cut on 29 Oct. and 27 Nov., 2009 and treated as above with distilled water (control), 100 μM KODA or 1% (w/v) (0.24 M) HC (registered as CX-10, Nippon Carbide Industries Co., Inc., Tokyo, Japan).

Data in 2006 was done with the Kruskal Wallis Test and Sheffe's Test. Data in 2007, 2008, and 2009, were analyzed for significant differences by analysis of variance (ANOVA) and least significant difference (LSD) values were calculated for comparison of means.

Results and Discussion

Generally, endodormancy of flower buds of Japanese pear is deepest around the mid to end of Oct. under natural conditions, gradually releasing as manifest by an increase in bud breaking (Fig. 6). We investigated effect of KODA on endodormancy breaking in 'Kosui' in 2006 and 2007. In 2006, 100 and 1000 μM of KODA promoted significant endodormancy breaking. KODA treatment at 10 μM also tended to promote breaking (Table 2). Based on 2006 results, in 2007 we evaluated treatments at different intensities

of endodormancy to determine the most effective timing for KODA application. Endodormancy breaking occurred earlier in flower buds treated with 100 μM KODA on 15 Oct., 30 Oct., 15 Nov., and 22 Nov. than in controls (Fig. 7A, B, C, D). Toward the last stage of endodormancy (On 29 Nov. and 6 Dec.), breaking tended to occur earlier with 100 μM KODA (Fig. 7E, F). Treatment with 10 μM KODA on 15 Oct. and 30 Oct. resulted in breaking flower bud endodormancy before the controls (Fig. 7A, B). Treatment on 29 Nov. and 6 Dec. with 10 μM KODA resulted in breaking on the same date as controls, but 100% of endodormancy breaking was attained earlier with KODA treatment (Fig. 7E, F). There was no observable difference in the final percentages of endodormancy breaking between 10 μM KODA treatment and control with CSSs cut and treated on 15 Nov. or 22 Nov. (Fig. 7C, D), but the reason for this is not yet known. Collectively, 100 μM was a more effective dose than 10 μM KODA for breaking endodormancy of flower buds among the concentrations tested.

One effect of seasonal timing on endodormancy breaking of 100 μM KODA-treated flower buds was that breaking of treated buds preceded breaking of the controls on 29 Oct., 11 Nov., and 26 Nov. (Fig. 8A, B, C), with the percentage of treated buds increasing more rapidly than the control, including the treatment on 3 Dec. (Fig. 8). This result was in agreement with the results from 2007. ‘Natsushizuku’ breaking tended to be the same as ‘Kosui’ (Fig. 9A, B, C), but ‘Hosui’ breaking was not advanced with 100 μM KODA on any date (Fig. 10). However, the final percentage in 100 μM KODA-treated flower buds was higher than in controls (Fig. 10A, B, D), except for the treatment on 26 Nov. (Fig. 10C). Thus, the effect of KODA, at least with the timing used in these experiments, was lower in ‘Hosui’ compared with ‘Kosui’ and ‘Natsushizuku’ (Fig. 10). Asano and

Okuno (1990) reported that the period required for endodormancy breaking corresponds to the chilling requirements was shorter in ‘Hosui’ than in ‘Kosui’ after cold treatment. ‘Kosui’ and ‘Natsushizuku’ have about the same breaking period (Sudo et al., 2009). ‘Hosui’ thus requires the least time at low temperature to break endodormancy among the three cultivars. This trait is likely to be related to the diminished effect of KODA on endodormancy breaking in ‘Hosui’. No phytotoxicity symptoms were observed during the course of these experiments.

HC is one of the most effective endodormancy breaking agents (Nir and Lavee, 1993; Shulman et al., 1983; Siller-Cepeda et al., 1992). KODA and HC-treated flower buds broke endodormancy before the control treatment on both 29 Oct. and 27 Nov. (Fig. 11), but KODA was generally inferior to HC for both timing and extent of bud break. However, the differences between HC and KODA decreased with the approach of normal endodormancy breaking (Fig. 11). In addition to the effect of advanced bud breaking, Bound and Jones (2004) reported that the flowering period from pink bud to full bloom in ‘Fuji’ apple was compressed by the HC application 40 d prior to the estimated bud breaking, while HC application did not always result in the compression of the flowering period especially under higher temperature conditions. In this study, although both treatments advanced bud breaking, no apparent compression of flowering period (from bud breaking to full bloom) was observed by both KODA and HC treatments (data not shown), which may be due to high temperature (25 ± 1.0 ºC) condition under phytotron as suggested by Bound and Jones (2004). The phytotoxicity of HC is well known, and is dependent on concentration and application date (Siller-Cepeda et al., 1992; Kuroda et al., 2002). Therefore, with refinement of application schedules and doses, KODA could

be the preferable treatment for endodormancy breaking for Japanese pear flower buds at late endodormancy stage. However, it should be noted that this work was done on CSSs cut from the trees in the orchard: therefore, it could be required to examine the effect of KODA on bud breaking using the potted trees.

Table 2. Effect of KODA on endodormancy breaking in flower bud of Japanese pear 'Kosui' (2006).

Treatment	Bud breaking (%)[z]
KODA 10 μM	78.9 ± 10.0 ab[y]
KODA 100 μM	98.0 ± 2.0 a
KODA 1000 μM	100 a
Control	30.4 ± 18.1 b

[z] Percentage of flower bud breaking 21 d post treatment.

[y] The data are presented as mean ± SE (n = 3-5). Different letters within a column indicate significant differences at $P < 0.05$ by the Scheffe's Test.

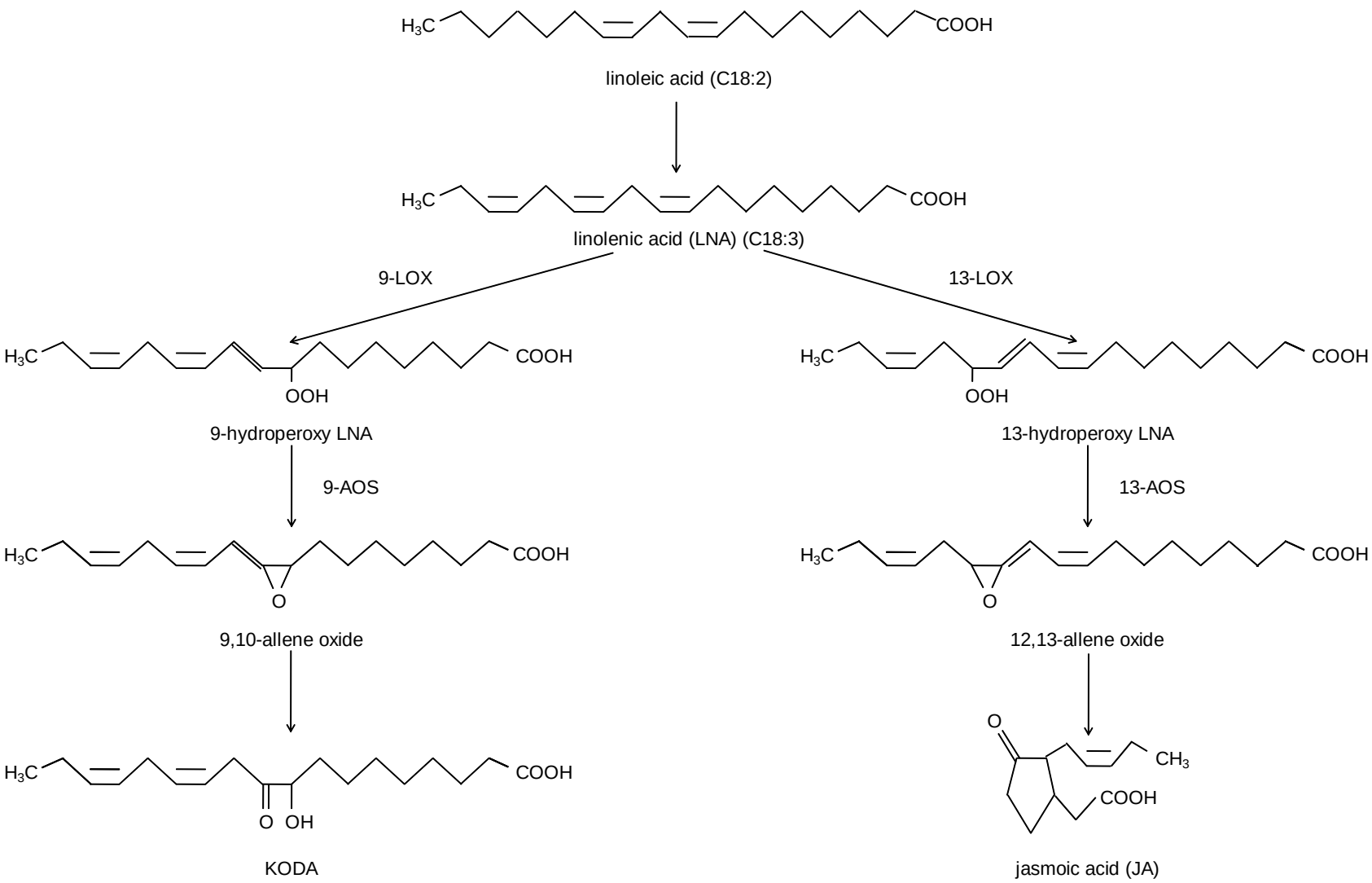

Fig. 5. Proposed biosynthesis pathways for KODA and jasmonic acid (JA).

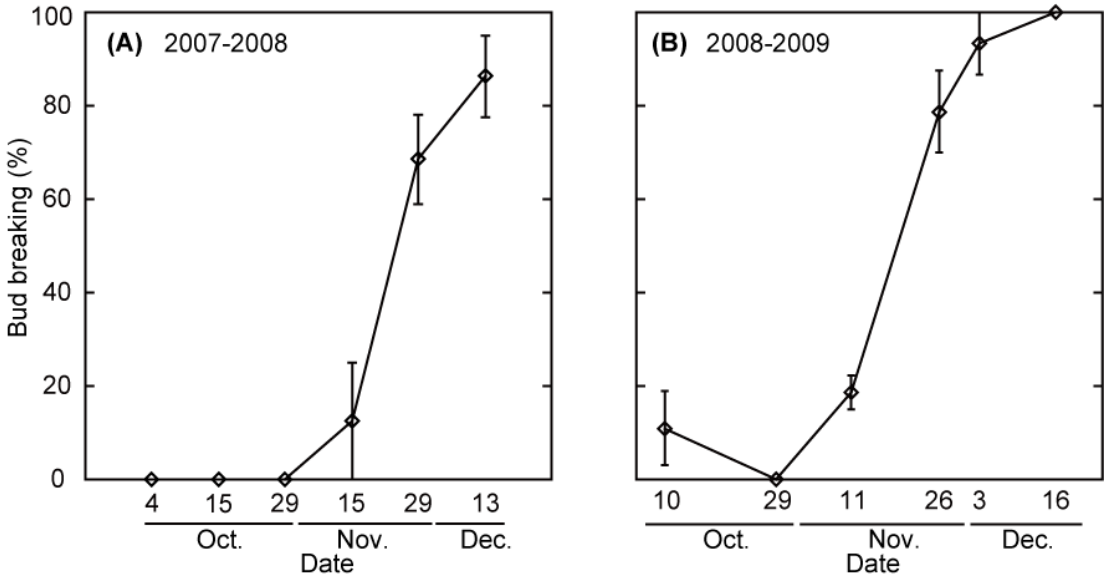

Fig. 6. Seasonal changes of endodormancy status in flower buds of Japanese pear 'Kosui' in 2007-2008 (A) and 2008-2009 (B). Vertical bars are the SE (n = 3-5).

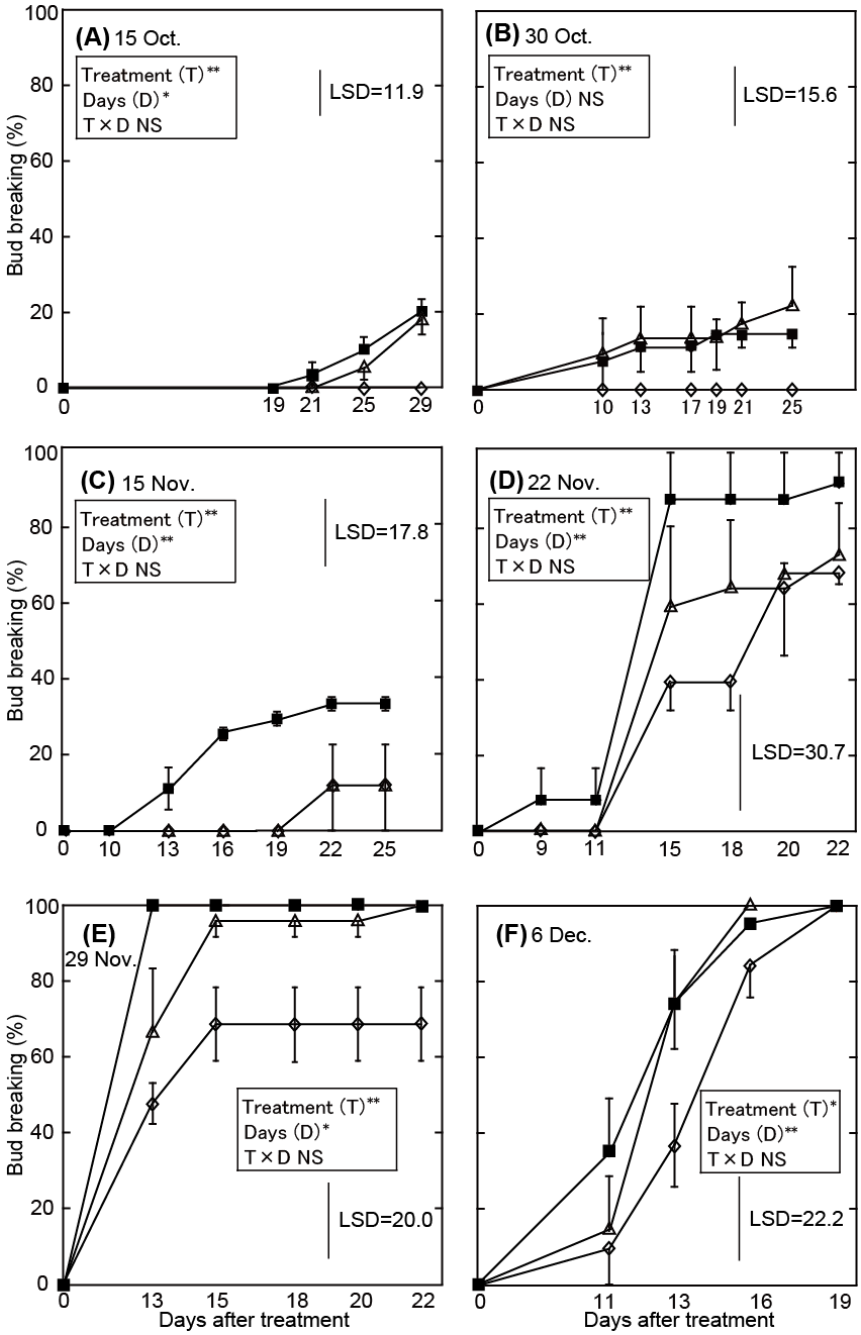

Fig. 7. Effect of concentration of KODA on endodormancy breaking in flower buds of Japanese pear 'Kosui' (2007). Treatment on 15 Oct.; (A), 30 Oct.; (B), 15 Nov.; (C), 22 Nov.; (D), 29 Nov.; (E), 6 Dec.; (F). Control; (◇), KODA 10 μM; (△), KODA 100 μM; (■). Values are means ± SE (n = 3). Vertical bars indicate LSD (P = 0.05). NS, *, and ** indicate not significant, or significant differences at $P < 0.05$ and 0.01, respectively, by ANOVA.

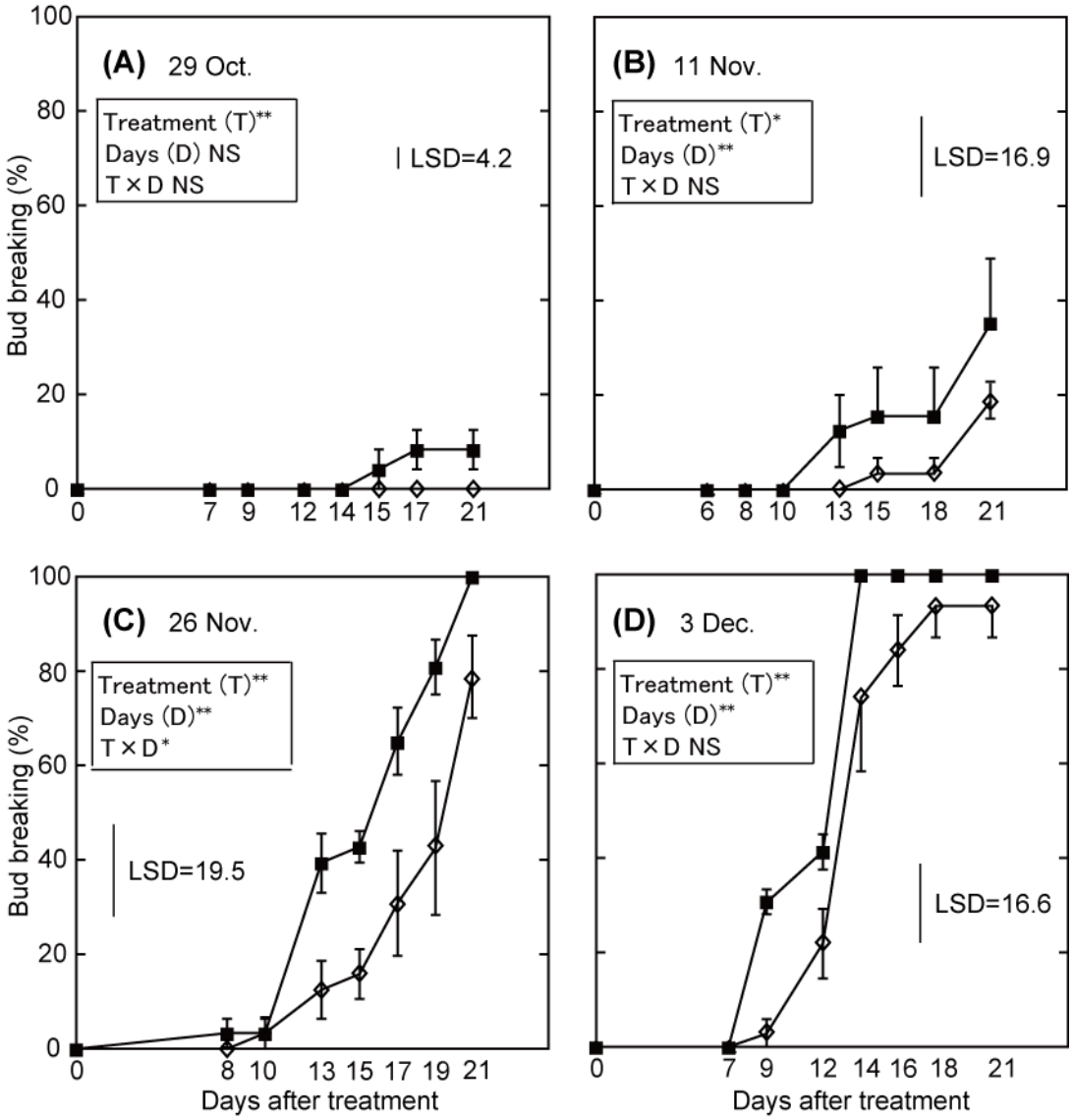

Fig. 8. Effect of KODA on endodormancy breaking in flower buds of Japanese pear 'Kosui' (2008). Treatment on 29 Oct.; (A), 11 Nov.; (B), 26 Nov.; (C), 3 Dec.; (D). Control; (◇), KODA 100 μM; (■). Values are means ± SE (n = 3-5). Vertical bars indicate LSD ($P = 0.05$). NS, *, and ** indicate not significant, or significant differences at $P < 0.05$ and 0.01, respectively, by ANOVA.

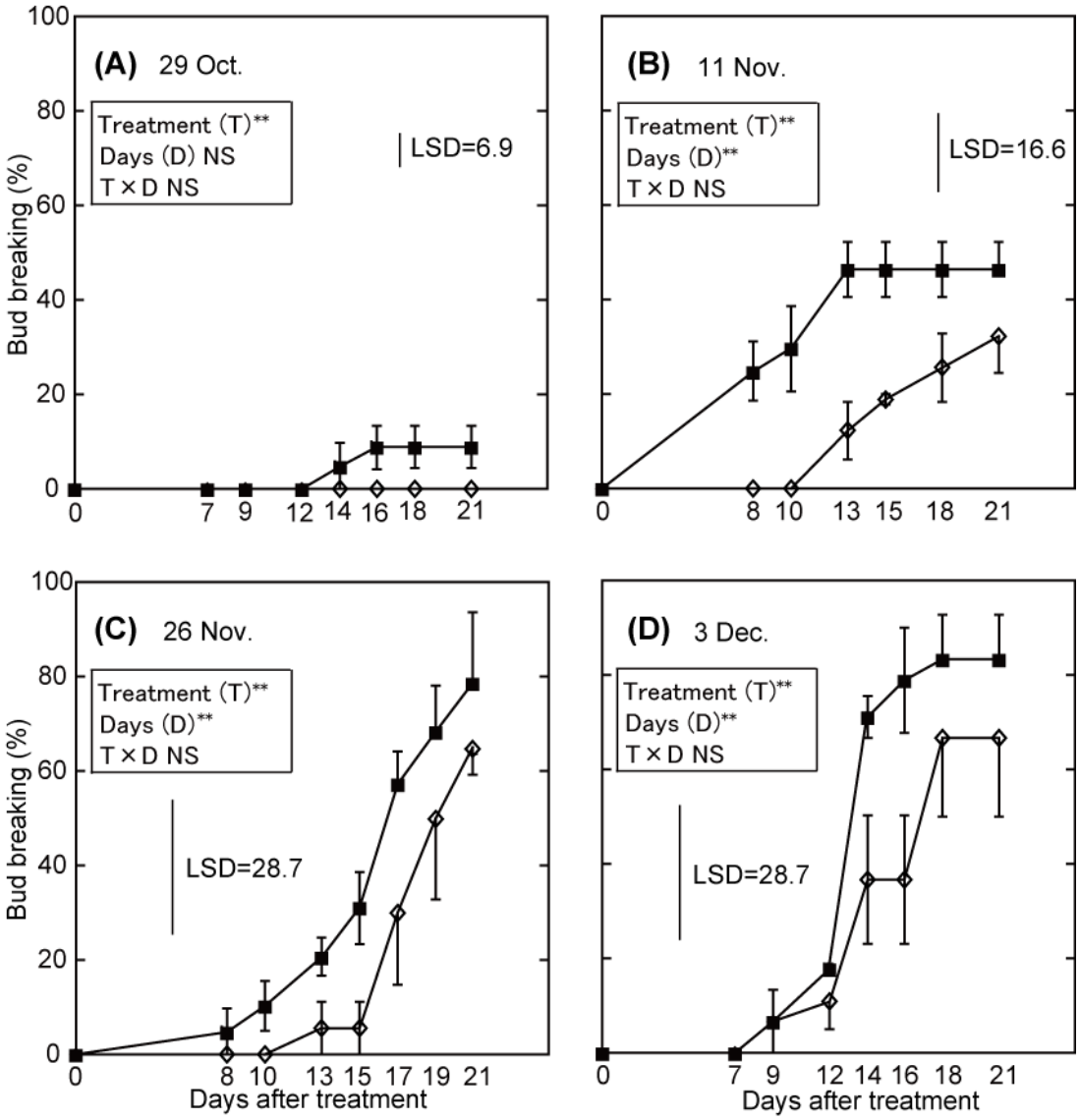

Fig. 9. Effect of KODA on endodormancy breaking in flower buds of Japanese pear 'Natsushizuku' (2008). Treatment on 29 Oct.; (A), 11 Nov.; (B), 26 Nov.; (C), 3 Dec.; (D). Control; (◇), KODA 100 μM; (■). Values are means ± SE (n = 3-5). Vertical bars indicate LSD (P = 0.05). NS, *, and ** indicate not significant, or significant differences at P < 0.05 and 0.01, respectively, by ANOVA.

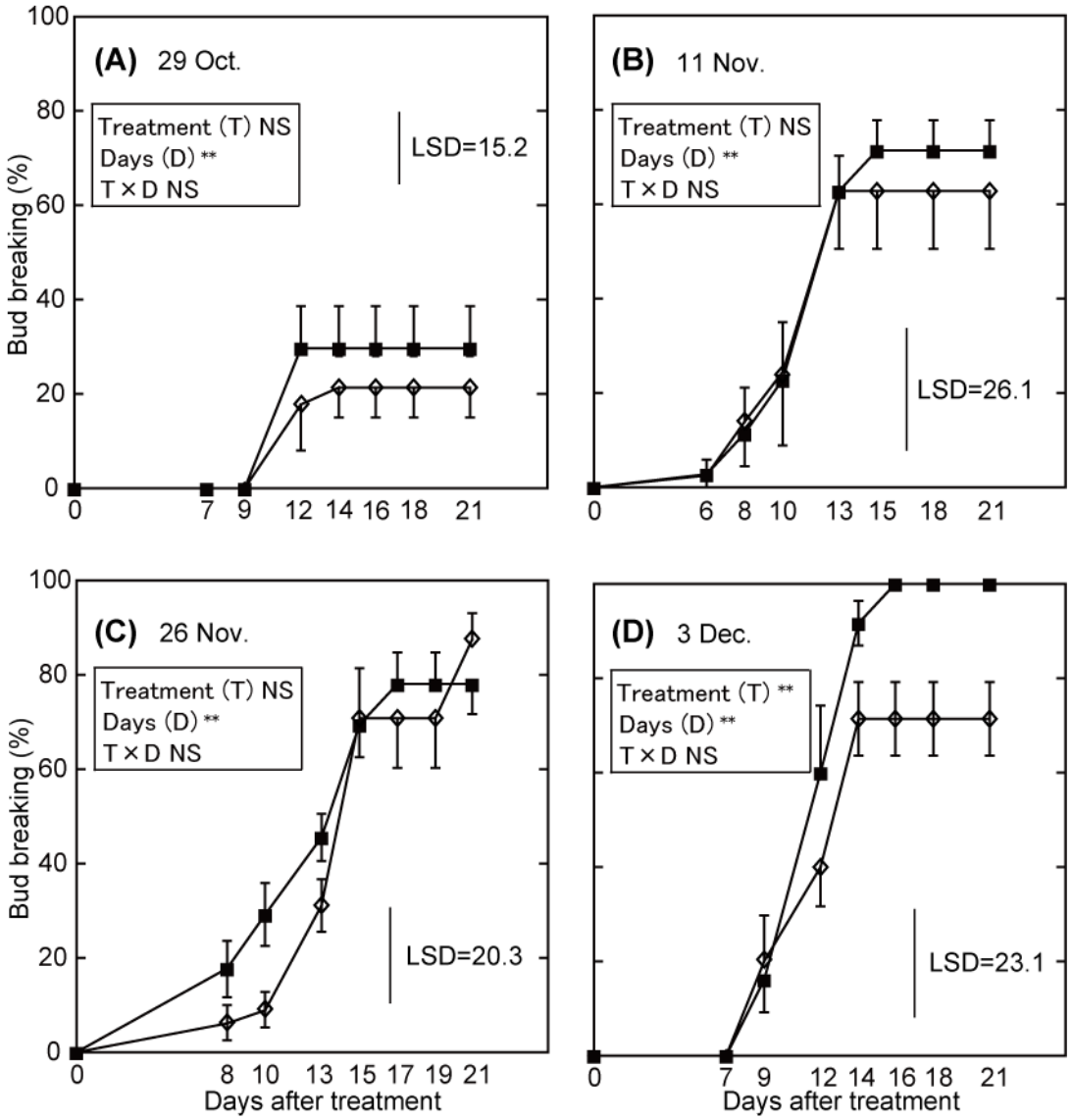

Fig. 10. Effect of KODA on endodormancy breaking in flower buds of Japanese pear 'Hosui' (2008). (A) Treatment on 29 Oct.; (A), 11 Nov.; (B), 26 Nov.; (C), 3 Dec.; (D). Control; (◇), KODA 100 μM; (■). Values are means ± SE (n = 3-5). Vertical bars indicate LSD ($P = 0.05$). NS, *, and ** indicate not significant, or significant differences at $P < 0.05$ and 0.01, respectively, by ANOVA.

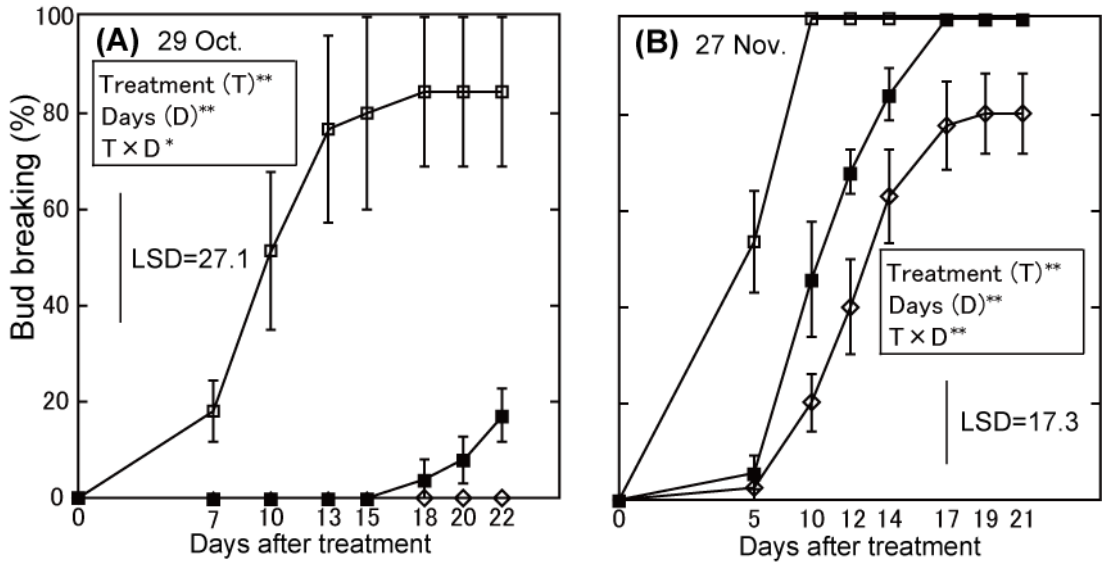

Fig. 11. Effect of KODA or HC on endodormancy breaking in flower buds of Japanese pear 'Kosui' (2009). Treatment on 29 Oct.; (A), 27 Nov.; (B). Control; (◇), KODA 100 μM; (■), HC 1% (w/v); (□). Values are means ± SE (n = 5). Vertical bars indicate LSD (P = 0.05). NS, *, **Nonsignificant or significant at P < 0.05 and 0.01, respectively, by ANOVA.

Section 2 Appropriate fertilizer application methods, including livestock waste compost, as a source of N supplementation to reduce dead flower buds in Japanese pear 'Kosui'

Introduction

The Japanese pear is widely cultivated from the northern (Hokkaido region) to the southern (Kyushu region) regions of Japan. Dead flower buds of Japanese pear 'Kosui' trees in the southwestern regions of Japan have recently been observed in the spring. Application of N fertilizers in the late fall season reduces cold hardiness and enhances the frequency of freezing injury in apples (Kuroda et al., 1984). In addition, Sakamoto et al. (2015b) reported that livestock waste compost (compost) as an N supplement reduces the freezing tolerance of chestnut trees. Nevertheless, it is customary for pear farmers to N-fertilize their orchards during the fall/winter season with compost in addition to chemical fertilizers (Ishizuka, 1984). Before 1999, compost was traditionally stored in the open air, leading to losses in the N content through leaching; however, in 1999 a Japanese law that prohibited the open-air storage of compost was enacted. This new law led to an increase in the total N content of compost of more than 1% compared with that before the law was enacted (Fujita, 2014). The total N content of the compost used in this study was approximately 2.0%. Given that this phenomenon tends to happen in years with a warmer fall-winter season and that excessive N application in the fall reduces the freezing tolerance of pears (Matsumoto et al., 2010), we hypothesized that the occurrence of dead flower buds was related to the timing of N application using compost. In this study, we fertilized Japanese pear ('Kosui') trees with compost in Oct. or Dec. and evaluated the

freezing tolerance, N, and sugar contents of trees to determine if these parameters influenced the incidence of dead flower buds.

Materials and Methods

Plant materials

For the controlled temperature experiment, 12 three or four-year-old potted trees (25 L in 2012/2013 and 20 L in 2013/2014) of the Japanese pear cultivar ‘Kosui’, growing in an experimental orchard at the NIFTS were used. The experiment was conducted during the winter seasons of 2012/2013 and 2013/2014. In both growing seasons, the warm fall and winter field conditions (from mid-Nov. to mid-Mar.) similar to those recorded in Satsumasendai, Kagoshima, where the incidence of dead flower buds was observed in 2008/2009, were simulated using greenhouses equipped with heat pump systems (SPW-AGP180E, E's Inc., Tokyo, Japan). Specifically, maximum air temperatures were set to 15 ºC (from 4 a.m. to 4 p.m.), and the average air temperature was set to 13 ºC (from 4 p.m. to 6 p.m.) and 7 ºC (from 6 p.m. to 4 a.m.). Air temperatures calculated from a 5-d moving average during the experiments in 2012/2013 and 2013/2014 simulated temperatures in Satsumasendai in 2008/2009 (Fig. 12).

For the field experiment, 9 mature Japanese pear ‘Kosui’ trees, growing in the experimental orchard at the Kagoshima Prefectural Institute for Agricultural Development, Hokusatsu Branch (Satsumasendai, Japan), located at 31º 5'N and 130º 2'E, were used. The experiment was conducted during the three fall-winter seasons of 2012/2013, 2013/2014, and 2014/2015. To analyze changes in freezing tolerance, the N

contents, and sugar contents of flower buds, we sampled three uniform CSSs (60-120 cm in length) with 5-10 flower buds per each CSS from Oct. to Mar. at about 2-4 week intervals, and then used the flower buds to analyze the parameters indicated below. As for fertilizer management before the experiments (2011), mature trees were managed according to the ordinary cultural practices used in Japanese pear orchards in Satsumasendai. Briefly, mixed organic (40%) – inorganic (60%) fertilizer [mikan-aki4gou, Japan Agricultural Cooperatives (JA) Butsuryu Kagoshima, Kagoshima, Japan] was applied in Sept. (20%, 3.6 kgN/10a), Oct. (50%, 9 kgN/10a), and Nov. (30%, 5.4 kgN/10a). In Dec. compost (2 t /10a) was applied.

Fertilizer treatment

For the controlled temperature experiment, we used three potted trees per treatment, in which 2 g of readily available chemical fertilizer (ammonium sulfate, Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) was applied to each pot at three different times (mid-Dec., mid-Jan., and mid-Feb.). One set of pots was used as a control and did not receive any fertilizer. The potted trees were first kept in an open field and then transferred to a greenhouse equipped with a heat pump system (SPW-AGP180E, E's Inc.) on 13 Nov. (2012) and were returned to an open field on 8 Mar. (2013). Similarly, for the 2013/2014 season, the pots were transferred to a greenhouse on 20 Nov. (2013) and were returned to an open field on 10 Mar. (2014).

For the field experiment, we used nine mature trees (three mature trees per treatment) and applied 18 kgN/10a of mixed organic-inorganic fertilizer and 2 t/10a of compost that was purchased from JA Kitasatsuma in Satsuma-cho, Kagoshima, every year according to the

following experimental design that is summarized in Table 3. Mixed organic-inorganic fertilizer (18 kgN/10a) was applied in Mar. in all experiments. In 2012/2013 and 2014/2015, compost was applied in Mar. (‘‘Compost in spring’’) or Dec. (‘‘Compost in winter’’). In 2013/2014, compost was applied in Mar. (‘‘Compost in spring’’) or Oct. (‘‘Compost in fall’’). The total N concentration of compost used in this study was approximately 2.0%.

Status of flower buds at flowering

The number of dead flower buds in the controlled temperature experiment was also investigated. During flowering, 6-33 flower buds (apical flower buds of the spur) on each of the three potted pear trees were examined. Similarly, 72-208 flower buds from each of the three trees in each treatment were randomly chosen during flowering (three biological replications each having 72-208 flower buds), and the number of dead apical flower buds on spurs and axillary flower buds of the CSSs were counted separately. The data were transformed using an arcsine transformation and then analyzed by a two-way ANOVA.

Freezing tolerance of flower buds in the field experiment

Freezing tolerance experiments using CSSs were conducted from Nov. to Mar. at about 4-week intervals. Fifteen CSSs from three mature trees for each treatment were randomly chosen and used to evaluate the freezing tolerance. Freezing tolerance was evaluated using an environmental test chamber (SU-642, ESPEC Corp., Osaka, Japan). Three CSSs with 15-26 flower buds were collected from each treatment, wrapped in polyethylene bags and precooled for 3 h at 0 ºC. After leaving the CSSs at selected temperatures, i.e., -5, −8, −12, −16, or −20 ºC, for 16 h, the samples were transferred to 0 ºC for 3 h and

then 5 ºC for 5 h. To evaluate the viability of flower buds after treatment, CSSs were placed in plastic containers with rockwool cubes submerged in distilled water in a chamber (MLR-351, SANYO Electric Co. Ltd., Osaka, Japan) set to 20 ºC under cycling conditions, 12 h/12 h (light/dark), for two weeks. Thereafter, injury due to freezing was evaluated visually by monitoring the browning of floral primordia and the number of buds that failed to sprout. Freezing tolerance was defined as the temperature at which half of the flower buds were dead and was expressed as the lethal temperature 50 (LT_{50}) using the Spearman-Kärber method that was previously used to evaluate blueberry flower buds and Japanese pear (Bittenbender and Howell, 1974; Honjo and Omura, 1987). Calculation of the LT_{50} for a treatment is 1 replication. Data were analyzed by a *t*-Test.

Flower bud total N and sugar contents in the field experiment

Total N and sugar contents were analyzed in the flower buds. Five to ten flower buds per tree were randomly chosen from three mature trees and were collected at about 2- or 3-week intervals from late-Nov. The flower buds including scales were dried (24 h at 80 ºC) using a constant temperature oven (DK600, Yamato Scientific Co. Ltd., Tokyo, Japan), and the total N content was analyzed using an NC analyzer (SUMIGRAPH, NC-220F, Sumika Chemical Analysis Service Ltd., Tokyo, Japan). Dried flower buds from each tree were also used for extraction and analyses of soluble sugars (sorbitol, sucrose, fructose, and glucose) as described by Ito et al. (2012). Data were analyzed by a *t*-Test.

Results

Status of flower buds at flowering

In the controlled temperature experiment (Table 4), the percentage of dead flower buds was significantly higher in the Dec. or Jan. treatments compared with the no fertilizer control. There were no significant differences in the percentage of dead flower buds between the Feb. treatment and the no fertilizer application control, but the Feb. treatments had higher numbers of dead flowers than the no fertilizer control in both years studied. In the field experiment (Table 5), the average percentage of dead flower buds increased with compost application in the fall and winter (‘‘Compost in winter’’ and ‘‘Compost in fall’’) compared with compost application in the spring (‘‘Compost in spring’’) for all types of flower buds (apical flower buds of spurs and axillary flower buds of the CSSs). Furthermore, when these data were analyzed by a two-way ANOVA, compost application in the fall and winter significantly increased the percentage of dead flower buds in all three experiments compared with the ‘‘Compost in spring’’ treatment. In addition, the percentage of dead flower buds was significantly higher in axillary flower buds of the CSSs than in apical flower buds of spurs in 2013 and 2015, but there were no significant differences in 2014 (Table 5).

Freezing tolerance of flower buds in the field experiment

The effect of the timing for compost application on freezing tolerance is shown in Table 6 and Figure 13. Freezing tolerance was assessed by measuring the $LT_{50,}$ defined as the temperature at which half of the flower buds were dead. The ‘‘Compost in winter’’ treatment had a significantly higher LT_{50} than that in the ‘‘Compost in spring’’ treatment during the experimental periods (Table 6, Fig. 13). Similarly, the ‘‘Compost in spring’’ treatment had a significantly higher freezing tolerance than the ‘‘Compost in fall’’ treatment during the experimental periods (Table 6, Fig. 13). In mid-Dec. and mid-Jan.,

freezing tolerance of flower buds in the ‘‘Compost in winter’’ treatment was lower than that of the ‘‘Compost in spring’’ treatment. Similar trends were observed in the ‘‘Compost in fall’’ treatment, in which the freezing tolerance was lower than that of the ‘‘Compost in spring” treatment in mid-Feb. (Fig. 13).

Flower bud total N and sugar content in the field experiment

The effect of the timing for compost application on the total N content of flower buds is shown in Table 6. Total N was significantly higher for the compost treatments in fall and winter over all three seasons. In addition, differences in the freezing tolerance (ºC) between the ‘‘Compost in spring ’’ and ‘‘Compost in winter’’ treatments or between the ‘‘Compost in spring ’’ and ‘‘Compost in fall’’ treatments per each sampling date in three years were significantly and positively correlated with the differences in the N concentration (%) between the ‘‘Compost in spring ’’ and ‘‘Compost in winter’’ treatments or between the ‘‘Compost in spring ’’ and ‘‘Compost in fall’’ treatments per month for each sample (Fig. 14).

There were significant differences in the total sugar content between the ‘‘Compost in spring’’ treatment and the ‘‘Compost in fall’’ treatment, but there were no significant differences between the ‘‘Compost in spring’’ treatment and the ‘‘Compost in winter’’ treatment (Table 6).

Discussion

Orimoto and Ishitsuka (1989) reported that excessive N chemical fertilizer application

caused an increase in dead buds of Japanese pear cultivars ‘Kosui’ and ‘Hosui’ in the following spring. In addition, application of N chemical fertilizers in the late-fall reduced cold hardiness by stimulating root activity and inversely increased the potential for freezing injury in apple buds (Kuroda et al., 1984). In our study, the N content of flower buds significantly increased after the fall-winter compost applications compared with compost application in the spring (Table 6). There is a possibility that excess N from compost application activated tree nutrient uptake in the winter, unlike the bud-sprouting period (Mar.) that usually increases the level of nutrient uptake when trees experience warm winter conditions. The depth of endodormancy was shallow in chestnut trees after excessive compost application at planting, resulting in inadequate freezing tolerance in the winter (Sakamoto et al., 2015b). In addition, ‘Hosui’, a low-chill cultivar compared with ‘Kosui’, is more sensitive to excessive N application than ‘Nijisseiki’, a high-chill cultivar. Thus, reduced cold hardiness and increased growth inhibition in the spring were reported in ‘Hosui’ (Matsumoto et al., 2010). Endodormancy in woody perennials affects both the absolute hardiness and the hardiness transitions during the annual cycles (Kalberer et al., 2007). These results suggest that endodormancy status is related to the acquisition of freezing hardiness in Japanese pear. The dead flower buds were induced not only by the readily available chemical fertilizer in potted trees, but also by compost application in the fall-winter season. Progression of freezing tolerance was significantly and positively correlated with the transition in the N concentration (Fig. 14). In addition, the difference in freezing tolerance depends on environmental conditions and weather conditions of the current year rather than being decided by the absolute N content (Table 6, Fig. 13). It is possible that the most suitable N content is different every year. These results may be ascribed to the remaining readily available N fertilizer in the compost.

Indeed, cold hardening in the fall delayed in trees receiving the ‘‘Compost in winter’’ treatment in 2012/2013, and trees treated with ‘‘Compost in fall’’ in 2013/2014 and ‘‘Compost in winter’’ in 2014/2015 had more significant cold de-hardening in the spring (Fig. 13). Our results also suggest that the flower buds died in the early-winter or early-spring.

The percentage of dead flower buds was higher for axillary flower buds of the CSSs than for apical flower buds of spurs (Table 5). The terminal inflorescences of apical buds of spurs differentiate in mid- to late-June (Banno et al., 1982; Horiuchi et al., 1973), whereas lateral primordia initiate their differentiation after the terminal inflorescence primordium has been formed (Banno et al., 1986). It is thus possible that axillary flower buds of the CSSs delayed the acquisition of freezing tolerance compared with apical flower buds of spurs via delayed CSS maturity, including bud development.

Significant differences in the sugar content among treatments were observed in the ‘‘Compost in fall’’ treatment; however, there were no significant differences between ‘‘Compost in spring’’ and ‘‘Compost in winter’’ treatments (Table 6). Therefore, we concluded that the involvement of sugar content in the acquisition of freezing tolerance in this study was unclear. However, in many deciduous fruit trees, freezing tolerance is positively correlated with the sugar content in the CSSs during the winter, as is the case for pear (Ito et al., 2013), apple (Kuroda et al., 1985), and chestnut trees (Sakamoto et al., 2015a). Thus, it is clear that there is a close relationship between sugar content and freezing tolerance. The basis for this observation remains to be elucidated in the near future.

Collectively, these results suggest that compost application during the fall-winter months can adversely affect freezing tolerance through an increase in the N content, thereby promoting dead flower buds.

Table 3. Experimental design for the controlled temperature experiment (upper) and the field experiment (lower).

Year	Treatment (location)	Abbreviation	Type	Oct.	Dec.	Jan.	Feb.	Mar.
Controlled temperature experiment using potted trees (Tsukuba)								
2012-2013	No fertilizer application	Cont.	—	—	—	—	—	—
	Chemical fertilizer applied in mid-Dec.	Dec.	Chemical	—	2 g[z]	—	—	—
	Chemical fertilizer applied in mid-Jan.	Jan.	Chemical	—	—	2 g	—	—
	Chemical fertilizer applied in mid-Feb.	Feb.	Chemical	—	—	—	2 g	—
2013-2014	No fertilizer application	Cont.	—	—	—	—	—	—
	Chemical fertilizer applied in mid-Dec.	Dec.	Chemical	—	2 g	—	—	—
	Chemical fertilizer applied in mid-Jan.	Jan.	Chemical	—	—	2 g	—	—
	Chemical fertilizer applied in mid-Feb.	Feb.	Chemical	—	—	—	2 g	—
Field experiment using mature trees (Kagoshima)								
2012-2013	Fertilizer[y] (0.8 kg[x] in Mar.) and compost in spring	Compost in spring	Compost	—	—	—	—	90 kg[w]
	Fertilizer (0.8 kg in Mar.) and compost in winter	Compost in winter	Compost	—	90 kg	—	—	—
2014-2015	Fertilizer (0.8 kg in Mar.) and compost in spring	Compost in spring	Compost	—	—	—	—	90 kg
	Fertilizer (0.8 kg in Mar.) and compost in winter	Compost in winter	Compost	—	90 kg	—	—	—
2013-2014	Fertilizer (0.8 kg in Mar.) and compost in spring	Compost in spring	Compost	—	—	—	—	90 kg
	Fertilizer (0.8 kg in Mar.) and compost in fall	Compost in fall	Compost	90 kg	—	—	—	90 kg

[z]2 g of readily available chemical fertilizer (ammonium sulfate) was applied to each pot.

[y]Mixed organic-inorganic fertilizer.

[x]18 kg/10a of N chemical fertilizer applied.

[w]2 t/10a of livestock (cow) waste compost applied.

Table 4. Effect of the timing of N fertilizer application on the occurrence of dead flower buds (%) in the controlled temperature experiment.

Year	Treatment (Abbreviation)	Dead flower bud (%)[z]
2012-2013	No fertilizer application (Cont.)	24.7 b[y]
	Chemical fertilizer applied in mid-Dec. (Dec.)	86.7 a
	Chemical fertilizer in mid-Jan. (Jan.)	86.2 a
	Chemical fertilizer in mid-Feb. (Feb.)	43.7 ab
2013-2014	No fertilizer application (Cont.)	27.4 b
	Chemical fertilizer applied in mid-Dec. (Dec.)	100 a
	Chemical fertilizer applied in mid-Jan. (Jan.)	100 a
	Chemical fertilizer applied in mid-Feb. (Feb.)	78.9 ab

[z]The status of the buds was investigated at flowering.

[y]Different letters within a column indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test.

Table 5. Effect of N supplementation from livestock waste compost during fall-winter on the occurrence of dead flower buds in the field experiment.

Year	Treatment	Abbreviation	Dead flower buds (%)[z] for each type of flower bud		Significance[y]		
			Apical flower buds on spurs	Axillary flower buds of the CSSs	Treatment	Types of flower buds	Interaction
2012-2013	Fertilizer[x] (0.8 kg in Mar.) and compost in spring	Compost in spring	0.0	2.1	*	**	NS
	Fertilizer (0.8 kg in Mar.) and compost in winter	Compost in winter	2.9	14.1			
2014-2015	Fertilizer (0.8 kg in Mar.) and compost in spring	Compost in spring	0.6	3.5	*	**	NS
	Fertilizer (0.8 kg in Mar.) and compost in winter	Compost in winter	2.8	4.3			
2013-2014	Fertilizer (0.8 kg in Mar.) and compost in spring	Compost in spring	0.3	2.1	**	NS	NS
	Fertilizer (0.8 kg in Mar.) and compost in fall	Compost in fall	8.1	6.6			

[z]72-208 flower buds from each tree were randomly chosen during flowering, and the status of the buds was investigated at flowering.

[y]NS, *, and ** indicate not significant, or significant differences at $P < 0.05$, and 0.01, by a two-way ANOVA (n = 3).

[x]Mixed organic-inorganic fertilizer.

Table 6. Effect of the N supplementation from livestock waste compost during fall-winter on the average freezing tolerance, N concentration, and total sugar content of flower buds during the fall-winter months[z] in the field experiment.

Year	Treatment	Abbreviation	Freezing tolerance (°C)		Total N concentration (mg · g^{-1} DW)		Total sugar content (mg · g^{-1} DW)	
2012-2013	Fertilizer[y] (0.8 kg in Mar.) and compost in spring	Compost in spring	-10.2	*[x]	8.8	*	50.3	NS
	Fertilizer (0.8 kg in Mar.) and compost in winter	Compost in winter	-7.9		9.0		49.4	
2014-2015	Fertilizer (0.8 kg in Mar.) and compost in spring	Compost in spring	-9.3	**	9.7	*	37.8	NS
	Fertilizer (0.8 kg in Mar.) and compost in winter	Compost in winter	-6.8		9.9		38.2	
2013-2014	Fertilizer (0.8 kg in Mar.) and compost in spring	Compost in spring	-10.1	*	8.8	*	49.7	*
	Fertilizer (0.8 kg in Mar.) and compost in fall	Compost in fall	-8.5		9.0		47.6	

[z]The average freezing tolerance of flower buds shows the mean value from mid-Nov. to mid-Mar., the average N concentration and total sugar content of flower buds shows the mean value (from late-Nov. to mid-Mar.).

[y]Mixed organic-inorganic fertilizer.

[x]NS, *, and ** indicate not significant, or significant differences at $P < 0.05$, and 0.01 as determined by a *t*-Test.

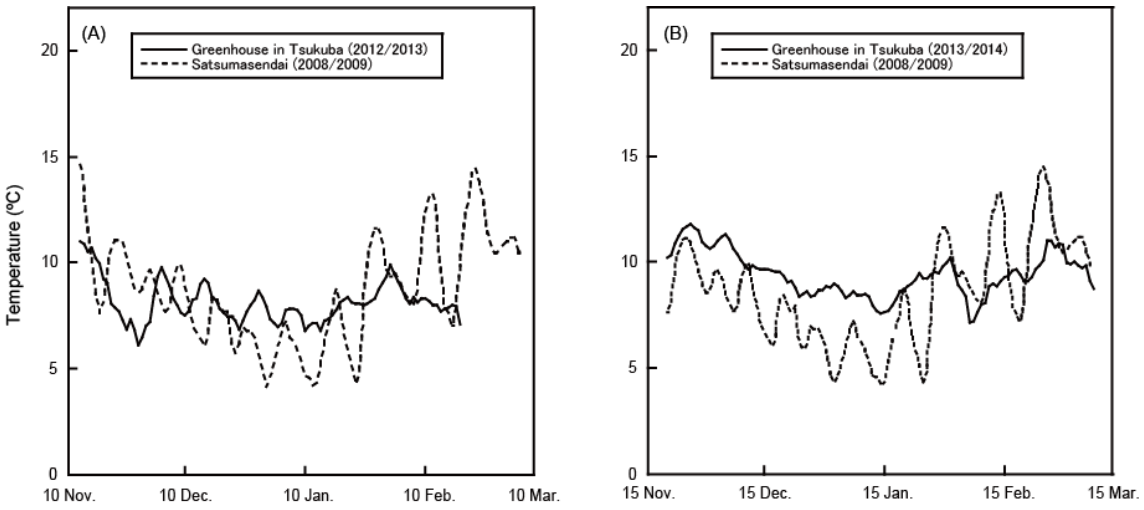

Fig. 12. Air temperature during experiments at Tsukuba.

Air temperature (5-d moving average) in the greenhouse during the experiments in 2012/2013 (A) and 2013/2014 (B) is plotted against that of Satsumasendai in 2008/2009. For 2012/2013, the average air temperature data after 23 Feb. in the greenhouse is missing.

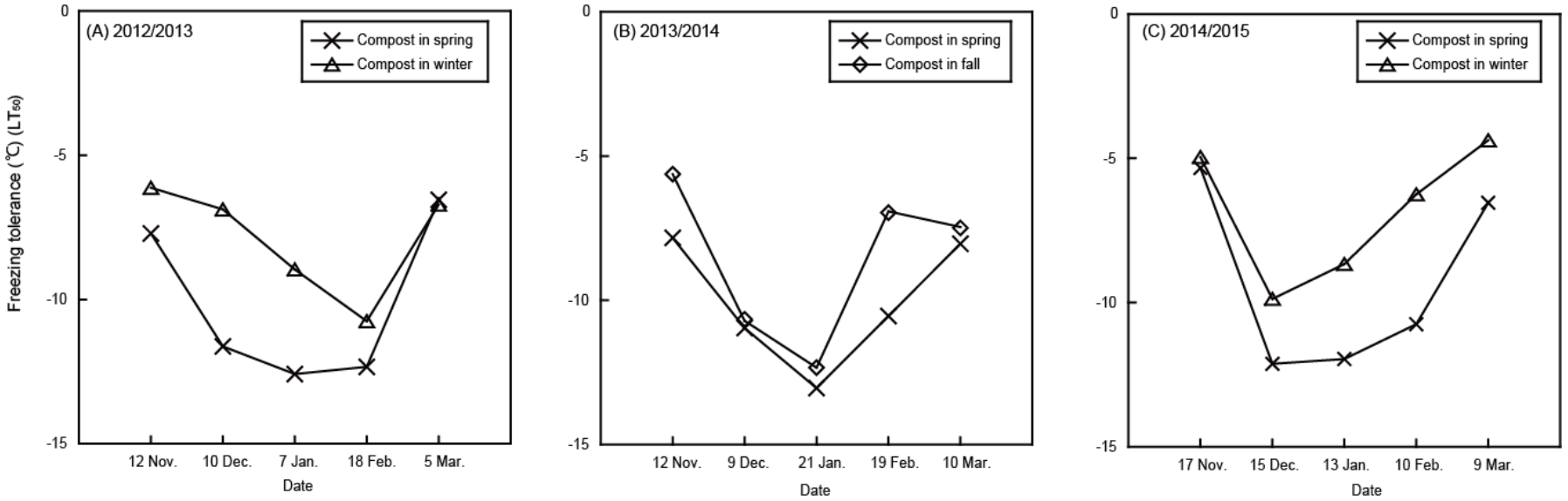

Fig. 13. The effect of the timing of livestock waste compost application on the freezing tolerance of 'Kosui' flower buds in 2012/2013 (A), 2013/2014 (B), and 2014/2015 (C). Compost in spring, mixed organic-inorganic fertilizer and compost in spring (×); Compost in winter, mixed organic-inorganic fertilizer in spring and compost in winter (△); Compost in fall, mixed organic-inorganic fertilizer in spring and compost in fall (◇). Freezing tolerance was defined as the temperature at which half of the flower buds were dead and was expressed as the LT_{50}.

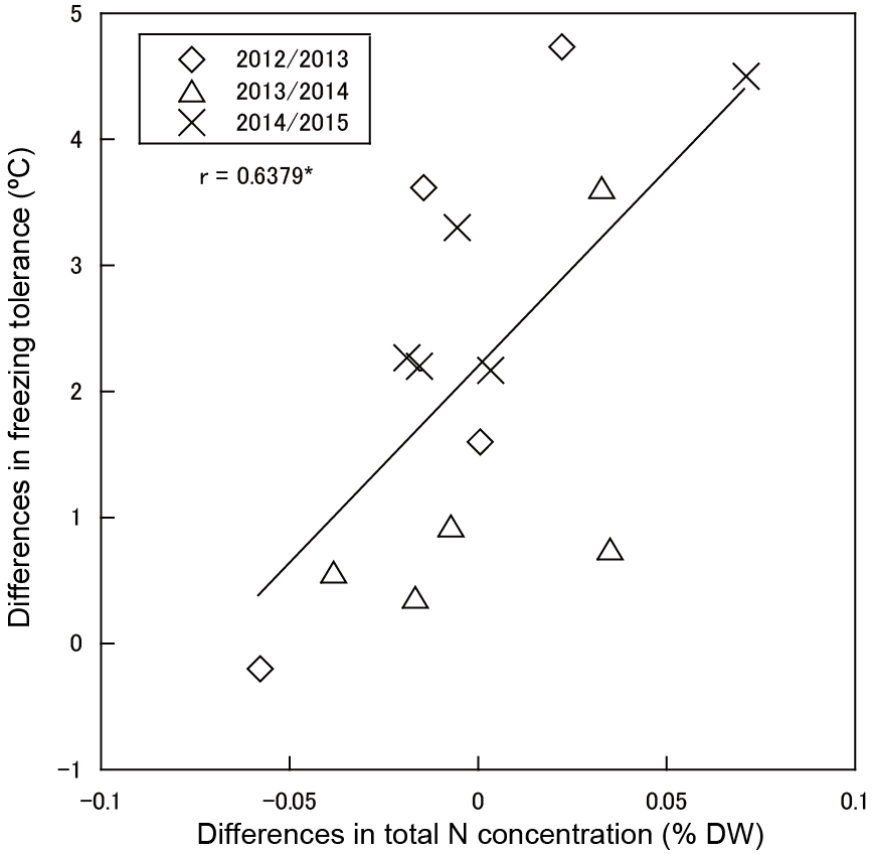

Fig. 14. The relationship between freezing tolerance and total N concentration in 2012/2013 (◇), 2013/2014 (△), and 2014/2015 (×). The value of a coordinate on the vertical axis represents the differences in the freezing tolerance between ‘‘Compost in spring’’ and ‘‘Compost in winter’’ treatments or ‘‘Compost in spring’’ and ‘‘Compost in fall’’ treatments per month in each sample. The value of a coordinate on the horizontal axis represents the differences in the N concentration between ‘‘Compost in spring’’ and ‘‘Compost in winter’’ or ‘‘Compost in spring’’ and ‘‘Compost in fall’’ per month in each sample. Lines represent statistically significant linear regressions. * indicates significant differences at $P < 0.05$.

Chapter 3

The use of KODA as a tool for regulating flower bud formation in Japanese pear

Introduction

For sustaining fruit production of Japanese pear, stable flower bud formation over many years is very important because flower buds are the basis of annual yield. Flower bud formation is influenced by environmental factors such as temperature and nutritional status including the balance between carbon and N that could cause year-to-year variation in flower bud number (Banno et al., 1984). In Japanese pear, flower buds usually are formed on the apical parts of spurs and CSSs. Axillary buds of the CSSs also develop into flower buds depending on the environmental and nutritional conditions. Among the three types of flower buds, apical flower buds on spurs and axillary flower buds of the CSSs are used for fruit production, but flower buds on spurs are preferable for fruit production because they can produce good quality fruit sustainably (Yoshioka and Matsunami, 2000). Flower bud differentiation of Japanese pear generally occurs in early summer (from mid-June to mid-July) in Japan (Banno et al., 1986). Flower buds of Japanese pear contain both floral and vegetative primordia (vegetative growing points and/or floral lateral inflorescences), meaning that flower buds are important for both fruit production in the current year and maintenance of flower buds for the next year. Typical flower buds of Japanese pear are composed of twelve scales, a terminal inflorescence (consisting of ten bracts and ten florets) and one or two lateral primordia that are the growing points (Banno et al., 1985). The lateral primordia initiate in the axil of the eleventh and twelfth scale

after the terminal inflorescence primordium is formed (Banno et al., 1986). Sometimes, flower buds contain one or two additional lateral inflorescences that differentiate from the lateral primordia (Fig. 15). When flower buds contain no lateral primordia (no growing point), they become blind buds after blooming. The number and kinds of lateral primordia (vegetative growing points and/or floral lateral inflorescences) in flower buds are not only affected by the developmental level of the buds, but also are genetically regulated depending on the type of Japanese pear cultivar.

Recently, the level of endogenous KODA was shown to transiently increase in immature flower buds in the flower-inducing phase of *Pharbitis nil* (Suzuki et al. 2003; Yokoyama et al. 2005). Furthermore, KODA facilitated an increase in flower bud formation in the apical parts of apple (Kittikorn et al., 2010). These previous studies encouraged us to investigate the influence of KODA on lateral primordia formation by the apical flower buds on spurs for two Japanese pear cultivars, ‘Kosui’ and ‘Shinsei’, that differed in their ability to form lateral primordia. The aim of this study is to clarify the effects of KODA on lateral primordial formation in the apical flower buds of Japanese pear.

Materials and Methods

Plant materials

Mature Japanese pear trees of ‘Kosui’ and ‘Shinsei’, grown at the NIFTS were used for all experiments. All mature Japanese pear trees were managed according to the ordinary cultural practices used in Tsukuba.

Effect of KODA on apical flower bud formation on spurs

Experiments were performed in 2007, 2008, and 2009. In 2007, to get preliminary information on concentration of KODA, both 10 and 100 μM KODA were applied to six spurs of 'Shinsei' per treatment. Resultantly, we found that the effects of 10 μM KODA on apical flower bud formation were superior to those of 100 μM KODA. Therefore, taking into consideration of the results in apple (Kondo, personal commun.) as well, we showed the result of 10 μM KODA in this study. Treatments consisted of 10 μM KODA combined with three different application dates (13 June, 3 July, and 19 July): i.e., a total of four treatments including a control (no application of KODA). In 2008, spurs of 'Shinsei' and 'Kosui' were used as materials. For 'Shinsei', 20 spurs were selected per treatment. Treatments consisted of 10 μM KODA combined with three different application dates (27 May, 4 July, and 4 Aug.): i.e., a total of four treatments including a control. For 'Kosui', 10 μM KODA was sprayed on 20 spurs on 4 July, and the control trees were not sprayed. In 2009, similarly, 10 μM KODA was sprayed on 20 'Shinsei' spurs and 30 'Kosui' spurs on 3 July. In addition, duplicate applications of 10 μM KODA on 3 July and 13 July were sprayed on 20 and 30 spurs for 'Shinsei' and 'Kosui', respectively. Control trees of both cultivars were not sprayed.

KODA stock solutions (16 mM) were initially prepared using distilled water, and then stock solutions were diluted to 10 μM with distilled water (without surfactant). In all experiments, KODA solutions were sprayed on the apices of spurs until run-off. The number of lateral primordia per apical flower bud and the number of lateral inflorescences per lateral primordia were counted after bud burst during the next spring season. In addition, the frequency of flower bud type in the apical flower buds on spurs was

investigated as shown in Figure 15.

Results

Effect of KODA on the number of lateral primordia per apical flower bud and the number of lateral inflorescences per lateral primordia

The numbers of lateral primordia (vegetative growing points and/or floral lateral inflorescences) in the control were relatively stable for both cultivars throughout the different years studied with fewer lateral primordia on 'Kosui' (0.43-0.50) than on 'Shinsei' (0.67-0.73) (Tables 7, 8). The number of lateral inflorescences on 'Shinsei' was stable (0.14-0.17) but varied on 'Kosui' (0.17-0.34) depending on the year (Tables 7, 8). On the other hand, KODA applications significantly increased the numbers of lateral primordia for both cultivars. In 'Shinsei', KODA treatment on 3 July and 19 July was more effective than the other KODA applications in 2007 (Table 7). Based on these results in 2007, KODA was applied to 'Shinsei' spurs on an extended range of dates (27 May, 4 July, and 4 Aug.) in 2008, and only KODA application on 4 July showed a significant increase in the numbers of lateral primordia. Single (3 July) and double (3 July and 13 July) applications of KODA in 2009 significantly increased of the numbers of lateral primordia. Concerning the number of lateral inflorescences on 'Shinsei', the effects of KODA varied depending on the year (Table 7). For 'Kosui', KODA application in early-July increased the number of lateral primordia (Table 8), a result consistent with similar experiments with 'Shinsei'. Double (3 July and 13 July) applications of KODA in 2009 also did not show an increase in the number of lateral primordia. Similar to the results found for 'Shinsei', the effects of KODA on the number of lateral inflorescences

were not stable in ‘Kosui’, showing a positive KODA effect in 2008, but not in 2009 (Table 8). Thus, KODA application in early-July tended to increase the number of lateral primordia in both cultivars, but the effects of KODA on increasing the number lateral inflorescences were not stable and depended on the year for both cultivars.

Effect of KODA on the frequency of flower bud type in the apical flower buds on spurs

In the cultivars used for this study, the apical flower buds on current spurs generally contain one terminal inflorescence and zero to two lateral primordia (vegetative growing points and/or floral lateral inflorescences). Thus, flowers could be classified into five types according to the number and kind of lateral primordia as indicated in Figure 15: i.e., 1) no lateral primordium, 2) one lateral vegetative primordium (one vegetative growing point), 3) one lateral floral primordium (one floral lateral inflorescence), 4) two lateral vegetative primordia (two vegetative growing points), and 5) one lateral vegetative primordium and one lateral floral primordium (one vegetative growing point and one floral lateral inflorescence), respectively.

For the ‘Shinsei’ control, the frequency (%) of each flower bud type was relatively stable for all three years (Fig. 16). The frequency of type 1, 2, 3, 4, and 5 flower bud types was 34-37 %, 44-50 %, 11-14 %, 0-3 %, and 3-4 %, respectively. For the ‘Kosui’ control, the frequency of type 1, 4, and 5 flower bud types was relatively constant for two years, but the frequency of type 2 and 3 flower bud types was different between 2008 and 2009, when the frequency of type 2 and 3 in 2008 was about twice and half of those in 2009, respectively (Fig. 17). When the frequency of flower bud type in the ‘Shinesi’ and ‘Kosui’ controls was compared, type 2 was most the prevalent type in ‘Shinsei’ (Fig. 16), but type

1 was the most prevalent flower bud type in ‘Kosui’ (Fig. 17). KODA applications affected the frequency of flower bud type in ‘Shinsei’ (Fig. 16). In 2007, KODA significantly decreased the frequency of flower bud type 1, but increased the number of type 4 and 5 flower buds. Application of KODA on 4 July in 2008 decreased the number of type 1 flower buds, but increased the number of type 4 and 5 flower buds, a result similar to that observed in 2007 (Fig. 16). However, for the applications on 27 May and 4 Aug., such a tendency was not observed. In 2009, single (3 July) and double (3 July and 13 July) applications of KODA increased the number of type 3 and 5 flower buds with a decrease in the number of type 1 flower buds. For ‘Kosui’, similar results to ‘Shinsei’ were obtained resulting from KODA application in 2008, when KODA decreased the number of type 1 flower buds, but increased the number of type 4 and 5 flower buds after application on 4 July (Fig. 17). Similarly, single (3 July) and double (3 July and 13 July) applications of KODA in 2009 decreased the number of type 1 flower buds, and especially increased type 5 flower buds in ‘Kosui’. Needless to say, all these changes in flower bud type observed in both cultivars are deeply connected with changes in the numbers of lateral primordia and lateral inflorescences in both cultivars; increase in type 4 and 5 flower buds led to a significant increase in the numbers of lateral primordia and sometimes of lateral inflorescences.

Discussion

In this study, we applied KODA to the apical buds on spurs to investigate the influence of KODA on flower bud formation in two Japanese pear cultivars. When KODA was applied to the spurs of both cultivars, the number of lateral primordia in the apical flower

buds increased, depending on the application date. Applications of KODA in July increased the numbers of lateral primordia significantly compared with the controls (Tables 7, 8). The terminal inflorescences of apical buds on spurs differentiate in mid- to late-June (Horiuchi et al., 1973; Banno et al, 1982) and lateral primordia initiate after the terminal inflorescence primordium has been formed (Banno et al., 1986). In Tsukuba, the terminal inflorescence primordia differentiated in mid- to late-June, and the florets developed in July (Ito et al., 2002). Applications of KODA in late-May (before differentiation of the terminal floral primordia) and early-Aug. (after formation of the terminal floral primordia) did not show significant effects on the number of lateral primordia compared with the 'Shinsei' control (Table 7). In addition, a double application of KODA in July to both cultivars did not result in additional effects compared with a single application (Tables 7, 8). These lesser effects of KODA might be ascribed to missing the suitable application stage since July was the most effective time for KODA application to promote the initiation of lateral primordia, a stage for the active formation of terminal floral primordia (Ito et al., 2002).

The numbers of lateral primordia in flower buds differed among cultivars (Tables 7, 8). 'Shinsei' had a larger number of lateral primordia in flower buds than 'Kosui'. Also, the numbers of flower bud types 1 and 3 that resulted in blind buds tended to be reduced (10-30%) by the application of KODA in July in comparison with the controls for both cultivars. The frequency of flower bud type after application of KODA compared to the control shifted from type 1 to type 2 or 3 and from type 2 or 3 to type 4 or 5 (Figs. 16, 17). As previously reported, the differentiation and development of Japanese pear flower buds occurs continuously from mid-June (Ito et al., 2002). The earliest visible change

from vegetative bud to terminal inflorescence, enlargement of the apical meristem, is followed by the formation of twelve scales. The terminal inflorescence primordia develop from the apical meristem and, afterwards, the lateral primordia initiate in the axil of the eleventh and twelfth scales (Banno et al., 1986). Sometimes, lateral inflorescences differentiate from lateral primordia. These observations suggested that all buds in Japanese pear have the potential to become flower buds, and flower buds gradually advance from type 1 to type 5 buds. Our results indicate that KODA may serve to push up this bud development. A similar effect of KODA was reported for Satsuma mandarin by Nakajima et al. (2011), where KODA increased the number of flowers by promoting axillary bud sprouting. Therefore, KODA promotes bud differentiation and development in addition to its effect on flower bud formation.

Table 7. Effect of KODA on apical flower bud formation on spurs of Japanese pear 'Shinsei'[z].

Year	Application date	Concentration (μM)	Number of lateral primordia	Number of lateral inflorescences
2007	13 June	10 μM	0.88 ± 0.06 *[y]	0.40 ± 0.05 **
	3 July	10 μM	1.15 ± 0.06 **	0.36 ± 0.05 **
	19 July	10 μM	1.15 ± 0.05 **	0.31 ± 0.05 *
	-	0 μM (Control)	0.67 ± 0.05	0.14 ± 0.03
2008	27 May	10 μM	0.60 ± 0.05 NS	0.26 ± 0.04 NS
	4 July	10 μM	0.89 ± 0.06 *	0.19 ± 0.04 NS
	4 Aug.	10 μM	0.79 ± 0.05 NS	0.21 ± 0.04 NS
	-	0 μM (Control)	0.69 ± 0.06	0.16 ± 0.04
2009	3 July	10 μM	0.90 ± 0.06 *	0.30 ± 0.05 *
	3 July, 13 July	10 μM	0.99 ± 0.06 **	0.28 ± 0.05 NS
	-	0 μM (Control)	0.73 ± 0.06	0.17 ± 0.04

[z]The number of lateral primordia of apical flower buds and lateral inflorescence per lateral primordia in the spring following the application. Values are the means ± SE (n = 78-120 (8-20 terminal bud per tree) in 2007, n = 99-108 (14-20 terminal bud per tree) in 2008, n = 95-99 (12-19 terminal bud per tree) in 2009.

[y]NS, *, and ** indicate not significant, or significant differences at $P < 0.05$, and 0.01, respectively, compared to the control by the Dunnett's Test.

Table 8. Effect of KODA on apical flower bud formation on spurs of Japanese pear 'Kosui'[z].

Year	Application date	Concentration (μM)	Number of lateral primordia	Number of lateral inflorescences
2008	4 July	10 μM	0.70 ± 0.08 **[y]	0.32 ± 0.05 *
	-	0 μM (Control)	0.43 ± 0.06	0.17 ± 0.04
2009	3 July	10 μM	0.72 ± 0.06 **	0.43 ± 0.05 NS
	3 July, 13 July	10 μM	0.72 ± 0.06 **	0.38 ± 0.04 NS
	-	0 μM (Control)	0.50 ± 0.05	0.34 ± 0.04

[z]The number of lateral primordia of apical flower buds and lateral inflorescence per lateral primordia in the spring following the application.
Values are the means ± SE (n = 82-84 (18-26 terminal bud per tree) in 2008, n = 118-121 (15-28 terminal bud per tree) in 2009.

[y]NS, *, and ** indicate not significant, or significant differences at $P < 0.05$, and 0.01, respectively, compared to the control by the Dunnett's Test.

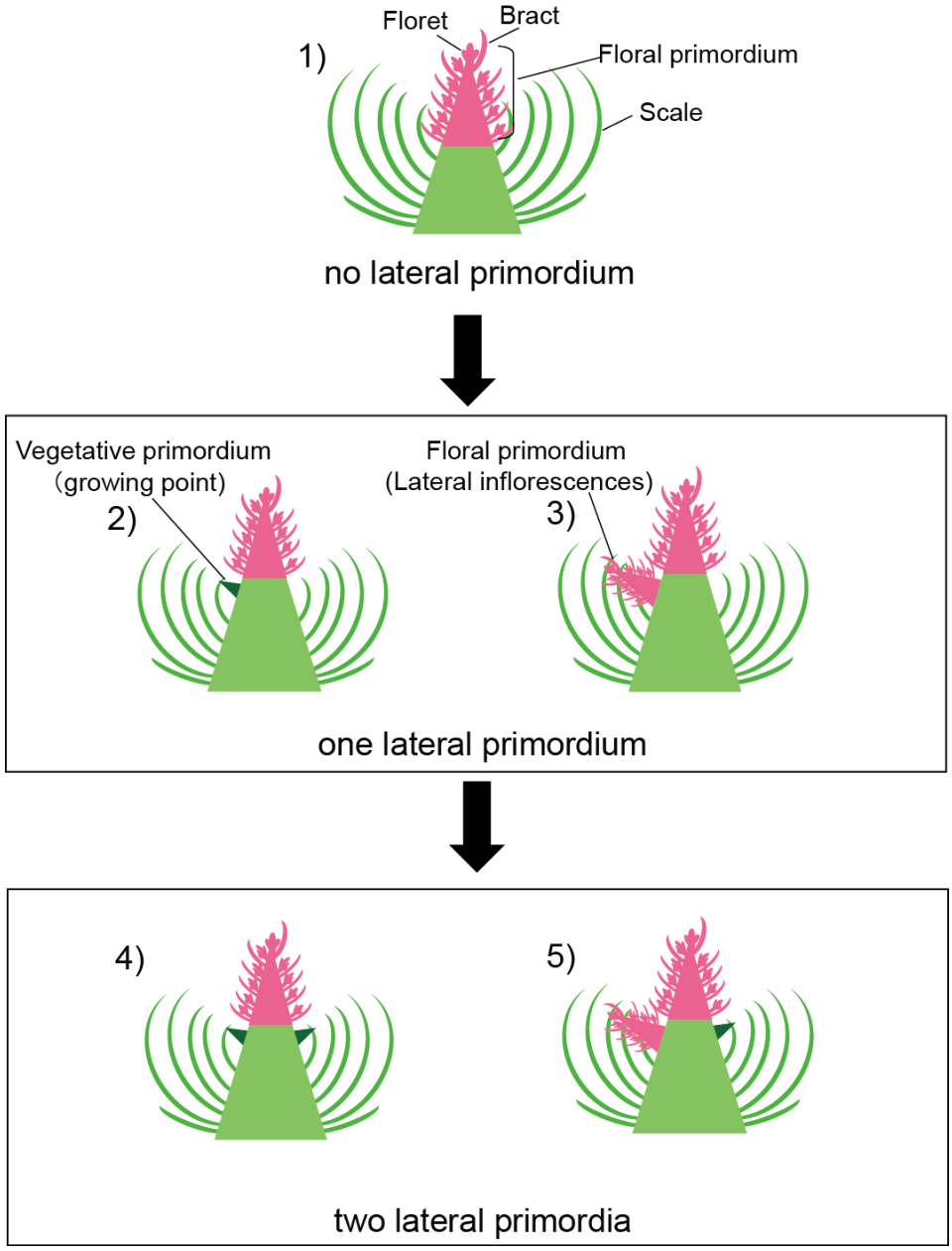

Fig. 15. Schematic diagram of differentiation and development of Japanese pear flower buds. Types of flowers were classified into five types according to the number and kind of lateral primordia as follows: 1) no lateral primordium**,** 2) one lateral vegetative primordium (one growing point), 3) one lateral floral primordium (one lateral inflorescence), 4) two lateral vegetative primordia (two growing points), and 5) one lateral vegetative primordium and one lateral floral primordium (one growing point and one lateral inflorescence), respectively.

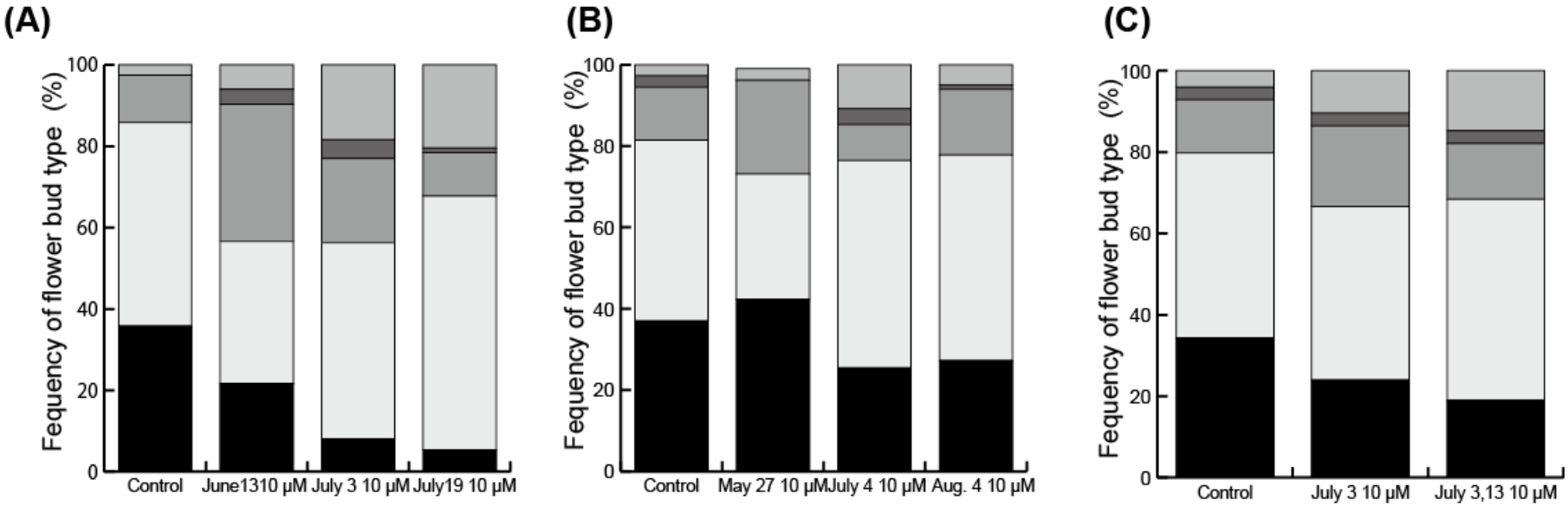

Fig. 16. Effect of KODA on the frequency of flower bud type in the apical flower buds on spurs of Japanese pear 'Shinsei' in 2007 (A), 2008 (B) and 2009 (C). Flower types were classified into five categories as shown in Figure 15: i.e., 1) no lateral primordium; (■), 2) one lateral vegetative primordium; (□), 3) one lateral floral primordium; (▩), 4) two lateral vegetative primordia; (▩), 5) one lateral vegetative primordium and one lateral floral primordium; (▩).

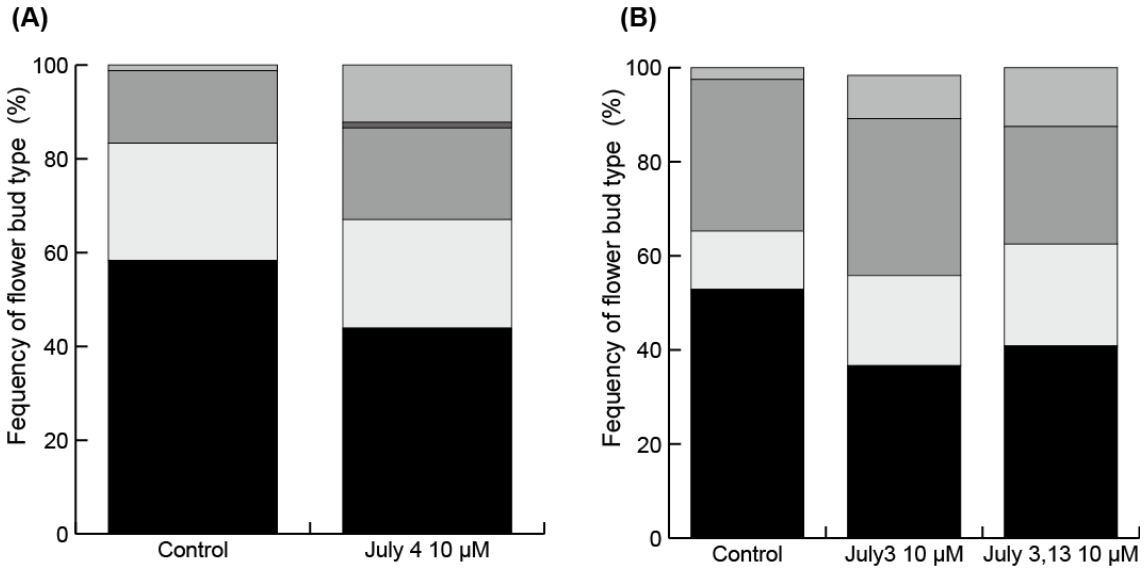

Fig. 17. Effect of KODA on the frequency of flower bud type in apical flower buds on spurs of Japanese pear 'Kosui' in 2008 (A), 2009 (B). Flower types were classified into five categories as shown in Figure 15: i.e., 1) no lateral primordium; (■), 2) one lateral vegetative primordium; (□), 3) one lateral floral primordium; (▣), 4) two lateral vegetative primordia; (■), 5) one lateral vegetative primordium and one lateral floral primordium; (□).

Chapter 4

Spray pollination as a labor-saving pollination system for Japanese pear

Section 1 Applicability of using spray pollination for 'Kosui'

Introduction

Japanese pear is one of the most important fruit crops in Japan. Most cultivars of Japanese pear show self-incompatibility. Therefore, artificial pollination by hand with compatible pollen using a conventional feathered stick (designated as "hand pollination" in this study) is usually carried out for commercial production. However, the effective period for artificial pollination is limited, and the success of hand pollination is dependent on environmental factors such as wind and rainfall. Furthermore, hand pollination is a labor-intensive process resulting in high labor costs. As an alternative technique, spray pollination using aqueous pollen grain solutions is expected to reduce labor and costs in fruit tree cultivation. Therefore, several attempts have been made to establish spray pollination methods. In a spray pollination of peach, a 10% sucrose solution and a wetting agent were used, but this yielded poor fruit set (Mizuno et al., 2002). In kiwifruit, a practical technique was successfully established through the development of a pollen grain suspension medium (Hopping and Simpson, 1982). More recently, a liquid pollen grain suspension medium thickened with agar was also developed for kiwifruit (Yano et al., 2007). It is generally accepted that xanthangum (XG), a kind of polysaccharide, is superior to agar with respect to its potent ability in holding peach pollen grains on the

stigma (Miyata et al., 2005). In addition, trials of the spray pollination using a 10% sucrose solution have been also conducted in Japanese pear, but a stable technique for practical application has not yet been developed (Inadomi, personal commun.).

It is generally known that it is difficult to maintain pollen grain viability in solution (Ohno et al., 1964). Boron compounds, such as boric acid and sodium borate (borax), can be effective in maintaining pollen grain viability. At concentrations of 10 to 100 ppm, boron was shown to stimulate *in vitro* pollen grain germination and promote growth of the pollen tubes in *Amaryllis hybrida* and *Pyrus communis* (Visser, 1955; Stanley and Lichtenberg, 1963). However, boron must be handled carefully because it is poisonous to the human body. On the other hand, it has been reported that pollen grains of *P. communis* release pectinase immediately after being placed in a germination medium (Stanley and Thomas, 1967). Furthermore, the addition of a low concentration of pectinase to the growth medium stimulated pollen tube growth *in vitro* (Roggen and Stanley, 1969). These results suggested that pectin-cleaving enzymes such as pectin methylesterase (PME) and polygalacturonase (PG) might be potentially useful for maintaining pollen grain viability. In previous report as for peach (‘Kawanakajima Hakuto’), the addition of PME or PG to the suspension media used for spray pollination was also useful for maintaining pollen grain viability, and as a result, these additions improved the percentages of fruit set (Sakamoto et al., 2008). These results encouraged us to investigate the use of PME and PG in spray pollination of Japanese pear.

Our goal for this study was to develop a suspension medium suitable for spray pollination in Japanese pear. We focused on the effects of PME and PG combined with either agar

or XG on pollen grain viability. We also analyzed the influences of spray pollination on fruit set and fruit quality in the Japanese pear 'Kosui'. Then, we evaluated the effectiveness of spray pollination from the point view of cost savings in labor and materials.

Materials and Methods

Effects of PME and PG on pollen grain viability

To prepare the pollen grains used for all experiments described in this paper, dry anthers including pollen grains (of cultivar 'Xue Hua Li') were refined with acetone and stored in a deep freezer (-30 ºC). Before use, the refined pollen grains were acclimatized for 12 h at room temperature. In order to assess the effects of PME and PG on pollen grain viability in solution, we measured pollen germination rates and pollen tube growth rates after incubation of the pollen grains for 2 h in media supplemented with PME or PG. The acclimatized pollen grains were suspended at 0.3% (w/v) in media containing 10% (w/v) sucrose and 0.1% (w/v) agar supplemented with 0, 0.01, 0.1, 1.0, and 10.0 $mg \cdot L^{-1}$ of either PME from orange peel (Sigma-Aldrich Co. LLC., Tokyo, Japan) or PG from *Aspergillus niger* (Sigma-Aldrich Co. LLC., Tokyo, Japan). These suspension media were kept at 20 ºC for 2 h, then aliquots (0.2 mL) of each pollen grain suspension were spread on the surfaces of agar plates [1% (w/v) agar and 10% (w/v) sucrose], and incubated at 25 ºC for another 2 h as described by Yano et al. (2007). Three plates were used for each suspension. After incubation, the germination rates and lengths of the pollen tubes were assessed under a microscope (Digital Fine Scope VC3500, OMRON Corp., Kyoto, Japan). The germination rate was determined using 150 pollen grains in each of

three view points per plate. Pollen tube length was measured for approx. 50 pollen tubes in each of three view points per plate, using image analysis software (Image-Ana LITE ver 4.1, OMRON Corp., Kyoto, Japan).

Spray pollination experiments in the field

Field pollination experiments were performed in 2005 to 2007 at the NIFTS using mature trees of Japanese pear 'Kosui'. Pollen grains ('Xue Hua Li') were prepared as described above. Spray pollination with the pollen suspensions was carried out using an electromotive-style sprayer provided by Matsushita Battery Industrial Co. Ltd (Osaka, Japan). As a control, hand pollination using pollen grains diluted to 1/5 (w/w) with *Lycopodium* spores was carried out using a conventional feathered stick. A total of 30 randomly selected flower clusters were used for each treatment. Since each flower cluster contained 8 to 10 flowers, we reduced the number of flowers per cluster to 5 by removing extra flowers at the balloon stage. Then, each flower cluster was covered with a paper bag to avoid natural pollination. At the time of flowering, pollinations were carried out, and the treated flowers were again covered with bags for about 1 month. We also included a set of flower clusters that were not pollinated, to be used as controls in the determinations of fruit and seed set, and fruit quality.

Suspension media for spray pollination

All media contained 10% (w/v) sucrose to prevent explosion of the pollen grains. Either agar at 0.1% (w/v) or XG at 0.04% (w/v) was used to improve the dispersion of pollen and the viscosity of the media (Miyata et al., 2005). The six kinds of media used for the experiment were as follows: i) "XG", ii) "XG + PME (0.1 $mg \cdot L^{-1}$)", iii) "XG + PG (0.1

mg·L $^{-1}$)", iv) "Agar", v) "Agar + PME (0.1 mg·L $^{-1}$)", vi) "Agar + PG (0.1 mg·L $^{-1}$)". Pollen grains were suspended in these media at final concentrations of 0.3% (w/v). In parallel with the pollination experiments in the field, pollen germination rates and pollen tube lengths were investigated for each medium tested, by taking aliquots at the beginning and at the end of each pollination procedure (pollination experiments took about 2 h), and incubating on agar plates as described above. Pollen germination rates and pollen tube lengths were also determined for the pollen used in hand pollination procedures. The pollen grains were scattered on agar plates and analyzed as described for the pollen in the suspension media.

Effects of the suspension media on fruit set and fruit quality

The percentages of fruit set were determined in the mid-May. After fruit set determination, trees were managed according to the usual commercial practices. Fruit were harvested at the end of Aug. and fruit quality was assessed as follows, using from 3 to 25 fruit for each treatment. Fruit weight, the index of irregular fruit shape (calculated using the ratio of the maximum and minimum diameters of the equatorial plane of the fruit), and the number of seeds per fruit were determined. Total soluble solids (^{o}Brix) and the pH of juice extracted from the flesh of opposite sides of the fruit were determined using a refractometer (PR-101, Atago Co. Ltd., Tokyo, Japan) for soluble solids and a pH meter (pHboy-P2, Shindengen Electric Manufacturing Co. Ltd., Tokyo, Japan) for pH.

Assessment of labor and pollen amounts required for spray pollination

In order to assess the time and pollen amounts required for spray pollination vs. hand pollination, the following experiment was carried out in 2007 at a commercial orchard

located in the Kumamoto prefecture (Japan) using mature trees of Japanese pear 'Niitaka'. The pollen suspension medium consisted of 10% (w/v) sucrose and 0.04% (w/v) XG. Pollen ('Xue Hua Li') was prepared, and spray pollination was performed using the electromotive-style sprayer, as described above. As a control experiment, hand pollinations were performed using 'Xue Hua Li' pollen grains that had been refined, stored in a deep freezer, and acclimatized, as described for the pollen used in spray pollinations. This pollen was diluted to 1/5 (w/w) with *Lycopodium* spores and the pollinations were carried out using a conventional feathered stick. Each treatment was carried out on a main stem with two replications. Based on the results of these experiments, the pollination times and the amounts of the pollen grain used were calculated for an area of 10a.

Results

Effect of PME or PG on pollen grain viability

When pollen grains were suspended in the medium containing 0.1- 10 mg·L^{-1} PME, there were no significant differences in the pollen germination rates compared with the controls (0 mg·L^{-1} PME; Fig. 18A). Pollen tube lengths in the media containing 0.1- 1 mg·L^{-1} PME were significantly greater than those of the controls. In particular, supplementing the medium with PME at 0.1 mg·L^{-1} resulted in the longest pollen tubes (about 1.5 times the average length of the controls) (Fig. 18B). When the pollen grains were suspended in the medium containing 0.1- 10 mg·L^{-1} PG, the pollen germination rates were not significantly different from those of the controls (0 mg·L^{-1} PG; Fig. 18C). PG at 10 mg·L^{-1} significantly repressed pollen tube growth, but media supplemented with 0.1- 1

mg·L^{-1} PG resulted in longer pollen tubes than the controls, with the longest pollen tubes (about 1.5 times the average length of the controls) in the medium containing 0.1 mg·L^{-1} PG (Fig. 18D).

Effects of the suspension media on pollen grain viability in the field

The pollen germination rates at the beginning of each pollination procedure revealed no significant differences among the treatments (Fig. 19A). At the end of each pollination procedure, the pollen germination rates after spray pollination were lower than those after hand pollination, irrespective of the type of suspension medium used. However, the medium "XG + PG" showed a significantly higher germination rate than the other suspension media at the end of the pollination procedure.

In aliquots taken at the beginning of each pollination procedure, the pollen tubes in the suspension media "XG + PME", "XG + PG" and "Agar + PG" were significantly longer than those of pollen that was used for hand pollination (Fig. 19B). The pollen tubes in the "Agar" suspension medium were significantly shorter than those of pollen samples to be used for hand pollination, even at the beginning of each pollination procedure. All of the pollen tubes in the suspension media were significantly shorter than those in the pollen sample that was used for hand pollination, at the end each pollination procedure. However, the addition of PME or PG seemed to encourage pollen tube growth, when compared with XG or agar alone.

Effects of spray pollination on fruit set and fruit quality

The average percentage of fruit set per flower cluster of five flowers for each treatment

is shown in Table 9. For all treatments and years, the data shown are the averages for 30 flower clusters. The percentages of fruit set after spray pollination seemed to be lower than those after hand pollination in 2005 and 2007, but the percentages of fruit set for spray and hand pollination were comparable in 2006. In 2005 and 2007, some consistent tendencies could be observed among the suspension media: the media containing either PME or PG gave apparently higher percentages of fruit set than the media without PME and PG, although these differences were not significant. Consequently, the media "XG + PME" and "XG + PG" gave slightly higher levels of fruit set than the other suspension media. In the flower clusters that were not artificially pollinated, the average percentages of fruit set were 44%, 10%, and 6% in 2005, 2006, and 2007, respectively. On the other hand, the number of fruit that could be harvested and used for analysis varied from 3 to 25 depending on the treatments; we could not get sufficient fruit from the controls (no pollination) or even from some pollination treatments due to fruit cracking and so on. Data for the average numbers of seeds produced per fruit after each pollination method are shown in Table 9. In 2005, fewer seeds per fruit were obtained after spray pollination than after hand pollination. However, the spray pollination resulted in more seeds per fruit than hand pollination in 2006 and 2007, although the differences were not significant.

The data showing fruit quality measurements after different pollination methods or after no pollination are shown in Table 10. No pollination resulted in smaller average fruit weights than other treatments; however, there were no significant differences in fruit weight among any of the pollination treatments. Furthermore, there were no significant differences among any of the treatments for the index of irregular fruit shape, the measure

of total soluble solids (^{o}Brix) or the pH in three years.

Time and amounts of pollen needed for spray pollination compared with hand pollination

The time needed for spray pollination was less than half of the time needed for hand pollination (Table 11). In addition, the amount of the pollen grain required for the spray pollination was about one-third of that needed for the hand pollination (Table 11).

Discussion

In most plant species, the pollen tube cell wall consists of two layers, the inner sheath of callose and outer coating containing mainly pectin with cellulose and hemicellulose. The pollen tube grows exclusively at its tip, where the newly synthesized cell wall is continually forming (Taylor and Hepler, 1997). Furthermore, a single pectin layer, lacking callose or cellulose, forms the tip cell wall (Ferguson et al., 1998). Generally, pectin can be demethylesterified by PME and subsequently cleaved by PG, which can lead to cell wall loosening (Micheli, 2001). Jiang et al. (2005) identified and characterized the gene *VANGUARD1* (*VGD1*) that encodes a PME-homologous protein in *Arabidopsis thaliana*, and showed that the pollen tubes from a *vgd1* mutant grew much more slowly than wild-type pollen tubes within the style. Thus, PME is known to play a central role in the growth of the pollen tube wall, which is composed mainly of pectin (Li et al., 1994; Ferguson et al., 1998). On the other hand, it is also known that PG activity is associated with pollen grain maturation and pollen tube growth (Pressey and Reger, 1989; Pressey, 1991), which, we deem, might be a result of the cleavage action of demethylesterified

pectin. Our results showed that the addition of PME or PG to the pollen suspension media at concentrations of 0.01-1 mg·L^{-1} improved pollen tube growth, and tended to increase the percentage of fruit set. Thus, both PME and PG could contribute to an improvement in pollen grain viability in suspension media through the functional roles discussed above.

With regard to XG versus agar, the suspension medium containing 0.04% XG gave a higher level of fruit set than the medium containing 0.1% agar, and the agar-containing medium tended to become gelatinous. Also, the number of pollen grains that landed on each stigma was significantly greater when using a 0.04% XG medium than a 0.1% agar medium (data not shown) and this was also observed in peach spray pollination (Miyata et al., 2005). Thus, XG is superior to agar for general use in spray pollination.

It is well known that the numbers and sizes of seeds affect fruit enlargement, and play important roles in nutrient competition among fruit, in many species (Grant and Ryugo, 1984; Nitsch, 1950). In this work, the number of seeds per fruit was lower after spray pollination than after hand pollination in 2005, but the numbers were comparable after both pollination methods in 2006 and 2007. In addition, there were no obvious differences in fruit weight, shape and quality among the treatments in three years. Thus, year-to-year differences, possibly due to different weather conditions, greatly affected the number of seeds produced. Furthermore, the partial parthenocarpic property of the ‘Kosui’ (Inomata et al., 1992) may also have affected our results. This remains to be elucidated in the near future.

The time required for spray pollination was less than half of the time needed for hand

pollination (Table 11). In kiwifruit, the time needed for spray pollination was also about half of the time needed for hand pollination (Yano et al., 2007). Furthermore, the amount of pollen needed for spray pollination was about one-third of the amount needed for hand pollination. In Japan, the pollen used in the artificial pollination of Japanese pear is usually imported from China, and constitutes a large part of the expense of artificial pollination. Therefore, the labor saving benefits of spray pollination may also contribute to the low-cost management of pear cultivation, because additional labor will not be required for pollination procedures, unlike the situation for hand pollination. Our results suggest that spray pollination is a practical system for reducing the costs of both materials and labor.

In summary, we suggest that a pollen suspension medium containing 0.1 $mg \cdot L^{-1}$ of either PME or PG, combined with 0.04% XG and 10% sucrose, could be suitable and practical for the spray pollination of Japanese pear ‘Kosui’.

Table 9. Numbers of seeds and fruit set after spray pollination with media containing PME or PG in Japanese pear 'Kosui' (2005-2007).

Pollination method	Suspension medium[z]	Fruit set (%) [y]			Number of seeds per fruit (n = 3-25)		
		2005	2006	2007	2005	2006	2007
Spray	XG	72 bc[x]	76 a	74 bc	2.7 ab (22)[w]	1.5 a (21)	2.2 a (25)
Spray	XG+PME	86 ab	—	76 ab	1.8 bc (24)	—	2.3 a (25)
Spray	XG+PG	—[v]	78 a	82 ab	—	1.9 a (24)	1.4 a (21)
Spray	Agar	—	72 a	60 c	—	2.6 a (21)	1.4 a (22)
Spray	Agar+PME	78 abc	—	66 bc	2.6 ab (23)	—	1.5 a (20)
Spray	Agar+PG	—	78 a	70 bc	—	1.9 a (23)	1.7 a (20)
Hand		94 a	74 a	90 a	3.5 a (22)	1.3 a (21)	1.4 a (19)
No pollination		44 c	10 b	6 d	0 (16)	0 (3)	0 (3)

[z] XG : 0.04%, Agar : 0.1%, PME : 0.1 mg·L^{-1}, PG : 0.1 mg·L^{-1}. All media included 10% sucrose.

[y] Average percentage of fruit set in each cluster of five flowers; number of flower clusters counted: 30.

[x] Different letters within a column indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test.

[w] Fruit number used for analysis is shown in parenthesis.

[v] Not tested.

Table 10. Effects of suspension media and pollination methods on fruit quality in Japanese pear 'Kosui' (2005-2007)[z].

Pollination method	Suspension medium[y]	Fruit weight (g)			Index of distortion of fruit shape[x]			○Brix			pH		
		2005	2006	2007	2005	2006	2007	2005	2006	2007	2005	2006	2007
Spray	XG	429.8 ab[w]	464.6 a	385.1 a	1.05 a	1.04 a	1.03 a	11.5 a	11.1 a	11.9 a	5.2 a	5.1 a	5.0 a
Spray	XG+PME	447.1 a	—	390.9 a	1.05 a	—	1.03 a	11.4 a	—	12.0 a	5.3 a	—	5.0 a
Spray	XG+PG	—[v]	393.7 ab	419.0 a	—	1.04 a	1.03 a	—	11.1 a	11.8 a	—	5.1 a	5.0 a
Spray	Agar	—	388.9 ab	400.0 a	—	1.04 a	1.04 a	—	11.5 a	11.8 a	—	5.1 a	5.0 a
Spray	Agar+PME	333.3 b	—	371.3 a	1.05 a	—	1.04 a	11.3 a	—	11.8 a	5.2 a	—	4.9 a
Spray	Agar+PG	—	396.5 ab	362.6 a	—	1.05 a	1.03 a	—	11.2 a	11.2 a	—	5.1 a	4.9 a
Hand		382.8 ab	332.3 b	374.8 a	1.06 a	1.04 a	1.03 a	11.1 a	11.4 a	11.7 a	5.2 a	5.0 a	5.0 a
No pollination		263.6 c	317.9 b	229.0 b	1.04 a	1.05 a	1.04 a	10.7 a	11.7 a	11.6 a	5.3 a	4.9 a	5.1 a

[z] Number of fruit number used for analysis in each treatment (n = 3-25) is indicated in Table 9.

[y] XG : 0.04%, Agar : 0.1%, PME : 0.1 mg・L^{-1}, PG : 0.1 mg・L^{-1}. All media included 10% sucrose.

[x] The ratio of maximum and minimum diameter of the equatorial plane of the fruit.

[w] Different letters within a column indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test.

[v] Not tested.

Table 11. Effect of difference in pollination method on pollination time and amount of pollen used.

Pollination method	Pollination time (h・10a^{-1})	Amount of pollen use (g・10a^{-1})
Spray[z]	2.7	8.1
Hand[y]	6.2	25.2
Significance[x]	**	**

[z]XG : 0.04% (w/v), Sucrose : 10% (w/v), Pollen : 0.3% (w/v).

[y]Pollen was diluted to 1/5 (w/w) with Lycopodium spores and the pollinations were carried out using a conventional feathered stick.

[x]** indicate significant differences at $P < 0.01$ as determined by a *t*-Test.

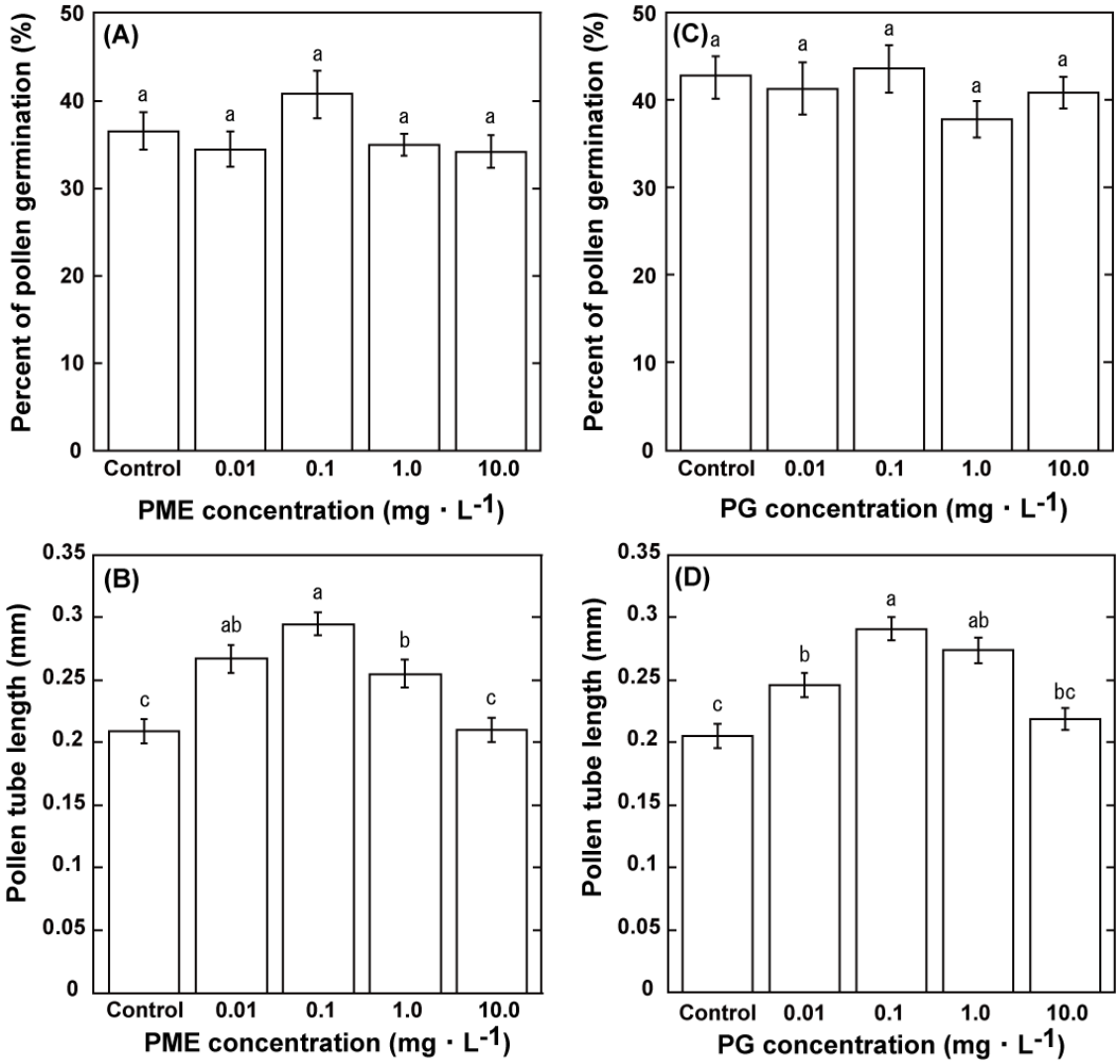

Fig. 18. Effects of PME (A, B) and PG (C, D) on pollen germination rate (A, C) and pollen tube length (B, D). Pollen grains were suspended in a sucrose-agar medium containing either PME or PG at the concentrations indicated, and incubated for 2 h at 20 ºC, then transferred to agar plates. After incubation for 2 h at 25 ºC, germination rates and pollen tube lengths were investigated. Control pollen was treated similarly, but the suspension medium contained no PME or PG. Different letters above bars within a graph indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test. Vertical bars indicate SE (n = 3)

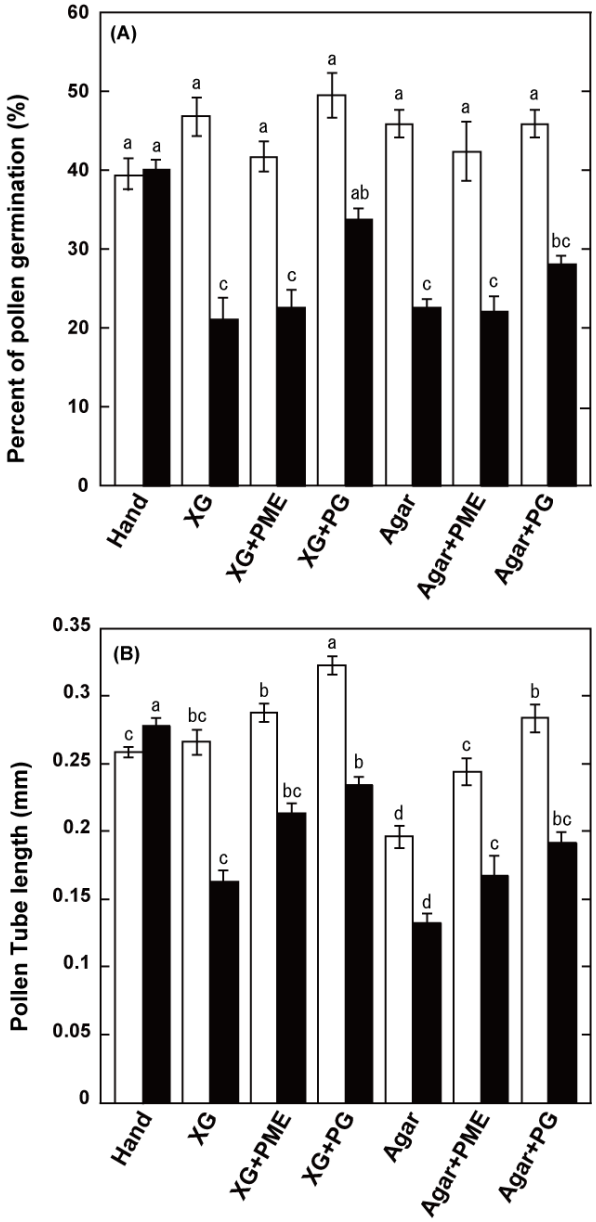

Fig. 19. Effects of the suspension media on pollen germination rates (A) and pollen tube length (B) at the beginning (white bars) and end (black bars) of pollination experiments performed in 2007. Different letters above bars within a graph indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test. Vertical bars indicate SE (n = 3). Hand, hand pollination; XG, 0.04% XG; XG + PME, 0.04% XG + 0.1 mg·L^{-1} PME; XG + PG, 0.04% XG + 0.1 mg·L^{-1} PG; Agar, 0.1% agar; Agar + PME, 0.1% agar + 0.1 mg·L^{-1} PME; Agar +PG, 0.1% agar + 0.1 mg·L^{-1} PG.

Section 2 Applicability of using spray pollination for ‘Hosui’

Introduction

As described in section 1, in the case of Japanese pear, a practical spray pollination technique for ‘Kosui’, the most popular cultivar in Japan, was successfully established and has been useful for reducing both material and labor costs. However, fruit set using spray pollination varied among the cultivars. Sakamoto et al. (2014) have been reported that the level of fruit set in ‘Akizuki’ and ‘Shuurei’ after spray pollination was almost the same as ‘Kosui’ (about 67 to 97% of hand pollinated fruit). But, ‘Hosui’ and ‘Niitaka’ yielded poor fruit set (less than 50% of hand pollinated fruit) with the same medium as used for ‘Kosui’ (Hiura, personal commun.). Therefore, a practical cultivar(s)-specific spray pollination technique needed to be developed.

The high fruit set of ‘Kosui’, ‘Akizuki’ and ‘Shuurei’ obtained by spray pollination may be due to the partial parthenocarpic properties of these cultivars (Sakamoto et al., 2014). Treatments with gibberellin and/or CPPU induce parthenocarpic properties in several fruit (Sotomayor et al., 2012; Watanabe et al., 2008), but gibberellin also inhibits pollen germination in grape (Ohara et al., 2005). Also, CPPU application before and during the flowering period induced partial parthenocarpic properties and resulted in higher levels of fruit set compared with no pollination in pear, apple, and kiwifruit (Ainalidou et al., 2015; Niu et al., 2015; Zhang et al., 2008).

Based on these factors, we investigated the effects of CPPU on pollen grain germination

and its influences on fruit set and fruit quality to establish a spray pollination protocol in which more than 70% of hand-pollinated flowers would set fruit in ‘Hosui’, the second most popular Japanese pear cultivar in Japan.

Materials and Methods

Effects of CPPU on pollen grain viability

The pollen grains used for all experiments in this study were prepared as follows. Purified pollen grains of ‘Xue Hua Li’ stored a few years in a deep freezer (-30 ºC) were used. Before use, purified pollen grains were acclimatized for 2 h at 15 ºC, 100% humidity. Before performing spray pollination experiments in the field, we carried out pollen germination tests to assess the effects of CPPU on pollen grain viability. The acclimatized pollen grains were suspended at 0.3% (w/v) in media containing 10% (w/v) sucrose and 0.1% (w/v) agar supplemented with 0, 2, or 10 mg·L^{-1} of CPPU (Kyowa Hakko Bio Co. Ltd., Tokyo, Japan). These suspension media were kept at 20 ºC for 0.5, 1, and 2 h. Aliquots (0.2 mL) of each suspension medium were subsequently spread on the surfaces of agar plates [1% (w/v) agar and 10% (w/v) sucrose] and the number of germinated pollen were counted after 2 h at 25 ºC as described by Yano et al. (2007). As a control, pollen grains (not in medium) kept at 20^{o}C for 0 and 2 h were also used in the pollen germination test as a control. Three plates were used for each treatment. The germination rates were assessed using a microscope (Digital Fine Scope VC3500, OMRON Corp.). The germination rate was determined from about 100 pollen grains in each of three areas per plate.

Spray pollination experiments in the field

Field pollination experiments were performed in 2010, 2012, and 2013 at the NIFTS using four (2010) and three (2012 and 2013) mature trees of Japanese pear 'Hosui'. Pollen grains were acclimated and then suspended in media at a concentration of 0.3 % (w/v). Details of the media used for the spray pollination experiments are shown in the next section. Spray pollination was carried out using a hand sprayer provided by Furupra Co. Ltd. (Tokyo, Japan). As a positive control, pollen grains diluted to 1/5 (w/w) with *lycopodium* spores were used for hand pollination using a conventional feathered stick. A total of 60 flower clusters were used for each treatment. Fifteen individual flower clusters were selected for each treatment per tree (four trees) in 2010, and 20 individual flower clusters for each treatment per tree (three trees) were selected in 2012 and 2013. Similar treatments were applied to flower clusters on the same tree but not to flower clusters on the same bearing shoot. Since each flower cluster contained 8 to 10 flowers, we reduced the number of flowers per cluster to 5 by removing extra flowers at the balloon stage. Then, each flower cluster was covered with a paper bag to avoid natural pollination. Pollinations were carried out at the time of flowering, and the treated flowers were again covered with bags for about 3 weeks. Air temperatures during the pollination treatments were 17.9 ºC in 2010, 16.9 ºC in 2012, and 17.4 ºC in 2013, and the average air temperatures 1 week after pollination were 10.0 ºC in 2010, 12.6 ºC in 2012, and 12.2 ºC in 2013, respectively. We also included a set of flower clusters that were not pollinated to be used as negative controls for determining fruit and seed set, and fruit quality.

Suspension media for spray pollination

All media contained 10% (w/v) sucrose to prevent the pollen grains from bursting due to

osmotic shock. Agar at 0.1% (w/v) was used to improve the dispersion of pollen and the viscosity of the media according to the method of section 1. As shown in Table 12, three kinds of media supplemented with 0, 2, and 10 mg·L^{-1} of CPPU were used in the 2010 experiment. In 2012 and 2013, two kinds of media supplemented with 0 and 2 mg·L^{-1} of CPPU were used in the experiments. In 2013, a medium supplemented with 2 mg·L^{-1} of CPPU and containing no pollen grains was used for the experiment in addition to the above described set of conditions. In parallel with the pollination experiments in the field, the germination rates of pollen in all media were checked at the beginning and the end of each pollination procedure (pollination experiments took about 3 h) by the pollen germination test, as described above. The germination rates of the pollen used for hand pollination were also checked.

Evaluating the effects of the suspension media on fruit set and fruit quality

The percentages of fruit set were determined about 21 d after flowering. After measuring fruit set, fruit was thinned to one fruit per flower cluster, and the fruit load per branch was adjusted with fruit that were not used for the study according to the usual commercial practices in Japan. Fruit from all treatments were harvested during the normal commercial harvest period (from the late-Aug. to the mid-Sept.). The quality of all harvested fruit for each treatment was assessed as follows. Fruit weight, the index of irregular fruit shape (calculated using the ratio of maximum width to height of the fruit), and the number of seeds per fruit were determined. Total soluble solids (^{o}Brix) and the pH of juice extracted from the flesh at opposite sides of the fruit were determined using a refractometer (PR-101α, Atago Co. Ltd.) and a pH meter (twin pH B-212, Horiba Ltd., Kyoto, Japan), respectively. In the CPPU treatment without pollen, only 8 fruit were harvested. For other

treatments, 35-57 fruit per treatment were harvested.

The data were analyzed by a one-way ANOVA for each year and tested for significant differences among treatments by the Tukey-Kramer Test.

Results and Discussion

Effect of CPPU on pollen grain germination

Before performing spray pollination experiments in the field, we carried out pollen germination tests to assess the effects of CPPU on pollen grain viability. The pollen germination rate of pollen grains (no medium) kept at 20 ºC for 2 h did not change with time (Fig. 20). When pollen grains were suspended in a medium without CPPU (0 mg·L $^{-1}$), the germination rates tended to decrease with time and, after 2 h, the rate was significantly lower than that of pollen grains not suspended in medium (Fig. 20). When pollen grains were suspended in medium with 2 mg·L $^{-1}$ CPPU, the pollen germination rates after 0.5, 1, and 2 h were significantly higher than for those in medium without CPPU (Fig. 20). Also, when pollen grains were suspended in a medium with 10 mg·L $^{-1}$ CPPU, the germination rates after 1 and 2 h were significant higher than for those in a medium without CPPU. The germination rates of pollen grains incubated for 2 h in both CPPU-containing media were almost the same as those for pollen grains not suspended in medium (Fig. 20).

According to several past reports, grape and almond pollen germination in the presence of a synthetic cytokinin was significantly lower than that of the control (Sotomayor et al.,

2012; Wang et al., 2013). In this experiment, CPPU contributed to the improved viability of Japanese pear pollen in suspension media. Possibly, the effect of this synthetic cytokinin on pollen grain germination may vary depending on the dose or timing of the treatment. These parameters remain to be elucidated in the near future.

Effects of suspension media on pollen grain viability in the field

The pollen germination rates at the beginning of each pollination procedure in the field revealed no significant differences among the treatments in 2012 and 2013 (Fig. 21). At the end of each pollination procedure (pollination experiments took about 3 h), the pollen germination rates were lower than those after hand pollination, irrespective of the type of suspension media used. However, the pollen in the medium with 2 $mg \cdot L^{-1}$ CPPU showed a significantly higher germination rate than that in the medium without CPPU at the end of the pollination procedure (Fig. 21). Therefore, CPPU may contribute to improved pollen grain viability in suspension media for practical use.

Effects of spray pollination on fruit set and fruit quality

The average percentage of fruit set per flower cluster containing five flowers for each treatment is shown in Table 12. For all treatments and years, the data shown are the averages for 60 flower clusters.

The percentages of fruit set after spray pollination without CPPU were lower than those after hand pollination in all 3 years and ranged from 48% to 72% of the hand pollinated rate. Temperatures during and after pollination influenced the variation in the percentage of fruit set by year. In particular, fewer pollen grains adhered to the stigmata with spray

pollination compared to hand pollination in grape (Kimura et al., 1998). We assumed that spray pollination was easily affected by temperature; however, media containing CPPU gave significantly higher percentages of fruit set than media without CPPU, ranging from 71% to 106% of the hand-pollinated rate (Table 12). These results suggested that the addition of CPPU stabilized fruit set for spray pollination. In 2010, the medium with 10 mg·L $^{-1}$ CPPU gave the highest levels of fruit set that was almost the same as that achieved by hand pollination. Nevertheless, fruit resulting from pollination with media containing 10 mg·L $^{-1}$ CPPU had abnormally thick and perpetually enlarged calyxes 21 d after pollination with a frequency of approximately 100% (Fig. 22D), even though the calyx usually falls off 'Hosui' fruit by the mid-May (Fig. 22A, B). During early stages of fruit growth, CPPU or high daytime temperatures will promote cell division that can induce the calyx-perpetual trait in fruit (Watanabe et al., 2003; Zhang et al., 2008). Additionally, media containing 10 mg·L $^{-1}$ CPPU tended to increase the degree of fruit deformation (Fig. 22D). On the other hand, fruit resulting from pollination with media containing 2 mg·L^{-1} CPPU also had a high frequency of the calyx-perpetual trait, but the calyxes were normal and the fruit shape tended to be less deformed unlike the fruit pollinated with 10 mg·L^{-1} CPPU (Fig. 22C). In this study, we did not recognize differences in fruit shape at harvest between treatments with 2 mg·L^{-1} and 10 mg·L $^{-1}$ CPPU; however, Niu et al. (2015) reported that a 20 mg·L^{-1} CPPU treatment resulted in abnormally shaped, large fruit with extraordinarily expanded calyx tubes. Thus, we conducted experiments focusing on media containing 2 mg·L^{-1} CPPU in 2012 and 2013. Media containing 2 mg·L^{-1} CPPU gave significantly higher levels of fruit set than media without CPPU in all years, and fruit set levels were similar to that for hand pollination in

2013.

Fruit quality measurements after spray pollination using media with/without CPPU in comparison with hand pollination are shown in Table 13. Calyx-perpetual fruit resulting from CPPU treatment significantly increased compared with the control. In addition, harvest time (average date between the first and last dates of the harvest) was delayed 3 - 7 d in fruit resulting from CPPU (2 mg·L^{-1}) treatment (Table 13). Notably, there were no significant differences in fruit weight among any of the pollination treatments. Furthermore, there were no significant differences among any of the treatments for the fruit shape index (except in 2012), the total soluble solids (^{o}Brix) or the pH in all 3 years. From these results, we concluded that a pollen suspension medium containing 2 mg·L^{-1} of CPPU was suitable for use in spray pollinating Japanese pear 'Hosui'.

Application of 20 to 100 mg·L^{-1} CPPU before and during the flowering period induced partial parthenocarpic properties in pear (Niu et al., 2015; Zhang et al., 2008). Accordingly, we also investigated treatments in which the flower clusters were not artificially pollinated or the flower clusters were treated with media supplemented with 2 mg·L^{-1} of CPPU only (no pollen). When the flower clusters were treated with 2 mg·L^{-1} CPPU only (no pollen) in 2013, the average fruit set was 29%. On the other hand, when the flower clusters were not artificially pollinated, fruit set was not observed in any of the years. From these results, we assumed that the flowers treated with CPPU could set fruit without seeds due to the induction of parthenocarpy by CPPU. Thus, we hypothesized that the level of fruit set after spray pollinating cultivars that have weak parthenocarpic properties, like 'Hosui', was improved by supplementing the pollination media with

CPPU. Usually, a high pollen germination rate is important to achieve high levels of fruit set. When pollen grains were suspended in media with CPPU, the germination rate of the pollen grains was maintained at a higher level than that without CPPU. However, the number of seeds per fruit obtained after spray pollination with CPPU was lower than that resulting from hand pollination for 2 years but not in 2013. In 2010, the number of seeds per fruit obtained after spray pollination with 10 $mg \cdot L^{-1}$ CPPU was the fewest, but the germination rate of the pollen grains after 2 h was the same as that using pollen grains. Our results suggest that the higher levels of fruit set by CPPU might not be due to the high germination rate of the pollen grains but due to the induction of partial parthenocarpic properties.

The numbers and sizes of seeds affect fruit enlargement and play important roles in nutrient competition among fruit in many species (Grant and Ryugo, 1984; Nitsch, 1950). However, in this study, there were no obvious differences in fruit weight, shape or quality among the treatments in all 3 years (Table 13). Thus, year-to-year differences, possibly due to different weather conditions, greatly affected the number of seeds produced.

In summary, we propose that a pollen suspension medium containing 2 $mg \cdot L^{-1}$ of CPPU is a suitable and practical method to use for the spray pollination of Japanese pear ‘Hosui’ and will result in fruit sets of greater than 70% of that achieved by hand pollination.

Table 12. Effects of suspension media and pollination methods on the rate of fruit set and number of seeds in Japanese pear ‘Hosui’ (2010, 2012 and 2013).

Pollination method	CPPU concentration	Fruit set (%)[z]			Number of seeds per fruit[y]		
	(mg・L^{-1})	2010	2012	2013	2010	2012	2013
Spray	0	36 c[x](48)[w]	49 c (57)	62 b (72)	3.3 b (38)[v]	4.9 b (36)	3.3 a (35)
Spray	2	58 b (73)	61 b (71)	81 a (94)	2.3 b (54)	4.8 b (37)	2.5 a (38)
Spray	10	82 a (106)	—[u]	—	1.8 c (57)	—	—
Hand		77 a (100)	86 a (100)	86 a (100)	4.6 a (55)	5.8 a (33)	2.9 a (43)
Spray (no pollen)	2	—	—	29 c (34)	—	—	0 (8)
No pollination		0	0	0			

[z]Average percentage of fruit set in each cluster of five flowers; number of flower clusters counted: 60.

[y]The number of seeds per fruit were determined at harvest time.

[x]Different letters within a column indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test.

[w]Fruit set of hand-pollinated flowers was set to 100: the other pollination methods are expressed as fold ratios compared with hand pollination and are shown in parentheses.

[v]Fruit number used for analysis is shown in parentheses.

[u]Not tested.

Table 13. Effects of suspension media and pollination methods on fruit quality in Japanese pear ‘Hosui’ (2010, 2012 and 2013).

Pollination	CPPU concentration	Harvest date[z]			Fruit weight (g)			Fruit shape index[y]			°Brix			pH			Calyx-perpetual fruit (%)		
method	(mg・L^{-1})	2010	2012	2013	2010	2012	2013	2010	2012	2013	2010	2012	2013	2010	2012	2013	2010	2012	2013
Spray	0	9 Sep.	10 Sep.	30 Aug.	435.5 a[x]	464.6 a	523.6 a	1.16 a	1.17 a	1.17 a	14.1 ab	12.2 a	12.4 a	4.9 a	4.8 a	4.8 a	10.1 a	5.6 a	8.6 a
Spray	2	16 Sep.	13 Sep.	2 Sep.	436.0 a	501.8 a	525.5 a	1.15 a	1.12 b	1.14 a	14.1 ab	12.3 a	12.2 a	4.9 a	4.8 a	4.8 a	60.1 b	75.6 b	89.5 b
Spray	10	14 Sep.	—[w]	—	483.9 a	—	—	1.16 a	—		13.8 b	—	—	4.9 a	—	—	97.0 c	—	—
Hand		9 Sep.	10 Sep.	30 Aug.	427.3 a	478.7 a	537.1 a	1.17 a	1.16 a	1.17 a	14.4 a	12.1 a	12.4 a	4.8 b	4.8 a	4.8 a	7.2 a	0.0 a	9.3 a
Spray (no pollen)	2	—	—	2 Sep.	—	—	335.7 b	—	—	1.21 a	—	—	12.1 b	—	—	4.8 a	—	—	87.5 b
No pollination																			

[z]Average date between the first and last dates of the harvest.

[y]The ratio of maximum width to height of the fruit

[x]Different letters within a column indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test.

[w]Not tested.

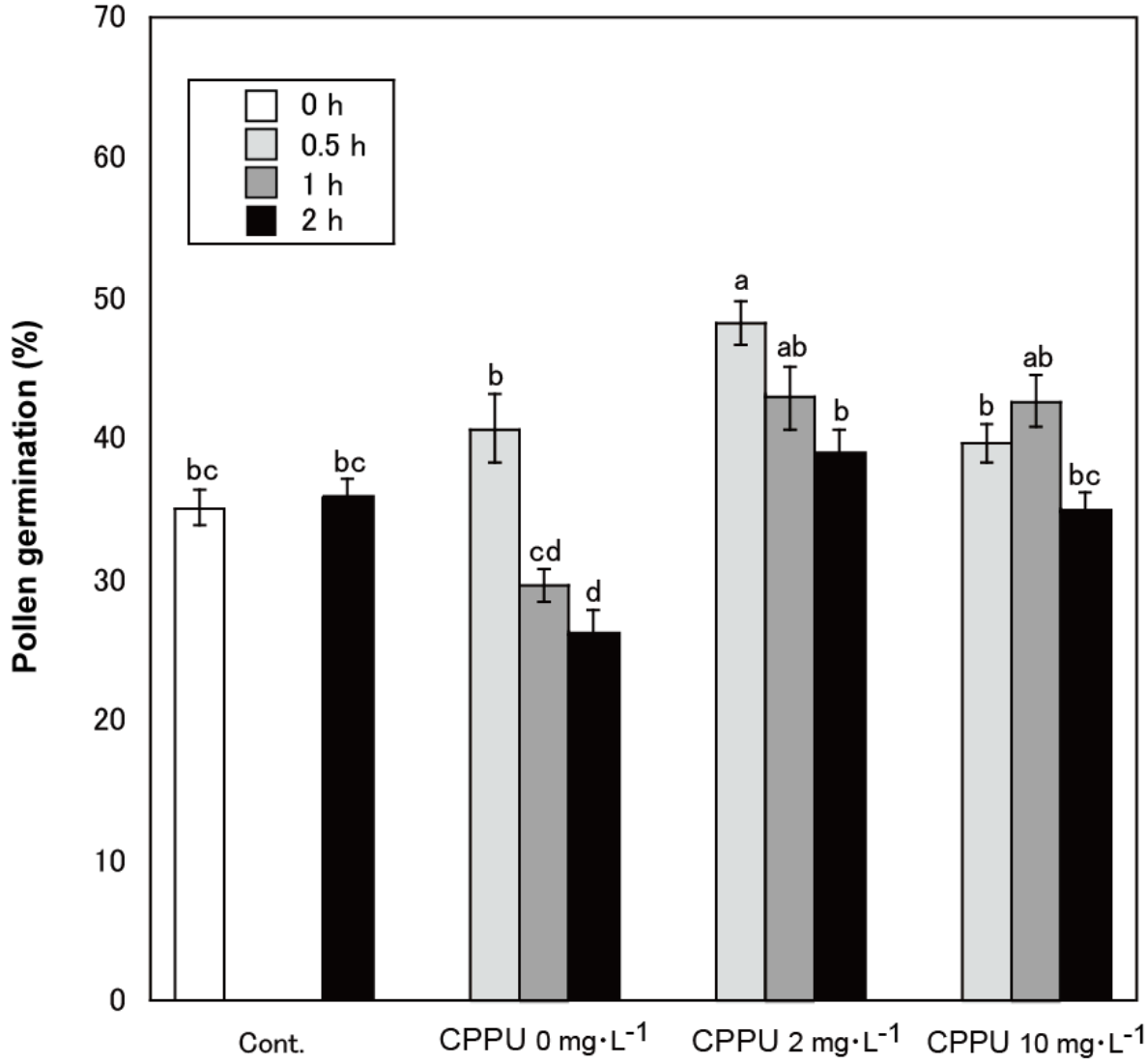

Fig. 20. Effects of CPPU addition on pollen germination rate. Different letters above the bars within a graph indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test. Vertical bars indicate SE (n = 3). Cont., pollen grains acclimatized only; CPPU 0 mg·L^{-1}, medium without CPPU; CPPU 2 mg·L^{-1}, medium with CPPU 2 mg·L^{-1}; CPPU 10 mg·L^{-1}, medium with CPPU 10 mg·L^{-1}.

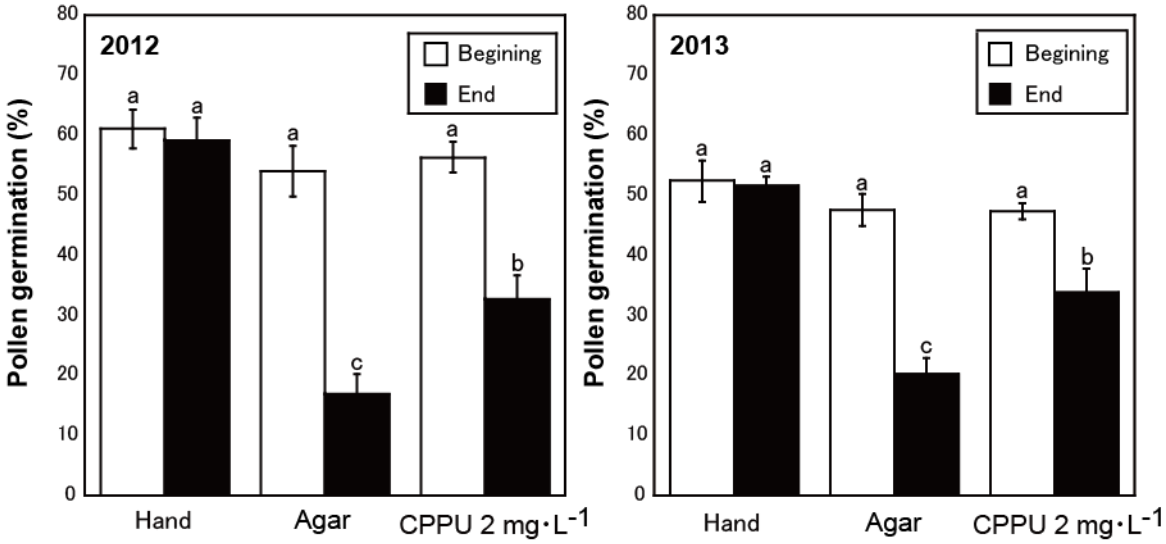

Fig. 21. Effects of CPPU concentration on pollen germination rates of in 2012 and 2013 at the beginning (white bars) and end (black bars) of pollination experiments (approximately 3 h). Different letters above the bars within a graph indicate significant differences at $P < 0.05$ by the Tukey-Kramer Test. Vertical bars indicate SE (n = 3). Hand, hand pollination; Agar, 0.1% agar; CPPU 2 mg·L^{-1}, 0.1% agar + CPPU 2 mg·L^{-1}.

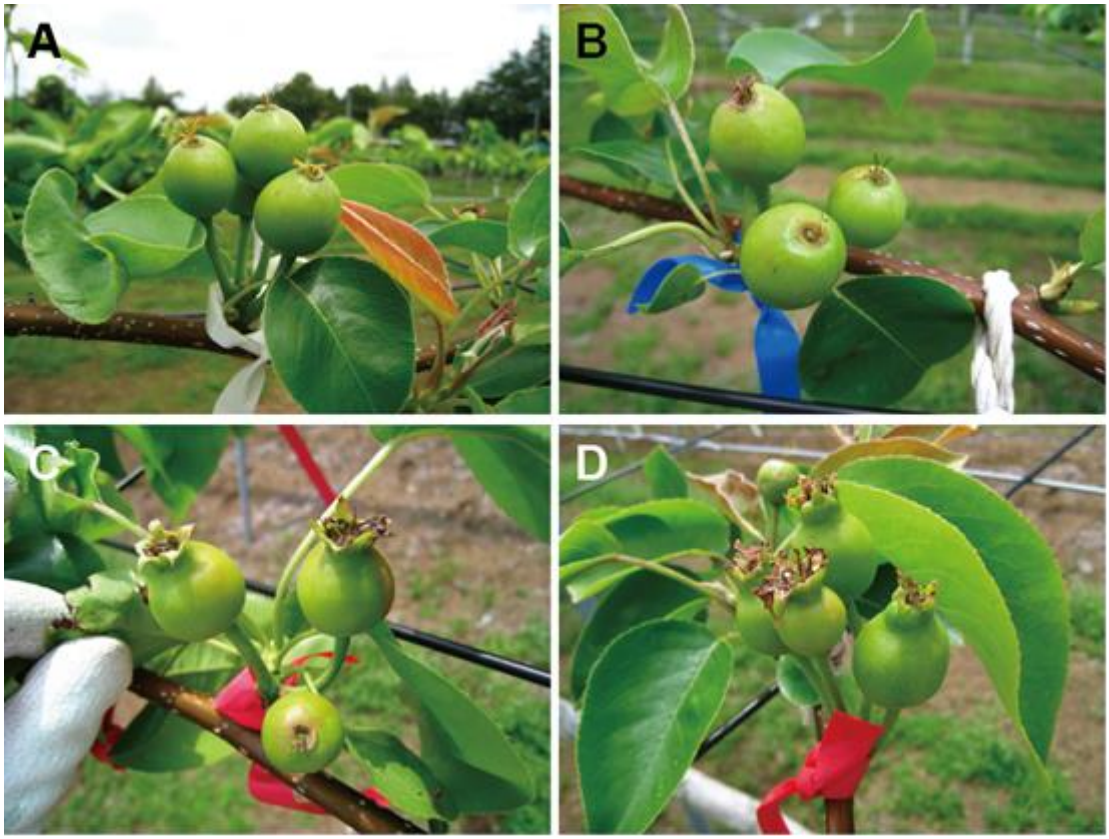

Fig. 22. Effects of CPPU concentration on the formation of calyx-perpetual fruit in Japanese pear ‘Hosui’. Hand pollinated (A); CPPU 0 mg·L^{-1} (B); CPPU 2 mg·L^{-1} (C); CPPU 10 mg·L^{-1} (D). All fruits were photographed at 26 d after full bloom.

Chapter 5

General discussion

The flat trellis training system, a traditional training method in our country, is superior to other methods for several reasons, most notably in its support of tree branches or fruits against frequent strong winds (e.g., typhoons). By using this training system, less damage occurs on plants and fruits compared with other training systems such as the central leader system or the modified open-center system. On the other hand, the productivity of 'Kosui', a cultivar that was originally planted in the 1970s and occupied 39.9% of the total growing area for Japanese pear in 2013 (MAFF, 2016), has started to decline; therefore, replanting other cultivars is highly desired. If the flat trellis training system is used for replanting, it will take a long time (about 10 years) for the orchard to mature to commercial use. Several studies have reported the development of alternative training and culturing systems to attain high-yielding fruit trees in a short period (Kagami et al., 2002; Matsuura, 2002; Oshida, 2002). One of these systems, the tree joint system, grafts the apical tips of each tree into the bent part of the main stem of the adjacent tree under a flat trellis. Use of the tree joint system is expected to produce high-yielding Japanese pears within 7 years. In addition, there are several other merits to the new system, such as uniform fruit distribution and superior fruit quality (Shibata et al., 2008). Through this superior management system, we can reduce risks caused by both natural and man-made disasters and hope to retain the sustainable production of Japanese pear. However, two new problems have arisen that may influence the sustainable cultivation of Japanese pear in our country. The first is the problem of global warming. Fruit trees are affected by temperature through the year; thus, unlike an annual crop, there are no countermeasures such as making a change in sowing time. As a result, fruit trees face very serious damage due to global warming.

Sugiura et al. (2012) reported that all 47 prefectures (100%) reported warming effects on fruit tree cultivation. This level of influence is quite high compared with rice (77%), cereals and soybeans (43%), vegetables and flowers (94%), and forage crops (32%). For Japanese pear, a few prefectures reported delays in endodormancy breaking or budding in forced culture. In fact, delayed and poor bud breaking has been observed in Japanese pears grown in the southwestern region of Japan during an unusually warm winter season (Fujikawa et al., 2012). Lack of chilling accumulation results in bud abortion during the following spring season and has a negative impact on subsequent tree growth. In addition, high temperatures in the summer inhibit flower bud initiation and consequently induce unstable fruit production (Tromp, 1976). In 2011, high temperatures during the previous summer were attributed as a factor in the poor flowering of apple, which like pear also is a member of the Rosaceae family (Tamai, personal commun.). Second, another problem threatening sustainable pear production is the decrease in the workforce. Most Japanese pear cultivars are self-incompatible, and pollinizers must be inter-planted in commercial orchards (Sawamura et al., 2013). Also, approximately one-fourth of the total annual labor time (about 320 h/10a) in Japanese pear production is spent on artificial pollination and fruit thinning (Fig. 4). Thus, reducing the amount of labor required for pollination of Japanese pear has become an urgent need. The work reported in this study was designed to provide solutions to these important problems affecting Japanese pear production.

In *Chapter 2*, we investigated the effect of KODA on endodormancy breaking in flower buds of Japanese pear in order to identify an endodormancy breaking agent with less toxicity for plants and humans. The optimal concentration of KODA (100 μM) for endodormancy breaking was established over a 2-year period during the endodormancy stage of 2006 and 2007 in three cultivars, ‘Kosui’, ‘Natsushizuku’, and ‘Hosui’. The effect of KODA on endodormancy breaking in flower buds was similar between ‘Natsushizuku’ and ‘Kosui’ but somewhat lower

in ‘Hosui’. These results indicate that KODA can be an effective agent for promoting endodormancy breaking of flower buds, at least in ‘Natsushizuku’ and ‘Kosui’. KODA was generally inferior to HC for both the timing and the extent of bud break; however, the differences between HC and KODA became minimal when they are applied toward the normal time for endodormancy breaking. KODA can be used to break endodormancy of Japanese pear flower buds at the late endodormancy stage without any symptoms of toxicity. On the other hand, a relationship was identified between the incidence of dead flower buds and application of livestock waste compost as a source of N supplementation in Japanese pear ‘Kosui’. Compost application in the fall-winter season significantly reduced the freezing tolerance of flower buds concomitant with a significant increase in the percentage of dead flower buds compared to compost application in the spring. Application of compost in the fall-winter season resulted in a significantly higher N content compared to that in the spring. These results suggest that compost application during the fall-winter season can adversely affect freezing tolerance through an increase in the N content, thus promoting dead flower buds. Collectively, these results led to the proposal that the timing of fertilization should shift from fall and winter to the spring.

In *Chapter 3*, we investigated the effects of KODA on the formation of lateral primordia in apical flower buds on their spurs. The maintenance of spurs year-to-year is easy in the Japanese pear cultivars ‘Shinsei’ and ‘Kosui’. Application of KODA (10 μM) in early-July increased the number of lateral primordia in the apical flower buds of both ‘Shinsei’ and ‘Kosui’, whereas the effects of KODA on the number of lateral inflorescences were not stable in either cultivar. Nevertheless, KODA tended to enhance bud differentiation and development in both cultivars, compared to the control, i.e., toward an increase in vegetative primordia and/or floral lateral inflorescences. These results suggest that application of KODA in July increased the number of

lateral primordia in flower buds on Japanese pear spurs; therefore, these results are potentially useful for decreasing the number of blind buds and promoting stable fruit production especially in ‘Kosui’.

In *Chapter 4*, we investigated the applicability of spray pollination in two Japanese pear cultivars, ‘Kosui’ and ‘Hosui’, to replace hand pollination. As for ‘Kosui’, the level of fruit produced after spray pollination using media containing 0.3% (w/v) pollen grains, 0.1% (w/v) agar and 10% (w/v) sucrose was almost the same as the level after hand pollination. In addition, the media containing agar or XG combined with either PME or PG had slightly better results for pollen grain viability and fruit set. However, fruit set using spray pollination varied between ‘Kosui’ and ‘Hosui’. In the case of ‘Hosui’, which yielded poor fruit set when spray pollinated with the same medium as used for ‘Kosui’, addition of CPPU to the spray pollination media increased fruit set. We hypothesized that the higher levels of fruit set by CPPU might be due to the induction of partial parthenocarpic properties in ‘Kosui’. The time required for spray pollination was less than one-half of that for hand pollination, and the amount of pollen grains required for spray pollination was less than one-third of that needed for hand pollination.

Application of KODA is associated with endodormancy breaking and flower bud formation. It remains to be demonstrated how KODA breaks endodormancy in Japanese pear. KODA is synthesized from linolenic acid by a 9-specific lipoxygenase, as opposed to the JA pathway (Fig. 5). Therefore, we proposed a mechanism underlying the potent ability for endodormancy breaking by KODA in terms of lipid metabolism. A major change in the phospholipid content was a large decrease in linoleic acid (C18:2); for example, a decrease in linoleic acid and an increase in linolenic acid in ‘Delicious’ apple buds is observed when the chilling requirement

is satisfied (Wang and Faust, 1990). Gemma et al. (1993) also reported that lipid metabolism in the buds of Japanese pear 'Kosui' was greatly accelerated during dormancy, resulting in an increase in phospholipids, especially linolenic acid. According to our preliminary experiment, linolenic acid tends to promote endodormancy breaking, whereas linoleic acid does not (Appendix 2). Also, Taniguchi et al. (2003) reported that the breaking of dormancy in apical and lateral buds in horse chestnut seedlings was promoted by treatment with JA. Considering these results, accumulation of either linolenic acid or its oxylipin metabolites, including KODA and JA, may be involved in endodormancy breaking. In addition, parametric analysis of gene-set enrichment suggested that genes related to JA and oxylipin biosynthesis and metabolic processes were significantly up-regulated by prolonged chilling in Japanese apricot (Habu et al., 2014). In contrast, Bai et al. (2013) suggested that a decrease in jasmonate levels may occur toward endodormancy release, on the basis of transcription analysis of Japanese pear flower buds transitioning through endodormancy. Further studies will be necessary to elucidate the detailed physiological functions of linolenic acid, its metabolites, or related compounds, like JA, for endodormancy breaking in Japanese pear.

In Japanese pear, flower initiation does not always correspond exactly with flower bud formation. When each bud develops to the floret stage before winter dormancy, the bud will flower in the spring, but when the bud does not reach the floret stage, it does not flower (Banno et al., 1985). Banno (1982) suggested that the length of the period from flower bud differentiation to dormancy induction controlled the fate of flowers. Possibly global warming has an impact on the succession of CSS elongation or the delay of dormancy induction. Consequently, global warming has both positive and negative effects for flower bud differentiation. Applications of 10 or 100 μM KODA during paradormancy promotes bud breaking in strawberry flower buds (Yokoyama, personal commun.). In this study, KODA

promoted the breaking of endodormancy of Japanese pear flower buds. Thus, these studies suggest that KODA promotes bud differentiation and development by regulating the dormancy stage (i.e., delay of dormancy induction, promotion of dormancy breaking). Based on this knowledge, better solutions for these problems may be found in the future by clarifying the relationship between lipid metabolism and paradormancy or endodormancy.

Regarding flower bud formation after KODA application, the expression of apple *TERMINAL FLOWER 1* (*MdTFL1*) just before the flower bud differentiation period was lower after KODA treatment than the untreated control (Kittikorn et al., 2011). Several studies have suggested functional roles for the flowering-related genes such as *FLOWERING LOCUS T* (*FT*) and *TFL1*. The expression of *TFL1* decreases prior to flower initiation; in contrast, expression of *FT* is induced by flower induction in apple (Mimida et al., 2009; Kotoda et al., 2010), grape (Carmona et al., 2007), and *Citrus* (Pillitteri et al., 2004; Endo et al., 2005). Down-regulation of *TFL1* prior to flower initiation was recognized without any notable up-regulation of *FT* during flower formation in Japanese pear (Saito, personal commun.). In addition, Kittikorn et al. (2013) suggested that endogenous KODA in the buds of apple trees with high degrees of flower bud formation increased before the initiation of flower bud formation, and the application of CKODA, an analog of KODA, increased the proportion of flower buds concomitant with a decrease in *MdTFL1* expression. Recently, Haberman et al. (2016) suggested that fruit load prevents flowering through reaccumulation of MdTFL1-2 in apple bourse shoot apices. Thus, KODA may be responsible for bud differentiation and development through suppression of *TFL1* expression in Japanese pear.

As for the cause of dead flower buds, we found positive correlations between i) an increase in the number of dead flower buds and low freezing tolerance and ii) low freezing tolerance and

N application in the fall-winter period. Several reports have shown that N fertilization in late fall delayed the development of cold acclimation (Matsumoto et al., 2010; Ouzounis and Lang, 2011). Sakai (1982) suggested that earlier endodormancy completion accelerates water uptake by the roots under warm winter conditions, which results in a rapid reduction in freezing tolerance. Dormancy often inhibits or prevents the de-acclimation process that accompanies resumption of growth (Arora and Rowland, 2011). Our results also indicate the possibility that the progression of endodormancy is related to the process of acquiring freezing hardiness. 'Rinka', a new Japanese pear cultivar, was released in 2014 by NIFTS (Saito et al., 2014) (Appendix 3) and originated from a cross between 269-21 ('Hosui' × 'Osa Nijisseiki') and 'Akiakari'. One of the interesting features of this cultivar is that it has fewer dead flower buds in the southern (Kyushu region) regions when the fall and winter are warm. The chilling requirement for 'Rinka' is almost the same as that for 'Kosui' (data not shown). When we investigated the effect of applying chemical N fertilizer during the winter using 'Rinka' potted trees, the percentage of dead flower buds tended to be higher in December compared with the no fertilizer control but was apparently less than that of 'Kosui' (Appendix 4). These results indicate that factors other than the chilling requirement may be involved in the reduced number of 'Rinka' dead flower buds in the fall and winter. Thus, investigation of nitrogen use during the fall-winter season using a ^{15}N tracer technique will shed light on the reason for fewer dead flowers on 'Rinka'. Furthermore, the mobilization and recycling of N during endodormancy release plays an important role in flower bud development in cultivars of peach, nectarine, and plum (González-Rossia et al., 2008). In our previous report about Japanese chestnut, we proposed that supplementation of N affected the degree of endodormancy, possibly due to stimulation of root activity (Sakamoto et al., 2015b). In fact, new fibrous roots appeared on apple and peach trees treated with NO_3 at a low temperature (7.2ºC) (Nightingale, 1935). Notably, nitrate is not only a major nitrogen source but also a signaling molecule that modulates

the expression of a wide range of genes that regulate growth and development (Konishi and Yanagisawa, 2013). However, the relationship between endodormancy progression and acquisition of freezing tolerance through N supplementation is not well understood at present and should be investigated in the near future.

With respect to spray pollination as a less labor-intensive pollination system, media containing agar combined with either PME or PG resulted in slightly better pollen grain viability and fruit set. However, enzymes such as PME or PG are difficult to handle under field conditions. In contrast, the spray pollination technique can be widely applied in combination with CPPU, a registered plant growth regulator for use with Japanese pear, to induce partial parthenocarpic properties. A small number of fruit without intact seeds formed on ‘Akizuki’, indicating that this cultivar possesses parthenocarpic or pseudo-parthenocarpic properties similar to those of ‘Kosui’ (Sakamoto et al., 2014). By contrast, ‘Hosui’ shows no such parthenocarpic property. It was apparent in this study, however, that parthenocarpy in ‘Hosui’ was induced by CPPU; thus, spray pollination using a medium with 2 $mg \cdot L^{-1}$ CPPU is an acceptable method for pollinating ‘Hosui’ Japanese pear. By exploiting this property of CPPU to induce parthenocarpy, it may be possible to use spray pollination with other plants such as apple. When we investigated the applicability of spray pollination using a medium containing 0.3% (w/v) pollen grains, 0.1% (w/v) agar and 10% (w/v) sucrose with two other Japanese pear cultivars, ‘Akizuki’ and ‘Shuurei’, the levels of fruit set in both cultivars were nearly the same as those that were hand pollinated. In addition, even if not pollinated, some fruits with intact seeds were set on ‘Akizuki’ and ‘Shuurei’ (Sakamoto et al., 2014). Hiratsuka and Zhang (2002) proposed that the expression of self-incompatibility is different among Japanese pear cultivars. Although neither cultivar was investigated for their expression of self-

incompatibility, we hypothesize that ‘Akizuki’ and ‘Shuurei’ are weakly self-incompatible, a property that enables these cultivars to partially self-pollinate in the absence of artificial pollination. Ordinarily, the level of fruit set after spray pollination would be inferior to that after hand pollination; however, we hypothesize that cultivars with the ability to partially self-pollinate can set fruit after spray pollination that is nearly at the same level as that after hand pollination. Fruit set using spray pollination varies among different kinds of fruit trees. The use of spray pollination with stone fruits, including peaches, plums, and sweet cherries, yielded poor fruit set in comparison with hand pollination (Hagihara et al., 2014). In kiwifruit, the applicability of spray pollination is recognized for many cultivars (Yano et al., 2007). Among the factors that are different among fruit trees when using spray pollination, the stigmatal area in kiwifruit is 7-10 times larger than those of Japanese pear or peach. Therefore, it is possible that the potent ability for holding pollen grains on the stigmata varies among different kinds of fruit trees (Yano, personal commun.). In the worst case, spray pollination tended to decrease fruit set compared with hand pollination (70% of fruit set achieved by hand pollination); however, this is not a problem at the practical level because such a fruit set frequency does not influence the final harvest (the ultimate percentage of fruit set is 3%). Conversely, decreases in fruit set may be a labor-saving benefit for thinning. On the other hand, it is still necessary to verify the amount of labor and pollen required for spray pollination since our study was conducted using a prototype model. Nevertheless, when spray pollination was carried out in this prototype model using commercially available products, the time needed for spray pollination was less than one-half of the time needed for hand pollination. Furthermore, the amount of pollen grains required for spray pollination of the prototype model was approximately 1.7 times of that needed for hand pollination (Appendix 5). So, development of a sprayer aimed at shortening the working time and reducing pollen consumption is necessary.

Collectively, this study provides effective and scientific strategies that will enable the sustainable production of Japanese pear under the threats of global warming and decreases in the workforce. Specifically, the following outcomes of this work are: 1) development of an agent to break endodormancy and produce stable Japanese pear flower buds using KODA, 2) establishment of appropriate fertilizer application methods to reduce dead flower buds in Japanese pear, and 3) development of a suspension medium suitable for spray pollination in Japanese pear.

This study offers an array of new methods for cultivating Japanese pear that will respond to eminent threats to the sustainable production of this fruit. Based on the factors that we have identified so far, it will be necessary to take additional action to develop self-compatible cultivars with a low chilling requirement that will be suitable for overcoming the increasing problems that are expected in the future.

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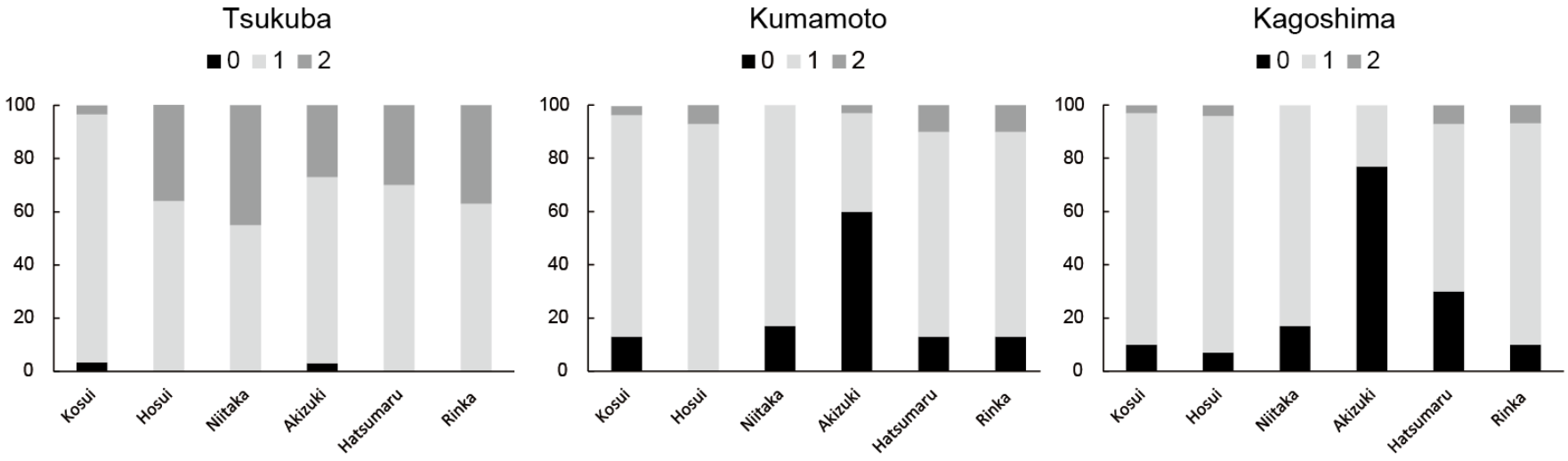

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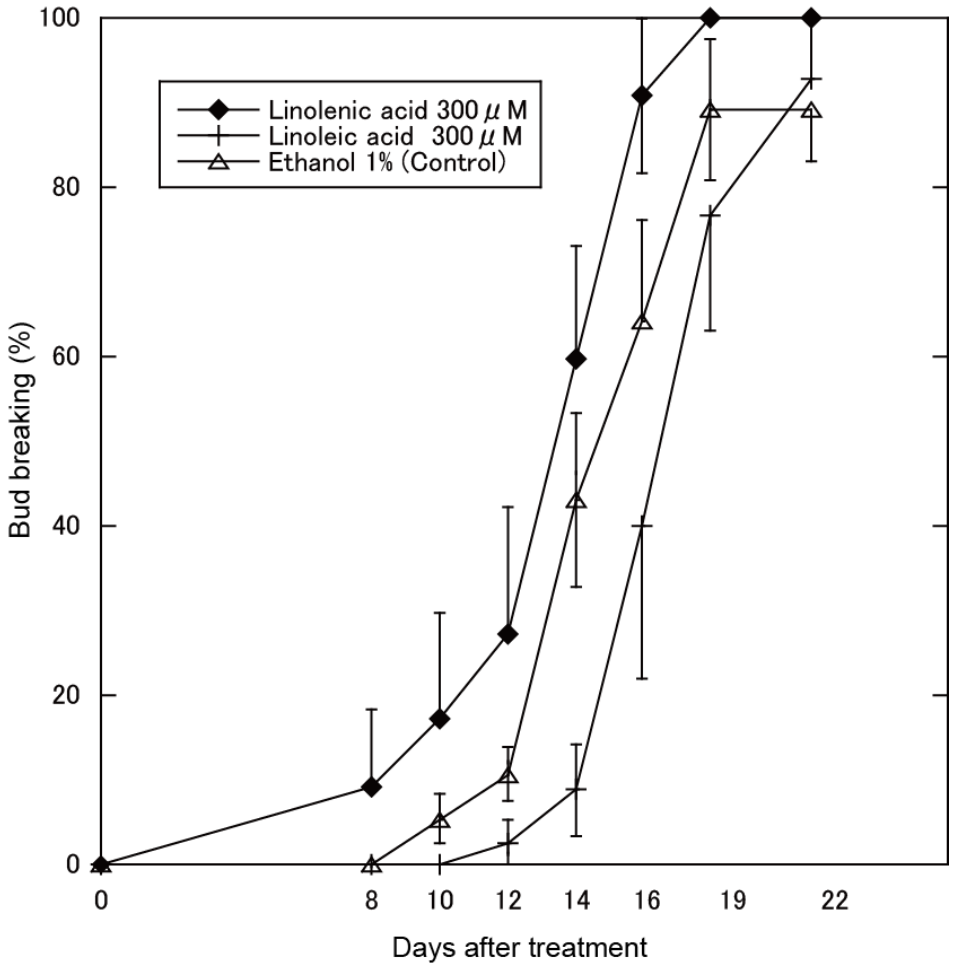
Appendix 1. Geographical effects on the number of lateral primordia in apical flower buds on spurs of Japanese pear cultivars in 2013.



Appendix 2. Effects of linolenic acid (C18:3) (1% (v/v) in ethanol) or linoleic acid (C18:2) (1% (v/v) in ethanol) on endodormancy breaking in flower buds of 'Natsushizuku' Japanese pear (2009). Treatment was on 16 Dec. Plants were transferred on 28 Dec. to a heated greenhouse (25 ± 2 ºC). Vertical bars are the SE (n = 3).

Appendix 3. Fruit of the Japanese pear cultivar ‘Rinka’.

Appendix 4. Effect of N fertilizer application on the occurrence of dead flower buds (%) in the controlled temperature experiment.

Treatment	Dead flower bud (%)[z]
No fertilizer application	1.2
Chemical fertilizer applied in mid-December	40.0
Significance[y]	NS

[z]The status of the buds was investigated at flowering.

[y]NS indicate not significant as determined by a *t* -Test.

Appendix 5. Effect of pollination method on the pollination time and amount of pollen used.

Pollination method	Pollination time (h・10a^{-1})	Amount of pollen use (g・10a^{-1})
Spray[z]	4.1	66.7
Hand[y]	10.6	39.9
Significance[x]	**	*

[z]Agar : 0.04% (w/v), Sucrose : 10% (w/v), Pollen : 0.3% (w/v).

[y]Pollen was diluted to 1/5 (w/w) with Lycopodium spores and the pollinations were carried out using a conventional feathered stick.

[x]* and ** indicate significant differences at $P < 0.05$, and 0.01 as determined by a *t* -Test.