

RESEARCH PAPER

Pollen density on the stigma affects endogenous gibberellin metabolism, seed and fruit set, and fruit quality in *Pyrus pyrifolia*

Caixi Zhang^{1,*}, Naoya Tateishi² and Kenji Tanabe²

¹ Department of Plant Science, School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200240, China

² Laboratory of Horticultural Science, Faculty of Agriculture, Tottori University, Tottori, 680-0855, Japan

* To whom correspondence should be addressed. E-mail: acaizh@sjtu.edu.cn

Received 15 March 2010; Revised 22 June 2010; Accepted 7 July 2010

Abstract

To clarify the relationship between pollen density and gametophytic competition in *Pyrus pyrifolia*, gametophytic performance, gibberellin metabolism, fruit set, and fruit quality were investigated by modifying *P. pyrifolia* pollen grain number and density with *Lycopodium* spores. Higher levels of pollen density improved seed viability, fruit set, and fruit quality. Treatments with the highest pollen density showed a significantly increased fruit growth rate and larger fruit at harvest. High pollen density increased germination rate and gave a faster pollen tube growth, both *in vivo* and *in vitro*. Endogenous gibberellin (GA) concentrations increased in pollen tubes soon after germination and the concentration of two growth-active GAs, GA₃, and GA₄, was positively correlated to final fruit size, cell numbers in the mesocarp, and pollen tube growth rate. These two GAs appear to be biosynthesized *de novo* in pollen tube and are the main pollen-derived bioactive GAs found after pollen germination. GA₁ levels in the pollen tube appear to be related to a pollen–style interaction that occurred after the pollen grains landed on the stigma.

Key words: Gametophytic competition, gibberellins, pollen density, pollination, *Pyrus pyrifolia*.

Introduction

Pollination is a critical phase of the production cycle for most speciality crops, including Japanese pear (*Pyrus pyrifolia* Nakai). Some observational studies have found positive correlations between floral density and pollinator visits, pollen loads, fruit set, and seed set (Knight, 2003). Well pollinated crops usually result in higher fruit set, produce larger and more even fruit, and improve grower profit in fruit crops, including blueberry (Suzuki *et al.*, 1998), cranberry (Cane, 2009), raspberry (Jennings and Topham, 1971), buckwheat (González *et al.*, 2006), cherimoya (Björkman, 1995), loquat (Freihat *et al.*, 2008), apple and pear (Jackson, 2003; Hayashi and Tanabe, 1991). Most varieties of *P. pyrifolia* are self-incompatible and require cross-pollination to produce heavy and regular crops (Hayashi and Tanabe, 1991). The reproductive success of *P. pyrifolia* largely depends on both the quantity and

quality of pollen transferred, therefore, more pollinators are needed for *P. pyrifolia* than for many other tree fruit species. Hayashi and Tanabe (1991) observed that increasing the pollen load on the stigma by hand pollination could improve fruit size and quality in *P. pyrifolia*. Thus, growers need to increase bee colony density or frequency of hand pollination until an adequate pollen loading is reached in Japanese pear (Hayashi and Tanabe, 1991).

For many plants, the amount of pollen that arrives on stigmas is far in excess of the amount required to fertilize all the ovules. Once a threshold of pollen grain number is reached, pollen competition can become important. Larger pollen loads can intensify selection among gametophytes and considerable evidence is now available that more intensive pollen competition will produce more vigorous progeny (Mulcahy and Mulcahy, 1975; Bertin, 1990;

Schlichting *et al.*, 1990; Snow, 1991; Björkman, 1995). Thus, many crop breeders manipulate the intensity of gametophytic competition by increasing the number of pollen grains on the stigma to increase the vigour of the subsequent sporophytic generation (Mulcahy, 1974; Janse and Verhaegh, 1993; Snow and Spira, 1991; Hormaza and Herrero, 1996a). However, some species, such as, European pear (*Pyrus communis*) and a member of the Plantaginaceae (*Collinsia heterophylla*) (Janse and Verhaegh, 1993; Lankinen *et al.*, 2009) have given results that indicate that the vigour of the progeny are not affected by an increased pollen load regime.

Jennings and Topham (1971) have suggested that pollen germination is conditioned by growth substances (PGRs) that are provided partly by the pollen grains and partly by the styles of the seed parent, and that there were interactions between the two sources of the PGRs. In *P. pyrifolia*, pollen germination and elongation is controlled by an *S*-allele in both the pollen and pistil and substantial progress in the molecular basis of self-(in)compatibility has been made in the past decade (Dai *et al.*, 2007; Yamane and Tao, 2009). However, little information is available in the literature about the mechanisms by which gametophytic competition bring about increased vigour of the progeny. Much of the past interest on gametophytic competition has focused on the effects on progeny vigour and performance (Mulcahy, 1974; Mulcahy and Mulcahy, 1975; Bertin, 1990; Schlichting *et al.*, 1990; Snow, 1991; Janse and Verhaegh, 1993; Hormaza and Herrero, 1996a; Björkman, 1995), with very few studies dealing with the question of how gametophytic competition leads to vigorous progeny and gives rise to a higher pollen germination percentage and faster pollen tube growth.

Evidence is accumulating that suggests that fertilization is not always a random process (Mulcahy and Mulcahy, 1987) and that mate choice can occur in plants (Marshall *et al.*, 2007). Some researchers are convinced that pollen competition has a chemical basis, and that pollen grains modify their chemical environment by secreting compounds which may have either positive or negative effects on pollen performance (Brewbaker and Majumder, 1961; Marshall *et al.*, 1996). Both flavonols and phytosulphokine- α were identified as possible factors that may be involved in density-dependent pollen germination (Taylor and Hepler, 1997; Chen *et al.*, 2000). From the above evidence it has been concluded that pollination is a very complex reproductive process, and one which needs further clarification.

Previous studies suggested that gibberellins (GAs) might be involved in pollen–pistil interaction and fruit set (Hiratsuka *et al.*, 1984; Kashyap and Gupta, 1989). Our previous reports also established that endogenous GAs may be the trigger for rapid fruit growth and development after pollination, and that the fertilized ovule is a likely source of gibberellin in *P. pyrifolia* (Zhang *et al.*, 2007a, 2008; Hayashi and Tanabe, 1991). Among various physiological events regulated by GAs, reproductive development is one of the most important events (Pharis and King, 1985). Several more recent studies also indicate that GAs are also

involved in pollen viability and pollen tube growth in *Arabidopsis* and rice (Singh *et al.*, 2002; Chhun *et al.*, 2007). Bioactive GAs, include GA₁, GA₃, GA₄, and GA₇, and these GAs have been identified and their roles investigated in post-fertilization events, fruit set and fruit development, are well documented in *P. pyrifolia* (Yuda *et al.*, 1981; Zhang *et al.*, 2005, 2007a, b, 2008, 2009). However, little information is available about the putative role of GAs in the pre-fertilization events, i.e. from the time pollen lands on the stigma to the stage when the pollen tube finally reaches the ovule.

We have hypothesized that an increase in pollen load can result in gametophytic selection through pollen grain competition, and that the interaction between pollen grains is mediated by PGRs, including GAs, in the pollen grain competition that occurs prior to fertilization in *P. pyrifolia*. The current work attempts to accomplish quantitative correlations between pollen densities, fruit set, fruit quality, and endogenous GAs concentrations under high and low pollen loads. It also attempts to clarify the roles of GAs in gametophytic competition in *P. pyrifolia*.

Materials and methods

Plant materials

Field experiments and other analyses were conducted in Watanabe Orchard and at Tottori University in Tottori, Japan (latitude 35°15', longitude 133°47'). Fifteen-year-old self-incompatible 'Gold Nijisseiki' (S2S4) pear trees were selected for the current studies. 'Gold Nijisseiki', a mutant of 'Nijisseiki' caused by gamma irradiation, comes from the old trees of 'Nijisseiki' (S2S4). It is a non-russet Japanese type and has resistance to black spot disease and was first released in 1991. All cultural management practices were the same as accorded to commercial production.

Pollen collection and hand pollination

Flowers were pollinated with 'Chojuro' (S2S3) pear pollen on 14 April 2007. To collect 'Chojuro' pollen, fruiting branches were pruned on 10 April and put into buckets of water at 40 °C in a greenhouse. As previously reported (Hayashi and Tanabe, 1991), the flowers were collected for pollen collection at the pink stage (20% full bloom). The collected flowers were put into an anther collector and the fresh anthers were separated from the other flower parts. The collected pollen was maintained at room temperature (25 °C) and a relative humidity of 30–35%. The pollen was separated with a pollen mill and the pure pollen grains were collected and stored at –20 °C for use in pollination.

Two controlled hand-pollinations were used in the orchard. One had a low pollen density of 10% and the other had a high density of 100% pure pollen grains. Both the number of pollen grains and germinating pollen tubes were greater than the number of ovules. To obtain a diluted pollen concentration of 10%, *Lycopodium* spores were added by volume (Hayashi and Tanabe, 1991; Janse and Verhaegh, 1993; Suzuki *et al.*, 1998; González *et al.*, 2006). Thus there were two pollen density treatments and for each treatment density 600 spurs were pollinated with each spur having 5–8 flowers. To prevent self-pollination and the influence of other pollen sources, the spurs were bagged before blooming. At 10% full bloom, flowers at the full white stage or recently opened flowers with fresh unopened anthers were pollinated with 100% pure pollen and 10% diluted pollen, respectively. Pollination was done as uniformly as possible, by applying similar amounts of

either the mixture or the undiluted pollen to the stigmas with brush pen. Any opened flowers with desiccated anthers were removed from the spur. The fruitlets were hand-thinned on 10 May 2007 and thereafter the fruitlets were bagged with a small white bag. The third to the fifth flowers from the bottom were left and the other fruit were hand-thinned and then bagged. A second fruit thinning was conducted before 5 June 2007 and only one fruit per spur was left to develop. Then the fruit was covered with a large bag to allow secure fruit development. The fruitlets were sampled at 0, 1, 4, 12, 24, and 72 h after pollination. Fruit length and diameter were measured with a digital caliper each week, and the mean daily increase in fruit length and diameter were calculated and used for a comparison of fruit growth patterns.

Pollen germination and pollen tube growth

In vitro germination was utilized for assessment and observation of pollen tube growth and also for endogenous GAs measurement. The growth medium used for pollen germination contained 10 g l⁻¹ agar and 100 g l⁻¹ sucrose (Hayashi and Tanabe, 1991). The prepared agar solution after micro-waving was transferred to 20 ml Petri dishes. Ten replications were conducted for each treatment. Five replications were used for pollen germination observation and the other five replications were extracted and analysed for endogenous GAs. Equal amounts of the mixture of diluted pollen or pure pollen grains were placed on each Petri dish. The germinated pollen grains were studied by using the VH-8000 digital HF microscope system (Keyence Co., Tokyo, Japan) and the length of the pollen tubes and germination rate were measured. For GA extraction and analysis, three Petri dishes per treatment were randomly selected at 1, 4, 12, 24, 36, and 48 h after planting in the controlled environment chamber at 25 °C.

In addition, samples of flowers pollinated with both diluted and undiluted pollen were fixed in FAA (80% ethanol:acetic acid:formalin, 90:5:5 by vol.) and then used to assess *in vivo* pollen germination. The flowers were washed three times in distilled water and then softened in 5 M NaOH overnight at room temperature. The flowers were then washed with distilled water and stained with 0.1% aniline blue in K₃PO₄ buffer (pH 8.5) for more than 3 h. The samples were mounted in 80% glycerol and were observed under UV epifluorescence. Pollen grains were scored as germinated if a pollen tube could be seen emerging from the pollen coat.

GA determination

GA levels of fruitlets in natural conditions and of the male gametophyte (the pollen grain and the tube) were quantified *in vitro* using Gas Chromatography-Mass Spectrometry-Selected Ion Monitoring (GC-MS-SIM) with deuterated internal standards. The GA extraction, purification, and determination were accomplished according to Zhang *et al.* (2007a) and described briefly below.

Samples of frozen fruitlets, or samples of agar medium with pollen grains were homogenized and extracted overnight in 80% cold aqueous methanol containing 0.02% butyl hydroxytoluene. At this point, 100 ng deuterated GAs {[17-²H₂]GA₁, [17-²H₂]GA₃, and [17-²H₂]GA₄} were included in the samples as internal standards for recovery estimation after purification. After extraction and filtration, the pooled extracts were evaporated *in vacuo* at 42 °C to an aqueous solution. The aqueous residue was adjusted to a pH of 6–7 and was partitioned against n-hexane. The pooled aqueous residue was then adjusted to pH 2.5 with HCl and partitioned against ethyl acetate. The pooled ethyl acetate phase was partitioned against a potassium phosphate buffer (pH 8.0). Then, insoluble polyvinylpyrrolidone (PVPP) was added to the combined aqueous solution and filtered. The filtered aqueous solution was adjusted to pH 2.5 with HCl and partitioned against ethyl acetate again. The pooled ethyl acetate solution was dried over Na₂SO₄ and evaporated *in vacuo*. The residue was dissolved

in 80% aqueous methanol and loaded onto a C₁₈ Pre Sep Cartridge (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and purification. The dried residue was dissolved in 45% methanol-water containing 0.1% acetic acid and further loaded onto a Bondesil DEA (diethylaminopropyl) column (Varian Associates, Palo Alto, CA). The sample was eluted successively with distilled water and methanol. The eluate was evaporated *in vacuo* and stored at -20 °C for further purification by high-performance liquid chromatography (HPLC).

The residue was subjected to a reverse-phase Senshu-Pak ODS (OctaDecyl-Silyl)-4253-D HPLC column (10 mm i.d.×250 mm; Senshu Scientific, Tokyo) and eluted with 0.1% acetic acid in 30% aqueous methanol (solvent A) and 100% aqueous methanol (solvent B) at 40 °C as follows: 0–3 min, elution with solvent A; 3–30 min, linear gradient of 0% to 100% solvent B; 30–50 min, elution with 100% solvent B. The flow rate of the solvent was 3 ml min⁻¹, and the eluate was collected every 1 min as one fraction. Fractions expected to contain GA₁, GA₃, and GA₄ were collected from the HPLC eluate for GC-MS-SIM analysis at 16 min, 14–15 min, and 26–28 min, respectively.

The grouped HPLC eluates were dried, dissolved in 100% methanol, transferred to a reaction vial, and again dried, all at room temperature. The samples were then methylated with ethereal diazomethane followed by trimethylsilylation with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (Sigma, St Louis) in glass vial at 80 °C for GC-MS (6890N network GC system; Agilent Technologies Santa Clara, CA) analysis. One microlitre of each silylated sample was injected into a DB-1 fused silica chemically bonded capillary column [15 m, 0.25 mm (i.d.), 0.25 µm film thickness; Agilent Technologies]. The GC oven temperature for GAs was programmed for 3 min, held at 80 °C, then increased at 15 °C min⁻¹ to 300 °C, followed by 5 min at 300 °C. Helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced with a mass selective detector with an interface and source temperature of 280 °C, an ionizing voltage of 70 eV, and a dwell time of 100 ms. The GC conditions were as follows: 3 min held at 60 °C, then increased at 20 °C min⁻¹ to 290 °C, and maintained at 290 °C for 5 min.

The identity of eluted GAs, according to retention times, was verified by monitoring diagnostic ions of both endogenous GAs and deuterated GAs. Levels of endogenous GAs were determined by measuring the abundance of the following ion pairs: *m/z* 506/508 for GA₁, *m/z* 504/506 for GA₃, and both *m/z* 418/420 and 284/286 for GA₄. Concentrations of endogenous GAs were expressed as either ng g⁻¹ FW of fruitlets or ng g⁻¹ of pollen grains applied to the agar plates.

Fruit quality analyses

Fruit were randomly selected for fruit quality analysis. Fruit diameter and length were measured by digital calipers and then fresh fruit weight was recorded. The numbers of normal shaped fruit and of misshapen fruit were counted. Flesh firmness was assessed using a Rheometer (RT-3010D, Rheotech, Japan) with a 0.8 mm diameter flat-tipped probe. The crosshead speed of the Rheometer was 100 mm min⁻¹, and driving depths were 10 mm. Values were expressed in Newtons. The peel colour (*L**, *a**, and *b**) was determined at three different points around the equator by using a hand-held colour meter (MR-3000, Nippon Denshoku Co., Tokyo, Japan). The degree of brightness, greenness, and yellowness were presented by *L**, *a**, and *b**, respectively. The soluble solids content (°Brix) of juice was assessed from an aliquot of expressed juice from a longitudinal slice from each fruit with a digital refractometer (Atago Co., Tokyo, Japan). Juice pH was measured with a digital pH meter (Shindengen Electric Manufacturing Co., Tokyo, Japan). Finally, seeds per fruit were collected for the evaluation of seed set. Unfertilized ovules always undeveloped and presented as an extremely small tissue (Hayashi and Tanabe, 1991; Zhang *et al.*, 2008).

Measurement of cell number and cell length of mesocarp

The measurement of cell number and cell length of the mesocarp was conducted according to Zhang *et al.* (2006). Ten fruit were collected and were cut along the equatorial region, then the diameter of the core was measured, and mesocarp width was calculated from the difference between the longest width of the transverse section of the fruit and core. Slices of the mesocarp were preserved in FAA for later histological analysis. A transverse slice of mesocarp was stained by rubbing softly with a cloth soaked in blue ink. The stained surface was observed under the above digital HF microscope system and an image from a CCD camera displayed on a monitor. Cell length, as an indicator of cell size, was measured from the length of seven contiguous cells from the core to the fruit surface: from these, the average cell length was calculated. Ten observation zones per section were measured. Cell number of the mesocarp along the equatorial region was then calculated by dividing the mesocarp width by the average cell length, and this was taken as an indicator of total cell number per fruit.

Statistical analysis

Statistical differences between treatments were analysed by Student's *t* test using Sigmaplot (Jandel Science, San Rafael, CA) software. A probability of $P > 0.05$ was considered to be non-significant.

Results

Pollen tube growth

Both pollen germination and elongation are density-dependent *in vivo* and *in vitro* and our results show differences in pollen performance (Figs 1, 2, 3). Thus, pollen dilution reduced pollen germination rate both *in vivo* and *in vitro*. One hour after pollen grain were planted, the undiluted controls exhibited longer pollen tube lengths than the diluted treatment (Fig. 1), and this enhanced pollen tube growth continued for 24 h. In fact, pollen tube length was reduced by 2/3 when the pollen was diluted by *Lycopodium* spores (Fig. 1). In the field, at 24 h after hand pollination, pollen tubes in the pistil for the undiluted control treatment grew faster than pollen tubes in the diluted pollen treatment. (Fig. 3). At the 24 h stage of pollen tube growth, control pollen tubes had almost reached the base of the style, while pollen tubes in the diluted treatment had only reached the upper section of the pistil.

Fruit set, seed set, and fruit shape

As shown in Fig. 4, fruit set was decreased by pollen dilution with fern spores, i.e. 82% fruit set in the undiluted control, but only 53% in the diluted pollen treatment. A subsequent investigation of seed set showed that the diluted treatment had an average of 4.6 viable seeds per fruit compared with 7.7 viable seeds per fruit for the control treatment (Fig. 5; Table 1). Interestingly, the average ovule number/fruit, based on the viable and non-viable seeds at harvest, was larger in the diluted pollen treatment than the control treatment, although the difference is not significant (Table 1). The shape of fruit was also pollen density-dependent. Thus, 43% misshapen fruit

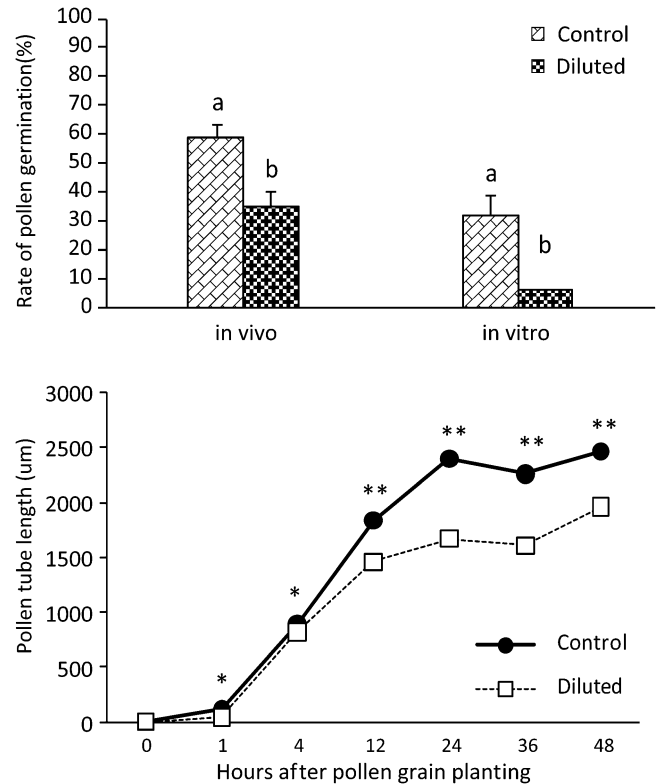


Fig. 1. Pollen germination and pollen tube growth in *Pyrus pyrifolia* cv. 'Gold Nijisseiki'. Pollen germination was measured 4 h after pollination (*in vivo*) and 1 h after planting in the medium (*in vitro*), and only pollen tube growth *in vitro* is shown. Values followed by different letters are significantly different at $P < 0.05$ in pollen germination; Non-significant (ns) or significant at $P < 0.05$ (*) or 0.01 (**) by *t* test in pollen tube growth.

was observed in the diluted treatment compared with only 18% in the control (Table 1). Fruit that contained *c.* 10 viable seeds usually showed an even and symmetrical shape. There was also a high correlation between poor seed viability and misshapen fruit (data not shown).

Fruit quality, cell number, and cell length

Control fruit showed no difference in length, relative to fruit from the diluted pollen treatment, but control fruit diameter was 12% greater than fruit from the diluted pollen treatment (Table 2). A reduced pollen density did not affect the single sigmoid pattern of fruit growth seen for control treatment fruit (data not shown). However, control treatment fruit grew more rapidly than fruit in the diluted pollen treatment during the final stage of fruit growth (Fig. 6). In addition, control treatment fruit were larger and weighed more, and also had a larger pedicel than fruit from the diluted pollen treatment (Table 2). Fruit weight distribution also showed differences, for example, about 60% of the fruit from the control treatment fell into 300–400 g category, but 60% of the fruit in the diluted pollen treatment was in the 200–300 g category (Fig. 7).

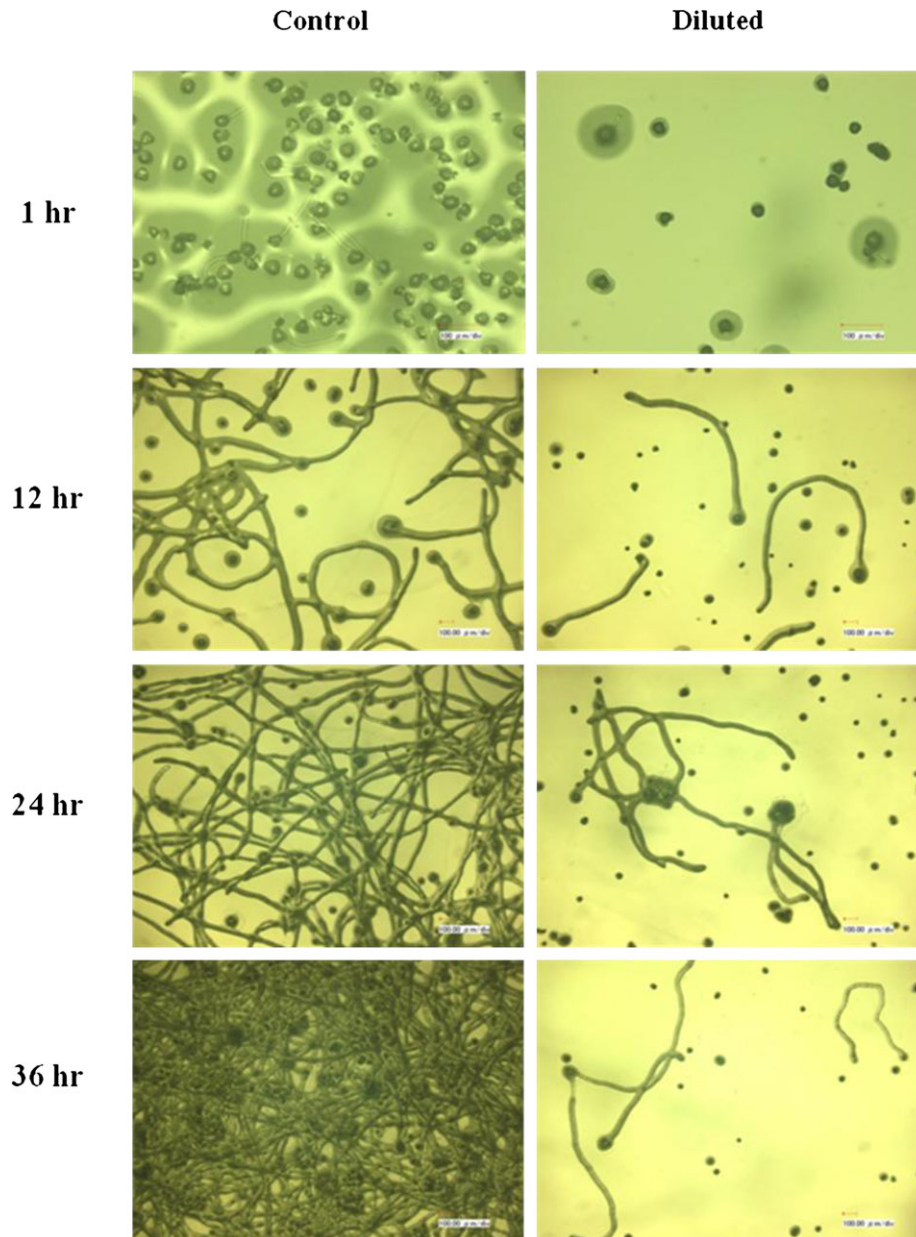


Fig. 2. *In vitro* pollen tube growth assays in *Pyrus pyrifolia* cv. 'Gold Nijisseiki' after planting on agar medium at 25 °C. Plates: left, the control treatment (100% pollen); right, the diluted pollen treatment (10% pollen).

Our histological study revealed that fruit in the diluted pollen treatment had decreased cell number and relatively smaller cell size relative to the fruit from the control treatment (Fig. 8). The concentration of sugar and acidity, together with flesh firmness, are important indices that characterize fruit ripeness, and are also important quality parameters. There were no differences in juice pH, firmness, and total soluble solid (°Brix) between the control and diluted pollen treatments (Table 2). The results of the peel colour by L^* , a^* , and b^* also showed that fruit in the pollen diluted treatment had a lower a^* than that fruit from the control treatment. This suggests that fruit maturation occurs earlier in the diluted pollen treatment.

Changing GAs levels in fruitlets samples relative to in vitro

Endogenous GA_1 levels in fruitlets from both treatments showed an increasing pattern following hand pollination. However, at 12 h, GA_1 concentration continued to increase in the fruitlets from the control treatment, whereas fruitlets from the diluted pollen treatment showed a reduction in GA_1 concentration (Fig. 9A). Both GA_3 and GA_4 levels in fruitlets from the control treatment peaked at 24 h after hand pollination. By contrast, significantly reduced GA_3 and GA_4 levels were observed for the pollen dilution treatment (Fig. 9B, C). Bioactive GAs (in total) in fruitlets from the control treatment increased sharply 4 h after hand

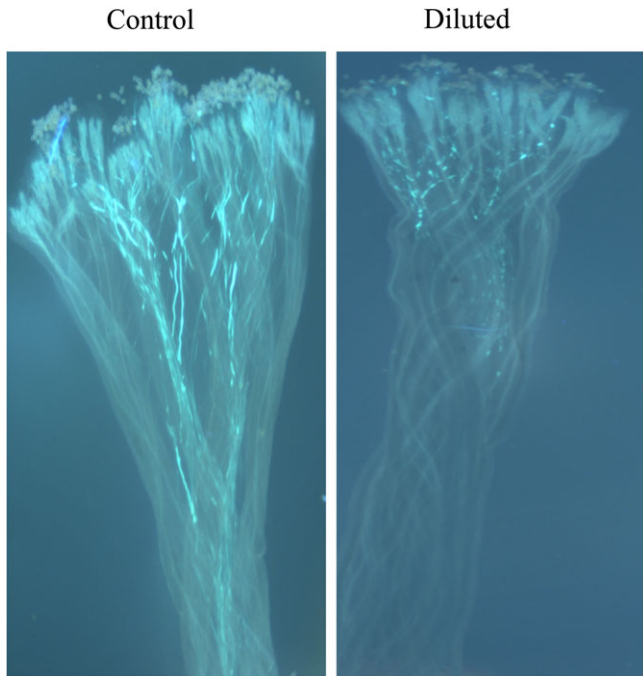


Fig. 3. Pollen tube growth in the pistil of *Pyrus pyrifolia*. Aniline blue staining at 24 h of the control (left) and the treatment where pollen was diluted with *Lycopodium* spores (right).

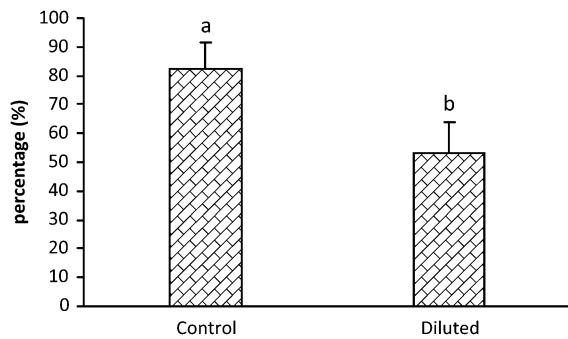


Fig. 4. Influence of pollen density on the stigma on final fruit set in *Pyrus pyrifolia* cv. ‘Gold Nijisseiki’. Values followed by different letters are significantly different at $P < 0.05$.

pollination, and then increased steadily to a peak at 24 h after pollination. Subsequently, there was a decline through 72 h after pollination. However, in the diluted pollen treatment, total bioactive GA concentration peaked 1 h after pollination, then remained stable until 12 h after pollination, after which it decreased steadily until 72 h (Fig. 9D).

Bioactive GAs levels in the germination medium exhibited totally different patterns compared with GAs in fruitlets from pollinated flowers (Figs 9, 10). The GA_1 level in the control germination medium was higher than that in the diluted pollen treatment and GA_1 levels in both treatments remained stable during the germination test (Fig. 10A). Interestingly, the GA_3 level for the control treatment medium reached a peak 1 h after pollen grain

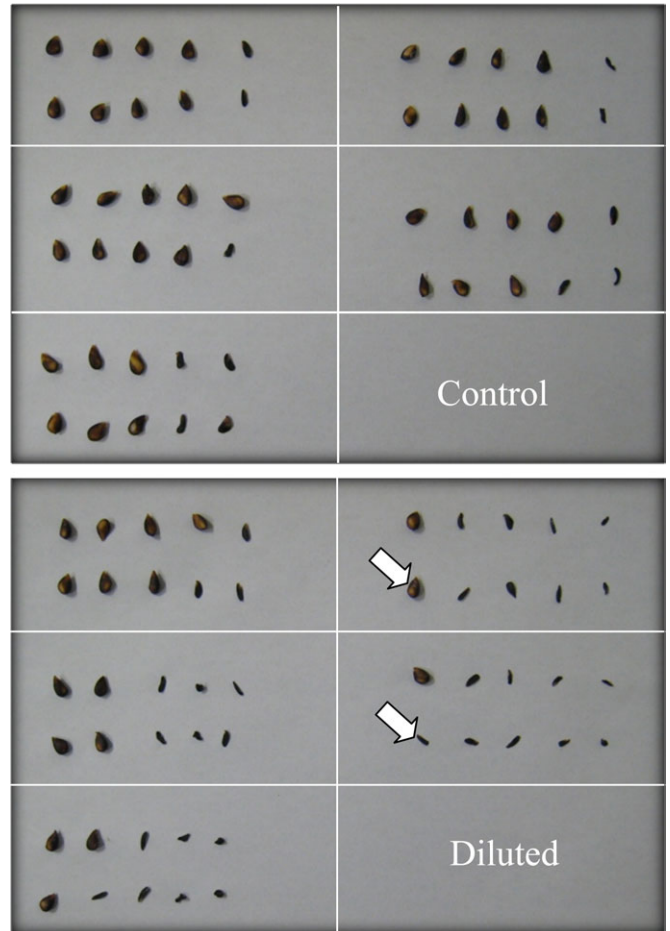


Fig. 5. Influence of pollen density on the stigma on seed set and viability at harvest in *Pyrus pyrifolia* cv. ‘Gold Nijisseiki’. Five typical fruit were selected for investigation for each treatment and arrows show the viable and aborted seeds or unfertilized ovules.

Table 1. Influence of pollen density on the stigma on seed set, and fruit shape at harvest in *Pyrus pyrifolia* cv. ‘Gold Nijisseiki’

Treatment	Seed set	Ovule number ^a	Fruit shape (%)	
	(No. fruit ⁻¹)	(No. fruit ⁻¹)	Normal	Misshapen
Control	7.7	9.3	82.0	18.0
Diluted	4.6	9.9	57.0	43.0
P^b	**	ns	*	*

^a Ovule number was determined based on the total number of viable and non-viable seeds in fruit at harvest

^b Non-significant (ns) or significant at $P < 0.05$ (*) or 0.01 (**) by *t* test.

planting in the medium, then decreased sharply (Fig. 10B). GA_4 levels in both treatments were extremely low compared with GA_1 and GA_3 levels. The GA_4 level in the control medium increased 1 h after planting and peaked at 4 h, then steadily declined to 48 h (Fig. 10C). Similarly, the GA_4 level in the medium of the diluted pollen treatment followed the same pattern change seen for the control,

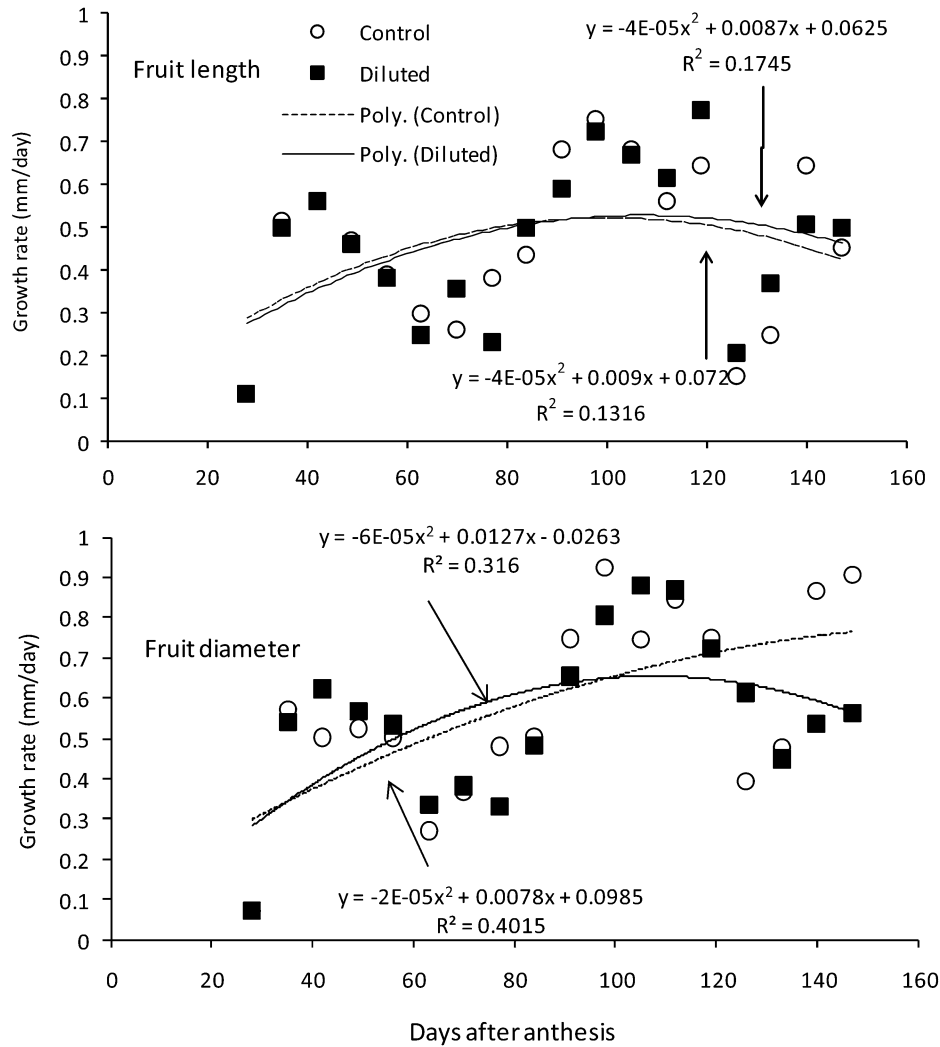


Fig. 6. Influence of pollen density on the stigma on the pattern of fruit growth (fruit length and diameter) in *Pyrus pyrifolia* cv. 'Gold Nijisseiki'. Data are presented as length d^{-1} (mm).

Table 2. Influence of pollen density on fruit quality at harvest in *Pyrus pyrifolia* cv. 'Gold Nijisseiki'

Parameter measured	Control	Diluted	P^a
Fruit weight (g)	367.2	268.1	**
Fruit diameter (mm)	89.3	79.4	*
Fruit length (mm)	72.0	71.5	ns
Total soluble solid ($^{\circ}$ Brix)	10.7	10.8	ns
Juice pH	4.55	4.55	ns
Firmness (N)	26.1	26.3	ns
Pedicle length (mm)	23.4	27.3	*
Pedicle diameter (mm)	4.6	4.1	*
Fruit colour			
L^*	62.2	61.6	ns
a^*	-8.9	-9.7	*
b^*	32.4	32.4	ns

^a Non-significant (ns) or significant at $P < 0.05$ (*) or 0.01 (**) by t test.

although the GA_4 concentration was significantly lower than that seen for the medium in the control treatment. Changes in the total amount of bioactive GAs in both treatments exhibited patterns similar to those seen for

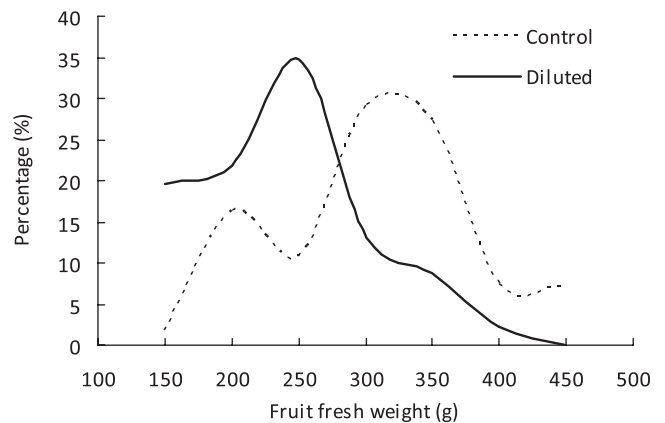


Fig. 7. Influence of pollen density on the stigma on final fruit fresh weight in *Pyrus pyrifolia* cv. 'Gold Nijisseiki'.

GA_3 . However, much higher total GA levels were seen in the medium of the control treatment, relative to total GA concentration in the medium of the diluted pollen treatment (Fig. 10D).

Discussion

Pollen density versus fruit set, seed set, and fruit quality

It is well known that successful fruit set and subsequent fruit development in plants with seeded fruit are dependent on both pollination and subsequent fertilization. This is because fertilization activates cell division and triggers fruit growth and hormone production, principally the production of GAs and auxins (Crane, 1964; Sponsel and Hedden, 2004). In the current study, it was apparent that fruit set of *Pyrus pyrifolia* cv. 'Gold Nijisseiki' was decreased by pollen dilution with *Lycopodium* spores (Fig. 4). This result is consistent with previous reports in cranberry (Cane, 2009), raspberry (Jennings and Topham, 1971), cherimoya (Björkman, 1995), and loquat (Freihat *et al.*, 2008). It has been suggested that reproductive success, in terms of quantity and/or quality of pollen grains (Chacoff *et al.*,

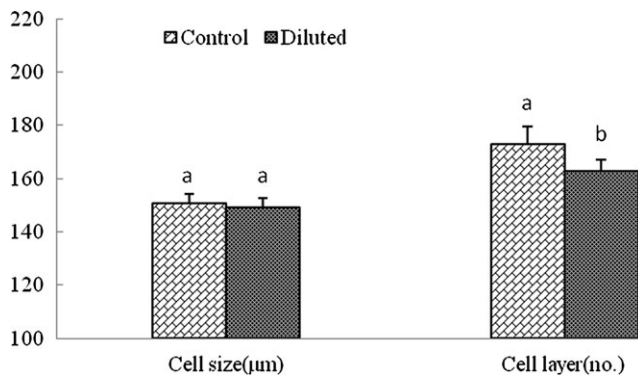


Fig. 8. Influence of pollen density on the stigma on final cell size and number of cell layers in *Pyrus pyrifolia* cv. 'Gold Nijisseiki'. The control (100%), diluted pollen (10%). Values followed by different letters are significantly different at $P < 0.05$.

2008), is closely linked to a high frequency of viable pollen grains. Effective cross-pollination between genotypes (different varieties) in *P. pyrifolia* is essential for the successful fertilization of the ovules, followed by subsequent initiation of seed development, and finally, fruit set. Pollination density has been postulated to influence the quality of seeds produced, in that high pollen loads intensify competition among male gametophytes, with the most vigorous gametophytes (perhaps those with the fastest-growing pollen tubes) effecting fertilization (Bertin, 1990). With low pollen loads, gametophytic competition should be reduced and the chance of fertilization by inferior male gametophytes should be increased. If this supposition is correct, the result will be an increased proportion and number of non-viable zygotes that soon abort. By contrast, when an excess of pollen grains are applied, gametophytic competition is intensified and the chance of fertilization yielding vigorous zygotes increases. In the present study, low fruit set in the diluted pollen treatment was accompanied by a low seed set and misshapen fruit (Figs 1, 5; Table 1). Previous reports have provided evidence that developing young seeds are an important sources of GAs (Nakagawa *et al.*, 1979; Hayashi and Tanabe, 1991), and these seed-produced GAs were postulated as a trigger of the entire fruit development process (Zhang *et al.*, 2007a). Fruit with increased numbers of viable seeds will presumably become stronger sinks for maternal resources. It is therefore shown that fruit growth of *P. pyrifolia* is strongly activated by increasing the number of pollen grains on the stigma, thereby leading to the enhancement of cell division and a larger fruit (Figs 6, 7, 8; Table 2). In raspberry, a reduction in pollen concentration affected several aspects of early fruit development, but quantitative differences in the amount of pollen applied did not reduce the adverse effects of using diploid pollen. This suggests that promotive effects do not arise through direct

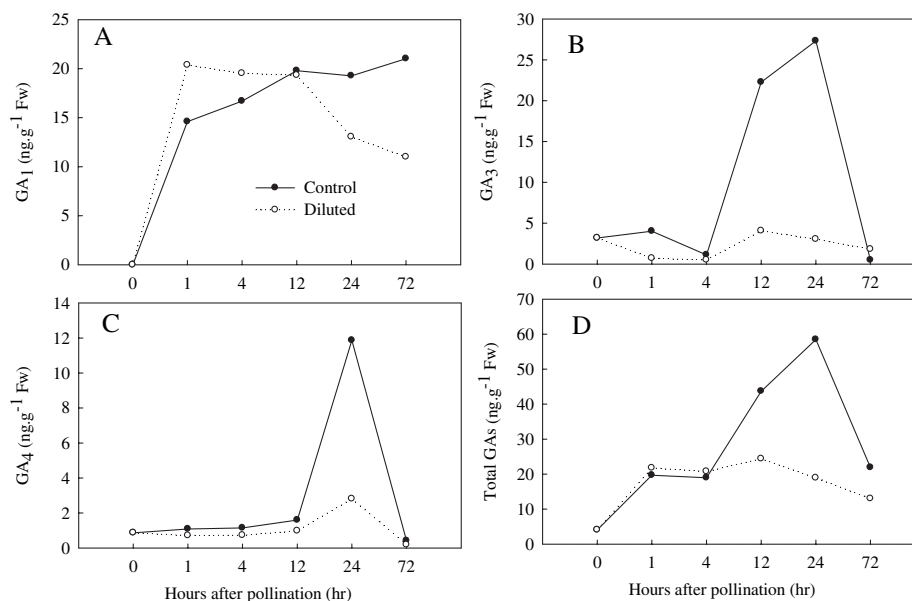


Fig. 9. Influence of pollen dilution on (A) endogenous GA₁ (A), GA₃ (B), GA₄ (C) and total bioactive GAs (D) levels in fruitlets of *Pyrus pyrifolia* cv. 'Gold Nijisseiki'.

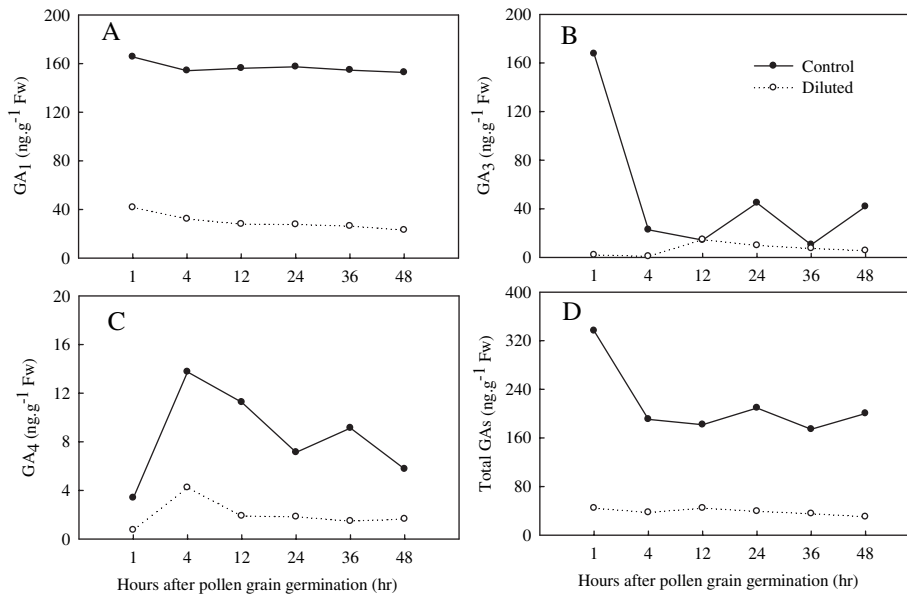


Fig. 10. Pollen-derived gibberellin concentrations on agar medium after germination, showing the differences between the diluted pollen (10%) and the control treatments using pollen of *Pyrus pyrifolia* cv. 'Gold Nijisseiki'. GA₁ (A), GA₃ (B), GA₄ (C), and total bioactive GAs (D).

action of the pollen on the maternal tissues (Jennings and Topham, 1971). Therefore, the low fruit set, higher non-viable seed ratios, and increases in misshapen fruit by a diluted pollen grain treatment should be focused on both pre-fertilization developmental events, including pollen germination, pollen tube growth, and the involvement of GAs, as well as post-fertilization events which would include seed and fruit growth.

Pollen density versus GA levels and pollen tube growth

Pollen grains undergo active metabolic changes in the course of pollination and fertilization. This includes signaling, cell recognition, and cell growth, and a number of investigations have focused on genes and proteins that are involved in the pollen–stigma interaction and rapid pollen tube growth (Dai *et al.*, 2007). Density-dependent pollen germination and tube growth *in vitro* is a well-documented phenomenon in many plant species and it has been called the pollen population effect (Brewbaker and Majumder, 1961; Lee, 1980; Tonutti *et al.*, 1991; Hormaza and Herrero, 1996b; Pasonen and Kapyla, 1998; Chen *et al.*, 2000). However, its physiological and molecular basis is still poorly understood. Brewbaker and Majumder (1961) suggested that the positive effect of increasing pollen density on germination is caused by water-soluble growth factors which they identified as calcium ions. Recently, flavonols (Taylor and Hepler, 1997) and phytosulphokine- α (Chen *et al.*, 2000) have been postulated to play important roles in density-dependent germination.

High pollen competitive ability, particularly pollen tube growth rate, is associated with high siring ability (Snow and Spira, 1991; Marshall, 1998; Aronen *et al.*, 2002). In the present study, pollen tube growth from both our *in vivo* and *in vitro* studies also showed that increased pollen density

leads to faster pollen tube growth and a higher pollen germination rate (Figs 1, 2, 3). Increased seed set in the control treatment showed that a higher pollen density allows for a higher frequency of ovule fertilization (Fig. 5; Table 1). In other words, it is apparent that an increased effective pollen number resulted in a higher seed set. Slow pollen tube growth in the diluted pollen treatment was also responsible for low seed set (Figs 2, 3; Table 1). Interestingly, pollen dilution significantly decreased the concentration of individual GAs and the total amount of GAs found during pollen tube growth, although the patterns of GA change were different between *in vivo* (fruitlet GA levels) and *in vitro* (pollen grains germinating on agar) studies (Figs 9, 10). The overall endogenous fruitlet GA levels increased dramatically almost immediately after hand pollination, implying that the fruitlet's production of GAs is closely involved with the processes of pollination, pollen tube growth, and fertilization events. The changes in fruit GA levels could be explained by the combined effect of more pollen tubes (Fig. 3) and more seeds set (Fig. 5; Table 1) in the control treatment. As an analogy, it has been suggested that GAs produced in the developing anthers of flowers are required for corolla development (Weiss *et al.*, 1997). GAs also appear to be present in developing pollen after the flower's anthesis. This is based on work that shows that GAs must be present for normal pollen tube growth (Singh *et al.*, 2002; Chhun *et al.*, 2007). Numerous studies have reported an effect of GA application on pollen tube growth *in vivo* and *in vitro* (Sponsel and Hedden, 2004). Depending on the species examined and the concentration used, applied GAs can promote, inhibit, or have no effect on pollen tube elongation *in vitro*. Our experiment on *in vitro* germination of pollen grains attempted to remove the possible effect of maternal side tissues. The fact that three bioactive GAs were found in relatively high concentrations

after germination suggests that *de novo* GA biosynthesis occurs in pollen tubes very soon after germination, i.e. less than 1 h for *P. pyrifolia*, a conclusion which is also supported by the work with *Arabidopsis* and rice (Singh *et al.*, 2002; Chhun *et al.*, 2007). Furthermore, the different patterns of GA₁ levels indicated that the sharp increase of GA₁ in fruitlets (Fig. 9A) is closely related to an interaction between pollen and pistil and this increase differed appreciably from the pattern seen *in vitro* (Fig. 10A). Also, the higher GA₃ and GA₄ levels within 1 h or 4 h after planting the pollen onto the germination medium (Fig. 10B), compared with low GA₃ and GA₄ levels in pollinated flowers (Fig. 9B), suggests that GA₃ and GA₄ were biosynthesized *de novo* in the growing pollen tube and, further, that they are the main pollen-derived bioactive GAs produced after pollen germination. Thus, it seems likely that pollen-derived GAs are a governing stimulus for pollen tube growth and subsequent fruitlet development prior to actual fertilization.

Pollen density versus pollen-derived GAs and gametophytic competition

Studies of the impact of pollen density on gametophytic competition have given consistent results in many crops, including maize (Sari-Gorla *et al.*, 1992), zucchini (Schlichting *et al.*, 1990), plum (Lee, 1980), sweet cherry (Tonutti *et al.*, 1991), blueberry (Suzuki *et al.*, 1998), cranberry (Cane, 2009), raspberry (Jennings and Topham, 1971), buckwheat (González *et al.*, 2006), cherimoya (Björkman, 1995), and loquat (Freihat *et al.*, 2008). However, the mechanisms that regulate it are still little known. One factor could be the differences in pollen competitive ability. It has been suggested that pollen and growing pollen tubes could release auxin, gibberellins, and perhaps other hormones. These hormones are known to stimulate fruit growth, and in some species are present at sufficient levels to ensure fruit production, even when no seeds are present (Nitsch, 1952; Zhang *et al.*, 2008). Jennings and Topham (1971) suggested that pollen germination was initiated by growth substances partly from the grains themselves and partly by the styles of the seed parent, and that there were interactions between the two sources. Thus, when more compatible pollen grains are present on a stigma than are needed to fertilize all available ovules, the outcome of mating may appear as a non-random event, either because pollen donors compete for access to those ovules or because the maternal tissue influences the outcome of mating (Mulcahy, 1974; Marshall *et al.*, 2000). Pollen grains with higher viability might contain higher levels of stimulus growth substance, thus becoming the most vigorous gametophytes with the fastest-growing pollen tubes and, thereby, finally fertilizing the ovules in the competition (Bertin, 1990). The following scenario might thus be a possible explanation of the relationship between pollen density and gametophytic competition. Increased pollen density obviously produced higher levels of pollen-derived GAs. These GAs would then be available to

promote more pollen grains to germinate, thereby enhancing overall pollen tube growth. Speculatively, this could result in increased endogenous GAs synthesis in the pistil, thereby increasing endogenous GAs levels in the developing fruitlet, prior to, and just after fertilization (Fig. 9). Therefore, the most vigorous pollen grain will have the highest opportunity to accomplish fertilization and initiate the development of a vigorous seed, thereby increasing the vigour of the subsequent sporophytic generation. Our previous reports have established that developing seeds are an important source of GAs found in fruit of *P. pyrifolia* (Nakagawa *et al.*, 1979; Hayashi and Tanabe, 1991; Zhang *et al.*, 2007a, 2008). Furthermore, it was found that GAs could increase sink strength in developing fruit, which then resulted in larger fruit size and better fruit quality (Zhang *et al.*, 2005, 2007a, b, 2008). A higher pollen load on the stigma will thus allow for increased levels of total GAs excreted by male gametophytes.

In summary, higher pollen density on the stigma of *P. pyrifolia* can improve fruit set and fruit quality at harvest. A *de novo* increase in the concentration of GAs in the male gametophytes was also shown *in vitro* and this implies increased levels of GA biosynthesis. Levels of pollen-derived GAs were closely related to enhanced pollen germination and increased pollen tube elongation. Applied GAs and, by implication, elevated levels of endogenous GAs, are causally implicated in early growth of the fruitlet and fruit. All of the above evidence indicates that GAs are a very important naturally occurring PGR that is involved in the pollen population effect and/or in gametophytic competition. In tree fruit production poor fruit set or low yields are often caused by poor pollination. A better understanding of the relationship between gametophytic competition and the GA-signalling pathway has potential applications in crop breeding, especially via improvements in pollination and fertilization success.

Acknowledgements

We thank Watanabe Orchard for providing materials, and Kumiko Okayama and Ryo Aoki for their technical assistance in gibberellins analysis. We also thank two anonymous referees for critical reading and comments for this manuscript. The work was supported by the Japanese Society of the Promotion of Science (No. P06196).

References

- Aronen T, Nikkanen T, Harju A, Tiimonen H, Häggman H. 2002. Pollen competition and seed-siring success in *Picea abies*. *Theoretical and Applied Genetics* **104**, 638–642.
- Bertin RI. 1990. Effects of pollination intensity on *Campsis radicans*. *American Journal of Botany* **77**, 178–187.
- Björkman T. 1995. The effect of pollen load and pollen grain competition on fertilization success and progeny performance in *Fagopyrum esculentum*. *Euphytica* **83**, 47–52.

- Brewbaker JL, Majumder SK.** 1961. Cultural studies of the pollen population effect and the self-incompatibility inhibition. *American Journal of Botany* **48**, 457–464.
- Cane JH.** 2009. Pollen viability and pollen tube attrition in cranberry (*Vaccinium macrocarpon* Aiton). *Acta Horticulturae* **810**, 563–566.
- Chacoff NP, García D, Obeso JR.** 2008. Effects of pollen quality and quantity on pollen limitation in *Crataegus monogyna* (Rosaceae) in NW Spain. *Flora–Morphology, Distribution, Functional Ecology of Plants* **203**, 499–507.
- Chen Y, Matsubayashi Y, Sakagami Y.** 2000. Peptide growth factor phyto-sulfokine- α contributes to the pollen population effect. *Planta* **211**, 752–755.
- Chhun T, Aya K, Asano K, Yamamoto E, Morinaka Y, Watanabe M, Kitano H, Ashikari M, Matsuoka M, Ueguchi-Tanaka M.** 2007. Gibberellin regulates pollen viability and pollen tube growth in rice. *The Plant Cell* **19**, 3876–3888.
- Crane JC.** 1964. Growth substances in fruit setting and development. *Annual Review of Plant Physiology* **15**, 303–326.
- Dai S, Wang T, Yan X, Chen S.** 2007. Proteomics of pollen development and germination. *Journal of Proteome Research* **6**, 4556–4563.
- Freihat NM, Al-Ghazawi A, Zaitoun S, Alqudah A.** 2008. Fruit set and quality of loquats (*Eriobotrya japonica*) as effected by pollinations under sub-humid Mediterranean. *Scientia Horticulturae* **117**, 58–62.
- González M, Baeza E, Lao JL, Cuevas J.** 2006. Pollen load affects fruit set, size, and shape in cherimoya. *Scientia Horticulturae* **110**, 51–56.
- Hayashi S, Tanabe K.** 1991. *Basic knowledge of fruit tree culture*. Tottori, Japan: Association Agriculture Press.
- Hiratsuka S, Hirota M, Takahashi E, Hirata N.** 1984. The amount of gibberellin-like substances in pistils when overcome self-incompatibility by bud pollination in Japanese pear. *Technology Bulletin of the Faculty of Horticulture, Chiba University* **34**, 1–7.
- Hormaza JI, Herrero M.** 1996a. Male gametophytic selection as a plant breeding tool. *Scientia Horticulturae* **65**, 321–333.
- Hormaza JI, Herrero M.** 1996b. Dynamics of pollen tube growth under different competition regimes. *Sexual Plant Reproduction* **9**, 153–160.
- Jackson JE.** 2003. *Biology of apples and pears*. Cambridge, UK: Cambridge University Press.
- Janse J, Verhaegh JJ.** 1993. Effects of varying pollen load on fruit set, seed set and seedling performance in apple and pear. *Sexual Plant Reproduction* **6**, 122–126.
- Jennings DL, Topham PB.** 1971. Some consequences of raspberry pollen dilution for its germination and for fruit development. *New Phytologist* **70**, 371–380.
- Kashyap R, Gupta SC.** 1989. The role of gibberellic-acid in the pollen–pistil interaction in sporophytic self-incompatible systems. *Plant Growth Regulation* **8**, 137–149.
- Knight TM.** 2003. Floral density, pollen limitation, and reproductive success in *Trillium grandiflorum*. *Oecologia* **442**, 557–563.
- Lankinen Å, Maad J, Armbruster WS.** 2009. Pollen-tube growth rates in *Collinsia heterophylla* (Plantaginaceae): one-donor crosses reveal heritability but no effect on sporophytic-offspring fitness. *Annals of Botany* **103**, 941–950.
- Lee CL.** 1980. Pollen germination, pollen tube growth and fertilization behaviour of *Prunus domestica*. II. Pollen tube growth in the style. *Gartenbauwissenschaft* **45**, 241–248.
- Marshall DL.** 1998. Pollen donor performance can be consistent across maternal plants in wild radish (*Raphanus sativus*, Brassicaceae): a necessary condition for the action of sexual selection. *American Journal of Botany* **85**, 1389–1397.
- Marshall DL, Folsom MW, Hatfield C, Bennet T.** 1996. Does interference competition among pollen grains occur in wild radish? *Evolution* **50**, 1842–1848.
- Marshall DL, Shaner MGM, Oliva J.** 2007. Effects of pollen load size on seed paternity in wild radish: the roles of pollen competition and mate choice. *Evolution* **61**, 1925–1937.
- Marshall DL, Avritt JJ, Shaner M, Saunders RL.** 2000. Effects of pollen load size and composition on pollen donor performance in wild radish, *Raphanus sativus* (Brassicaceae). *American Journal of Botany* **87**, 1619–1627.
- Mulcahy DL.** 1974. Correlation between speed of pollen tube growth and seedling height in *Zea mays* L. *Nature* **249**, 491–493.
- Mulcahy DL, Mulcahy GB.** 1975. The influence of gametophytic competition on sporophytic quality of *Dianthus chinensis*. *Theoretical and Applied Genetics* **46**, 277–280.
- Mulcahy DL, Mulcahy GB.** 1987. The effects of pollen competition. *American Science* **75**, 44–50.
- Nakagawa S, Matsui H, Yuda E, Murofushi N, Takahashi N.** 1979. Biologically active gibberellins in immature seeds of *Pyrus serotina*. *Phytochemistry* **18**, 1695–1697.
- Nitsch JP.** 1952. Plant hormones in the development of fruits. *Quarterly Review of Biology* **27**, 33–57.
- Pasonen HL, Kapyla M.** 1998. Pollen–pollen internations in *Betula pendula* in vitro. *New Phytologist* **138**, 481–487.
- Pharis RP, King RW.** 1985. Gibberellins and reproductive development in seed plants. *Annual Review of Plant Physiology* **36**, 517–568.
- Sari-Gorla M, Pé ME, Mulcahy DL, Ottaviano E.** 1992. Genetic dissection of pollen competitive ability in maize. *Heredity* **69**, 423–430.
- Schlichting CD, Stephenson AG, Small LE.** 1990. Pollen loads and progeny vigor in *Cucurbita pepo*: the next generation. *Evolution* **44**, 1358–1372.
- Singh DP, Jermakow AM, Swain SM.** 2002. Gibberellins are required for seed development and pollen tube growth in *Arabidopsis*. *The Plant Cell* **14**, 3133–3147.
- Snow AA.** 1991. Effects of pollen-load size on sporophyte competitive ability in two *Epilobium* species. *American Midland Naturalist* **125**, 348–355.
- Snow AA, Spira TP.** 1991. Pollen vigor and the potential for sexual selection in plants. *Nature* **352**, 796–797.
- Sponsel VM, Hedden P.** 2004. Gibberellin biosynthesis and inactivation. In: Davies PJ, ed. *Plant hormones: biosynthesis, signal transduction, action!* Dordrecht, The Netherlands: Kluwer Academic Publishers, 63–94.

- Suzuki A, Shimizu T, Aoba K.** 1998. Effects of leaf/fruit ratio and pollen density on highbush blueberry fruit quality and maturation. *Journal of the Japanese Society for Horticultural Science* **67**, 739–743.
- Taylor LP, Hepler PK.** 1997. Pollen germination and tube growth. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 461–491.
- Tonutti P, Ramina A, Cossio F, Bargioni G.** 1991. Effective pollination period and ovule longevity in *Prunus avium* L. *Advances in Horticultural Science* **5**, 157–162.
- Weiss D, Izhaki A, Ben-nissan G, Dagan Y, Borochoy A.** 1997. GA-induced gene expression in petunia flowers. *Plant Physiology* **114**, Supplement, S160.
- Yamane H, Tao R.** 2009. Molecular basis of self-(in)compatibility and current status of S-genotyping in Rosaceous fruit trees. *Journal of the Japanese Society for Horticultural Science* **78**, 137–157.
- Yuda E, Nakagawa S, Matsui H, Wada K, Imai T.** 1981. The effect of 15- β -OH gibberellins on the fruit set and development of pears. *HortScience* **16**, 436.
- Zhang C, Lee U, Tanabe K.** 2008. Hormonal regulation of fruit set, parthenogenesis induction and fruit expansion in Japanese pear. *Plant Growth Regulation* **55**, 231–240.
- Zhang C, Tanabe K, Lee U, Kang S, Tokunaga T.** 2009. Gibberellins and N-(2-chloro-4-pyridyl)-N'-phenyl urea improve retention force and reduce water core in pre-mature fruit of Japanese pear 'Housui'. *Plant Growth Regulation* **58**, 25–34.
- Zhang C, Tanabe K, Tamura F, Itai A, Yoshida M.** 2007b. Role of gibberellins in increasing sink demand in Japanese pear fruit during rapid fruit growth. *Plant Growth Regulation* **52**, 161–172.
- Zhang C, Tanabe K, Tamura F, Matsumoto K, Yoshida A.** 2005. ¹³C-photosynthate accumulation in Japanese pear fruit during the period of rapid fruit growth is limited by the sink strength of fruit rather than by the transport capacity of the pedicel. *Journal of Experimental Botany* **56**, 2713–2719.
- Zhang C, Tanabe K, Tani H, Nakajima H, Mori M, Sakuno E.** 2007a. Biologically active gibberellins and ABA in fruit of two late-maturing Japanese pear (*Pyrus pyrifolia* Nakai) cultivars with contrasting fruit size. *Journal of the American Society for Horticultural Science* **132**, 452–458.
- Zhang C, Tanabe K, Wang S, Tamura F, Yoshida A, Matsumoto K.** 2006. The impact of cell division and cell enlargement on the evolution of fruit size in *Pyrus pyrifolia*. *Annals of Botany* **98**, 537–543.