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Troubles in pear pollination: Effects of collection and storage method on pollen viability and fruit production

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ABSTRACT

Pear trees require insect pollination among compatible cultivars to set fruit. However, pollen transfer limitation is the main cause of poor yields in this species, due to low visitation rates and few pollen transfer among compatible cultivars. Assisted pollination using "pollen dispensers" containing precollected compatible pollen fitted into the opening of a beehive could increase yield. We investigated whether stored pollen collected from flowers or bee visitors is suitable for pollinating two European pear (*Pyrus communis*) cultivars, 'Conférence' and 'Doyenné du Comice'. Pollen collected directly from flowers and indirectly from insects was stored for up to 1 year at 4 °C and −20 °C. Pollen stored at −20 °C showed high viability for both cultivars (60–72%). Consequently, fruit set and fruit diameter using this pollen for pollination was similar to that of fresh pollen. However, the viability of pollen that had been stored at 4 °C was low (6%–14%) and fruit diameter was 6.5% lower when flowers were pollinated with pollen stored at 4 °C compared to fresh pollen. The viability of bee-collected pollen was up to 62% lower than that of pollen collected from flowers. The use of bee-collected pollen as a pollen source resulted in a 48% lower fruit set and 95% lower seed set in 'Conférence' compared to fresh pollen, whereas it did not affect 'Doyenné du Comice' fruit and seed sets. The fruit diameter was smaller when flowers were pollinated with bee-collected pollen than with fresh pollen in both 'Conférence' (54.7 vs. 60.6 mm) and 'Doyenné du Comice' (62.5 vs. 70.0 mm). Therefore, pear pollen collected from flowers and stored at −20 °C could successfully be used for alternative pollination without affecting fruit production.

1. Introduction

The majority of flowering plants, including 75% of crop species, depends on animals for pollen transfer and successful reproduction (Hanley et al., 2015; Ollerton et al., 2011). This essential ecosystem service plays a key role in biological diversity and agricultural productivity (Hanley et al., 2015). The economic value of pollination worldwide was estimated to amount between US$215 billion and US$ 361 billion although these figures should be considered with caution (Gallai et al., 2009; Hanley et al., 2015; Lautenbach et al., 2012). Loss of pollinators therefore has dramatic consequences for ecosystems and agricultural productivity. Concerns and evidences of the decline of insects in general and pollinators in particular have accumulated in recent decades (Hanley et al., 2015; Sánchez-Bayo and Wyckhuys, 2019). Need of alternative pollination systems for entomophilous crop species may thus increase in the next years due to pollinator decline. Such artificial pollination by humans already exist in several highly pollinator-dependent crops due to peculiarities of their reproductive system, a lack of specialized pollinators, or particular conditions of cultivation (Sáez et al., 2019; Slavković et al., 2016; Westerkamp and Gottsberger, 2000; Wongsheree et al., 2013).

Like most rosaceous species, European pear (*Pyrus communis*) tree is a self-incompatible entomophilous species (De Franceschi et al., 2012) whose fruit production relies on compatible intercultivar pollination and fertilization. Fertilization and seed development increase fruit diameter and, consequently, the commercial value of the fruit (Monzón et al., 2004; Quinet and Jacquemart, 2017; Theron, 2011). The main pear pollinators are honeybees although bumblebees and solitary bees are also efficient pear pollinators (Quinet et al., 2016b; Quinet and Jacquemart, 2017). Moreover, cultivar placement in the orchard was also shown to affect pollination efficiency in this species (Quinet and Jacquemart, 2017). To promote intercultivar pollination, compatible pollinizer cultivars are planted in orchards in addition to the main cultivar (Delaplane and Mayer, 2000). In Belgium, 'Conférence' accounts for 90% of pear production, and 'Doyenné du Comice' is commonly used as a pollinizer. Cultivars must flower synchronously and be attractive to insects to allow for effective pollen transfer (Stern et al., 2007). Most pear cultivars flower in early spring and their flowering

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period lasts approximately 10 days (Monzón et al., 2004; Quinet et al., 2016b; Quinet and Jacquemart, 2017; Webster, 2002). Asynchronous flowering between cultivars and an inadequate number of pollinators during the flowering period limit pollen transfer onto stigmas and, therefore, fertilization and fruit production (Gemeda et al., 2018; Monzón et al., 2004; Quinet et al., 2016b; Quinet and Jacquemart, 2017; Webster, 2002; Zisovich et al., 2012).

Insufficient pollination, a common cause of poor yields in pears, could be explained by poor visitation rates and pollen transfer limitations (Monzón et al., 2004; Quinet and Jacquemart, 2017). Indeed, pear flowers are not as attractive as those of other fruit trees due to the low volume of nectar secreted per flower (< 3 μL) and its low sugar concentration (< 25%) (Delaplane and Mayer, 2000; Farkas et al., 2002; Quinet et al., 2016b, 2019abib_Quinet_et_al_2019abib_Quinet_et_al_2016b; Quinet and Jacquemart, 2017). Moreover, bad weather conditions or asynchronous flowering between cultivars may limit insect visits and pollen transfer (Monzón et al., 2004; Quinet et al., 2016a).

Alternative pollination systems have been developed to sustain pollination in fruit tree species and ensure successful fruit set (Quinet et al., 2016a; Shivanna, 2015). Assisted pollination can be performed using “pollen dispensers” containing precollected compatible pollen fitted into the opening of a beehive (Hatjina, 1998; Townsend et al., 1957). Honeybees or bumblebees are forced to walk through the pollen dispensers, become covered with pollen during their exit from the hive, and transport compatible pollen when foraging on the target flowers (Dag et al., 2000; Hatjina, 1998; Quinet et al., 2016a; Sharma et al., 2014). Another assisted pollination method involves spraying precollected pollen powder onto the target flowers using pollen blowers (Awad, 2010; Hopping and Jerram, 1980; Sakamoto et al., 2009). These techniques could overcome pollination deficits in self-incompatible species and limit the need for pollinizer cultivars and/or insect visitors (Shivanna, 2015). However, their usefulness remains under debate depending on the studies and fruit species (Dag et al., 2000; Hatjina, 1998; Sharma et al., 2014; Shivanna, 2015; Townsend et al., 1957).

The efficient use of pollen dispensers or pollen spraying requires the selection of suitable pollen (Townsend et al., 1957). Suitable pollen should be compatible with the target flowers and have high pollen viability (Bieniasz et al., 2017; Kuroki et al., 2017; Sakamoto et al., 2009; Townsend et al., 1957; Webster, 2002). The pollen used for assisted pollination is often collected by hand, which constitutes a high cost (Dag et al., 2000; Hatjina, 1998; Sakamoto et al., 2009; Townsend et al., 1957). Pollen collection from flowers is indeed time-consuming and expensive and poses a major restriction to the use of alternative pollination systems (Hopping and Jerram, 1980; Sakamoto et al., 2009). A more economical method for pollen collection involves installing pollen traps at the entrance of the hives to collect pollen from bee corbicular structures (Gemeda et al., 2018; Mauriello et al., 2017; Townsend et al., 1957). Pollen loads are usually considered as lost pollen for pollination since corbicular pollen did not touch the stigma when bees visit flowers. Although the viability of bee-collected pollen was reported to decrease more rapidly than hand-collected pollen (Townsend et al., 1957), some authors consider that the quality of bee-collected pollen is sufficient for use in artificial pollination (Griggs and Vansell, 1949; Okada et al., 1983 cited in Alspach et al., 1992).

Preserving the viability of pollen for subsequent use in artificial pollination has recently emerged as a focus of interest (Bhat et al., 2012; Imani et al., 2011; La Franchie, 2002). Storage conditions of floral pollen have been investigated in several fruit trees such as apple (*Malus pumila*), pear, quince (*Cydonia oblonga*), sweet cherry (*Prunus avium*), almond (*Prunus dulcis*), and apricot (*Prunus armeniaca*) while storage conditions of bee-collected pollen were little investigated (Alburquerque et al., 2007; Bhat et al., 2012; Imani et al., 2011; Martínez-Gómez et al., 2002; Pereira et al., 2014; Perveen and Khan, 2008; Sharafi, 2011; Sharafi and Bahmani, 2011). Floral pollen stored at 4 °C for 1–3 months is usually viable, depending on the fruit tree species, but longer storage periods required usually temperatures below 0 °C (La Franchie, 2002; Martínez-Gómez et al., 2002). Regardless of the species, pollen viability following cold storage varies among cultivars of the same species (Alburquerque et al., 2007; Bhat et al., 2012; Kuroki et al., 2017; Martínez-Gómez et al., 2002; Sharafi, 2011; Sharafi and Bahmani, 2011).

Although the influence of storage temperature on floral pollen viability has been investigated in several fruit tree species, little is known about how different storage methods would affect fruit production and quality (Pereira et al., 2014). Most studies investigating the impact of pollen storage on pollen quality have involved the analysis of pollen viability and/or pollen germination percentages *in vitro* (Alburquerque et al., 2007; Imani et al., 2011; Martínez-Gómez et al., 2002; Perveen and Khan, 2008; Sharafi, 2011; Sharafi and Bahmani, 2011). However, pollen tube growth *in vivo* and subsequent fruit production and quality have not been investigated. Likewise, the feasibility of using bee-collected pollen has yet not been assessed for pear pollination.

In this study, we investigated for the first time whether stored pollen and pollen collected by bees are suitable for European pear tree pollination and affect fruit production and quality in the two main cultivated cultivars in Belgium, ‘Conférence’ and ‘Doyenné du Comice’. To this aim, we addressed the following questions: (i) Do pollen storage methods and duration affect pollen viability, fruit production, and quality? and (ii) Is bee-collected pollen suitable for pollination?

2. Material and methods

2.1. Site and plant material

This study was conducted between 2014 and 2016 in a 0.6-ha orchard at Centre Fruitier Wallon (CEF) in Wasseiges (50° 38′ 31″ N; 5° 0′ 15″ E), Belgium. ‘Conférence’ (on Quince C rootstock) was planted at 3.4 × 1.3 m (2262 trees/ha), and ‘Doyenné du Comice’ was present as the pollinizer cultivar. The pollinizer cultivar was planted in the same row as the main cultivar at a mean ratio of 1 ‘Doyenné du Comice’ pollinizer tree to 16 ‘Conférence’ trees. At the beginning of the flowering period, one honeybee (*Apis mellifera*) hive (~40,000 individuals/hive) and one bumblebee (*Bombus terrestris*) hive (multihive, Biobest, Westerlo, Belgium; ~450 individuals/hive) were introduced into the orchard.

2.2. Pollen collection, storage, and viability assessment

Two collection methods were compared: floral pollen and bee-collected pollen. Floral pollen was hand-collected from ‘Conférence’ and ‘Doyenné du Comice’ using hundreds of newly opened flowers during three spring seasons (2014, 2015, and 2016). Bee-collected pollen was removed from the corbicular structures of approximately 10 honeybees and 10 bumblebees visiting ‘Conférence’ and ‘Doyenné du Comice’ flowers in 2014 and 2015. After the bees had been immobilized in a bee-marking cage, one of the two pollen loads present in the corbiculae was carefully removed with a toothpick. We checked that the majority of the insect pollen loads comprised pear pollen via light microscopy (Eclipse E400; Nikon, Amsterdam, The Netherlands) before viability tests and/or storage treatments.

To test storage conditions, floral pollen collected in 2014 and 2015 was divided into three samples: fresh (control), stored at 4 °C, and stored at −20 °C. Pollen viability was assessed immediately after collection, after 2 months, and after 1 year of storage.

Pollen viability was assessed using fluorescein diacetate staining according to Dafni et al. (2005). This method was previously successfully used to assess pear pollen viability (Quinet et al., 2016a, 2019abib_Quinet_et_al_2016abib_Quinet_et_al_2019a). For floral pollen, a minimum of five samples per storage treatment and 200 pollen grains per sample were observed. For bee-collected pollen, a minimum of 200 pollen grains were observed per pollen load. The percentage of pollen viability was calculated as the ratio between the number of fluorescent grains and the total number of grains counted under a microscope (Eclipse E400; Nikon, Amsterdam, The Netherlands).

2.3. Pollination trials

Pollination trials were carried out in 2016. Due to self-incompatibility within a cultivar, 'Conférence' flowers were hand-pollinated with 'Doyenné du Comice' pollen, while 'Doyenné du Comice' flowers were hand-pollinated with 'Conférence' pollen. Hand pollination was performed with (i) floral pollen (freshly collected from the orchard), (ii) floral pollen stored for 1 year at 4 °C (only for 'Conférence'), (iii) floral pollen stored for 1 year at −20 °C, and (iv) bee-collected pollen stored for 1 year at −20 °C. Pollen loads collected on 'Doyenné du Comice' were used to pollinate 'Conférence' flowers and vice versa. Hand-pollinations were performed in the orchard on 10 to 15 inflorescences on three trees per treatment (minimum of 200 flowers). Flowers were emasculated at the balloon stage to avoid incompatible self-pollination as emasculation has no effect on stigma receptivity. The emasculated flowers were then bagged during the flowering period to avoid insect visits. Pollen from the male parent was deposited onto the stigmas of the female parent. Each flower was pollinated twice (2 days apart) to increase pollination success.

Three days after pollination, 10 flowers per treatment were harvested, and the styles were stained with 0.1% (w/v) aniline blue solution to determine pollen tube growth according to Jacquemart et al. (2006). Fruit initiation was recorded 2 weeks after pollination and fruit set was recorded in September. At harvest, the fruit "quality" i.e. fruit weight, length, diameter, sugar content, and the seed set i.e. number of viable and aborted seeds per fruit were quantified using 30 fruits per cultivar as described by Quinet and Jacquemart (2017) and Quinet et al. (2019b).

2.4. Statistical analyses

Statistical analyses were conducted using SAS Enterprise Guide 7.1. The normality of the data was checked using Shapiro–Wilk tests, and homoscedasticity was verified using Levene's tests. The data were transformed when required to ensure normal distributions, and Welch's correction was applied when homoscedasticity was not met. Pollen viability was compared among treatments using analyses of variance (ANOVA). Fruit parameters (seed sets, fruit weight, length, size, and sugar content) were analyzed by ANOVA and were weighted by the number of fruits per tree at harvest. Post-hoc analyses were performed using Tukey's tests to investigate the differences among treatments. Pollen tube growth, fruit initiation, and fruit set were compared among pollination treatments for each cultivar using chi-square tests. The data are presented as means ± standard errors unless otherwise indicated.

3. Results

3.1. Does pollen storage affect fruit production and quality?

The viability of fresh floral pollen was > 85% for both cultivars and strongly decreased after storage at 4 °C (Table 1). Pollen viability decreased by 93% in 'Conférence' and 85% in 'Doyenné du Comice' after 1 year of storage at 4 °C. However, floral pollen viability remained high after storage at −20 °C, with values > 60% after 1 year of storage at −20 °C for both cultivars (Table 1). Pollen viability did not differ between cultivars ($F_{1,376} = 1.74$, $p = 0.1881$).

Pollen storage decreased *in vivo* pollen tube growth in 'Conférence' regardless of temperature ($\chi^2 = 61.397$, df = 4, $p < 0.0001$, Fig. 1A), but this negative impact was mainly observed at 4 °C. Indeed, few pollen tubes were observed in the styles of flowers pollinated with pollen stored at 4 °C compared to the other treatments. However, pollen storage did not affect fruit initiation ($\chi^2 = 0.027$, df = 2, $p = 0.986$, Fig. 1C) or fruit set ($\chi^2 = 0.45$, df = 3, $p = 0.9859$, Fig. 1C). Pollen storage at 4 °C and −20 °C reduced viable seed set by 97% and 79%, respectively, compared to fresh pollen ($F_{2,69} = 13.67$, $p < 0.0001$, Fig. 1C). Pollen storage at −20 °C did not affect the fruit quality parameters of 'Conférence' (Fig. 2 A, C, E, G), and storage at 4 °C only resulted in a reduction of fruit diameter in this cultivar (56.8 mm vs. 59.4 mm, $F_{2,69} = 60.90$, $p < 0.0001$, Fig. 2E) compared to fresh pollen.

Table 1
Effects of storage methods and durations on pollen viability in 'Conférence' and 'Doyenné du Comice' pear cultivars.

Storage	Duration	'Conférence'		'Doyenné du Comice'	
		n	viability (%)	n	viability (%)
Fresh		10	86.62 ± 2.56 a	10	96.34 ± 0.99 a
4 °C	2 months	5	60.02 ± 11.80 b	5	37.00 ± 8.78 c
	1 year	4	6.04 ± 2.32 c	7	13.70 ± 3.42 d
−20 °C	2 months		–	5	93.28 ± 1.07 a
	1 year	8	60.13 ± 7.35 b	9	72.04 ± 4.09 b
ANOVA2	Storage	$F_{1,151} = 118.65$, $p < 0.0001$		$F_{1,225} = 427.23$, $p < 0.0001$	
	duration	$F_{1,151} = 302.82$, $p < 0.0001$		$F_{1,225} = 85.57$, $p < 0.0001$	
	interaction	–		$F_{1,225} = 0.033$, $p = 0.9856$	

Values followed by different letters for the same cultivar are significantly different according to Tukey's tests ($p < 0.05$).

In vivo pollen tube growth was also reduced by pollen storage at −20 °C in 'Doyenné du Comice', although it remained sufficient, since 33% of the flowers contained more than 10 pollen tubes per style ($\chi^2 = 22.71$, df = 2, $p < 0.0001$, Fig. 1B). Pollen storage at −20 °C did not affect fruit initiation ($\chi^2 = 0.136$, df = 1, $p = 0.712$, Fig. 1D), fruit set ($\chi^2 = 0.06$, df = 1, $p = 0.806$, Fig. 1D) or seed set ($F_{1,19} = 0.16$, $p = 0.6915$; $F_{1,19} = 0.10$, $p = 0.7527$ for viable and aborted seeds, respectively, Fig. 1F) in this cultivar. 'Doyenné du Comice' fruit weight ($F_{1,19} = 1.93$, $p = 0.1527$, Fig. 2B), length ($F_{1,19} = 0.02$, $p = 0.8769$, Fig. 2D), diameter ($F_{1,19} = 0.31$, $p = 0.5829$, Fig. 2F), and sugar content ($F_{1,19} = 0.55$, $p = 0.4663$, Fig. 2H) were not affected by pollen storage at −20 °C.

3.2. Is bee-collected pollen suitable for pear pollination?

The viability of pollen collected from flowers sometimes differed from that of pollen collected and packed in pollen loads by bees, depending on the year ($F_{1,112} = 191.03$, $p < 0.0001$). Specifically, pollen viability was ~60% lower for bee-collected pollen than for floral pollen in 2014, whereas no difference in pollen viability was observed in 2015 regardless of the insect type (Table 2). The viability of bee-collected pollen decreased by 20% after 1 year of storage at −20 °C.

In 'Conférence', fewer pollen tubes were observed in the styles after pollination with bee-collected pollen compared to floral pollen ($\chi^2 = 61.397$, df = 4, $p < 0.0001$, Fig. 1A). Pollination with bee-collected pollen did not affect fruit initiation ($\chi^2 = 0.13$, df = 2, $p = 0.9369$, Fig. 1C), but it decreased fruit set by 55% compared to floral pollen ($\chi^2 = 10.51$, df = 2, $p = 0.0052$, Fig. 1C). Bee-collected pollen strongly reduced seed set, decreasing both the number of viable ($F_{2,69} = 13.63$, $p < 0.0001$) and aborted ($F_{2,69} = 8.01$, $p = 0.0007$) seeds. Viable seed set dropped from 20% for floral pollen to less than 1% for bee-collected pollen (Fig. 1E). Pollination with bee-collected pollen also reduced fruit weight ($F_{2,69} = 4.57$, $p = 0.0137$, Fig. 2A), length ($F_{2,69} = 4.16$, $p = 0.0196$, Fig. 2C), and diameter ($F_{2,69} = 8.11$, $p = 0.0007$, Fig. 2E) by 10%, while it increased fruit sugar content by 5% ($F_{2,69} = 6.08$, $p = 0.0037$, Fig. 2G) compared to floral pollen.

In 'Doyenné du Comice', the percentage of styles with growing pollen tubes differed depending on the pollination treatment

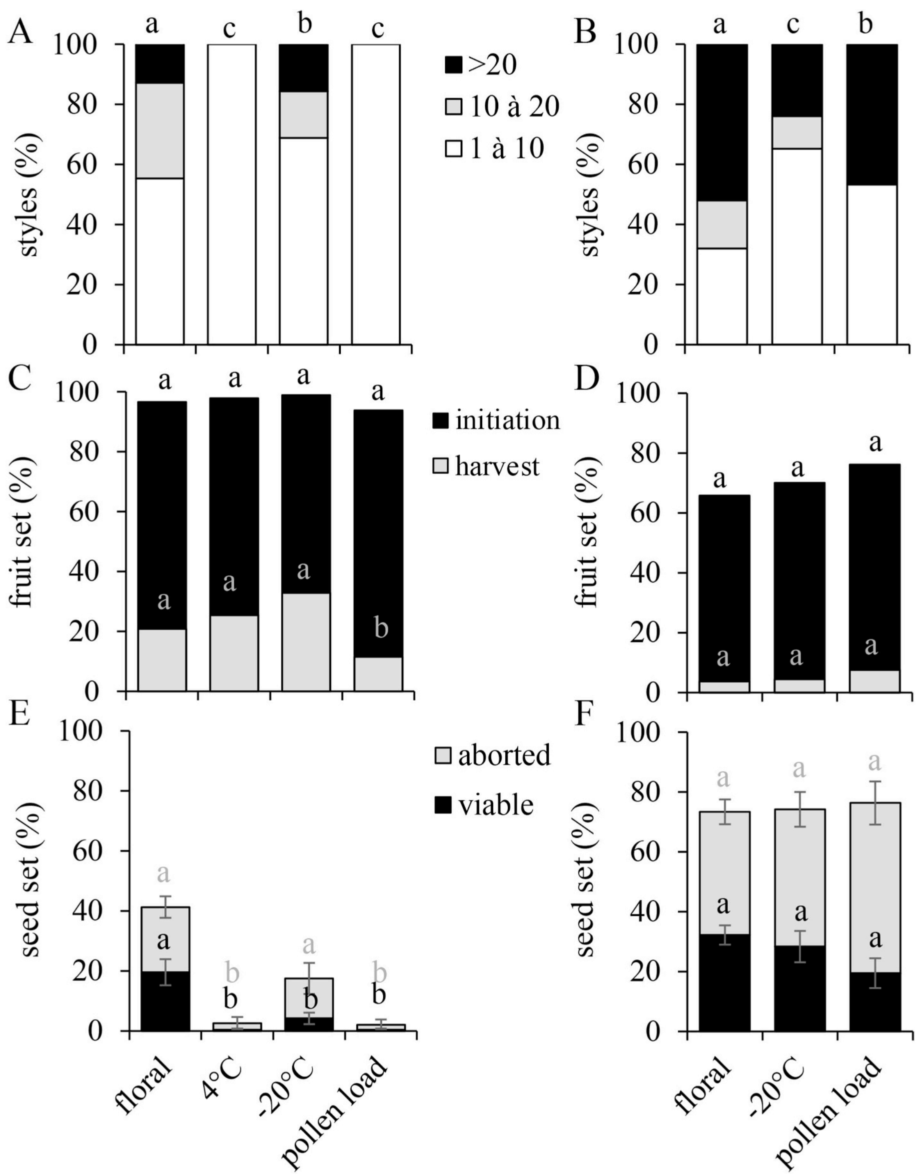

Fig. 1. Effects of pollen storage method on pollen tube growth, fruit, and seed set in 'Conférence' (A, C, E) and 'Doyenné du Comice' (B, D, F) pear cultivars. 'Conférence' or 'Doyenné du Comice' flowers were pollinated with compatible floral pollen (floral), compatible pollen stored for 1 year at 4 °C (4 °C) or −20 °C (−20 °C), or pollen collected by honeybees and bumblebees (pollen loads). (A–B) Percentage of styles with growing pollen tubes according to the number of pollen tubes per style. (C–D) Fruit set, calculated as the percentage of flowers that initiated fruit development 2 weeks after pollination (initiation) and that resulted in a fruit at harvest (harvest). (E–F) Seed set, the percentage of viable and aborted seeds per fruit at harvest with respect to the number of ovules. Values followed by different letters are significantly different at $p < 0.05$.

($\chi^2 = 37.089$, df = 4, $p < 0.0001$, Fig. 1B), but 47% and 52% of the styles contained more than 20 pollen tubes after pollination with bee-collected and floral pollen, respectively. Pollination treatment did not affect fruit initiation ($\chi^2 = 0.773$, df = 2, $p = 0.679$, Fig. 1D), fruit set ($\chi^2 = 1.663$, df = 2, $p = 0.435$, Fig. 1D) or seed set ($F_{2,37} = 1.15$, $p = 0.3266$; $F_{2,37} = 0.98$, $p = 0.3845$ for viable and aborted seeds, respectively, Fig. 1F). Pollination with bee-collected pollen reduced fruit weight by 25% ($F_{2,37} = 4.55$, $p = 0.0171$, Fig. 2A) and fruit diameter by 10% ($F_{2,37} = 5.76$, $p = 0.0066$, Fig. 2E) but did not affect fruit length ($F_{2,37} = 1.50$, $p = 0.2363$, Fig. 2D) or fruit sugar content ($F_{2,37} = 1.60$, $p = 0.2148$, Fig. 2G) compared to floral pollen.

4. Discussion

4.1. Pollen storage at −20 °C does not affect fruit production

Our results demonstrate that pollen stored for 1 year at −20 °C, but not at 4 °C, remained highly viable. Indeed, pollen viability decreased after storage at 4 °C for only 2 months in both 'Conférence' and 'Doyenné du Comice' cultivars, whereas pollen viability was not affected after 2 months of storage at −20 °C. Similar results were previously obtained for other pear tree cultivars (Bhat et al., 2012; Sharafi, 2011). Pear pollen has low viability, is highly sensitive to desiccation, and loses its viability rapidly when stored at room temperature (Bhat et al., 2012).

The longevity of pollen is related to its desiccation tolerance and its susceptibility to damage from drying (Du et al., 2019; Fonseca and Westgate, 2005; Giovannini et al., 2015; Song and Tachibana, 2007). Pollen dehydration might lead to defects in membrane permeability and irreversible membrane damage during imbibition (Fonseca and Westgate, 2005). Storage at low temperature reduces metabolism and enzyme activity, which slows the rate at which pollen viability declines (Du et al., 2019). Notably, a decline in pear pollen viability with an increased storage period was previously observed over a range of storage temperatures (Bhat et al., 2012). Pollen deterioration due to aging has been reported for several plant species, even when the pollen was stored at low temperatures (Du et al., 2019; Fonseca and Westgate, 2005; Song and Tachibana, 2007).

Even though the precise cause of the loss of pollen viability is not well understood, deleterious physical and chemical changes occur gradually, including disrupted intracellular integrity, decreased enzyme activity, the accumulation of free radicals, and reduced protein synthesis (Song and Tachibana, 2007). Pollen from almond and apple trees could be stored for longer periods at 4 °C compared to pear pollen

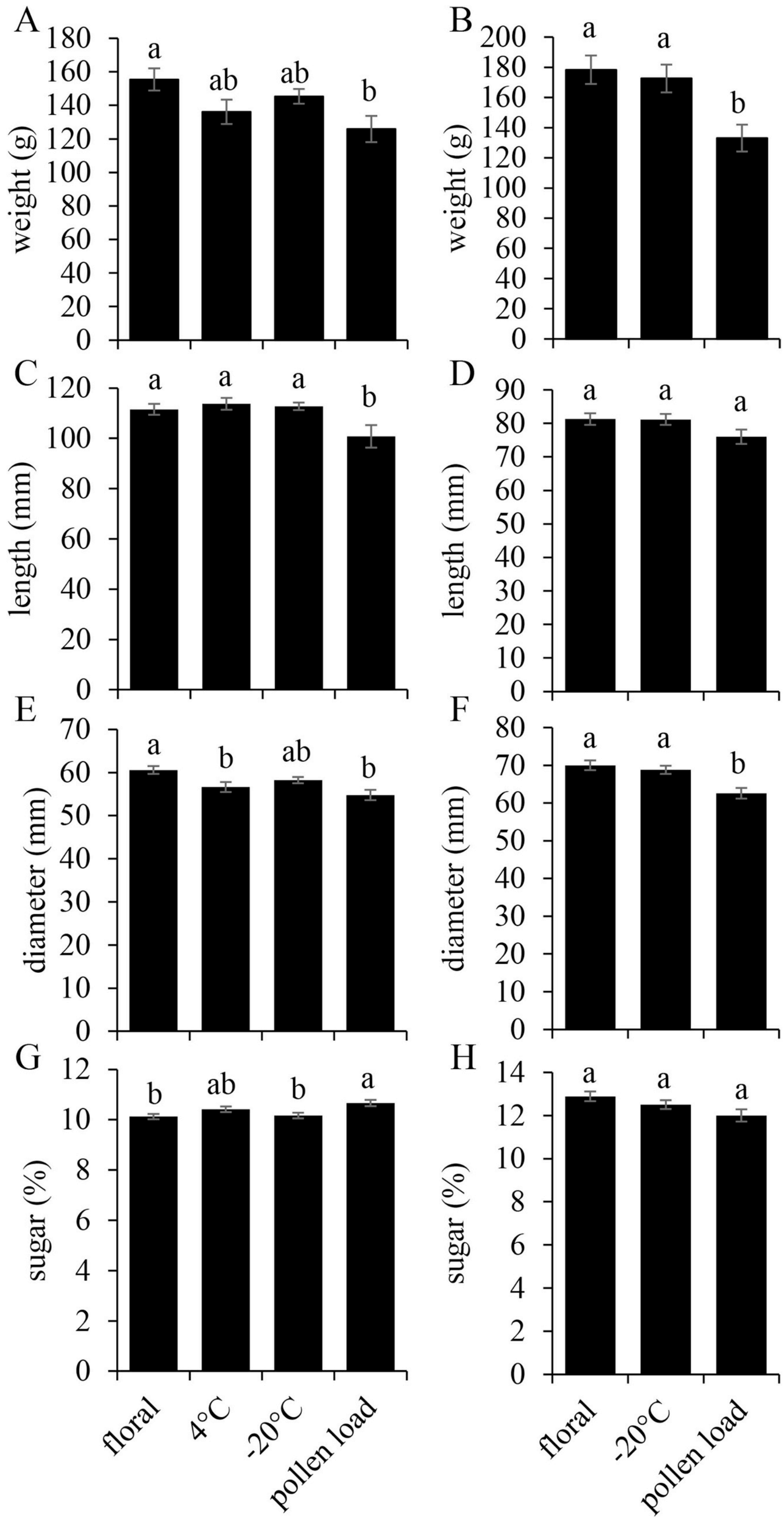

Fig. 2. Effect of pollen storage method on fruit parameters in 'Conférence' (A, C, E, G) and 'Doyenné du Comice' (B, D, F, H) pear cultivars. 'Conférence' or 'Doyenné du Comice' flowers were pollinated with compatible floral pollen (floral), compatible pollen stored for 1 year at 4 °C (4 °C) or −20 °C (−20 °C), or pollen collected by honeybees and bumblebees (pollen loads). (A–B) Fruit weight. (C–D) Fruit length. (E–F) Fruit diameter. (G–H) Fruit sugar content. Values followed by different letters are significantly different at $p < 0.05$.

Table 2
Comparison of pollen viability percentages (based on fluorescein staining) of floral pollen and pollen collected by honeybees and bumblebees.

Origin	2014		2015	
	n	viability (%)	n	viability (%)
Floral	10	98.32 ± 1.63 a	20	91.48 ± 1.74 a
Honeybee	28	37.86 ± 2.37 b	20	90.21 ± 1.30 a
Bumblebee	22	35.95 ± 2.55 b	20	90.60 ± 1.21 a
ANOVA1		$F_{2,57} = 86.80$, $p < 0.0001$		$F_{2,57} = 0.15$, $p = 0.8620$

Values followed by different letters for the same cultivar are significantly different according to Tukey's tests ($p < 0.05$).

(Martínez-Gómez et al., 2002; Perveen and Khan, 2008; Sharafi and Bahmani, 2011). Nevertheless, for long (> 1 year) storage periods, temperatures of −20 °C or lower are recommended for fruit tree species (Alburquerque et al., 2007; Imani et al., 2011; Martínez-Gómez et al., 2002; Perveen and Khan, 2008; Sharafi and Bahmani, 2011). Regardless of the species, repeated freezing and thawing of pollen grains reduces pollen viability (Bhat et al., 2012).

The temperature at which pollen is stored also affects subsequent fruit production and quality. In this study, storage for 1 year at 4 °C significantly decreased pollen viability, and the use of this pollen for pollination trials limited pollen tube growth, seed set, and fruit diameter in 'Conférence'. However, the viability of 'Conférence' and 'Doyenné du Comice' pollen stored at −20 °C for 1 year remained high enough to be used for pollination without affecting fruit set or fruit size. Nevertheless, storage at −20 °C decreased pollen tube growth in both cultivars compared to fresh pollen and decreased viable seed set in 'Conférence' fruits. In Asian pear, La Franchie (2002) showed that storing pollen for 1 year at −18 °C reduced fruit set only slightly compared to fresh pollen, while after 2 years of storage, fruit set significantly decreased. Fruit weight also decreased with increasing duration of pollen storage (La Franchie, 2002). Differences in fruit weight, fruit diameter, and number of seeds per fruit due to different pollen storage treatments have also been observed in sugar apple (*Annona squamosa*) (Pereira et al., 2014).

4.2. Bee-collected pollen decreases fruit quality

In pear tree, the viability of bee-collected pollen was lower than that of floral pollen, and pollen viability differed according to the year. In Asian pear, La Franchie (2002) reported that the viability of bee-collected pollen was < 27%. Several studies have shown that pollen from pollen loads loses its viability more rapidly than hand-collected pollen (Kremer, 1949; Singh and Boynton, 1949), although other studies have suggested that bee-collected pollen is satisfactory for use in artificial pollination (Griggs and Vansell, 1949; Okada et al., 1983 cited in Alspach et al., 1992). Bees collect pollen from flowers and moisten it with nectar and mouth secretions, allowing it to adhere to their rear legs and form pollen loads (Mauriello et al., 2017). The formation of pollen loads affects the biochemical composition of pollen (Roulston and Cane, 2000; Vanderplanck et al., 2014), and we therefore cannot exclude the possibility that this process also decreases pollen viability. As the viability of pear floral pollen decreases by more than half after 2 h of storage at room temperature (La Franchie, 2002), bee-collected pollen might quickly lose its viability in this species. The differences in the viability of bee-collected pollen between years could be explained by environmental conditions in the orchard, as the mean daily temperature was 3 °C higher in 2014 than in 2015 during the flowering period. Temperature increase was indeed shown to decrease pollen viability even for fresh floral pollen (Descamps et al., 2018).

In this study, we assessed bee-collected pollen with 70% pollen viability in pear tree pollination trials. In 'Conférence', pollination with bee-collected pollen resulted in reduced pollen tube growth, fruit set, and seed set compared to floral pollen, while in 'Doyenné du Comice', the pollen source did not affect fruit or seed set. This discrepancy could be explained by a higher proportion of 'Conférence' than 'Doyenné du Comice' pollen in the bee pollen loads and thus at a higher deposit of non-compatible pollen on 'Conférence' stigma. Indeed, as there was more 'Conférence' trees in the orchard than 'Doyenné du Comice' trees, we may not exclude that bees collected on 'Doyenné du Comice' contained 'Conférence' pollen in their pollen loads. However, pollination with bee-collected pollen reduced fruit weight and fruit diameter in both cultivars. Thus, for both cultivars, bee-collected pollen resulted in less fruit production and/or a lower fruit quality than floral pollen; therefore, this pollen is not recommended for pear pollination. Pollen loads are often considered as lost pollen for pollination since corbicular pollen did not touch the stigma when bees visit flowers. However, our results suggest that even if pollen loads touch stigma during insect visit, it would not be suitable for successful fertilization.

In apple tree, Johansen (1956) showed that although the viability of bee-collected pollen was more than adequate for pollination (78%), its use resulted in very poor fruit set. Indeed, the use of hand-collected pollen led to ~250% greater fruit set than bee-collected pollen in pollination trials (Johansen, 1956), perhaps because the pollen tubes from bee-collected pollen were unable to penetrate through the style and successfully fertilize the ovules (Johansen, 1956). Such behavior was not observed for 'Doyenné du Comice' in this study. When bee-collected pollen was used in kiwi (*Actinidia deliciosa*) trials, the effectiveness of pollination increased with an increasing rate of pollen application (Alspach et al., 1992). Therefore, the efficiency of bee-collected pollen appears to be species dependent.

Alternative pollination methods using bee-collected pollen or stored pollen have to be tested in other crops and particularly in fruit tree species if pollinator decline continues at this rate (Hanley et al., 2015; Sánchez-Bayo and Wyckhuys, 2019) and if orchards continue to be planted in monotypic blocks to facilitate management (Kron et al., 2001; Quinet and Jacquemart, 2017). Moreover, in the frame of climate change, pollen viability and thus pollination success may decrease in response to temperature increase (Descamps et al., 2018) resulting in a lower fruit production.

5. Conclusions

Pollen stored at −20 °C for up to one year was highly viable, resulting in high fruit production and fruit quality when used in pollination trials. However, floral pollen stored at 4 °C and bee-collected pollen had lower pollen viability, resulting in low fruit production and/or fruit quality in pollination trials. These findings suggest that floral pollen stored at −20 °C could successfully be used for alternative pollination and breeding programs. This result presents an opportunity to develop new pollination techniques in pear orchards. Development of pollen dispensers containing stored floral pollen could be combine with strategies aiming to increase abundance and diversity of insect pollinators in order to improve fruit production while maintaining insect diversity. Nevertheless, studies comparing alternative pollination using floral pollen versus bee-collected pollen, as well as economic studies, are needed to optimize the balance between low cost and high fruit yield in pear tree.

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