Methods for Determining Compatibility and Pollinator Efficiency in Temperate Fruit Species

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ABSTRACT

Fruit yields depend on the successful achievement of a series of sequential processes from floral induction to fruitlet retention. For the successful set of an optimum crop load of most fruit cultivars, pollination and fertilization of flowers must be effective. In this paper, I describe the most commonly used methods for testing self-incompatibility and pollinator efficiency in the main fruit trees. Estimation of self-incompatibility can be done experimentally or genetically. Experimentally, comparisons following self- vs. cross- pollinations can be performed sequentially: (1) difference in fruit or seed sets; (2) different pollen germination and tube growth in the styles (self-incompatibility syndrome); (3) difference in ovule development (ovarian self-incompatibility) or cessation of embryo development after self-pollination (late-acting self-incompatibility). If seed set differences are not explained by differences in these pre-zygotic steps, inbreeding depression can be responsible. The genetic approach of incompatibility with the characterization of *S*-alleles can assess the compatibility level between cultivars. Finally, pollination effectiveness and pollinator efficiency can be assessed with direct and indirect measures. Direct measures focus on pollen deposition and retrieval on virgin flowers. Indirect measures include pollinator guild, relative abundance and behaviour, pollen carryover capacity and gene flow.

CONTENTS

INTRODUCTION

Context

In 2005, overall fruit production in Europe (UE25) reached 74.7 million t (FAOSTAT 2005). Main productions concern grapes (29.8 million t), apples (16.5 million t), *Citrus* sp. (oranges, mandarines, etc) (9.8 million t) and pears (3.7 million t). Fruit yields depend on the successful achievement of a series of sequential processes from floral induction to fruitlet retention. Each of these essential physiological processes is influenced by genetic, environmental, physiological and tree management factors (Barbier 1986; Free 1993; Webster 2002). For the successful set of an optimum crop load of most fruit cultivars, pollination and fertilization of flowers must be effective. For a majority of fruit crops, animal (mainly insect) pollination is important or even essential (McGregor 1976; Free 1993; Klein *et al*. 2006, **Table 1**). For 70% of tropical crops out of 1330 species, the production of at least one variety per species is improved by animal pollination (Roubick 1995). For European crops, Williams (1994) concluded that 84% (out of 264 species) depend at least to some extent upon animal pollination. However, detailed studies of the crop pollination systems

Invited Review

Table 1 Floral biology and pollinators in the main fruit crops. Pollinator effect on yield is defined as essential (production reduction of 90% or more without flower visitors), great (40 to <90% reduction), modest (10 to <40% reduction, in currants) or little (<10%) according to Klein *et al.* 2006. (World production data according to FAOSTAT 2005; pollen and nectar from McGregor 1976 and Free 1993; pollinators from Free 1993 and Klein *et al.* 2006).

| Fruit type	World production (Mt, 2004)	Pollen/flower (mg)	Nectar production /day/flower (mg)	Main pollinators	Pollinator effect on yield
Almond	1 725 638	1.14-1.95	0.77-4.40	Honeybees, bumblebees, solitary bees (*Osmia*), diptera	great
Apple	63 3999 721	0.57-2.05	0.74-7.09	Honeybees, bumblebees, solitary bees (*Andrena*, *Anthophora*, *Osmia*), syrphids (*Eristalis*)	great
Apricot	2 788 008	0.61-1.68	-	Honeybees, bumblebees, solitary bees (*Osmia*), diptera	great
Citrus	110 911 127	-	-	Honeybees, bumblebees	little
Peach	15 346 666	1.10-2.23	2.20-6.40	Honeybees, bumblebees, solitary bees (*Osmia*), diptera	great
Pear	18 680 513	0.62-1.88	0.84-0.85	Honeybees, bumblebees, solitary bees (*Osmia*), syrphids (*Eristalis*)	great
Plum	9 633 255	0.39-1.96	0.80-3.40	Honeybees, bumblebees, solitary bees (*Osmia*), diptera	great
Cherries	1 830 309	0.21-1.95	0.81-4.59	Honeybees, bumblebees, solitary bees (*Osmia*), diptera	great
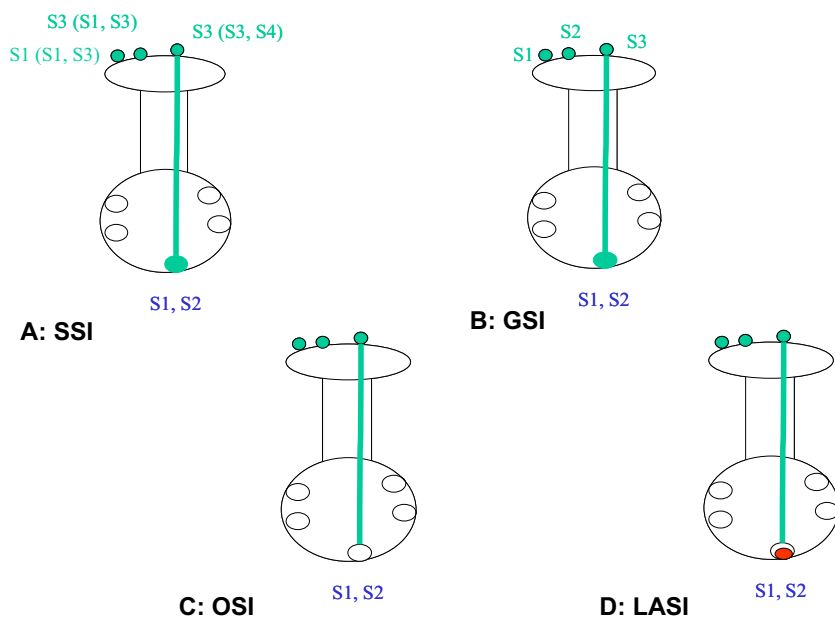
Fig. 1 Schematic representation of the different self-incompatibility (SI) systems. (A) Sporophytic SI, (**B**) gametophytic SI, (**C**) ovarian SI, (**D**) late-acting SI. Haploid *S*-alleles are indicated in green (for sporophytic SI, the paternal diploid genotype is given under brackets). Maternal *S*-alleles are indicated in blue. Ovule colour indicated if it is not fertilized (white), fertilized (green) or aborted (red).

are often incomplete or out of date.

Self-incompatibility

Self-incompatibility (SI) prevents the production of seed following self-pollination. A great number of species and cultivars are self-incompatible or produce more and/or better fruit when cross-pollinated, which requires the transfer of pollen between different cultivars planted within the orchard (Taseï 1984; Free 1993; Webster 2002). Basic knowledge about the self-compatibility of a cultivar is thus essential for fruit growers.

Self-incompatibility may result from a variety of mechanisms. Self-pollen may not adhere to the stigma, adhere but not germinate, or germinate but be unable to penetrate or grow down the style. SI is generally considered to be a pre-zygotic mechanism and does not include seed abortion or reduced viability of inbred offspring (de Nettancourt 2001). Incompatibility systems are broadly classified as gametophytic or sporophytic depending on whether the incompatibility reaction with the maternal tissue is mediated by the genotype of the haploid pollen grain (gametophytic SI) or by the genotype of the diploid anther that produced the pollen (sporophytic SI; **Fig. 1A, 1B**).

Self-incompatibility is estimated to be present in at least half of all angiosperm species. The most widespread system, gametophytic SI (GSI) means that the phenotype of the pollen is determined by its own haploid genotype. Pollen tube growth is prevented if the pollen tube has an S-allele in common with one of the two *S*-alleles in the style. GSI is present in several families including Rosaceae, which includes most of the fruit trees. In almond (*Prunus dulcis*), apple (*Malus domestica*), apricot (*Prunus armeniaca*), pear (*Pyrus communis*), plum (*Prunus domestica*) and

sour and sweet cherries (*Prunus avium*, *P. cerasus*, *P. fruticosa*) the majority of the cultivars are self-incompatible (refs. in **Table 2**). Only peach (*P. persica*) is a self-compatible species (Hegedus *et al.* 2006). Sporophytic SI (SSI) means that the phenotype of the pollen is determined by the diploid genotype of the pollen parent. SSI is found in Brassicaceae and Asteraceae. The concept of SI has been expanded to include apparent pre-zygotic ovarian SI (OSI, Sage *et al.* 1994) and post-zygotic ovarian SI (**Fig. 1C, 1D**). In pre-zygotic OSI ovule development differs according to self- or cross-pollination. In post-zygotic SI, all embryos stop their development at the same stage (on the contrary, inbreeding depression leads to abortion at different stages during embryo development: Seavey and Bawa 1986; Sage *et al.* 1994; de Nettancourt 2001).

In the Solanaceae, the Scrophulariaceae and the Rosaceae, two separate genes at the highly polymorphic *S*-locus control self-incompatibility interactions (Sijacic *et al.* 2004). The *S*-locus products in the pistil are basic glycoproteins having ribonuclease (RNase) activity, called *S*-RNases (Sassa *et al.* 1992; de Nettancourt 2001). These enzymes present a mechanism of blocking self pollen tube growth through the style (Kao and McCubbin 1996). At anthesis, after *S*-RNase is expressed in the style, it invades the germinating pollen grain and inhibits growth. The Rosaceae *S*-RNase differs partly from that of the Solanaceae and it is considered to be another class in the T2/S-RNase superfamily (Sassa *et al.* 1996). The basic structure of the Rosaceae *S*-RNase comprises five highly conserved regions (C1 to C5) and one hypervariable region (RHV) that includes an intron (Broothaerts *et al.* 1996; Romero *et al.* 2004). Pollen-expressed F-box genes (SLF and SFB) showing allelic sequence polymorphism have been identified near to the *S*-RNase gene (Entani *et al.* 2003; Ushijima *et al.* 2003; Sijacic *et al.* 2004;

Table 2 Main references for the sequential tests for self-incompatibility and insect pollinator efficiency for the major temperate fruit tree species. "Pollinations and fruit set" refers to hand pollinations, "Pollen tubes" to observation of pollen tube growth, "SI alleles" to the description of *S*-alleles by means of PCR-RFLP and "Insect pollination" to observations and experiments with insects in orchards.

	Pollinations fruit set	Pollen tubes	SI Alleles	Insect pollination
Almond (*Prunus dulcis*)	Rovira *et al*. 1998; Dicenta *et al*. 2002; Lopez *et al*. 2004	Certal *et al*. 2002; Ortega *et al*. 2002; Dicenta *et al*. 2002; Lópéz *et al*. 2004, 2006	Lopéz *et al*. 2004, 2006; Ortega *et al*. 2006	
Apple (*Malus domestica*)	Komori *et al*. 1999; van Nerum *et al*. 2000	Modlibowska 1945; Marcucci and Visser 1987; Vezuaei 1998; van Nerum *et al*. 2000	Broothaerts *et al*. 1996; Ishimizu *et al*. 1996; Goldway *et al*. 1999; van Nerum *et al*. 2001; Kim *et al*. 2004; Cheng *et al*. 2006	Fourez 1995; Stern *et al*. 2001; Schneider *et al*. 2002
Apricot (*Prunus armeniaca*)	Burgos *et al*. 1993, 1997; Andrés and Durán 1998	Burgos *et al*. 1997; Andrés and Durán 1998; Austin *et al*. 1998	Andrés and Durán 1998; Romero *et al*. 2004; Vilanova *et al*. 2006; Feng *et al*. 2006	Austin *et al*. 1996
Cherries (*Prunus avium, P. cerasus, P. fruticosa*)		Cerović and Ružić 1972; Kuehn 1988; Hauck *et al*. 2002	Yamane *et al*. 2000; Hauck *et al*. 2002; Tobutt *et al*. 2004; Wunsch and Hormaza 2004; Williams *et al*. 2004; Bošković *et al*. 2006; Schueler *et al*. 2006	Bosh *et al*. 2006
Pear (*Pyrus communis, P. pyrifolia*)	Nyéki and Soltész 1998; Michotte-van der Aa and Jacquemart 2003; Falk Kühn and Bertelsen 2004	Modlibowska 1945; Marcucci and Visser 1987; Hiratsuka *et al*. 2001; Jacquemart *et al*. 2006	Sassa *et al*. 1992; Ishimizu *et al*. 1996; Hiratsuka *et al*. 2001; Zuccherelli *et al*. 2002; Kim *et al*. 2004; Zisovich *et al*. 2004; Sanzol *et al*. 2006	Benedek and Ruff 1998; Benedek *et al*. 1998; Farkas *et al*. 2002; Monzon *et al*. 2004; Stern *et al*. 2004
Plum (*Prunus domestica*)			Beppu *et al*. 2002, 2003; Sapir *et al*. 2004	Calzoni and Speranza 1998

Cheng *et al*. 2006; Vilanova *et al*. 2006). Two cultivars can be fully compatible (e.g. 'pollen receiver' S_1S_2 x 'pollen donor' S_3S_4) if neither haploid pollen grain shares the 'pollen receiver' alleles, semi-compatible (S_1S_2 x S_1S_3) if only half the pollen grains (S_3) can germinate and fertilize, or incompatible (S_1S_2 x S_1S_2, **Fig. 1B**). The compatibility level (full or semi) is a main factor affecting the fertilization potency among self-incompatible cultivars in the orchard. When planning an orchard, at least two fully intercompatible cultivars must be used to ensure successful pollination and consistently acceptable yield. The *S*-genotype of each cultivar thus needs to be determined in order to establish the group of its best pollinizers. Determining cultivar *S*-genotype was traditionally based on field crossings, which are ambiguous due to environmental and physiological effects and make it difficult to differentiate between the fully compatible and the semi-compatible crosses. DNA sequence analysis of genes encoding for *S*-RNase may thus serve for the identification of the *S*-locus and hence determine the compatibility level between cultivars (see refs. in **Table 2**).

The available information regarding the *S* alleles of self-incompatibility in many fruit species has not been yet compiled, since it is comprised in a variety of publications, many having a limited readership. In addition, the determination of the alleles through stylar ribonucleases analysis is occasionally contradictory due to the electrophoresis technique used, and possible misnames in cultivars (Williams *et al*. 2004; López *et al*. 2006). Nowadays, self-incompatibility is thus re-evaluated for a increasing number of varieties (see for example, López *et al*. 2006 for almond).

Pollination

Pollination is a very critical factor in self-incompatible and very early-flowering species or cultivars. The shape and weight of valuable fruits depend on the presence of seeds and thus on pollination events (Williams 1970; Borneck 1984; Taseï 1984; Nyéki and Soltész 1998).

To ensure pollination, rows of pollinizer cultivars are usually planted among rows of the main cultivar in orchards, and insects (mainly honey bee, *Apis mellifera* L.) are introduced to ensure adequate pollen transfer (McGregor 1976; Free 1993; Fourez 1995; Austin *et al*. 1996; Schneider *et al*. 2002; Stern *et al*. 2004). However, for the majority of the fruit crops, the number of visits by insect pollinators to the flowers for optimum cross-pollination has not been determined. Numerous species of insects may visit the flowers, including Hymenoptera, Diptera, Lepidoptera, and other major groups (McGregor 1976; Klein *et al*. 2006).

In many fruit species, the primary attractants for insects are nectar and pollen (McGregor 1976; Benedek *et al*. 1998; Farkas *et al*. 2002). Compared with the management of several wild bees, honeybees are versatile, cheap and convenient, but for some crops they are not the most effective pollinators (see Bosh and Blas (1994) for almond; Javorek *et al*. (2002) for blueberry; Bosh *et al*. (2006) for cherry, or Monzon *et al*. (2004) for pear; other refs in **Table 2**). *Apis mellifera* workers often visit rosaceous flowers mainly for pollen and they often switch to other fruit tree species or other more attractive plants for nectar (Free 1993; Benedek and Ruff 1998; Farkas *et al*. 2002; Monzon *et al*. 2004). Moreover, the weather conditions in early spring strongly limit their foraging activity. The importance of bumble bees and other wild bees for crop pollination has thus been increasingly emphasized (Jacob-Remacle 1989; Fussell 1992; Free 1993; Williams 1995; Walther-Hellwig and Frankl 2000; Monzon *et al*. 2004). Differences in pollination effectiveness have been observed among Apidae for many different species and pollinators other than honeybees are increasingly recommended (Macfarlane *et al*. 1994; Hedtke 1996; Meynie and Bernard 1997; Dag and Kammer 2001; Javorek *et al*. 2002; Gupta 2005).

Bumble bees, for example, exhibit a daily activity in the field that differs and complements that of honeybees. They spend less time flying, forage on flowers clumped on inflorescences for longer periods each day, visit flowers most frequently in the evening or in early morning or during adverse weather and deposit more pollen than do honeybees (Lundberg 1980; Westerkamp 1991; Hedkte 1996; Stout *et al*. 1998; Dag and Kammer 2001; Kwon and Saeed 2003; Klein *et al*. 2006). They are highly recommended in buzz pollinated species as they can sonicate the anthers (in *Vaccinium* or *Solanum* for example, Jacquemart 1993; Dag and Kammer 2001; Javorek *et al*. 2002). Some other wild bees (*Osmia*, *Andrena*) visit flowers at lower temperatures than do honeybees and their flying period (February-April) overlaps with the blooming of many fruit crops as well (McGregor 1976; Chagnon *et al*. 1993; Bosh and Blas 1994; Calzoni and Speranza 1998; Monzon *et al*. 2004; Cane 2005; Bosh *et al*. 2006; Slaa *et al*. 2006). In the majority of cases, a diverse pollinator assemblage, including different order or genera, conducts to the best pollination results as many different visitor species can act together to ensure adequate pollination and fertilization by means of their different foraging behaviour, morphology and pollen carry-over and deposition capacities (Thomson and Thomson 1992; Herrera 2005; Herrera *et al*. 2006; Jacquemart *et al*. 2007).

Table 3 Experimental conditions for field evaluation of self-incompatibility for several species.

Species	Number cv.[1]	Units	Bags	Treatments	Pollen	Results	Reference
Almond	6	Branches	Bags	Hand self Hand cross	-	Initial fruit (30 d), final fruit (60 d)	Dicenta *et al.* 2002
Almond	14, 133	Branches	Paper bags	Hand self Hand cross	-	Fruit (40-60 d)[2]	Rovira *et al.* 1998; Lopéz *et al.* 2004
Apple	1	Trees	Cages	(emasculated) Hand self Hand cross	Dried	Initial fruit, final fruit, number of seeds	van Nerum *et al.* 2000
Apricot	19	Branches 60 cm	Bags	Free Hand self	Dried 48 h – stored 4°C with $CaCl_2$	Fruit: 25-30 d after	Andrés and Durán 1998
Pear	3, 1	Branches	Bags	Bagged (no pollination) Hand self Hand cross	-	Fruit set, fruit length, fruit width, seed set[3]	Michotte-van der Aa and Jacquemart 2003; Falk Kühn and Bertelsen, 2004

[1] Cultivar.
[2] The cultivar is considered self-compatible if selfed fruit set >4%.
[3] Many pear cultivars are parthenocarpic.

Even if highly informative, comparative self and cross-pollination[1] assays in fruit crops are still quite scarce (see **Table 3**).

Objectives

In this paper, the most commonly used methods for testing self-incompatibility and pollinator effectiveness in the main fruit trees will be described. The review focuses more particularly on tree fruit species (**Table 1**). Estimation of self-incompatibility can be done experimentally or genetically. Experimentally, comparisons following self- vs. cross-pollinations can be performed sequentially (**Fig. 2**): (1) Is there any difference in fruit or seed set? If no differences are detected, the cultivar does not suffer from self-incompatibility nor inbreeding depression; (2) Are pollen germination and tube growth in the styles different (self-incompatibility)?; (3) Is there any difference in ovule development (e.g. OSI, ovarian self-incompatibility) or some cessation of embryo development after self-pollination (late-acting SI)? If seed set differences are not explained by differences in these pre-zygotic steps, inbreeding depression can be responsible (**Fig. 2**).

The genetic approach of incompatibility with the characterization of *S*-alleles can assess the compatibility level between cultivars.

Five methods can be useful for genotype identification: (1) controlled pollinations by bagging flowers in the field; (2) controlled pollinations and microscopy in the laboratory; (3) DNA isolation and *S* allele specific PCR; (4) stylar ribonuclease elctrophoresis; and (5) nucleotides sequencing.

Finally, pollinator efficiency can be assessed with direct and indirect measures and these two types of approaches are presented, including molecular marker tools for gene flow estimation.

SELF-INCOMPATIBILITY TESTS

Experimental approaches

For all the experimental tests of SI or pollinator efficiency, assessed with pollen germination or fruit set, hand pollinations are necessary (**Table 3**). Controlled pollinations in the field require a device to exclude pollinators. Pollination bags are most commonly used. They are made of white very fine mesh bridal veil preventing insect visits. The main objective is to minimize alterations in the immediate floral environment. Plastic bags must be avoided as they cause the greatest variation in temperature and humidity (Wyatt *et al.* 1992). Paper bags are quite difficult to use in wet temperate climates as they collapse after rain. The effect of bagging needs to be evaluated (by means of comparisons of

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[1] Here, I still use the terms self- and cross-pollination even if in many fruit crops, this refers to intra- and inter-cultivar pollination.
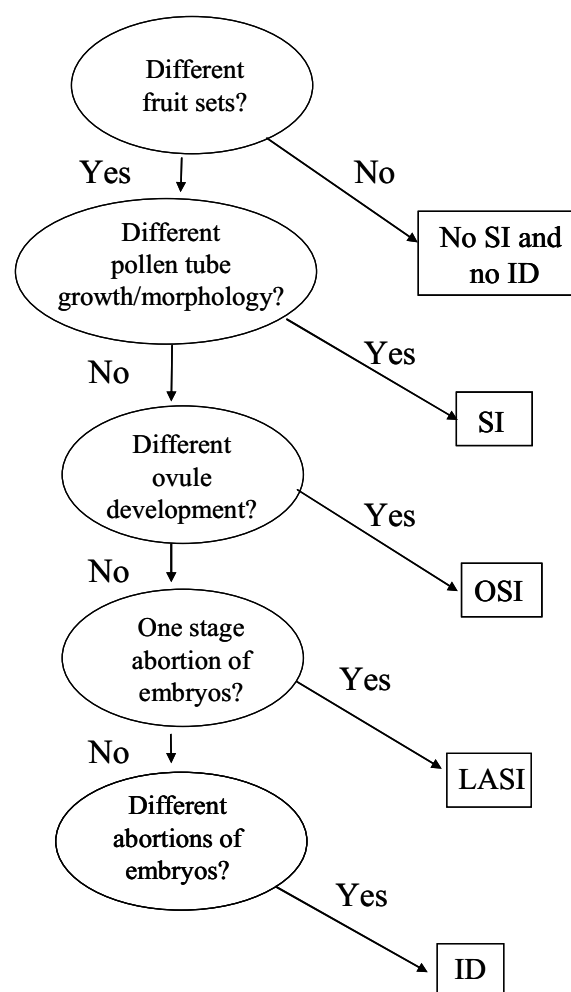

Fig. 2 Sequential tests to distinguish among self-incompatibility (SI), ovarian self-incompatibility (OSI), late-acting self-incompatibility (LASI) and inbreeding depression (ID). The comparison is done between hand self- and cross- pollinated flowers.

the same pollination treatment without any bag and under different bags). Pollination bags are placed on plants just prior to anthesis ('balloon' stage for many rosaceous species) and removed after stigmatic receptivity has ended. Bags are placed on individual flowers or flowering units (clusters) although entire plants may be covered. A tutor supporting a circle made with metallic wire can be introduced along the peduncle or the branch. Bags are closed by means of a runner in the lower part. In some cases, some rubber can be placed around the stem or peduncle to prevent ant visits (they can damage the flowers).

Tents are useful to cover entire (small) trees or several

Table 4 Experimental conditions for laboratory evaluation of self-incompatibility for several species (pollen tube observations).

Species	Number cv.	Pollination units	Pollination timing	Time[1] (h)	Fixation	Softening	Aniline blue solution	Reference
Almond	6 + 4	Branches 5% sucrose 22°C	day +1 after emasculation	24, 48, 72, 96	FAA[2]	Autoclaved 30 min 1 kg/cm² 5% Na$_2$SO$_3$	0.1 % in 0.1 N K$_3$PO$_4$	Dicenta et al. 2002; Ortega et al. 2002
Almond	14 + 133	Pollen dried 4°C	Flowers floated in water trays	72	FAA	Autoclaved 10 min 1.2 kg/cm² 5% Na$_2$SO$_3$	0.1 % in 0.1 N K$_3$PO$_4$	Lopez et al. 2004; 2006
Almond	2	Trees in 150 L pots	day +2 after emasculation	6, 12, 24, 48, 72, 96, 144	Carnoy's fluid fixative[3] + rehydrated	NaOH 8 N 60°C	0.1 % in 0.1 N K$_3$PO$_4$	Vezvaei 1998
Apple	1	Trees	? emasculation	144	FAA	NaOH 8N 30 min	0.05 % in K$_2$HPO$_4$ (pH 10)	van Nerum et al. 2000
Apricot		Branches 5% sucrose 20°C	day +1 after emasculation	12, 48, 72, 96	FAA	NaOH 8 N 60°C 1 h	0.1 % in 0.1 N K$_3$PO$_4$	Andrés and Durán 1998
Cherry	3	Pollen dried 24h and frozen –20°C	day +1 after emasculation	72	CEA[4] 24h, conservation in 100% Ethanol	NaOH 10 N 5-6 h	0.1 % in 33 mM K$_3$PO$_4$	Hauck et al. 2002
Pear	1	Branches	day +1 after emasculation	96	70% Ethanol	NaOH 1.0 M 1 h	0.1 % in 1.0 M K$_2$HPO$_4$ (pH9)	Jacquemart et al. 2006

[1] Duration between pollination and flower picking.
[2] 40% formalin - 90% acetic acid - 70% alcohol 1:1:18 (v/v).
[3] Absolute alcohol - chloroform - acetic acid 6:3:1(v/v).
[4] Chloroform 95% - ethanol - glacial acetic acid 1:3:1 (v/v).

individuals (for example to introduce a specific pollinator, see below).

Flowers should be tagged at the time of bagging. Because seed set is often resource limited with limitations depending on floral position (Brown and McNeil 2006) and competition may occur between crossed and selfed flowers, care must be taken in determining the location and number of flowers pollinated throughout the season and in a flowering unit. The same number of flowers is usually pollinated per flowering unit, the unpollinated should be removed after the pollination.

In self-compatible species, floral buds are emasculated to avoid self pollen deposition, and the following day (coinciding with anthesis) flowers are pollinated. The effect of emasculation (sometimes highly deleterious) needs to be estimated. For example, in compatible species, selfing with and without emasculation can be compared.

Pollinations can most effectively be conducted by gently touching the stigma with freshly dehisced anthers removed from the appropriate flower with fine tweezers. Pollination may be realized with a thin brush or gently rubbing one flower over another one (all methods in Kearns and Inouye 1993). The best way is to pollinate all stigmas as even apparently fused styles can be actually functionally distinct (Sanzol et al. 2003). A headband binocular magnifier can be used to assist pollinations at a higher magnification. If possible, it is advisable to perform pollinations in an environmentally controlled greenhouse or growth chamber concurrent with pollinations in the field as extrinsic factors influence pollination and fertilization (temperature, humidity…). For pollen germination and pollen tube growth, flowers can be pollinated from branches taken into the laboratory, placed in water or in 5% sucrose solution and maintained at constant temperature and relative humidity, if viability of flowers remains correct (**Table 4**). In some species, pollen can be collected and stored during 1 to 7 days at 4°C (Andrés and Durán 1998; Hauck et al. 2002) in the presence or not of CACl$_2$ as humidity absorbent. Pollen viability needs to be checked before pollination, as a possible loss of germinating capacity can result from pollen ageing or even because some cultivars are characterized by poorly viable pollen (**Box 1**).

Fruit set

The first step in sequential SI tests include fruit and seed set following self- and cross- hand pollinations (**Fig. 2**). In fruit trees, initial fruit set (fruits developing/flowers pollinated ratio) can be determined 20-30 days after pollination. Final fruit set is determined later, after physiological fruit drop

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BOX 1 Pollen viability tests.

There are direct (germination in situ or in vitro) and indirect (staining) measures of pollen viability (Kearns and Inouye 1993). Authors distinguish between pollen stainability, fertility, germinability, vigor and viability (Dafni et al. 2005). Considering compatible mating types, hand-pollinating flowers and assessing seed production ("pollen fertilization ability") seems to be the most accurate method of determining pollen viability but the amount of pollen deposited per stigma and many environmental factors can alter the results; moreover the method is time consuming (Stone et al. 1995; Dafni et al. 2005). In vitro germination (or "pollen germinability") is also time consuming and can also be influenced by many environmental factors (temperature, humidity, etc). The staining procedures include several dyes and methods (Kearns and Inouye 1993). The staining with Alexander's red (Alexander 1969) allows the distinction between aborted and non-aborted pollen (Kearns and Inouye 1993; Dafni et al. 2005). For direct methods in the field, Dafni and Firmage (2000) recommended MTT stain, followed by X-Gal test, Baker's solution and isatin. As the results can vary among methods, authors are recommended to perform several different tests to assess viability. The fluorochromatic procedure (Heslop-Harrisson and Heslop-Harrisson 1970) is the easiest and most widely used test. It assesses pollen viability by enzymatically (esterase activity) induced fluorescence. When the cell (pollen grain) membrane is intact, the fluoresceine accumulates in the cell and the cell fluoresces. Fluorescein diacetate (FDA) is dissolved in acetone (2 mg per ml acetone) and added to 15-30% sucrose solution. Sucrose concentration (w/w) should be the lowest concentration preventing pollen bursting. After 10 min in a drop of this FDA-sucrose solution, viable pollen fluoresces in bright golden-yellow under a fluorescent microscope (Kearns and Inouye 1993; Dafni et al. 2005). FDA and in vitro germination are highly correlated, supporting the idea that the ability to germinate is dependent on the condition of the plasmalemma; FDA being easier and faster (La Porta and Roselli 1991; Kearns and Inouye 1993).

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has taken place (see **Table 3**). Mature fruits can be harvested and measured (weight, shape, number of viable and of aborted seeds …). If no differences are detected, the cultivar does not suffer from self-incompatibility nor inbreeding depression. For example, an almond cultivar is considered as self-compatible if selfed fruit set is higher than 4 % (López et al. 2006). If selfing results in lower fruit or seed set, more tests for self-incompatibility and inbreeding depression should be performed.

Pollen germination and self-incompatibility

Microscopic study of pollen tubes allows assessment of pol-
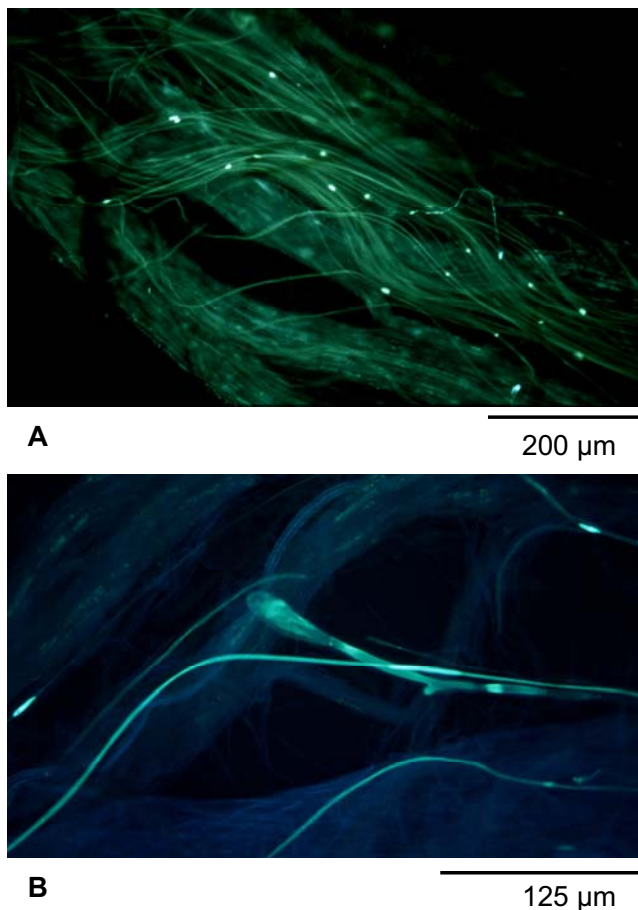
A 200 µm

B 125 µm

Fig. 3 Pollen tubes in styles of *Pyrus communis* cv. 'Conférence' observed under fluorescence microscopy. (A) Compatible pollen tubes containing a lot of small and regularly distributed callosic plugs; **(B)** incompatible tubes showing much longer and frequent callosic plugs and becoming swollen at their tip with the occurrence of loops and branching. Photograph A. Michotte-Van der Aa.

len germination, speed of pollen growth and pollen tube attrition. The presence of pollen tubes at the base of the styles enables to quantify pollination effectiveness after a few hours to a few days according to the studied species (Davis 1992; Kearns and Inouye 1993; Sage *et al*. 2005). Several trials may be needed, with collecting styles at several different times after pollination, to determine the time needed before pollen tube growth is observed in a given species or under the specific conditions of the experiment. For example, in pear (*Pyrus communis*) pollen tubes reach the base of the styles after 4-5 days (Jacquemart *et al*. 2006) while in buckwheat (*Fagopyrum esculentum*) pollen tubes reach the ovary in only one hour (Cawoy *et al*. 2006). In almond and apricot 72-96 h are required for complete pollen growth (Andrés and Durán 1998; Dicenta *et al*. 2002; Ortega *et al*. 2002). As a consequence, in several rosaceous species, pistils are usually collected at 12, 24, 48, 72, 96, and 144 h after pollination (see **Table 4**). These intervals can be tried in a first time and then reduced or increased if not adapted to the studied species. Care must be taken in choosing environmental conditions as, for example, temperature has a great influence on pollen tube growth, with low temperature (<15°C) decreasing pollen tube growth speed (Cerović and Ružić 1972; Vasilakakis and Porlingis 1985; Austin *et al*. 1998).

Pollen tube growth can be assessed by counting the percentage of pollen tubes that reach the ovary or by scoring the number of tubes that reach a given zone of the style (zones are arbitrarily determined by the distance from the stigma, or as some fraction of style length, Andrés and Durán 1998). The length of pollen tubes in the styles depends on the type of pollen (compatible vs. incompatible) placed on the stigma. For example, incompatible pollen tubes do

not reach the mid part of the styles in pears. Thus, in pear, the presence of pollen tubes in the third part of the styles is a reliable histological indicator of the compatibility of pollination (Jacquemart *et al*. 2006). Several measures of pollen performance and compatibility can be performed: number of pollen adhering to the stigmas, number of germinating grains, mean tube length determined from the number of tubes present at the different zones of the style, number of tubes at the base of the style and number of tubes entering the ovules (Andrés and Durán 1998; Certal *et al*. 2002). As high numbers (more than 100) can not be exactly counted, the number of pollen grains and pollen tubes can be estimated and classified in different categories of abundance (Jacquemart *et al*. 2006). All styles of each flower need to be observed as pollen deposition can vary among stigmas.

The most widely used procedure for looking at pollen tubes involves the use of aniline blue stain and examination of pollen tubes under fluorescence microscopy (Martin 1959). This method is easy, convenient for routine counts, measurements and identification of pollen tubes. For other methods, see Kearns and Inouye (1993). As they grow down the style, pollen tubes from many species periodically deposit callose plugs that may serve to separate the protoplast in the tip from the empty tube above. Aniline blue stain with a pH of 6-7 has a great affinity to callose and fluoresces under ultraviolet light. Fluorescence microscopy thus allows observation of the stained callose. The callosic content of the pollen tube wall and deposits (pollen tube plugs) fluoresces heavily under UV-excitation (λ = 365 nm). Compatible and incompatible pollen tubes can easily be distinguished (Modlibowska 1945; Lewis 1979). Compatible pollen tubes usually contain a lot of small and regularly distributed callosic plugs in the first half of the style (**Fig. 3A**). On the other hand, incompatible tubes show much longer and frequent callosic plugs, and can present swollen tip (**Fig. 3B**), with the occurrence of loops and branching (Cerović and Ružić 1972). These procedures and distinctions work well with pear and apple (Marcucci and Visser 1987; Jacquemart *et al*. 2006), almond (Certal *et al*. 2002; Ortega *et al*. 2002) or

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BOX 2 Pollen tube growth observation.

Pollinated pistils are fixed immediately after harvest to prevent further pollen tube growth. Three main solutions are used to fix the styles (FAA is the most commonly used (40% (v/v) formaldehyde, 90% (v/v) glacial acetic acid and 70% (v/v) ethanol in a ratio 1:1:18).

Long styles can be cut into pieces to facilitate examination and thick ovaries or styles sometimes need to be cut longitudinally.

Some tissues require softening before staining. This can be easily obtained by placing pistils (rinced in water) in 1.0 M NaOH during 1 hour or more (Kho and Baër 1968) or by heating in NaOH at 60°C (Andrés and Durán 1998). Other authors use autoclaving in 5 to 10% (w/v) sodium sulphite (Na_2SO_3) for 10 to 30 min at 121°C or at a pressure of 1 kg/cm^2 (see **Table 4**). Softened tissues are then washed in water.

Dissolving aniline blue in KH_2PO_4 or K_3PO_4 decolorizes it, after one night at room temperature, the blue solution becomes pale yellowish (Currier 1957). The dye decolorizes more or less rapidly depending on the supplier. The tissues are rinsed in water and placed in 0.1% aniline blue solution (for example, in KH_2PO_4 1.0 M pH 9.0) during 2 h to overnight depending on the species, the dye supplier, and the author (Martin 1959; Kho and Baër 1968; Sage *et al*. 2005; see **Table 4**). Pistils are then mounted on microscope slides, covered with a coverslip, and delicately crushed before their examination with fluorescence microscope. Many epi-illumination fluorescence microscope systems are available, and microscope companies will provide information to set up a system that best suits the specific needs. For example, mercury vapour lamps can be replaced by less expensive halogens.

For all species, samples can be stored in aniline blue at 4°C for many months. The mounted specimens are stored by sealing the edge of the coverslips with fingernail polish or petroleum jelly for example.

apricot (Burgos *et al*. 1997; Austin *et al*. 1998, **Table 2**). There are several variations on the fluorescence procedure that may produce different results with different species (**Table 4**). The basic steps comprise fixing, softening and clearing tissues, staining and viewing under epifluorescence (**Box 2**).

Late-acting self-incompatibility

If there are no qualitative and quantitative differences observed between self- and cross- pollen tube growth, but seed set following selfing is very low, ovarian self-incompatibility (OSI, Sage *et al*. 1994), late-acting self-incompatibility (de Nettancourt 2001), cryptic self-incompatibility (Bertin and Sullivan 1988) or early-acting inbreeding depression (Seavey and Bawa 1986) may be at play (**Fig. 1**).

Pre-zygotic OSI will result in failure of ovule development prior to pollen tube entry into ovules. Light microscopy or differential interference contrast optics (Shaw and Rawlins 1994) can be used to examine ovules from dissected ovaries at each sampling time to determine if there is differential pre-zygotic development following self- vs. cross- pollination (Sage *et al*. 2005). In this case, pollen tubes fail to enter the micropyle or fertilization does not take place. The delayed action of OSI is expressed immediately before, during or just after the double fertilization (de Nettancourt 2001). The abortion takes place at a single stage of development (contrary to inbreeding depression, Sage *et al*. 1994). If examination of cleared ovules reveals that double-fertilization has taken place as indicated by the presence of a resting zygote or young embryo and endosperm, then post-zygotic or inbreeding depression may be the cause of self-sterility. More pollinations are necessary to harvest at various stages of development up to seed maturity. Post-zygotic OSI can be assessed following histological observations (**Box 3**). Distinction between late acting SI, cryptic SI and inbreeding depression is difficult (Sage *et al*. 1994; de Nettancourt 2001).

Genetic study of self-incompatibility

The gametophytic self-incompatibility (GSI) system in Rosaceae has been shown to be controlled by two genes located at the *S*-locus: an *S*-RNase and a recently-described, pollen-expressed, *S*-haplotype-specific *F-box* gene (SLF or SFB) (Entani *et al*. 2003; Ushijima *et al*. 2003; Sijacici *et al*. 2004; Romero *et al*. 2004; Vilanova *et al*. 2006). The divergence of *S*-alleles occurred shortly after the divergence into subfamilies in the Rosaceae, as Maloideae *S*-alleles are highly similar among them and diverge from Prunoideae *S*-alleles (Zisovich *et al*. 2004) and *vice versa* (Hauck *et al*. 2002). In recent years, an increasing number of *S*-genotypes of many cultivars have been determined by PCR-RFLP or RT-PCR (**Table 2, Box 4**). The number of S-alleles differ among species as 26 alleles were described in almond (Certal *et al*. 2002; Vilanova *et al*. 2006; López *et al*. 2006); 29 alleles were described in apple (Ishimizu *et al*. 1996; Goldway *et al*. 1999; van Nerum *et al*. 2001; Kim *et*

BOX 3 Post-zygotic OSI.

To test for post-zygotic OSI, harvest selfed and crossed flowers for fixation at regular daily intervals throughout seed maturation. The fixed ovaries of different developmental stages are rehydrated (50% ethanol, 25% ethanol, 2 x distilled water 1 h each) before staining 1-2 days with Mayer's hematoxylin solution. After destaining for 1-2 days with 0.5% acetic acid, they are dehydrated in ethanol series (25%, 50%, 70%, 95%, 2 x 100% for a minimum of 1 h at each step) and infiltrated with methyl salicylate (using 2:1:0.5 ethanol: methyl salicylate followed by 2 x 100% methyl salicylate). The dissected ovules, mounted in methyl salicylate, are observed under microscope (Sage *et al*. 2005). Other techniques are presented in Palser *et al*. (1989).

BOX 4 Genetic analysis of S-alleles.

1. S-specific PCR

For PCR-RFLP, young leaves are collected, frozen in liquid nitrogen and stored at -80°C until use. Genomic DNA is usually isolated by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987; Kim *et al*. 2004; Zisovich *et al*. 2004) or DNeasy Plant Mini Kit (Qiagen, de Cuyper *et al*. 2005). Conserved oligonucleotide primers are synthesized using sequence information for the described *S*-Rnases (Sutherland *et al*. 2004; Takasaki *et al*. 2006). PCR amplification is performed according to the conditions developed for each species. For all details, the best way is to refer to the original publications (see **Table 2**). The amplified PCR products are then digested with restriction enzymes and the restriction fragments are separated by electrophoresis usually on agarose. The PCR product can also be cloned and sequenced in both directions.

2. RT-PCR

S-RNase activity may also be analysed using RT-PCR method (Raspé and Kohn 2002; Tobutt *et al*. 2004). Styles of flower buds or freshly opened flowers are collected, ground on dry ice and stylar RNA are isolated using TRIZOL (Gibco BRL) or SuperScriptII RnaseH-reverse transcriptase (Invitrogen) and resuspended in 20 µl of water (Richman *et al*. 1995). cDNA synthesis is performed with the RNA solution, using kits (Invitrogen, Qiagen). PCR amplification is performed and the alleles are then cloned using cloning kits (Invitrogen TA). The different cloned alleles are then identified by RFLP and sequenced (Janssens *et al*. 1995; Raspé and Kohn 2002; Sijacic *et al*. 2004).

3. Stylar ribonuclease analysis

A more controversial technique, used in almond, concerns stylar ribonuclease analysis. Styles are frozen and stored at –80°C. Whole protein stylar extracts are prepared and separated using NEpHGE (non-equilibrium pH gradient) as described in Bošković *et al* (1997). RNase activity is detected by gel incubation with RNA followed by staining with toluidine blue. Protein extracts from other cultivars with known S-phenotypes are used as standards (Certal *et al*. 2002; López *et al*. 2004). The determination of the alleles through stylar RNase analysis is contradictory due to differences due to the electrophoresis used (isoelectric focusing IEF or NEpHGE), gel length and thickness, gel composition and migrating conditions (López *et al*. 2006).

4. Cloning and sequencing of the genomic fragment of S-alleles

Bands corresponding to the S-alleles in the agarose gels are purified and cloned into plasmid vectors. The presence of the inserts is confirmed by PCR and plasmid DNA is isolated by alkaline lysis method. Sequencing is carried out using dye terminator cycle sequencing with fluorescent-labelled by terminators on a DNA sequencer. Nucleotide sequences are determined in both directions. Genomic DNA sequences are aligned using for example, the neighbour-joining method of CLUSTALX (http://www2.ebi.ac.uk/clustalw/) or DNASTAR software (Zuccherelli *et al*. 2002; de Cuyper *et al*. 2005). The sequence can be aligned against all published *S*-alleles sequences on the Gen Bank database (López *et al*. 2004).

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al. 2004; Broothaerts *et al*. 1996, 2004; Cheng *et al*. 2006); 27 alleles in apricot (Vilanova *et al*. 2006; Feng *et al*. 2006); 13 alleles in sweet cherry and 6 alleles in sour cherry (Yamane *et al*. 2000; Hauck *et al*. 2002; Tobutt *et al*. 2004; De Cuyper *et al*. 2005; Bošković *et al*. 2006); and 9 alleles in pear (Sassa *et al*. 1992 ; Ishimizu *et al*. 1996 ; Hiratsuka *et al*. 2001; Zuccherelli *et al*. 2002; Zisovich *et al*. 2004; Kim *et al*. 2004; Sanzol *et al*. 2006). The nucleotide sequences of primers are highly conserved so this genetic method is applicable to identify *S*-RNases and test for inter-compatibility among varieties (**Box 4**). In pear, for example, cv. 'Spadona' shares an *S*-allele with all the pollinizer cultivars planted in Israel ('Gentile', 'Coscia', 'Spadochina'), which can explain the low observed fruit set (Zisovich *et al*. 2004). The same

'varietal semi-compatiblity' was detected in apple and in almond (Goldway *et al*. 1999; Ortega *et al*. 2002; Schneider *et al*. 2005; López *et al*. 2006).

POLLINATOR EFFICIENCY

Klein *et al*. (2006) reviewed insect pollination impact on fruit crops: on a total of 32 fresh temperate and tropical fruits, 27 showed an increase of their production due to insect pollination, 2 showed no increase and the impact on the last 5 species remained unknown. Insect pollinators' impact on fruit production is considered as essential (e.g. production reduced by 90% or more without flower visitors: for cocoa, melon, kiwifruit, passion fruit). Impact is considered as great (40 to less than 90% reduction: in apple, mango, pear, all the *Prunus*, *Rubus* and *Vaccinium* species). Impact of pollinators is modest (10 to <40% reduction, in currants) or little (<10%, in *Citrus* sp.), depending on the breeding system of the studied species (review in Klein *et al*. 2006). Honeybees (*Apis mellifera* L.) are the most economically valuable pollinators in a majority of cases, followed by bumblebees and other Apidae species (see Klein *et al*. 2006, **Table 1**; or Bosh and Blas 1994; Fourez 1995; Austin *et al*. 1996; Monzon *et al*. 2004; **Table 2**). Only grapes, which rely on passive self- and wind- pollination, do not need any insect for their pollination (McGregor 1976; Free 1993; Klein *et al*. 2006). However, the relative contribution and efficiency of the different insect visitors are rarely estimated. Inadequate information is available on pollinator requirements of many crops, especially when considering modern varieties, and the efficiency of different pollinators (Klein *et al*. 2006).

'Pollinator efficiency' has been used in different ways and with different meanings by different researchers (see Inouye and Kearns 1993; Gross 2005). Authors use undiscriminately pollinator efficiency or effectiveness, pollination efficiency or effectiveness. Not all floral visitors are pollinators and not all pollinators are equally effective in their pollination activities. The most effective pollinator is not always the most abundant floral visitor (Herrera 1990) and even the most abundant visitors may not be pollinators at all (Navarro 2000). Direct measurements of pollinator efficiency provide a more robust set of conclusions than do indirect measures. Nevertheless, indirect measures can contribute to the understanding of the mechanics of the studied system.

'Pollinator efficiency' can be defined as the trade-off between the benefits and costs to the plant from a single visit by an animal to a flower or floral unit (Waser 1983; Gross 2005). Benefits include seed set and pollen removal and costs might include nectar, pollen and ovule consumption, foreign or incompatible pollen deposition and clogged stigmas or damage incurred to floral attractiveness from spoil pollinators. A review of the terminology and the parameters can be found in Kearns and Inouye (1993). 'Pollination effectiveness' is defined as the ratio of the number of pollen grains from a single flower deposited on receptive conspecific stigmas, over the total ('total pollination effectiveness') or available ('partial pollination effectiveness') pollen grains of the original flower. 'Pollination efficiency' is defined as the ratio of the number of pollen grains deposited on a receptive conspecific stigma in a single visit by a pollinator, over the number of pollen grains carried by the vector (Kearns and Inouye 1993).

If only total pollen deposition is important and not the knowledge of individual insect efficiency, indirect measures can be sufficient. Indeed, the amount of compatible pollen needed to maximize ovule fertilization typically exceeds the number of ovules in a ovary 5- to 10-fold (Mitchell 1997; Harder and Routley 2006). If stigmas do not receive enough compatible pollen, pollinator identity and abundance can constitute the first step of the study (Lindsey 1984). The second step includes efficiency estimation for the most common visitors.

Pollination experiments and direct measures

Pollinator exclusion tents made of a metallic frame covered by a white windbreak net are placed before flower set (Kearns and Inouye 1993). A special care needs to be taken for the tent nets as anti-UV fabrics have a profound effect on insects (no activity or a huge decrease, B. Vaissière, pers. com.; Vorobyev *et al*. 1997).

Tents can be devoted to inter-cultivar pollinations if the two tested varieties can be introduced in the same tent. Sometimes branches of the other cultivar are placed in bottles of water and hung in the trees if introducing mature trees is not feasible. Even with several trees from different cultivars inside the same tent, transfer of pollen could be intra- and inter- cultivar, and the pollination needs thus to be considered as mixed if the flowers are not emasculated.

To test an effect of pollinator visits in these tents, flowering units on each tree will be tagged and covered with exclusion bags to prevent pollinator visit.

Honeybee colonies usually comprise approximately 10,000-30,000 bees, while hives of mason bees comprise only 100 cocoons and hives of bumble bees only approximately 50 workers (Calzoni and Speranza 1998; Stern *et al*. 2001; Jacquemart *et al*. 2006). The difference in insect numbers does not influence the results if pollinator efficiency is measured after only one insect visit. But pollinator number may influence overall pollination success. The exclusion bag is removed from a flower cluster and the flowers are observed until one single insect visit. The visited flowers are noted and after the insect leaves, all unvisited flowers are removed from the flower cluster and the branch is rebagged.

The units of measurement may include the number of pollen grains deposited, pollen removed, seeds or fruit produced.

The most useful method for discerning efficiency of floral visitors is to offer new virgin flowers. Pollen removal, deposition, pollen tube growth and seed set can be measured (Gross 2005). A virgin stigma has no pollen deposited on it. Flowers are bagged before anthesis and only unbagged while being observed, or flowers that are opening for the first time and have been observed since they opened. In many cases, flower buds have to be emasculated to ensure that no self-pollen is deposited before the observed pollinator visit, except in self-incompatible species. Visual inspection of stigmas after a visit may be sufficient if the pollen grains are large enough, otherwise lens or a microscope may be used. Virgin picked flowers may be presented to foragers: this works well with honeybees, bumblebees and flies (Kearns and Inouye 1993). Several hours or days (depending on the studied species) after pollination, pollinated flowers are collected, fixed in FAA and can be used to count pollen grains onto stigmas and evaluate pollen tube growth in the styles (see above). Two components to pollen deposition can be assessed: intensity (number of pollen grains) and purity (compatibility) (Beattie 1971; Waser 1986; Davis 1992). Pollen removal can be assessed by picking the anthers after a single visit. The number of pollen grains removed per dehisced anther is estimated by subtracting the amount remaining per dehisced anther of each sample from the average number of grains per undehisced anther of the variety. For example, *Apis* and *Bombus* did not differ in their removal of pollen in apple, except in 'Delicious' cv. and under certain circumstances (Goodell and Thomson 1997).

The relative efficiency of a single pollinator needs to be judged when other types of sympatric pollinators are taken into account (Thomson and Thomson 1992). For example, a pollinator that removes lots of pollen but deposits little on stigmas may be valuable when it is the sole species. However, when a second pollinator that deposits much pollen on stigmas is also present, the first type of pollinator may actually be a liability, wasting much pollen that would effect fertilizations. Because flowers received multiple pollinator visits, reproductive success is dependent upon the sequence of the visitors (Kearns and Inouye 1993; Morris *et al*. 1995).

Moreover, pollinator responses may differ between plant communities and plant-pollinator studies that ignore community context can therefore misrepresent real pollinator efficiency (Geber and Moeller 2006). "Pollinator-community composition" varies according to geographical, environmental and annual differences and these differences need to be taken into account (Herrera 2005; Herrera *et al*. 2006).

Indirect measures

If no information is available on the insect guild, quantitative collections by capturing and killing all insect visitors within one standard area of plants and during several periods per day for several days may be realized.

Moreover, during the same days, data about insect foraging behaviour on the flowers and on the plants (foraging for nectar, for pollen, mating or resting) can be collected. The number of each visiting taxa, the time spent per flower, per inflorescence, per plant, and the number of visited flowers are usually recorded (Primack and Silander 1975; Lindsey 1984; Kearns and Inouye 1993).

For pollinator behaviour estimations, the main floral visitors can be described as belonging to categories when species are not identifiable in the field (for example: honeybees, bumblebees, solitary bees, syrphids (one or more categories according to the body size)).

The indirect measures do not determine the contribution made by each visitor on the plant reproductive success. They try to determine the relative abundances and importance of visitors. Moreover the amount (and position) of pollen on floral visitors, visitation rates to flowers and relative amount of pollen transferred to stigmas are assessed (Primack and Silander 1975; Lindsey 1984; Harder and Barrett 1995; Gupta 2005).

Indirect measures include insect carryover capacity. Pollen carrying capacity can be analysed with collection of pollen on killed insect bodies (Bernhardt 2005). Pollen carried on flower visitors is removed and examined under microscope (number and diversity of pollen grains). The most widely used method involves use of stained glycerine jelly (Beattie 1971). Small cubes of the jelly are used to pick the pollen off of an insect. The method allows the distinction among the different parts of insect integument (code numbers for all parts in Kearns and Inouye 1993). The cube can then be dissolved in a drop of lactophenol with cotton blue on a microscope slide (Motten 1986). Less interestingly, the overall pollen load can be evaluated with "bathing" the killed insect body in ethanol, removing the insect and allowing ethanol to evaporate before microscope count (Bernhardt 2005). In all cases, pollen grains may then be counted and/or identified. These methods allow some knowledge about the carryover capacity which is different to the deposition capacity of this particular visitor, as there is a progressive reduction in the number of pollen from counts of pollen grains picked up by an insect to pollen deposited on a stigma due to transport loss, including pollen packed in corbiculae (Harder and Routley 2006).

Other less used measures of pollen carryover include the use of pollen stains, pollen-mimicking dye powders (Waser 1986; Thomson *et al*. 1988; Campbell 1991) and radioactive labelling of pollen (Kearns and Inouye 1993). These pollen analogues (particularly coloured dyes) are used to infer patterns of pollen movement and to estimate both male (pollen removal) and female (pollen deposition) reproductive success. Specific individuals or flowers are marked with a dye and the transfer of this dye to other individuals or flowers is recorded to estimate pollen flow. Multiple individuals can be tracked simultaneously with the use of different types or colours of dyes. For example, histochemical stains (brilliant green, Bismarck brown, methylene aniline blue, orange G, rhodamine, trypan red) were successfully used for monitoring pollen flow in the orchid *Prasophyllum fimbria* (Peakall 1989) as well as fluorescent dyes in *Stellaria pubera* (Campbell 1985), *Delphinium nel-*

sonii (Waser 1988) or in the heterostylous *Gelsemium sempervirens* (Adler and Irwin 2006). The main criticisms against these dyes concern the induced different visitor behaviour compared to 'real' pollen carryover (Thomson *et al*. 1988). The value of fluorescent powders depends on the similarity of powder and pollen transport and this similarity needs to be tested (Kearns and Inouye 1993).

Sometimes, morphological markers can permit to evaluate gene flow or pollinator efficiency as in *Cucumis melo* and *Cucumis sativus* (Handel 1982, 1983) or in apple (Reim *et al*. 2006). Nevertheless, such morphological markers are rare and genetic markers should be preferred.

Increasingly useful carryover measures include molecular markers (Morris *et al*. 1995; Granger 1997; Sharifani and Jackson 2001; Schueler *et al*. 2003; Granger 2004; Garcia *et al*. 2005; Schueler *et al*. 2006; Koopman *et al*. 2007). Genetic analysis are performed to evaluate gene flow and to compare pollinator efficiency. Most plant species have several described polymorphic genetic markers (allozymes, DNA polymorphisms like SSR, etc). Co-dominant markers allow a direct discrimination of genotypes. Isozyme markers have bee used for analyses of genetic relatedness but they tend to detect a relatively low level of polymorphism and may depend on the physiology of the plant at the time of analysis. Since the early 1990s, molecular (DNA) markers have become popular tools for investigating the genetic diversity within a population or among cultivars and species.

Isozyme markers have been successfully used, for example, to estimate indirect pollinator efficiency in 'Packham Triumph' pear and two other cultivars ('Josephine and 'Lemon Bergamot') using only two isozymes (ADH and GPI, extracted from seeds) which discriminated among cultivars (Sharifani and Jackson 1997, 2001). In a first step, crossings between cultivars allowed to determine the best segregating isozymes. Isozyme analysis allows the determination of pollen source leading to successful fertilization. Seeds were ground in extraction buffer, cellulose acetate gel was used and two enzymes, ADH and GPI showed segregation patterns between cultivars. In a second step, honeybees were introduced in the orchard and fruits were picked at the end of the season on both the East and West sides of each tree of 'Packham Triumph'. The number of specific bands reflects the number of successful fertilization events by 'Josephine' pollen, the remaining bands were taken as being fertilized by 'Lemon Bergamot' pollen (Sharifani and Jackson 2001). The same technique was used in almond with seven isozyme systems and showed that honeybee fidelity was high (80%) with bees visiting only one cultivar during any particular flight, along one row for example (Vezvaei and Jackson 1997).

Molecular (DNA) markers are powerful tools that may help in gene flow and pollinator efficiency. Among genetic markers, microsatellites (or SSR, Single sequence repeat) have become valuable because they present extremely high levels of polymorphism together with codominant inheritance. They are increasingly used for analysis of plant genetic diversity, population structure, seed dispersal patterns, genetic linkage, molecular mapping and cultivar characterization. Microsatellite markers are being developed for almost all fruit species (see **Table 5**). Their flanking regions are well conserved across a genus, a sub-family (Maloideae, Amygdaloideae, etc) or a family. SSR markers developed in *Prunus* or even in Rosaceae for example are used to identify and characterize cultivar genotypes in many related species. The same process occurs in other families or genera and references are easily found. Test of 10 to 30 primer pairs allows to detect numerous alleles and a combination of primers unequivocally distinguish the different genotypes or cultivars. Ten primer pairs were sufficient to detect 85 alleles that distinguish 44 apricot cultivars (Maghuly *et al*. 2006); 85 peach cultivars were distinguished with 42 primer pairs (Wunsch *et al*. 2006) or only seven primer pairs were sufficient to distinguish the 28 North American pear cultivars (Ghosh *et al*. 2006). In an orchard with different cultivars, pollinator efficiency can thus be measured by genoty-

Table 5 Most recent references for available molecular (DNA) markers and their use in cultivar (Cv.) characterization.

Species	Marker type	Use	Reference
Almond	RAPD and SSR	Cv. characterization	Shiran *et al.* 2007
	SSR	Variation	Xie *et al.* 2006
Apple	S-RNase and SSR	Cv. characterization	Fernández-Fernández *et al.* 2004; Kitahara *et al.* 2005
	SSR	Cv. characterization	Galli *et al.* 2005; Guarino *et al.* 2006
Apricot	SSR	Cv. characterization	Krichen *et al.* 2006; Maghuly *et al.* 2006
		Heterozygosity	Sanchez-Perez *et al.* 2006
Cherry	SSR	Cv. characterization	Pedersen 2006; Marchese *et al.* 2007
	SSR and SI locus	Within population structure	Schueler *et al.* 2006
Peach	SSR	Genetic diversity and phylogeography	Yoon *et al.* 2006
		Cv. characterization	Wunsch *et al.* 2006
Pear	SSR	Isolation	Fernández-Fernández *et al.* 2006
		Cv. characterization	Ghosh *et al.* 2006
Prunus in general	RAPD and SSR	Genetic diversity	Baranek *et al.* 2006
	Functional markers	Mapping	Sargent *et al.* 2007

ping the seeds. All details about material and protocols are available in the literature (**Table 5**). Moreover, *S*-allele genotyping can also be used for pollinator efficiency if the orchard includes cultivars with different *S*-alleles (see above).

In conclusion, the development of molecular tools should offer new and precise opportunities for self-incompatibility and pollinator efficiency studies in an increasing number of fruit species. In a near future, this field of research will expand to many other species and for diverse purposes.

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REFERENCES

Adler LS, Irwin RE (2006) Comparison of pollen transfer dynamics by multiple floral visitors: experiments with pollen and fluorescent dye. *Annals of Botany* **97**, 141-150

Alexander P (1969) Differential staining of aborted and non-aborted pollen. *Stain Technology* **44**, 117-122

Andrés MV, Durán JM (1998) Self-incompatibility in Spanish clones of apricot (*Prunus armeniaca* L.) tree. *Euphytica* **101**, 349-355

Austin PT, Hewett EW, Noiton DA, Plummer JA (1996) Cross pollination of 'Sundrop' apricot (*Prunus armeniaca* L) by honeybees. *New Zealand Journal of Crop and Horticultural Science* **24**, 287-294

Austin PT, Hewett EW, Noiton D, Plummer JA (1998) Self incompatibility and temperature affect pollen tube growth in 'Sundrop' apricot (*Prunus armeniaca* L.). *Journal of Horticultural Science and Biotechnology* **73**, 375-386

Baranek M, Raddova J, Pidra M (2006) Comparative analysis of genetic diversity in *Prunus* L. as revealed by RAPD and SSR markers. *Scientia Horticulturae* **108**, 253-259

Barbier E (1986) La pollinisation des cultures. Pourquoi? Comment? Barbier, E. Avignon, France, 480 pp

Beattie AJ (1971) Pollination mechanism in *Viola*. *New Phytologist* **70**, 343-360

Benedek P, Ruff J (1998) Flower constancy of honeybees and its importance during pear pollination. *Acta Horticulturae* **475**, 427

Benedek P, Ruff J, Nyéki J (1998) Comparison of honey bee visitation of pear cultivars. *Acta Horticulturae* **475**, 429-430

Beppu K, Yamane H, Yaegaki H, Yamaguchi M, Kataoka I, Tao R (2002) Diversity of *S*-RNase genes and *S*-haplotypes in Japanese plum (*Prunus salicina* Lindl.). *Journal of Horticultural Science and Biotechnology* **77**, 658-664

Beppu K, Takemoto Y, Yamane H, Yaegaki H, Yamaguchi M, Kataoka I, Tao R (2003) Determination of *S*-haplotypes of Japanese plum (*Prunus salicina* Lindl.) cultivars by PCR and cross-pollination tests. *Journal of Horticultural Science and Biotechnology* **78**, 315-318

Bernhardt P (2005) Pollen transport and transfer by animal pollinators. In: Dafni A, Kevan PG, Husband BC (Eds) *Practical Pollination Biology*, Cambridge, Ontario, Canada, pp 371-380

Borneck R (1984) L'apiculture au service de l'arboriculture et des producteurs de semences. In: Pesson P, Louveaux J (Eds) *Pollinisation et Productions*

Végétales, INRA, Paris, France, pp 57-563

Bosh J, Blas M (1994) Foraging behaviour and pollinating efficiency of *Osmia cornuta* and *Apis mellifera* on almond (Hymenoptera, Megachilidae and Apidae). *Applied Entomology and Zoology* **29**, 1-9

Bosh J, Kemps WP, Trostle GE (2006) Bee population returns and cherry yields in an orchard pollinated with *Osmia lignaria* (Hymenoptera, Megachilidae). *Journal of Economical Entomology* **99**, 408-413

Bošković RI, Tobutt KR, Battle I, Duval H (1997) Correlation of ribonuclease zymograms and incompatibility genotypes in almond. *Euphytica* **97**, 167-176

Bošković RI, Tobutt KR, Duval H, Battle I, Dicenta F, Vargas FJ (1999) A stylar ribonuclease assay to detect self-compatible seedlings in almond progenies. *Theoretical and Applied Genetics* **99**, 800-810

Bošković RI, Wolfram B, Tobutt KR, Cerović R, Sonneveld T (2006) Inheritance and interactions of incompatibility alleles in the tetraploid sour cherry. *Theoretical and Applied Genetics* **112**, 315-326

Broothaerts W, Verdoodt L, Keulemans J, Janssens GA, Broekaert WF (1996) The self-incompatibility gene in apple and determination of the *S*-genotype of apple cultivars by PCR. *Acta Horticulturae* **423**, 103-109

Broothaerts W, van Nerum I, Keulemans J (2004) Update on and review of the incompatibility (*S*-) genotypes of apple cultivars. *HortScience* **39**, 943-947

Brown AO, McNeil JN (2006) Fruit production in cranberry (Ericaceae: *Vaccinium macrocarpon*): a bet-hedging strategy to optimize reproductive effort. *American Journal of Botany* **93**, 910-916

Burgos L, Berenguer T, Egea J (1993) Self and cross-compatibility among apricot cultivars. *HortScience* **28**, 148-150

Burgos L, Ledbetter CA, Perez Tornero O, Ortin Parraga F, Egea J (1997) Inheritance of sexual incompatibility in apricot. *Plant Breeding* **116**, 383-386

Calzoni GL, Speranza A (1998) Insect controlled pollination in Japanese plum (*Prunus salicina* Lindl.). *Scientia Horticulturae* **72**, 227-237

Campbell DR (1985) Pollinator sharing and seed set of *Stellaria pubera*: competition for pollination. *Ecology* **66**, 544-553

Campbell DR (1991) Comparing pollen dispersal and gene flow in a natural population. *Evolution* **45**, 1965-1968

Cane, JH (2005) Pollination potential of the bee *Osmia aglaia* for cultivated red raspberries and blackberries (*Rubus*: Rosaceae). *HortScience* **40**, 1705-1708

Cawoy V, Deblauwe V, Halbrecq B, Ledent JF, Kinet JM, Jacquemart A-L (2006) Morph differences and honeybee morph preference in the distylous species *Fagopyrum esculentum* Moench. *International Journal of Plant Science* **167**, 853-861

Cerović R, Ružić D (1972) Pollen tube growth in sour cherry (*Prunus cerasus* L.) at different temperatures. *Journal of Horticultural Science* **67**, 333-340

Certal AC, Almeida RB, Bošković R, Oliveira MM, Feijó JA (2002) Structural and molecular analysis of self-incompatibility. *Sexual Plant Reproduction* **15**, 13-20

Chagnon M, Gingras J, de Oliveira D (1993) Complementary aspects of strawberry pollination by honey and indigenous bees (Hymenoptera). *Journal of Economical Entomology* **86**, 416-420

Cheng JH, Han ZH, Xu XF, Li TZ (2006) Isolation and identification of the pollen-expressed polymorphic *F*-box genes linked to the *S*-locus in apple (*Malus × domestica*). *Sexual Plant Reproduction* **19**, 175-183

Clarke JB, Ortega E, Sutherland B, Marchese A, Tobutt KR (2004) Some new cherry microsatellites and their transferability to other stone fruits. *Acta Horticulturae* **663**, 83-86

Currier HB (1957) Callose substance in plant cells. *American Journal of Botany* **44**, 478-488

Dafni A, Firmage D (2000) Pollen viability and longevity. *Plant Systematics and Evolution* **222**, 113-132

Dafni A, Pacini E, Nepi M (2005) Pollen and stigma biology. In: Dafni A, Kevan PG, Husband BC (Eds) *Practical Pollination Biology*, Cambridge Ontario, Canada, pp 83-147

Dag A, Kammer Y (2001) Comparison between the effectiveness of honey bee (*Apis mellifera*) and bumble bee (*Bombus terrestris*) as pollinators of greenhouse sweet pepper (*Capsicum annuum*). *American Bee Journal* **141**, 447-

448

Davis AR (1992) Evaluating honey bees as pollinators of virgin flowers of *Echi-um plantagineum* L. (Boraginaceae) by pollen tube fluorescence. *Journal of Apicultural Research* **31**, 83-95

de Cuyper B, Sonneveld T, Tobutt KR (2005) Determining self-incompatibility genotypes in Belgian wild cherries. *Molecular Ecology* **14**, 945-955

de Nettancourt D (2001) *Incompatibility and Incongruity in Wild and Cultivated Plants*, Springer-Verlag, Berlin, Germany, 322 pp

Dicenta F, Ortega E, Cánovas JA, Egea J (2002) Self-pollination vs. cross-pollination in almond: pollen tube growth, fruit set and fruit characteristics. *Plant Breeding* **121**, 163-167

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11-15

Entani T, Iwano M, Shiba H, Che FS, Isogai A, Takayama S (2003) Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: identification of a pollen-expressed *F*-box gene with allelic diversity. *Genes Cells* **8**, 203-213

Falk Kühn B, Bertelsen M (2004) Pollination experiment with the pear cultivar 'Clara Frijs'. *Acta Hortulturae* **663**, 375-379

FAOSTAT data 2005 Data available at http://faostat.fao.org; Agricultural Data/ Agricultural production/Crops primary.

Farkas A, Orosz-Kovacs Z, Szabo LG (2002) Insect attraction of flowers in pear cultivars. *Acta Horticulturae* **596**, 773-776

Feng JR, Chen XS, Wu Y, Liu W, Liang Q, Zhang LJ (2006) Detection and transcript expression of *S*-RNase gene associated with self-incompatibility in apricot (*Prunus armeniaca* L.). *Molecular Biology Reports* **33**, 215-221

Fernández-Fernández F, Clarke JB, Tobutt KR (2004) Preferential amplification of microsatellite alleles: an example in apple. *Acta Hortulturae* **663**, 87-90

Fernández-Fernández F, Harvey NG, James CM (2006) Isolation and characterization of polymorphic microsatellite markers from European pear (*Pyrus communis* L.). *Molecular Ecology Notes* **6**, 1039-1041

Fourez A (1995) Impact of pollination by bees (*Apis mellifera* L.) on the production of apples (cv. Jonagold). *Belgian Journal of Zoology* **125**, 135-141

Free JB (1993) *Insect Pollination of Crops*, Academic Press, London, UK, 684 pp

Fussell M (1992) Diurnal patterns of bee activity, flowering, and nectar reward per flower in tetraploid red clover. *New Zealand Journal of Agricultural Research* **35**, 151-156

Galli Z, Halasz G, Kiss E, Heszky L, Dobranszki J (2005) Molecular identification of commercial apple cultivars with microsatellite markers. *HortScience* **40**, 1974-1977

Garcia C, Arroyo JM, Godoy JA, Jordano P (2005) Mating patterns, pollen dispersal, and the ecological maternal neighbourhood in a *Prunus mahaleb* L. population. *Molecular Ecology* **14**, 1821-1830

Geber MA, Moeller DA (2006) Pollinator responses to plant communities and implications for reproductive character evolution. In: Harder LD, Barrett SCH (Eds) *Ecology and Evolution of Flowers*, Oxford University Press, New York, USA, pp 102-120

Ghosh AK, Lukens LN, Hunter DM, Strommer JN (2006) European and Asian pears, Simple sequence repeat-polyacrylamide gel electrophoresis-based analysis of commercially important North American cultivars. *HortScience* **41**, 304-309

Goldway M, Shai O, Yehuda H, Matityahu A, Stern RA (1999) 'Jonathan' apple is a lower-potency pollinizer of 'Topred' than 'Golden delicious' due to partial *S*-allele incompatibility. *Journal of Horticultural Sciences and Biotechnology* **74**, 381-385

Goodell K, Thomson JD (1997) Comparisons of pollen removal and deposition by honey bees and bumblebees visiting apple. *Acta Horticulturae* **437**, 103-107

Granger AR (1997) Pollen gene flow in south Australian cherry (*Prunus avium* L) orchards. *Australian Journal of Experimental Agriculture* **37**, 583-589

Granger AR (2004) Gene flow in cherry orchards. *Theoretical and Applied Genetics* **108**, 497-500

Gross CG (2005) Pollination efficiency and pollinator effectiveness In: Dafni A, Kevan PG, Husband BC (Eds) *Practical Pollination Biology*, Cambridge Ontario, Canada, pp 354-365

Guarino C, Santoro S, De Simone L, Lain O, Cipriani G, Testolin R (2006) Genetic diversity in a collection of ancient cultivars of apple (*Malus* x *domestica* Borkh.) as revealed by SSR-based fingerprinting. *Journal of Horticultural Science and Biotechnology* **81**, 39-44

Gupta JK (2005) An overview of insect pollinators of temperate fruit crops. *Acta Horticulturae* **696**, 589-591

Handel SN (1982) Dynamics of gene flow in an experimental population of *Cucumis melo* (Cucurbitaceae). *American Journal of Botany* **69**, 1538-1546

Handel SN (1983) Contrasting gene flow patterns and genetic subdivision in adjacent populations of *Cucumis sativus* (Cucurbitaceae). *Evolution* **37**, 760-771

Harder LD, Barrett SCH (1995) Pollen dispersal and mating patterns in animal-pollinated plants. In: Lloyd DG, Barrett SCH (Eds) *Floral Biology. Studies on Floral Evolution in Animal-Pollinated Plants*, Chapman and Hall, New York, USA, pp 140-191

Harder LD, Routley MB (2006) Pollen and ovule fates and reproductive performance by flowering plants. In: Harder LD, Barrett SCH (Eds) *Ecology and Evolution of Flowers*, Oxford University Press, New York, USA, pp 61-81

Hauck NR, Yamane H, Tao R, Iezzoni AF (2002) Self-compatibility and incompatibility in tetraploid sour cherry (*Prunus cerasus* L.). *Sexual Plant Reproduction* **15**, 39-46

Hedtke C (1996) Heimfinden von *Apis mellifera* und 4 *Bombus*-Arten im Vergleich. *Apidologie* **27**, 320-323

Hegedus A, Szabo Z, Nyeki J, Halasz J, Pedryc A (2006) Molecular analysis of *S*-haplotypes in peach, a self-compatible *Prunus* species. *Journal of the American Society for Horticultural Science* **131**, 738-743

Herrera CM (1990) Daily pattern of pollinator activity, differential pollinating effectiveness and floral resource availability in summer-flowering Mediterranean shrub. *Oikos* **58**, 727-788

Herrera CM (2005) Plant generalization on pollinators: species property or local phenomenon? *American Journal of Botany* **92**, 13-20

Herrera CM, Castellanos MC, Medrano M (2006) Geographical context of floral evolution: towards an improved research programme in floral diversification. In: Harder LD, Barrett SCH (Eds) *Ecology and Evolution of Flowers*, Oxford University Press, New York, USA, pp 278-295

Heslop-Harrisson JS, Heslop-Harrisson Y (1970) Evaluation of pollen viability by enzymatically induced fluorescence: intracelular hydrolysis of fluorescein diacetate. *Stain Technology* **45**, 115-120

Hiratsuka S, Zhang SL, Nakagawa E, Kawai Y (2001) Selective inhibition of the growth of incompatible pollen tubes by S-protein in the Japanese pear. *Sexual Plant Reproduction* **13**, 209-215

Ishimizu T, Sato Y, Saito T, Yoshimura Y, Norioka S, Nakanishi T, Sakiyama F (1996) Identification and partial amino acid sequences of seven *S*-RNases associated with self-incompatibility of Japanese pear, *Pyrus pyrifolia* Nakai. *Journal of Biochemistry* **120**, 326-334

Jacob-Remacle A (1989) Comportement de butinage de l'abeille domestique et des abeilles sauvages dans des vergers de pommiers en Belgique. *Apidologie* **20**, 271-285

Jacquemart A-L (1993) Floral visitors of *Vaccinium* species in high Ardenne, Belgium. *Flora* **188**, 263-273

Jacquemart A-L, Michotte-van der Aa A, Raspé O (2006) Compatibility and pollinator efficiency tests on *Pyrus communis* L. cv. 'Conference'. *Journal of Horticultural Science and Biotechnology* **81**, 827-830

Jacquemart A-L, Gillet C, Cawoy V (2007) Floral visitors and the importance of honey bee on buckwheat (*Fagopyrum esculentum* Moench) in central Belgium. *Journal of Horticultural Science and Biotechnology* **82**, 104-108

Javorek SK, Mackenzie KE, van der Kloet SP (2002) Comparative pollination efficiency among bees (Hymenoptera: Apoidea) on lowbush blueberry (Ericaceae: *Vaccinium angustifolium*). *Annals of the Entomological Society of America* **95**, 345-351

Kao TH, McCubbin A (1996) How flowering plants discriminate between self and non-self-pollen to prevent inbreeding. *Proceedings of the National Academy of Sciences USA* **93**, 12059-12065

Kearns CA, Inouye DW (1993) *Techniques for Pollination Biologists*, University Press of Colorado, Niwot, USA, 583 pp

Kim HT, Hirata Y, Shin YU, Hwang HS, Hwang JH, Shin IS, Kim DI, Kang SJ, Kim HJ, Shin DY (2004) A molecular technique for selection of self-compatible varieties of Japanese pear (*Pyrus pyrifolia* Nakai). *Euphytica* **138**, 73-80

Kitahara K, Matsumoto S, Yamamoto T, Soejima J, Kimura T, Komatsu H, Abe K (2005) Molecular characterization of apple cultivars in Japan by *S*-RNase analysis and SSR markers. *Journal of the American Society for Horticultural Science* **130**, 885-892

Klein AM, Vaissière BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T (2006) Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences* **274**, 303-313

Kho YO, Baër J (1968) Observing pollen tubes by means of fluorescence. *Euphytica* **17**, 298-302

Komori S, Soejima J, Ito Y, Bessho H, Abe K, Kotoda N (1999) Establishing self-incompatibility genotypes of several apple cultivars by analyzing progenies derived by back-crossing. *Journal of the Japanese Society for Horticultural Science* **68**, 569-577

Koopman WJM, Li YH, Coart E, De Weg EV, Vosman B, Roldan-Ruiz I, Smulders MJM (2007) Linked vs. unlinked markers: multilocus microsatellite haplotype-sharing as a tool to estimate gene flow and introgression. *Molecular Ecology* **16**, 243-256

Krichen L, Mnejja M, Arus P, Marrakchi M, Trifi-Farah N (2006) Use of microsatellite polymorphisms to develop an identification key for Tunisian apricots. *Genetic Resources and Crop Evolution* **53**, 1699-1706

Kuehn BF (1988) Examination of reasons for poor fruit set in the sour cherry cultivar "stevnsbaer" by means of fluorescence microscopy. *Tidsskrift for Planteavl* **92**, 169-174

Kwon YJ, Saeed S (2003) Effect of temperature on the foraging activity of *Bombus terrestris* L. (Hymenoptera: Apidae) on greenhouse hot pepper (*Capsicum annuum* L.). *Applied Entomology and Zoology* **38**, 275-280

La Porta N, Roselli G (1991) Relationship between pollen germination *in vitro* and fluorochromatic reaction in cherry clone F12/1 (*Prunus avium* L.) and some of its mutants. *Journal of Horticultural Science* **66**, 171-175

Lewis D (1979) *Sexual Incompatibility in Plants, Studies in Biology*, 10, Edward Arnold, USA, 230 pp

Lindsey AH (1984) Reproductive biology of Apiceae. I. Floral visitors to *Thaspium* and *Zizia* and their importance to pollination. *American Journal of Botany* **71**, 375-387

Lopéz M, Mnejja M, Rovira M, Collins G, Vargas FJ, Arus P, Batlle I (2004) Self-incompatibility genotypes in almond re-evaluated by PCR, stylar ribonucleases, sequencing analysis and controlled pollinations. *Theoretical and Applied Genetics* **109**, 954-964

Lopéz M, Vargas FJ, Batlle I (2006) Self-(in)compatibility almond genotypes, A review. *Euphytica* **150**, 1-16

Lundberg H (1980) Effects of weather on foraging-flights of bumble bees (Hymenoptera, Apidae) in a subalpine/alpine area. *Holartic Ecology* **3**, 104-110

Macfarlane RP, Royce LA, Wyatt BKW, Mayer DF (1994) Evaluation of commercial bumble bee colonies for cranberry pollination. *Melanderia* **50**, 13-19

Maghuly F, Fernández EB, Zelger R, Marschall K, Katinger H, Laimer M (2006) Genetic differentiation of apricot (*Prunus armeniaca* L.) cultivars with SSR markers. *European Journal of Horticultural Science* **71**, 129-134

Marchese A, Tobutt KR, Raimondo A, Motisi A, Bošković RI, Clarke J, Caruso T (2007) Morphological characteristics, microsatellite fingerprinting and determination of incompatibility genotypes of Sicilian sweet cherry cultivars. *Journal of Horticultural Science and Biotechnology* **82**, 41-48

Marcucci MC, Visser T (1987) Pollen tube growth in apple and pear styles in relation to self-incompatibility, incongruity and pollen load. *Advances in Horticultural Science* **1**, 90-94

Martin FW (1959) Staining and observing pollen tubes in the style by means of fluorescence. *Stain Techniques* **34**, 125-128

McGregor SE (1976) Insect pollination of cultivated crop plants, USDA. Available online: http://gears.tucson.ars.ag.gov/book

Messina R, Lain O, Marrazzo MT, Huang WG, Cipriani G, Testolin R (2004) Isolation of microsatellites from almond and apricot genomic libraries and testing for their transportability across different *Prunus*. *Acta Horticulturae* **663**, 79-82

Meynie S, Bernard R (1997) Pollinator efficiency of some insects in relation to wild species populations of *Helianthus* L. *Agronomie* **17**, 43-51

Michotte-van der Aa A, Jacquemart AL (2003) Les principales variétés de poirier cultivées en Belgique (*Pyrus communis* L., var. Conférence, var. Doyenné et var. Durondeau) sont-elles compatibles intra-variété ? *Le Fruit Belge* **503**, 107-112

Mitchell RJ (1997) Effects of pollination intensity on *Lesquerella fendleri* seed set: variation among plants. *Oecologia* **109**, 382-388

Modlibowska I (1945) Pollen tube growth and embryo-sac development in apples and pears. *Journal of Horticultural Science* **62**, 31-36

Monzon VH, Bosch J, Retana J (2004) Foraging behavior and pollinating effectiveness of *Osmia cornuta* (Hymenoptera: Megachilidae) and *Apis mellifera* (Hymenoptera: Apidae) on "Comice" pear. *Apidologie* **35**, 575-585

Morris WF, Mangel M, Adler FR (1995) Mechanisms of pollen deposition by insect pollinators. *Evolutionary Ecology* **9**, 304-317

Motten AF (1986) Pollination ecology of the spring wildflower community of a temperature deciduous forest. *Ecological Monographs* **56**, 21-42

Navarro L (2000) Pollination ecology of *Anthyllis vulneraria* subsp. *vulgaris* (Fabaceae): Nectar robbers as pollinators. *American Journal of Botany* **87**, 980-985

Nyéki J, Soltész M (1998) The variation of seed content of fruits in pear varieties, also as function of different conditions of fertilization, as open pollination, natural autogamy and allogamy. *Acta Horticulturae* **475**, 237-250

Ortega E, Egea J, Cánovas JA, Dicenta F (2002) Pollen tube dynamics following half- and fully-compatible pollinations in self-compatible almond cultivars. *Sexual Plant Reproduction* **15**, 47-51

Ortega E, Bošković RI, Sargent DJ, Tobutt KR (2006) Analysis of S-RNase alleles of almond (*Prunus dulcis*), characterization of new sequences, resolution of synonyms and evidence of intragenic recombination. *Molecular Genetics and Genomics* **276**, 413-426

Palser BF, Rouse JL, Williams EG (1989) Coordinated timetables for megagametophyte development and pollen tube growth in *Rhododendron nuttallii* from anthesis to early postfertilization. *American Journal of Botany* **76**, 1167-1202

Peakall R (1989) A new technique for monitoring pollen flow in orchids. *Oecologia* **79**, 361-365

Pedersen BH (2006) DNA fingerprints of 51 sweet and sour *Prunus* accessions using Simple Sequence Repeats. *Journal of Horticultural Science and Biotechnology* **81**, 118-124

Primack RB, Silander JA (1975) Measuring the relative importance of different pollinators to plants. *Nature* **255**, 143-144

Prŷs-Jones OE (1986) Foraging behaviour and the activity of substrate cycle enzymes in bumble bees. *Animal Behaviour* **34**, 609-611

Raspé O, Kohn JR (2002) S-allele diversity in *Sorbus aucuparia* and *Crataegus monogyna* (Rosaceae: Maloideae). *Heredity* **88**, 458-465

Reim S, Flachowsky H, Michael M, Hanke MV (2006) Assessing gene flow in apple using a descendant of *Malus sieversii* var. *sieversii* f. *niedzwetzky* an identifier for pollen dispersal. *Environmental Biosafety Research* **5**, 89-104

Richman AD, Kao TH, Schaeffer SW, Uyenoyama MK (1995) S-allele sequence diversity in natural populations of *Solanum carolinense* (Horsenettle). *Heredity* **75**, 405-415

Romero C, Vilanova S, Burgos L, Martinez-Calvo J, Vicente M, Llacer G, Badenes ML (2004) Analysis of the *S*-locus structure in *Prunus armeniaca* L. Identification of S-haplotype specific *S*-RNase and *F*-box genes. *Plant Molecular Biology* **56**, 145-157

Roubick DW (1995) Pollination of cultivated plants in the tropics. Food and Agriculture Organization of the United Nations, Rome, Italy, Bulletin **118**, 198 pp

Rovira M, Clavé J, Romero M, Santos J, Vargas FJ (1998) Self-compatibility in almond progenies. *Acta Horticulturae* **470**, 66-71

Sage TL, Bertin RI, Williams EG (1994) Ovarian and other late-acting self-incompatilibity systems. In: Williams AG, Clarke AE, Knox RB (Eds) *Genetic Control of Self-Incompatibility and Reproductive Development in Flowering Plants*, Kluwer, Dordrecht, NL, pp 116-140

Sage TL, Strumas F, Cole WW, Barrett SCH (1999) Differential ovule development following self- and cross-pollination: the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *American Journal of Botany* **86**, 855-870

Sage TL, Husband BC, Routley MB (2005) Intrinsic attributes of the breeding system. In: Dafni A, Kevan PG, Husband BC (Eds) *Practical Pollination Biology*, Cambridge Ontario, Canada, pp 30-56

Sánchez-Pérez R, Martínez-Gómez P, Dicenta F, Egea J, Ruiz D (2006) Level and transmission of genetic heterozygosity in apricot (*Prunus armeniaca* L.) explored using simple sequence repeat markers. *Genetic Resources and Crop Evolution* **53**, 763-770

Sanzol J, Rallo P, Herrero M (2003) Asynchronous development of stigmatic receptivity in the pear (*Pyrus communis*, Rosaceae) flower. *American Journal of Botany* **90**, 78-84

Sanzol J, Sutherland BG, Robbins TP (2006) Identification and characterization of genomic DNA sequences of the *S*-ribonuclease gene associated with self-incompatibility alleles *S-1* to *S-5* in European pear. *Plant Breeding* **125**, 513-518

Sapir G, Stern RA, Eisikowitch D, Goldway M (2004) Cloning of four new Japanese plum *S*-alleles and determination of the compatibility between cultivars by PCR analysis. *Journal of Horticultural Science and Biotechnology* **79**, 223-227

Sargent DJ, Rys A, Nier S, Simpson DW, Tobutt KR (2007) The development and mapping of functional markers in *Fragaria* and their transferability and potential for mapping in other genera. *Theoretical and Applied Genetics* **114**, 373-384

Sassa H, Hirano H, Ikehashi H (1992) Self-incompatibility-related RNases in styles of Japanese pear (*Pyrus serotina* Rehd.). *Plant Cell Physiology* **33**, 811-814

Sassa H, Nishio T, Kowyama Y, Hirano H, Koba T, Ikehashi H (1996) Self-incompatibility (*S*) alleles of the Rosaceae encode members of a distinct class of the T2/S ribonuclease superfamily. *Molecular and General Genetics* **250**, 547-557

Schneider D, Stern RA, Eisikowitch D, Goldway M (2002) The relationship between floral structure and honeybee pollination efficiency in 'Jonathan' and 'Topred' apple cultivars. *Journal of Horticultural Science and Biotechnology* **77**, 48-51

Schneider D, Stern RA, Goldway M (2005) A comparison between semi- and fully compatible apple pollinators grown under suboptimal pollination conditions. *Hortscience* **40**, 1280-1282

Schueler S, Tusch A, Schuster M, Ziegenhagen B (2003) Characterization of microsatellites in wild and sweet cherry (*Prunus avium* L.) – markers for individual identification and reproductive processes. *Genome* **46**, 95-102

Schueler S, Tusch A, Scholz F (2006) Comparative analysis of the within-population genetic structure in wild cherry (*Prunus avium* L.) at the self-incompatibility locus and nuclear microsatellites. *Molecular Ecology* **15**, 3231-3243

Seavey SR, Bawa SK (1986) Late-acting self incompatibility in angiosperms. *The Botanical Review* **52**, 195-219

Sharifani MM, Jackson JF (1997) Isozyme inheritance in controlled crosses with "Packham Triumph" pear. *Acta Horticulturae* **437**, 169-174

Sharifani MM, Jackson JF (2001) Estimation of gene flow by pollen in a part of "Packham Triumph" pear orchard. *Acta Horticulturae* **561**, 53-59

Shaw PJ, Rawlins DJ (1994) An introduction to optical microscopy for plant cell biology. In: Harris N, Oparka KJ (Eds) *Plant Cell Biology. A Practical Approach*, Information Press Ltd, Oxford, UK, pp 1-26

Shiran B, Amirbakhtiar N, Kiani S, Mohammadi S, Sayed-Tabatabaei BE, Moradi H (2007) Molecular characterization and genetic relationship among almond cultivars assessed by RAPD and SSR markers. *Scientia Horticulturae* **111**, 280-292

Sijacic P, Xi W, Skirpan AL, Yan W, Dowd PE, McCubbin AG, Shihshieh H, Teh-hui K (2004) Identification of the pollen determinant of *S*-RNase-mediated self-incompatibility. *Nature* **429**, 302-305

Slaa EJ, Sánchez CLA, Malagodi-Braga KS, Hofstede FE (2006) Stingless bees in applied pollination. Practice and perspectives. *Apidologie* **37**, 293-315

Stern RA, Eisikowitch D, Dag A (2001) Sequential introduction of honeybee colonies and doubling their density increases cross-pollination, fruit-set and yield in 'Red Delicious' apple. *Journal of Horticultural Science and Biotechnology* **76**, 17-23

Stern RA, Goldway M, Zisovich AH, Shafir S, Dag A (2004) Sequential intro-duction of honeybee colonies increases crosspollination, fruit-set and yields of 'Spadona' pear (*Pyrus communis* L.). *Journal of Horticultural Science and Biotechnology* **79**, 652-658

Stone JL, Thomson JD, Dent-Acosta SJ (1995) Assessment of pollen viability in hand-pollination experiments: a review. *American Journal of Botany* **82**, 1186-1197

Stout JC, Allen JA, Goulson D (1998) The influence of relative plant density and floral morphological complexity on the behaviour of bumble bees. *Oecologia* **117**, 543-550

Sutherland BG, Robbins TP, Tobutt KR (2004) Primers amplifying a range of *Prunus S*-alleles. *Plant Breeding* **123**, 582-584

Takasaki T, Moriya Y, Okada K, Yamamoto K, Iwanami H, Bessho H, Nakanishi T (2006) cDNA cloning of nine *S* alleles and establishment of a PCR-RFLP system for genotyping European pear cultivars. *Theoretical and Applied Genetics* **112**, 1543-1552

Taseï JN (1984) Arbres fruitiers des régions tempérées. In: Pesson P, Louveaux J (Eds) *Pollinisation et Productions Végétales*, INRA, Paris, France, pp 348-372

Thomson JD, Price MV, Waser NM, Stratton DA (1988) Comparative studies of pollen and fluorescent dye transport by bumble bees visiting *Erythronium grandiflorum*. *Oecologia* **69**, 561-566

Thomson JD, Thomson BA (1992) Pollen presentation and viability schedules and their consequences for reproductive success through animal pollination. In: Wyatt R (Ed) *Ecology and Evolution of Plant Reproduction: New Approaches*. Chapman and Hall, New York, USA, pp 1-24

Tobutt KR, Bošković R, Cerović R, Sonneveld T, Ružić D (2004) Identification of incompatibility alleles in the tetraploid species sour cherry. *Theoretical and Applied Genetics* **108**, 775-785

Ushijima K, Sassa H, Dandekar AM, Gradziel TM, Tao R, Hirano H (2003) Structural and transcriptional analysis of the self-incompatibility locus of almond: identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. *Plant Cell* **15**, 771-781

van Nerum I, Incerti F, Keulemans J, Broothaerts W (2000) Analysis of self-fertility in transgenic apple lines transformed with an *S*-allele either in sense or antisense direction. *Acta Horticulturae* **538**, 625-629

van Nerum I, Geerts M, van Haute A, Keulemans J, Broothaerts W (2001) Re-examination of the self-incompatibility genotype of apple cultivars containing putative 'new' *S*-alleles. *Theoretical and Applied Genetics* **103**, 584-591

Vasilakakis M, Porlingis IC (1985) Effect of temperature on pollen germination, pollen tube growth, effective pollination period, on fruit set of pear. *HortScience* **20**, 733-735

Vezvaei A, Jackson JF (1997) Gene flow by pollen in an almond orchard as determined by isozyme analysis of individual kernels and honey bee pollen loads. *Acta Horticulturae* **437**, 75-81

Vezvaei A (1998) Pollen tube growth in nonpareil almond in relation to pollen genotype, temperature and competition among mixed pollen. *Acta Horticulturae* **470**, 251-261

Vilanova S, Romero C, Burgos L, Martinez-Calvo J, Vicente M, Llacer G, Badenes ML (2006) Self-(in)compatibility in *Prunus armeniaca* L.: analysis of the *S*-locus structure and identification of *S*-haplotype specific *S*-RNase. *Acta Horticulturae* **717**, 213-216

Vorobyev M, Gumbert A, Kunze J, Giurfa M, Menzel R (1997) Flowers through insect eyes. *Israel Journal of Plant Sciences* **45**, 93-101

Walther-Hellwig K, Frankl R (2000) Foraging habitats and foraging distances of bumble bees, *Bombus* spp. (Hym., Apidae), in an agricultural landscape.

Journal of Applied Entomology **124**, 299-306

Waser NM (1983) The adaptive nature of floral traits: ideas and evidence. In: Real L (Ed) *Pollination Biology*, Academic Press, Orlando, Florida, USA, pp 242-305

Waser NM (1986) Flower constancy: definition, cause, and measurement. *The American Naturalist* **127**, 593-603

Waser NM (1988) Comparative pollen and dye transfer by pollinators of *Delphinium nelsonii*. *Functional Ecology* **2**, 41-48

Webster AD (2002) Factors influencing the flowering, fruit set and fruit growth of European pears. *Acta Horticulturae* **596**, 699-709

Westerkamp C (1991) Honey bees are poor pollinators - Why? *Plant Systematics and Evolution* **177**, 71-75

Williams CS (1995) Conserving Europe's bees: why all the buzz? *Trends in Ecology and Evolution* **10**, 309-310

Williams EG, Ramm-Anderson S, Dumas C, Mau SL, Clarke AE (1982) The effect of isolated components of *Prunus avium* L. styles on *in vitro* growth of pollen tubes. *Planta* **156**, 517-519

Williams IH (1994) The dependences of crop production within the European Union on pollination by honey bees. *Agricultural and Zoology Review* **6**, 229-257

Williams KJ, Willsmore KL, Granger AR (2004) Evaluation of DNA based techniques for the determination of sweet cherry (*Prunus avium* L.) self-incompatibility genotypes. *Acta Horticulturae* **663**, 661-665

Williams RR (1970) Factors affecting pollination in fruit trees. In: Luckwill LC, Cohung CV (Eds) *Physiology of Tree Crops*, Academic Press, London, UK, pp 193-206

Wunsch A, Hormaza JI (2004) S-allele identification by PCR analysis in sweet cherry cultivars. *Plant Breeding* **123**, 327-331

Wunsch A, Carrera M, Hormaza JI (2006) Molecular characterization of local Spanish peach (*Prunus persica* (L.)) germplasm. *Genetic Resources and Crop Evolution* **53**, 925-932

Wyatt R, Broyles SB, Derda GS (1992) Environmental influences on nectar production in milkweeds (*Asclepias syriaca* and *A. exaltata*). *American Journal of Botany* **79**, 636-642

Xie H, Sui Y, Chang FQ, Xu Y, Ma RC (2006) SSR allelic variation in almond (*Prunus dulcis* Mill.). *Theoretical and Applied Genetics* **112**, 366-372

Yamane H, Tao R, Murayam H, Sugiura A (2000) Determining the *S*-genotypes of several sweet cherry cultivars based on PCR-RFLP analysis. *Journal of Horticultural Science and Biotechnology* **75**, 562-567

Yoon JH, Liu DC, Song WS, Liu WS, Zhang AM, Li SH (2006) Genetic diversity and ecogeographical phylogenetic relationships among peach and nectarine cultivars based on simple sequence repeat (SSR) markers. *Journal of the American Society for Horticultural Science* **131**, 513-521

Zhang SL, Hiratsuka S (1999) Analysis of varietal differences in self- and cross-incompatibility reactions of Japanese pears using stylar culture technique. *Journal of the Japanese Society of Horticultural Science* **68**, 373-383

Zisovich AH, Stern RA, Shafir S, Goldway M (2004) Identification of seven *S*-alleles from the European pear (*Pyrus communis*) and the determination of compatibility among cultivars. *Journal of Horticultural Science and Biotechnology* **79**, 101-106

Zisovich AH, Stern RA, Shafir S, Goldway M (2005) Fertilisation efficiency of semi- and fully-compatible European pear (*Pyrus communis* L.) cultivars. *Journal of Horticultural Science and Biotechnology* **80**, 143-146

Zuccherelli S, Tassinari P, Broothaerts W, Tartarini S, Dondini L, Sansavini S (2002) *S*-allele characterization in self-incompatible pear (*Pyrus communis* L.). *Sexual Plant Reproduction* **15**, 153-158