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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.

Keywords: Apideae, fluorescence, genetic methods, hand pollination, pollination effectiveness, pollen tubes, pollen viability, Rosaceae, *S*-alleles, self-incompatibility

Abbreviations: CTAB, cetyltrimethylammonium bromide; **FAA**, formalin-acetic acid-alcohol; **FDA**, fluoresceine diacetate; **GSI**, gametophytic self-incompatibility; **OSI**, ovarian self-incompatibility; **PCR**, polymerase chain reaction; **RAPD**, random amplified polymorphic DNA; **RFLP**, restriction fragment length polymorphism; **RNase**, ribonuclease; **SFB**, *S-F* box gene; **SI**, self-incompatibility; **SSI**, sporophytic self-incompatibility; **SSR**, single sequence repeat

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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

Received: 22 February, 2007. Accepted: 22 March, 2007.

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

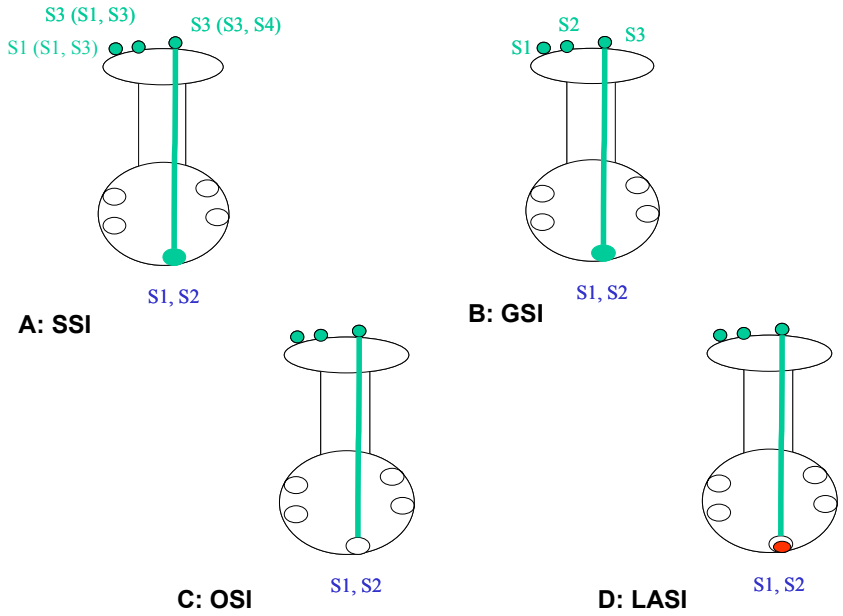

Fig. 1 Schematic representation of the different self-incompatibility (SI) systems. (**A**) Sporophytic SI, (**B**) gametophytic SI, (**C**) ovarian SI, (**D**) late-acting SI. Haploid *S*-alleles are indicated in green (for sporophytic SI, the paternal diploid genotype is given under brackets). Maternal *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 polymerphism 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; Lopéz *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 carryover 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 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

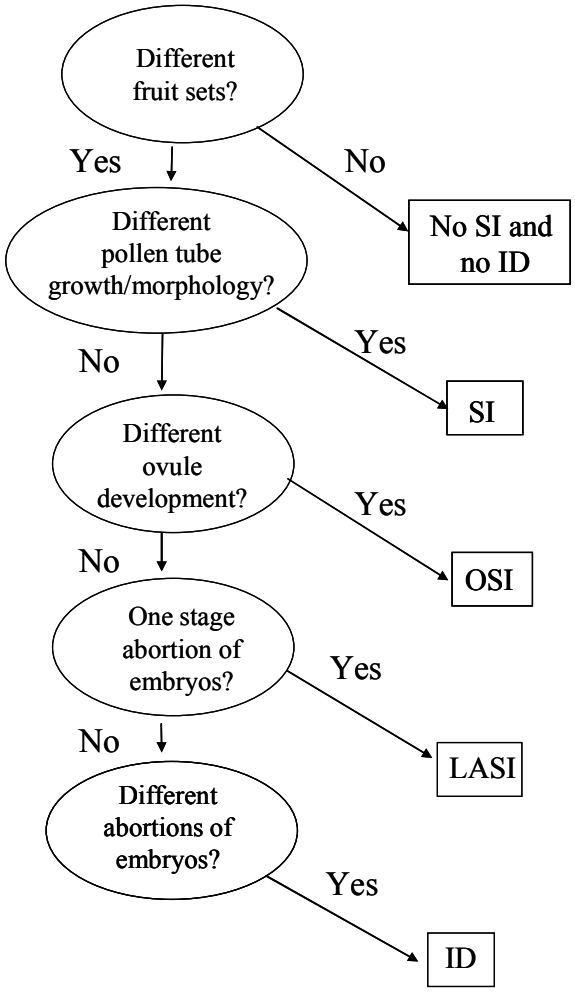

Fig. 2 Sequential tests to distinguish among self-incompatibility (SI), ovarian self-incompatibility (OSI), late-acting self-incompatibility (LASI) and inbreeding depression (ID). The comparison is done between hand self- and cross- pollinated flowers.

[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.

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_2SO_3	0.1 % in 0.1 N K_3PO_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_2SO_3	0.1 % in 0.1 N K_3PO_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_3PO_4	Vezvaei 1998
Apple	1	Trees	? emasculation	144	FAA	NaOH 8N 30 min	0.05 % in K_2HPO_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_3PO_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_3PO_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_2HPO_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**).

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).

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 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-

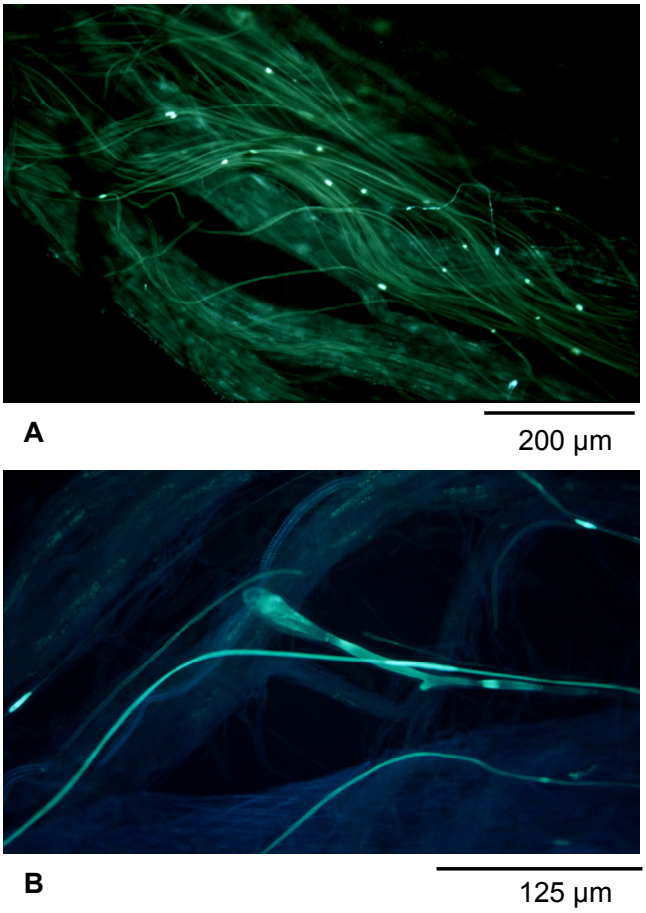

Fig. 3 Pollen tubes in styles of *Pyrus communis* cv. 'Conférence' observed under fluorescence microscopy. (**A**) Compatible pollen tubes containing a lot of small and regularly distributed callosic plugs; (**B**) incompatible tubes showing much longer and frequent callosic plugs and becoming swollen 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

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 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

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).

'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 effecttiveness') 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 nelsonii* (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, Amygdaloidae, 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.

ACKNOWLEDGEMENTS

Many thanks to Agnès Michotte-Van der Aa for the photographs under fluorescence microscopy; and to Olivier Raspé, Marie Pairon, Arnaud Vervoort, and Renate Wesselingh for their critical review of the manuscript and English improvement. This is publication number 113 of the Biodiversity Research Centre, Université catholique de Louvain.

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