RESEARCH ARTICLE

Running title:

Fruit set in apple

Title:

The influence of the progamic phase for fruiting in the apple tree

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Abstract

Final fruit production is the result of a number of processes, over which several environmental circumstances interact. But it is often difficult to disentangle the part played by each of these factors in the final crop. The aim of this work is to evaluate the influence of the progamic phase for fruiting in the apple tree. For this purpose we track back the process that goes from flower to fruit, identifying the inflection points where the final crop is reduced. We evaluate early versus late fruit development, pollination versus non pollination, and the effect of the progamic phase that goes from pollination to fertilization. From flowers to fruits fifteen weeks elapsed, but the final fruit set settled eight weeks after flowering, and the main flower-fruit drop occurred three-four weeks after flowering. Differences between dropped fruits and those that remained in the tree emerged earlier, and the onset of fruiting started seven days after pollination. This time was coincident with the time lapse of the progamic phase. These results show that fruiting gets established well ahead of cropping, but also that the progamic phase is the main determinant of the final fruit set in apple trees.

Keywords: apple; fruit drop, fruit set; fruiting; *Malus;* pollen tube growth; progamic phase.

Introduction

Angiosperms usually produce much less fruits than the initial flower number (Klein *et al*., 2007). This low fruit set has been largely described in plants growing in natural conditions (Marshall & Ellstrand, 1986), and also in cultivated plants such as fruit trees (Rodrigo & Herrero, 2002; Hedhly *et al*., 2009). The fact that seed-fruit set may account for crop productivity has a particular relevance in agricultural important species. Seeds and fruits are the result of a series of complex interactions initiated at pollination (Maeshwari, 1950; Herrero, 1992; Lord & Russell, 2002). A number of external and internal factors affect fruit set (Sutherland, 1988). Environmental effects as temperature (Hedhly et al, 2009; Hedhly, 2011), and pollen abundance (Weber & Goodwillie, 2009; Bianchi & Cunningham, 2012) may limit fruit set. But also a number of internal processes do play a clear part. Thus, erratic fruit set has been associated with stigmatic receptivity (González *et al*., 1995; Sanzol & Herrero, 2001; Sanzol *et al*., 2003), the stage of ovule development (Ruiz & Egea, 2008), or flower quality (Williams, 1965; Rodrigo *et al*., 2009, Alcaraz *et al*. 2010). Further down fruitlet competition and the so-called June drop also contribute to a reduction in fruit set (Goldwin, 1992).

While all these factors have shown to play a part in the final crop in different species and circumstances, it is often difficult to disentangle the part played by each of these factors in the final crop. Mainly because what we face is the final result of a series of biological processes. Still to know the main responsible of crop production is an active field of research and the possible contribution of the genotype and the environment have received much attention (Slafer, 2003). In this context fruit trees have the advantage that cultivars are of clonal origin and thus genetically identical and also that they remain located at the same area. Work in *Prunus* species shows that the progamic phase, defined as the time lasting from pollination to fertilization (Linskens, 1975), plays a

major part in the establishment of fruit set (Rodrigo & Herrero, 2002; Hedhly *et al*., 2007). But little is known on the relevance that this phase has in on apple trees (*Malus* x *domestica),* which is the main temperate fruit tree.

Apple crop production has focused much attention since it has strong economical importance in agricultural systems. Because of that, it has been studied for more than 50 years using agronomic approaches (Byers, 1993). At flowering time, apples bloom in groups of inflorescences called corymbs (Pratt, 1988). However, within the corymb some flowers have more ability than others to set a fruit (Bangerth, 2000), and a strategy has been put forward for the whole corymb that may facilitate setting a minimum number of fruits per inflorescence under different environmental conditions (Losada & Herrero, 2013). Still, to achieve larger fruits, chemical or manual thinning has usually been a common agricultural practice (Ebert & Bangerth, 1982). More recently, the genetic control of fruit maturation is also an active field (Seo & Kim, 2009), and the apple genome has recently been sequenced (Troggio *et al*., 2012) enabling new breeding strategies (Kumar *et al*., 2012). Concerning the reproductive process, the Effective Pollination Period was first defined in this species as the time a pollination is effective resulting in fertilization (Williams, 1966); and recently, it has been put forward what confers a stigma the capacity to be receptive (Losada & Herrero, 2012). Also, much is known about self incompatibility in apple (Kim *et al*., 2009). But surprisingly the information relating to compatible crosses is far scarcer and there is a paucity of information relating the progamic phase with fruit production in this species.

The aim of this work was to evaluate the influence of the progamic phase for fruiting in apple trees. For this purpose we track back the process that goes from flower to fruit, identifying the inflection points where the final crop is reduced. The results obtained not

only show that fruiting gets established well ahead of cropping, but also that the progamic phase is the main determinant of the final crop.

Materials and methods

Plant material and fruit set measurements

Apple trees cv Golden Delicious Spur grown for 20 years in the province of Huesca (Spain), 461m above sea level were used in this study. Since Golden Delicious is self incompatible, the cultivar Royal Gala was in the same plantation as a pollinator cultivar. Fruit set measurements were carried during two non-consecutive years, 2008 and 2010, both with good bearing. Ten trees were used per experiment, labelling branches in all orientations: North, South, East and West. To monitor natural fruit set, flower/fruits in these branches were weekly counted until fruit ripening, with a minimum of 1.000 flowers per treatment. Flower/fruit changes were also followed and photographs taken with an OLYMPUS μ760, 7.1 megapixels.

Supplemental pollination

To evaluate the impact of pollination, a supplemental pollination experiment was conducted in the second year. For this purpose a similar number of branches oriented to all directions were selected and only flowers at the balloon stage were left. This stage occurs one day prior to anthesis, and is easily discernible by the inflated appearance of the petals. The following day, these just opened flowers were hand pollinated with a paint brush with pollen from the compatible cv Royal Gala. Fruit set was controlled by weekly counts of flowers/fruits until cropping.

To obtain pollen, anthers were collected from flowers at the balloon stage -one day before anthesis- and left to dry on a paper at room temperature around 20ºC for 24-48 h

until dehiscence. Pollen was sieved with a mesh with a diameter pore of 0.26 mm and then stored at -20ºC until required.

Flower weight measurements

To evaluate flower/fruitlet growth in pollinated and unpollinated flowers, in both years 200 flowers were selected at the balloon stage in branches oriented to all directions. Flower buds at the balloon stage were depetalled to avoid insect pollination (Free, 1964). The following day, half of the flowers were hand pollinated with a paint brush with pollen from cv. Gala, and half of the flowers were left without pollination. Five pollinated and five unpollinated flowers per day were weighed sequentially for a period of 20 days after anthesis, when unpollinated flowers shed.

Microscope preparations

To monitor nuclei movement during the first 24 hours of pollen grains-stigma contact, five flowers from two, four, six, and eight hours after pollination were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH7.3 overnight. Then they were washed three times in PBS 10X for one hour in each wash, and the pistils stained in a mixture of 1 µg/ml 4',6-diamidino-2-phenylindole-DAPI in water and 1% TritonX for 30 minutes in the dark (modified from González-Melendi *et al*., 2005). Styles were then washed in PBS again and squashed onto glass slides and visualized under a fluorescence microscope LEICA DM2500 with 340/400 nm filter. Photographs were taken with a LEICA DFC320 camera linked to the software Leica Application Suite.

On the other hand, five flowers were daily sampled from 24 hours after pollination until petal fall and fixed in FAA (formalin: acetic acid: 70% ethanol) (1:1:18) (Johansen, 1940). Following fixation, the pistils were washed three times in distilled water for one

hour each, and left in 5% sodium sulphite for 24 h. Samples were autoclaved for 10 min at 1Kg cm^{-2} in 5% sodium sulphite (Jefferies & Belcher, 1974), and then the individual styles were squashed onto glass slides with 0.1% aniline blue in 0.1 N K_3PO_4 to visualize callose (Currier, 1957) and pollen tubes (Linskens & Esser, 1957). The arrival of pollen tubes to the different pistil structures was timed with a LEICA DM2500 epifluorescence microscope with 340/400 nm filter. Photographs were taken with a CANON Power Shot S50 camera linked to the CANON Remote Capture software.

Statistical analysis

To evaluate drop patterns with time, repeated measurements were statistically compared among weeks using one-way ANOVA assuming a randomized experiment, for each year separately. The second year of experiments, same tests were used, plus additional one-way ANOVAS served to evaluate differences between supplemental pollination and open pollination. Finally, early pollination effect on flowers was evaluated by one-way ANOVA comparison between pollinated and un-pollinated gynoecia weights for each year independently. Correlation and regression analyses were performed to identify and evaluate the degree of relationship among the percentage of flowers remaining in the tree along weeks after anthesis, as well as for gynoecium mean weights with days after pollination. All Statistical analyses were performed with the SPSS 17.0 software (SPSS Inc., Chicago, USA) at a $P \leq 0.05$ significant level.

Results

Fruit set and fruit drop

Apple flowers showed slight differences in blooming time between both years of the experiments, but the time interval from flowering to fruit maturation matched in both

years. Observation of inflorescences from anthesis, which is the day the petals open, (Fig. 1A) showed that petal fall occurred about one week after anthesis (Fig. 1B). First differences in flower/fruits growth inside the corymb were apparent two to three weeks after anthesis (Fig. 1C). Four weeks later, only a few fruits per corymb continued to grow (Fig. 1D), resulting later in an average of only two fruits per corymb (Fig. 1E). Finally, those fruits continued growing, and maturation-harvest occurred some fifteen weeks after flowering (Fig. 1F).

Flowers/fruits dropped from two to eight weeks after flowering leading to a final fruit set of 15% of the initial flowers in open pollination conditions in the first year of the experiments (Fig. 2A), and the data fitted to a logarithmic curve using a non-linear regression protocol (r^2=0.918; $P \leq 0.05$). Significant differences in percentages of dropped flower/fruits were observed between the third and fourth weeks after anthesis ($P \leq 0.05$). Relative fruit drop showed two significant peaks of flower/fruit drop (Fig. 2B), a first one four weeks after anthesis leading to the initial fruit set; and a second wave of drop seven weeks after flowering, that correspond to the June drop and resulted in the final fruit set. The presence of seeds was examined in aborted fruits from the first drop, to evaluate if fertilization had occurred. These fruits showed a mean number of 1.82 plump seeds (±1.04 SE), while an average of seven plump seeds are found in mature fruits of this cultivar (Losada, unpublished).

Drop patterns were similar the second year of the experiments, with a final fruit set also of 15% in the open pollinated flowers (Fig. 3A). Supplemental hand pollination of flowers resulted in a slightly higher final fruit set (23%), but the same drop pattern; and similar logarithmic regression curves than open pollinated flowers (r^2_{open}= 0.907; r^2_{suppl}= 0.930, $P \leq 0.05$). Differences were significant, between percentages of flower/fruit drop in both pollination conditions, three weeks after anthesis ($P \leq 0.05$),

and also seven weeks after anthesis, when final fruit set was defined. While the first relative fruit drop (Fig. 3B) was similar between both open pollinated (38%) and pollen supplemented flowers (43%), in the final fruit drop, pollen supplemented flowers showed a minor fruitlet drop, resulting in a higher percentage of remaining fruits in the tree (23%) than in open pollination conditions (15%).

Receptacle enlargement in apple flowers

At the initial fruit drop, three weeks after anthesis, just fallen fruits weighed less than 100mg, as compared to 700mg of set fruits. To evaluate when these differences arose, flower/fruit weight was sequentially compared between pollinated and unpollinated flowers, for the first 20 days after anthesis. Although clear differences in gynoecia weight were observed for same type of flowers between years, gynoecia development followed a similar pattern both years of experiments (Fig. 4). Unpollinated flowers only slightly changed their weight both years, showing a weak regression fit between flower weights and time after blooming (r^2_{2008}=0.357; r^2_{2010}=0.141; $P \leq 0.05$). On the contrary, hand pollinated flowers clearly increased weight following an exponential curve (r^2_{2008}=0.939, r^2_{2010}=0.680, $P \leq 0.05$). Additionally, ANOVA comparison between un-pollinated and pollen supplemented flower weights showed significant differences ($P \leq 0.05$) between both kinds of flowers, which started, in the first and second year of the experiments, on the six and seven day respectively (Fig. 4A, 4B). These results raised the question of when fertilization had occurred and how long the progamic phase lasted.

Pollen tube kinetics in the apple pistil

The sequential evaluation of pollen tube growth along the apple pistil showed that two hours after pollination, pollen grains were completely hydrated on the stigma surface

and nuclei visible inside (Fig. 5A). Pollen grains germinated and the nuclei migrated into the pollen tube on the stigma by six hours after pollination (Fig. 5B). But the highest germination rate on the stigma was observed 24 hours after pollination (Fig. 5C). First pollen tubes reached the base of the style 72 hours after pollination (Fig. 5D), and grew on the obturator surface, located at the base of the ovary locule, one day later (Fig. 5E). Finally, the first pollen tubes entering the ovule were observed six days after pollination (Fig. 5F), and 10 days after pollination several endosperm nuclei and a young embryo were apparent.

Discussion

The evaluation of the influence of pollination, the onset of fruiting, and the progamic phase, sheds light on the part played by each of them. While from flowering to cropping almost four months elapsed, final fruit set settled two months after flowering. But first differences between set and unset flowers could be seen three weeks after flowering. Finally, the onset of gynoecium growth in pollinated flowers started as early as one week after pollination, a time that matches with the duration of the progamic phase.

Pollination and final fruit set

Final fruit set in apple was low (15%), but resulted in an adequate cropping. Low flower-fruit ratio is commonly reported also in other species and related with breeding systems (Sutherland, 1988), or pollen density (Zhang *et al*., 2010). However, in this work supplemental pollination in apple trees only resulted in a slight increase of the final fruit set (23%) showing that insect pollination in the orchard was effective.

After pollination, two great waves of fruitlet drop were observed. These two waves have been previously reported in apple and also in other species, (Kester & Griggs, 1959;

Sedgley & Griffin, 1989; Rodrigo & Herrero, 2002; Hedhly *et al*., 2007). Fruit drop in apple has been associated to competence among fruits (Goldwin, 1992) and seed number (Brault & de Oliveira, 1995; Sheffield *et al*., 2005), with it is implications on hormone production (Bottom *et al*., 2010). In support of this, our results show that the second fruit drop in apple was reduced following hand supplemental pollination. Also few plump seed were present in the first fruitlets dropped. Taken together these data show that, with adequate pollination condition in the orchard, further pollination does not rescue the big proportion of flowers that drop; and that the main fruit drop occurs in early fruit development. This led us to focus on the onset of fruiting.

The onset of fruiting

The first flower/fruit drop took place two or three weeks after pollination, depending on the years. But dropped flower/fruitlets arrested their development well ahead, and one week after pollination thee first differences between pollinated and unpollinated flowers were apparent. Moreover, only a proportion of pollinated flowers initiated growth, while others did not grow and eventually dropped. The situation is similar to what has been described in apricot (Rodrigo & Herrero, 2002) or sweet cherry (Hedhly *et al*., 2007), where an early flower/fruit drop has also been recorded. The fact that these were hand pollinated flowers rules out pollen shortage as the cause of this arrested growth, suggesting that either poor fertilization or strong resource limitation would lead to this early fruit abortion.

The term flower quality reflects the inherent capacity of the flower to set a fruit (Williams, 1965) and differences in this capability have been reported in apple (Williams, 1970). It has been further put forward that both the stage of development of the pistil (Rodrigo *et al*., 2009; Julian *et al*., 2010), and the nutritional status of the

flowers (Rodrigo *et al*., 2000, 2009, Alcaraz *et al*., 2010) affect this flower quality and hence flower/fruit drop. An initial developmental of the ovary could be observed in both unpollinated and pollinated flowers in apricots (Rodrigo & Herrero, 2002), but in apple flowers only enlarged the receptacle following pollination. To evaluate this effect the progamic phase was timed.

The part played by the progamic phase on fruiting

The progamic phase lasted for seven days after pollination. While the duration of this phase is variable and depending on temperature (Hedhly *et al*., 2009), a similar timing has been shown in other temperate fruit trees, as apricot (Rodrigo & Herrero, 2002) or sweet cherry (Hedhly *et al*., 2004). In contrast, others showed longer periods, as peach (Herrero & Arbeloa, 1989), or plum trees (Thompson & Liu, 1973). Some authors proposed that style length could play a part in the duration of this phase (Mulcahy, 1979; Owens, 1992), and indeed it is rather short in an early angiosperm lacking a long style (Lora *et al*., 2010). But the main factor contributing to the duration of the progamic phase appears to be pistil maturation at anthesis, and this has been put forward in unrelated species (Herrero & Arbeloa, 1989, Sogo & Tobe, 2005).

In this work, the initiation of fruitlet growth closely followed fertilization. This strongly suggests that the progamic phase plays a pivotal role in the establishment of fruit set in apple trees. This argument is also backed by the fact that no ovary growth occurred in unpollinated flowers. However, this initial gynoecium growth could respond to fertilization or to close pre or post fertilization events. In pear flowers pollination induces a prolongation of ovule and flower viability (Herrero & Gascon, 1987). And in some parthenocarpic fruits the reproductive process and the onset of fruiting are uncoupled (Distefano *et al*., 2011). However, in apples, unpollinated flowers did not

grow pointing to that pollination is required for this gynoecium growth. Alternatively, early post fertilization abortion could also occur, but this seems unlikely giving the timing of gynoecium growth in relation to endosperm and young embryo development. This work opens a number of questions, but focus to the part played by the progamic phase in the final crop of apple trees. The fact that male-female synchrony is required for a successful mating (Herrero, 2003), together to the vulnerability of this process to a changing climate (Hedhly *et al*., 2009) drives the focus to a short period of time with clear implications in final fruit crop in apples.

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References:

Alcaraz L., Hormaza J.I., Rodrigo J. (2010) Ovary starch reserves and pistil development in avocado (*Persea americana*). *Physiologia plantarum,* **140,** 395-404.

Bangerth F. (2000) Abscission and thinning of young fruit and their regulation by plant hormones and bio regulators. *Plant Growth Regulation,* **31,** 43-59.

Bianchi F.J.J.A., Cunningham S.A. (2012) Unravelling the role of mate density and sex ratio in competition for pollen. *Oikos,* **121,** 219-227.

Bottom A., Eccher G., Forcato C., Ferrarini A., Begheldo M., Zermiani M., Moscatello S., Battistelli A., Velasco R., Ruperti B., Ramina A. (2010) Signalling pathways mediating the induction of apple fruitlet abscission. *Plant Physiology,* **155,** 185-208.

Brault A.M., de Oliveira D. (1995) Seed number and an asymmetry index of `McIntosh´ apples. *Hortscience,* **30,** 44-46.

Byers R.E. (1993) Controlling growth of bearing apple trees with ethephon. *Hortscience,* **28,** 1103-1105.

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

Distefano G., Gentile A., Herrero M. (2011) Pollen-pistil interactions and early fruiting in parthenocarpic citrus. *Annals of Botany,* **108,** 499-509.

Dorcey E., Urbez C., Blázquez M.A., Pérez-Amador M.A. (2009) Fertilization-dependent auxin response in ovules triggers fruit development through the modulation of gibberellin metabolism in *Arabidopsis*. *Plant Journal,* **58,** 318-332.

Ebert A., Bangerth F. (1982) Posible hormonal modes of action of three apple thinning agents. *Scientia Horticulturae,***16,** 343-356.

Free J.B. (1964) Comparison of the importance of insect and wind pollination of apple trees. *Nature,* **201,** 726–727.

Goldwin G.K. (1992) Environmental and internal regulation of fruiting, with particular reference to Cox´s Orange Pippin apple. In *Fruit and seed production,* pp. 75-100. Ed. Marshall C.and Grace J. Cambridge, UK: University Press.

González-Melendi P., Ramírez C., Testillano P.S., Kumlehn J., Risueño M.C. (2005) Three dimensional confocal and electron microscopy imaging define the dynamics and mechanisms of diploidisation at early stages of barley microspore-derived embryogenesis. *Planta,* **222,** 42-57.

González M.V., Coque M., Herrero M. (1995) Stigmatic receptivity limits the Effective Pollination Period in kiwifruit. *Journal of the American Society for Horticultural Science,* **120,** 199−202.

Hedhly A. (2011) Sensitivity of flowering plant gametophytes to temperature fluctuations. *Environmental and Experimental Botany,* **74,** 9-16.

Hedhly A., Hormaza J.I., Herrero M. (2004) Effect of temperatura on pollen tube kinetics and dynamics in sweet cherry, *Prunus avium* (Rosaceae). *American Journal of Botany,* **91,** 558-564.

Hedhly A., Hormaza J.I., Herrero M. (2007) Warm temperatures at bloom reduce fruit set in sweet cherry. *Journal of Applied Botany and Food Quality,* **81,** 158-164.

Hedhly A., Hormaza J.I., Herrero M. (2009) Global warming and sexual plant reproduction. *Trends in Plant Science,* **14,** 30-36.

Herrero M. (1992) From pollination to fertilization in fruit trees. *Plant Growth Regulation,* **11,** 27-32.

Herrero M., Gascon M. (1987) Prolongation of embryo sac viability in pear (*Pyrus communis* L.) following pollination or treatment with gibberellic acid. *Annals of Botany,* **60**, 287-293.

Herrero M., Arbeloa A. (1989) Influence of the pistil on pollen tube kinetics in peach (*Prunus persica* (L.) Batsch). *American Journal of Botany,* **176,** 1441-1447.

Herrero M. (2003) Male and female synchrony and the regulation of mating in flowering plants. *Philosophical Transactions of the Royal Society Biological Sciences,* **358,** 1019-1024.

Jefferies C.J., Belcher A.R. (1974) A fluorescent brightener used for pollen tube identification in vivo. *Stain Technology,* **49,** 199-202.

Johansen D.A. (1940) *Plant microtechnique*. Ed. McGraw-Hill, New York, NY, USA.

Julian C., Herrero M., Rodrigo J. (2010) Flower bud differentiation and development in fruiting and non-fruiting shoots in relation to fruit set in apricot (*Prunus armeniaca* L.). *Trees-Sructure and Function,* **5,** 833-841.

Kester D.E., Griggs W.H. (1959) Fruit setting in the almond: the pattern of flower and fruit drop. *Proceedings of the American Society for Horticultural Sciences,* **74,** 214-219.

Kim H., Kakui H., Kotoda N., Hirata Y., Koba T., Sassa H. (2009) Determination of partial genomic sequences and development of a CAPS system of the S-RNase gene for the identification of 22 S haplotypes ofn apple (*Malus* x *domestica* Borkh). *Molecular Breeding,* **23,** 463-472.

Klein A.M., Vaissière B.E., Cane J.H., Steffan-Dewenter I., Cunningham S.A., Kremen C., Tscharntke T. (2007) Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society Biological Sciences,* **1608,** 303-313.

Kumar S., Bink M.C.A.M., Volz R.K., Bus V.G.M., Chagne D. (2012) Towards genomic selection in apple (*Malus* x *domestica* Borkh.) breeding programmes: Prospects, challenges and strategies. *Tree Genetics and Genomes,* **8**, 1-14.

Linskens H.F. (1975) Incompatibility in Petunia. *Proceedings of the Royal Society Biological Sciences,* **188,** 299–311.

Linskens H.F., Esser K.L. (1957) Über eine spezifische anfärbung der pollenschläuche im griffel und die zahl der kallosepfropfen nach selbstung und fremdung. *Die Naturwissenschaften*, **44,** 16-16.

Lora J., Hormaza J.I., Herrero M. (2010) The progamic phase o fan early divergent angiosperm *Annona cherimola* (Annonaceae). *Annals of Botany,* **105,** 221-231.

Lord EM, Russell S (2002) The mechanisms of pollination and fertilisation in plants. *Annual Review of Cell and Developmental Biology,* **18,** 81-105.

Losada J.M., Herrero M. (2012) Arabinogalactan-protein secretion is associated with the acquisition of stigmatic receptivity in the apple flower. *Annals of Botany,* **110,** 573–584.

Losada J.M., Herrero M. (2013) Flower strategy and stigma performance in the apple inflorescence. *Scientia Horticulturae,* **150,** 283–289.

Maheshwari P. (1950) *An introduction to the embryology of angiosperms*. Ed. McGraw-Hill, New York, NY, USA.

Marshall D.L., Ellstrand N.C. (1986) Sexual selection in *Raphanus sativus*: experimental data on non random fertilization, maternal choice, and consequences of multiple paternities. *The American Naturalist,* **127,** 446-461.

Mulcahy D.L. (1979) The rise of the angiosperms: a genecological factor. *Science,* **206,** 20-23.

Owens S.J. (1992) Pollination and fertilisation in higher plants. In *Fruit and seed production*, pp. 33-56. Ed. Marshall C and Grace J. Cambridge, UK. University Press.

Pratt C. (1988) Apple flower and fruit: morphology and anatomy. *Horticultural Reviews,* **12,** 265-305.

Rodrigo J., Herrero M. (2002) The onset of fruiting in apricot (*Prunus armeniaca). Journal of Applied Botany and Food Science,* **76,** 13-19.

Rodrigo J., Herrero M., Hormaza, J.I. (2009) Pistil traits and flower fate in *Prunus armeniaca* (Rosaceae) *Annals of Applied Biology,* **154,** 365-375.

Rodrigo J., Hormaza J.I., Herrero M. (2000) Ovary starch reserves and flower development in apricot (*Prunus armeniaca*). *Physiologia Plantarum,* **108,** 35-41.

Ruiz D., Egea J. (2008) Analysis of the variability and correlations of floral biology factors affecting fruit set in apricot in a Mediterranean climate. *Scientia Horticulturae,* **115,** 154-163.

Sanzol J., Herrero M. (2001) The "effective pollination period" in fruit trees. *Scientia Horticulturae,* **90,** 1-17.

Sanzol,J., Rallo, P., Herrero, M. (2003) Stigmatic receptivity limits the effective pollination period in 'Agua de Aranjuez' pear. *Proceedings of the American Society for Horticultural Science,* **128**, 458-462.

Sedgley M., Griffin A.R. (1989) *Sexual reproduction of tree crops*. Academic Press, London, UK.

Seo Y.S., Kim W.T. (2009) A genomics approach using Expressed Sequence Tags and microarrays in ripening apple fruit (*Malus domestica,* Borkh.). *Journal of Plant Biology,* **52,** 35-40.

Sheffield C.S., Smith R.F., Kevan P.G. (2005) Perfect syncarpy in apple (*Malus* x *domestic*a `Summerland McIntosh´) and its implications for pollination, seed distribution and fruit production (Rosaceae: Maloideae). *Annals of Botany*, **95,** 583-591.

Slafer G.A. (2003) Genetic basis of yield as viewed from a crop physiologist perspective. *Annals of Applied Biology,* **142**, 117-128.

Sogo A., Tobe H. (2005) Intermitent pollen-tube growth in pistils of alders (*Alnus*). *Proceedings of the National Academy of Sciences USA,* **102,** 8770-8775.

Sutherland S. (1988) Patterns of Fruit-Set: What Controls Fruit-Flower Ratios in Plants? *Evolution,* **40,** 117-128.

Thompson M.M., Liu L.J. (1973) Temperature, fruit set, and embryo sac development in `Italian´ prune. *Journal of the American Society for Horticultural Science,* **98,** 193-197.

Troggio M., Gleave A., Salvi S., Chagne D., Cestaro A., Kumar S., Crowhurst R.N., Gardiner S.E. (2012) Apple, from genome to breeding. *Tree Genetics and Genomes,* **8**, 509-529.

Weber J.J., Goodwillie C. (2009) Evolution of the mating system in a partially Self-Incompatible species: reproductive assurance and pollen limitation in populations that differ in the timing of Self-Compatibility. *International Journal of Plant Sciences*, **170,** 885-893.

Williams R.R. (1965) Effect of summer nitrogen applications on quality of apple blossom. *Journal of Horticultural Science,* **40,** 31-41.

Williams R.R. (1966) Pollination studies in fruit trees III. The effective pollination period for some apple and pear varieties. *Report Long Ashton Research Station,* 1965, 136−8.

Williams R.R. (1970) Factors affecting pollination in fruit trees. In *Physiology of Tree Crops*, pp. 193–207. Ed. Luckwill LC and Cutting CV, London, UK: Academic Press.

Zhang C., Tateishi N., Tanabe K. 2010. Pollen density on the stigma affects endogenous gibberelin metabolism, seed and fruit set, and fruit quality in *Pyrus pyrifolia*. *Journal of Experimental Botany,* **61,** 4291-4302.

Figure legends:

Figure 1 From flower to fruit in apple. (A) Flower at anthesis, week 0 (W0). (B) Most flowers of the corymb showed petal fall one week after anthesis (W1). (C) Initial fruit set in the apple corymb three weeks after anthesis (W3). (D) Final fruit set in the apple corymb at June drop some six-seven weeks after anthesis (W6-7). (E) After the last fruit drop, an average of two fruits remained per corymb nine weeks after anthesis. (F) Fruits continued to enlarge until harvest, some fifteen weeks after anthesis (W15)

Figure 2 Fruit drop in Golden Delicious apple in open pollination conditions. (A) Fruit drop pattern followed a logarithmic curve, and final fruit set (15%) was settled eight weeks after anthesis. But a clear shift could be observed between the third and fourth weeks after anthesis. (B) Relative fruit drop showed two peaks of fruit drop, the first one four weeks after anthesis, and the second one seven weeks after anthesis. One-way ANOVA significant differences (asterisks) between absolute (A) and relative (B) percentages of remaining flowers/fruits with time are shown by asterisks ($P \leq 0.05$)

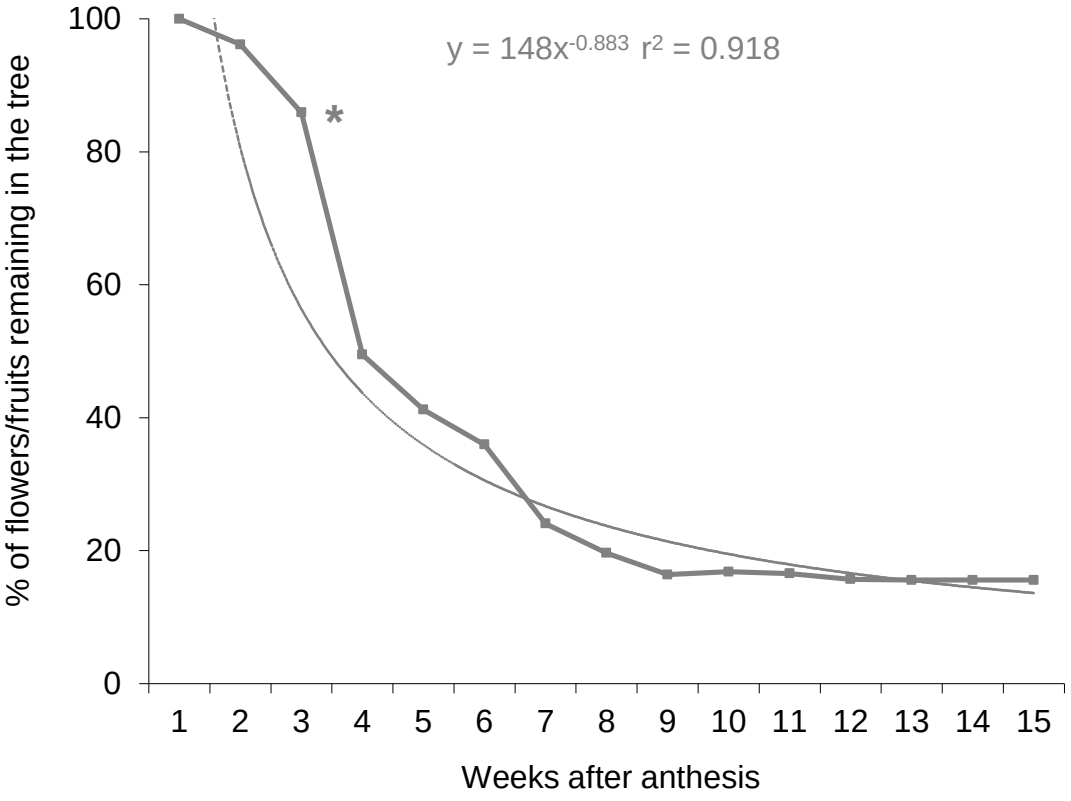
Figure 3 Fruit drop in Golden Delicious apple in open and supplemental pollination conditions. (A) Fruit drop followed the same pattern in both pollination conditions, adjusting to a logarithmic curve. While open pollination (grey line) showed a final fruit set of 15%, pollen supplemented flowers (black line) showed a slightly higher final fruit set (23%) seven weeks after anthesis. One-way ANOVA showed significant changes of flower/fruit drop at the third and the seventh week after anthesis in each treatment separately (asterisks); (B) Relative fruit drop. While the first fruit drop was similar in both pollination conditions, one-way ANOVA showed that over pollinated flowers had a significant lower percentage (asterisks) of fallen fruits in June drop ($P \leq 0.05$).

Figure 4 Gynoecium weight in pollinated (light grey triangles) and unpollinated flowers (dark circles) for the first 20 days after anthesis for the first (A) and the second year (B) of the experiments. Unpollinated flower weights mean values were year dependent, but followed a conserved pattern both years. Pollinated flowers followed an exponential continuous increase in gynoecium weight (r^2_{2008}=0.939, $P \leq 0.05$; r^2_{2010}=0.680, $P \leq 0.05$). One-way ANOVA significant differences ($P \leq 0.05$) between pollinated and unpollinated flowers were apparent from the six and the seven day after anthesis onwards, in the first and second year respectively.

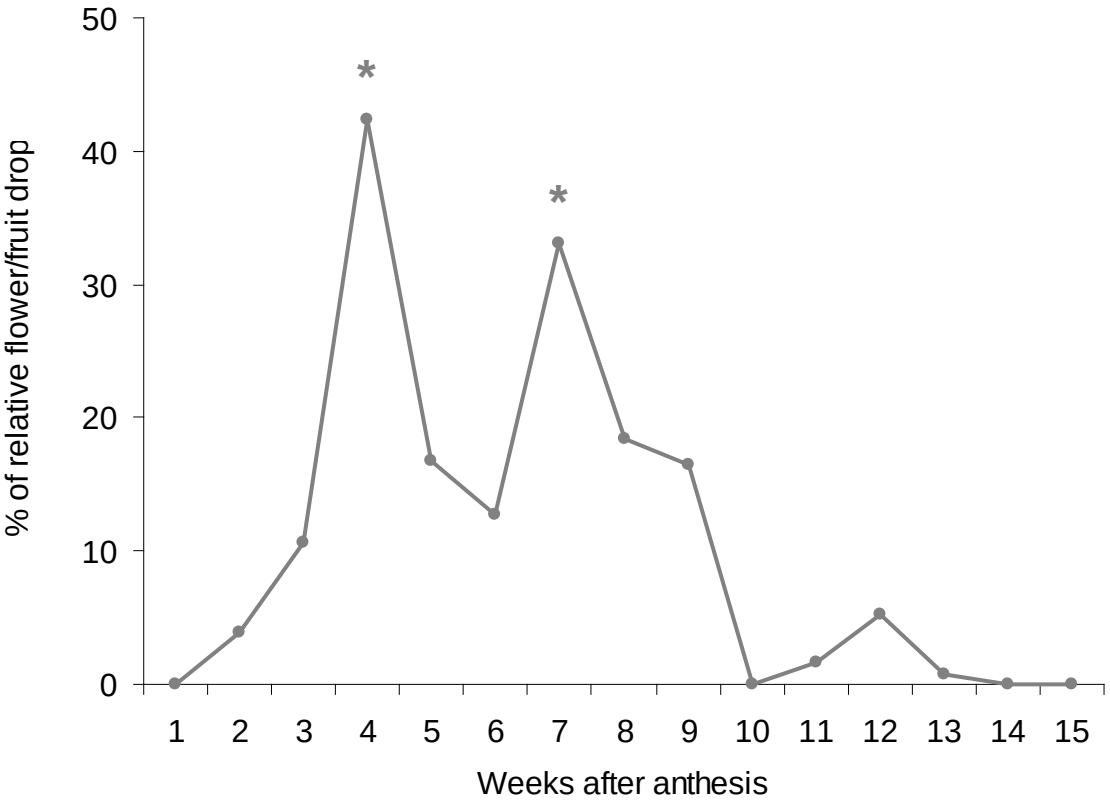
Figure 5 Pollen tube growth in the apple pistil. (a) Hydrated pollen grains on the stigmatic surface showing nuclei (arrow) two hours after pollination. (b) Germinated pollen tube six hours after pollination, with nucleus (arrow) migrating into the pollen tube. (c) High pollen germination rate on the stigma surface 24 hours after pollination. (d) First pollen tubes at the stylar base three days after pollination. (e) Pollen tubes growing on the obturator surface four days after pollination. (f) Pollen tube entering the ovule six days after pollination. Squash preparations of Paraformaldehyde (a,b) and FAA (c-f) fixed pistils, stained with DAPI (a,b), and aniline blue (c-f). ob (obturator), ov (ovule). Scale bars = 10µm

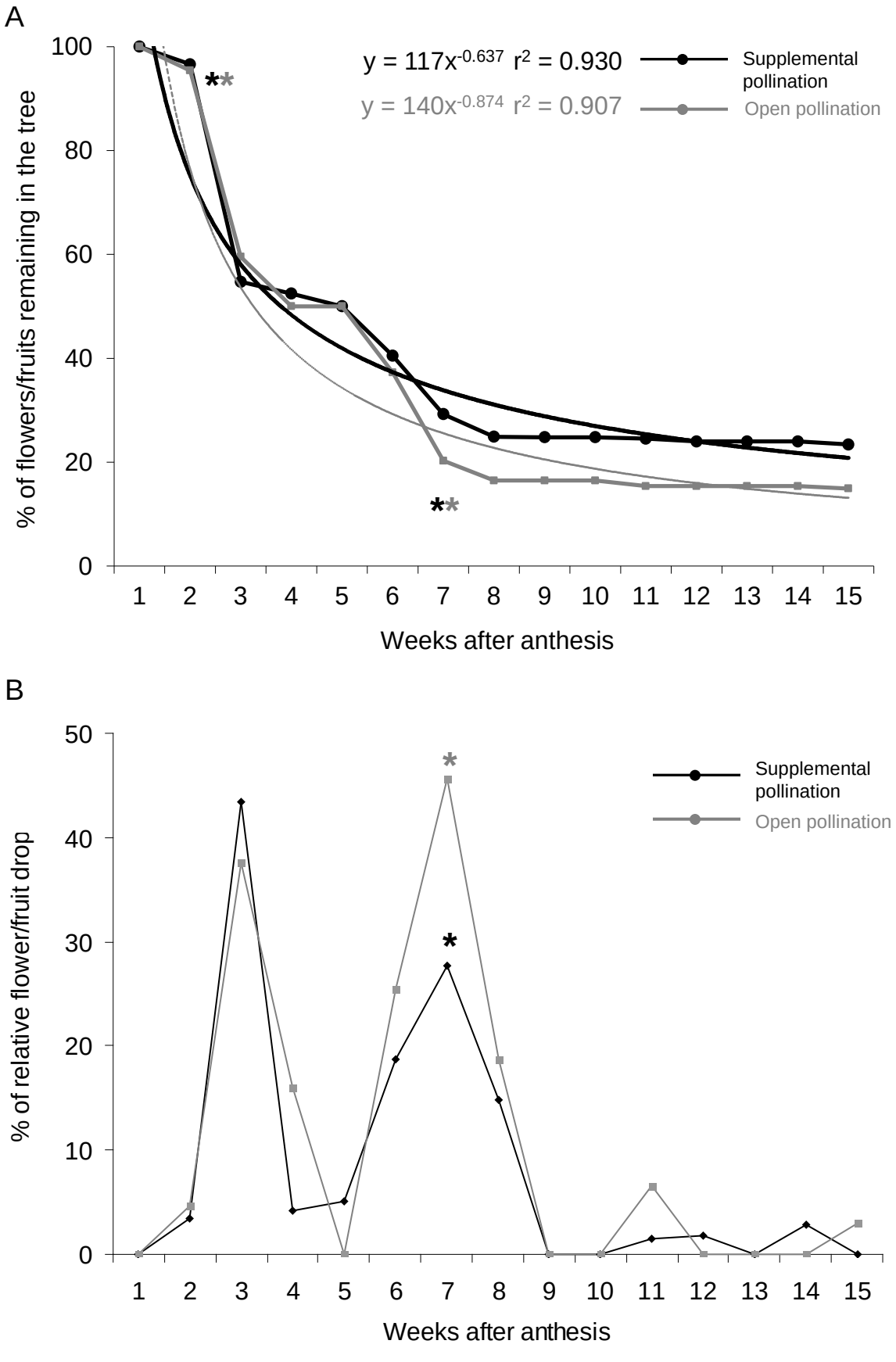

A
W0
B
W1
C
W3
D
W6-7
E
W9
F
W15

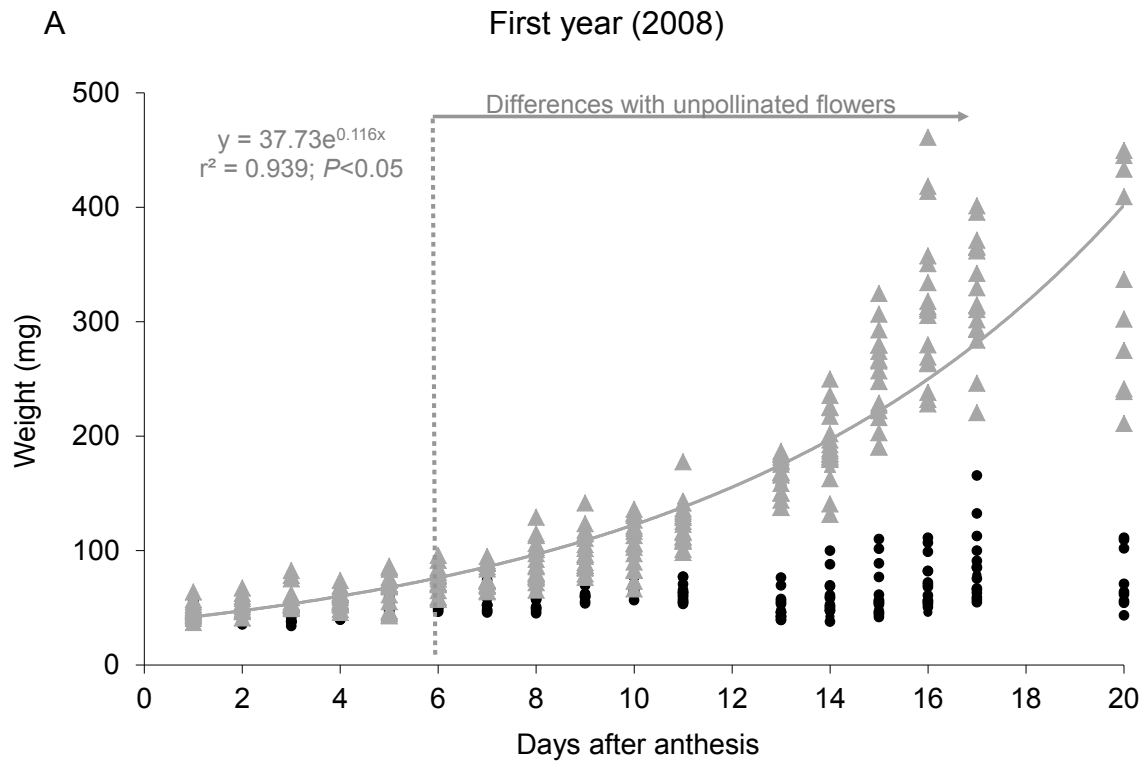
A

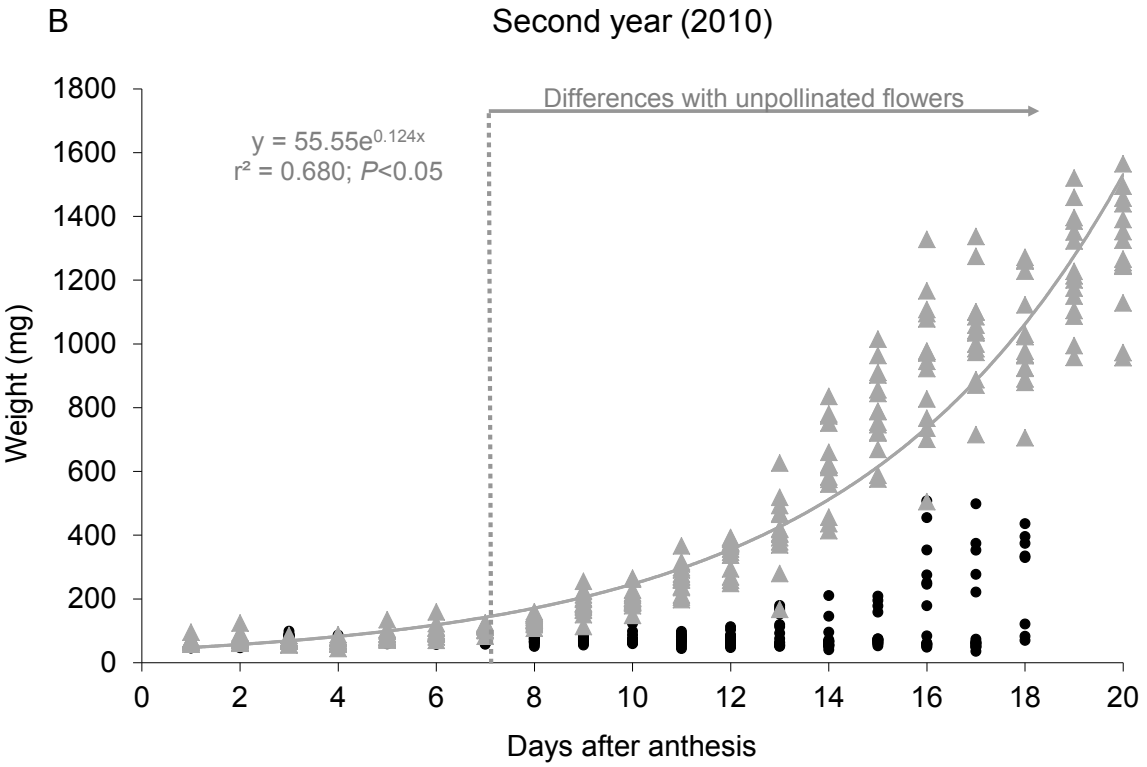

y = 148x-0.883 r2 = 0.918
*
% of flowers/fruits remaining in the tree
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Weeks after anthesis

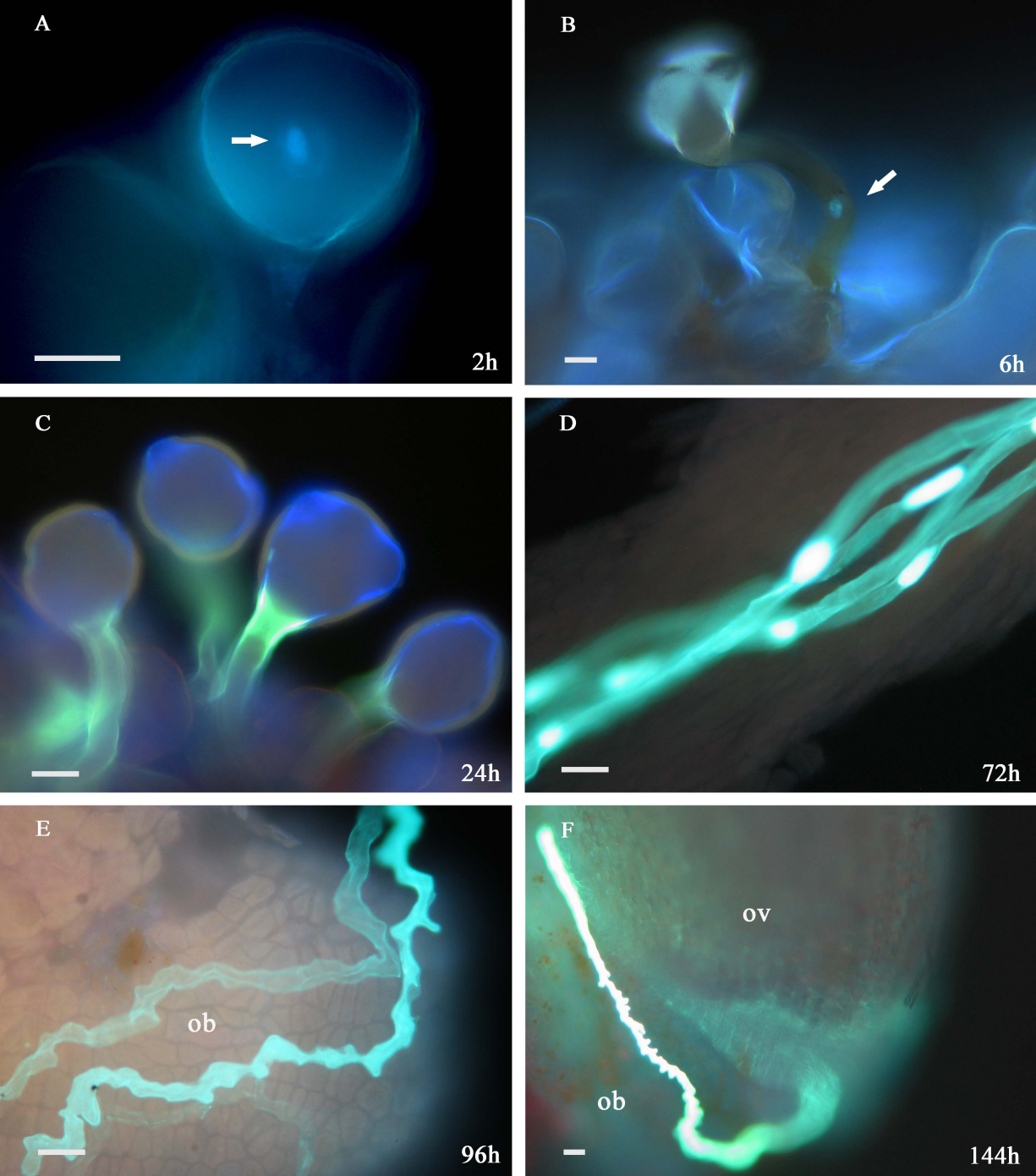
B


*
*
% of relative flower/fruit drop
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Weeks after anthesis


A
y = 117x-0.637 r2 = 0.930
y = 140x-0.874 r2 = 0.907
Supplemental pollination
Open pollination
% of flowers/fruits remaining in the tree
Weeks after anthesis
B
Supplemental pollination
Open pollination
% of relative flower/fruit drop
Weeks after anthesis


A
First year (2008)
Differences with unpollinated flowers
y = 37.73e0.116x
r² = 0.939; P<0.05
Weight (mg)
Days after anthesis
0
100
200
300
400
500
0
2
4
6
8
10
12
14
16
18
20


B
Second year (2010)
Differences with unpollinated flowers
y = 55.55e0.124x
r² = 0.680; P<0.05
Weight (mg)
Days after anthesis
0
200
400
600
800
1000
1200
1400
1600
1800
0
2
4
6
8
10
12
14
16
18
20


A
2h
B
6h
C
24h
D
72h
E
ob
96h
F
ov
ob
144h