Chestnut reproductive biology and orchard design

C. Larue[1,2,a] and R.J. Petit[1]

[1]Univ. Bordeaux, INRAE, BIOGECO, 33610 Cestas, France; [2]INVENIO, Maison Jeannette, 24140 Douville, France.

Abstract

Without reliable pollination services, it is not possible to have high-yielding orchards. In chestnut, results obtained in recent years call into question traditional orchard conception. To design well-pollinated orchards, it is important to include enough genetic diversity to ensure effective cross-fertilization. Traditional use of as few cultivars as possible in chestnut orchards has been counterproductive. We found a strong positive effect on cultivar diversity on pollination success. However, not only the number and proportion of cultivars used and their spatial distribution in the orchard matter but also their identities. In European chestnut (*Castanea sativa*) and in its hybrids, many trees are male-sterile: they have aborted stamens, allowing them to escape the negative consequences of self-pollination. In male-fertile trees, on the contrary, many female flowers are wasted due to ovule usurpation following self-pollination, thus reducing orchard yield. To illustrate the effect of pollen donor diversity, spatial distribution of the trees in the orchard and self-pollen interference, we are currently developing a predictive Bayesian model to evaluate pollination success as a function of various orchard designs, with a focus on the proportion of male-sterile cultivars deployed. Here we give some first indications of the main conclusions and propose a few rules for designing well-pollinated orchards. In particular, we strongly encourage the use of male-sterile cultivars to increase yield.

Keywords: spatially explicit mating model, paternity analyses, emasculation experiment, female advantage, gynodioecy, fruit set, ovule discounting, sexual interference

INTRODUCTION

Fruit production depends on pollination services (Reilly et al., 2020). In entomophilous species, two services need to be considered: pollen transport by pollinating insects (pollinators) and production of pollen by compatible pollen donors (pollenizers). If there are not enough pollinating insects (pollinator limitation) or pollen-producing plants (pollenizer limitation), pollination service, and therefore fruit production, will not be optimal. Contrary to what was originally thought, chestnuts are not wind-pollinated but are entirely pollinated by wild insects: fruit set decreases by -94% when insects are excluded (Rodger et al., 2021; Larue et al., 2021a; Petit and Larue, 2022; Larue and Petit, in press). Moreover, chestnuts are self-incompatible (Stout, 1926; Xiong et al., 2019), implying that female flowers of a given tree must be fertilized by pollen produced by another tree to form a fruit. Hence, orchards should include sufficient compatible pollenizer cultivars to ensure pollination service (Breisch, 1995).

The first discussions on spatial arrangement of trees in chestnut orchards are quite old: the relative advantages of planting in square or in staggered rows were already discussed at the beginning of the 20th century (Lavialle, 1906). To optimize orchard production, advices on orchard composition were provided early on, supported by compatibility experiments between cultivars relying on manual pollen applications (Solignat and Chapa, 1975). However, these experiments are time-consuming and quite tricky, as the pollen used must be viable and free from contamination. Moreover, without relying on genetic analyses, it is not possible to identify with certainty the cultivars producing pollen responsible for fruit production. With the development of molecular markers, paternity analyses have become increasingly popular to study pollen exchanges between trees (Larue and Petit, 2023a). For example, in chestnut,

---

[a]E-mail: clement.larue@inrae.fr

Nishio et al. (2019) studied the effect of distance between mother and pollen donor trees on fruit production. However, these paternity analyses also have limitations. In particular, they do not provide indications on the mechanisms responsible for pollination failure. Ideally, we should investigate both male and female reproductive success. This can be achieved using Bayesian spatially explicit mating models, thus allowing to identify mechanisms affecting fruit formation (Klein et al., 2008; Lagache et al., 2014; Larue et al., 2023).

In late spring, chestnuts display a huge number of white to yellowish male flowers that emit a strong semen-like odor (Larue et al., 2021a). Despite synchronous blooming, chestnuts are nearly fully outcrossed. Although self-pollination is high, selfing is low as a consequence of both pre- and postzygotic barriers (Xiong et al., 2019). Hasegawa et al. (2009) estimated that 90% of pollen received by female flowers is self-pollen while self-fertilized fruits represent less than 1% of the fruits produced. This raises the question of the possible negative consequences of self-pollination on fruit production. Here we review studies on orchard pollination success as a function of orchard composition and design. We then explore female advantage of male-sterile trees using new analyses and discuss the implications of these findings for the design of productive and resilient chestnut orchards.

TOO FEW POLLENIZERS

In southwestern France, chestnut orchards have been simplified as much as possible. Very few cultivars are typically planted, making pollenizer limitation an important but widely underappreciated issue. Chestnut orchards are often surrounded by chestnut forests. A persisting misconception is that there is no need for much diversity within orchards as wild chestnuts will guarantee sufficient pollination. To reevaluate the importance of orchard diversity, we have recently compared fruit set in five contrasted orchards in southwestern France (Larue and Petit, 2023a). In each orchard, for each fruit-producing cultivar planted, at least five mother trees were selected. From each mother tree, 10 fruits were harvested for paternity analysis. We measured fruit set as well as the percentages of fruit fertilized by cultivars planted in orchards versus by chestnut trees located outside the orchards, including by forest trees. The number and diversity of pollenizer trees within the orchard determined to a large extent female flowers fertilization rates and thus fruit production. We explain this result by the generally short-range dispersal of chestnut pollen by pollinating insects. Moreover, nearby European chestnut forests can play only a minor role in fertilizing hybrid cultivars due to interspecific barriers (Larue and Petit, 2023a; Larue et al., 2023). For instance, our results show that, for the cultivar ‘Marigoule’, a hybrid male-fertile cultivar, fruit set is multiplied by five between a monovarietal orchard producing on average one fruit every three burrs and a diversified orchard with several pollenizer cultivars in which fruit set reaches up to two fruits per burr (Larue and Petit, 2023a). With male-sterile cultivars, fruit set also benefits from orchard diversity (Larue and Petit, 2023a). However, regardless of the orchard diversity, fruit set of male-sterile cultivars is much higher. We therefore recommend that future orchards rely to a large extent on such cultivars.

As chestnut pollinators consist of moderately mobile insects such as calyptrate flies or beetles, and not of highly mobile insects such as honeybees (Larue et al., 2021a; Pauly et al., 2023), pollenizers must not be located too far from the recipient trees, which implies that they are evenly distributed in the orchard. In a *C. crenata* orchard with several consecutive rows of the same cultivar, Nishio et al. (2019) showed that fruit yield drops rapidly as the distance to pollen donor row increases. Fruit set was 66% for the row adjacent to pollenizers and 42, 34 and 25% for rows 2, 3 and 4. In other words, fruit set decreased almost three-fold when the pollenizers were located 16 m away from the recipient seed trees rather than 4 m away. The diversity of cultivars planted in orchards and their spatial distribution is therefore crucial for pollination success, as it is the only guarantee of high and regular fruit production. Once an orchard is established, improving orchard design a posteriori implies removing a subset of trees or grafting them with other cultivars, a tedious and uncertain task. It is therefore of utmost importance to conceive the best possible orchards that guarantee maximal pollination success. This implies not only high genetic diversity in orchards and optimal spatial arrangement but also the inclusion of cultivars that will achieve maximal fruit set under these conditions. To select such cultivars, we need to consider carefully chestnut reproductive biology.

A VARIABLE POLLEN PRODUCTION CAPACITY

Chestnut flowers are grouped into inflorescences called catkins that develop on younger twigs located at the periphery of the canopy. Chestnuts have two types of inflorescences: unisexual male catkins and bisexual catkins. The unisexual catkins bear only male flowers gathered in glomerules. In contrast, bisexual catkins consist of a male catkin associated with one or two female inflorescences. Unisexual male catkins are much more numerous than bisexual ones. We previously estimated the proportion of both types of male inflorescences in 16 trees from eight cultivars, half of which were *C. sativa* and half hybrids (including 'Marigoule' and 'Bouche de Bétizac'), and found an average of 30 unisexual catkins for each bisexual catkin (Larue et al., 2021a).

Male fertility varies greatly across cultivars: all cultivars do not have the same capacity to fertilize surrounding trees (Larue et al., 2023). In fact, two genders can be easily distinguished: cosexual trees, which are fully male-fertile; and female trees, which are completely or largely male-sterile (Solignat and Chapa, 1975; Larue et al., 2023) (Figure 1). Cosexual trees, which produce large amounts of pollen, are called 'longistaminate' trees in the literature, whereas male-sterile trees are subdivided into three categories depending on their ability to produce pollen. 'Astaminate' trees have fully aborted stamens and produce no pollen, 'brachystaminate' trees have dysfunctional stamens and produce scarce amounts of mostly non-functional pollen and 'mesostaminate' trees are partly male sterile (Solignat and Chapa, 1975; Bounous et al., 1992). Within a given tree, and among grafted trees belonging to the same clonal cultivar, all the flowers are of the same type (astaminate, brachystaminate, etc.). Moreover, according to Silva et al. (2020), male flowers of unisexual and bisexual catkins are similar and produce pollen of the same quality. An interesting point to note is that even if they no longer produce pollen, sterile male catkins still produce nectar and attract pollinators, thus ensuring effective pollination (Larue et al., 2021a).

Male-sterile trees are frequent in *C. sativa* or in interspecific hybrids when the mother is a *C. sativa* (Soylu, 1992). Male fecundity variation has also been detected in natural chestnut forests, making the European chestnut one of the few gynodioecious tree species (Larue and Petit, 2023b). These male-sterile trees can no longer sire seeds.

FEMALE ADVANTAGE

Fruit set is an important component of the productivity of chestnut orchards. In chestnuts, female flowers are grouped together by three to form small female inflorescences that later become spiny burrs. With few exceptions, burrs contain three fruits. If a female flower is fertilized, it produces a filled fruit including usually a single seed. If the absence of fertilization, it produces an empty fruit consisting of a pericarp of maternal origin with no seeds inside. A straightforward measure of fruit set is the proportion of developed fruits per burr. However, empty burrs can abort during the summer. Hence, a statistical correction is needed to account for this and to derive a reliable estimate of the quality of pollination service (Larue et al., 2023; Larue and Petit, 2023b).

To test for the existence of a female advantage in male-sterile trees, we used the INRAE chestnut germplasm collection located in Villenave d'Ornon, in southwestern France (Larue et al., 2021b). This collection includes 242 trees (ramets) corresponding to 92 different genotypes (clones) assigned to different chestnut species: *C. sativa*, *C. crenata*, *C. mollissima* and their interspecific hybrids. Among the clones, 62 are cosexuals and 30 are female, corresponding to 156 (64%) cosexual and 86 (36%) female ramets. This collection includes genotypes of forest trees as well as some cultivars selected for fruit production.

We analysed the complete fruit set data for years 2018 and 2019 in our intensive study plot (Larue et al., 2021b). Fruit set estimates are based on 176 trees in 2018 (110 cosexuals and 66 females) and on 206 trees in 2019 (134 cosexuals and 72 females). We harvested on average 22 burrs tree^{-1} in 2018 and 36 burrs in 2019. In 2018, the average fruit set of cosexual trees was 61% and that of female trees was 82% (Figure 2a), a highly significant difference

(PERMANOVA, $\mathrm{p}<10^{-16}$). In 2019, the average fruit set of cosexual trees was 55% and that of female trees was 83% (Figure 2b), again a very significant difference (PERMANOVA, $p<10^{-16}$). When the species effect is also taken into account in a two-way PERMANOVA, there is no difference in average fruit set between species in either 2018 ($p>0.06$) and 2019 ($p>0.94$). In contrast, fruit set of female trees is significantly higher than fruit set of cosexual trees, regardless of the studied year ($p<10^{-16}$). Male-sterile trees therefore have much higher fruit set than male-fertile ones. The only obvious difference between genders is pollen production.

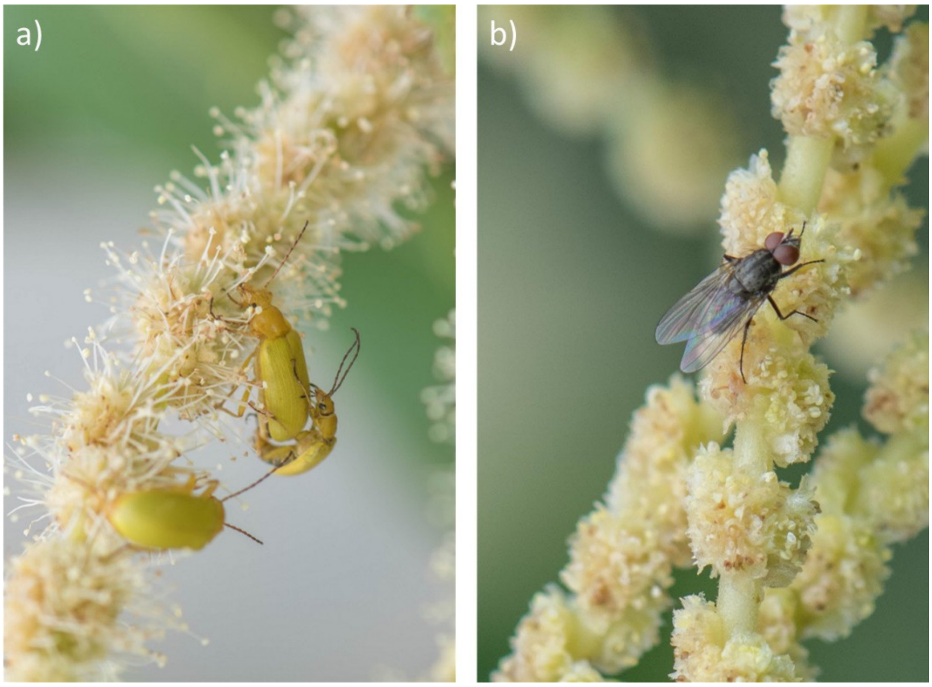

Figure 1. Close-up photos of chestnut male catkins. a) Cosexual trees: the numerous stamens harbor tiny anthers located at the tip of long filaments; b) Male-sterile trees in full bloom: stamens are typically aborted and do not produce pollen.

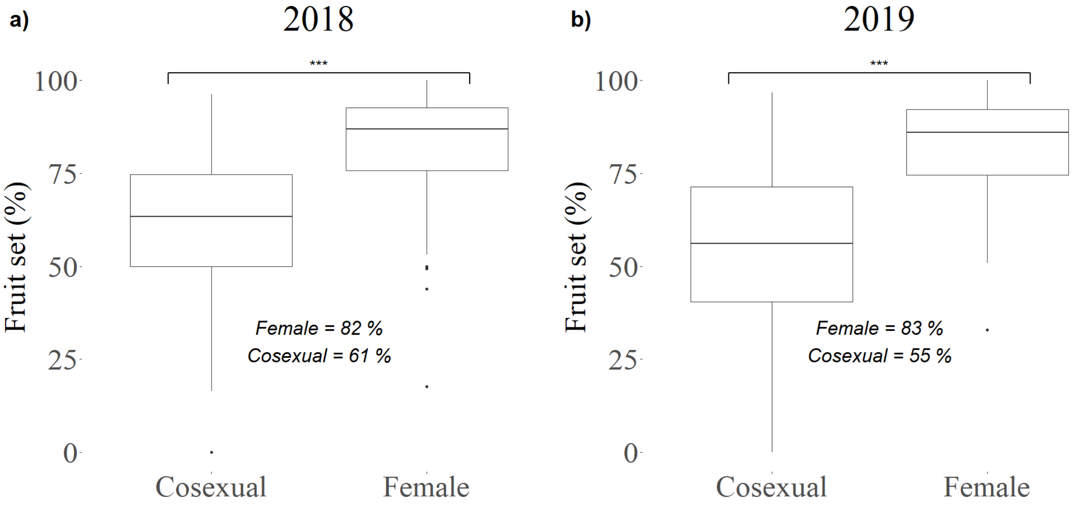

Figure 2. Fruit set of monoecious and female trees from INRAE chestnut collection. a) estimates for 2018; b) estimates for 2019.

SELF-POLLEN INTERFERENCE

One potential mechanism of female advantage is the release from sexual interference. The interference between maternal and paternal functions in cosexual plants has started to be studied only relatively recently and remains understudied (Lloyd and Webb, 1986; Barrett, 2002). Sexual interference can lead to gamete wastage and reduced seed set, even in self-incompatible species (de Jong et al., 1993). At least two mechanisms can be involved. First, self-pollen can clog stigmas, occupying the available space, thereby reducing the chances for compatible pollen grains to establish on stigmas and germinate. For example, Galen et al. (1989) showed, with the help of a pollen chase experiment, that pollen clogging reduces seed set of *Polemomium viscosum* by 40%. Second, self-pollen might germinate and pollen tubes could penetrate the ovules and make them abort: this results in ovule discounting, i.e., the elimination of ovules rendered non-functional following interactions with self-pollen (Barrett et al., 1996). For example, Waser and Price (1991), applying a mix of self and outcross pollen on flowers of *Ipomopsis aggregate*, showed that seed set decreased by 42% compared to when only outcross pollen is applied. These negative effects of self-pollen on seed set were reported in species with late-acting self-incompatibility systems (Seavey and Bawa, 1986), making chestnut an ideal model species to test if the avoidance of self-interference can explain female advantage.

To test this hypothesis, we developed a new type of Bayesian mixed effect mating model to study jointly mating system using paternity analyses and fruit set measurements (Larue et al., 2023). We performed this work in 2018, in the same INRAE chestnut germplasm collection located in Villenave d’Ornon (Larue et al., 2021b). We geolocated, genotyped and phenotyped all chestnut trees in our intensive study site. For each tree, the flowering phenology was described in details and different traits affecting male and female fecundity were evaluated. In addition, fruit set was measured on 216 trees. Fruits were collected from 43 mother trees, representing a total of 1924 mating events. In this mating model, self-pollen can be counter-selected at two levels. First, there might be different competitive abilities between self and outcrossed pollen in the style prior to fertilization. Second, at the fertilization stage, the late-acting self-incompatibility mechanism can cause the abortion of self-fertilized embryos or can result in early inbreeding depression.

The results demonstrate that self-pollen interference needs to be considered to accurately predict fruit set. Thanks to the model, the proportion of self-pollen received by each mother tree can be estimated at different stages of the reproductive cycle. The first stage corresponds to pollen deposition on stigmas (Figure 3a; Larue et al., 2023). On average, we predict that 74% of the pollen pool received by male-fertile mother tree is self-pollen. The second stage corresponds to events taking place in the styles. Thanks to the 5-fold difference in competitive ability between self- and cross-pollen tubes, the proportion of self-pollen tubes reaching ovules decreases and we estimate that only 48% of self-pollen tubes grow up to the ovules (Figure 3b; Larue et al., 2023). The third stage takes place at the time of fertilization or afterwards, and corresponds to the processes of late acting self-incompatibility and early inbreeding depression. Due to all these barriers, the proportion of self-pollinated fruits falls down to 5% (Figure 3c; Larue et al., 2023). In particular, flower abortion caused by interference with self-pollen has a major effect on fruit set: 46% of all ovules produced by male-fertile trees are eventually wasted (95 of 48%).

IMPLICATIONS FOR ORCHARD DESIGN

Testing new orchard designs experimentally is costly and time-consuming because trees take several years to mature. We are currently testing an alternative approach based on modeling. Using our previously published spatially explicit Bayesian model (Larue et al., 2023), we developed a predictive model that allows us to evaluate quantitatively the consequences of various orchard designs on fruit production. The input file is an orchard map where the characteristics of each tree are informed. These characteristics make it possible to estimate the probability that the different trees mate with each other and that fruits are produced. Several processes affect the pollination service. In particular, the identity and the position of nearby trees together with the pollen dispersal kernel play an important role in the quantity and quality of pollen received by each tree. Tree gender (male-fertile or male-sterile) has a particularly strong impact on pollination success model because those trees that produce most pollen are those that suffer most from self-pollen interference.

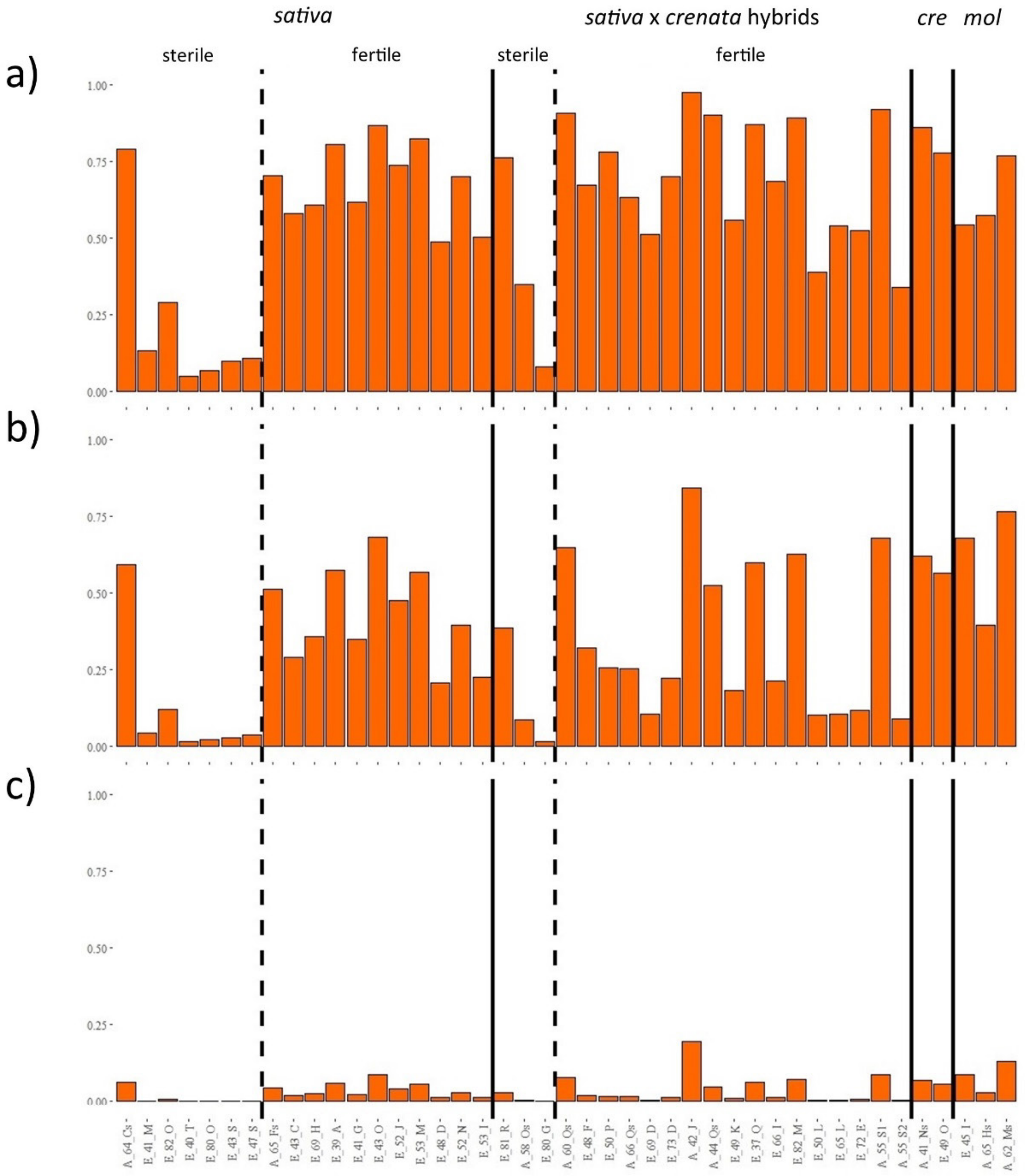

Figure 3. Predicted proportion of self-pollen on each mother tree at different stages of reproduction cycle. a) Expected proportion of self-pollen arriving on the stigmas of female flowers; b) Expected proportion of self-pollen after the operation of pollen competition; c) Expected proportion of self-pollinated zygotes after abortion of self-pollinated ovules or selfed embryos.

Several lessons can already be drawn from the simulations that are currently being performed. Self-pollination causes an inevitable decrease in pollination success. This means that male-sterile cultivars should be favoured in new orchards. However, if only male-sterile cultivars are planted in an orchard, few fruits will be produced, as female cultivars produce little or no pollen and will therefore not pollinate each other. Furthermore, pollen produced by nearby forest trees will provide only very limited pollination insurance due to low pollen dispersal ability by insects and the existence of reproductive barriers with forest trees when hybrid cultivars are used. On the other hand, if the new orchards are composed solely of male-fertile cultivars, all trees will be self-pollinated, which will cause a significant waste of female flowers, especially in few cultivars are used, thus limiting fruit production. Overly simple designs with just one male-sterile, fruit-producing cultivar and one pollen-producing cultivar perform poorly. In these ultra-simplified orchards, pollination of the male-sterile cultivar is acceptable in some years but fluctuates across years depending on the flowering phenology of the cultivars. More importantly, its long-term stability is not guaranteed as its pollination depends entirely on a single pollen-emitting cultivar. At the same time, in such simplified orchard, the pollination of the male-fertile cultivar is catastrophic due to the absence of compatible pollen donors. Ideally, all trees in an orchard should produce fruits. Male-sterile cultivars, owing to their very high fruit set, should account for the majority of trees, but the orchard must also include several cosexual cultivars that produce the pollen.

CONCLUSIONS

To build a well-pollinated orchard producing abundant fruits, diversity is the key. This will ensure that compatible pollen reaches most stigma in the face of inter-annual fluctuation in pollen delivery and uneven susceptibility of cultivars to possible pathogen outbreaks. The effects of self-pollination on fruit set and eventually on yield in outcrossed species have long been underappreciated. Chestnut trees produce pollen in large quantities. Due to self-pollen interference, many self-pollinated female flowers abort and a large proportion of ovules are wasted. Male-sterile trees avoid self-pollen interference and benefit from a much greater pollination success. Their use should be encouraged in new orchards. However, they will not contribute pollen to the orchard and hence they cannot be used alone: several male-fertile cultivars also need to be included to provide a reliable pollen source within the orchard. Chestnut growers do not have to rely on specialized pollen-donor cultivars: there are enough productive male-fertile cultivars to ensure that all trees in the orchard will contribute to fruit production.

In a fast-changing world, our model-based method makes it possible to choose rapidly the most productive and resilient orchard designs, without having to wait several years for the trees to grow. Focusing on the main mechanisms controlling chestnut pollination success, we can now explore different scenarios to create diversified, resilient and productive orchards.

ACKNOWLEDGEMENTS

Many thanks to Etienne K. Klein for developing the MEMM model. We thank T. Barreneche (administrator), and INRAE experimental unit Vigne Bordeaux (UEVB) in Villenave d’Ornon who manage the INRAE chestnut germplasm collection. We thank our colleagues of Biogeco, Y. Mellerin, B. Dencausse and A. Ducousso, C. Helou, C. Lalanne, J. Dudit, M. Martin-Clotte and our many great students for their invaluable assistance. SNP development and genotyping were performed at the Genome Transcriptome Facility of Bordeaux (PGTB) with the help of E. Guichoux, M. Massot, A. Delcamp, C. Boury and L. Dubois.

The results presented here are based on experiments carried out during the Ph.D. and postdoc of CL. These works (Conventions Industrielles de Formation par la Recherche) was supported by the ANRT (Association Nationale de la Recherche de la Technologie, convention Cifre N°2018/0179). It was funded by Invenio, the Région Nouvelle-Aquitaine (Regina project N°22001415-00004759 and chestnut pollination project N°2001216-00002632), by INRAE (Institut National de Recherche pour l’Agriculture et l’Environnement) and by ANR (project ANR-21-PRRD-0008-01).

Literature cited

Barrett, S.C.H. (2002). The evolution of plant sexual diversity. Nat Rev Genet *3* (*4*), 274–284 https://doi.org/10.1038/nrg776. PubMed

Barrett, S.C.H., Lloyd, D.G., and Arroyo, J. (1996). Stylar polymorphisms and the evolution of heterostyly in *Narcissus* (Amaryllidaceae). In Floral Biology, D.G. Lloyd, and S.C.H. Barrett, eds. (Boston, MA: Springer US), p.339–376.

Bounous, G., Paglietta, R., and Peano, C. (1992) Methods for observing chestnut pollen viability, germinability and pollen tube growth. Paper presented at: International Chestnut Conference (Morgantown, USA).

Breisch, H. (1995). Châtaignes et Marrons (Paris: Ctifl).

de Jong, T.J., Waser, N.M., and Klinkhamer, P.G. (1993). Geitonogamy: the neglected side of selfing. Trends Ecol Evol *8* (*9*), 321–325 https://doi.org/10.1016/0169-5347(93)90239-L. PubMed

Galen, C., Gregory, T., and Galloway, L.F. (1989). Costs of self-pollination in a self-incompatible plant, *Polemonium viscosum.* Am. J. Bot. *76* (*11*), 1675–1680 https://doi.org/10.1002/j.1537-2197.1989.tb15152.x.

Hasegawa, Y., Suyama, Y., and Seiwa, K. (2009). Pollen donor composition during the early phases of reproduction revealed by DNA genotyping of pollen grains and seeds of *Castanea crenata.* New Phytol *182* (*4*), 994–1002 https://doi.org/10.1111/j.1469-8137.2009.02806.x. PubMed

Klein, E.K., Desassis, N., and Oddou-Muratorio, S. (2008). Pollen flow in the wildservice tree, *Sorbus torminalis* (L.) Crantz. IV. Whole interindividual variance of male fecundity estimated jointly with the dispersal kernel. Mol Ecol *17* (*14*), 3323–3336 https://doi.org/10.1111/j.1365-294X.2008.03809.x. PubMed

Lagache, L., Klein, E.K., Ducousso, A., and Petit, R.J. (2014). Distinct male reproductive strategies in two closely related oak species. Mol Ecol *23* (*17*), 4331–4343 https://doi.org/10.1111/mec.12766. PubMed

Larue, C., and Petit, R.J. (2023a). Strong pollen limitation in genetically uniform hybrid chestnut orchards despite proximity to chestnut forests. Ann. For. Sci. *80* (*1*), 37 https://doi.org/10.1186/s13595-023-01188-6.

Larue, C., and Petit, R.J. (2023b) Harmful self-pollination drives gynodioecy in European chestnut, a self-incompatible tree. 2022.08.01.502348. https://doi.org/10.1101/2022.08.01.502348

Larue, C., and Petit, R.J. (in press). Insect pollination in chestnut: an organized mess? Sci. Hortic. (Amsterdam).

Larue, C., Austruy, E., Basset, G., and Petit, R.J. (2021a). Revisiting pollination mode in chestnut (*Castanea* spp.): an integrated approach. Bot. Lett. *168* (*3*), 348–372 https://doi.org/10.1080/23818107.2021.1872041.

Larue, C., Barreneche, T., and Petit, R.J. (2021b). An intensive study plot to investigate chestnut tree reproduction. Ann. Sci. *78* (*4*), 90 https://doi.org/10.1007/s13595-021-01104-w.

Larue, C., Klein, E.K., and Petit, R.J. (2023). Sexual interference revealed by joint study of male and female pollination success in chestnut. Mol Ecol *32* (*5*), 1211–1228 https://doi.org/10.1111/mec.16820. PubMed

Lavialle, J.-B. (1906). Le Châtaignier (Paris: Vigot Frères).

Lloyd, D.G., and Webb, C.J. (1986). The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. N. Z. J. Bot. *24* (*1*), 135–162 https://doi.org/10.1080/0028825X.1986.10409725.

Nishio, S., Takada, N., Terakami, S., Kato, H., Inoue, H., Takeuchi, Y., and Saito, T. (2019). Estimation of effective pollen dispersal distance for cross-pollination in chestnut orchards by microsatellite-based paternity analyses. Sci. Hortic. (Amsterdam) *250*, 89–93 https://doi.org/10.1016/j.scienta.2019.02.037.

Pauly, G., Larue, C., and Petit, R.J. (2023). Adaptive function of duodichogamy: why do chestnut trees have two pollen emission phases? Am J Bot *110* (*8*), e16204 https://doi.org/10.1002/ajb2.16204. PubMed

Petit, R.J., and Larue, C. (2022). Confirmation that chestnuts are insect-pollinated. Bot. Lett. *169* (*3*), 370–374 https://doi.org/10.1080/23818107.2022.2088612.

Reilly, J.R., Artz, D.R., Biddinger, D., Bobiwash, K., Boyle, N.K., Brittain, C., Brokaw, J., Campbell, J.W., Daniels, J., Elle, E., et al. (2020). Crop production in the USA is frequently limited by a lack of pollinators. Proc Biol Sci *287* (*1931*), 20200922 https://doi.org/10.1098/rspb.2020.0922. PubMed

Rodger, J.G., Bennett, J.M., Razanajatovo, M., Knight, T.M., van Kleunen, M., Ashman, T.L., Steets, J.A., Hui, C., Arceo-Gómez, G., Burd, M., et al. (2021). Widespread vulnerability of flowering plant seed production to pollinator declines. Sci Adv *7* (*42*), eabd3524 https://doi.org/10.1126/sciadv.abd3524. PubMed

Seavey, S.R., and Bawa, K.S. (1986). Late-acting self-incompatibility in angiosperms. Bot. Rev. *52* (*2*), 195–219 https://doi.org/10.1007/BF02861001.

Silva, D.M., Zambon, C.R., Techio, V.H., and Pio, R. (2020). Floral characterization and pollen germination protocol for *Castanea crenata* Siebold & Zucc. S. Afr. J. Bot. *130*, 389–395 https://doi.org/10.1016/j.sajb.2020.01.027.

Solignat, G., and Chapa, J. (1975). Biologie Florale du Châtaignier. In Châtaignes et marrons (Invuflec).

Soylu, A. (1992). Heredity of male sterility in some chestnut cultivars (*Castanea sativa* Mill.). Acta Hortic. *317*, 181–186 https://doi.org/10.17660/ActaHortic.1992.317.21.

Stout, A.B. (1926). Why are chestnuts self-fruitless? Journal of the New York Botanical Garden *27*, 154–158.

Waser, N.M., and Price, M.V. (1991). Reproductive costs of self-pollination in *Ipomopsis aggregata* (polemoniaceae):

are ovules usurped? Am. J. Bot. *78* (*8*), 1036–1043 https://doi.org/10.1002/j.1537-2197.1991.tb14511.x.

Xiong, H., Zou, F., Guo, S., Yuan, D., and Niu, G. (2019). Self-sterility may be due to prezygotic late-acting self-incompatibility and early-acting inbreeding depression in Chinese chestnut. J. Am. Soc. Hortic. Sci. *144* (*3*), 172–181 https://doi.org/10.21273/JASHS04634-18.