BOTANY LETTERS
https://doi.org/10.1080/23818107.2022.2088612

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Confirmation that chestnuts are insect-pollinated

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ABSTRACT

We have performed new insect-exclusion experiments to test if chestnuts are insect-pollinated, as proposed in an earlier study. We used double rather than simple nets to ensure that erect styles do not emerge outside of the nets while allowing most airborne pollen to penetrate. Our findings indicate that ≥94% of chestnut flowers are pollinated by insects. Therefore, chestnut ought to be considered as one of the most important entomophilous tree genus of the northern temperate and subtropical flora.

ARTICLE HISTORY
Received 7 April 2022
Accepted 31 May 2022

KEYWORDS
Castanea; insect pollination; wind pollination; comparative study; nectar resources; forest tree; fruit tree

This letter is a follow-up on our article on chestnut pollination published last year in this journal (Larue et al. 2021a). Although the paper was well received (Nadot and Jabbour 2022), our finding that chestnuts are insect-pollinated remained controversial. The belief that chestnuts are mainly wind-pollinated is firmly anchored in the scientific community (but see Oh and Manos 2008; Hasegawa et al. 2015) and in the farming/agronomy community (e.g. Breisch et al. 1995; Abrol 2015). The fact that chestnut trees produce huge amounts of tiny pollen grains that can be transported by wind over long distances (e.g. Peeters and Zoller 1988), sometimes generating allergies (e.g. Astray et al. 2016), makes the proposal that chestnut is mainly insect-pollinated particularly counter-intuitive. A related issue is whether chestnuts are ambophilous, i.e. whether they have a dual pollination system involving both wind and insects. Ambophily is predicted to be a rare condition as adaptation to either wind or insect pollination should involve widely different strategies, resulting in trade-offs difficult to overcome. Stebbins (1970) recognized this problem early on. He proposed in his most effective pollinator principle that plants should evolve to increase the efficiency of their main pollinating agent. In line with this principle, Friedman and Barrett (2009) doubt that ambophily could in fact represent an evolutionarily stable strategy. It is therefore of importance to confirm that chestnuts are entomophilous and not anemophilous or ambophilous.

Recent comparative studies exploring the relation between pollination mode and other variables of ecological importance have all classified chestnuts in the wind-pollinated category. For instance, Bastl et al. (2020) have performed an aerobiological study in Austria with pollen traps. They compared 39 plant species classified as insect-pollinated with 43 classified as wind-pollinated. They included the European chestnut in the wind-pollinated category. They report that pollen of wind-pollinated plants is more abundant and dispersed earlier in the season than pollen of insect-pollinated plants. In their study, the European chestnut, which flowers in June and July, appears as an outlier, as it flowers much later than most wind-pollinated taxa and its pollen is quite rare in the pollen traps. Their analysis also leads them to conclude that allergenic taxa are necessarily wind-pollinated, therefore failing to realize that one of the allergenic species, chestnut, is in fact insect-pollinated.

Similarly, Garcia et al. (2021) have analysed long-term data on perennial crop yield compiled by the FAO (1961–2018). They compared 21 cultivated plants classified as insect-pollinated and six classified as wind-pollinated, among which they included the European chestnut. They found that wind-pollinated taxa, not insect-pollinated ones, tend to bear fruits massively only during some years. Their results comfort previous findings showing that masting is more common and more pronounced in wind-pollinated taxa than in animal-pollinated taxa (Kelly and Sork 2002; Bogdziewicz et al. 2020). In the study of Garcia et al. (2021), chestnut was an outlier, because it had one of the lowest coefficient of variation for yield across years, a characteristic typical of animal-pollinated taxa.

In another study, Deguines et al. (2014) have investigated yield stability of 54 crop plants in France as a function of their pollination mode. They showed that the yield benefits of intensive agriculture are lower in insect-pollinated than in wind-pollinated crops. In contrast to the two above-mentioned studies, they relied on an index

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Published online 14 Jun 2022

of crop pollinator dependence, not on a binary classification of pollination mode (biotic/abiotic). They considered that chestnuts depend on pollinators for only 25% of their reproduction events. In our study (Larue et al. 2021a), we have obtained a diametrically opposite result, with a minimum level of dependence of chestnuts on pollinators ranging from 70% to 91% depending on the variety.

Clearly, the value of these comparative studies depends on a correct classification of plant pollination systems, if possible using an accurate quantitative index of pollinator dependence (Klein et al. 2007). Whereas misclassification of a single species might not change the main conclusion of a comparative study, it is worrying that the pollination of an important fruit and forest tree genus such as chestnut, widely distributed throughout the Northern hemisphere and often included in comparative studies, is incorrectly interpreted.

One possible criticism of our recently published work in which we concluded that chestnuts are insect-pollinated is that fine nets used to protect the flowers from pollinating insects could also somewhat restrain access of airborne chestnut pollen to female flowers (e.g. Ramsay et al. 2003; Bartomeus et al. 2014). Clearly, this would result in the underestimation of wind as pollination agent in chestnuts. Another possible criticism is that our study was not replicated across years and across sites (note however that similar experiments had been performed in Europe and in the USA, in which results similar to ours were obtained). We therefore decided to repeat the experiment two more years in another site while exploring new procedures to limit access of visiting insects to flowers without affecting wind pollination potential.

New experiments

All studied trees are located in one of the two intensively studied orchards (orchard A) of the INRAE chestnut collection located in Villenave d'Ornon, southwestern France (Larue et al. 2021b). In 2020, we selected five trees for the insect exclusion experiments: A42J and A44Qs (both trees belonging to "Marigoule", a longistaminate male-fertile variety), A55S2 ("Vignols", longistaminate), A45E ("Merle", longistaminate) and A58Os ("Bouche de Bétizac", an astaminate male-sterile variety). We again studied five trees in the following year (2021), including two previously studied trees (A45E and A58Os), and three others: A66Qs ("Marlhac", a mesostaminate, partly male-sterile variety), A60Qs ("Maridonne", longistaminate), and A71Rs ("Marsol", longistaminate).

In 2020, for each of the five trees, we set up the following pollination treatments before the onset of flowering:

- 10 control branches for the open pollination treatment;
- 20 branches covered with fine nets. The nets (Diatex F550P, https://www.diatex.com/fr/diatex-produit/f510/) are the same as those used in 2019 (Larue et al. 2021a). They have a mesh size of 700 × 400 µm, much larger than chestnut pollen, which measures about 15 × 11 µm (Larue et al. 2021a). Their permeability is higher than 15,000 l/m^2/s at 200 Pa;
- 10 branches with relatively rigid insect-proof nets with large mesh size (2 × 6 mm), fully permeable to incoming airborne pollen.

In 2021, for each of the five trees, we set up the following pollination treatments before flowering:

- 15 control branches for the open pollination treatment;
- 15 branches with the same fine net treatment as described above;
- 15 branches covered with a net having medium-sized meshes (1050 × 1050 µm). These nets (Diatex F510; https://www.diatex.com/fr/diatex-produit/f510-filets-pollinisation/) are designed specifically for pollination studies and allow on average 75% of incoming airflow, according to manufacturer's information (Bartomeus et al. 2014; Chabert et al. 2020);
- 5 branches with a fine-meshed net combined with a large-meshed rigid net (same specifications as above);
- 5 branches with a medium-meshed net combined with a large-meshed rigid net (same specifications as above).

In the fall, we counted all burrs and developed fruits and computed mean fruit set for each treatment, by dividing the number of developed fruits by the total number of fruits (empty fruits + developed fruits).

The mean fruit set in the controls was 40% in 2020 and 47% in 2021 (Table 1). Nets with large and medium-sized meshes were not well suited when used alone. First, some important pollinators of chestnut such as soldier beetles (*Rhagonycha fulva*, Cantharidae) could penetrate inside the nets with the larger meshes. Second, a few of the erect and tapered styles of female flowers were protruding from the nets having either medium or large-sized meshes. Insects walking on the outer side of these nets could therefore pollinate the enclosed flowers (Figure 1). Nevertheless, the results indicate strongly reduced fruit set when using these nets. With the largest meshes, pollinating success dropped from 40% in the

Table 1. Fruit set in control versus in netted treatments (large-, medium-, or fine-meshed nets or combinations of nets).

Trees	Year	Control	Large	Medium	Fine	Medium + Large	Fine + Large
A58Os	2020	89 % (31)[1]	49 % (19)	/	5 % (39)	/	/
A42J	2020	8 % (41)	4 % (25)	/	1 % (56)	/	/
A44Qs	2020	30 % (11)	11 % (29)	/	4 % (60)	/	/
A55S	2020	28 % (25)	2 % (16)	/	2 % (63)	/	/
A45E	2020	42 % (11)	6 % (47)	/	1 % (105)	/	/
Mean	2020	40 % (119)	15 % (136)	/	2 % (323)	/	/
A58Os[3]	2021	87 % (35)	/	11 % (51)	9 % (35)	6 % (6)	0 % (11)
A60Qs	2021	30 % (59)	/	8 % (62)	1 % (75)	0 % (4)	4 % (16)
A66Qs	2021	66 % (129)	/	22 % (139)	6 % (124)	5 % (49)	6 % (57)
A71Rs	2021	22 % (30)	/	9 % (42)	7 % (67)	9 % (15)	3 % (11)
A45E	2021	31 % (26)	/	16 % (60)	8 % (65)	5 % (14)	0 % (15)
Mean	2021	47 % (279)	/	13 % (354)	6 % (366)	5 % (88)	3 % (110)

[1]Burr number is provided in brackets after fruit set.

Figure 1. Tapered erect styles emerging from the nets (nets with medium-sized meshes, 1050 × 1050 µm), visited by a sulphur beetle (*Cteniopus sulphureus*, Tenebrionidae). This situation has prompted us to use double nets to limit such contamination when assessing the relative importance of wind versus insect pollination.

control to 15%; with medium-sized meshes, it dropped from 47% to 13%. With the finest nets, the reduction was much stronger, dropping from 40% to 2% in 2020 and from 47% to 6% in 2021. By combining two nets, thereby even more effectively isolating female flowers from insects, the reduction in fruit set dropped from 47% to 3–5% (Table 1 and Figure 2).

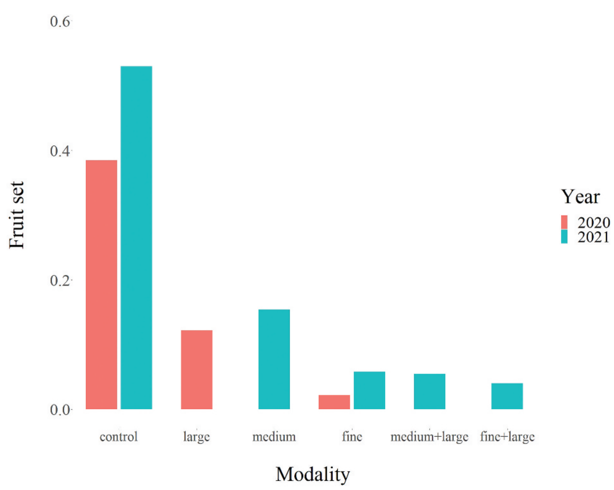

Figure 2. Fruit set measured using different types of nets.

Even with poorly suited nets that let some insects get in or through which the styles can protrude, fruit set dropped considerably compared to that measured in the controls. According to manufacturers' information, these nets allow at least 75% of incoming airborne pollen to get through. The new insect exclusion experiments therefore comfort our previous findings: when female flowers are isolated from insects, fruit set collapses (Larue et al. 2021a). With double nets (nets with fine or medium meshes associated with a solid net with large meshes), we observed an even lower fruit set, representing as little as 6% of that of the control. The only difference with the previous treatments is the addition of a net with large meshes fully permeable to incoming airflow. The results therefore show that these double nets treatments exclude more thoroughly pollinators. This comforts our hypothesis of a crucial, nearly exclusive role for insects in chestnut pollination. When using simple nets set up around flowering branches, without frame to hold them away from the flowers, it is indeed likely that pollination of erect female flowers in direct contact with the nets take place occasionally, even when the net has very fine meshes. Using double nets creates a buffer space that reduces the likelihood that insects walking on the outside surface of the nets will come into contact with the erect female flowers. This would explain the more than two-fold reduction of fruit set when using an additional relatively rigid large-meshed net (fruit set dropped from 13% to 5% when combining such nets with nets having medium-sized meshes, and from 6% to 3% when combining them with fine-meshed nets).

Klein et al. (2007) proposed to classify crops as a function of their level of dependence on animal-mediated pollination. Insect pollination is considered essential when crops have their production decreasing by 90% or more in the absence of pollinators. Chestnut, with an estimated dependence on pollinators of 94% (considering the double nets treatment with the finest net), falls within this category.

This strengthens our original conclusion (Larue et al. 2021a) that the role of wind in chestnut pollination is negligible. Indeed, some of the remaining fruits found in the nets could originate from self-pollination, which is rare but not impossible in chestnut (Larue 2021). Moreover, in rare cases, some nets (especially the finest ones) can be torn apart after being set up, possibly resulting in some pollen contamination, which could also account for some of the fruits found inside of the nets. Finally, high-concentration pollen released by insects flying over the nets could also in principle pollinate a few remaining flowers through the nets (Pierre et al. 2010). All this leaves very little room for wind-pollination *sensu stricto* in chestnut. As stressed previously (Larue et al. 2021a), the extremely reduced stigmatic surface of female chestnut flowers makes them particularly unfit for the capture of highly diluted airborne pollen. Moreover, the powerful odour emitted during flowering (Larue et al. 2021a), and the presence of abundant pollenkitt (Hesse 1978), which facilitates pollen clumping and adherence to insect body parts, fit well with a predominantly insect pollination mode (Friedman 2011).

Outlook

Correctly ascertaining chestnut pollination mode is important. Chestnut species occupy a broad range across the northern hemisphere, under both subtropical and temperate climates. Moreover, several chestnut species are widely cultivated across the world, including outside of their native range (Larue et al. 2021c). In some regions, such as in France, the demonstration that chestnut is insect-pollinated makes it by far the most abundant insect-pollinated tree, covering much larger areas than willows, limes or Rosaceae trees and shrubs, for instance. It represents therefore a major pollen and nectar resource for a large variety of wild insects as well as for honeybees, at a critical period of the year, when such resources are getting scarce (Balfour et al. 2018). Proper consideration of chestnut pollination mode is therefore crucial on many different grounds. It is also important to check claims for ambophily as this mixed pollination syndrome is predicted to be excessively rare in view of the potential underlying tradeoffs. We hope that the additional experiments presented here will help correctly classify the pollination mode of chestnuts in the future for academic studies including comparative approaches and for effective management of this tree in agriculture and conservation.

Acknowledgments

These experiments would not have been possible without the maintenance of the orchards by the INRAE experimental unit (UE1442 Vignes Bordeaux) in Villenave d'Ornon (33), and X. Capdevielle of UMR Biogeco. We thank Céline Lalanne, Olivier Bonnard, Eva Austruy, Julien Bonnier, Tanguy Menthonnex, Marie Rheinheimer, Grégoire Pauly and Catherine Bodénès for help with setting up the nets or with fruit set measurements. We also thank the referee as well as the editor, Guillaume Besnard, for useful advices on a previous version of the manuscript.

Author contributions

Both authors contributed to the design of the experiments. *RJP* and *CL* set up the nets in spring with the help of students and staff (see acknowledgements). *RJP* (2020 and 2021) and *CL* (2020) estimated fruit set in the fall. Both authors analyzed the data. The first draft in French was written by *CL* as part of his PhD. *RJP* wrote the English version which was revised by *CL*.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Funding for the work was provided by ANRT (Association Nationale de la Recherche de la Technologie, convention Cifre) [# 2018/0179], by Invenio, by the Regional Council of Nouvelle -Aquitaine [project Regina # 22001415-00004759 and project chestnut pollination # 22001216-00002632], by INRAE, and by ANR [project ANR-21-PRRD-0008-01].

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