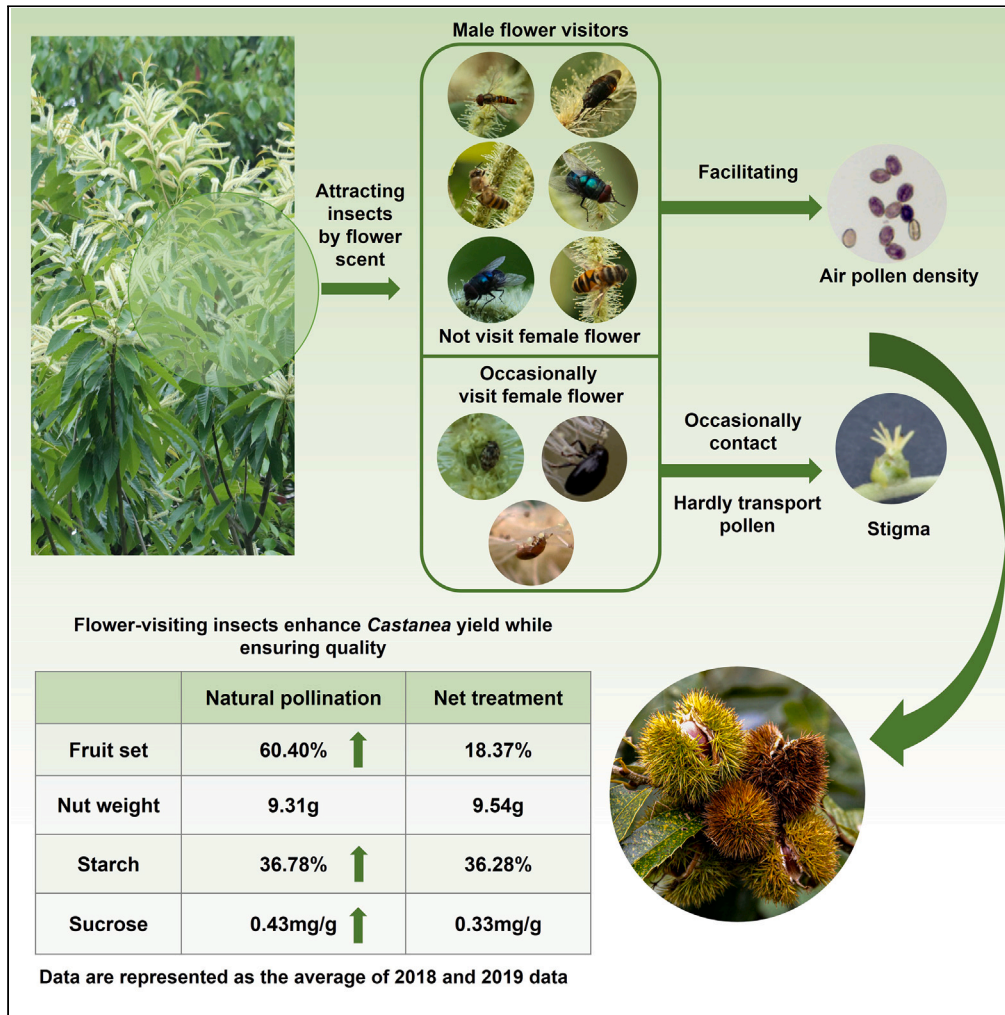Article

Managing flower-visiting insects is essential in *Castanea*: Enhance yield while ensuring quality

Bin Yuan, Yi-huan Li, Jia-qi Zhang, Xiao-xiao Zhang, Fu-liang Hu, De-yi Yuan, Xiao-ming Fan

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Highlights

Insects are essential for *Castanea henryi* yield while ensuring quality

Chestnut odor attracts insects while they hardly transport pollen by contacting the stigma

Insects can enhance orchard fruit production as facilitators of wind pollination

Insect management is also important for plants can be pollinated by wind

Article

Managing flower-visiting insects is essential in *Castanea*: Enhance yield while ensuring quality

Bin Yuan,[1,2,3,4] Yi-huan Li,[1,2,4] Jia-qi Zhang,[1,2] Xiao-xiao Zhang,[1,2] Fu-liang Hu,[3] De-yi Yuan,[1,2] and Xiao-ming Fan[1,2,5,*]

SUMMARY

The role of insects in the agriculture of plants that can rely on wind for successful pollination has been a mystery. We studied the contributions of wind-, insect-, and self-pollination in *Castanea henryi* (which can receive fruits relying on the wind). The fruit set under open pollination was significantly higher than other treatments. Insects contributed 40% of fruit by their indirect or direct pollination and did not decrease fruit quality. When the stigma was receptive, floral fragrance attracted numerous insects to visit male flowers and carry pollen; however, insects rarely visit female flowers and hardly transport pollen by contacting the stigma. The flower-visiting insects density was positively correlated with air pollen density. Therefore, insects, as facilitators of wind pollination, enhance orchard fruit production. Not emphasizing the importance of wind pollination to chestnut but reminding us that insect management is also extremely important for the agriculture of plants that can be harvested by the wind.

INTRODUCTION

Pollinating insects greatly impact crop yield and quality in agroecosystems.[1,2] In recent years, the global decline in pollinating insects has raised concerns about the robustness of the yields of insect-pollinated plants.[3,4] However, whether plants that can reproduce successfully by wind (including wind-pollinated or ambophilous plants) will be affected by such change remains unclear. Increasingly, wild wind-pollinated plants are reported to have insect flower visitors, and some are even identified as ambophilous.[5] In addition, small insects are considered to have the potential to perform pollination services for wind-pollinated plants.[6] Consequently, a decline in pollinating insect numbers may also cause a decline in the yield of orchards of wind-pollinated or ambophilous plants. Nevertheless, insect pollination is not their sole method of pollination.

Moreover, insects visit wind-pollinated or ambophilous plants for food resources; however, such behavior does not mean that they are efficient in facilitating fruit production.[7] More importantly, the plant communities in orchards are quite distinct from those in the wild, and the pollination strategies of wild populations may have limited application in orchard production.[8] Plant individuals in the wild usually develop directly from seeds and may have high diversity, whereas orchard populations typically consist of a few varieties, which means low diversity. Therefore, the role of insects in the production of wind-pollinated or ambophilous plants needs to be evaluated further. Particularly, considering the ongoing rapid decline in pollinator populations, the impact of insect populations on the production of wind-pollinated or ambophilous crops in agroecosystems should be explored. If there is evidence that insects can facilitate the production of wind-pollinated or ambophilous crops in orchards, pro-active insect management of wind-pollinated or ambophilous crop orchards should be integrated in basic management plans to preserve and promote beneficial insect populations.[9]

The genus *Castanea* Miller (Fagaceae), commonly known as chestnut, has an extensive global distribution and is economically valuable as its nuts are an important nutritional source.[10] The plants of the genus have mixed inflorescences and male-only inflorescences, which are borne on the same branch; the mixed inflorescences distributed at the tips of the branches, and the male-only inflorescences are arranged parallel behind the mixed inflorescences.[11] The front end of the mixed inflorescence is the male flower and the base is the female flower. In the inflorescence, the female flower blooms before the male flower in the mixed inflorescence and at the same time as the male-only inflorescence. Female flowers are in the stigma receptivity stage when male inflorescences bloom, and stigma receptivity is lost when male flowers on mixed inflorescences open. Because the female flowers are distributed in the outer layer of the crown, they receive more alien (non-self) pollen, which is beneficial for maintenance of population diversity and vigor.[12,13] Both female and male flowers have no petals, leaving them exposed continuously, with numerous long and well-exposed stamens hanging freely from catkins producing large quantities of small pollen
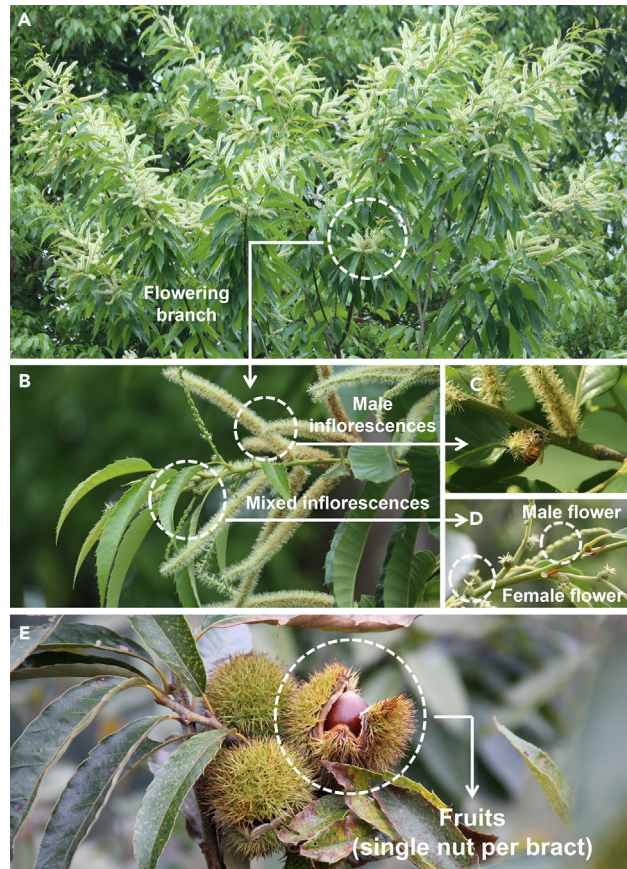
Figure 1. *Castanea henryi* description
(A and B) branches at flowering; (C) male-only inflorescences; (D) mixed inflorescences; (E) fruits.

grains, in addition to the long gynophores with acicular and sticky stigmas. Because of some wind-pollination traits, it has often been considered a wind-pollinated plant in the past.[14] Although it can achieve reproduction success by wind, it is not an entirely wind-pollinated plant. It has recently been reported that flowers in a related genus, *Quercus*, are visited frequently by insects.[15] In addition, Zhang et al.[16] observed that dipteran species visited *C. mollissima* flowers. *C. sativa* has also been reported to be pollinated by small insects.[17] Although a visit by insects does not represent pollination success, such flower visits highlight a need to explore whether insects could promote production in plant that can achieve reproduction success via wind pollination. Furthermore, understanding of the contribution rate of insects to fruit set in *Castanea* and its effect on fruit quality is unclear. The findings of such studies could be exploited to increase crop fruit yields.

In the present study, a monoecious plant *C. henryi* (Skam) Rehder and Wilson (Figure 1), which produces one of the most popular chestnuts in China, which is favored for its good taste and nutritional value, was selected as the research subject.[18,19] While there is increasing attention on the relationship between insects and plants that can rely on wind to produce fruit (including wind-pollinated or ambophilous plants), no in-depth studies have been conducted of the impact of insect populations on the production of wind-pollinated or ambophilous crops. Here, we distinguished and analyzed the contribution of an insect population on *C. henryi* productivity.

RESULTS

Importance of flower visitors for *C. henryi* production

Our results over two years showed that there was a significant difference in fruit set among the three treatments (Figure 2A, *p < 0.05*). In the open pollination treatment (CK, insects not excluded), the fruit set was significantly higher than in the other two treatments, increased by about 50%; the fruit set of the bagging treatment was significantly lower than that of the netting treatment (9.80–10.46% and 17.01–19.74%, respectively). By comparing the contribution of wind, insect, and self-pollination to fruit yield over two years, it was found that the individual contribution of insects to fruit yield was significantly higher than that of the other two treatments, in fact, four times that of wind and self-pollination. The contribution of wind to the fruit setting of *C. henryi* was essentially equal to, or even lower than, that of self-pollination (Figure 2B). It is worth noting that the contribution of insects in this result does not just represent the contribution of direct insect pollination, but may also include its indirect contribution to wind pollination. In addition, we found that the netting impeded about 30∼40% of the wind flow. However, as the wind speed increased, the blocking effect decreased following the expression $y = -0.0077x + 0.4039$ (Figure 2C).
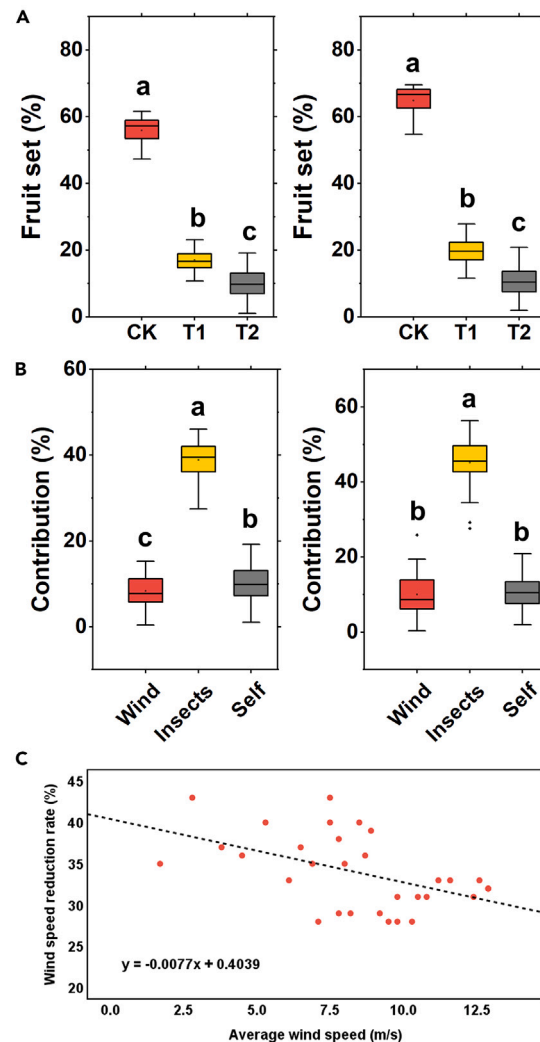
Figure 2. Effects of wind-, insects- and self-pollination on the chestnut fruit set

(A) fruit sets of different pollination treatments; (B) the contribution rates of wind, insects, and self-pollination in 2018 and 2019; (C) influence of mesh bag (40 mesh size) on wind speed. CK stands for natural treatment, T1 for treatment excluding insects, and T2 for self-crossing. The different letters indicate significant differences at *p < 0.05*. Data are represented as mean ± SEM.

Importance of flower visitors for nut quality

Overall, the nut quality of *C. henryi* in the three pollination treatments showed little difference; however, some indices exhibited significant differences ($p < 0.05$). In 2018, the nut weight of the open pollination treatment (9.16 ± 0.92 g) was significantly lower than that of the other two treatments; however, in 2019, no significant difference in nut weight was observed among the three treatments (Figure 3A). Consistent with the nut weight, the nut horizontal (50.63 ± 1.37 mm) and vertical (53.36 ± 1.14 mm) lengths of the open pollination treatment in 2018 were significantly lower than in the other two treatments. In 2019, the nut vertical length in the open pollination treatment remained the shortest; however, its horizontal length was between the other two treatments (Figures 3B and 3C). The water content of the nuts in the open pollination treatment was significantly lower than that of the other two treatments in both years, whereas no significant difference in reducing sugar content was observed in the nuts harvested in either year (Figures 3D and 3E). The soluble sugar content of the open pollination treatment was significantly lower than that of the other two treatments, yet its starch content (37.20 ± 0.63%) was significantly higher in 2018 (Figures 3F and 3G). Finally, the test results for sucrose showed that the sucrose content of the nuts from open pollination was the highest each year, at 0.48 mg/g and 0.37 mg/g, respectively (Figure 3H).

Flower-visiting insect characteristics

A total of 15 species of flower-visiting insects were recorded in the field, coming from the orders Hymenoptera, Diptera, Coleoptera, and Hemiptera (Figure 4 IA–O). Details of their specific flower visits were also recorded (Table S2). Most of the insects were observed only during
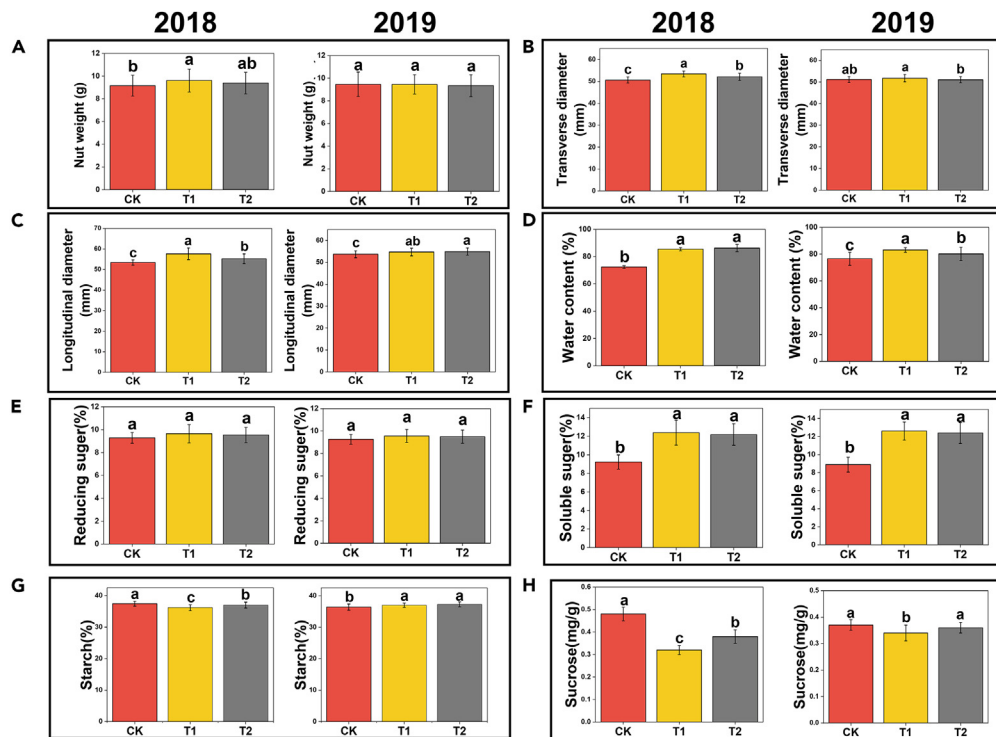
Figure 3. Quality of nuts harvested from three treatments in 2018 and 2019

(A) nut weight; (B) transverse diameter; (C) longitudinal diameter; (D) water content; (E) Reducing sugar content; (F) soluble sugar content; (G) starch content; (H) sucrose content. CK stands for natural treatment, T1 for treatment excluding insects, and T2 for self-crossing. The different letters indicate significant differences at $p < 0.05$. Data are represented as mean $\pm$ SEM.

the day (6:00-18:00), with only three species (*Stomorhina obsolete*, *Chrysochus asclepiadeus*, and *Subgenus fluviatilis*) being active at night, *S. fluviatilis* was also observed in the early morning. Most of the visiting insects exhibited a marked preference for male flowers, with significantly higher visits to male flowers than to female flowers ($p < 0.01$). Only *Chrysochus asclepiadeus*, *A. verbasci*, and *Aulacophora femoralis* were observed occasionally visiting female flowers. However, visits of female flowers by insects other than *A. verbasci* were found to be invalid in the statistical analysis. The remaining insects did not visit female flowers. *Neomyia timorensis*, *Eristalis cerealis*, *Lucilia sericata*, and *Apis cerana* were the most active visitors, while the visits of *Chrysochus asclepiadeus*, *A. verbasci*, and *Aulacophora femoralis* were relatively infrequent (Figure 4 IA–O). To further evaluate the role of the insects in pollination, we analyzed the pollen load of insects with visiting numbers >50 and repeated female flower stigma contact. The results showed that *A. verbasci* carried almost no pollen, while the pollen load of five pollinating insects with higher flower-visiting density was >4000 gains (Figure 4 II).

Insect visits also are influenced by flower fragrance. The ratio of the visits of insects in branches sprayed with mixed fragrance reagent to untreated branches showed that the branches sprayed with the fragrance mixture attracted more insects. Compared with the branches sprayed with solvent only, the visit volume of insects increased 10-fold, and increased 3-fold as compared to the branches without any treatment (Figure 4 III, $p < 0.001$). We provide the experimental results of a single species in the Supplemental Materials (Table S3), showing that the actual effect of the mixture is the best, followed by those of Xylene and 1,3,5-trimethylene ($p < 0.05$).

Effect of insects and wind on air pollen density

The changing trend of air pollen density was highly consistent with flower-visiting insect density, and both their peaks appeared between 14:00 and 14:30. There was also a small peak between 11:00 and 11:30, which is consistent with the peak time of wind speed. The wind speed and flower-visiting insect density began to increase after 6:00, however, the pollen density only modestly increased until 11:00. The air pollen density and flower-visiting insect density continued to rise and reached the highest point between 14:00 and 14:30 and then began to decrease. In contrast, the wind speed first decreased during the period of 12:00 to 12: 30, but then showed a slight rise between 14:00 and 14: 30. The wind speed began to drop continuously after 16:00. In this process of decline, the pollen density decreased the fastest, reaching almost the lowest at 17: 00, but the wind speed and flower-visiting insect density did not reach their lowest level until 22:00 (Figure 5A).

There was a significant correlation between air pollen density with flower-visiting insect density and wind speed (Figure 5B). However, stepwise regression and a generalized mixed linear model found that only flower-visiting insect density had a significant linear correlation with pollen density (Table 1).
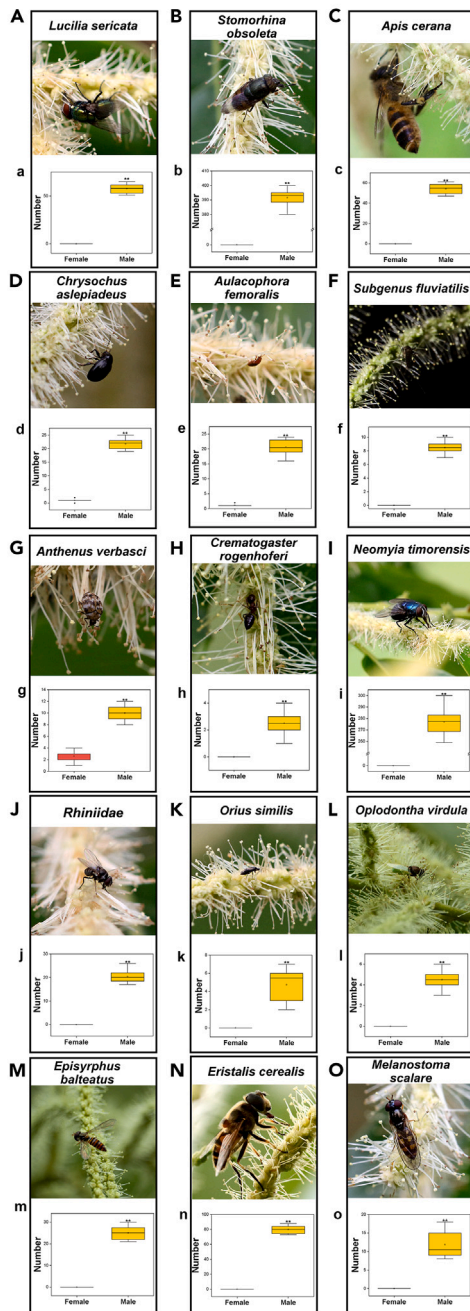
Figure 4. The relationship between insects and flowers

(I) flower-visiting insects (A–O) and the number visiting male vs. female flowers (a–o); (II) pollen load of six species of insects with visiting number greater than 50 and frequent contact with female flower stigma; (III) the attractive ability of floral substances to insects.** indicate significant differences at *p < 0.01,* *** indicate significant differences at *p < 0.001.* Data are represented as mean ± SEM.
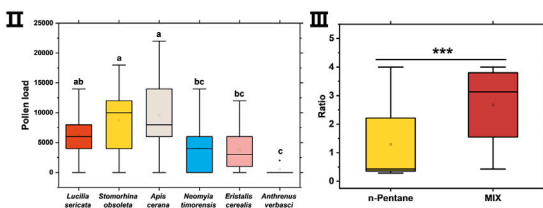
Figure 5. The relationship between visitors, wind speed, and air pollen density

(A) air pollen density, flower-visiting insect density, and wind speed for *Castanea henryi*, (B) the correlation analysis of each factor. Data are represented as mean ± SEM.

DISCUSSION

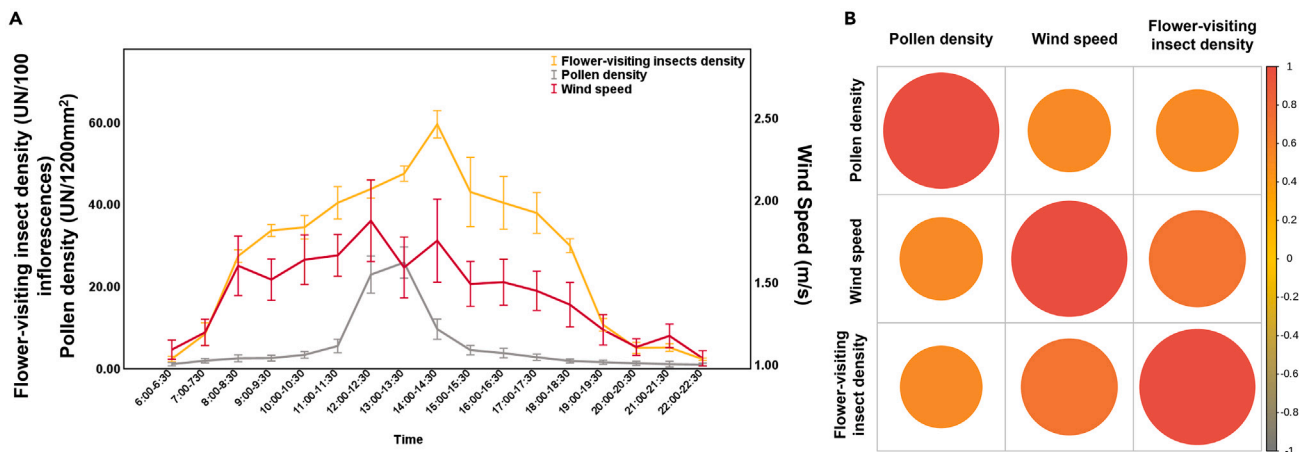Insects are vital for *C. henryi* production. When netted or bagged, the fruit-set rate of *C. henryi* was significantly lower than that in the natural state, which is consistent with the findings of previous studies.[17,20] Although the method of setting up the net could explain the findings clearly, in the present study, we still attempted the method of setting up the net to isolate the potential contribution of insects more strongly, and the results showed that the fruit-set rates of trees with net frame decreased significantly (Figure S1). All this evidence supports the importance of insects in chestnut yield, and the enhancement of chestnut fruit yield by insects does not lead to a decrease in fruit quality. In general, there was no significant decline fruit quality obtained under any of the three treatments, although in some cases the fruits under the net treatment were heavier, which may be attributed to the decreased number of fruits produced under the netting. With increased fruit number, plants must allocate limited nutrients to as many fruits as possible to ensure that most of the fruits can develop normally, resulting in smaller individual fruits. Nevertheless, this only occurs when the fruit yield is very high.[21] Notably, the starch and sucrose content of nuts obtained under open pollination increased, although water and soluble sugar content decreased slightly. Chestnut is a type of edible starch nut; therefore, the observation was quite surprising.[22]

Insects can help plants transfer pollen both directly and indirectly.[23] In insect direct pollination, insects transfer pollen via contact with stigma.[24] Indirect pollination relies on other mediums for pollen transfer, such as wind.[25] Insect direct pollination services are usually the focus of many studies; however, insect indirect pollination is also important, at least in *C. henryi* because *C. henryi* flower-visiting insects rarely touch stigma, and insects that touch stigma do not carry a lot of pollen. Larue et al.[17] observed a peak of insect visits to *C. sativa* female flowers when male flowers of mixed inflorescences bloomed. Such a flowering or pollen release strategy (unisexual flowers bloom first, male flowers in mixed inflorescences bloom later) may be effective in wild populations because of differences in flowering periods among individuals, which ensures adequate pollination.[6] However, it is not applicable in *C. henryi* orchards because the flowering periods of each individual in overlap orchards. When the second pollen release peak (when male flowers of mixed inflorescences bloom) occurs, most of the stigma receptivity of female flowers in orchards could have disappeared.[11] Therefore, fruit production in orchards may not benefit from stigma contact of insects at the second pollen release peak. Hence, insect pollination services for *C. henryi* fruit production in orchards occur mainly at the first flowering stage (male-only inflorescences flowering stage). At this stage, the odor released by male flowers attracts a large number (including 15 species) of daytime insects to visit male flowers; only *Chrysochus asclepiadeus, A. verbasci,* and *Aulacophora femoralis* touch female flowers occasionally. However, insects visiting female flowers are few in number and carry almost no pollen, and their successful pollination service depends on chance. Bees and flies, which carry numerous pollen, did not visit stigmas. Therefore, the indirect pollination service of visitors may be the main avenue via which insects enhance *C. henryi* fruit production.[26] Correlation analysis results for wind speed, air pollen density, and insect visitation support the capacity of insects to enhance chestnut yield by increasing pollen density in the air. In addition, the stickiness of

Table 1. Correlation analysis and generalized linear mixed model of related factors with pollen density

Related factors	Stepwise regression		Generalized linear model		
	r	P	F	df	P
Flower-visiting insect density	0.62	<0.01[a]	165.34	1	<0.01[a]
Wind speed	–	0.142	1.23	16	0.33

[a]Indicates significant correlation $p < 0.01$;—indicates exclusion from the stepwise regression model.

chestnut pollen decreases over time, which enables insects to release it into the air.[27] Notably, insect population composition may vary across orchards due to varying land use and management practices as cultivated plants spread.[28] Direct pollination may not be achieved in some orchards because pollinators that make contact with stigma may be missing. Nevertheless, indirect pollination is not limited to certain insect species. Bees and flies actively visit flowers and carry high amounts of pollen in various *Gramineae* plants; therefore, managed pollinators could increase yield in chestnut orchards.[29,30] In addition, *Salix* plant production has been reported to benefit from insects, although the mechanism of the benefit is unclear.[31]

As human demand for food increases, the stability of fruit production in cultivated plants is a major concern.[32] Insects are valued for their pollination services. However, their role in plants that can be pollinated successfully by wind or several agents has been rarely discussed. The findings of the present study suggest that indirect pollination services by visitors in orchards increase fruit yield without compromising fruit quality. Therefore, insects may be more critical for the sustainability of global agricultural systems than previously thought, especially under conditions of considerable environmental (including wind) variability.[33] Conservation of beneficial insect diversity is necessary for sustainable fruit production across insect-pollinated, wind-pollinated, or ambophilous plants.[31,34] From a production perspective, grassland management could be enhanced to provide a habitat for insects.[35] Furthermore, such initiative could improve plant growth and exploit the root nodules of Fagaceae plants, improve orchard productivity, while protecting ecosystems.[36]

Limitations of the study

A key limitation of the present study is that although we have used two methods to test the contributions of insects to fruit production, bagging branches and netting trees, very consistent results were observed from the two methods. However, we still cannot rule out the possibility of insects releasing pollen in the air, and the existence of the net weakens the contribution of wind. This still makes it impossible for us to quantify the contribution of wind and insects, especially the direct and indirect contributions of insects. Another limitation of this study is that correlation can only be used to speculate the role of insects in fruit production, and cannot fundamentally attribute the increase in concentration of pollen in the air to insects.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Xiao-ming Fan (fan_xiaoming001@163.com).

Materials availability

This study does not involve ethical approval and human research.

Data and code availability

- All data produced in this study are included in the published article.
- This work does not report the original code.
- Any datasets generated during and/or analyzed during the current study are available upon request from the primary contact.

AUTHOR CONTRIBUTIONS

Formal analysis, B.Y.; validation, B.Y., F.L.H., D.Y.Y. and X.M.F.; writing—original draft, B.Y. and Y.H.L.; methodology, Y.H.L.; picture shooting, J.Q.Z.; writing—review and editing, J.Q.Z. and X.M.F.; visualization, X.X.Z., B.Y., and Y.H.L. supervision, project administration, conceptualization, resources, X.M.F.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS

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- o T-test
- o Correlation analysis
- o Stepwise regression model
- o Generalized linear model
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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Castanea henryi	This paper	N/A
Lucilia sericata	This paper	N/A
Stomorhina obsoleta	This paper	N/A
Apis cerana	This paper	N/A
Chrysochus asclepiadeus	This paper	N/A
Aulacophora femoralis	This paper	N/A
Subgenus fluviatilis	This paper	N/A
Anthrenus verbasci	This paper	N/A
Crematogaster rogenhoferi	This paper	N/A
Neomyia timorensis	This paper	N/A
Orius similis	This paper	N/A
Rhiniidae	This paper	N/A
Oplodontha virdula	This paper	N/A
Episyrphus balteatus	This paper	N/A
Eristalis cerealis	This paper	N/A
Melanostoma scalare	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Acetophenone	Adamas,99%+	Cat# 01128337
Styrene	Dr.E,99%+	Cat# 04497518
Xylene	Greagent,99%+	Cat# 01018114
1,3,5-trimethylene	Adamas,98%+	Cat# 01152905
Quinoline	Adamas,99%+	Cat# 01124247
2-hydroxyl-3-methyl-2-cyclopropene-1-ketonesfc	Adamas,98%+	Cat# 01116888
1,2,3-trimethylene	Adamas,98%+	Cat# 01480885
3-penten-2-one	Adamas,95%+	Cat# 01098186
Pentane	Sigma-Aldrich, ≥99%	Cat# 01291305
Ethyl acetate	Sigma-Aldrich, ≥99%	Cat# 676810
Ethanol	Supelco, ≥99%	Cat# 1.00974
Critical commercial assays		
0.3% MTT	Sigma-Aldrich, ≥98%	Cat# 475989
Reducing sugar (3,5-dinitrosalicylic acid colorimetry)	Sigma-Aldrich, ≥98%	Cat# 128848
Soluble sugar (anthrone colorimetry)	Sigma-Aldrich, ≥97%	Cat# 319899
Starch (anthrone colorimetry)	Supelco	Cat# SA20
Sucrose (resorcinol method)	Supelco	Cat# ScA20
Experimental models: organisms/strains		
Castanea henryi	Hongjiang, Huaihua, Hunan Province, China	N/A
Software and algorithms		
Origin	OriginLab, Massachusetts, USA	version 2024
SPSS	SPSS Inc., Chicago, IL, USA	version 26

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The study was conducted in Hongjiang, Huaihua, Hunan Province, China (27°11′45″ N, 109°49′44″ E), which has a subtropical monsoon climate. The mean annual rainfall ranges between 1136.1 and 1779.0 mm and the rainy and hot seasons occur simultaneously. The tree growth state in this area is relatively consistent (with an age range of 8–12 years). No pruning or fertilization took place during the experiment in more than 750 acres of *C. henryi* orchards concentrated on the mid-slope and hilltop areas. The dominant varieties included 'Huali 3' and 'Huali 4' as well as certain exceptional clones (for which protective measures have been proposed).

METHOD DETAILS

Pollination treatments

We investigated the reproductive success of *C. henryi* in 2018 and 2019 using different pollination treatment experiments. Its flowers (including female and male flowers) can bloom for 12 days, which provided ample time for experiments. We randomly selected nine healthy target trees with similar growth in each experimental site. Three days prior to the early flowering stage in early May, various treatments were carried out according to the flowering time (all through May) of the experimental site, as well as field observations. Each treatment was implemented on four branches of an individual tree; this was repeated three times on three separate trees ($n = 36$). There are at least 6 female flowers, that is, at least 3 mixed inflorescences, on each branch. The various treatments were as follows: Control group (CK) – natural pollination; net treatment (T1) – each branch of individual trees was covered with a PVC mesh bag (40 mesh), permitting wind and rain to pass through but blocking insects; bag treatment (T2) – each branch was covered with a transparent paper bag, preventing both wind and insects from entering. The fruit set was calculated as a total number of fruit/total number of female flowers × 100%.

To assess the effect of different pollination types, we used the fruit set per treatment as a measure of yield per plant for each experimental treatment. We then estimated the insect contribution (including indirect contributions) by computing the difference between the fruit set of the treatment which excluded insects (T1) and the control (CK), which allowed all pollination; the direct wind contribution was calculated as the difference between the fruit set of the treatment allowing only self-pollination (T2) and the treatment which excluded insects (T1); the self-pollination contribution was determined as the fruit set of T2. Since it is impossible to directly quantify the contribution of the three cases on a single branch, it can only be calculated using different branches, which means that each branch has its own insect contribution, wind contribution and self-pollination contribution. This inevitably leads to some negative calculated results when comparing treatments on different branches, and since a "negative" contribution is not theoretically possible, these negative values were arbitrarily set to 0; this adjustment did not affect the results.[37]

Harvesting and yield measurements

To compare the quality of nuts following the different pollination treatments, all ripe nuts were harvested on the same day after fruit ripening, 5 months after treatment, and stored separately by treatment. As *C. henryi* produces a single nut per bract, the results of each nut test can accurately reflect the quality of each prickly fruit. The weight of each nut ($n = 50$) was measured using an analytical balance (0.01 g), and its transverse and longitudinal diameter ($n = 50$) were measured with a Vernier caliper (0.01 mm). Additionally, the water content of the nuts ($n = 30$) was determined by the drying method; the reducing sugar content was determined by 3,5-dinitrosalicylic acid colorimetry ($n = 30$), the soluble sugar and starch content ($n = 30$) were evaluated by anthrone colorimetry, and the sucrose content ($n = 30$) was determined by the resorcinol method.

Influence of netting on wind speed

We tested the influence of the net on wind flow to better understand the influence of the net treatment on fruit set. We fixed a 40 mesh PVC bag in the middle of a bellows and set up two anemometers (Testo 410-2, Germany), one on each side of the net, in an empty room. Wind, at different speeds, was introduced from the left and the influence of the netting on wind speed was calculated as the difference between the anemometer readings on each side of the net. A total of 30 replicates were recorded.

Abundance and foraging behavior of flower-visiting insects

To identify the species composition of insect visitors of female and male *C. henryi* flowers, we observed the species and abundance of flower-visiting insects on seven sunny days during flowering, from 6:00 to 22:30, since the chestnut flower has no petals and is open all day. Abundance was calculated as the total number of individuals of an insect species in each area.[38,39] Additionally, we captured different *C. henryi* floral visitors using a sweep net, euthanized them in a bottle with ethyl acetate fumes and stored them in 80% ethanol solution for future identification. We utilized the literature and expert assistance to identify each species of floral visitor, though some insects could only be identified to the genus level.[40] Furthermore, to better assess the contribution of flower-visiting insects to productivity, we recorded the female and male flower-visiting number of each insect species separately on three trees during 12:00–15:00 on eight clear days. The plants were changed every day, and only the insects in contact with the stigma of female flowers were recorded as flower-visiting insects of female flowers. To determine the effect of the number of flower visitors on the pollen density in the air, insect samples with visiting number greater than 50 and frequent female flower stigma visits were also collected to determine the individual pollen load ($n > 20$ each species).

Floral attraction test

To evaluate whether *C. henryii* can attract insects actively, we carried out fragrance attraction experiments. Following previous determinations of volatile components of chestnut flowers using GC-MS at the Wuhan Punes Biotechnology Co., Ltd (Wuhan, China), nine kinds of aromatic volatiles with the highest content (>0.03 g/g) were selected and standard samples were purchased (Table S1). A 5 mL solution of each was prepared with n-pentane according to Table S1, and 1 mL of each was combined into a mixed reagent (MIX). The experiment was carried out from 14:00 to 14:30 (the most active time for flower-visiting insects). The number of flower visits on a single inflorescence in 10 min was recorded after spraying, and only the male inflorescence was observed. Flowers sprayed with n-pentane alone were used as the negative control, and the unsprayed flower next to the branches of the experimental group as the positive control. Each process was repeated on a different tree at least three times, for a total of 750 observation minutes. At the end of the experiment, the ratio of the number of flower-visiting insects in the untreated branches to the number in the reagent-sprayed branches was calculated.[41]

Pollen and flower-visiting insect density

We selected seven sunny days during the flowering period to investigate insect visits and the pollen density in the air from 6:00 to 22:30 at the site. Each survey lasted half an hour, with a total of 17 surveys per day. For flower-visiting insect density, strip sampling was carried out in three selected planting bands. In each observation period, the number of flower-visiting insects on male and female flowers on 100 *C henryi* inflorescences was recorded as the flower-visiting insect density (number/100 inflorescences), and the three planting bands were repeated in triplicate.[42] For pollen density, the park was evenly divided into six regions to ensure that each area contained at least one *C. henryi*. Then bamboo poles were inserted in the middle of each area, thin Vaseline-coated slides were hung and replaced every half an hour. Vaseline was melted from the slides with an alcohol lamp and dyed with 0.3% MTT for 30 min, sealed, and taken to the laboratory.[43] The pollen number of *C. henryi* under the cover glass was recorded by microscope observation, combined with staining results and pollen morphology. The formula for calculating pollen density was: Pollen density = pollen number under cover glass/(50 mm $\times$ 24 mm).

QUANTIFICATION AND STATISTICAL ANALYSIS

SPSS version 26 (SPSS Inc., Chicago, IL, USA) is for statistical analysis, and Origin version 2024 (OriginLab, Massachusetts, USA) is for drawing figures. Averaged data from individual experiments are presented as mean $\pm$ standard deviation (SD), and the number of samples (n) of each experiment is shown in the part method details.

One-way analysis

The fruit set and nut quality of pollination treatments, the contribution of different pollination avenues, and attraction of flower scent were analyzed using the Tukey HSD test analysis of variance. Statistical significance was set at $p < 0.05$.

T-test

The T-test was used to compare the visits to female and male flowers of each species of flower-visiting insect. Statistical significance was set at $p < 0.05$.

Correlation analysis

The correlation between relative factors (visiting-insect density and wind speed) and pollen density was analyzed, and the factors most strongly related to pollen density were obtained.[44]

Stepwise regression model

To explore the linear relationship between pollen density and relative factors (visiting-insect density and wind speed), we took the relative factors as independent variables, the pollen density as dependent variables, and statistical significance was set at $p < 0.01$. The most relevant factors of pollen density were obtained by the stepwise regression method.[45]

Generalized linear model

To explore whether there are other linear relationships between the relative factors (visiting-insect density and wind speed) and pollen density, we took the pollen density as the target and the visiting-insect density and wind speed as the fixed effect. We used the generalized linear mixed model to obtain their significance ($p < 0.01$).

ADDITIONAL RESOURCES

This study has not generated or contributed to a new website/forum and it is not part of a clinical trial.