Agriculture, Ecosystems and Environment 330 (2022) 107887

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

Importance of maternal resources in pollen limitation studies with pollinator gradients: A case study with sunflower

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ARTICLE INFO

Keywords:
Entomophilous crop
Pollen supplementation
Pollen limitation
Pollinator gradient
Seed yield
Sunflower (*Helianthus annuus*)

ABSTRACT

Pollen limitation studies are scarce in entomophilous crops, as it can be very tedious to supplement plants with hand pollination at the relevant plant scale. To overcome this, recent studies have used pollinator gradients over several fields to assess whether crops were pollen limited. But the plant maternal resources and thus the yield potential may vary between fields of the same crop. If these resources are not properly controlled, it may affect the conclusions of the study.

We investigated the relationship between yield, measured as seed number, seed mass, and seed oil content per head, and pollinator density in sunflower (*Helianthus annuus* L.) by using a gradient of pollinator densities set both across and within fields. We analyzed the yield and the % of yield potential, the latter being assessed with pollen supplementations applied at the whole plant scale. We analyzed the data both with and without random effects including year, cultivar, field and sampling plot. We also compared the yields of open and pollen-supplemented sunflowers to sunflowers isolated under tulle bags to assess autonomous self-fertilization and self-production rates in each field.

Without random effects included in models, yields increased with pollinator density but only up to 0.3 bees per head, implying pollen limitation only below this threshold, while with random effects included, the yields were marginally pollen limited across the whole range of pollinator densities observed ($P = 0.072$ and 0.037). Yet, on the other hand, there was no relationship between bee density and % of yield potential (assessed with pollen supplementation treatments), with or without random effects included, implying no pollen limitation across the complete range of bee densities observed in our study (0.1–1 bees per sunflower head). These last results are explained by variation in the yield potential between and within fields, and by the positive correlation found between yield potential and pollinator density. Insect pollination contributed on average to 53.7% and 42.8% of the seed number and mass per head, respectively, and also increased the seed oil content by 5.5% on average.

Our study showed that plant maternal resources and random effects are crucial to consider when quantifying pollen limitation using pollinator gradients and yields. Pollinator gradients on a per flower basis are a useful tool to identify target pollinator densities to maximize yields, but should be combined with pollen supplementation treatments at the relevant scale in order to correctly assess pollen limitation.

1. Introduction

For a given ovule number, seed and fruit production in flowering plants require pollen and resources (e.g. water, nutrients), and are thus necessarily limited by the least available of these two components (Harder and Routley, 2006; Harder and Aizen, 2010). Resource limitation occurs when a plant does not have enough resources to develop all the ovules or ovaries that are fertilized into seeds or fruits. On the other

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https://doi.org/10.1016/j.agee.2022.107887
Received 12 July 2021; Received in revised form 24 January 2022; Accepted 25 January 2022

hand, pollen limitation takes place when an inadequate quantity or quality of pollen is received on stigmas to fertilize all the ovules or ovaries into seeds or fruits given the available resources for fruit and seed development. Pollen limitation is a common and widespread phenomenon encountered in a majority of wild angiosperms, especially in human-modified landscapes (Knight et al., 2005; Bennett et al., 2018, 2020). However, pollen limitation studies are scarce in crops, as pollen limitation is recommended to be studied at the whole plant scale and across several reproductive events in polycarpic species (Knight et al., 2006; Webber et al., 2020). Applying pollen supplementations at these scales, a method typically used to assess pollen limitation, can be very tedious in crops as individual plants often bear a large number of flowers.

Recent studies highlighted pollen limitation in entomophilous crops by using gradients of pollinator densities, hereafter referred to as pollinator gradients (e.g. Garibaldi et al., 2016; Catarino et al., 2019; Rollin and Garibaldi, 2019; Reilly et al., 2020). In these studies, if crop yields increased with pollinator density up to a potential plateau, the crop was considered pollen limited below this plateau (conceptualized by Reilly et al., 2020). This study design has the advantage of being able to identify a threshold of pollinator visitation beyond which all ovules are fertilized in the crop (Garibaldi et al., 2020; Reilly et al., 2020). This can help to provide guidelines to growers on the number of pollinators to introduce into a field to supplement those already present and thereby achieve integrated crop pollination (Isaacs et al., 2017). These target values may also enable growers to avoid pollination deficits without introducing too many managed pollinators, thereby saving on input costs and avoiding potentially deleterious effects of excessive managed pollinators on pollination (Morales et al., 2017; Aizen et al., 2020) and wild entomofauna (Geslin et al., 2017; Mallinger et al., 2017; Russo et al., 2021).

With this approach, pollen limitation is determined simply by comparing the yields over several fields varying in pollinator density, with often several cultivars grown across fields and without controlling for the yield potential of each field or without relating a pollinator density to a flower number. Yet, the plant maternal resources and thus both flower number and yield potential can vary greatly between fields, even for the same cultivar, depending on crop management. For instance, radiation and photosynthesis levels, water and nutrient availabilities in the soil, and pest control can all affect the yield potential (Bos et al., 2007; Boreux et al., 2013; Garratt et al., 2018; Tamburini et al., 2019). Additionally, pollination requirements in terms of number of insect visits required per flower to reach full ovule fertilization can vary substantially between cultivars for a given crop (Kendall et al., 2020). To control for these differences in yield potential and pollination requirements between fields, one possibility is to establish a pollinator gradient within a field of a given cultivar, called 'pollinator front' (Vaissière et al., 2011), as maternal resources and pollination requirements are likely to be more homogeneous within a single field. Another possibility is to apply pollen supplementations to determine the difference between open and pollen supplemented treatments for each cultivar and each field. As pollen supplementations can be tedious to apply at the whole plant scale, it could be applied at a lower relevant plant scale such as the branch or flower cluster (see Wesselingh, 2007; Webber et al., 2020).

This study aimed to test if the use of a pollinator gradient set both across and within fields can reliably determine the degree of pollen limitation without also including pollen supplementation treatments. The results excluding pollen supplementation treatments were compared to those including pollen supplementation treatments in order to answer our study question. We used cultivated sunflower (*Helianthus annuus* L.; Asteraceae) as our model crop, which has the advantage of being easily pollen supplemented at the whole plant scale and over its entire lifespan as it is monocarpic with flowers concentrated in one large inflorescence. This crop is largely self-compatible (Gandhi et al., 2005; Sun et al., 2012), but, as it is protandrous, insect pollinators can contribute to pollination by carrying pollen from staminate florets to pistillate florets. Indeed, while sunflower crop can be largely self-fertile, depending on the cultivar, the site, or the year (e.g. Mallinger and Prasifka, 2017a; Mallinger et al., 2019), pollinator-dependency varies from 0 to over 90% of seed set, with most studies reporting intermediate levels of dependency (Dag et al., 2002; see references in Chamer et al., 2015). Some studies also showed that insect pollination can increase seed oil content in oilseed sunflowers (e.g. Mahmood and Furgala, 1983; Bartual et al., 2018). But overall, no study has identified a threshold of pollinator density beyond which there is no longer pollen limitation in this crop. We expected to see a difference in our conclusions as to the degree of pollen limitation observed in the fields when including or excluding pollen supplementation treatments. Indeed, pollen limitation cannot be properly assessed without including pollen supplementation treatments.

2. Materials and methods

2.1. Study sites and experimental design

The study was performed in 2015 and 2017 near Toulouse (France). It took place in nine sunflower fields, with four F_1 hybrid oilseed cultivars: four fields with cv. 'Extrasol' in 2015, three fields with 'SY Talento' in 2017, one field with 'SY Rialto' in 2017, and one field with a coded cv. 'X' in 2017. Extrasol could not be sampled again in 2017 as 2015 was its last year of marketing. These fields were at least 280 m long and 75 m wide. The locations and features of these fields are provided in Table A1 (Appendix A). The features of the cultivars are provided in Table A2 (Appendix A).

2.1.1. Honey bee colonies and pollinator source

Honey bee (*Apis mellifera* L.) colonies were provided by one beekeeper each year at a stocking rate of approximatively 2 colonies/ha and distributed along one of the two shortest edges in each field (Fig. 1), except for the field 'Extrasol 4' which did not receive any colony (Appendix A, Table A1). The colonies were placed along the field edge with the maximum amount of semi-natural habitats nearby (wood, hedgerow, river, lakeside) in order to achieve the maximum density of both honey bees and wild pollinators coming from the same field edge considered as the pollinator source (PS), with the gradient extending from this field edge into the centre of each field (Fig. 1). Indeed, semi-natural habitats are a source of wild pollinators (Ricketts et al., 2008). We made sure that there were no uncontrolled honey bee colonies in a radius of ca. 1 km around each study field, and that fields were at least 2 km apart for independence.

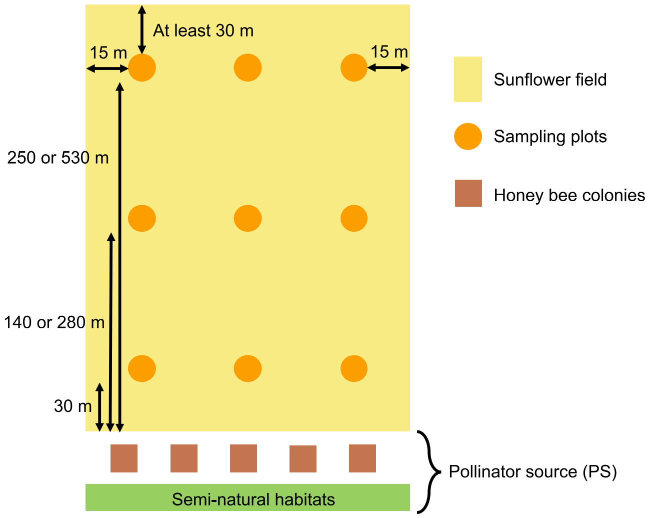

Fig. 1. Schematic representation of the sampling design.

2.1.2. Sampling plots

Nine sampling plots of 15–16 plants each were identified per field at three distances from the PS (Fig. 1). The sampling distances from the PS differed between the two years as well as across fields within a year due to size differences between the fields. Along the wide axis of the fields, at each distance from the PS, the three plots were distributed as follows: one plot in the centre, and the two others placed equidistant from the centre and 15 m from the two longest field edges. Field 'Extrasol 1' was an exception in which we sampled 12 plots at four distances from the PS. In 2015, 3 plots each were placed 30 m, 140 m, and 250 m away from the PS in each field (+ the distance of 500 m for 'Extrasol 1'). In 2017, 3 plots each were placed 30 m, 280 m, and 530 m away from the PS in each field. In 2015, in the smaller field 'Extrasol 4', plots were placed 30, 85, and 140 m from the PS (Appendix A, Table A1).

2.2. Pollination treatments

In each sampling plot, 5 or 6 sunflower heads chosen at random were isolated under tulle bags of 1.05 mm mesh size (fabric F510, Diatex, France) just before the onset of flowering and during the whole flowering period to prevent insect pollination. These heads were therefore pollinated only by autonomous self-pollination and wind. Since the wind carries very little sunflower pollen (e.g. Radford et al., 1979a; Low and Pistillo, 1986), we used this treatment to assess the rate of autonomous self-fertilization (i.e. following autonomous self-pollination) of plants in each field.

In each sampling plot, 5 other heads chosen at random were left uncovered for open pollination, and therefore pollinated by autonomous self-pollination, wind, and insects. Finally, in each sampling plot in 2015, and only in each central plot at each distance from the PS in 2017 in order to decrease sampling effort, 5 other heads chosen at random received a treatment of pollen supplementation in addition to open pollination. Pollen supplementation consisted of brushing the pollen from the florets at the staminate stage of a head onto the open stigmas of the nearby florets at the pistillate stage of the same head with a paint brush. We did not use donor pollen from other plants within the same field as all fields consisted of only a single F_1 hybrid cultivar. Indeed, Chamer et al. (2015) found in two cultivars that the seed number, seed mass, and oil content did not differ in heads supplemented with pollen coming from the same plant or from other plants of the same cultivar. Pollen supplementations were repeated every two days in the morning over the flowering period of each flower head.

2.3. Pollinator counts

Pollinators were counted on 100 (in 2017) or approximatively 200 (in 2015) heads chosen at random on each sampling plot in 2017, and only in each central plot at each distance from the PS in 2015 in order to decrease sampling effort, over the whole flowering period (defined as when at least 10% of plants were in bloom). These counts were repeated between 4 and 6 dates per field, between 07:00 and 15:00 UTC during insect pollinator activity, and only under suitable weather conditions for insect foraging, i.e. without rain, with low wind, temperature > 15 °C and dry vegetation (Westphal et al., 2008). The sampling order of the plots changed across dates for each field.

Each head was scanned once by counting instantly all the pollinators foraging on it at the time of observation (Vaissière et al., 2011; Garibaldi et al., 2016). The scan sampling method enabled us to assess pollinator density across a large number of heads (at least 900 per field and observation day). Additionally, by scanning pollinators from a distance of about 1 m ahead of the observer, we avoided disrupting pollinators before they could be counted. We did not use the method of persistent observation during several minutes to a focal flower group (Fijen and Kleijn, 2017; Garibaldi et al., 2020), because it can prevent pollinators from visiting the focal flower group as the observer stays in close proximity to the flowers, and furthermore the observer samples only a small fraction of the flowers. As well, we did not use colored pan traps, as the bee specimens collected with pan traps do not necessarily visit the flowers of the crop within which the pan traps have been placed (Popic et al., 2013; Gibbs et al., 2017; O'Connor et al., 2019). At last, netting was not used either as it can miss small bee specimens (Boyer et al., 2020; Krahner et al., 2021; Pei et al., 2021).

The pollinators were recorded in 5 groups: honey bees with pollen pellets in their corbiculae, honey bees without pollen pellets, bumble bees, other wild bees, and syrphid flies.

2.4. Measures of seed yield components: seed number, seed mass and seed oil content

Sunflower heads were harvested at physiological maturity just before the commercial harvest. They were put in a drying room for 48 h at 60 °C to lower their moisture content to 9%, before processing them with a thresher to separate the filled seeds from the empty hulls. The filled seeds were counted with a seed counter (Contador, Pfeuffer, Germany) and weighed to get the seed number and the seed mass per head. The seed oil content was measured for each head with a 6–10 g seed sample using a nuclear magnetic resonance (NMR) device (minispec mq7.5, Bruker, UK).

2.5. Data analysis

2.5.1. Pollinator gradient

To detect if the experimental design resulted in a pollinator gradient in the fields, the number of honey bees, wild pollinators (including bumble bees, other wild bees and syrphid flies), and pollinators as a whole (honey bees + wild pollinators) counted on 100 or ca. 200 heads were analyzed with a generalized linear mixed effect model (GLMM), with a quadratic negative binomial regression (NB2) chosen for the residual distribution (Hilbe, 2014), as overdispersion was detected with a Poisson regression (Zuur et al., 2009). The distance from the PS was set as a fixed quantitative explanatory variable, and after a top-down model selection, some factors including year, cultivar nested in year, field nested in cultivar (and including a random slope to allow the effect of distance from the PS to vary across fields), and plot nested in field were set as random effects (see Appendix B, Table B1). As the number of sample heads varied across observations in both years, this number was added as an offset.

2.5.2. Relation between pollinator density and seed yields: test for pollen limitation

To test if the crop was pollen limited in our study, we used analyzes from Reilly et al. (2020). We used three mechanistic models, (i) a null model implying no relation between pollinator density and seed yields (Eq. (1)), (ii) a positive linear relation (Eq. (2)), and (iii) a piecewise relation with one threshold below which there is a positive linear relation and beyond which there is no relation (Eq. (3)). These three functions were written as follows:

$$Y = i \tag{1}$$

$$Y = i + sP \tag{2}$$

$$\left\{ \begin{array}{ll} P < t, & Y = s(P - t) + m \\ P \geq t, & Y = m \end{array} \right\} \tag{3}$$

where Y is the seed yield, P is the pollinator density (= the number of pollinators per head), i is the y-intercept, s is the slope, m is the maximum yield, and t is the threshold of pollinator density beyond which m is reached. Eq. (1) implies that the crop is not pollen limited anywhere, Eq. (2) implies that the crop is pollen limited everywhere, and Eq. (3) implies that the crop is pollen limited only below the threshold of pollinator density t.

To test if the pollinator gradient was a reliable method to assess pollen limitation using yield measurements, we first analyzed the seed number, the seed mass, and the seed oil content per open pollinated head with the three models shown in Eqs. (1)–(3). Next, we analyzed the same yields measured from the open pollinated heads, but divided by the corresponding yield potential measured in each field and at each distance from the PS to calculate the % of yield potentials. The yield potential was determined by the mean seed number, mean seed mass, and mean seed oil content per head of the pollen-supplemented heads in each field and at each distance from the PS. For models shown in Eqs. (1)–(3), the yields were related to the mean pollinator number per sunflower head in each field and at each distance from the PS.

The parameters *i*, *s*, *m*, and *t* were estimated with either linear or nonlinear least squares (Bates and Watts, 1988; Bolker, 2008), using the *lm* function in the software R for Eqs. (1) and (2), and the *nls* function for Eq. (3). But, as there were repeated measurements per year, cultivar, field and plot, some of these four factors were also added as random effects in an additional analysis following a top-down model selection (Appendix B, Table B2). With random effects, the parameters *i*, *s*, *m*, and *t* were estimated with maximum likelihood, using the *lmer* function (linear mixed effect models; R package *lme4*) for Eqs. (1) and (2), and the *nlme* function (nonlinear mixed effect models; Lindstrom and Bates, 1990; R package *nlme*) for Eq. (3). For Eq. (3), standard deviations of random effects were assessed for the parameter *m*, which is the equivalent of the intercept in the formula. The parameters *s* and *t* were fixed because the pollinator gradients observed in each field or cultivar were not large enough to be able to estimate these parameters independently for each field or cultivar. For each seed yield variable *Y*, the most parsimonious model between the three equations, and with or without random effects, was identified with the AIC and its relative weight of evidence (Burnham and Anderson, 2002). However, Eq. (3) was estimated only when the algorithms of the *nls* and *nlme* functions could converge given the data structure, which was not the case with oil content, nor with % of yield potential with random effects.

In addition, we tested if the yield potential varied between fields and within each field depending on the distance from the PS. With this aim, we applied a top-down model selection from the beyond optimal models including the year, cultivar nested in year, field nested in cultivar, the distance from the PS nested in field, and plot nested in the distance from the PS all set as random effects with seed number, seed mass, and seed oil content per pollen-supplemented head as response variables. In this procedure, the random effects were removed one by one from the beyond optimal model, and the nested models were compared with the beyond optimal model with a χ^2 test in order to test each random effect independently from each other. If the χ^2 tests gave a $P > 0.005$ for at least one removed random effect, the random effect with the χ^2 test giving the highest *P* was removed from the beyond optimal model, and the top-down selection process started again with this new nested model (see chapter 5 in Zuur et al., 2009). The random effects not removed at the end of the procedure were considered significant. As the number of seeds per head is a non-independent count (as seeds are grouped within heads), implying overdispersion, the NB2 was used for the residual distribution. The mass of seeds and seed oil content per head are continuous quantitative variables and were thus analyzed with the normal distribution.

Finally, we tested if the yield potential and average pollinator density per field and per distance from the PS were correlated with Pearson correlation tests for the three yield components (seed number, seed mass, seed oil content).

2.5.3. *Rate of autonomous self-fertilization and self-production, and pollinator contribution to seed yields and oil content*

We compared the yield components, i.e. the seed number, seed mass and seed oil content per head, between the three pollination treatments (i.e. the heads isolated under tulle bags, those open pollinated and those pollen-supplemented) with mixed effect models. As previously, the seed number was analyzed with a NB2, while the seed mass and oil content were analyzed with a normal distribution. The pollination treatment was set as a fixed qualitative explanatory variable, and after a top-down model selection, some factors including year, cultivar nested in year (and including a random slope to allow the effect of the pollination treatment to vary across cultivars), field nested in cultivar (and including a random slope to allow the effect of the pollination treatment to vary across fields), and plot nested in field were set as random effects (see Appendix B, Table B3). In addition, to assess the effect of the pollination treatment for each field separately, the same analyzes were conducted for each field but with only the plot set as a random effect.

The rate of autonomous self-fertilization, i.e. following autonomous self-pollination, was calculated in each field by dividing the mean seed number of the heads isolated under tulle bags by the mean seed number of the pollen-supplemented heads (Rodger et al., 2021). In the same way, we calculated the rate of autonomous self-production in each field by dividing the mean seed mass of the heads isolated under tulle bags by the mean seed mass of the pollen-supplemented heads. The remaining percentages of seed yield were attributed to insect pollination. The increase in seed oil content due to insect pollination was calculated in each field by subtracting the mean seed oil content of the heads isolated under tulle bags from the mean oil content of the open pollinated heads. The mean rate of autonomous self-fertilization, the mean % of yield potential of the heads under tulle bags, and the mean increase in oil content due to insect pollination were calculated over all the fields from the predictions of the three models described previously.

2.5.4. *Software, packages and P-value threshold*

All the statistics were computed with the software R, version 4.0.3 (R Core Team, 2020). The linear mixed effects models were computed with the package *lme4*, version 1.1–26 (Bates et al., 2015). The *P*-values of the linear mixed effects models were obtained with the package *lmerTest*, version 3.1–3 (Kuznetsova et al., 2017). The NB2 regression was used with the package *MASS*, version 7.3–53 (Venables and Ripley, 2002). The nonlinear mixed effect models were computed with the package *nlme*, version 3.1–149 (Pinheiro et al., 2020). The asymptotic 95% confidence intervals of the parameters of the mechanistic models were estimated with the package *nlstools*, version 1.0–2 (Baty et al., 2015). The chosen *P*-value threshold for statistical significance was set at 0.005, as recommended to increase the reproducibility of scientific studies (Johnson, 2013; Benjamin et al., 2018). When the *P*-value was included between 0.005 and 0.1, the associated effect was considered to be *marginally* significant.

3. Results

3.1. *Pollinator community and gradient*

Overall, the pollinator community visiting the sunflower heads was largely dominated by honey bees without pollen pellets in their corbiculae (91.8% of the 14,888 recorded visits), with few wild pollinators (Appendix A, Table A3). The predicted mean number of honey bee visits per head decreased over all the fields with distance from the PS, by 25.5% at 250 m from the PS, and by 44.5% at 500 m from the PS, and with differences in visitation rates across fields (Fig. 2a; Appendix A, Table A4). There were very few wild pollinator visits overall, and no effect of the distance from the PS on wild pollinator visitation rates (Fig. 2b; Appendix A, Table A4). But as wild pollinators made up only a small part of the visiting pollinator community, total insect pollinator visitation rates between fields and in relation to distance from the PS showed a similar pattern as that of honey bees (Fig. 2c; Appendix A, Table A4).

3.2. *Relation between pollinator density and seed yields*

Without random effects, the most parsimonious models selected by

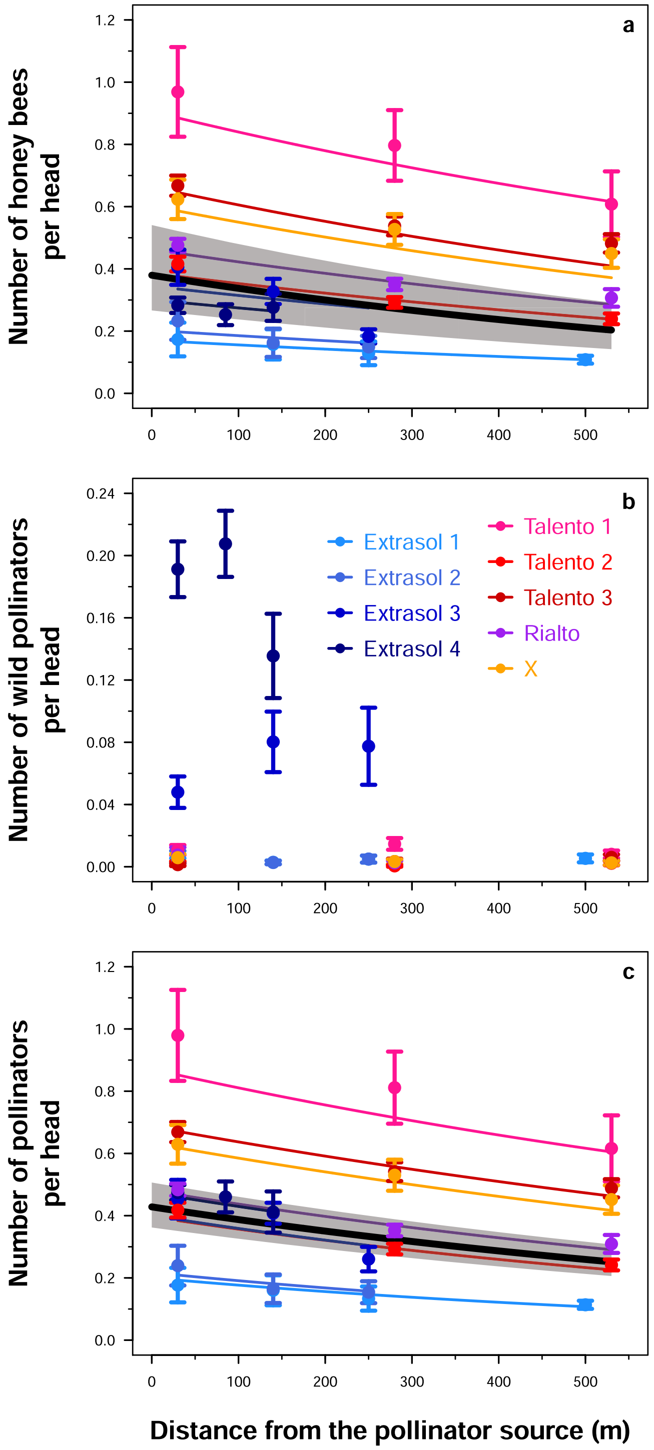

Fig. 2. Density of honey bees (a), wild pollinators (b), and pollinators as a whole (honey bees + wild pollinators) (c) in relation with the distance from the pollinator source per field (mean ± SE). Thick black lines are the GLMM mean predictions (± SE, shaded area; Appendix A, Table A4), and thin colored lines are the GLMM predictions for each field. Legend: the names Extrasol, Talento, Rialto and coded X are cultivars, and the numbers 1, 2, 3 and 4 are the replicated field numbers per cultivar.

AIC for the seed number and seed mass per open pollinated head were by far the piecewise models (ΔAIC > 44 and > 24, respectively, with the next best models; Table 1), followed by the linear models (ΔAIC > 72 and > 32 with the least supported models, Eq. (1)). These yield measurements increased with pollinator density up to 0.3 pollinators per head (Table 2; Fig. 3a,d). When including the random effects, the most parsimonious models were the linear models (ΔAIC > 12 and > 9, respectively, with the next best models; Table 1), followed by the null models (ΔAIC > 1 and > 4 with the least supported models, Eq. (3)): both seed number and mass increased continuously with pollinator density, but only marginally ($P = 0.072$ and 0.037, respectively; Table 2; Fig. 3a,d).

On the other hand, the piecewise and linear models did not perform better than the null models for both % of seed number potential and % of seed mass potential, without random effects included (Table 1); i.e. there was no increase of the % of yield potential across the complete range of pollinator densities observed from 0.11 to 0.98 bees per head (Fig. 3b,e). When including the random effects, the linear models performed better than the null models for both yield measurements (ΔAIC > 4 and > 5, respectively; Table 1), but their respective estimated slopes were not different from 0 ($P = 0.64$ and 0.40; Table 2; Fig. 3b,e).

For seed oil content, the linear models were more parsimonious than the null models, both for when random effects were excluded or included (ΔAIC > 11 and > 3, respectively; Table 1). However, while the estimated slope was negative without random effects, it was not different from 0 with random effects included ($P < 0.005$ and $= 0.38$, respectively; Table 2; Fig. 3g). On the other hand, for % of oil content potential, the linear model did not perform better than the null model without random effects included (Table 1); i.e. there was no increase of the % of oil content potential across the complete range of pollinator densities observed (Fig. 3h). When including the random effects, the linear model performed better than the null model, but with an estimated slope not different from 0 ($P = 0.47$; Tables 1 and 2, Fig. 3h).

In addition, the yield potential varied between fields for seed oil content per pollen-supplemented head, from a mean of 43.4% in ‘Extrasol 4’ to 47.8% in ‘Extrasol 2’, as well as within fields with the distance from the PS for seed number and seed mass per pollen-supplemented head, from 802 seeds and 33 g in average in ‘Extrasol 1’ at 140 m from the PS to 1818 seeds and 125 g in field ‘X’ at 30 m from the PS (Appendix A, Table A5; Figs. A1 and A2). The % of seed number potential was positively correlated with pollinator density ($r = 0.52$; 95% CI = ± 0.23; $P < 0.005$; Fig. 3c), the % of seed mass potential was marginally positively correlated with pollinator density ($r = 0.32$; 95% CI = ± 0.30; $P = 0.096$; Fig. 3f), and the % of seed oil content was marginally negatively correlated with pollinator density ($r = -0.60$; 95% CI = ± 0.53; $P = 0.030$; Fig. 3i).

3.3. *Rate of autonomous self-fertilization and self-production, and pollinator contribution to seed yields and oil content*

Over all fields, the open pollinated heads had higher seed number, seed mass, and seed oil content per head than those isolated under tulle bags (Fig. 4; Appendix A, Table A6). On the other hand, yield measurements including seed number, seed mass, and oil content were not different between open and pollen-supplemented heads. The mean rate of self-fertlization was 46.3% for the seed number per head, and the mean rate of self-production was 57.2% for the seed mass per head, with the remaining yield percentages being attributed to insect pollination (53.7% and 42.8%, respectively). These rates varied between fields, even within a given cultivar, and between cultivars (Table 3; Appendix A, Table A7; Fig. A1a,b). The mean increase in oil content due to insect pollination was 5.5%, but also varied across fields and cultivars (Table 3; Appendix A, Table A7; Fig. A1c).

4. Discussion

4.1. *Assessing pollen limitation with yields versus % of yield potentials across pollinator gradients*

The relationship between yield and pollinator density, and thus whether the crop was pollen limited or not, depended on whether yields

Table 1
AIC values of the three mechanistic models computed per seed yield component, and with or without random effects included.

Response variable per open pollinated head	Random effects included	Model	Eq.	Random effects			Parameters				k	AIC	Model rank	ΔAIC	w_i
				Cultivar	Field	Plot	i	s	m	t					
Seed number	No	null	1				X				2	5937.54	3	117.16	< 0.001
		linear	2				X	X			3	5865.36	2	44.98	< 0.001
		piecewise	**3**					**X**	**X**	**X**	**4**	**5820.39**	**1**	**0**	**1.000**
	Yes	null	1		X	X	X				4	5762.42	2	12.99	0.002
		linear	**2**		**X**	**X**	**X**	**X**			**5**	**5749.43**	**1**	**0**	**0.998**
		piecewise	3	X		X		X	X	X	6	5766.72	3	17.29	< 0.001
Seed mass	No	null	1				X				2	3862.71	3	57.65	< 0.001
		linear	2				X	X			3	3829.82	2	24.75	< 0.001
		piecewise	**3**					**X**	**X**	**X**	**4**	**3805.06**	**1**	**0**	**1.000**
	Yes	null	1		X	X	X				4	3745.37	2	9.80	0.007
		linear	**2**		**X**	**X**	**X**	**X**			**5**	**3735.57**	**1**	**0**	**0.988**
		piecewise	3		X	X		X	X	X	5	3746.46	3	10.89	0.004
Seed oil content	No	null	1				X				2	1449.53	2	11.51	0.003
		linear	**2**				**X**	**X**			**3**	**1438.02**	**1**	**0**	**0.997**
	Yes	null	1		X	X	X				3	1344.62	2	3.37	0.156
		linear	**2**		**X**	**X**	**X**	**X**			**4**	**1341.25**	**1**	**0**	**0.844**
% of seed number potential	No	**null**	**1**				**X**				**2**	**3749.86**	**1**	**0**	**0.555**
		linear	2				X	X			3	3751.14	2	1.28	0.292
		piecewise	3					X	X	X	4	3752.44	3	2.57	0.153
	Yes	null	1			X	X				3	3707.08	2	4.11	0.113
		linear	**2**			**X**	**X**	**X**			**4**	**3702.97**	**1**	**0**	**0.887**
% of seed mass potential	No	**null**	**1**				**X**				**2**	**4235.18**	**1**	**0**	**0.404**
		linear	2				X	X			3	4235.22	2	0.03	0.398
		piecewise	3					X	X	X	4	4236.62	3	1.43	0.198
	Yes	null	1			X	X				3	4177.97	2	5.87	0.050
		linear	**2**			**X**	**X**	**X**			**4**	**4172.10**	**1**	**0**	**0.950**
% of oil content potential	No	**null**	**1**				**X**				**2**	**1385.28**	**1**	**0**	**0.572**
		linear	2				X	X			3	1385.86	2	0.58	0.428
	Yes	null	1			X	X				3	1348.26	2	4.15	0.111
		linear	**2**			**X**	**X**	**X**			**4**	**1344.11**	**1**	**0**	**0.889**

Eq.: equation number; k: number of estimated parameters; i: y-intercept; s: slope; m: maximum yield; t: threshold of pollinator density beyond which m is reached; ΔAIC: AIC gap with the model having the lowest AIC; w_i: AIC relative weight of evidence. Models in bold are the most parsimonious models, i.e. with the lowest AIC. See Fig. 3 for graphical representation.

or % of yield potentials were analyzed as well as whether random effects were included or not in the analyzes. Using yields for seed number and seed mass led to the conclusion that sunflower was pollen limited below 0.3 bees per head without random effects, or were marginally pollen limited on the whole range of pollinator density observed when including the random effects. On the other hand, using % of yield potentials assessed through pollen supplementation treatments led to the conclusion that the crop was not pollen limited for both seed number and mass anywhere within the range of pollinator densities encountered, from 0.1 to 1 bees per head, whether including the random effects or not in the models. These differing conclusions are attributed to the differences in yield potential observed between and within fields, and to the positive correlations observed between pollinator density and yield potential measured with both seed number and seed mass. These positive correlations may be explained by the fact that plants that are less resource limited, which yield therefore more seeds, can also display more florets, or secrete more nectar per floret, both resulting in attracting more pollinators per head (Mallinger and Prasifka, 2017b; Prasifka et al., 2018).

While our results are clear and consistent for seed number and seed mass, seed oil content did not necessarily follow these same patterns. However, seed oil content was not measured in pollen-supplemented heads in 2017, or it was measured in a very low number of open pollinated heads in 2017, probably explaining why there are contrasting results between analyzes made with or without random effects, and between analyzes made using seed oil content versus % of oil content potential. We expect that with larger sample sizes, we may be able to make the same conclusions using seed oil content as a yield component in comparison to seed number and seed mass.

Our results highlight the importance of considering plant maternal resources and resulting differences in yield potential when assessing degrees of pollen limitation using pollinator gradients. It also highlights the importance of including random effects in the analyzes when there are repeated measurements, e.g. within the same years, cultivars, fields, or sampling plots. These considerations could modify some of the conclusions made by previous studies which used pollinator gradients and found pollinator and pollen limitation in crops without controlling for the yield potential with pollen supplementation treatments (Benjamin and Winfree, 2014; Garibaldi et al., 2016; Catarino et al., 2019; Mallinger et al., 2021), including studies on sunflower (Mallinger and Prasifka, 2017a; Perrot et al., 2019), or without considering repeated measurements in fields (Reilly et al., 2020). Therefore, it is highly recommended to control for the level of maternal resources with pollen supplementation treatments in future studies investigating pollen limitation with pollinator gradients. Furthermore, some of these studies did not record the pollinator visitation rate in relation to flower number. Yet, yields and pollinator number can both increase simply because plants produce more flowers. Thus, it is also recommended to always associate pollinator density to flower density in pollen limitation studies in entomophilous crops.

Indeed, when pollen supplementation treatments are applied and yield potentials are compared with open pollination (as in Holland et al., 2020; Webber et al., 2020), pollen limitation seems to be less common. This seems to be especially the case for sunflower for which pollen-supplemented heads did not have higher yields than open pollinated heads in some studies (Langridge and Goodman, 1981; Mallinger and Prasifka, 2017a; Bartual et al., 2018; but see Holland et al., 2020). Radford et al. (1979b) theoretically calculated that 0.24 bees are needed per sunflower head to prevent pollen limitation, while Lecomte (1962) assessed from the Russian literature a pollination deficit only when the bee density fell below 0.15 bees per head. Yet, the pollinator densities generally observed in sunflower fields very rarely fall below 0.2 bees per

Table 2
Estimated parameters for the most parsimonious model per seed yield component, with or without random effects included.

Response variable per open pollinated head	Random effects included	Most parsimonious model	Random effect σ		Parameter	Estimate	± 95% CI	*t*	*P*
			Field	Plot					
Seed number	No	piecewise			slope	2563	± 765	6.59	< 0.005
					maximum yield	1281	± 33	76.51	< 0.005
					threshold of pollinator density	0.299	± 0.038	15.37	< 0.005
	Yes	linear	156	105	*y*-intercept	1068	± 185	11.87	< 0.005
					slope	317	± 356	1.85	0.072
Seed mass	No	piecewise			slope	159	± 67	4.66	< 0.005
					maximum yield	69.8	± 2.9	47.49	< 0.005
					threshold of pollinator density	0.293	± 0.052	11.06	< 0.005
	Yes	linear	10.5	11.4	*y*-intercept	51.7	± 13.8	7.24	< 0.005
					slope	31.9	± 27.9	2.20	0.037
Seed oil content	No	linear			*y*-intercept	48.0	± 1.2	80.31	< 0.005
					slope	-6.17	± 3.3	-3.71	< 0.005
	Yes	linear	2.58	2.41	*y*-intercept	44.3	± 4.9	20.32	< 0.005
					slope	4.71	± 12.90	0.91	0.384
% of seed number potential	No	null			*y*-intercept	97.5	± 2.2	88.78	< 0.005
	Yes	linear		11.6	*y*-intercept	96.2	± 6.8	27.65	< 0.005
					slope	3.5	± 14.9	0.46	0.644
% of seed mass potential	No	null			*y*-intercept	103.6	± 3.9	52.52	< 0.005
	Yes	linear		22.6	*y*-intercept	99.0	± 12.8	15.18	< 0.005
					slope	12.1	± 28.0	0.85	0.400
% of oil content potential	No	null			*y*-intercept	100.3	± 1.1	181.5	< 0.005
	Yes	linear		4.9	*y*-intercept	101.6	± 4.0	49.41	< 0.005
					slope	-4.8	± 13.0	-0.73	0.471

See Fig. 3 for graphical representation.

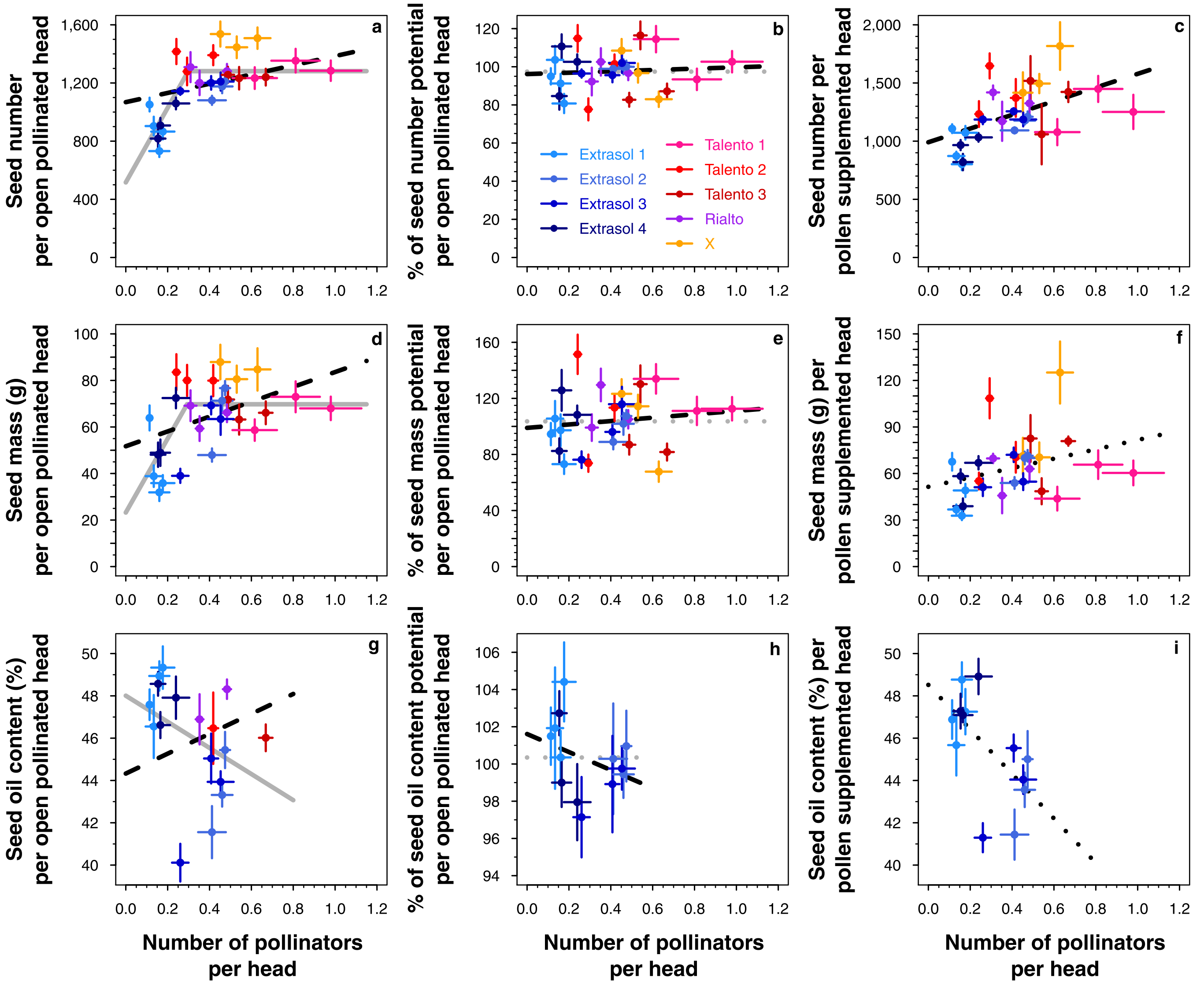

Fig. 3. Yields of open pollinated heads (a, d, g), % of yield potentials of open pollinated heads (b, e, h), and yields of pollen-supplemented heads (c, f, i) in relation with pollinator density (mean ± SE). a, b, d, e, g, h: black (resp. grey) lines are the most parsimonious models with (resp. without) random effects, selected with AIC (Table 1); solid (resp. dashed) lines are slopes with $P < 0.005$ (resp. $P > 0.005$; Table 2); dotted lines are null models. c, f, i: dashed (resp. dotted) lines are correlations with $P < 0.005$ (resp. $0.005 < P < 0.1$). Legend: the names Extrasol, Talento, Rialto and coded X are cultivars, and the numbers 1, 2, 3 and 4 are the replicated field numbers per cultivar.

head, as in our study (e.g. Radford et al., 1979b and references therein; Langridge and Goodman, 1981; Degrandi-Hoffman and Chambers, 2006; Garibaldi et al., 2016). Further studies are needed to be able to properly identify a threshold of pollinator density below which sunflower is pollen limited, but clearly, research suggests that this threshold is very low. In our study, the experimental design enabled us to get a pollinator gradient, but this gradient was not low enough to identify a threshold. Furthermore, the number of visits required per head could vary between cultivars (e.g. see Kendall et al., 2020 with blueberry) or even between fields of the same cultivar, depending on the rate of autonomous self-fertilization or on the number of florets per head requiring insect pollination.

4.2. Pollinator contribution to seed yields and oil content

Although the crop was not pollinator limited when taking yield potential into consideration with pollen supplementation treatments in analyzes, the heads isolated from pollinators showed nevertheless that entomophilous pollination increases seed number and seed mass, illustrating that bees are required to maximize yields even if their required density is low. Our results also showed that the rate of autonomous self-fertilization and the pollinator contribution to yields may not only vary with the cultivar, but also across fields for a given cultivar. The differences in autonomous self-fertilization rates observed between cultivars can be at least partly explained by differences in level of self-fertility and in floret morphological characteristics, such as floret or style length (Segala et al., 1980; George, 1982; Sun et al., 2012), or the contact surface between the receptive part of the stigma and the self-pollen attached to the anthers as observed in a Campanulaceae protandrous wild species (Koski et al., 2018). In addition, if pollen performance is affected by high and low temperatures (Rosbakh et al., 2018), but the amount of pollen deposited on stigmas following autonomous self-pollination remains the same regardless of temperature, the rate of self-fertilization could be affected by temperature as suggested by Degrandi-Hoffman and Chambers (2006) and thereby potentially explain differences in self-fertilization rates and pollinator contributions between fields within cultivars.

The overall lower pollinator contribution observed for seed mass

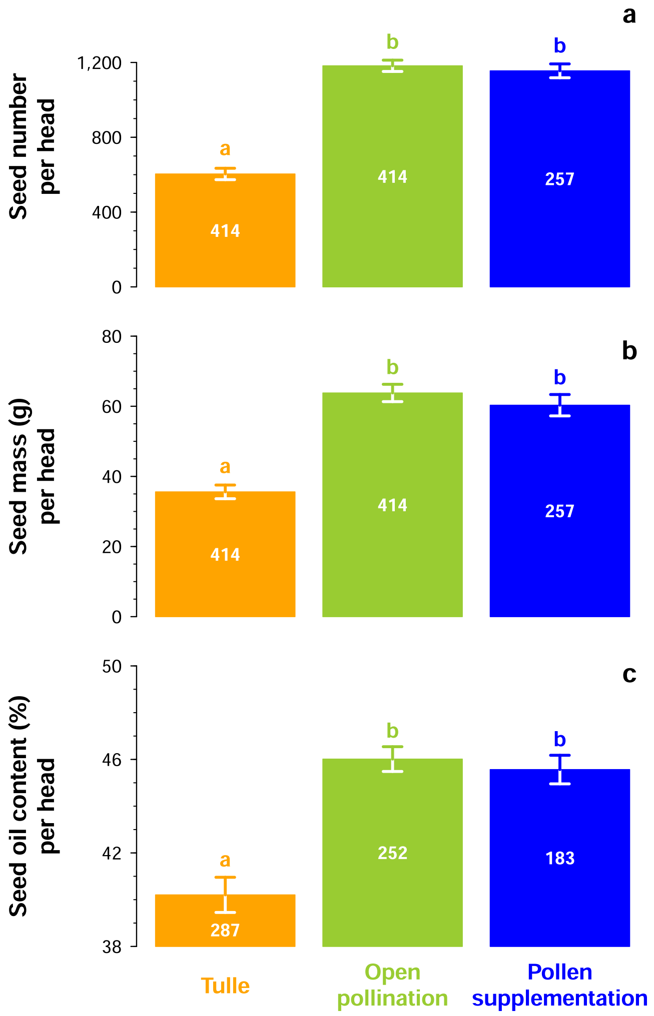

Fig. 4. Seed yield components according to the pollination treatment across fields (mean ± 95% CI). A different letter indicates a difference between pollination treatments with $P < 0.005$ (Appendix A, Table A6). Numbers in bars are numbers of sample heads per modality.

compared to seed number is explained by the partial compensation effect that exists between seed set and seed size (Sadras, 2007). Insect pollination also led to an increase in seed oil content, which depended on the cultivar and the field, as found in other studies (e.g. Langridge and Goodman, 1981; Mahmood and Furgala, 1983; Nderitu et al., 2008; Bartual et al., 2018).

4.3. Pollinator community and introduction of managed pollinators

The insects visiting sunflower heads were mainly composed of honey bees, especially nectar foragers, as has already been observed repeatedly. Indeed, honey bees often constitute > 80–90% of sunflower visits worldwide (e.g. Radford et al., 1979a; Nderitu et al., 2008; Carvalheiro et al., 2011; Sáez et al., 2012; Rollin et al., 2013; Pisanty et al., 2014; Hevia et al., 2016; Holland et al., 2020; see other references in Brown and Cunningham, 2019). This broad dominance of honey bees enabled us to get the pollinator gradient we were looking for within the fields by moving away from the PS, defined by where honey bee colonies were placed. However, in areas where sunflower is native (eastern North America; Blackman et al., 2011), honey bees often visit heads in small numbers compared to wild bees (e.g. Posey et al., 1986; Bennett and Isaacs, 2014; Todd et al., 2016; Mallinger and Prasifka, 2017a; Mallinger et al., 2019). In Asia, some other *Apis* species also visit sunflower fields in large numbers (e.g. Jadhav et al., 2011; Ali et al., 2015; Said et al., 2017; Devaramane et al., 2018). Indeed, crops are visited by more pollinator taxa within their native range than outside their native range, because of the inclusion of specialized pollinators in native ranges (Brown and Cunningham, 2019). The preponderance of nectar foragers in sunflower has also been observed in other studies (Lecomte, 1962; Free, 1964; Langridge and Goodman, 1981; Chambó et al., 2011). But as pollen release occurs in the three hours following sunrise in sunflower (Creux et al., 2021), pollen foragers could have been found in higher proportions if counts had focused only on those very early hours of the day.

Therefore, to reach the pollinator density required to maximize yields, it may be necessary to complement the wild pollinator community with managed pollinators when crops are grown outside their native range. This could be achieved with honey bees if there is not enough spontaneous beekeeping activity in the vicinity of the crop. On the other hand, the addition of managed pollinators should be less necessary when crops are within their native range, to avoid unnecessary input costs and potential negative impacts on the wild entomofauna.

5. Conclusions

Our results showed that yield potential can vary substantially between and within fields for a given crop, and thus plant maternal resources must be considered if one wants to properly assess pollen limitation with pollinator gradients which have to be measured on a per flower basis. Our results also showed the importance of including random effects when there are repeated measurements over years, cultivars, fields, or sampling plots. We advocate the use of pollinator gradients implemented over large distances in combination with pollen supplementations applied at the relevant scale in order to determine the local yield potential. This could help to establish the thresholds of pollinator density needed to maximize yields for different crops. Not exceeding this threshold would help to avoid potential negative effects

Table 3
Rate of autonomous self-fertilization (seed number), rate of autonomous self-production (seed mass), and increase in seed oil content due to insect pollination for each field and the overall averages across fields.

Field	Seed number Rate of autonomous self-fertilization (%)	Seed mass Rate of autonomous self-production (%)	Seed oil content increase due to insect pollination (%)
Extrasol 1	67.7	68.2	2.6
Extrasol 2	51.4	52.0	9.0
Extrasol 3	34.6	41.8	7.5
Extrasol 4	35.8	39.2	7.0
Talento 1	78.2	98.6	
Talento 2	70.7	85.2	2.7
Talento 3	50.6	60.0	3.9
Rialto	47.5	61.8	5.4
X	16.0	19.2	
Mean	**46.3**	**57.2**	**5.5**

of managed pollinators on the wild entomofauna and decrease the expense of securing managed pollinators at the same time. While we could not determine precisely this threshold for sunflower, we found that bee densities in the range of 0.1–1 bees per head were enough to maximize yields.

Funding sources

This work was supported by Syngenta (France), Arterris Innovation (France) and Agrosolutions (France). These sponsors contributed to the study design and to the data collection.

Authors' contribution

B.E.V., C.S. and A.F. conceived the study, O.G., V.G., S.L., C.M., and J.P. performed the field work and the laboratory analyzes, S.C. analyzed the data and wrote the original draft of the manuscript, R.E.M. and B.E. V. reviewed and edited the manuscript. All authors gave final approval for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the beekeeper Maurice Morlières for providing us with the colonies to carry out this study, as well as all the farmers for allowing us to sample their sunflower fields. We thank Edith Caumes from Arterris and Charlotte Cenier from Agrosolutions who helped to find the study fields. We also thank four anonymous reviewers for their constructive comments that enabled us to greatly improve the manuscript.

Appendix. Supporting information

Appendices A and B, as well as raw data associated with this article can be found in the online version at doi:10.1016/j.agee.2022.107887.

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