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Pollination benefits are maximized at intermediate nutrient levels

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Yield production in flowering crops depends on both nutrient resource availability and pollination, but their relative roles and potential interactions are poorly understood. We measured pollination benefits to yield in sunflower, combining a gradient in insect pollination (0, 25, 50, 100%) with a continuous gradient in nitrogen (N) fertilization (from 0 to 150 kg N ha^{-1}) in an experiment under realistic soil field conditions. We found that pollination benefits to yield were maximized at intermediate levels of N availability, bolstering yield by an approximately 25% compared with complete pollinator exclusion. Interestingly, we found little decrease in yield when insect visits were reduced by 50%, indicating that the incremental contribution of pollination by insects to yield is greater when the baseline pollination service provision is very low. Our findings shed light on the processes that drive crop production, providing evidence for nonlinear relationships between pollination and resource availability. Our results support ecological intensification as a promising strategy for sustainable management of agroecosystems. In particular, we found optimal level of pollination to potentially compensate for lower N applications.

1. Introduction

Animal-mediated pollination (hereafter pollination) is a pivotal ecosystem service to agriculture supporting yield in 75% of all crops [1,2]. The global decline in wild and managed pollinators [3,4] has aroused great concerns about potential negative impacts on the provision of pollination services in agroecosystems and on food production [5–7]. However, to what extent and under which environmental conditions pollinator scarcity might affect yield is still largely unknown. Pollination benefits have predominantly been evaluated by measuring yield losses due to the complete lack of pollinators. This approach, even if informative for understanding the ecological and economical role of pollination in agriculture, disregards the variation in pollinator density that exists in nature (e.g. [8], but see [9,10]). Moreover, pollination provision is expected to decrease in response to human disturbance rather than be completely nullified [11]. The estimation of the incremental contribution of pollination service to crop production is therefore considered a paramount step towards a more sustainable and effective management of agroecosystems [12,13]. However, quantitative information regarding the effects of pollinator decline on yield production for the majority of crops is still lacking.

Pollination has often been studied in isolation and its contribution considered independent to other co-acting processes. However, a growing number of recent studies empirically showed that pollination benefits depend on other resources, such as nutrients and water, indicating that generally the benefits of pollination increased at higher levels of resources available to plants [14–17]. When resource availability is low, the limited carrying capacity of the plant can restrain seed provisioning and hence pollination benefits [18]. Nevertheless, plant compensatory capacity might also play a decisive role in shaping the way pollination and resources interactively affects yield. Marini *et al.* [19], for example, found increased pollination benefits at lower nitrogen (N) inputs in oilseed rape, whereas under high N availability plants compensated for the lack of pollinators by developing a larger number of flowers and fruits. Although these results appear contradictory, previous studies have been conducted under different experimental conditions (e.g. crop species and resource type) and usually investigating only two levels in resource availability (e.g. [17,19]). The potential presence of nonlinear effects can strongly affect our ability to predict the impact on crop yield of pollination deterioration under variable environment conditions.

Among plant resources, N availability is a key factor shaping crop production. During the last 60 years, N fertilization allowed an unprecedented increase in world food production [20]. However, it has also dramatically impacted the environment, enhancing greenhouse gas emissions and eutrophication of soil and water bodies [21,22]. Nitrogen applications also represent a major cost in modern agriculture. Improving N use efficiency is therefore considered a pivotal achievement to both decreasing production costs and protecting environmental quality [23,24]. The intensification of ecological processes such as pollination has been suggested as a sustainable solution to maximize yields (ecological enhancement, e.g. [25,26]) or to replace external inputs (ecological replacement, e.g. [19]). Despite the growing number of evidence supporting this novel management approach (e.g. [25]), there is still a substantial knowledge gap to fill if a transition to ecological intensification is to be implemented.

To test the hypothesis that crop yield may respond nonlinearly to both pollination and resource availability, we measured pollination benefits to sunflower yield, combining a gradient in insect pollination (four levels: 0, 25, 50, 100%) with a continuous gradient in N fertilization (eight levels: from 0 to 150 kg N ha^{-1}) in an experiment under realistic soil field conditions. We hypothesized that (i) the gain in pollination benefits would vary with pollination asymptotically, with the highest benefit at low levels of service provision (e.g. [27,28]) and (ii) the two factors would interact such as pollination benefits would be maximized only at certain levels of N availability.

2. Material and methods

(a) Study system

Modern sunflower (*Helianthus annuus* L.) cultivars are F1 hybrids selected to display high levels of auto-compatibility. Nevertheless, several studies showed that sunflower hybrids largely benefit from cross-pollination provided by insects [29,30]. Hybrid cultivars typically produce one inflorescence per plant (head) composed by hundreds of florets. Each head flowers for about 6–10 days. The outer whorl of disc florets opens first. Successive whorls of one to four rows of florets open daily for 5 or more days. Each floret is male first and then female [29]. Insect visits are therefore required to effectively transfer pollen from male-phase to female-phase florets of different plants. If pollination occurs, each floret produces one seed (achene filled with a kernel; full seed), whereas when it fails, the floret results in an empty achene (without a kernel; empty seed). The experiment was performed on the hybrid sunflower cultivar Marciano ST (Strube, Italy).

(b) Experimental design and fertilization treatment

The study was performed during the 2016 growing season at the Experimental Farm of the University of Padova (northeast Italy, Legnaro; 45°21′ N; 11°58′ E; 6 m.a.s.l.) in 80 plots organized in 10 blocks (eight plots per block). Each plot measured 1 × 1 m (1.5 m apart from each other) and it was delimited and isolated from the surrounding soil by a concrete parallelepiped structure dug 1 m into the ground, constituted by four welded panels (1.2 × 1.2 m, individual panel width: 10 cm). The open underside allowed water from precipitation to percolate. The structure recreated realistic field soil conditions but also allowed reducing superficial run-off of nutrients. Soil fertility was measured in 2016 (available Olsen P: 16 mg kg^{-1}). In late April, nine pairs of sunflower seeds were sown directly in the soil at 2.5 cm depth. Plots were watered once immediately after sowing to favour plant establishment. After emergence, the best performing plant per pair was selected and the other clipped and removed in order to achieve the crop density of nine plants per plot (9 plants m^{-2}). Plant density was similar to that normally used in the study region. We checked plants daily for water stress throughout the experiment. Irrigation was unnecessary.

The fertilization treatment started five weeks after sowing (growing stage V12, six leaf pairs unfolded [31]). Eight levels of N fertilizer were applied to plots in doses corresponding to 0, 15, 30, 45, 60, 90, 120 and 150 kg N ha^{-1}. These levels were selected in order to cover a wide gradient in N availability and to detect potential nonlinear effects of the treatments on seed production. As the maximum recommended N application for sunflower crop in the region is 90 kg ha^{-1}, our two highest levels are higher than common field N application. Nitrogen was added in the form of ammonium nitrate pellets that were first dissolved in 10 l of water and then watered into the plots. Unfertilized plots (0 kg N ha^{-1}) received the same amount of water. One plot per block was randomly assigned to each fertilization level (randomized complete block design; a total of 10 replicates per fertilization level).

(c) Pollination treatment

Pollination treatment started just before the onset of flowering. For each plant, flower phenology was checked daily in order to detect the beginning of the anthesis and to set up the pollination treatment accordingly. Within each plot, plants of similar vigour were selected and randomly assigned to four levels of insect pollination: 0, 25, 50 and 100%. At least one plant per plot (and a maximum of two) was assigned to each pollination level (e.g. electronic supplementary material, figure S1). The different pollination levels were achieved by manipulating the number of days during which pollinators had access to flowers: complete exclusion (0% pollination), 1 day access followed by 3 days of exclusion (25%), 1 day access followed by 1 day of exclusion (50%) and all days open pollination (100%). Hence, during a hypothetical flowering period of 8 days, pollinators could visit the flower heads 0, 2, 4 and 8 days, respectively. The first day of pollinator exclusion for 25 and 50% pollination treatment was set when at least one to two whorls of female-phase florets were open. The treatment started according to the single plant phenology. Pollinator exclusion for different amount of time has been considered as a proxy for different levels of pollination service delivery because it affects the total number of pollinator visits each flower receives (e.g. [32,33]). For details about pollination exclusion treatment, weather conditions and plant phenology, see the electronic supplementary material. As meteorological conditions during flowering were optimal for insect activity (electronic supplementary material, figure S2) and pollinator exclusion increased flowering duration (electronic supplementary material, figure S5), all the (female) florets have been exposed at least 2 days to insect visitation (electronic supplementary material, figure S4). However, pollination treatment levels (0, 25, 50 and 100%) might slightly differ to the real decrease in insect visitation. Exclusion was performed by the mean of tulle bags (mesh size 1 mm) placed over sunflower heads. Bag removal and placement was performed daily between 08.00 and 10.00. As flower heads expand during the flowering, bags were periodically adjusted to avoid contact with florets. When anthesis was completed, tulle bags were also placed on all inflorescences in order to prevent damage by birds and keep the same microclimatic conditions during ripening.

(d) Yield parameters and visitation rate

At physiological maturity (R9 stage) [31], flower heads were harvested and put in paper bags to dry. Full seeds were mechanically extracted from each inflorescence, dried at 65°C and the total full seed weight was measured (yield). Additionally, a subset of 32 randomly selected inflorescences (one head per treatment combination) was manually inspected to count the total number of seeds (fruits either with or without kernel) and to calculate the proportion of full seeds (seed set; full seeds/total number of florets). We further estimated the weight of 1000 seeds using the average individual seed weight calculated for each plant (yield/number of full seeds × 1000).

Nutrient availability can affect floral traits (e.g. flower size) altering attractiveness to pollinators (e.g. [34]) and hence visitation rate, potentially influencing reproductive outcomes. Therefore, during the flowering period, flower-visiting honeybees, bumblebees and solitary bees (as main sunflower pollinators [35]) were recorded. Other, minor flower-visiting insect groups were so scarce that were not included in the study. At each of the six observation rounds, flower visits were assessed by an observer who spent 3 min per one '100% pollination' plant per plot. Different pollinator guilds spend different time on inflorescences per single visit: few visits of a pollinator that spends more time on the flower disc per visit might therefore have a stronger impact on pollination than more visits by a less efficient pollinator [36]. In order to account for guild-specific pollination behaviour, we recorded the number of florets visited on a subset of 20 randomly selected heads during one visitation event, for each pollinator guild (honeybees, bumblebees and solitary bees). The average number of florets visited per visitation event was then calculated for each guild and it was used to estimate the total number of florets visited per plant. Moreover, the number of observations per plant varied according to fertilization level, because of differences in flowering onset and duration (average number of observation rounds per plant: 3.8, min 1, max 6). We therefore calculated the weighted number of visited florets per plant (total number of visited florets/number of observation rounds). All observations were carried out between 09.30 and 16.00 under sunny weather conditions with temperature above 17°C.

(e) Statistical analysis

All statistical analysis was performed using R. We used linear mixed-effect models with a normal error distribution using the 'lme4' package to test whether the cover of MFCs affected pollinator densities and whether the effects were consistent across landscapes varying in their cover of SNHs.

We used generalized additive mixed models (GAMMs [37]) to test the effect of pollination, fertilization and their interaction on yield, related parameters and visitation rate. GAMMs were applied because the large number of factor levels made difficult to detect complex interactive nonlinear effects using generalized linear mixed models. Both pollination and fertilization were considered as continuous variables. GAMMs were fit using the 'gamm' function in the 'mgcv' package [38]. Cubic regression spline smoothers with 'shrinkage' were applied for each explanatory variable in the GAMMs. 'Shrinkage' is a method to minimize the degree of smoothing in the model for each explanatory variable, reducing each relationship to a linear function where possible [37]. The model for yield included block and plot ID as random factors, whereas those for seed-set, total number of seeds, weight of 1000 seeds and visitation rate included only block ID (one measure per plot). The analysis of visitation rate (weighted number of visited florets) included only fertilization as fixed factor. Standard diagnostic plots were inspected to evaluate the fit of the five models. Yield and the weighted number of visited florets (visitation rate) were log-transformed.

In order to visualize the effect size of fertilization on pollination benefits to yield, we used model yield predictions (from the GAMM described above) to calculate the estimated yield gain due to pollinators for different pollination levels at each fertilization level as:

$$\text{Estimated yield gain (\%)} = \frac{Y_{\text{FertA,PollB}} - Y_{\text{FertA,Poll0\%}}}{Y_{\text{FertA,Poll0\%}}},$$

where Y is the estimated yield at fertilization level A and pollination level B compared to pollinator exclusion (Poll0%) at the same level of fertilization.

Table 1. Summary of the results of GAMMs (yield, seed-set, total number of seeds, weight of 1000 seeds and visitation rate analyses) testing the effects pollination and nitrogen fertilization (N fert) and their interactive effect on response variables. Degrees of freedom (d.f.) for each variable refer to the complexity of the additive curve. *P*-values in italics are statistically significant ($p < 0.05$).

	d.f.	*F*-value	*p*-value
yield			
pollination	2.29	24.16	*<0.0001*
N fert	1.66	5.39	*<0.0001*
pollination × N fert	2.82	0.97	*0.0124*
seed set			
pollination	2.57	25.53	*<0.0001*
N fert	0.79	0.34	0.0905
pollination × N fert	3.14	1.19	*0.0231*
total number of seeds			
pollination	0.19	0.07	0.2340
N fert	0.97	0.56	*0.0473*
pollination × N fert	0.74	0.13	0.2180
weight of 1000 seeds			
pollination	1.86	4.33	*0.0037*
N fert	0.91	0.42	0.0780
pollination × N fert	1.90	0.55	0.0732
visitation rate (weighted number of visited florets)			
N fert	1.00	0.001	0.9790

In one plot, plants failed to establish. The analyses regarding yield and visitation rate were thus based on data from 79 plots. Moreover, a wind storm during the flowering peak damaged 15 plants. Data from a total of 339 plants were thus used for the analyses. For visualization, we used splines to show the significant interactive effects of predictors on explanatory variables. All statistical analyses were performed in R.

3. Results

Pollination and fertilization treatments influenced seed production processes (table 1). Yield displayed a nonlinear response to both predictors (figure 1). Pollination benefits to yield (25, 50 and 100 versus 0% pollination) peaked at intermediate levels of N fertilization (12.5, 21.7 and 25.3% estimated yield gain respect to pollinator exclusion, at 90, 60 and 60 kg N ha^{-1}, respectively, figure 2). The presence of pollinators generally increased yield from 0 to 50% pollination and then yield tended to stabilize between 50 and 100%. Similar levels of yield were estimated at pollination

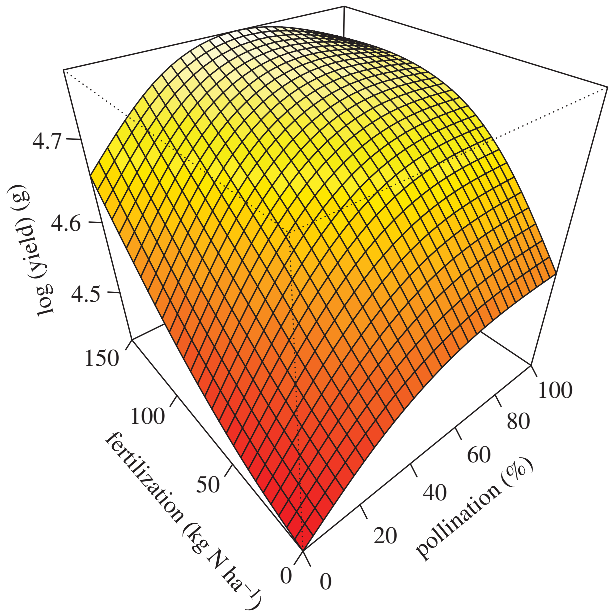

Figure 1. Spline fit of log-transformed seed yield per plant of sunflower in relation to pollination and fertilization treatments. (Online version in colour.)

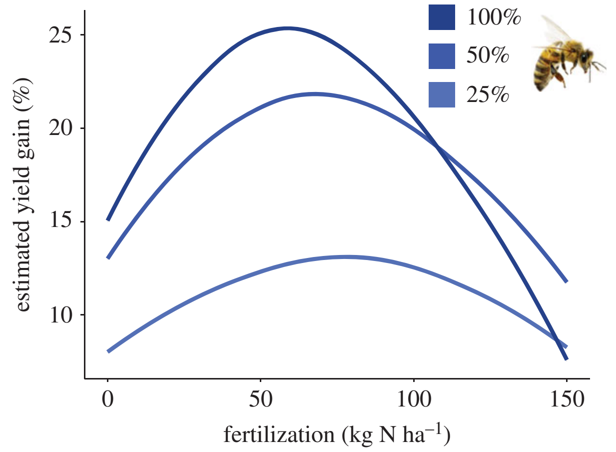

Figure 2. Pollination benefits to yield (estimated yield gain compared to pollinator exclusion) at 25, 50 and 100% pollination. (Online version in colour.)

levels higher than approximately 50% and fertilizer inputs greater than approximately 90 kg N ha^{-1} (electronic supplementary material, table S1). Simultaneous high levels of pollination and fertilization (100% and 150 kg N ha^{-1}) seemed to partially depress yield. The seed set increased with pollination, but its effect was slightly modulated by fertilization (figure 3*a*). The total number of seeds produced by each plant increased together with the amount of N input (figure 3*b*). Increasing levels of pollination strongly decreased the weight of 1000 seeds. However, we found a marginal significant interaction between pollination and fertilization ($p = 0.077$), where the weight of 1000 seeds increased at high level of N input but only when pollinator were excluded (figure 3*c*).

4. Discussion

Our study shows that pollination benefits to yield are maximized at intermediate levels of N availability, bolstering yield by an approximately 25% compared with complete pollinator exclusion. Interestingly, we found little decrease in yield when insect visits were reduced from 100 to 50% (max. approx. 4% reduction in yield benefits), indicating that the incremental contribution of pollination by insects to yield is greater when the baseline pollination service provision is very low. Our findings shed light on the processes that drive crop production, providing evidence for nonlinear relationships between pollination and resource availability. Moreover, our results support ecological intensification as a promising strategy for sustainable management of agroecosystems. In particular, we found that optimal levels of pollination can potentially compensate for lower N applications.

(a) Incremental contribution of pollination to crop production

We found a strong relationship between the level of pollination and seed production, confirming that insect pollination is a fundamental service for sunflower [17,30]. However, yield increased nonlinearly with pollination reaching the maximum value already at 50% of the maximum number of visits. The same patterns were visible also for seed set and the weight of 1000 seeds. Although we did not directly measure pollen limitation (hand pollination treatment), our findings suggest that nearly half of the insect visits received by sunflower plants were redundant in terms of pollen deposition and ovule fertilization. Our findings are in line with previous studies that showed how pollinator contribution to female reproduction saturate as the number of visits to a flower increases, because the amount of pollen needed to fertilize all the flower's ovules is finite [9,32,39,40]. Reproduction in flowering crops might therefore be positively related to pollinator density only when that density is low, suggesting that abundance fluctuations in healthy pollinator communities might have very little effect on crop production [13]. Nevertheless, the documented decline in both wild and domestic pollinators worldwide (e.g. 59% loss of colonies between 1947 and 2005 in USA [41]) indicates that severe loss of pollination services and consequent impacts on crop production might be expected, especially in intensively managed agricultural landscapes [4].

(b) Pollination benefits and resource availability

We found pollination benefits to crop production to strongly depend on resource availability. At both low and high levels of N fertilization, insect pollination led to a limited increase in yield in comparison to complete pollinator exclusion. Pollination benefits were instead maximized at intermediate levels of N fertilization that corresponded to the common field application rate. Resource availability is known to affect plant reproduction by changes in attractiveness to pollinators through floral trait modification, and/or by altering resource allocation strategy and fruit development process [15,18]. However, visitation rate was not affected by fertilization treatment, probably because of the small scale of the experiment and the random spatial arrangement of the treatments. Nitrogen fertilization therefore directly influenced the physiological processes involved in seed formation. When N availability is limited, early fruit abortion can decrease the proportion of fertilized ovules that can mature as the result of

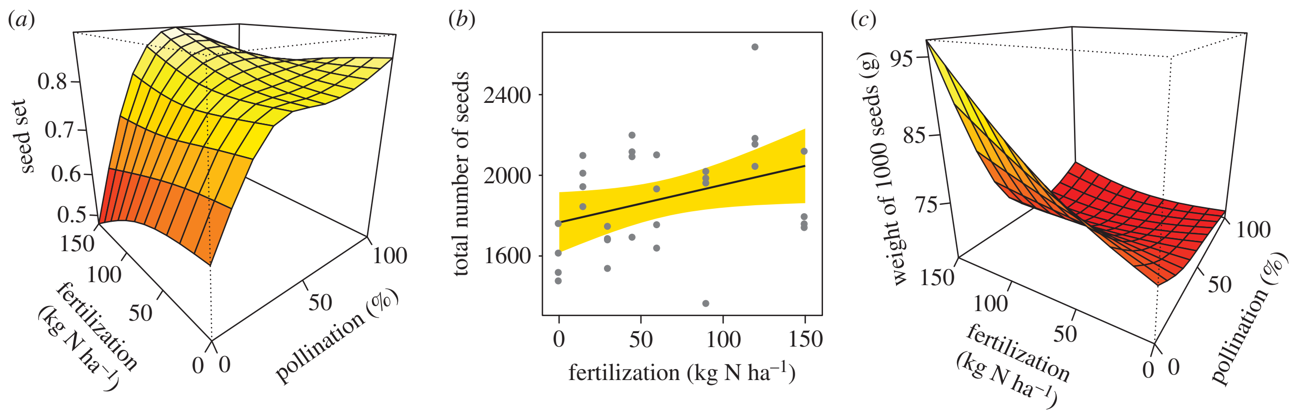

Figure 3. Effect of treatments on seed set (*a*), total number of seeds (*b*) and weight of 1000 seeds (*c*). Interaction between pollination and fertilization for the weight of 1000 seeds is only partially significant ($p = 0.073$, table 1). (Online version in colour.)

competition for resources, therefore reducing the potential benefits of pollination [17,42,43]. At optimal fertilization levels, plants probably had enough resources to develop all the fertilized ovules (higher when flower visitation occurs) fully manifesting the benefits of pollination [18]. At high levels of nitrogen inputs instead, pollination benefits decreased because plants compensated for the lack of pollinators producing much heavier seeds: the more abundant resources could have been in fact allocated to individual seeds increasing their weight (see also [19,44]).

As the result of the nonlinear contribution of pollination and fertilization to reproduction, high levels of seed yield were observed already at 50% pollination and 90 kg N ha^{-1}. Interestingly, we found that plants that received 120 kg N ha^{-1} at 25% pollination scored the same yield as those at 60 kg N ha^{-1} at 100% pollination, suggesting that insect pollination might play a fundamental role in shaping resource allocation and N use in flowering crops. The decrease in yield at simultaneous high levels of pollination and fertilization is probably due to the fact that excessive fertilization (150 kg N ha^{-1}, 40% higher than the maximum regional recommended N application) can unbalance plant resource allocation in favour of growth, compromising yield gain (e.g. [45]).

(c) Implications for management: options for ecological enhancement and replacement?

Ecological intensification of agroecosystems aims at either maximizing yield or replacing external inputs through the enhancement of ecological processes underpinning crop production. Our results provide relevant knowledge valuable to implement sound strategies for both ecological enhancement and replacement. We found that the incremental yield benefit associated with increasing pollinator visits is greater when the baseline pollination service provision is low. Therefore, the benefits deriving from interventions to sustain pollinator communities (such as the enhancement of semi-natural habitats around the fields) are expected to be higher in landscapes characterized by highly degraded pollination services. On the other hand, as suggested by Garibaldi *et al.* [13], those benefits would become extremely small in landscape characterized by average-to-high delivery of pollination services, hence questioning both the ecological and the economical efficacy of those interventions. Nevertheless, how and under which environment conditions, pollinator scarcity leads to pollen limitation is still unknown for the majority of crops.

Our results indicate that optimal levels of pollination might potentially compensate for lower N applications. Fertilizer applications of 60 kg N ha^{-1} at 100% pollination and of 90 kg N ha^{-1} (the highest recommended in the region for sunflower crop) at 25% pollination achieved similar levels of yield (electronic supplementary material, table S2). This means that interventions to sustain pollinator communities in highly degraded landscape (25% pollination) might potentially allow a reduction in N input of about 30%, without compromising yield. The majority of the studies regarding the physiological mechanisms governing seed production in flowering crops have usually overlooked the potential interactive effects between different processes [46]. Therefore, current strategies to increase yield production mainly focus on the management of external inputs. Moreover, considering the remarkable environmental and economic impacts that the use of fertilizers cause in agroecosystems (e.g. [47]), the integration of pollination and resource (input) management strategies might result in considerable advantages to both farmers and local administrators. Novel strategies to support sustainable crop production in agroecosystems necessitate a deeper understanding of the potential interactions between different processes involved in yield formation (e.g. pollination, nutrient and water availability, herbivory, pest control, climate change). We stress the importance of exploring the incremental contribution of these processes as an innovative approach to improve our ability to predict the impact of changing environmental conditions on crop production.

Data accessibility. Data available from the Dryad Digital Repository: http://datadryad.org/review?doi=doi:10.5061/dryad.42d2r [48].

Authors' contributions. G.T. and F.L. performed the study. G.T. performed data analysis and led the writing. L.M. participated in data analysis, results' interpretation and drafting the manuscript. G.T. and L.M. conceived and designed the study.

Competing interests. We declare we have no competing interests.

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