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EFFECT OF EUTROPHICATION ON PLANT-POLLINATOR INTERACTIONS OF CROP SPECIES

Dissertação no âmbito do Mestrado em Ecologia, orientada pela Doutora Mariana Oliveira e Castro e Doutora Sílvia Raquel Cardoso Castro Loureiro e apresentada ao Departamento de Ciências da Vida.

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"If I have seen further, it is by standing upon the shoulders of giants"
- Sir Isaac Newton

"All models are wrong, but some are useful"
- George E.P. Box

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Abbreviations

AIC – Akaike information criterion

am – From Latin *ante merīdiem*, meaning “before midday”

CO_2 – Carbon dioxide

df – Degrees of freedom

FAO – Food and Agriculture Organization of the United Nations

FDR – False discovery rate

e.g. – From Latin *exemplī grātiā*, meaning “for example”

emmeans – Estimated marginal means

et al. – From Latin *et alia*, meaning “and others”

ESAC – Escola Superior Agrária de Coimbra

g – Gram (unit of mass)

GLMM – Generalized linear mixed models

ha – Hectare (unit of area)

i.e. – From Latin *id est*, meaning “that is”

K – Potassium

kg – Kilogram (unit of mass)

km – Kilometer (unit of length)

L – Liter (unit of volume)

LM – Linear models

LMM – Linear mixed models

m – Meter (unit of length)

mg – Milligram (unit of mass)

min – Minutes (unit of time)

ml – Milliliter (unit of volume)

N – Nitrogen

n.s – Non-significant

P – Phosphorus

p-value – Probability value

pm – From Latin *post merīdiem*, meaning “after midday”

RD – Recommended dosage

SIRO - Sistemas Integrados de Reciclagem Orgânica (company)

sp. – From Latin *species*, meaning “species”

T - Treatment

var. – From Latin *varietas*, meaning “variety”

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Abstract

The increased use of agrochemicals, mainly fertilizers and pesticides, is becoming a worrying trend in the precarious balance between ecological stability and agricultural expansion. The increased land use and agricultural intensification can impact drastically floral composition and nesting sites suitability and thus, mediate changes in plant-pollinator interactions. Not only wildflowers depend on pollinators but also crops, being that most of the crop species in the world are pollinated by insects and a disruption in this mutualism can also translate into disruption in crop production. An increase in nutrient availability in the soil can impact adjacent ecosystems, e.g., freshwater ecosystems, due to nutrient runoff, enhancing eutrophication. Unfortunately, there are quite some gaps in the knowledge we currently possess when it comes to fertilizer effects in pollinator behavior and health. Therefore, the objective of this study was to assess how different levels of nitrogen (N) and phosphorus (P) can affect flower characteristics, such as flower production, and subsequently affect pollinator visitation rates and yield in crop species. Crops were selected based on their nitrogen-fixing ability and thus, nitrogen-fixing (*Vicia faba* and *Phacelia tanacetifolia*) and non-nitrogen fixing (*Solanum lycopersicum*) species were used. Treatments consisted in a full factorial design between three levels of N and two levels of P. It was found that fertilizers effects on crops are species-specific and dose-specific: in *S. lycopersicum*, pollinator visits were higher in plants growing in soils with recommended dosages of N and P; while *V. faba* and *P. tanacetifolia* showed no significant differences. It was also found that fruit set was not affected by visitation rates, despite seed set can be potentially benefited from increased pollination. Therefore, increased use of fertilizers for maximum crop production might not be the best approach, as plant yield will also depend on insect visitation. In order to guarantee that our dietary needs are met, while also maintaining ecological stability, we need to further investigate how the different drivers surrounding plant-pollinator interactions and eutrophication act in various crop species.

Keywords: crop species; eutrophication; fertilizers; plant-pollinator interactions; visitation rates

Resumo

O uso crescente de agroquímicos, sobretudo fertilizantes e pesticidas, torna-se cada vez mais um fator preocupante no que toca ao ténue equilíbrio entre estabilidade ecológica e expansão agrícola. Ainda mais quando a expansão de terras agrícolas e intensificação agrícola podem causar um impacto drástico na composição floral e disponibilidade de locais de nidificação e, por sua vez, alterar as interações planta-polinizador. Não são só as flores silvestres que dependem dos polinizadores, mas também as espécies agrícolas, já que uma esmagadora parte das espécies agrícolas são polinizadas pelos insetos e uma disrupção neste mutualismo também poderá causar uma disrupção na produção agrícola. Um aumento de nutrientes disponíveis no solo pode afetar os ecossistemas contíguos, e.g., sistemas de água doce, devido ao *runoff* de nutrientes, aumentando a eutrofização. Infelizmente, faltam-nos conhecimentos no que diz respeito ao impacto dos fertilizantes no comportamento e saúde de polinizadores. Como tal, o objetivo deste estudo foi avaliar como é que diferentes níveis de azoto (N) e fósforo (P) podem afetar características florais, como a produção de flores, e subsequentemente alterar as taxas de visita e produtividade em espécies agrícolas. As espécies foram selecionadas com base na sua capacidade de reterem azoto, portanto foram usadas espécies fixadoras de azoto (*Vicia faba* e *Phacelia tanacetifolia*) e não-fixadoras de azoto (*Solanum lycopersicum*). Os tratamentos resultaram de um design fatorial, entre três níveis de azoto (N) e dois de fósforo (P). Os resultados indicam que os efeitos dos fertilizantes são específicos das espécies e das doses usadas: em *S. lycopersicum*, os polinizadores visitaram mais plantas em solos com a dose recomendada de N e P; ao passo que *V. faba* e *P. tanacetifolia* não demonstraram diferenças significativas. Também foi demonstrado que a produção de frutos não foi afetada pelas taxas de visita dos polinizadores, apesar da produção de sementes poder potencialmente beneficiar do aumento de polinização. Deste modo, um uso crescente de fertilizantes para obter uma produção ótima poderá não ser a melhor abordagem a ter, já que a produção também vai depender de outros fatores, como a polinização. Para que possamos satisfazer as nossas necessidades alimentares, ao mesmo tempo que se salvaguarda a estabilidade ecológica, é necessário investigar mais como é que os diferentes fatores que envolvem as interações planta-polinizador e a eutrofização interagem nas diferentes espécies agrícolas.

Palavras-chave: espécies agrícolas; eutrofização; fertilizantes; interações planta-polinizador; taxas de visita

Introduction

Human activity has been a predominant driving force of changes in biodiversity across the globe, leading to shifts in the way animals and plants provide ecosystem services. Such impact led to the coinage of a new epoch, the Anthropocene (Crutzen and Stoermer, 2000; Lewis and Maslin, 2015). Although the term is not officially recognized as an epoch on the geological time scale, it is evident that humans already left their mark on the planet. Climate change due to increased emissions of CO_2, ocean acidification, increased land use, overexploitation of resources and the establishment of invasive species are only but a part of the anthropogenic activities that impact biodiversity (Steffen et al., 2011; Corlett, 2015). The degree at which biodiversity is affected is immensely varied, as it can be perceived at various levels of complexity. Depending on the disturbance registered, animal and plant species can be differentially affected, with some species being more affected than others (Potts et al., 2003; Newbold et al., 2015). While some species may face increased rates of extinction, e.g., vertebrates and invertebrates in freshwater ecosystems (Young et al., 2016), others can endure and potentially increase their numbers, e.g., plant communities (Ellis et al., 2012; Vellend et al., 2017). Therefore, this variance relies in the type of biodiversity assessed (richness and abundance) and the spatial level in which the assessment occurs, i.e., a range between the local and global scale (McGill et al., 2015). Furthermore, it should be considered that extinction is accompanied by speciation, especially in plants. This higher turnover rate is achieved due to human influence, in the artificial selection of agricultural and horticultural species and in the establishment of invasive species in foreign habitats (Thomas, 2015). But looking at species effect alone is insufficient if the main objective is portraying the impact of anthropogenic activities in the ecosystems. It should be also analyzed the subsequent impact of those pressures in the network structures that compose a community and the ecosystem services provided by the affected species (Morris, 2010). A decline in important species like bees could possibly impair pollination services (Larsen et al., 2005) and a decline in microbial communities in stream ecosystems could affect the nitrogen cycle (Qu et al., 2017), being nitrogen an important component in plant metabolism and growth, especially in crop species (Tilman, 1999).

Throughout the years, agriculture has become an activity of much interest and investment opportunities, providing food, fiber and fuel to existing humanitarian needs (Swinton et al., 2007). In order to cater the crescent world population numbers, agricultural land area increased and agricultural practices had to be intensified. Practices such as fertilization, irrigation and pesticide use, are constantly improved in order to produce the maximum yield possible by crops. As nitrogen, phosphorus and potassium use increases (FAO, 2017), such anthropogenic activities carry negative effects to the environment, enhanced by habitat loss, nutrient runoff and pesticide poisoning (Zhang et al., 2007). Aided by rainfall, this nutrient excess ends up in freshwater ecosystems, creating algal blooms and stagnant waters (Keatley et al., 2011; Withers et al., 2014). It also impoverishes soil quality, influencing floral composition at several scales of observation (Badía et al., 2008; Penuelas et al., 2009). Thus, agricultural intensification enhances crop productivity at the expense of increasing eutrophication. The response to eutrophication, characterized by fertilizer enrichment and land use, by animals and plants is of course varied, as not all species react in the same manner. Nitrophilous plants can potentially benefit from nitrogen deposition, decreasing competition with nitrophobous plants (Hedin et al., 2009; Hietz et al., 2011) and endangered species can possibly benefit from nitrogen enrichment, as phosphorus enrichment impairs their viability (Wassen et al., 2005). Accordingly, effects of soil eutrophication can progress into higher trophic levels, e.g., generalist insects or specialists in nitrophilous plants are favored, as these same plant species are favored in the community, thus affecting pollination services (Pöyry et al., 2016). However, eutrophication had also negative effects decreasing ecosystem stability and potentiating biodiversity losses. If such biodiversity losses impact key species viability, important for various ecosystem services, e.g., pollination, those same services and functions are at risk of being impacted to some degree (Cardinale et al., 2012). Many important crop species are depending on animal pollination to produce a maximum yield (Klein et al., 2007), so if pollinator dependence increases, agricultural intensification can hinder crop productivity in the long term. This means that, agricultural intensification can increase the yield of crops with low pollinator dependence, but it also can decrease the yield of crops that depend largely on insect pollinators, as habitat conditions deteriorate over time (Deguines et al., 2014).

Pollination is a vital ecosystem service. Mediated by wind and animals, especially insects, it ensures plants properly disperse their genetic information (Schulze et al., 2019). Therefore, pollinators assume a critical role in plants diversity. A decline in pollinators richness and abundance could potentially affect plant-pollinator interactions and ecosystem functions (Kevan and Viana, 2003; Ollerton, 2017). Pollinators are equally important in agricultural fields as they are in natural ecosystems, by providing pollination benefits in crop species (Bos et al., 2007; Klein et al., 2007), and by maintaining the delicate balance of ecosystems networks. Invasive species put the community stability at risk, by introducing new interactions and competition with native species, potentiating losses in more fragile species, that rely in specialist diets (Bartomeus et al., 2013; Vanbergen et al., 2018). Also, equally important, pollination mediates trait evolution in plants, through selective pressures pollinators exert on floral trait development (Fenster et al., 2004). In a matter of improving their fitness, plants select for the most efficient pollinators, that enable a better chance of dispersing their genes to their conspecifics. The mechanisms that promote pollinator attraction are immense and act on many senses, be it olfactory via the production of distinct fragrances (Raguso et al., 2003), visual via the production of a multitude of colors (Rausher, 2008) or simply by producing dietary rewards, i.e., pollen and nectar, vital to their diet (Hanley et al., 2008). It is that mutualism that promotes the existence of various pollination syndromes and a myriad of floral traits and resources (Schiestl and Johnson, 2013).

Overall, there is irrefutable evidence that insects are being threatened globally, due to the nefarious actions of industrialization and human expansion, culminating into the more recent effects of climate change (Conrad et al., 2006; Potts et al., 2010; Habel et al., 2016; Hallman et al., 2017). In a recent and extensive review done by Wagner (2020), the author describes a widespread decline in flying, ground and aquatic insects mainly attributed to habitat degradation, deforestation, agricultural intensification, land use change, insecticide use, climate change, pollution, and establishment of introduced species. The author also mentions the lack of evidence for the various taxonomic groups that compose entomofauna in the tropics, while most documented cases originate in Western Europe. This also constitutes a problem in the scientific literature as it makes much harder to predict what impact can biodiversity declines have at the global scale, regarding ecosystem functions, being that each driver of such decline, as mentioned above, will have a different influence depending on the region assessed, e.g. warmer temperatures in colder areas (mid to high latitudes) could accelerate flight periods in insects, thus improving their chances of survival by increasing the amount of time they can forage for resources and reproduce (Frazier et al., 2006; Robinet and Roques, 2010; Stange and Ayres, 2010). Losses in insect biodiversity could also imply disastrous effects on ecosystem services and crop species (Klein et al., 2007; Potts et al., 2010). Being that the agricultural sector is one of the most profitable globally and that pollination holds much value in crop productivity (Gallai et al., 2009), wildflower patches could help maintain pollinators population numbers by providing them nutritional resources between fragmented habitats (Garibaldi et al., 2011; Blaauw and Isaacs, 2014). By keeping natural patches of assorted plants, most known as weeds, in the vicinity of farmland, attraction of pollinators is higher, which in turn increases the chances of crop species being pollinated (Carvalheiro et al., 2011; Bommarco et al., 2013). Thus, minimizing effects of subsequent eutrophication. Nevertheless, insect preservation should be highly accounted for when it comes to establish conservation measures on affected communities and ecosystems, especially pollinators.

Besides considering effects of fertilization on soil and freshwater ecosystems as a whole, researchers and agricultural technicians also have dedicated a great effort in recognizing how does nutrient addition affect plants and their development. Although it is general rule that nitrogen, phosphorus and potassium are vital nutrients in plant growth and their addition does have positive effects in most species (LeBauer et al., 2008; Xia and Wan, 2008), there is high inter-specific variance. Regarding plant physiology, fertilizer use can significantly increase maximum crop yield, growth and photosynthetic rate evenly across all species, up to a certain extent (Lee and Dunton, 2000; Zubillaga et al., 2002; Wang and Li, 2004; Chen et al., 2005; Roy et al., 2011). It can also have noticeable effects on plant morphology, increasing maximum leaf area (Trápani et al., 1999; Lee and Dunton, 2000), increasing root collar diameter (Razaq et al., 2017), increasing plant height (Bi et al., 2008; Amanullah et al., 2009) and altering leaf pigmentation (Schulze et al., 2019). But in an ecological setting, plant phenology holds more weight when considering changes in plant development, as floral traits and resources are the bridge between plants and pollinators, and changes in this aspect can cascade through the ecosystems. Similarly, fertilizer mediated changes in plant phenology are highly species-specific. Nutrient addition can increase flower size while having no effect in flower production in perennials, (e.g., *Dactylorhiza lapponica*; Sletvold et al., 2017), delay the flowering period in grasses or even accelerate flowering in forbs (Cleland et al., 2006). Furthermore, in a study done by Burkle and Irwin (2009), it was evidenced how life-history traits in plants contribute to this variance in response. Two species were assessed, *Ipomopsis aggregata* and *Linum lewisii*, a monocarp and a perennial, respectively. *Ipomopsis aggregata* responded to nutrient addition more rapidly and effectively, increasing flower production, bloom duration, corolla width and nectar production, while *Linum lewisii* had a less pronounced response to fertilization, only increasing bloom duration. In the following year, *L. lewisii* exhibited greater aboveground biomass, seeds per fruit, and seeds per plant, evidencing delayed effects of nutrient enrichment. Therefore, plants life expectancy can give us insights on how plants deal with resource availability.

Nonetheless, with the study of changes in plant development, regarding floral traits and resources, comes the study of indirect effects of nutrient enrichment in pollination services, i.e., how fertilizers affect pollinator preferences by potentially altering the shape, color and scent of flowers, in conjunction with alterations in sugar, protein content and presence of essential amino acids in pollen and nectar (Schemske and Bradshaw, 1999; Gardener and Gillman, 2001; Hoover et al., 2012; Ceulemans et al., 2017). This indirect effect can be easily measured by assessing pollinator visitation rates in experiments that establish a fertilizer gradient, further assessing how pollinators respond, from low levels of nutrient enrichment to high levels. Only a few studies have been done in this topic and the scientific literature reveals mixed results when it comes to pollinator responses. Similarly, to studies done regarding effects of fertilization in plant development, the results seem to be species-specific. While some studies indicate that nitrogen addition does not have any effect on pollinator visitation rate (Burkle and Irwin, 2010; Tamburini et al., 2016; Tamburini et al., 2017), there are some studies that provide positive evidence, showing increased rates of pollinator visits in plants enriched with nitrogen in varying dosages (Muñoz et al., 2005; Dupont et al., 2018; Ramos et al., 2018; Banaszak-Cibicka et al., 2019). Some authors suggest that the increase in visits can occur due to an increase in floral abundance, again due to increased rates in nitrogen availability (Power and Stout, 2011; Dupont et al, 2018). Despite the conflicting results and the lack of knowledge in plant-pollinator interactions, there is still hope that trends in pollinator visits can be evidenced based on functional groups.

In a review article done by David, Storkey and Stevens (2019), these gaps regarding plant-pollinator interactions are properly and thoroughly addressed. One of the main gaps that currently holds plenty of relevance is whether pollinators are affected by increased nutrient availability or not and at what limit does nutrient availability is detrimental to pollinator's viability and health. Possibly, assessing nutrient response thresholds will represent quite an arduous task, i.e., assessing how different species react to different quantities of fertilizers and subsequent effects on trophic levels, considering that on the different species used in studies described above, no study used the same quantity of nutrients. For example, Munoz et al. (2005) used 30 g of nitrogen in the form of urea pellets dissolved in 2 L of water (15 mg ml^{-1}), once per growing season, and Banaszak-Cibicka et al. (2019) used various dosages of nitrogen (N), phosphorus (P) and potassium (K) (0, 90 and 141 kg N ha^{-1}, 60 kg P ha^{-1} and 120 kg K ha^{-1}). There is also the need to better assess how pollinators of various habitats, in various latitudes, are possibly affected by nutrient addition, being that if soil quality and subsequent plant communities differ in ecosystems across the globe, pollinators may also respond differently to nutrient availability. Overall, there is a need to better understand how the phenology is altered due to changes in soil nutrient quality and how can these changes affect ecosystem services like pollination. Possibly, in the near future, meta-analysis studies could unveil the trends in nutrient mediated plant-pollinator interactions.

It is important to know how flora and fauna will respond to our progressive shaping of the world, as our industrial processes involve severe drawbacks to the species that share the ecosystems with us. The study of this topic, fertilizer addition and plant-pollinator responses, does not only encompass effects in biodiversity and ecosystem stability but also crop productivity and maintenance of food stocks to the general population. Its understanding is vital to us on many levels and it is something that requires the involvement of many researchers in many fields, such as botany, entomology, evolution and climatology. Therefore, we need to be able to assess how are pollinators affected by indirect action of nutrient enrichment, i.e., in what manner does fertilizers used in modern agricultural practices affect the floral traits and resources in crop species, what repercussions does these alterations have in pollinator visitation and what fertilizer quantities are enough to trigger responses by flora and entomofauna alike. With this in mind, the aim of this study is to assess 1) if fertilizer addition induces changes in plant characteristics, in this case flower production, 2) if pollinator visits in crop species are altered by the addition of nitrogen and phosphorus, through changes in flower production and 3) if plant yield (fruit and seed sets) is affected by changes in pollinator visitation rates due to these fertilizers addition. According to the literature available, the working hypothesis present in this study is that the increase of nitrogen and phosphorus in soil fertility will alter visitation rates in the crop species used: if nutrient addition can provide beneficial alterations to flower production, such as increasing the number of flowers available to pollinators, possibly decreasing competition over a certain plant, it can alter pollinator preference and increase pollinator visitation, up to a certain extent. Additionally, nutrient addition will also alter fruit and seed set in crops used, as increased visitation rates can potentiate flower fertilization and increase fruit and seed production in the plant. The various responses will then help us formulate proper conservation tactics, appropriate to each species affected.

Material and Methods

Plant species

The plant species used in this experiment were *Vicia faba* var. *major* (fava beans), *Phacelia tanacetifolia* var. *stala* (lacy phacelia) and *Solanum lycopersicum* var. *cereza* (tomato). The plants mentioned were selected primarily for their nitrogen fixing ability, their entomophilous nature and their agricultural relevance in Portugal, being used as food (fava beans and tomatoes), animal feed (fava beans) or to attract pollinators in agricultural fields (phacelia), enhancing production in surrounding crops (Carreck et al., 1999; Carreck and Wiliams, 2002). *Vicia faba* and *P. tanacetifolia* are nitrogen fixing crops, and as such, might not be so dependent on nitrogen to grow and produce reproductive structures, whereas *S. lycopersicum* is a non-fixing crop which in turn may be more impacted by the availability of nitrogen in the soil. The pollinator community in *V. faba* and *P. tanacetifolia* is diverse and pollinators vary from honeybees (*Apis mellifera* and *A. florea*), species of the genus *Bombus*, such as *B. terrestris* and *B. pascuorum*, hoverflies such as *Sphaerophoria scripta* and *Eupeodes corollae*, large sized bees (e.g., *Xylocopa* sp., *Eucera* sp.) to beetles (e.g., *Oxythyrea* sp.) (Petanidou, 2003; Aouar-Sadli et al., 2008; Marzinzig et al., 2018; Owayss et al., 2020). *Solanum lycopersicum* also benefits from *Bombus* species (e.g., *B. terrestris* and *B. pascuorum*), *Lasioglossum* sp., *A. mellifera* and *Hylaeus gibbus*, for example (Teppner, 2005; Santos et al., 2014).

Experimental set up

The experimental set up was made to simulate the conditions of the Center region of Portugal, mainly Beira Litoral. The experiment focused on the potential effects of nitrogen (N) and phosphorus (P) in agricultural crops and pollinator communities, applied in six different treatments (T1 to T6), in a full factorial design, consisting of three dosages of N (half the recommended dosage – **½RD N**, recommended dosage – **RD N** and double that amount – **2xRD** N) and two levels of P (no phosphorous – **No P** and the recommended dosage of phosphorous – **RD P**) (Table 1). Fertilizer dosages were based on the recommended dosages used in Portugal, for varying crops (LQARS, 2006) and are presented in Table 2.

In total, 270 plants were used. Ninety plants per crop and 15 plants per treatment. Seeds were obtained from local retail stores and the substrate was bought from SIRO. The seeds were sown in cuvettes with SIRO Germe substrate and maintained in the greenhouse of the Botanical Garden of the University of Coimbra, between December of 2020 and February of 2021, until the development of the first pair of leaves. Afterwards, the seedlings were transplanted into 12L plastic pots, and maintained in a bigger greenhouse at InProPlant – University of Coimbra, until the beginning of flowering. The substrate used at this phase (SIRO 30-0) was also obtained from SIRO and is characterized by having a high percentage of humus, some peat, a pH between 5.5 and 6.5 and a N and P concentration of 50 to 100 mg/L. Plants were watered weekly, using a 220ml cup to ensure each plant was watered equally.

At the beginning of the flowering season, the plants were moved to five different sampling sites to monitor plant-pollinator interactions independently in five locations (due to logistic problems, one of the sites was replaced, see Figure 1 for more details). The five sampling sites were located inside the limits of the city of Coimbra (three in ESAC – Escola Superior Agrária de Coimbra), one in InProPlant and one in the Botanical Garden of the University of Coimbra (see Figure 2 for details). The sites were located at least 1 km apart from each other or had major physical barriers to pollinator movement (e.g., a dense forest or a highway in between), to ensure independent samples, i.e., so the pollinator communities in the sites were different (Ramos et al., 2018). The sites are characterized as open spaces, and the surrounding vegetation was properly cleared, so that wildflowers would not interfere in the assessment of pollinator visitation. Pots were displayed in triplets (three replicates per treatment per site), therefore, each species consisted of 18 plants laid out as exemplified in Figure 2. Plants were watered regularly as described above. Weed control covers (10 x 5 m) were also used, to prevent the growth of weeds in the site.

Table 1. Combinations between different dosages of nitrogen and phosphorus.

P treatment	**N treatment**		
	½RD N	**RD N**	**2xRD N**
No P	T1	T3	T5
RD P	T2	T4	T6

Table 2. Fertilizer values for each species used.

	Fertilizer dosages per pot (g)					**Reference values (kg/ha)**	
	N			**P**		**N**	**P**
Species	**½**	**RD**	**2x**	**No addition**	**RD**		
Vicia faba	0.147514	0.295028	0.590056	0	1.00528	30	30
Solanum lycopersicum	0.688398	1.376797	2.753593	0	2.211616	120	140
Phacelia tanacetifolia	0.147514	0.295028	0.590056	0	1.407392	30	100

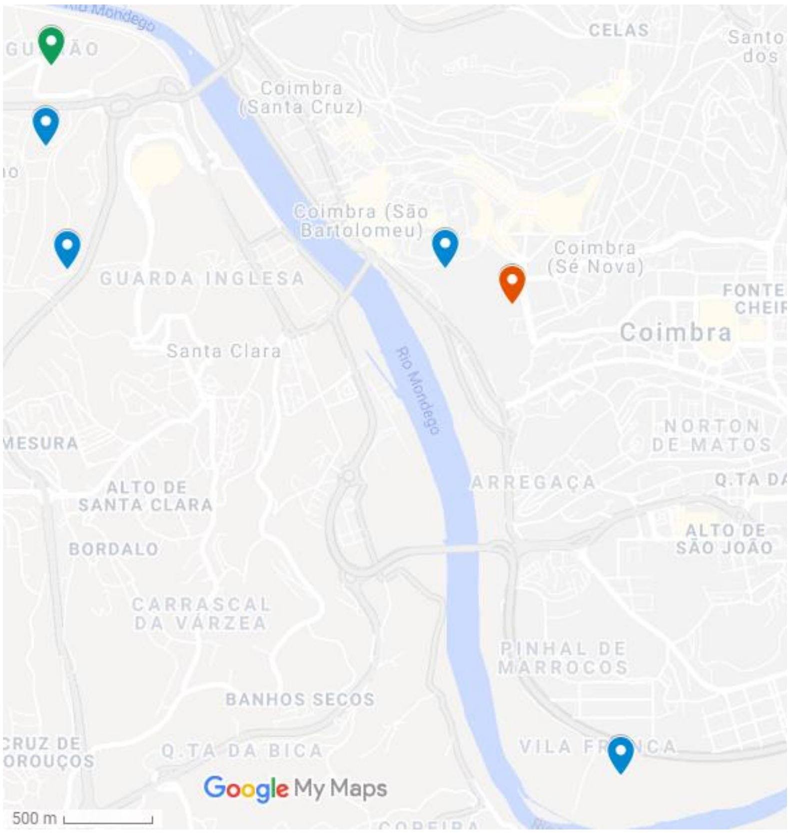

Figure 1. Location of sampling sites in the city of Coimbra. In May of 2021, point 1 of ESAC (green) was replaced by point 2 in the Botanical Garden (orange) due to logistic problems in the monitoring of the plants.

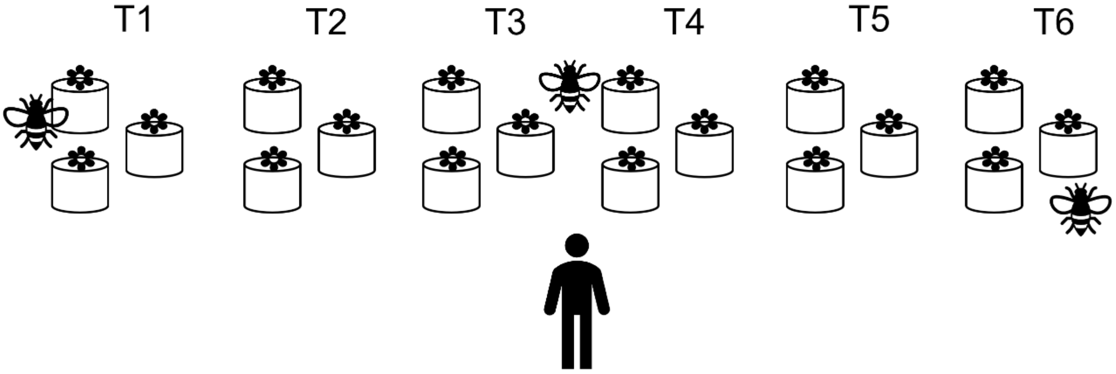

Figure 2. Census representation example. Notice that each treatment is composed of three plants (triplets) and distanced equally from each other.

Pollinator sampling

Direct observations (i.e., census) were employed to assess plant-pollinator interactions. Census were performed at the peak of flowering of each crop, when climatic conditions were favorable to pollinators foraging (i.e., rainless, little to no wind and warm temperatures). Census, which normally occurred between 10 am to 5 pm, consisted in periods of 10-minute observations and were performed to record pollinating bouts, i.e., only visits where insects were in contact with the sexual organs of the flower (Figure 3). The observer defined the set of plants being monitored and recorded all pollinator insects and the number of visits performed to each plant and treatment. Pollinators were identified *in situ*. However, when identification could not be completed to the species level, photographs were taken, and specimens collected for subsequent identification. The number of open flowers was also registered at the end of each day, to assess visitation rates for each plant. Visitation rates were calculated as the ratio between the number of visits per 10-min intervals and the number of open flowers per plant.

Figure 3. Pollination bouts. *Bombus pascuorum* pollinating a *Vicia faba* flower (left), *Xylocopa violacea* pollinating *Phacelia tanacetifolia* flowers (middle) and *Lasioglossum malachurum* pollinating a *Solanum lycopersicum* flower (right).

Plant and yield data

Regarding plant performance, data collection was done at flower and fruiting stages, thus flower production, fruit set, and seed set were calculated, as measures of plant fitness. Flower production was analyzed, as an indirect measurement of plant fitness, being that individual fitness increases if flower production also increases (Burkle and Irwin, 2010; Power and Stout, 2011). In *Vicia faba* and *Solanum lycopersicum*, the total number of flowers and total number of fruits produced per plant were counted. Fruit set was then calculated as the ratio between the total number of fruits and the total number of flowers. In *Phacelia tanacetifolia*, flower and fruit production are very high and thus were inferred for each plant. For that, the number of flowers and fruits produced per plant were first counted in three inflorescences per plant; then the total number of inflorescences was counted and multiplied by the mean number of flowers and fruits per inflorescence. Seed set in *P. tanacetifolia* was also inferred using the average number of seeds per fruit (between 0 and 4) in the inflorescences counted, multiplied by the number of fruits and then calculated by dividing the total number of mature seeds by the total number of seeds (mature and aborted). In *V. faba* and *S. lycopersicum*, seed set was done by counting the seeds produced and then applying the ratio between mature seeds and total number of seeds.

Statistical analysis

Data analysis was performed in R 3.6.3. (R Core Development Team, 2020). The models used were linear models (LM) and linear mixed models (LMM), for normally distributed datasets, and generalized linear mixed models (GLMM) for count data. These models were used due to the sampling sites and the various treatments acting as nested random effects. For linear models, the response variables were flower production, fruit set and seed set, while the explanatory variables were the varying degrees of fertilizer applied (in a factorial design, N and P as grouping factors). Using the lme4 and msme packages (Bates et al., 2015; Hilbe and Robinson, 2018) for generalized linear mixed models, with sampling locations and treatments acting as random effects (in a nested design), the response variables were number of visits and visitation rate by pollinators and the explanatory variables were the fertilizer treatments (also in a factorial design). Overdispersion was assessed and as such, a negative binomial distribution, with a log link function, produced the best fit for our data. Homoscedasticity, residuals, and dispersion were inspected, using the DHARMa package (Hartig, 2020) to select appropriate models. AIC was the deciding factor when more than one model was adequate. To assess group differences, multiple comparisons tests (estimated marginal means, with the aid of emmeans package; Lenth, 2021) were employed. In order to control false positives (Type I error) and false negatives (Type II error) more accurately, since Tukey HSD was too conservative in these comparisons for an α of 0.05 (as illustrated in Appendix 1), and due to many tests being performed at once (15 tests for 6 groups, resulting from the combinations of N and P), false discovery rate (FDR) was used as a p-value adjustment method (Benjamini and Hochberg, 1995; Verhoeven et al., 2005; Waite and Campbell, 2006; Pike, 2011; Midway et al., 2020). Lettering in multiple comparisons was done with the cld function, also present in the emmeans package (Lenth, 2021). Plots were built with ggerrorplot functions and pwpp functions, contained in ggplot2, ggpubr and emmeans packages, respectively (Wickham, 2016; Kassambara, 2020; Lenth et al., 2021).

Results

Flower production

Vicia faba and *Phacelia tanacetifolia* (the two nitrogen-fixing crops) did not exhibit significant differences in flower production for N and P levels (nor their interaction; Table 3). Although non-significant, the highest flower production was observed at Double N and No P for *V. faba* and in *P. tanacetifolia*, the highest flower production occurs without P for each N level and increases with increased N level (Figure 4). On the other hand, *Solanum lycopersicum* (non-nitrogen fixing), showed significant differences in flower production between different levels of N and P (while no differences were detected for their interaction; Table 3). Overall, increasing levels of N resulted in increased flower production, and RD of P resulted in higher flower production in comparison to treatments without P (Figure 4). T4 (RD N + RD P) and T6 (Double N + RD P) produced significantly more flowers in relation to the rest of the treatments, T1 (Half N + No P) produced significantly less flowers that the remaining treatments, while T2, T3 and T5 produced an intermediate number of flowers (Figure 4).

Table 3. LM and LMM results regarding the effects of nitrogen (N), phosphorous (P) and their interaction (N:P) on flower production in *V. faba*, *P. tanacetifolia* and *S. lycopersicum*. Degrees of freedom (df), F-values and p-values are presented. Significant p-values are marked in bold.

	Vicia faba			***Phacelia tanacetifolia***			***Solanum lycopersicum***		
Effect	df	*F*-value	*p*-value	df	*F*-value	*p*-value	df	*F*-value	*p*-value
N	2	1.3084	0.2758	2	2.0154	0.1583	2	4.949	**0.0094**
P	1	1.9679	0.1644	1	0.6041	0.4456	1	13.842	**0.0004**
N:P	2	0.5514	0.5783	2	0.2028	0.8181	2	0.187	0.8298

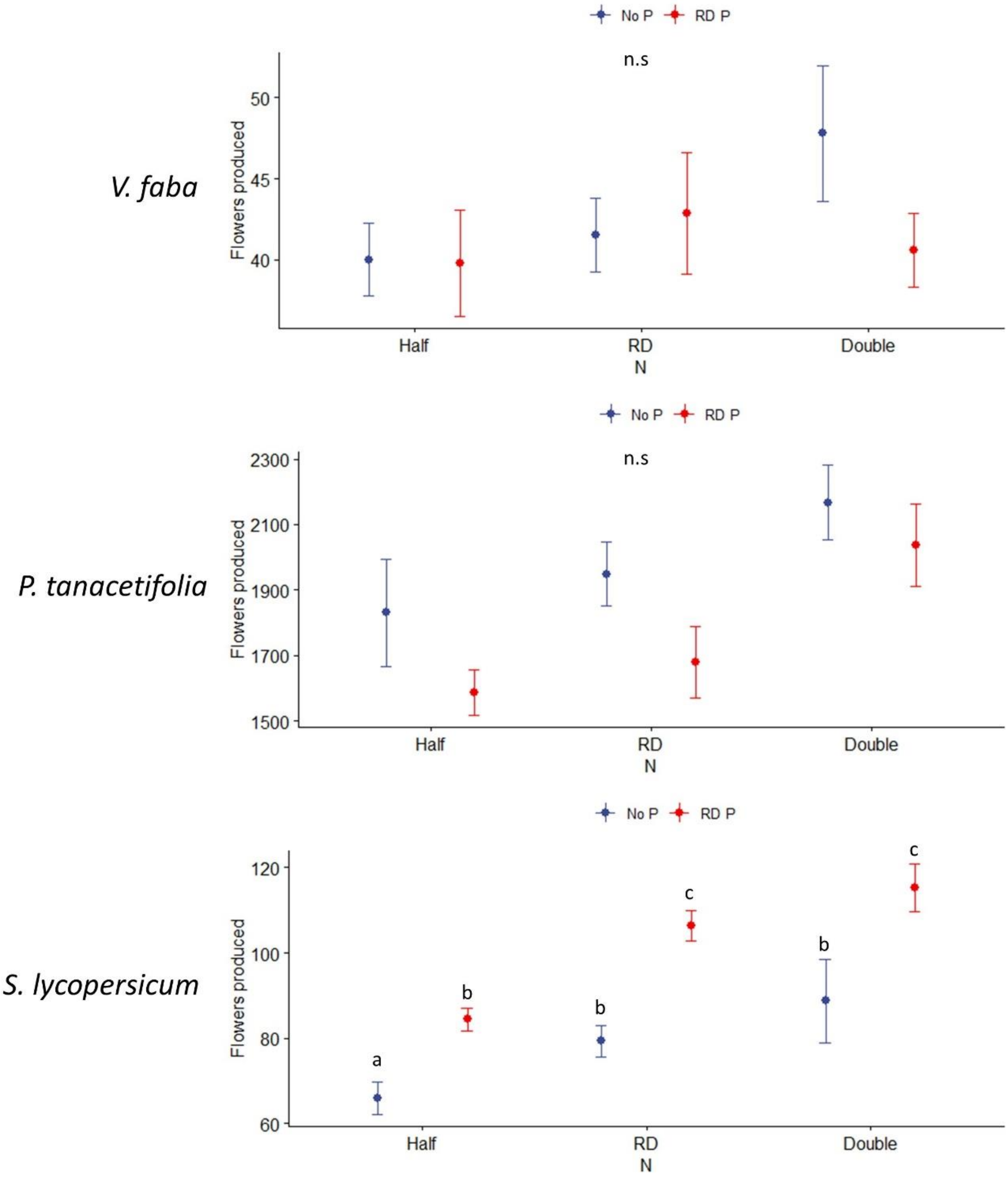

Figure 4. Flower production in crops species, in relation to different levels of N and P. Nitrogen is displayed in three levels: Half - half of the recommended dose; RD - recommended dose of nitrogen; and Double - double of the recommended dose of nitrogen, while phosphorus is displayed in two levels: Blue - No P - without phosphorous, and Red - RD P - recommended dose of phosphorous. Means and standard errors depicted. Estimated marginal means were calculated with FDR (false discovery rate) as a p-value adjustment method. Groups that share a letter are not statistically significant from each other. Species that do not show significant differences whatsoever are marked as n.s – non-significant.

Pollinator communities

Pollinator communities and main pollinator species varied in the three crop species studied (see Appendix 2). Firstly, regarding pollinator diversity, we recorded a total of 495 pollinators (from 17 species and 2 orders, Hymenoptera and Diptera) in *V. faba*, 2152 pollinators in *P. tanacetifolia* (from 55 species and 5 orders, Hymenoptera, Diptera, Coleoptera, Lepidoptera and Hemiptera) and 39 pollinators in *S. lycopersicum* (from 5 species and 2 orders, Hymenoptera and Diptera). In *V. faba*, the most frequent insect pollinator was *Anthophora plumipes* (31.9% of the insects recorded), responsible for 24.6% of total number of interactions, followed by *Eucera codinai* (25.9%), which performed 40.3% of all visits. For *P. tanacetifolia*, *Anthophora plumipes* accounted for 36.3% of all insects, followed by *Apis mellifera* (10.1%). Most visits were performed by *A. plumipes* (30.1%), followed by *A. mellifera* (15.9%) and *Bombus terrestris* (15.7%). Lastly, for *S. lycopersicum*, *Lasioglossum malachurum* was the most frequent pollinator (61.5%) and performed 55.6% of all visits.

Visitation rates

Vicia faba and *Phacelia tanacetifolia* did not present significant differences in both variables, the number of visits and visitation rate, among N and P treatments (nor for N:P interaction effect; Tables 4 and 5). *Solanum lycopersicum* displayed not only significant differences among N levels but also for the interaction between N and P levels in both variables (Tables 4 and 5). For both variables (number of visits and visitation rate), plants from T4, received significantly more visits than plants from T3, with the remaining treatments presenting intermediate and non-significant visitation levels (Figures 5 and 6).

Table 4. GLMM results regarding the effects of nitrogen (N), phosphorous (P) and their interaction (N:P) on number of visits in *V. faba*, *P. tanacetifolia* and *S. lycopersicum*. Degrees of freedom (df), chi-square values and p-values are presented. Significant p-values are marked in bold.

	Vicia faba			***Phacelia tanacetifolia***			***Solanum lycopersicum***		
Effect	df	χ2	*p*-value	df	χ2	*p*-value	df	χ2	*p*-value
N	2	0.4728	0.7895	2	1.8420	0.3981	2	7.9237	**0.0190**
P	1	0.4364	0.5088	1	0.0061	0.9377	1	1.4275	0.2322
N:P	2	1.9964	0.3686	2	3.8227	0.1479	2	10.9281	**0.0042**

Table 5. GLMM results regarding the effects of nitrogen (N), phosphorous (P) and their interaction (N:P) on visitation rates in *V. faba*, *P. tanacetifolia* and *S. lycopersicum*. Degrees of freedom (df), chi-square values and p-values are presented. Significant p-values are marked in bold.

	Vicia faba			***Phacelia tanacetifolia***			***Solanum lycopersicum***		
Effect	df	χ2	*p*-value	df	χ2	*p*-value	df	χ2	*p*-value
N	2	0.4447	0.8007	2	1.6996	0.4275	2	8.4289	**0.0148**
P	1	0.4428	0.5058	1	0.0157	0.9003	1	1.5805	0.2087
N:P	2	1.6524	0.4377	2	4.3801	0.1119	2	10.9260	**0.0042**

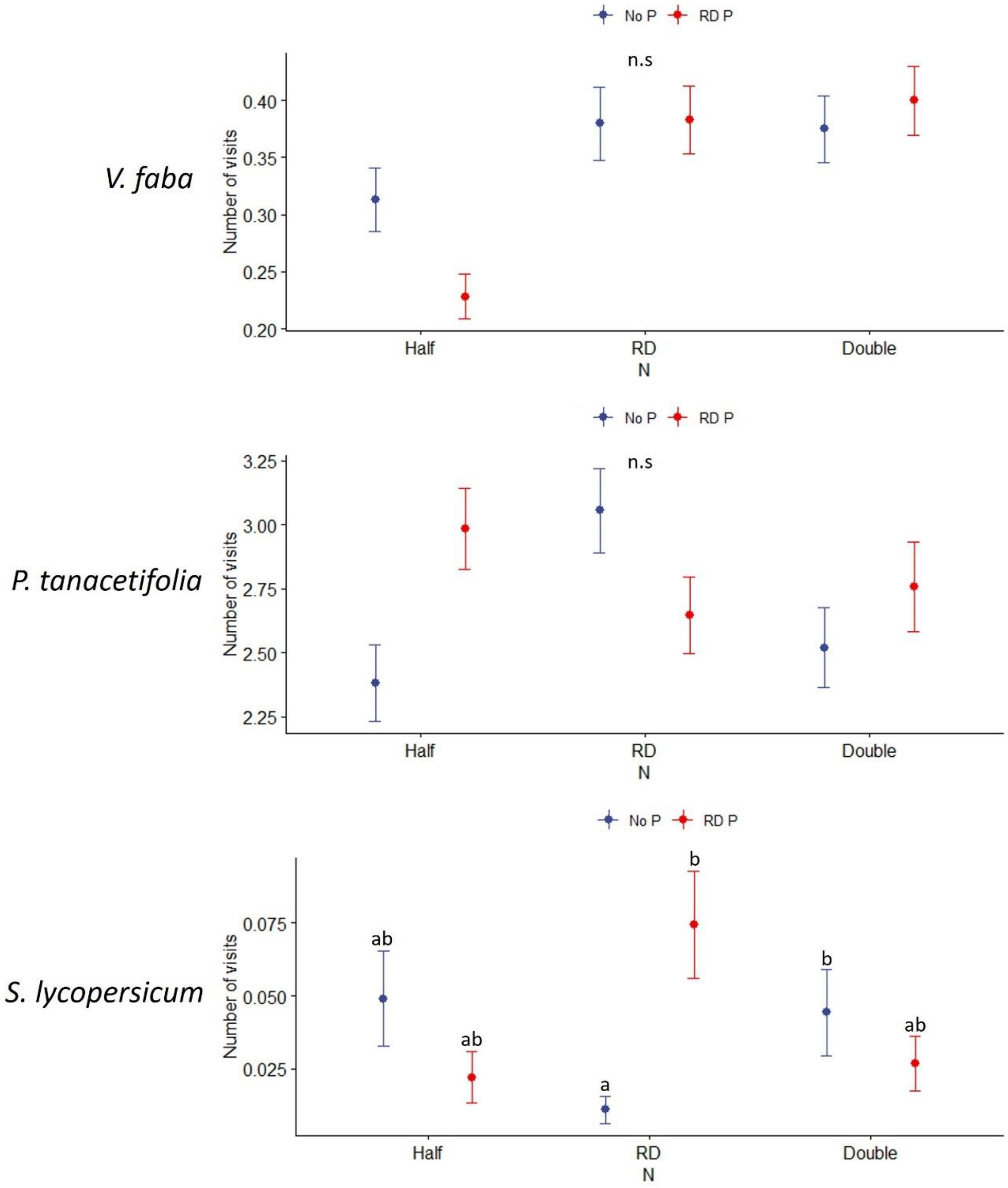

Figure 5. Number of visits by pollinators in crops species, in relation to different levels of N and P. Nitrogen is displayed in three levels: Half - half of the recommended dose; RD - recommended dose of nitrogen; and Double - double of the recommended dose of nitrogen, while phosphorus is displayed in two levels: Blue - No P - without phosphorous, and Red - RD P - recommended dose of phosphorous. Means and standard errors depicted. Estimated marginal means were calculated with FDR (false discovery rate) as a p-value adjustment method. Groups that share a letter are not statistically significant from each other. Species that do not show significant differences whatsoever are marked as n.s – non-significant.

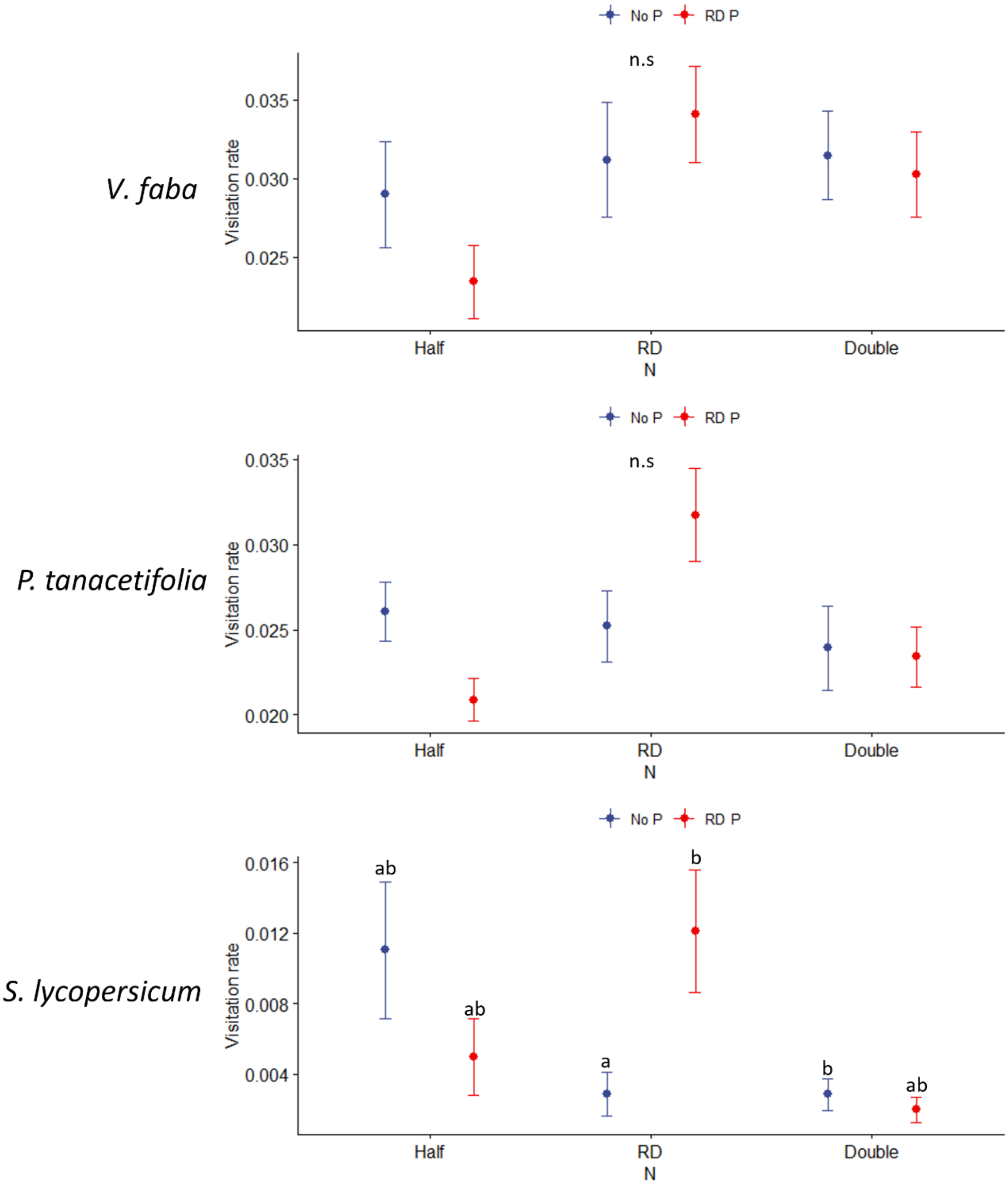

Figure 6. Visitation rates by pollinators in crops species, in relation to different levels of N and P. Nitrogen is displayed in three levels: Half - half of the recommended dose; RD - recommended dose of nitrogen; and Double - double of the recommended dose of nitrogen, while phosphorus is displayed in two levels: Blue - No P - without phosphorous, and Red - RD P - recommended dose of phosphorous. Means and standard errors depicted. Estimated marginal means were calculated with FDR (false discovery rate) as a p-value adjustment method. Groups that share a letter are not statistically significant from each other. Species that do not show significant differences whatsoever are marked as n.s – non-significant.

Fruit set and seed set

Regarding fruit set, no significant differences were observed between N and P levels, nor in their interaction, for *V. faba* and *S. lycopersicum*. Differences were only found in *P. tanacetifolia* between P levels (Table 6), with RD P producing more fruits that without P (Figure 7). Significant differences were registered between T1 and T6 (Half N + No P – Double N + RD P), T1 and T2 (Half N + No P – Half N + RD P) and between T3 and T6 (RD N + No P – Double N + RD P).

Concerning seed set, no differences were found for any of the factors in *V. faba* (Table 7). Both, *P. tanacetifolia* and *S. lycopersicum*, demonstrated significant differences among N levels, while P and the interaction factor were non-significant (Table 7). According to the multiple comparisons test, in *P. tanacetifolia*, T1 is statistically different from T2, T3, T4 and T6. As for *S. lycopersicum*, only T5 and T6 are statistically significant from T1 (see Figure 8).

Table 6. LMM results regarding the effects of nitrogen (N), phosphorous (P) and their interaction (N:P) on fruit set in *V. faba*, *P. tanacetifolia* and *S. lycopersicum*. Degrees of freedom (df), F-values and p-values are presented. Significant p-values are marked in bold.

	Vicia faba			***Phacelia tanacetifolia***			***Solanum lycopersicum***		
Effect	df	*F* -value	*p* -value	df	*F* -value	*p* -value	df	*F* -value	*p* -value
N	2	0.7215	0.4891	2	2.6379	0.0778	2	3.1608	0.0637
P	1	1.8681	0.1754	1	8.9616	**0.0037**	1	4.0807	0.0567
N:P	2	1.6642	0.1957	2	0.8282	0.4406	2	1.6868	0.2104

Table 7. LMM results regarding the effects of nitrogen (N), phosphorous (P) and their interaction (N:P) on seed set in *V. faba*, *P. tanacetifolia* and *S. lycopersicum*. Degrees of freedom (df), F-values and p-values are presented. Significant p-values are marked in bold.

	Vicia faba			***Phacelia tanacetifolia***			***Solanum lycopersicum***		
Effect	df	*F* -value	*p* -value	df	*F* -value	*p* -value	df	*F* -value	*p* -value
N	2	0.6347	0.5327	2	4.2983	**0.0169**	2	8.3611	**0.0021**
P	1	0.0619	0.8042	1	3.0743	0.0834	1	0.7786	0.3875
N:P	2	0.1690	0.8448	2	2.2198	0.1154	2	1.5388	0.2392

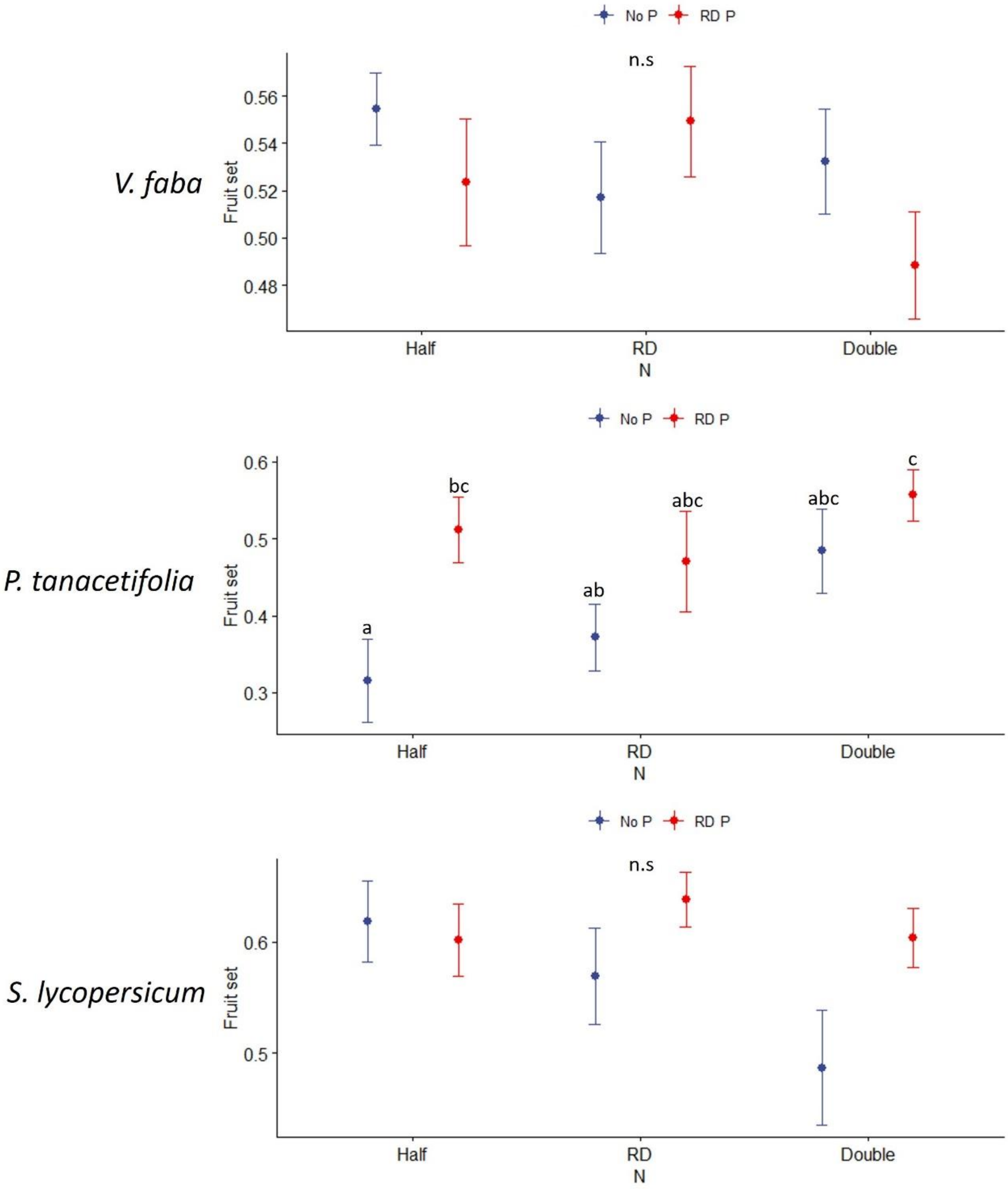

Figure 7. Fruit set in crops species, in relation to different levels of N and P. Nitrogen is displayed in three levels: Half - half of the recommended dose; RD - recommended dose of nitrogen; and Double - double of the recommended dose of nitrogen, while phosphorus is displayed in two levels: Blue - No P - without phosphorous, and Red - RD P - recommended dose of phosphorous. Means and standard errors depicted. Estimated marginal means were calculated with FDR (false discovery rate) as a p-value adjustment method. Groups that share a letter are not statistically significant from each other. Species that do not show significant differences whatsoever are marked as n.s – non-significant.

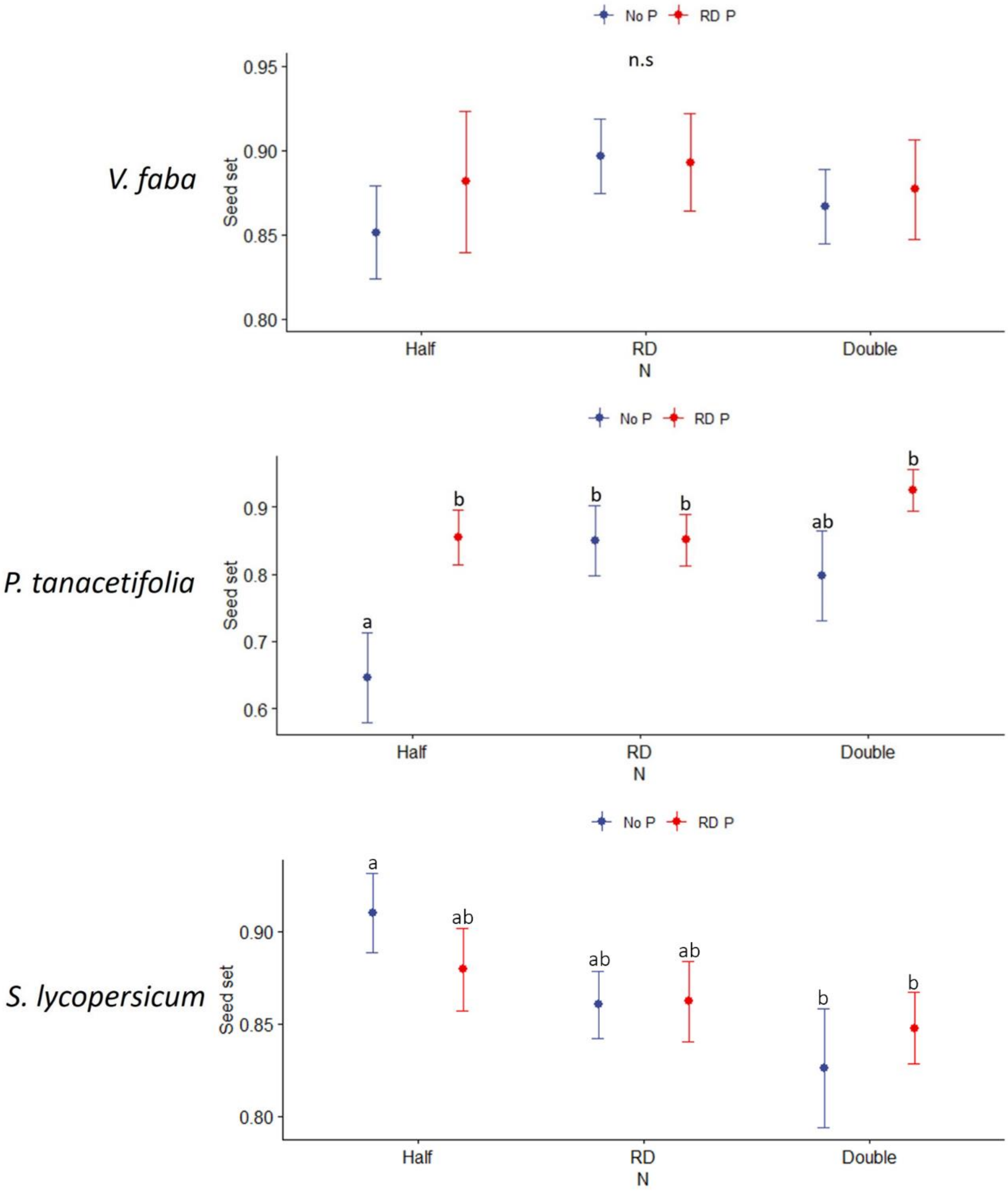

Figure 8. Seed set in crops species, in relation to different levels of N and P. Nitrogen is displayed in three levels: Half - half of the recommended dose; RD - recommended dose of nitrogen; and Double - double of the recommended dose of nitrogen, while phosphorus is displayed in two levels: Blue - No P - without phosphorous, and Red - RD P - recommended dose of phosphorous. Means and standard errors depicted. Estimated marginal means were calculated with FDR (false discovery rate) as a p-value adjustment method. Groups that share a letter are not statistically significant from each other. Species that do not show significant differences whatsoever are marked as n.s – non-significant.

Discussion

The rising numbers in world population during the last century led to an increase in agricultural efforts and subsequently, an increase in fertilizer use as well (FAO, 2020). Excessive fertilization not only damages the surrounding environment, diminishing soil and freshwater quality, but it can also impact plant-pollinator interactions, as alterations in soil nutritional values will likely impact plant development and plant traits linked with pollinator attraction (Ceulemans et al., 2017; David et al., 2019; Russo et al., 2020). Therefore, it is invaluable to properly ascertain how the rising trend in fertilizer use affects pollinator behavior and subsequent crop yield. In this study, we determine the effect of N and P in pollinator visitation rates, in crop species, as well as its effects in subsequent fruit and seed production. This is one of the first works that perform such data analysis across multiple crop species, in relation to plant-pollinator interactions and plant fitness. We observed that the impact of eutrophication might be crop dependent with some species being more impacted than others. Increased N availability led to increased flower production in crop species, even though only tomato registered significant p-values. Overall, recommended doses of N and P tended to have higher pollinator visitation rates. Additionally, in fruit set and seed set, no clear pattern was observed. Regarding fruit set, *V. faba* and *S. lycopersicum* did not report any significant differences, while in *P. tanacetifolia*, fruit set increased with the addition of P. In respect to seed set, *P. tanacetifolia* showed an increase as N increased, while in *S. lycopersicum*, seed set decreased with the increase of N. *Vicia faba* did not register significant differences regarding seed set. Findings are discussed below in more detail.

Flower production

Species are differently affected by changes in nutrients concentration, with consequences at several levels. One of the most affected organs are flowers, not just in flower size (Russo et al., 2020) or nectar and pollen quality (Ceulemans et al., 2017), but also in the number itself (Muñoz et al., 2005; Burkle and Irwin, 2010). In fact, our results seem to suggest that the effects of fertilizers on flower production are species-specific, dosage-dependent, and fertilizer-dependent. Generally, flower production increased with an increase in N, mostly in non-nitrogen fixing species (*S. lycopersicum*), where this pattern was accentuated by P addition. Although not significant, this general trend of increased flower production with N was also observed in the nitrogen-fixing *P. tanacetifolia*, with the addition of P having different consequences according to the crop. While in *S. lycopersicum*, the lack of P produced less flowers than the RD of P, the opposite pattern was observed in *P. tanacetifolia*. Similarly, *V. faba* showed no differences on flower production across treatments.

These findings are in alignment with current literature, with plant species exhibiting various responses to nutrient addition. For example, the lack of effect of N on floral production per plant that was observed in *Gymnadenia conopsea* (Gijbels et al., 2015) and *Ipomopsis aggregata* (Burkle and Irwin 2010). According to Phoenix et al. (2012), in *Calluna vulgaris*, a dominant shrub in European heathland, high levels of atmospheric N deposition, in the soil stimulated flowering, while in forbs in calcareous grasslands (*Gentianella amarella* and *Potentilla erecta*) led to a reduction in flowering rates at higher levels of N, further demonstrating species-specific differences in flower production in response to N changes in the soil. Burkle and Irwin (2009) also found that, depending on the species life-history, e.g., semelparity vs iteroparity, plants reacted differently to nutrient deposition, as *Ipomopsis aggregata* (semelparous) produced more flowers when exposed to fertilizers, while *Linum lewisii* (iteroparous) was not affected. Although in our study we only use annual crops, differences were expected between nitrogen-fixing and non-nitrogen fixing plants. Nitrogen-fixing plants are adapted to soils with lower N levels, promoting the efficient use of this nutrient (Kraiser et al., 2011; Ngom et al., 2016). In contrast, non-nitrogen fixing plants are more susceptible to changes in N concentration in the soil (Chen and Markham, 2021). It should be expected then that nitrogen-fixing plants and non-nitrogen fixing plants exhibited different responses to N deposition regarding flower production, which was not verified in this study. But it should also be noted that species did exhibit different responses regarding P addition, which might imply that there are more mechanisms at play, when it comes to fertilizer effects in nitrogen-fixing and non-nitrogen fixing plants.

Although not assessed here, through changes on other developmental stages of the plants, fertilization could also have impacts in plant growth affecting for example plant communities and phenological patterns, which will also directly or indirectly determine pollinator foraging patterns. Phenological alterations mediated by N addition, remain a complex field of study, as some plant species might get their flowering periods delayed, while other species may react in an opposite manner, anticipating their flowering periods, potentially causing plant-pollinator mismatches (Carvalheiro et al., 2021). Plant species richness may also contribute to altered visitation rates, as the number of flowering species increases, providing more foraging opportunities to pollinators (Ebeling et al., 2008; Lázaro et al., 2009). Thus, while assessing the impact of nutrient addition in crops provides important details, the effects of fertilizers in plant-pollinator interactions should be studied in a holistic manner, encompassing all its effects in the ecosystem.

Plant-pollinator interactions

Pollinator foraging behavior can be affected by many parameters. Several authors argued for the possibility that changes in floral characteristics and/or in floral rewards as consequence of nutrient addition could impact pollinators' choice (Burkle and Irwin, 2009, 2010; Dupont et al., 2018; Ramos et al., 2018; Banaszak-Cibicka et al., 2019; Russo et al., 2020). In our case, once again, we observed that the effect of nutrient concentration in pollinator attractivity is species-specific. The absence of significant differences in number of visits and visitation rates in nitrogen-fixed crops is in accord with the lack of significant differences in the number of flowers across treatments. Probably, the different levels of N and P did not lead to differences in morphology and/or physiology in flowers that can be detected by pollinators. In *S. lycopersicum* (non-nitrogen fixing species), the differences in visitation patterns could result from a combination of multiple traits as no direct relation was observed between the increase in flower number and pollinator visitation. However, pollinator behavior did change with alterations in nutrient levels, as pollinators reacted better at recommended dosages of N and P.

Overall, flower production may be a factor that impacts pollinator visitation (Burkle and Irwin, 2010; Power and Stout, 2011), but other factors should also be accounted for when plant-pollinator interactions are being discussed, e.g., flower size (Schemske and Bradshaw 1999; Burkle and Irwin, 2009; Russo et al. 2020) and nectar and pollen quality (Schemske and Bradshaw 1999; Burkle and Irwin, 2009; Ceulemans et al., 2017). Hoover et al. (2012) made one of the first articles evaluating the possible relation between nutrient addition and changes in sugars and amino acids in crops. Using *Cucurbita maxima* 'Little Cutie', the authors found that bumblebees (*Bombus terrestris*) demonstrated a preference for N-enriched plants when compared with control plants. The authors argue that the higher sucrose to hexose ratios present in N-enriched nectar are more appealing to bumblebees, even when N-enriched nectar cut their longevity by 22%. Despite not being found the cause for this decrease in survivability, authors alert for the existence of multiple drivers for plant attractiveness to pollinators, that could potentially have nefarious consequences to pollinators. Other works have researched further on how can floral resources impact insect longevity and offspring, e.g., the pollen's origin (plant species), chemistry and quantity used to rear the brood, which can certainly impact their health condition and consequently, alter offspring mortality rate (Roulston et al., 2000; Potts et al., 2003; Sedivy et al., 2011; Vanderplanck et al., 2014). Wild bees can also forage in a selective manner, recognizing flowers that carry pollen with higher amounts of essential amino acids (Cook et al., 2003). More recently, Goulnik et al. (2020), have explored the role of nitrogen and phosphorus, a nutrient whose role is not as studied, on floral traits and resources. It was found that while phosphorus negatively impacted flower area and color, nitrogen impacted positively flower area and nectar production per flower, further adding that interaction frequency by pollinators increased with nectar production. Nectar concentration in flowers can be, therefore, an important driver in pollinator attraction in plants, along with nectar amino acid concentration, which can vary by fertilizer dosages, with higher nutrient levels exhibiting higher levels of some amino acids, also enhancing bee visitation (Leach, 2018). Additionally, Ceulemans et al. (2017) reported that N and P addition could alter nectar and pollen's chemistry in *Succisa pratensis*; it was shown that bumblebees' larvae that fed on nutrient-enriched resources died at a higher rate than those that fed on control plants. However, the study could not establish if this higher mortality rate was due to changes in nectar and pollen composition or to another underlying factor. Contrastingly, Burkle and Irwin (2010) observed similar pollen production per flower with N addition. Nonetheless, in *Ipomopsis aggregata*, low N levels (10 kg N ha^{-1} year) enabled an increase in flower production, that in turn resulted in a higher pollen production per plant, while in *Potentilla pulcherrima*, flowers' production decreased at high N levels (200 kg N ha^{-1} year), which consequently decreased global pollen production. Lau and Stephenson (1993) had a different approach, reporting an increase in size by 5% in *Cucurbita pepo* pollen grains in soils with high nitrogen levels. Thus, there seems to exist some foundations to the argument that nutritional changes in the soil can decisively impact nectar and pollen production and composition, while the extent of such impact is not so certain, as literature that focuses on this topic is still scarce.

Although our results look robust, there is some difficulty in assessing truthful post hoc differences in group comparisons. In *S. lycopersicum*, it should be expected that the groups that were visited the least (T5, T6 and T3) would not exhibit any group differences in a post hoc comparison. Such was not the case here, as it was denoted that T5 was statistically different from T3, but not from T6. This is quite possibly a case of type I error. Due to tomato's lower pollinator attractiveness, it is expected that, for *S. lycopersicum* to have the same number of plant-pollinator interactions as the other species had, it would need more hours of census in order to produce a more robust visitation pattern. In this study we tried to balance the hours of observations in all species and all have roughly the same. Even so, multiple comparison tests are bound to have type I and type II errors, especially in studies with high sample size and many group comparisons, such as this study (Midway et al., 2020).

Plant reproductive fitness

The importance of pollinators on world crop production is widely recognized (Klein et al., 2007). In fact, 90% of flowering plants are pollinator dependent (Gill et al., 2016), and many of the crop species need pollinators to achieve an optimal production (Klein et al., 2007). The increasing demand of food, which partly comes from insect-pollinated crops, is transforming the production systems in a way to maximize production, with intensive systems where the use of fertilization and other agrochemicals is high (FAO, 2020). It is widely known that an increased use of fertilizers in agricultural settings can boost fruit production (Ruffo et al., 2003; Özer et al., 2004; Habbasha et al., 2007; Iqbal et al., 2011; Kashem et al., 2015; Adak and Kibritci, 2016; Popović et al., 2018). Yet, the available information of the effects of eutrophication in crop production mediated by pollinator behavior are scarce and limited to a few studies (Power and Stout, 2011; Klatt et al., 2013; Motzke et al., 2015; Eeraerts et al., 2017; Garrat et al., 2018).

In our study, in the case of *V. faba*, we observed that fertilizers did not affect this crop in fruit or seed production. In *P. tanacetifolia*, we found that in treatments with the recommended dosage of P, fruit set was not statistically different across N levels, while in treatments without P, fruit set increased as N increased. Seed set was not statistically different across most treatments, except for the treatment with the lowest dosage of N and without P. In *S. lycopersicum*, fruit set was not affected by fertilizer dosages, while seed set behaved in two patterns: in treatments with the recommended dosage of P, there are no differences among treatments but in treatments without P, seed set decreased with the increase of N levels. This non-significance in tomato's fruit set can be possibly explained by tomato's exceedingly low pollinator dependency (Carr and Davidar, 2015). However, seed set in *S. lycopersicum* demonstrates a possible relation between higher levels of insect pollination and higher production of viable seeds. This finding also aligns with the premise that increased visits help plants produce a higher number of seeds (Kunin, 1993; Steffan-Dewenter and Tscharntke, 1999; Bartomeus et al., 2014; Wu et al., 2021), reinforcing the importance of pollinators not only in fruit production but also in fruit quality.

For example, using trees of *Malus domestica* var. *Fuji*, Wu *et al*. (2021) proved the pollinator-dependence in apple culture, as fruits exhibited higher quality (more seeds and less deformation) when pollinated by insects, with a positive effect of bee abundance on the number of seeds per fruit. However, and more surprising, this expected positive correlation was lost in orchards with high concentration of soil nitrogen (>1.5 g Kg^{-1}). Marini et al. (2015), also contributed to our understanding of nitrogen-pollination relations and effects on crop production by using cage experiments in oilseed rape, *Brassica napus*. It was shown that nitrogen had an almost significant effect on bee visitation, with high N levels reporting more visits compared to low N levels. It was also shown that cultivar had a significant effect on pollination, with insect visitation increasing seed yield only on the open pollinated cultivar (Catalina). Insect pollinated plants also produced 19% more seed yield compared to other treatments. Finally, Bartomeus et al. (2014), also provide a comprehensive article denoting the importance of insect pollination on production. In comparison to other articles presented, here landscape was used as a variable indicating eutrophication, instead of direct nutrient addition. Using oilseed rape (*Brassica napus*), field bean (*Phaseolus vulgaris*), strawberry (*Fragaria x ananassa*) and buckwheat (*Fagopyrum esculentum*), it was shown that open pollination improved yield for all crops, as visitation rates increased, with simple landscapes (mainly agricultural land) presenting lower species richness compared to more complex landscapes that provided more nesting places and foraging opportunities to insects. Crop quality also improved, as oil content increased in oilseed rape, empty seeds decreased in buckwheat and commercial quality increased in strawberries. Overall, visitation rates were higher in complex landscapes, analogous to fields with lower soil N values. Albeit existing differences in how crops react to nutrient levels, there is a common pattern. As reported above, the current literature implies that higher nutrient levels enhance fruit production, but pollination can play a decisive role in the production of viable seeds in crops, improving fruit quality. In that case, agricultural landscapes with pollinator-dependent crops, along with lower nutrient values, should maintain high levels of crop production, at the same time maintaining profits and improving ecological stability.

Future perspectives

Although this kind of studies offers us a glimpse on pollinator viability in ecosystems, future studies can surely improve on the knowledge we currently possess in this very important topic. Pollinators benefit from crop species by harvesting floral resources needed for their diet and brood raising. In turn, pollinators help us maintain a steady production of fruits and vegetables that our diet relies on (Klein et al., 2007). If we engage in agricultural practices that diminish pollinator visitation, both humans and insects are bound to suffer. This study is only able to answer how nutrient addition may affect pollinators' attraction in three examples of crops present in our agroecosystems. Additionally, it is important not only to expand to other crops, but also to know how nutrient addition affects pollinators' health and survivability. Hoover et al. (2012) provide useful insights on how increased fertilizer availability can impact pollinators' health, possibly cutting pollinators' (*Bombus terrestris*) survival rate by 22%. This is quite concerning as these pollinators are generalists and support agricultural production. Therefore, it is equally important for us to assess how nutrient deposition impacts smaller and bigger species of bees. Our understanding of how N and P addition affects nectar and pollen quality and quantity can also be improved, in order to determine how increased levels of fertilizers can alter sugar and amino acid quantity and composition, in pollen and nectar (Gardener and Gillman, 2001; Ceulemans et al., 2017; reviewed in David et al., 2019). Studies focus on changes in nectar's and pollen's amino acid concentrations and composition and its impact on pollinators taxa, will provide the role of the various amino acids in pollinators' development and its susceptibility to nutrient deposition. Our current knowledge is also lacking in the assessment of these types of changes in the various taxa of pollinators besides bees, e.g., Diptera, Coleoptera, Lepidoptera, as some types of pollinators are extremely important in particular crops, e.g., midges' (Diptera) role in pollinating cacao (Klein et al., 2008). This study could certainly be improved by examining more extensively fruit quality parameters, such as firmness and shelf life, and how those parameters behave in relation to pollination rates, possibly adding to the market value. The experiments can also be replicated in several years, thus potentially determining if fertilizer effects on plant-pollinator interactions are changeless in a species, or can even be performed in different countries, with different climatic conditions in order to assess how results can differ depending on the region assessed and pollinator taxa, and its impact on the overall network of drivers. Pollinator efficiency, i.e., the number of seeds formed per visit, could also be assessed, so we can properly ascertain how different species of pollinators interfere in crop productivity and how fertilizers can potentially affect different species. Different crop species should also be used to assess general patterns that might exist between plant species, so we can determine what species are more vulnerable to pollinator loss. In order to produce more robust models, comparisons between crops should also take into account pollinator attraction of these crops, as to minimize skews in visitation. Lastly, studies should continue to assess how fertilizers affect plant characteristics (e.g., plant size, floral display size and floral color), resources (e.g., floral rewards' sugar and amino acid content and concentration) and phenology (e.g., duration of bloom period and plant-pollinator mismatch), as well as its consequences on pollinator attractivity and behavior, so it will increase our knowledge of pollination services' importance in agroecosystems.

Concluding remarks

In this study, we found that fertilizers may have direct effects in crop species, altering flower production, and indirect effects in pollinators, altering pollinator attraction in crop species, due to the impact in floral characters. These effects may influence plant fitness by altering fruit and seed production. Overall, the results align with the main core of studies supporting that crop species react more positively at median levels of nutrient addition (Cardoza et al., 2012; Marini et al., 2015; Dupont et al., 2018; Ramos et al., 2018), that the increased visits may be promoted by beneficial alterations to floral characteristics (e.g., increase of the number of available flowers; Power and Stout, 2011; Dupont et al., 2018) and that pollinator visitation may contribute to increased seed production, improving fruit quality (Bartomeus et al., 2014; Wu et al., 2021). Thus, farmers and producers should consider that increased nutrient availability does not always translate into increased fruit production (Hünicken et al., 2020). Instead, pollinators should be held more into account when it comes to merge profitability and sustainability in agroecosystems (Basu et al., 2011; Elias et al., 2017).

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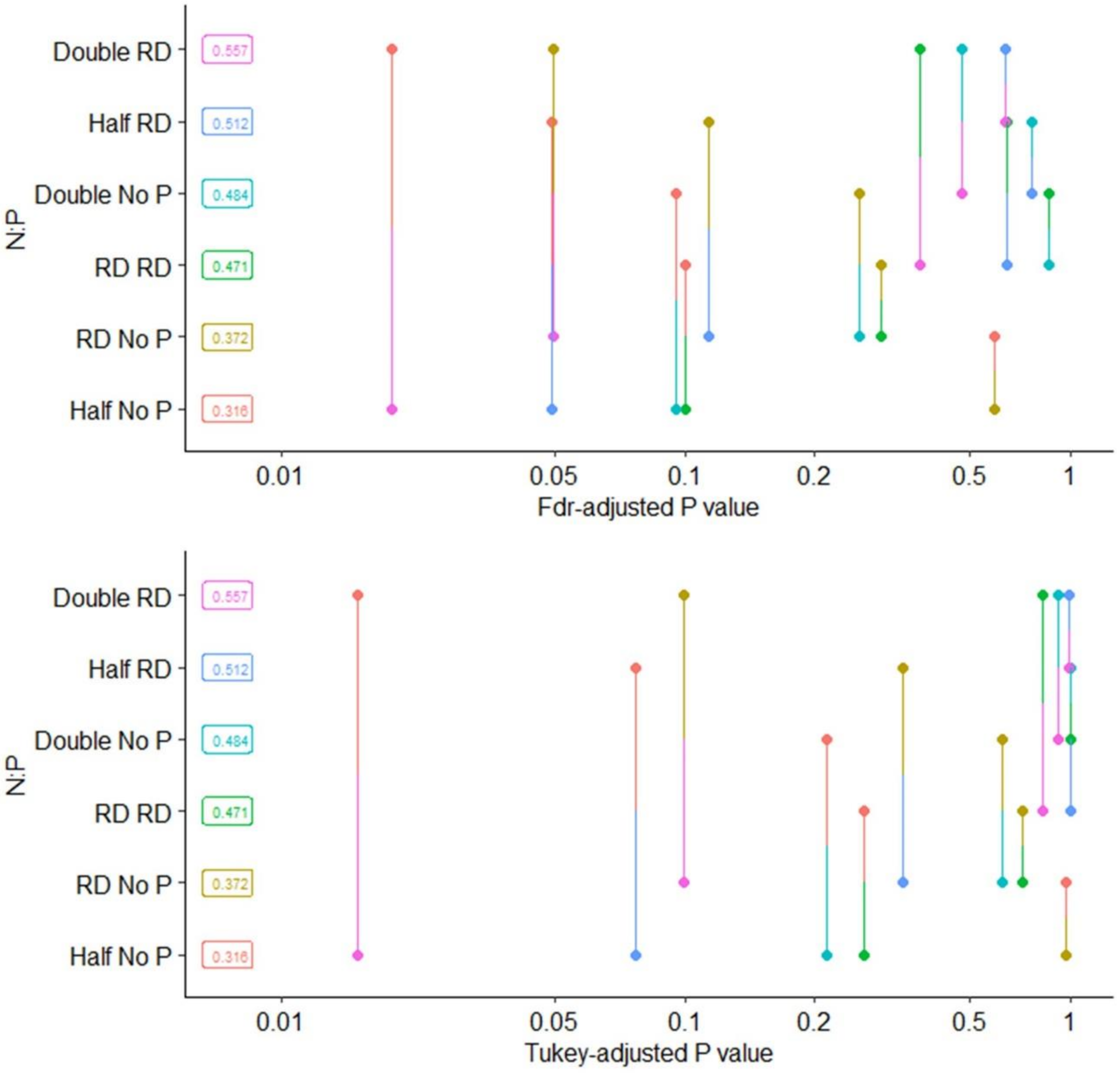
Appendices



Appendix 1. Example of difference between FDR and Tukey HSD power at α = 0.05, for fruit set analysis in *Phacelia tanacetifolia*, accounting for possible Type II error.

Appendix 2. List of pollinator species observed during sampling of pollination bouts. Species are divided by corresponding family and order and ordered alphabetically within each division. Number of insects observed per species (Insects) and number of visits performed per species (Visits) is indicated below each crop species.

			Vicia faba		***Phacelia tanacetifolia***		***Solanum lycopersicum***	
Order	**Family**	**Species**	**Insects**	**Visits**	**Insects**	**Visits**	**Insects**	**Visits**
Coleoptera	Cetoniidae	*Oxythyrea funesta*	-	-	11	17	-	-
	Chrysomelidae	*Altica* sp.	-	-	1	4	-	-
		Longitarsus sp.	-	-	1	3	-	-
	Melyridae	*Psilothrix* sp.	-	-	1	2	-	-
	Nitidulidae	*Nitidulidae* sp.	-	-	5	8	-	-
	Staphylinidae	*Staphylinidae* sp.	-	-	1	5	-	-
Diptera	Anthomyiidae	*Anthomyiidae* sp.	-	-	3	5	-	-
	Empididae	*Empis* sp.	-	-	28	162	-	-
	Muscidae	Muscidae	-	-	2	2	-	-
	Scathophagidae	*Scathophaga* sp.	-	-	2	4	-	-
	Syrphidae	*Epysirphus balteatus*	-	-	7	30	1	2
		Eristalis sp.	1	2	4	13	-	-
		Eristalis taeniops	-	-	3	9	-	-
		Eristalis tenax	3	11	4	6	-	-
		Eupeodes sp.	-	-	14	78	-	-
		Melanostoma sp.	-	-	1	5	-	-
		Sphaerophoria scripta	-	-	109	249	-	-
Hemiptera	Pentatomidae	*Aelia* sp.	-	-	1	1	-	-
Hymenoptera	Andrenidae	*Andrena flavipes*	3	9	76	717	-	-
		Andrena limata	-	-	3	66	-	-
		Andrena pilipes	-	-	20	135	-	-
		Andrena sp.	-	-	15	152	-	-
		Andrena thoracica	-	-	1	20	-	-
		Andrena vulcana	-	-	3	23	-	-
		Andrena wilkella	4	12	-	-	-	-
	Apidae	*Anthophora atroalba*	-	-	16	214	-	-
		Anthophora plumipes	158	703	782	3395	10	48
		Anthophora sp.	-	-	1	1	-	-
		Apis mellifera	13	56	217	1796	-	-
		Bombus hortorum	7	42	-	-	-	-
		Bombus pascuorum	22	174	4	49	-	-
		Bombus terrestris	19	81	178	1768	-	-
		Ceratina cyanea	-	-	1	2	-	-

cont.

Hymenoptera		*Ceratina cucurbitina*	-	-	50	91	-	-
		Eucera notata	-	-	55	223	-	-
		Eucera codinai	128	1149	24	123	-	-
		Eucera nigrilabris	8	14	1	3	-	-
		Eucera sp.	63	392	-	-	-	-
		Eucera sp. 1	-	-	4	16	-	-
		Eucera sp. 2	-	-	10	66	-	-
		Melecta albifrons	-	-	37	168	-	-
		Xylocopa cantabrita	16	40	5	36	-	-
		Xylocopa violacea	18	35	20	180	-	-
	Colletidae	*Colletes cunicularius*	-	-	1	2	-	-
	Halictidae	*Halictus* sp.	-	-	5	19	-	-
		Halictus fulvipes	-	-	6	25	-	-
		Halictus scabiosae	-	-	13	33	-	-
		Lasioglossum malachurum	5	19	97	326	24	80
		Lasioglossum pauxillum	-	-	1	2	-	-
		Lasioglossum sp.	-	-	95	385	2	11
	Megachilidae	*Osmia caerulescens*	9	24	92	268	2	3
		Osmia bicornis	-	-	11	32	-	-
		Osmia niveata	-	-	16	48	-	-
		Osmia latreillei	-	-	16	65	-	-
		Osmia sp.	-	-	66	191	-	-
	Vespidae	Vespidae	-	-	2	2	-	-
		Vespula vulgaris	18	89	-	-	-	-
Neuroptera	Chrysopidae	*Chrysoperla carnea*	-	-	2	6	-	-
Lepidoptera	Pieridae	*Pieris brassicae*	-	-	8	26	-	-