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ABSTRACT

Nutrient deficiency is known to constrain plant growth in numerous ways, but how it impacts floral displays and pollination success remains unclear. Here we investigate how insufficient availability of sulphur – a vital plant nutrient that is a limiting factor in natural and agricultural regions throughout the world – influences the production of floral displays in *Brassica rapa*, *Physalis philadelphica* and three *Petunia* species with differently coloured flowers. Sulphur deficiency led to a drastic reduction in the number of open flowers, an aberrant flower morphology and smaller pollen with an altered mineral nutrient content. Intriguingly, sulphur deprivation also led to a clear reduction in pigmentation of yellow flowers, but not in flowers with white, purple and red colours. The pale yellow flower colour was due to decreased amounts of violaxanthin, lutein and other carotenoids, suggesting that the carotenoid synthesis pathway is particularly susceptible to sulphur deficiency. Additional experiments with nitrogen and phosphorus depletion confirmed that observed colour and morphological changes were not a general nutrient limitation response, but could be ascribed to sulphur depletion specifically. Taken together, our results showed that (mild) sulphur deficiency deteriorates a suite of floral traits, and that the effects may cascade to pollinators and so have the potential to undermine (agro-)ecosystem functioning.

1. Introduction

Floral visual signals are vital for plants to attract pollinators, that in turn are needed for reproduction in the vast majority of angiosperms. The number, size and colouration of flowers determine a flower's visual display and how well pollinators can detect flowers (Ohashi and Yahara, 2001; Spaethe et al., 2001; van der Kooi et al., 2019). Abiotic factors can alter floral displays in various ways. At the macro-ecological level, abiotic stressors, such as drought or solar radiation, may select for particular flower colours (Arista et al., 2013; Dalrymple et al., 2020; Koski et al., 2020; Verloop et al., 2020). At the population or individual plant level, abiotic factors can also affect floral displays and reproductive fitness (Strauss and Whittall, 2006; Koski and Ashman, 2015; Caruso et al., 2019; Sapir et al., 2021). Particularly the effects of drought are known to impair production of floral displays and plant reproduction (reviewed by Descamps et al., 2021). For example, Gallagher and Campbell (2017) showed that in *Mertensia ciliata* a limited water availability decreased floral display size (i.e., the number of open flowers per plant), nectar production, visits by pollinating bumblebees and seed set. In other species reduced water availability also led to a decreased nectar quality and quantity, and a decreased pollen quality (Descamps et al., 2018; Göttlinger and Lohaus, 2020; Wilson Rankin et al., 2020). Such reductions in the quality and/or quantity of floral resources may cascade to pollinators that rely on these resources (e.g., Wilson Rankin et al., 2020).

In natural as well as agricultural conditions, nutrient deficiency is a common abiotic stressor, but how floral displays are affected by nutrient deprivation remains unclear. Several studies investigated how high and low nutrient conditions change floral displays (e.g., Caruso et al., 2005; Friberg et al., 2017), but as these studies generally used nutrient mixtures, the importance of individual nutrients cannot be disentangled. The impacts of nitrogen and phosphorus availability were studied to some degree, and these nutrients may potentially affect floral traits in species-specific ways (Lau and Stephenson, 1993, 1994; Poulton et al., 2002; Muñoz et al., 2004; Burkle and Irwin, 2009; Sletvold et al., 2016; Majetic et al., 2017; David et al., 2019). Whether and how nutrient

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deficiency changes flower colour remains largely unstudied (but see Majetic et al., 2017).

Sulphur is a vital nutrient that is widely limiting plant growth in agricultural and natural areas. Plants mainly acquire sulphur via the root as sulphate, which is subsequently used for the synthesis of proteins and other essential organic compounds (reviewed by Takahashi et al., 2011). In regions across the globe, plants suffer from sulphur deficiency. For instance, various subtropical and tropical ecosystems (e.g., savannas) experience sulphur deficiency (Pasricha and Fox, 1993), and modelling studies estimated that temperate forest ecosystems require more sulphur than what is available in the soil (Johnson, 1984; Zhao et al., 2008). Schnug and Haneklaus (1994) stated that as much as 50 % of forests in Germany may suffer reduced plant growth due to limited sulphur availability. Similar to natural ecosystems, agricultural plants are often prone to sulphur deficiency (Schnug and Haneklaus, 1994, 2005; Zhao et al., 2008). For example, 50 % of agricultural land across longitudinal transects in the United States appeared sulphur deficient (Fox et al., 2006) and in Germany there are virtually no unfertilised fields that supply sufficient sulphur to rapeseed, *Brassica napus* (Schnug and Haneklaus, 1994). Sulphur fertilisation of field-grown *B. napus* and *Triticum aestivum* in the United Kingdom increased seed yield by 40–50 % indeed (Blake-Kalff et al., 2000). However, since sulphur fertilisers are still not sufficiently applied, deficiency problems are lingering (Ausma and De Kok, 2019).

In this experimental study, we investigate how sulphur deficiency influences floral displays and pollen traits in *Brassica rapa* (Brassicaceae) and *Physalis philadelphica* (Solanaceae) that both have yellow flowers, and in three *Petunia* species (Solanaceae) with purple, red and white flowers. Based on anecdotal field observations that suggested that sulphur-deprived *B. napus* plants feature aberrant floral displays (Schnug and Haneklaus, 2005; Haneklaus et al., 2007), we predict that insufficient sulphur supply will limit plants to produce large, colourful floral displays. We additionally test whether the observed responses to sulphur deficiency are a general nutrient limitation response or specific for sulphur deficiency by subjecting plants to nitrogen and phosphorous limitation. We found that in all species sulphur – and not nitrogen or phosphorous – limitation severely reduces the number of flowers, changes flower dimensions, and alters the morphology and chemical profile of pollen. Sulphur limitation impairs carotenoid synthesis, which leads to a marked decrease in the colouration of yellow flowers.

2. Materials and methods

2.1. Plant material and growing conditions

A fast-flowering variety of *B. rapa* (cv. Fast Plants, University of Wisconsin-Madison, Wisconsin, USA) as well as non-domesticated varieties of *Ph. philadelphica, Petunia nyctaginiflora* (Radboud University Nijmegen, Nijmegen, The Netherlands), *P. exserta* (Plant World Seeds, Newton Abbot, UK) and *P. integrifolia* (B and T Seeds, Aigues-Vives, France) were germinated in vermiculite in a climate-controlled room. Air temperature was 23 °C (±1 °C), relative humidity was 60–70 % and the photoperiod was 16 h at a photon fluency rate of 300 ± 20 μmol m^{-2} s^{-1} (within the 400−700 nm range) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules.

After the formation of the first non-cotyledon leaves, which was after 7 days for *B. rapa* and after 28 days for the other species, seedlings were transferred to a 30 L container holding a 25 % Hoagland nutrient solution that was aerated (for details on solution composition, see Shahbaz et al., 2013). After the formation of the first flower buds, which was after 7 days, plants were transferred to a fresh 25 % Hoagland nutrient solution at 0.5 mM (+S, sulphur-sufficient) or 0 mM sulphate (-S, sulphur-deprived). For *B. rapa* we also investigated whether observed floral changes were specific for sulphur limitation or a general response to nutrient stress. We thus also transferred plants to solutions containing 0 mM nitrate (-N, nitrogen-deprived) or 0 mM phosphate (-P, phosphorus-deprived). The deprived solutions were made by substituting the respective anion with chloride. Nutrient solutions were refreshed weekly. Containers with plants were placed in a climate-controlled room with settings detailed above.

2.2. Nutrient deficiency

After 14–16 (nitrogen-deprived *B. rapa*) or 28–30 days (sulphur- and phosphorus-deprived *B. rapa* and sulphur-deprived *Petunia* spp. and *Ph. philadelphica*), it was verified that the plants had become nutrient deficient. Nutrient deprivation may alter the shoot-to-root ratio, in favour of the root, to optimise access to mineral nutrients (De Kok et al., 1997; Castro et al., 2008; Lopez-Arredondo et al., 2014; Ausma and De Kok, 2019). This may be associated with a (visible) decrease in leaf pigment content (De Kok et al., 1997; Gaude et al., 2007). Additionally, sulphate deprivation may decrease the content of the sulphur-containing amino acids cysteine and methionine, which commonly results in a lowered protein synthesis and consequently the accumulation of free, non-sulphur-containing, amino acids (De Kok et al., 1997). Prolonged nutrient deprivation may reduce plant growth (Castro et al., 2008; Lopez-Arredondo et al., 2014; Ausma and De Kok, 2019).

The shoot and root were separated, weighted and the shoot-to-root ratio was calculated by dividing shoot with root biomass. Additionally, fully-expanded leaves were frozen at −80 °C, ground with mortar and pestle and used for the determination of pigment and free amino acid levels. Chlorophyll *a*, *b* and carotenoids were extracted in cold 96 % ethanol:water (*v/v*). After centrifugation at 4 °C for 20 min at 3.000 rpm and a 1:10 dilution, pigment levels in the samples were quantified photometrically as per Lichtenthaler and Buschmann (2001). Free amino acids were extracted in cold demi-water. After deproteinization in a boiling water bath for 10 min and centrifugation at 4 °C for 15 min at 16.000 rpm, amino acid levels were quantified via their reaction with ninhydrin, following Rosen (1957).

2.3. Flower size and number of open flowers

Simultaneously with the analysis of nutrient deficiency, floral traits of most plants were analysed. Only the floral traits of the nitrogen-deprived plants were analysed one week after the determination of these plants' nutrient deficiency. Flower length, flower width and corolla tube length were measured using a digital calliper on flowers that were 2–3 days old (this also holds for the flower colour and pollen analyses detailed below). The total number of open flowers per plant was also counted.

2.4. Flower colour

Reflectance spectra of flowers were obtained with a bifurcated reflection probe, using a deuterium-halogen lamp (AvaLight-D(H)-S, Avantes, Apeldoorn, The Netherlands) as a light source and a white tile (Avantes WS-2) as a reference. The probe illuminated an area with a diameter of ~1 mm and captured the reflected light in a small spatial angle. For the figures, the reflectance spectra were normalised in the long wavelength range, to enable comparing the modulation of the spectrum.

Flowers of *Brassica* species are commonly pollinated by bees and flies (Steffan-Dewenter, 2003; Rader et al., 2013; Doyle et al., 2020). To interpret the reflectance spectra of these plants with a "pollinator-subjective view", we applied two established vision models - the colour hexagon (Chittka, 1992) and the receptor noise-limited model (Vorobyev and Osorio, 1998) - using honeybee spectral sensitivities (van der Kooi et al., 2021a) and common daylight (D65). Achromatic (green) contrast was calculated as the green photoreceptor excitation difference between the flower and background (Spaethe et al., 2001; van der Kooi et al., 2019). Non-normalised spectra were used for the vision modelling. The resulting model values scale with a flower's contrast against a

green background as perceived by bees under ambient daylight.

2.5. Pigment composition

Total carotenoid levels were quantified using a photometer (Hitachi, U-3000, Tokyo, Japan), following Lichtenthaler and Buschmann (2001); violaxanthin and lutein levels were determined using high-performance liquid chromatography (HPLC), following an adapted version of the method of Pfeifhofer (1989). −80 °C frozen petals were lyophilized in the dark for 24 h. Lyophilized petals were next immersed in 500 μL cold 90 % acetone:water (*v/v*) and one ~2 mm steel bead was added. The samples were then homogenized in a mixer mill (Retsch MM400, Haan, Germany) at 30 Hz frequency for 2 min, incubated at 4 °C for 30 min, homogenized in the mixer mill for 30 s, incubated at 4 °C for 1 h and finally shaken in an overhead shaker at 4 °C for 1 h at 5 rpm (all in the dark). After centrifugation at 4 °C for 30 min at 14.000 rpm, 140 μL supernatant was saponified in the dark. After evaporating the samples by a 15 min exposure to a gentle N_2 flow, samples were resuspended in 300 μL 2.5 % methanolic KOH (*w/v*), vortexed (*viz.* 30 s at 1.400 rpm), incubated in an ultrasonic bath for 10 min and incubated at room temperature for 2 h. Next, 600 μL cold *n*-hexane was added and the samples were vortexed. Phase separation was achieved by adding 300 μL saturated NaCl. Samples were subsequently vortexed and centrifuged at 22 °C for 10 min at 14.000 rpm. The upper *n*-hexane phase was collected and the lower phase was treated again with 300 μL *n*-hexane. The *n*-hexane phases were then combined and washed three times with ultrapure water by vortexing. Neutral pH in the final washing water proved sufficient washing. After the washed *n*-hexane was evaporated by a 7 min exposure to a gentle N_2 flow, samples were resuspended in 350 μL 90 % acetone:water (*v/v*) by a brief mixing and a 10 min incubation in an ultrasonic bath. After an overnight incubation at 4 °C, samples were incubated for 5 min in the bath, vortexed and centrifuged at 4 °C for 30 min at 14.000 rpm.

Carotenoids were separated using an Eurosphere II 100−5 250 × 4 mm C18 column (particle size 5 μm, Knauer, Berlin, Germany), equipped with a pre-column, which were kept at 28 °C. The autosampler chamber was kept at 8 °C. A linear solvent gradient was applied of solvent A, consisting of 20:2:1 acetonitrile:water:methanol (*v/v/v*) and B, consisting of 2:1 acetone:ethyl acetate (*v/v*). Solvents were filtered through 0.45 μm Teflon before use. The linear mobile phase gradient increased from 10 % to 80 % solvent B within 20 min. The mobile phase was then isocratic at 80 % B for 6 min, followed by returning to 10 % B within 1 min and an equilibration for 9 min. The flow rate was 1.0 mL min^{-1}. For each sample 20 μL was injected under the control of an external computer. Carotenoids were detected using diode-array spectroscopy at 445 nm (UltiMate 3000, ThermoFischer Scientific/Dionex, Waltham, MA, USA). Data were recorded and processed by Chromeleon software (version 6.80). Violaxanthin and lutein contents were quantified using standards (DHI Lab Products, Hørsholm, Denmark), kindly provided by Dr Willem H. van de Poll (Energy and Sustainability Research Institute, University of Groningen). These contents were corrected for losses during saponification by comparing total carotenoid levels before and after saponification.

2.6. Size and nutrient composition of pollen

Anthers were collected and subsequently dried at room temperature. After pollen were released from the dried anthers via centrifugation and suspended in a 1:1 water:glycerol mixture, pollen were observed with a Nikon Diaphot 300 Inverted Microscope and photographed using a Nikon D3200 digital camera (at a 100x magnification). For the determination of pollen diameter, per plant, 5–10 randomly-picked pollen were measured using ImageJ's Fiji, and their average diameter was regarded as one biological replicate in figures and statistics.

To assess pollen mineral composition, pollen from different plants were pooled per treatment per species. The resulting pooled sample was digested overnight in concentrated HNO_3 and minerals in the supernatant were quantified with an Agilent 7700 inductively coupled plasma mass spectrometer (ICP-MS, Agilent Technologies, Santa Clara, CA, USA, Almario et al., 2017).

2.7. Statistics

Data was checked for normality using a Shapiro-Wilk test at the $P \leq 0.05$ level. The treatment means of normally- and not-normally-distributed data were compared using, respectively, an independent two-tailed Student *t*-test and a Wilcoxon signed-rank test at the $P \leq 0.05$ level. Reported sample sizes refer to individual plants per treatment (except for the pooled pollen sample of the mineral nutrient analysis).

3. Results

3.1. Verification of nutrient deprivation

Nutrient deprivation resulted in a significantly decreased shoot-to-root ratio in all species and a decreased total plant biomass in *B. rapa* and *Ph. philadelphica* (Figs. S1 and S2). Sulphate deprivation further strongly decreased total chlorophyll and carotenoid contents in the leaves of all plants (Tables S1 and S2). Phosphate deprivation of *B. rapa* also lowered total chlorophyll levels (Table S1). The chlorophyll *a*:*b* ratio was furthermore decreased in sulphate-deprived *Ph. philadelphica* and *P. exserta* and nitrate-deprived *B. rapa* (Tables S1 and S2). Upon sulphate deprivation amino acid levels in the foliage were enhanced in all species, except for *B. rapa* (Tables S1 and S2). These results show that the plants were (mildly) nutrient deprived.

3.2. Sulphur deprivation drastically reduces floral display size

Sulphur depletion decreased the number of open flowers per individual plant (Figs. 1 and 2). The number of open flowers was reduced by 35 % in *B. rapa*, and reductions up to 80 % and 90 % were observed for *P. nyctaginiflora* and *Ph. philadelphica*. A time-series analysis in *B. rapa* indicated that this decrease was not due to sulphur-deprived plants flowering earlier than sulphur-sufficient ones (Table S3). The length and width of sulphur-deprived *B. rapa* petals were approximately 35 % reduced compared to those of sulphur-sufficient plants (Fig. 1). Consequently, sulphur-deprived *B. rapa* featured an as much as 60 % reduction in individual flower size. Individual flower size was also reduced by sulphur deficiency in *P. integrifolia* and *P. nyctaginiflora* by approximately 25 % and 20 %, respectively (Fig. 2). Furthermore, whereas sulphur-deficient *P. exserta* plants featured a 10 % shorter corolla tube, the corolla tube in *P. integrifolia* was 20 % increased and that in *P. nyctaginiflora* unaffected (Fig. 2). Sulphur deprivation finally resulted in reductions of pollen size by 10–30 % (Figs. 1 and 2) and an altered pollen's mineral nutrient composition (Table S4). In addition to profoundly reducing pollen sulphur and potassium contents across all species, sulphur deprivation consistently and strongly enhanced molybdenum contents (Table S4).

To investigate whether observed changes were a general response to nutrient stress or specific to sulphur deficiency, we tested how nitrogen and phosphorous deficiency impact floral traits in *B. rapa*. Phosphorus deprivation (slightly) increased the number of open flowers, but nitrogen- and phosphorus deprivation hardly affected flower and pollen morphology (Fig. 1).

3.3. Sulphur deprivation reduces carotenoid pigment content

The yellow flowers of sulphur-deprived *B. rapa* and *Ph. philadelphica* plants were visibly paler (Figs. 3 and 4). Reflectance spectra of the yellow flowers were more variable and showed a much weaker modulation, which is a sign of a decreased concentration of (carotenoid) pigments (van der Kooi, 2021). In the ultraviolet and blue wavelength

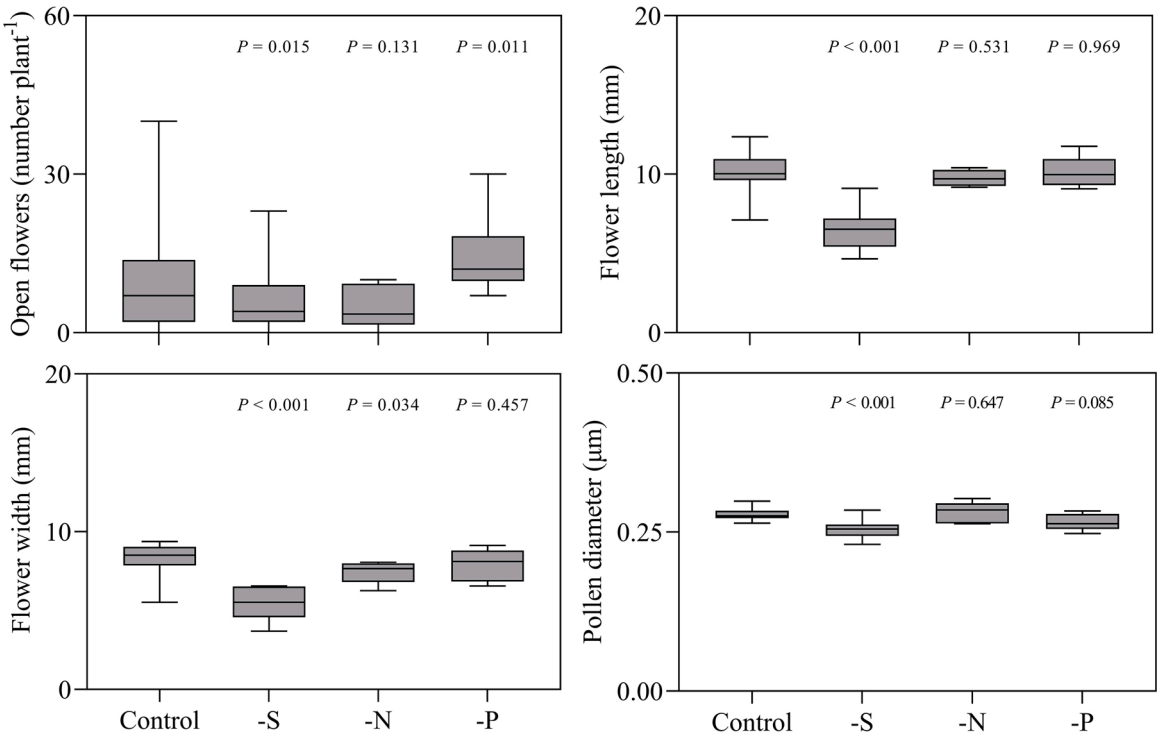

Fig. 1. Impact of sulphur, nitrogen and phosphorus deprivation on flower traits of *B. rapa*. Sample sizes are 5-15 (flower size), 10-78 (number of open flowers) and 5-10 (pollen diameter). *P*-values were obtained by comparing the control (nutrient-sufficient) treatment with the -S, -N and -P treatment.

ranges (300−500 nm), reflectance spectra of sulphur-deficient flowers were much more variable than in the control treatment (Figs. 3 and 4). The spectra of the white, purple and red *Petunia* flowers were virtually identical between the treatments, though in *P. exserta* there may be a small difference between the treatments (Fig. 4). The decreased petal yellowness was a specific response to sulphur deprivation, because nitrogen- and phosphorus deprivation did not affect petal reflectance spectra (Fig. 3). To assess how sulphur-deprived and sulphur-sufficient yellow petals differ in pigment composition, we quantified carotenoid levels in *B. rapa* petals. Sulphur deprivation resulted in a 45 % decreased total carotenoid level, and this decrease was, at least partly, due to decreased violaxanthin and lutein levels (Fig. 5).

Vision modelling revealed that flowers of sulphur-deprived *B. rapa* plants were much less colourful in the eyes of their pollinators than flowers of sulphur-sufficient plants. Colour contrast as per the hexagon model was 0.21 versus 0.11 hexagon units for sulphur-sufficient and -deprived *B. rapa* petals, respectively ($W = 3$, $P < 0.001$, $n = 10$). Colour contrast as per the receptor-noise-limited model was 3.3 versus 1.9 RNL units for sulphur-sufficient and -deprived *B. rapa*, respectively ($W = 0$, $P < 0.001$, $n = 10$). Achromatic (green) contrast, which can be important for long-distance attraction (Spaethe et al., 2001; van der Kooi et al., 2019), was also affected: 0.23 versus 0.17 for sulphur-sufficient and sulphur-deprived *B. rapa*, respectively ($W = 11$, $P = 0.002$, $n = 10$).

4. Discussion

In natural and agricultural regions across the globe, plant growth is limited by sulphur deficiency (Johnson, 1984; Pasricha and Fox, 1993; Blake-Kalff et al., 2000; Schnug and Haneklaus, 1994, 2005; Fox et al., 2006; Zhao et al., 2008), but the consequences of sulphur limitation for floral traits are poorly understood. We investigated how sulphur deprivation changes floral visual traits and pollen traits in rapeseed (*B. rapa*) and in tomatillo (*Ph. philadelphica*), two widely grown crop species, as well as in three *Petunia* species.

A suite of floral traits was negatively impacted by sulphur deficiency. The observed trait changes are a specific response to sulphur deficiency – and not a general nutrient deficiency response – given that nitrogen- and phosphorus-deprived plants did not show similar changes (Figs. 1 and 3). Additionally, since the nitrogen-deprived plants exhibited oxidative stress, as indicated by a strongly reduced chlorophyll *a*:*b* ratio (Table S1), the flower trait changes upon sulphur deficiency cannot be attributed to oxidative stress disrupting metabolism, meaning that imposed sulphur stress was comparatively mild.

The aberrant floral displays and pollen associated with sulphur limitation reduce the plant's attractiveness to pollinators, which likely has repercussions for plant reproduction and pollinator fitness. One of our main findings is that sulphur limitation dramatically reduced floral display size in all studied species (Figs. 1 and 2). Further, sulphur limitation led to aberrant flower morphologies and up to 60 % reductions in individual flower size. A reduction in the number and size of flowers is a commonly observed response to abiotic stress (Caruso et al., 2005; Majetic et al., 2017; Descamps et al., 2021). Large flowers generally receive more visits by pollinators than small flowers (reviewed by Ohashi and Yahara, 2001), and reductions in flower size drastically increase the time that bees need to detect a flower (Spaethe et al., 2001). The observed changes in the (relative) dimensions of the flower (length, width and corolla tube depth) may have consequences for the mechanical fit of pollinators and flowers as well as the way pollen are placed onto the pollinator's body (Minnaar et al., 2019; van der Kooi et al., 2021b). Flower production is hormonally controlled with e.g., auxin, ethylene and cytokines, enhancing floral display size and flower size (Krizek and Anderson, 2013). Whereas in vegetative tissues sulphur deprivation may upregulate auxin and ethylene production and downregulate cytokinin levels (Wawrzynska et al., 2015; Koprivova and Kopriva, 2016), the impact of sulphur deprivation on hormone profiles at the (pre-)flowering stage should be studied to understand how sulphur deprivation impacts flower development.

An intriguing observation is that sulphur limitation led to a significantly paler flower colour in *B. rapa* and *Ph. philadelphica*, which have carotenoid-based pigmentation (Figs. 3 and 4), but not in plants with other flower colours (Fig. 4). Sulphur-deprived *B. rapa* petals featured a significantly decreased carotenoid content, which was partly due to

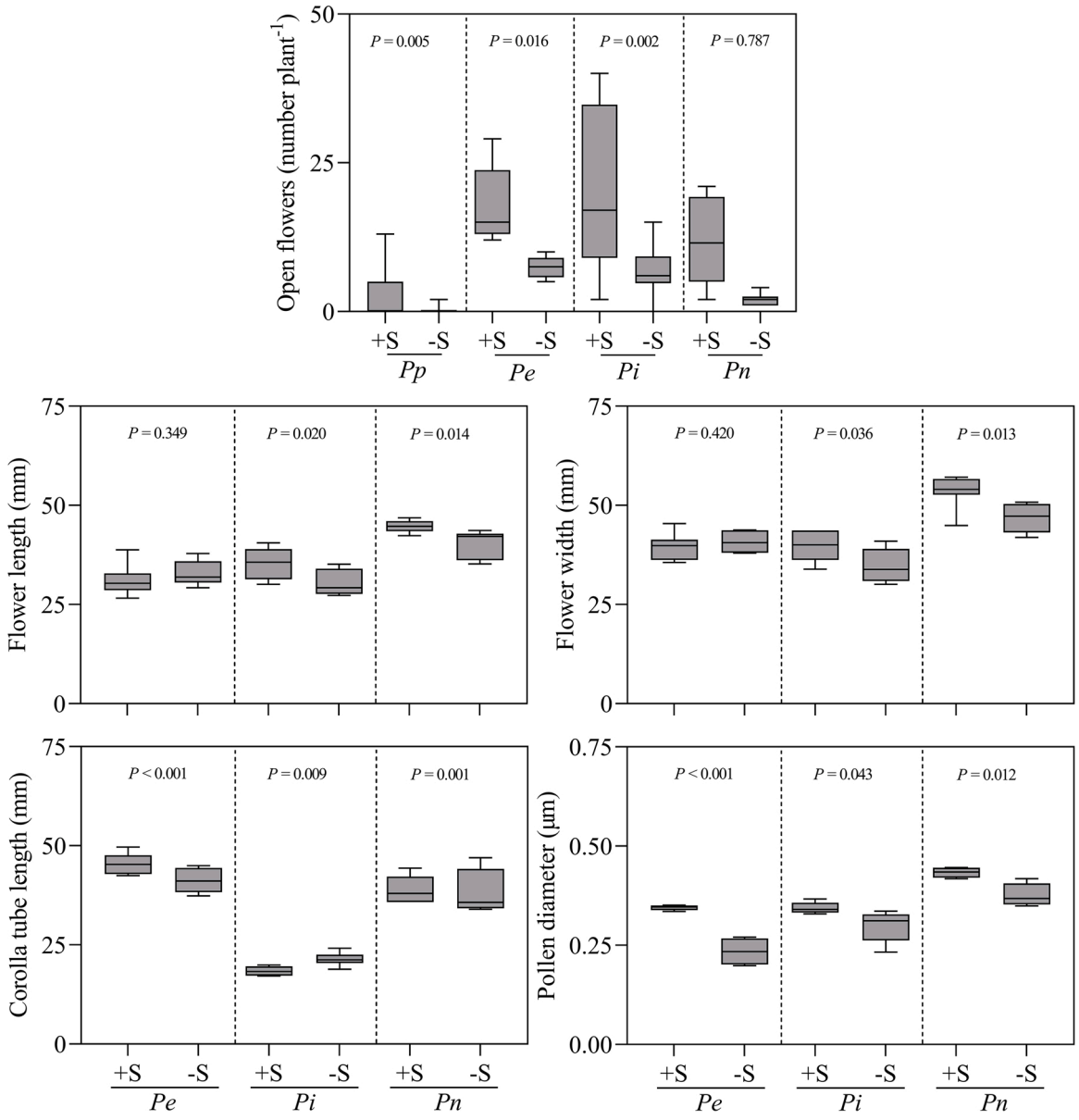

Fig. 2. Impact of sulphur deprivation on flower traits of *Ph. philadelphica* (*Pp*), *P. exserta* (*Pe*), *P. integrifolia* (*Pi*) and *P. nyctaginiflora* (*Pn*). Sample sizes are 6-7 (flower and corolla size), 9-23 (number of open flowers) and 4-5 (pollen diameter).

decreased violaxanthin and lutein contents (Fig. 5). Variation in these carotenoid levels has previously been associated with petal colour variation (Zhang et al., 2015, 2020). The observed fading of flower colour reduces visibility to pollinators, as suggested by the strong reductions in colour contrast (Results), which scale with contrast as perceived by pollinators (Dyer and Chittka, 2004; Spaethe et al., 2001; van der Kooi et al., 2019). In addition to reducing the flower's detectability, sulphur deprivation may also provoke bees to confuse unpollinated, sulphur-deficient flowers for pollinated, sulphur-sufficient ones, at least in *B. rapa*, the flowers of which fade and shrink after pollination (Schnug and Haneklaus, 2005). Our results may explain why in field experiments with ample pollinators, sulphur-deprived *B. napus* plants exhibit much lower seed set (Haneklaus et al., 2005; Schnug and Haneklaus, 2005).

The flowers of the studied *Petunia* species that contain various types of flavonoids were not markedly different between sulphur-sufficient and -deprived conditions (Fig. 4). For the red-flowered *P. exserta*, a very small effect of sulphur deficiency on colouration cannot be ruled out (Fig. 4), although the colour difference was much smaller than for yellow-flowered *B. rapa* and *Ph. philadelphica*. As opposed to *P. integrifolia* and *P. nyctaginiflora*, *P. exserta* flowers contain significant levels of delphinidin (Murakami et al., 2002) and, perhaps sulphur deprivation slightly affected the homeostasis of this anthocyanin. The finding that flavonoid pigmentation was hardly affected by sulphur deficiency is in line with a study where nitrogen depletion did not affect the flower colour of *Petunia hybrida* (Majetic et al., 2017), and is probably is related to flavonoid-based pigmentation being regulated by different genetic pathways and cellular processes, such as vacuolar pH, than carotenoid-based pigmentation (Mol et al., 1998; Stavenga et al., 2021).

A remaining open question is why particularly carotenoid-based pigmentation is impacted so strongly by sulphur limitation. Sulphur deprivation is known to significantly hamper lipid synthesis, likely because acetyl-CoA and the acyl carrier protein, two sulphur-containing molecules, are involved in lipid production (De Kok et al., 1997; Nikiforova et al., 2005). Carotenoids are synthesised and stored in plastids, which are lipid-rich organelles and hence restrictions in lipid production may result in restrictions in plastid biogenesis (Tanaka et al., 2008; Hölzl

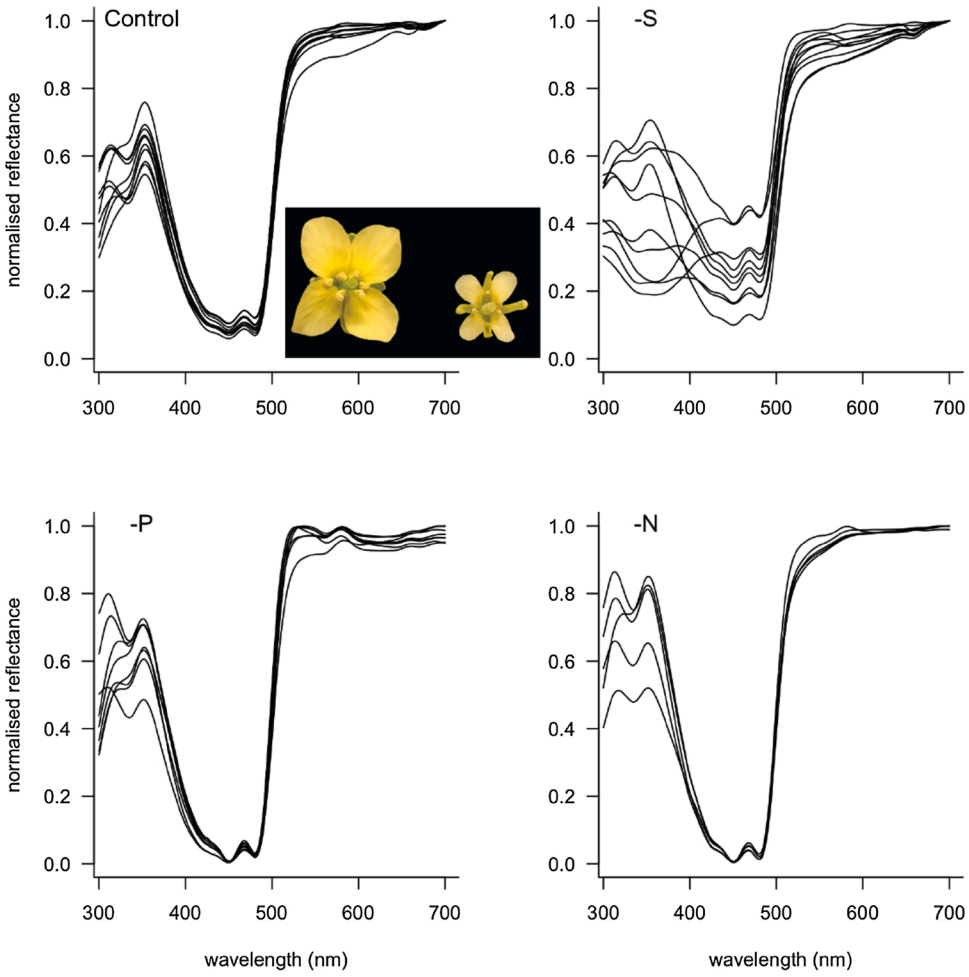

Fig. 3. Impact of sulphur, nitrogen and phosphorus deprivation on flower colours of *B. rapa*. The picture in the top left panel shows a flower in the control (nutrient-sufficient; left) and in the sulphur-deprived treatment (right). Flowers in the phosphorous- and nitrogen-limitation treatment were visibly indifferent from the control flowers.

and Dörmann, 2019). Interestingly, in sulphur-deprived vegetative leaves carotenoid (and chlorophyll) levels are usually also lower (Table S1 and S2; De Kok et al., 1997), despite the absence of transcriptional downregulation of carotenoid biosynthesis genes (Dietzen et al., 2020). By contrast, the content of anthocyanins, which are synthesized in the cytosol and stored in vacuoles, is generally unaffected or even enhanced in sulphur-deprived leaves (Nikiforova et al., 2005; Tanaka et al., 2008).

The quantity of pollen and nectar per flower may be similar in sulphur-sufficient and sulphur-deprived plants (Haneklaus et al., 2005), but sulphur-deprived plants featured smaller pollen with an altered mineral nutrient composition (Figs. 1 and 2; Table S4). These changes likely are detrimental for the plant and for pollinators that rely on pollen as resource. Pollen size is considered to positively correlate with energy storage, and hence with pollen fitness (Lau and Stephenson, 1993, 1994; Sarkissian and Harder, 2001; Cruden, 2009), meaning that smaller pollen may be less fertile. The reductions in pollen sulphur content upon sulphur deprivation may further lower pollen fertility (Zechmann et al., 2011). Pollinators require a specific ratio of mineral nutrients in their (pollen) food (Roulston and Cane, 2000; Kaluza et al., 2018; Filipiak, 2019; Ruedenauer et al., 2019; Vaudo et al., 2016) and sulphur deprivation affected these ratios in pollen, since it drastically decreased the pollen sulphur and potassium content across different species (Table S4). Experiments with *Osmia bicornis* demonstrated that bee fitness is particularly affected by low amounts of minerals – including potassium (Filipiak, 2019) – suggesting that sulphur deprivation indeed negatively affects pollinators.

The observed changes in mineral composition of pollen mimic those in vegetative tissues (Maillard et al., 2016; Reich et al., 2016; Zuidersma et al., 2019). The increased molybdenum content in pollen upon sulphur deprivation may arise from the chemical analogy between sulphate and molybdate (Maillard et al., 2016; Zuidersma et al., 2019). The activity of sulphate transporters is upregulated upon sulphate deprivation and these transporters may transport molybdate, which may explain the sulphate-deprived increase in molybdate levels (Maillard et al., 2016; Reich et al., 2016; Zuidersma et al., 2019). Furthermore, sulphate contents decrease upon sulphur deprivation. Sulphate is an anion and sulphate-deprived plants may show reduced contents of cations, including potassium, to preserve charge balance (Reich et al., 2016).

Sulphur deficiency probably also causes changes in floral scent (Bloem et al., 2010), further reducing plant reproductive success. Various sulphur-containing volatiles in floral scents, including dimethyl sulphide (DMS), are thought to attract pollinators (von Helversen et al., 2000; Shuttleworth and Johnson, 2010). Interestingly, in *Zea mays* seedlings, the availability of substrates for DMS production by the foliage drastically decreased upon sulphur deprivation (Ausma et al., 2017; T. Ausma and J. Stefels, unpublished data), so it is likely that DMS emissions similarly reduce.

In summary, sulphur deprivation, which occurs in natural and agricultural regions worldwide, drastically alters floral visual displays and pollen traits. This will likely reduce the reproductive output of plants and it may also reduce pollinator fitness. The observations that (mild)

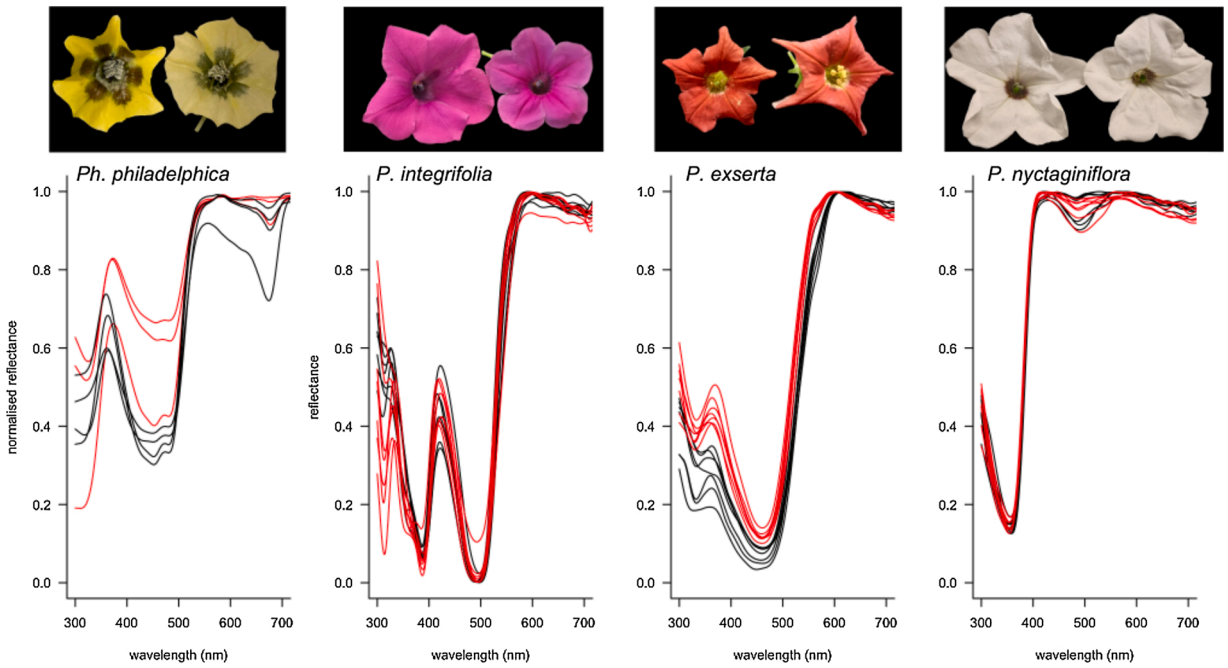

Fig. 4. Impact of sulphur deprivation on the flower colours of *Ph. philadelphica*, *P. exserta*, *P. integrifolia* and *P. nyctaginiflora*. Reflectance spectra of sulphur-sufficient flowers are shown in black curves and sulphur-deprived flowers in red curves. The pictures above each graph show a sulphur-sufficient (left) and -deprived (right) flower for each species (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

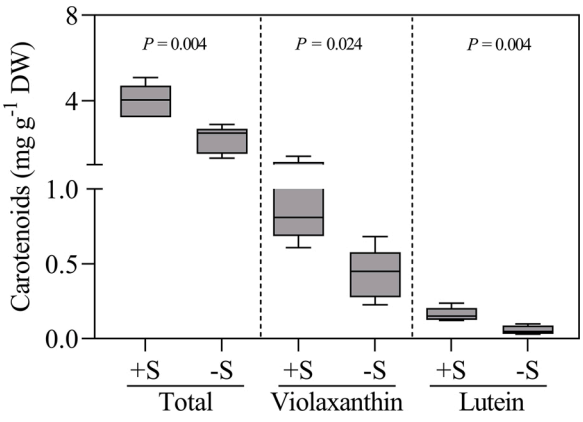

Fig. 5. Impact of sulphur deprivation on the total carotenoid, violaxanthin and lutein content of *B. rapa* petals. Sample size was 5.

sulphur deprivation has such clear effects on floral displays and that the effects on colour depend on the type of floral pigment, open new avenues for studies on how nutrient deprivation affects the regulatory pathways of flower (colour) production. Finally, our study paves the way for studies on how sulphur deprivation affects the fitness of plants, pollinators and organisms at different trophic levels, so to eventually obtain a better understanding of the bottom-up impact of nutrient deficiency on whole (agro-)ecosystem functioning.

Authors' contributions

T.A., V.B., L.J.D.K. and C.J.v.d.K. conceived and designed the study. T.A., V.B., M.K., A.C.M.V., A.G., M.M. and S.K. collected and analyzed the data. T.A. and C.J.v.d.K. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2021.104656.

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