Pollen limitation and xenia effects in a cultivated mass-flowering tree, *Macadamia integrifolia* (Proteaceae)

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- **Background and Aims** Pollen limitation is most prevalent among bee-pollinated plants, self-incompatible plants, and tropical plants. However, we have very little understanding of the extent to which pollen limitation affects fruit set in mass-flowering trees despite tree crops accounting for at least 600 million tons of the 9,200 million tons of annual global food production.

- **Methods** We determined the extent of pollen limitation in a bee-pollinated, partially self-incompatible, subtropical tree by hand cross-pollinating the majority of flowers on mass-flowering macadamia (*Macadamia integrifolia*) trees that produce about 200,000–400,000 flowers. We measured tree yield and kernel quality and estimated final fruit set. We genotyped individual kernels by MassARRAY to determine levels of outcrossing in orchards and assess paternity effects on nut quality.

- **Key Results** Macadamia trees were pollen limited. Supplementary cross-pollination increased nut-in-shell yield, kernel yield and fruit set by as much as 97%, 109% and 92%, respectively. The extent of pollen limitation depended upon the proximity of experimental trees to trees of another cultivar because macadamia trees were highly outcrossing. Between 84% and 100% of fruit arose from cross-pollination, even at 200 m (25 rows) from orchard blocks of another cultivar. Large variations in nut-in-shell mass, kernel mass, kernel recovery and kernel oil concentration were related to differences in fruit paternity, including between self-pollinated and cross-pollinated fruit, thus demonstrating pollen-parent effects on fruit quality, i.e. xenia.

- **Conclusions** This study is the first to demonstrate pollen limitation in a mass-flowering tree. Improved pollination led to increased kernel yield of 0.31–0.59 tons per hectare, which equates currently to higher farm-gate income of approximately $US3,720–$US7,080 per hectare. The heavy reliance of macadamia flowers on cross-pollination and the strong xenia effects on kernel mass demonstrate the high value that pollination services can provide to food production.

Key words: Breeding system, cross-pollination, kernel, macadamia, *Macadamia integrifolia*, mating system, nut, pollen limitation, pollination, Proteaceae, self-incompatibility, xenia.

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INTRODUCTION

Most flowering plants are pollinated by animals (Ollerton *et al*., 2011) and many major crops are highly dependent on pollinators for optimal fruit or seed production (Thomson and Goodell, 2001; Klein *et al*., 2007, 2018; Aizen *et al*., 2009; Perrot *et al*., 2019). Global declines in wild bee populations are placing pressure on food production and the supply of managed bee hives may not be keeping pace with the demand for agricultural pollination services (Aizen and Harder, 2009; Gallai *et al*., 2009; Potts *et al*., 2016; Klein *et al*., 2018; Patel *et al*., 2021). Anthropogenic disturbance is limiting plant reproductive output by reducing the quantity or quality of pollen deposited on the stigma of flowers (Bennett *et al*., 2020). Pollen limitation is related to the intensity of human land use and is greatest in urban landscapes but also evident in managed and natural landscapes (Bennett *et al*., 2020). Pollen limitation tends to be most severe for species that rely on one pollinator (Knight *et al*., 2005; Bennett *et al*., 2020) or, in natural landscapes, for species that are pollinated exclusively by bees (Bennett *et al*., 2020). Pollen limitation is also more prevalent in tropical than temperate species (Larson and Barrett, 2000) and more prevalent in self-incompatible than self-compatible species (Burd, 1994; Larson and Barrett, 2000; Knight *et al*., 2005).

The standardized approach to quantifying pollen limitation is to compare fruit set or seed set between flowers receiving natural pollination and flowers receiving supplementary pollination (Burd, 1994; Larson and Barrett, 2000; Ashman *et al*., 2004; Knight *et al*., 2005, 2006; Aizen and Harder, 2007; Wesselingh, 2007; Bennett *et al*., 2020). Experimental studies ideally hand-pollinate and assess fruit set or seed set from most of the flowers on a plant, because maternal resources for fruit or seed set could be reallocated from naturally-pollinated flowers to hand-pollinated flowers if only a small subset of flowers received supplementary pollination (Zimmerman and Pyke, 1988; Ashman *et al*., 2004; Knight *et al*., 2006; Wesselingh, 2007). Pollen supplementation is usually only feasible on most flowers if the

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plant is small or produces few flowers. Pollen supplementation on most flowers has almost never been attempted for tree species (Knight *et al*., 2006). Instead, pollen supplementation is usually performed on a small subset of flowers on the tree, with the flowers sometimes separated on different branches to try to minimize resource reallocation between different parts of the tree (Russell *et al*., 1998; Wesselingh, 2007; Chacoff *et al*., 2008; Pearse *et al*., 2015; Khanduri *et al*., 2019). This type of spatial separation might be effective experimentally if the branches function as independent physiological units (Watson and Casper, 1984; Wesselingh, 2007). However, fruit set, even on many evergreen tropical and subtropical trees, is supported from stored carbohydrates in the trunk and roots, and so their branches cannot be considered as completely independent physiological units (Scholefield *et al*., 1985; Trueman and Turnbull, 1994b; Stassen and Janse Van Vuuren, 1997; Hieke *et al*., 2002; Dovis *et al*., 2014). Pollen limitation in tree species is, therefore, poorly understood despite tree crops (excluding palm oil) accounting for at least 600 million tons of the 9,200 million tons of annual global food production (International Nut & Dried Fruit Council, 2019; FAO, 2020; Statista, 2021).

Mass-flowering trees often produce many more female or hermaphrodite flowers than the number of fruit that could be supported from the available maternal resources (Stephenson, 1981; Trueman and Turnbull, 1994b; Amin *et al*., 2015; Li *et al*., 2015; Pearse *et al*., 2015; Boldingh *et al*., 2016). Mass flowering could function, for example, to attract pollinators to the tree or allow the selective abortion of genetically inferior fruitlets (Stephenson, 1981; Burd, 1998). Most fruitlet abscission occurs during the first half of fruit development (Stephenson, 1981; Trueman and Turnbull, 1994a,b; Trueman and Wallace, 1999; Mahouachi *et al*., 2009; Boldingh *et al*., 2016; Garner and Lovatt, 2016), possibly representing an adaptive adjustment of crop load prior to the main period of maternal resource investment in fruit growth. Farmers who produce fruit on mass-flowering trees refer to this fruitlet

abscission as "premature fruit drop". They sometimes cite premature fruit drop as evidence that yield is not pollen-limited, contending that trees are setting more fruitlets than could be supported to maturity from the maternal plant resources. Macadamia farmers, for example, report more-easily observable factors such as pests, diseases, tree health and adverse climatic conditions as the major limitations to yield (Department of Agriculture & Fisheries, 2019). However, heavy fruitlet abscission does not necessarily demonstrate that trees are not pollen limited, because final fruit set could still be related to the number of successful pollination events on a tree even though many fruitlets are shed during the period of premature fruit drop.

Supplementary pollination experiments that incorporate most flowers on a plant have not been feasible for mass-flowering fruit trees because of the challenges in hand-pollinating flowers on multiple replicate trees, which could each be producing hundreds of thousands of flowers. However, cultivated trees of macadamia (*Macadamia integrifolia*, *M. tetraphylla* and hybrids) provide a rare opportunity to investigate pollen limitation on whole mass-flowering trees. Macadamia is an evergreen subtropical tree that produces up to 3,500 cylindrical racemes annually (Moncur *et al*., 1985; Moncur, 1988; McFadyen *et al*., 2011; Olesen *et al*., 2011), each bearing between 100 and 300 flowers (Storey, 1985; Joubert, 1986; Trueman and Turnbull, 1994a,b; Grass *et al*., 2018). Flowering occurs *en masse*, more-or-less simultaneously across cultivars, usually in late winter or early spring (Nagao *et al*., 1994; Trueman and Turnbull, 1994a,b; Wallace *et al*., 1996; Wilkie *et al*., 2009). Macadamia flowers are bee-pollinated, mainly by honey bees (*Apis mellifera*) but also, in Australia, by stingless bees (*Tetragonula* spp.) (Urata, 1954; Heard, 1994; Wallace *et al*., 1996; Howlett *et al*., 2015; Grass *et al*., 2018; Willcox *et al*., 2019). The flowers are partially self-incompatible, with greater pollen tube growth and higher fruitlet set after cross-pollination by pollen from a different cultivar than self-pollination by pollen from the same cultivar

(Sedgley, 1983; Sedgley *et al*., 1990; Meyers *et al*., 1995; Sacramento *et al*., 1999; Howlett *et al*., 2019). Each flower contains two ovules but only one pollen tube reaches the ovary and only one seed is formed (Sedgley, 1981, 1983; Trueman and Turnbull, 1994a). We developed a test-tube method to pollinate macadamia racemes manually and have shown that supplementary cross-pollination of individual racemes on a tree increases pollen deposition, fruitlet production, final fruit set, nut mass and kernel mass (Trueman and Turnbull, 1994a; Wallace *et al*., 1996). This rapid pollination method provides an exceptional opportunity to test pollen limitation, and the potential consequences for tree yield, by hand-pollinating the majority of flowers on a mass-flowering tree.

We hypothesized that macadamia, as a subtropical, bee-pollinated, and partially self-incompatible tree, would be pollen limited. We aimed to determine how manually cross-pollinating the majority of flowers on macadamia trees would affect nut yield, kernel yield and nut quality when compared with control trees that received natural levels of orchard pollination. We also aimed to determine the realized mating system of macadamia by performing progeny array analyses of the kernels using unique homozygous single nucleotide polymorphisms (SNPs) that we identified from each cultivar. These progeny array analyses also allowed us to assess relationships between individual nut paternity and nut quality. This study provides unique results on the extent of pollen limitation in a mass-flowering tree. The results also provide valuable information on the impacts of enhanced pollination on food production and food quality.

<div align="center">MATERIALS AND METHODS</div>

Study site

The experiment was conducted in a commercial macadamia orchard near Bundaberg, Queensland, Australia. The experimental site ($24°47'53''$ S $152°17'36''$ E) contained 11-year old trees of cultivars '816' and 'Daddow', planted in single-cultivar blocks that were 42 and

48 rows wide, respectively (Fig. 1). Further rows of 'Daddow' and then '816' trees were planted immediately west-south-west of the wide '816' block. Some storm-damaged trees in the orchard had been replaced by trees of other cultivars including '741'. Another orchard, which contained a wider range of macadamia cultivars, was located 1.2 km north-north-west of the experimental site. Honey bee hives had been introduced to the orchard prior to flowering (Fig. 2a) at a stocking rate of 5 hives per hectare, consistent with the recommended rate for Australian orchards of 5–8 hives per hectare (Department of Primary Industries, 2018).

Experimental design

We selected and tagged 20 trees of each cultivar ('816' and 'Daddow') in the first row adjacent to the other cultivar (Fig. 2a) and 20 trees of each cultivar in the twenty-fifth (25th) row away from the other cultivar. Macadamia trees are propagated clonally by grafting scions of a cultivar onto seedling rootstocks (Trueman, 2013). Pollen transfer within a cultivar, therefore, represents self-pollination whereas pollen transfer between cultivars represents cross-pollination. Cultivar '816' trees had mean ($\pm$ SE) trunk circumference at 10 cm above the graft union of 57.5 ± 1.0 cm and and height of 5.39 ± 0.08 m (n = 40). Cultivar 'Daddow' trees had trunk circumference of 52.0 ± 0.8 cm and height of 4.98 ± 0.07 m (n = 40). The first experimental tree in each row was the 5th tree from the northern end of the row. Racemes on the 5th, 15th, 25th… 95th tree in each row (and all non-experimental trees) received no supplementary pollination. These ten trees per row were designated as control trees ('Control'). We manually cross-pollinated racemes on the 10th, 20th, 30th… 100th tree in each row using pollen from the adjacent cultivar. These ten trees were designated as receiving supplementary pollination ('Suppl.').

We used a test-tube pollination method developed previously for macadamia (Trueman

and Turnbull, 1994a; Wallace *et al*., 1996). We collected pollen by rubbing the inside of a test tube (25-mm internal diameter) over at least four donor racemes that bore freshly opened flowers (Fig. 2b), until pollen was visible in streaks on the inside of the tube. Supplementary cross-pollination was performed by rubbing the inside of the test tube over receiver racemes of the adjacent cultivar that bore freshly opened flowers (Fig. 2c). Each test tube was used to pollinate a maximum of ten racemes, before it was washed with 70% (v/v) aqueous ethanol, rinsed with water, and dried in sunlight. We hand-pollinated each tree every second day during the flowering period, commencing on 8 and 9 Sep. 2018 and concluding on most trees on 18 or 19 Sep. 2018. The last hand-pollination of any tree was performed on 24 Sep. 2018. Typically, two to four people pollinated each tree on each occasion, attempting to reach all racemes with open flowers up to about 2.4 m above ground level. We estimated that this represented approximately 70% of the racemes on each tree. The '816' and 'Daddow' trees each possessed, on average, 1,940 and 2,140 racemes, respectively, based on extrapolations from the percentage of racemes that we could reach and the average number of test tubes used to pollinate each tree. Racemes of these two cultivars possessed 199 ± 5 and 104 ± 3 flowers $(n = 40)$, respectively, so that trees of '816 and 'Daddow' each produced approximately 386,060 and 222,560 flowers, respectively (Fig. 2d).

Floral visitors

We counted the numbers of (a) honey bees, (b) stingless bees, (c) syrphid flies, (d) other insects, and (e) other animals that contacted a flower within a 5-min period in a 1 m^3 quadrat on the illuminated side of each hand-pollinated tree between 0800 H and 1600 H on three days (10, 12 and 14 Sep 2018) during peak flowering. We calculated the number of flower visitors within this 15-min period for each tree.

Sample collection and nut quality

We harvested and weighed all fruit under the canopy of each tree on nine occasions throughout the harvesting season from February to June 2019. A subsample of the fruit from each harvest of each tree was weighed, dehusked, and dried at 37°C for 2 d, 45°C for 2 d, and 57°C for 2 d (Meyers *et al*., 1999). The dried subsample was then re-weighed, and the yield of each tree was calculated on a dried nut-in-shell (NIS) basis. NIS yield per tree was converted to NIS yield per hectare based on the orchard tree spacing of 8 m between rows and 4 m within rows.

We selected 20 dried nuts from each tree for assessment of nut quality and paternity. The number of nuts sampled from each harvest was in proportion to the total mass of nuts collected at each harvest, but the nuts within each harvest were sampled randomly. We recorded NIS mass for each nut, before cracking the nut using a manual nutcracker (T.J's, Morayfield, Australia). The kernel was weighed, and kernel yield per tree and kernel yield per hectare were calculated. Kernel recovery was calculated as the percentage of each NIS that was comprised of kernel. The oil concentration of a subsample of each kernel was determined by measuring its specific gravity using a pan immersed in 95% (v/v) aqueous ethanol (Trueman *et al*., 2000):

O_k (%) = 284.7 − 212.57 × G_s, where O_k is the kernel oil concentration and G_s is the specific gravity, and

G_s = (0.7995 × M_a) / (M_a − M_e), where M_a is the mass in air and M_e is the mass in 95% ethanol.

We calculated the number of fruit per tree by dividing the NIS yield per tree by the mean NIS mass per tree. We estimated fruit set by dividing the number of fruit per tree by the estimated number of flowers on each tree of that cultivar, i.e. 386,060 and 222,560 for cultivars 816 and Daddow, respectively.

Kernel genotyping by MassARRAY

Ten of the 20 kernels per tree were selected randomly, a subsample of each of these kernels was crushed, and a portion of at least 30 mg was used to determine its paternity. DNA extraction followed the glass-fibre plate DNA extraction protocol for plants (Ivanova *et al*., 2008). We used disposable 2.3 mm and 0.1 mm zirconia/silica beads prior to shaking on a TissueLyser II (Qiagen, Hilden, Germany). A ddRADseq approach was used to screen 53 samples from eight macadamia cultivars for private alleles: '741' (leaf samples from 6 individual trees), '660' (7), '344' (7), '842' (6), '816' (9), 'A4' (6), 'A203' (6), and 'Daddow' (6). The highly-similar cultivars, '741', '660' and '344', produced no private alleles and so further analyses were performed treating '741', '660' and '344' as a single group. Double-digest RADseq is commonly performed using 75-bp reads. We opted for longer reads (150 bp) to support downstream assay development for MassARRAY genotyping assays. Sequences extracted for private alleles from each cultivar were imported into Agena Assay Designer software, AgenaCX (Agena Bioscience, San Diego, CA). All proximal variants identified by Stacks were annotated onto the sequences, and preference was given to sequences with low degrees of variation. Standard design parameters were used except for the following changes to improve multiplexing: false priming threshold (0.8), primer dimer threshold (0.8), amplicon length variation (0.9), PCR primer T_m variation (0.9) and maximum pass iteration base (200). The design produced a single multiplex containing primer pairs and extension primers for nine assays (Supplementary data Tables S1 and S2).

High-throughput genotyping was performed using the Agena MassARRAY platform (Agena Bioscience, San Diego, CA) to assign paternity. Briefly, the extracted kernel DNA (2 μL; ~10 ng ul^{-1}) was amplified in 5 μL multiplex PCR reactions containing 1 U of Taq, 2.5 pmol of each PCR primer, and 500 μM of each dNTP (PCR Accessory and Enzyme Kit,

Agena Bioscience, San Diego, CA). Thermocycling was performed at 94°C for 4 min followed by 45 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 3 min. Unincorporated dNTPs were deactivated using 0.5 U of shrimp alkaline phosphatase (37°C for 4 min, 85°C for 5 min). Primer extension was initiated by adding 1.3 U of iPLEX GOLD, dideoxy nucleotide terminators and extension primers. The reaction conditions were 95°C for 30 s, 40 cycles of 95°C for 5 s plus five inner cycles of 52°C for 5 s and 80°C for 5 s, and a final extension at 72°C for 3 min. A cation exchange resin was added to remove residual salt, and 7 nL of the purified primer extension product was loaded onto the matrix pad of a SpectroCHIP (Agena) using an RS1000 nanodispenser. The extension products were analysed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using a MassARRAY Analyser 4 (Agena). Mass spectra (4,300 to 9,000 Daltons) were interpreted with TYPER 4.0 software (Agena) to identify the alleles and genotype the samples.

Effects of paternity on nut quality were assessed within those orchard rows that provided sufficient paternal diversity for analysis. The minimum number of nuts used to analyse relationships between paternity and quality was fixed at 27 for each pollen parent. No other pollen parent provided more than 10 nuts for analysis.

Statistical analysis

We assessed the effect of pollination treatment on NIS yield, kernel yield, number of fruit per tree, NIS mass, kernel mass, kernel recovery, oil concentration and paternity within each experimental row by random-block analysis of variance (random-block ANOVA). We also assessed the effect of paternity on NIS mass, kernel mass, kernel recovery and oil concentration by random-block ANOVA. Each block comprised two consecutive experimental trees along the row, i.e. a control tree and a hand-pollinated tree, because the

rows were oriented down a slight slope and the trees were slightly smaller at the bottom (southern) end of each row. Means were regarded as significantly different at P<0.05. Means are reported with standard errors.

RESULTS

Floral visitors

Most flower visitors at the experimental site were honey bees (*Apis mellifera*), with medians of 0.5–1.5 honey bee visitors per 15 min in a 1-m^3 section of the tree canopy (Fig. 3). Occasional visits were observed by stingless bees (*Tetragonula* spp.), syrphid flies, other insects including ants and other flies, and other animals, mainly spiders.

Nut-in-shell and kernel yield

Supplementary cross-pollination increased NIS yield from 1.26 ± 0.17 to 2.48 ± 0.17 t ha^{-1} in the middle of the '816' block (Fig. 4a) and from 2.12 ± 0.32 to 2.74 ± 0.31 t ha^{-1} in the '816' row next to the 'Daddow' block (Fig. 4b). Supplementary cross-pollination increased NIS yield from 3.07 ± 0.43 to 3.95 ± 0.22 t ha^{-1} in the 'Daddow' row next to the '816 block' (Fig. 4c) and from 2.75 ± 0.24 to 3.85 ± 0.19 t ha^{-1} in the middle of the Daddow block (Fig. 4d). These values represented increases in NIS yield of 97% and 29% in the middle and border rows of the '816' block, and 29% and 40% in the border and middle rows of the 'Daddow' block, respectively.

Similarly, supplementary cross-pollination increased kernel yield from 0.54 ± 0.07 to 1.13 ± 0.08 t ha^{-1} in the middle of the '816' block (Fig. 4e) and from 0.99 ± 0.15 to 1.30 ± 0.15 t ha^{-1} in the '816' row next to the 'Daddow' block (Fig. 4f). Supplementary cross-pollination increased kernel yield from 1.19 ± 0.17 to 1.54 ± 0.09 t ha^{-1} in the 'Daddow' row

next to the '816 block' (Fig. 4g) and from 1.03 ± 0.10 to 1.43 ± 0.08 t ha^{-1} in the middle of the Daddow block (Fig. 4h). These values represented increases in kernel yield of 109% and 31% in the middle and border rows of the '816' block, and 29% and 39% in the border and middle rows of the 'Daddow' block, respectively.

Nut quality

The mass of individual NIS and kernels often did not differ significantly between fruit on control trees and manually cross-pollinated trees (Table 1). However, supplementary cross-pollination increased kernel mass by 7.8% in the middle of the '816' block. Conversely, it decreased NIS mass by 3.2% and kernel mass by 8.5% in the middle of the 'Daddow' block'.

Supplementary cross-pollination increased kernel recovery in three of the four orchard rows (Table 1). Kernel recovery was increased by 2.4% (in absolute terms rather than relative terms) in the middle of the '816' block, by 1.0% in the '816' row adjacent to the 'Daddow' block, and by 0.5% in the 'Daddow' row adjacent to the '816' block.

Supplementary cross-pollination increased oil concentration slightly in the middle of the '816' and 'Daddow' blocks, respectively (Table 1). Oil concentration was increased by 0.4% (in absolute terms) by supplementary cross-pollination in both of these rows.

Final fruit set

Supplementary cross-pollination increased the number of mature fruit per tree in all orchard rows (Table 2). Fruit number was increased by 92% and 33% in the middle and border rows of the '816' block and by 32% and 51% in the border and middle rows of the 'Daddow' block, respectively. Estimated final fruit sets, based on the average number of

flowers per tree in each cultivar, ranged from 0.14–0.29% in '816' and 0.51–0.85% in 'Daddow' trees, depending on the orchard row and pollination treatment (Table 2).

Fruit paternity

Supplementary cross-pollination did not significantly affect the proportions of mature fruit arising from cross- versus self-pollination in any of the four orchard rows (Fig. 5). Most fruit arose from cross-pollination. The proportions of cross-pollinated fruit on control trees and manually cross-pollinated trees, respectively, were $84 \pm 6\%$ and $85 \pm 3\%$ in the middle of the '816' block (Fig. 5a) and $94 \pm 4\%$ and $96 \pm 2\%$ in the '816' row next to the 'Daddow' block (Fig. 5b). The same respective proportions were 100% and 100% in the 'Daddow' row next to the '816' block (Fig. 5c) and $95 \pm 2\%$ and $96 \pm 2\%$ in the middle of the 'Daddow' block (Fig. 5d).

However, supplementary cross-pollination altered the relative contributions of different cross-pollen parents to fruit paternity in the middle row of both the '816' and 'Daddow' blocks (Table 3). That is, supplementary cross-pollination with 'Daddow' pollen increased the proportion of fruit fathered by 'Daddow' in the middle of the '816' block, and supplementary pollination with '816' pollen increased the proportion of fruit fathered by '816' in the middle of the 'Daddow' block, when compared with the proportions on control trees. Supplementary cross-pollination did not significantly affect the relative contributions of different cross-pollen parents to fruit paternity in the adjoining border rows of the '816' and 'Daddow' blocks (Table 3). The other main cross-pollen parents were cultivars '741' and 'A203'. Some fruit (<4%) were fathered by '842', '849' or 'A4'.

Paternity effects on nut quality

Fruit of cultivar '816' that were fathered by 'Daddow' (i.e. '816' × 'Daddow') had 28% higher NIS mass and 36% higher kernel mass than fruit arising from self-pollination (Table 4). They had 2.8% higher kernel recovery and 2.3% higher kernel oil concentration (in absolute terms) than fruit arising from self-pollination. Fruit from '816' × 'Daddow' also had 17% higher NIS mass, 24% higher kernel mass and 3.1% higher kernel recovery than '816' × '741' fruit.

Fruit of cultivar 'Daddow' that were fathered by 'A203' (i.e. 'Daddow' × 'A203') had 18% higher NIS mass and 20% higher kernel mass than 'Daddow' × '816' fruit (Table 4). They also had 9% higher NIS mass, 15% higher kernel mass and 1.7% higher kernel recovery than 'Daddow' × '741' fruit. Fruit from 'Daddow' × '741' had 0.8% higher kernel oil concentration than 'Daddow' × '816' fruit.

DISCUSSION

Our study is the first to demonstrate that yield is pollen limited in a mass-flowering tree. Macadamia trees each possessed about 200,000–400,000 flowers but less than 1% of the flowers produced a mature fruit. Supplementary cross-pollination of most racemes on each tree increased final fruit set by as much as 92% and increased nut-in-shell yield by as much as 97%. This demonstrates that trees had sufficient maternal resources to produce more fruit, but that fruit set was pollen limited even though honey bee hives were introduced into the orchard at recommended stocking rates.

The extent of pollen limitation depended on the proximity of experimental trees to trees of the other cultivar because macadamia flowers were heavily dependent on cross-pollination for mature fruit production. The realized mating system of cultivars '816' and 'Daddow' was

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highly outcrossing, even at 200 m (25 rows) from the other cultivar, with 84–100% of fruit arising from cross-pollination. High levels of outcrossing (80–100%) have also been found in commercial orchards of macadamia cultivars 'A4' and 'A16' and in individual trees of other cultivars in multiple-cultivar research plots (Vithanage *et al*., 2002; Langdon *et al*., 2019; Richards *et al*., 2020). Macadamia flowers are partially self-incompatible (Sedgley, 1983; Sedgley *et al*., 1990; Howlett *et al*., 2019) and they initially produce more fruitlets after cross-pollination than self-pollination (Urata, 1954; Sedgley *et al*., 1990; Meyers *et al*., 1995). The very high levels of outcrossing suggest that final fruit set could be constrained by both pollen-pistil self-incompatibility and selective abortion of selfed fruitlets. Mass flowering of macadamia trees could function to (i) attract pollinators to the tree, (ii) provide an opportunity for selective abortion of genetically-inferior fruitlets, (iii) produce a heavy load of young fruitlets as a strategy against subsequent predation, disease or extreme climatic events, and (iv) father more seeds on other trees following pollen dispersal (Stephenson, 1981; Burd, 1998). The high levels of pollen limitation and outcrossing in this bee-pollinated, subtropical tree species add evidence to the concept that pollen limitation tends to be more severe or prevalent among species that are self-incompatible rather than self-compatible (Burd, 1994; Larson and Barrett, 2000; Knight *et al*., 2005), that are pollinated exclusively by bees (Bennett *et al*., 2020), and that are tropical rather than temperate (Larson and Barrett, 2000).

The main visitors to macadamia flowers were honey bees, which are the primary pollinators of cultivated macadamia trees (Urata, 1954; Heard, 1994; Heard and Exley, 1994; Wallace *et al*., 1996; Grass *et al*., 2018; Willcox *et al*., 2019). The predominance of honey bees was not surprising because managed honey bee hives were placed around the orchard at recommended stocking rates. Few stingless bees were observed at the site, similar to recent observations from other macadamia orchards in the same region (Willcox *et al*., 2019).

Stingless bees may also have a shorter foraging range than honeybees in macadamia orchards (Evans *et al*., 2021). The surrounding agricultural landscape was typical of many Australian macadamia orchards, in this case being used for broad-acre crops such as sugarcane and sweet potato. Proximity to forest has been related positively to pollinator abundance in macadamia orchards (Heard and Exley, 1994; Blanche *et al*., 2006). Forest sites provide greater plant species richness and, therefore, a floral resource supply that is both more continuous and of higher nutritional quality for stingless bees than that found in macadamia plantations (Kaluza *et al*., 2018; Trinkl *et al*., 2020; Wilson *et al*., 2021). Long-term monitoring is required to determine whether stingless bee populations are declining in the intensively-managed agricultural landscapes around macadamia orchards, reflective of trends among wild bee populations globally (Potts *et al*., 2010; Drossart and Gérard, 2020). The high levels of pollen limitation and the heavy reliance on honey bees for pollination, therefore, also add evidence to the concept that pollen limitation is greatest for species that rely on a single pollinator (Knight *et al*., 2005; Bennett *et al*., 2020).

Supplementary cross-pollination did not significantly affect mean kernel mass in the macadamia trees that were planted immediately adjacent to another cultivar. Almost all fruit from both the control trees and the hand-pollinated trees in these two rows were fathered by pollen from the neighbouring cultivar that was planted only 8 m away. However, supplementary pollination altered mean kernel mass in trees that were planted in the middle of the wide blocks, where the trees were 200 m away from the other cultivar. This was because supplementary cross-pollination by 'Daddow' or '816' increased the proportion of '816' × 'Daddow' or 'Daddow' × '816' kernels, respectively, in these orchard rows and because the mass of '816' and 'Daddow' kernels was influenced by their cross-pollen parentage, as it is in cultivars 'A16' and '246' (Herbert *et al*., 2019a,b). Kernels of '816' × 'Daddow' were 24% heavier than kernels of the other cross-parent combination, '816' ×

'741', in the middle row of the '816' block. As a result, supplementary pollination with 'Daddow' increased mean kernel mass of '816' in this row. In contrast, 'Daddow' × '816' kernels were 17% less heavy than kernels of another cross-parent combination, 'Daddow' × 'A203', in the middle row of the 'Daddow' block. As a result, supplementary pollination with '816' decreased mean kernel mass of 'Daddow' in this row.

Pollen parent effects were not limited to kernel mass but were also evident on nut-in-shell mass, kernel recovery and kernel oil concentration. Importantly, we found that self-pollinated '816' fruit had lower nut-in-shell mass and kernel oil concentration than cross-pollinated fruit, regardless of the cross-pollen source. However, only cross-pollination by 'Daddow' (rather than '741') increased kernel recovery and kernel mass. Kernel recovery and kernel mass are both higher following supplementary cross-pollination of individual racemes of 'A4' by '246', '246' by 'A4', '660' by '333', and '660' by '246', when compared with either open-pollination or supplementary self-pollination (Trueman and Turnbull, 1994a; Wallace *et al*., 1996). The current results, though, suggest that increased kernel recovery and kernel mass may not be a universal effect of cross-pollination in macadamia, but may sometimes depend on the parental combination. Paternity effects might be lower when the pollen-parent cultivar is closely related to the mother cultivar (Forrest *et al*., 2011; Takeuchi and Diway, 2021). However, none of the cultivars, '816', 'Daddow', '741' or 'A203', appear to be closely related to each other (Alam *et al*., 2018, 2019).

Pollen parent effects on fruit characteristics are termed xenia (Denney, 1992), but it is sometimes difficult to distinguish between the potentially confounding effects of seed paternity and seed number when attempting to identify xenia effects (de Jong and Scott, 2007). Macadamia, though, is a single-seeded fruit and so the pollen parent effects on nut-in-shell mass, kernel mass, kernel recovery and kernel oil concentration can be attributed fully to seed paternity. Fruit characteristics have also been attributed to seed paternity in other

single-seeded fruit such as almond, hazelnut, lychee and mango (Degani *et al*., 1995; Dag *et al*., 1999; Kodad *et al*., 2009; Fattahi *et al*., 2014). Macadamia processors trade kernels under a range of "styles" that provide higher returns for larger kernels, and growers receive premiums for supplying nuts with higher kernel recovery (Penter *et al*., 2008; Australian Macadamia Society, 2017, 2018; Department of Agriculture & Fisheries, 2019). The effects of pollen source on macadamia kernel mass and kernel recovery, therefore, demonstrate how xenia effects can strongly influence the financial value of an agricultural crop.

An interesting finding from our study was the high percentage of fruit in the middle of the adjoining blocks of '816' and 'Daddow' that were pollinated by other cultivars, mostly '741' and 'A203'. The main cross-pollen cultivar (i.e. 'Daddow' or '816') was 200 m away, across 25 rows of trees. Some storm-damaged trees in the orchard had been replaced by '741' trees, and their pollen may have been contributing to fruit set in the rows that were 200 m away from another cross-pollen source. The nearest other macadamia orchard was 1.2 km from the study site, although this was well within the potential flight range of honey bees (Beekman and Ratnieks, 2000; Hagler *et al*., 2011; Ratnieks and Shackleton, 2015). Our fruit paternity results demonstrate that individual honey bee workers were foraging on trees of more than one cultivar or that pollen from different cultivars was transferred at the hive between workers. In-hive transfer of pollen has been demonstrated between individual bees that were foraging on mango trees either inside or outside of large cages (Dag *et al*., 2001). Analyses of pollen loads on bees suggest that in-hive pollen transfer also occurs between bees foraging on almond, apple, cotton and sunflowers (DeGrandi-Hoffman *et al*., 1986, 1992; DeGrandi-Hoffman and Martin, 1993; Loper and DeGrandi-Hoffman, 1994). Pollen from storm-damage replacement trees or in-hive pollen transfer partly explain the yields of cross-pollinated nuts produced by macadamia trees at 200 m from the other main cultivar. However, these trees were clearly pollen limited and highly dependent on outcrossing, which suggests that closer

interplanting of cultivars and better management of bee hives are required to maximize nut production.

In conclusion, supplementary cross-pollination of macadamia trees increased fruit set by approximately 32–92 %, nut-in-shell yield by 29–97% and kernel yield by 29–109% when compared with control trees that received no additional pollination. This is the first time that pollen limitation has been demonstrated in a mass-flowering tree, and it occurred even though honey bee hives were introduced into the orchard at recommended stocking rates. The results support findings that pollen limitation is often greater or more prevalent for plant species that: (a) rely on a single pollinator (Knight *et al*., 2005; Bennett *et al*., 2020) (b) are exclusively bee-pollinated (Bennett *et al*., 2020); (c) are self-incompatible rather than self-compatible (Burd, 1994; Larson and Barrett, 2000; Knight *et al*., 2005); or (d) have a tropical rather than temperate distribution (Larson and Barrett, 2000). The extent of pollen limitation in macadamia depended on the distance to trees of the other main cultivar because fruit production was highly dependent on the transfer of cross-pollen by bees from one cultivar to another. Between 84% and 100% of the fruit arose from cross-pollination. Furthermore, large variations in kernel mass and kernel quality between fruit could be attributed to differences in fruit paternity. The reliance of bee-pollinated macadamia flowers on cross-pollination for fruit set and the strong xenia effects on kernel mass and oil concentration demonstrate the high value that pollination services can provide to food production and food quality. Improved pollination led to increases in macadamia kernel yields of 0.31–0.59 tons per hectare, which equate to increases in farm-gate income of $US3,720–$US7,080 per hectare at current prices of about $US12,000 per ton of kernel.

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FIGURE CAPTIONS

FIG. 1. Schematic diagram of the macadamia orchard, showing cv. '816' and 'Daddow' trees planted in wide single-cultivar blocks that were 42 and 48 rows wide, respectively. Each tree is indicated by a dot. Experimental 816 trees were either 25 rows (R25) or 1 row (R1) from the wide 'Daddow' block. Experimental 'Daddow' trees were either 1 row (R1) or 25 rows (R25) from the wide '816' block. Further rows of 'Daddow' and '816' trees were located WSW of the wide blocks, and another macadamia orchard was located 1.2 km NNW of the study blocks. The approximate positions of a total of 160 honey bee (HB) hives are indicated.

FIG. 2. (a) Trees of macadamia cvv. '816' (rows on the left) and 'Daddow' (row on the right) with hives of honey bees (centre left); (b) macadamia cv. '816' racemes, which each possess about 200 flowers; (c) hand-pollinating macadamia racemes using 25-mm internal-diameter test tubes; (d) macadamia trees during the flowering period, with each tree producing about 2000 racemes.

FIG. 3. Number of honey bees, stingless bees, syrphid flies, other insects and other animals observed in a 15-min period making contact with flowers in a 1 m^3 quadrat on the illuminated side of macadamia cv. '816' and 'Daddow' trees planted in adjacent single-cultivar blocks that were 42 and 48 rows wide, respectively. '816' trees were either (a) 25 rows or (b) 1 row from the 'Daddow' block. 'Daddow' trees were either (c) 1 row or (d) 25 rows from the '816' block. Medians are presented with 25th and 75th percentiles (boxes), 10th and 90th percentiles (whiskers), and outliers (n = 10 trees).

FIG. 4. (a–d) Nut-in-shell yields and (e–h) kernel yields of macadamia cv. '816' and 'Daddow' trees planted in adjacent single-cultivar blocks that were 42 and 48 rows wide, respectively. '816' trees were either (a, e) 25 rows or (b, f) 1 row from the 'Daddow' block. 'Daddow' trees were either (c, g) 1 row or (d, h) 25 rows from the '816' block. Trees received no additional pollination (Control) or received supplementary pollination using pollen from the neighbouring cultivar (Suppl.). Means (+ SE) within an orchard row with different letters are significantly different (random block ANOVA, P<0.05, n = 10 trees).

FIG. 5. Levels of cross-paternity and self-paternity among fruit of macadamia cv. '816' and 'Daddow' trees planted in adjacent single-cultivar blocks that were 42 and 48 rows wide, respectively. '816' trees were either (a) 25 rows or (b) 1 row from the 'Daddow' block. 'Daddow' trees were either (c) 1 row or (d) 25 rows from the '816' block. Trees received no additional pollination (Control) or received supplementary pollination using pollen from the neighbouring cultivar (Suppl.). Means (+ SE) for cross-paternity or for self-paternity within an orchard row do not differ significantly (random block ANOVA, P>0.05, n = 10 trees).

TABLE 1. *Nut-in-shell mass, kernel mass, kernel recovery and kernel oil concentration in macadamia cvv. '816' and 'Daddow'. Trees of '816' and 'Daddow' were planted in adjacent single-cultivar blocks that were 42 and 48 rows wide, respectively. '816' trees were either 25 rows or 1 row from the 'Daddow' block. 'Daddow' trees were either 1 row or 25 rows from the '816' block. Trees received no additional pollination (Control) or received supplementary pollination using pollen from the neighbouring cultivar (Suppl.).*

Parameter	Cultivar, orchard row and pollination treatment							
	'816' Row 25		'816' Row 1		'Daddow' Row 1		'Daddow' Row 25	
	Control	Suppl.	Control	Suppl.	Control	Suppl.	Control	Suppl.
Nut-in-shell mass (g)	$7.37 \pm 0.12a$	$7.54 \pm 0.09a$	$7.74 \pm 0.09a$	$7.52 \pm 0.09a$	$6.56 \pm 0.10a$	$6.41 \pm 0.12a$	$7.55 \pm 0.11a$	$6.92 \pm 0.12b$
Kernel mass (g)	$3.19 \pm 0.06a$	$3.44 \pm 0.05b$	$3.58 \pm 0.05a$	$3.55 \pm 0.05a$	$2.52 \pm 0.04a$	$2.49 \pm 0.05a$	$2.81 \pm 0.04a$	$2.57 \pm 0.05b$
Kernel recovery (%)	$43.0 \pm 0.5a$	$45.4 \pm 0.4b$	$46.4 \pm 0.3a$	$47.4 \pm 0.3b$	$38.3 \pm 0.4a$	$38.8 \pm 0.6b$	$37.1 \pm 0.2a$	$37.0 \pm 0.5a$
Oil concentration (%)	$78.2 \pm 1.1a$	$78.6 \pm 0.4b$	$79.0 \pm 0.4a$	$79.2 \pm 0.7a$	$78.3 \pm 1.0a$	$78.2 \pm 1.2a$	$78.4 \pm 0.6a$	$78.8 \pm 1.2b$

Means $\pm$ SE within an orchard row with different letters are significantly different (random block ANOVA; $P<0.05$, n = 200 nuts)

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TABLE 2. *Number of mature fruit per tree and estimated final fruit set (%) in macadamia cvv. '816' and 'Daddow'. Trees of '816' and 'Daddow' were planted in adjacent single-cultivar blocks that were 42 and 48 rows wide, respectively. '816' trees were either 25 rows or 1 row from the 'Daddow' block. 'Daddow' trees were either 1 row or 25 rows from the '816' block. Trees received no additional pollination (Control) or received supplementary pollination using pollen from the neighbouring cultivar (Suppl.).*

Parameter	Cultivar, orchard row and pollination treatment							
	'816' Row 25		'816' Row 1		'Daddow' Row 1		'Daddow' Row 25	
	Control	Suppl.	Control	Suppl.	Control	Suppl.	Control	Suppl.
Number of fruit per tree	$525 \pm 72a$	$1009 \pm 65b$	$854 \pm 130a$	$1136 \pm 139b$	$1445 \pm 205a$	$1901 \pm 111b$	$1136 \pm 120a$	$1719 \pm 88b$
Estimated fruit set (%)	0.14	0.26	0.22	0.29	0.65	0.85	0.51	0.77

Means $\pm$ SE for number of fruit set within an orchard row with different letters are significantly different (random block ANOVA; $P<0.05$, n = 10 trees)

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TABLE 3. *Levels of 'Daddow' or '816' cross-paternity among mature fruit of macadamia cvv. '816' and 'Daddow'. Trees of '816' and 'Daddow' were planted in adjacent single-cultivar blocks that were 42 and 48 rows wide, respectively. '816' trees were either 25 rows or 1 row from the 'Daddow' block. 'Daddow' trees were either 1 row or 25 rows from the '816' block. Some trees in the orchard had been replaced by '741' trees. Another orchard was 1.2 km away. Trees received no additional pollination (Control) or received supplementary pollination using pollen from the neighbouring cultivar (Suppl.).*

Mother cultivar × pollen parent	Cultivar, orchard row and pollination treatment							
	'816' Row 25		'816' Row 1		'Daddow' Row 1		'Daddow' Row 25	
	Control	Suppl.	Control	Suppl.	Control	Suppl.	Control	Suppl.
'816' × 'Daddow' (%)	52 ± 6a	66 ± 5b	86 ± 7a	90 ± 3a	—	—	—	—
'Daddow' × '816' (%)	—	—	—	—	93 ± 3a	94 ± 3a	28 ± 6a	68 ± 5b

Means ± SE within an orchard row with different letters are significantly different (random block ANOVA; P<0.05, n = 10 trees)

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TABLE 4. *Nut-in-shell mass, kernel mass, kernel recovery and kernel oil concentration of macadamia cv. '816' and 'Daddow' fruit with different pollen parents. Trees of '816' and 'Daddow' were planted in adjacent single-cultivar blocks that were 42 and 48 rows wide, respectively. '816' trees were 25 rows from the 'Daddow' block. 'Daddow' trees were 25 rows from the '816' block. Some storm-damaged trees in the orchard had been replaced by '741' trees. Another orchard was 1.2 km away.*

Parameter	Cultivar, orchard row and pollen parent					
	'816' Row 25			'Daddow' Row 25		
	× '816'	× 'Daddow'	× '741'	× '816'	× '741'	× 'A203'
Nut-in-shell mass (g)	6.22 ± 0.17c	7.98 ± 0.17a	6.84 ± 0.18b	6.74 ± 0.10b	7.28 ± 0.27b	7.94 ± 0.19a
Kernel mass (g)	2.63 ± 0.10b	3.58 ± 0.09a	2.88 ± 0.11b	2.51 ± 0.04b	2.62 ± 0.11b	3.02 ± 0.09a
Kernel recovery (%)	42.1 ± 0.9b	44.9 ± 0.7a	41.8 ± 0.8b	37.2 ± 0.2ab	36.0 ± 0.6b	37.7 ± 0.4a
Oil concentration (%)	76.8 ± 0.3b	78.9 ± 0.3a	78.1 ± 0.5a	78.4 ± 0.1b	79.2 ± 0.3a	78.9 ± 0.2ab

Means ± SE within an orchard row with different letters are significantly different (random block ANOVA; $P<0.05$; n = 26–143 nuts)
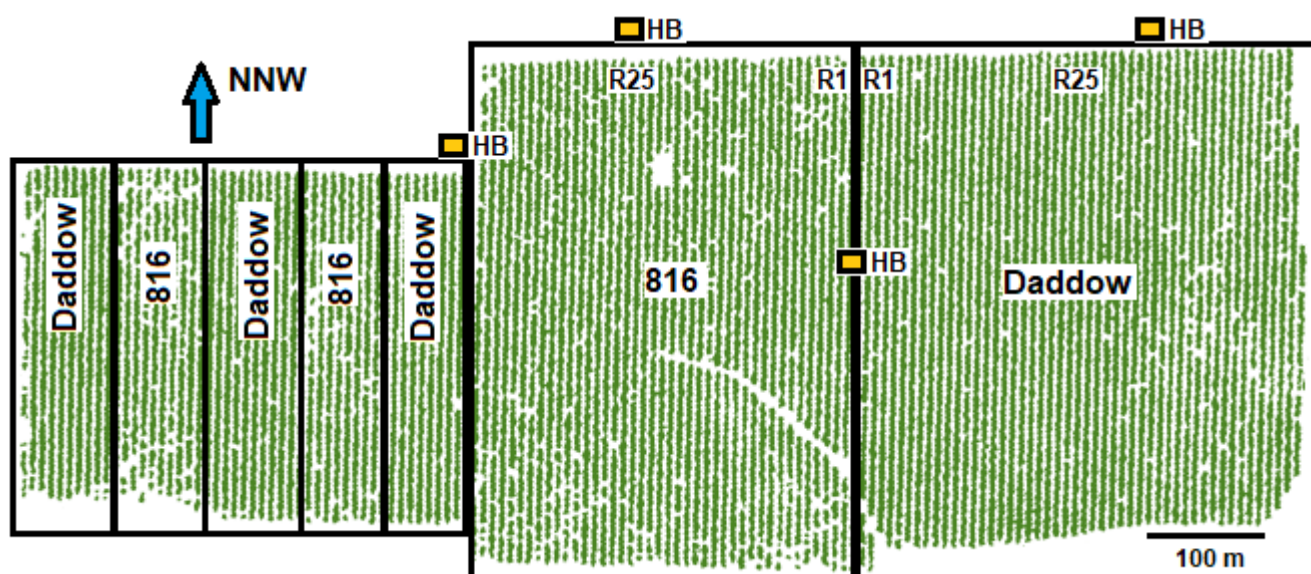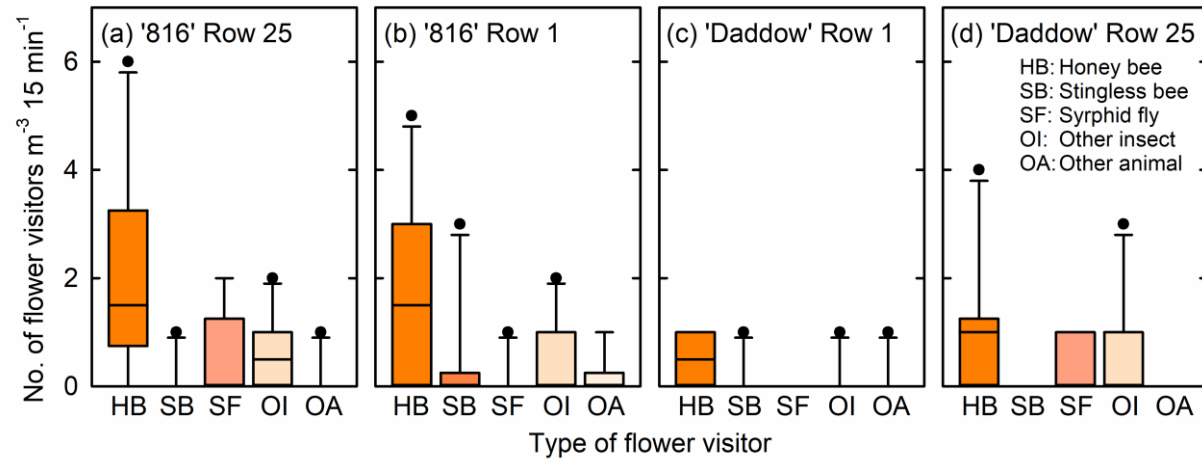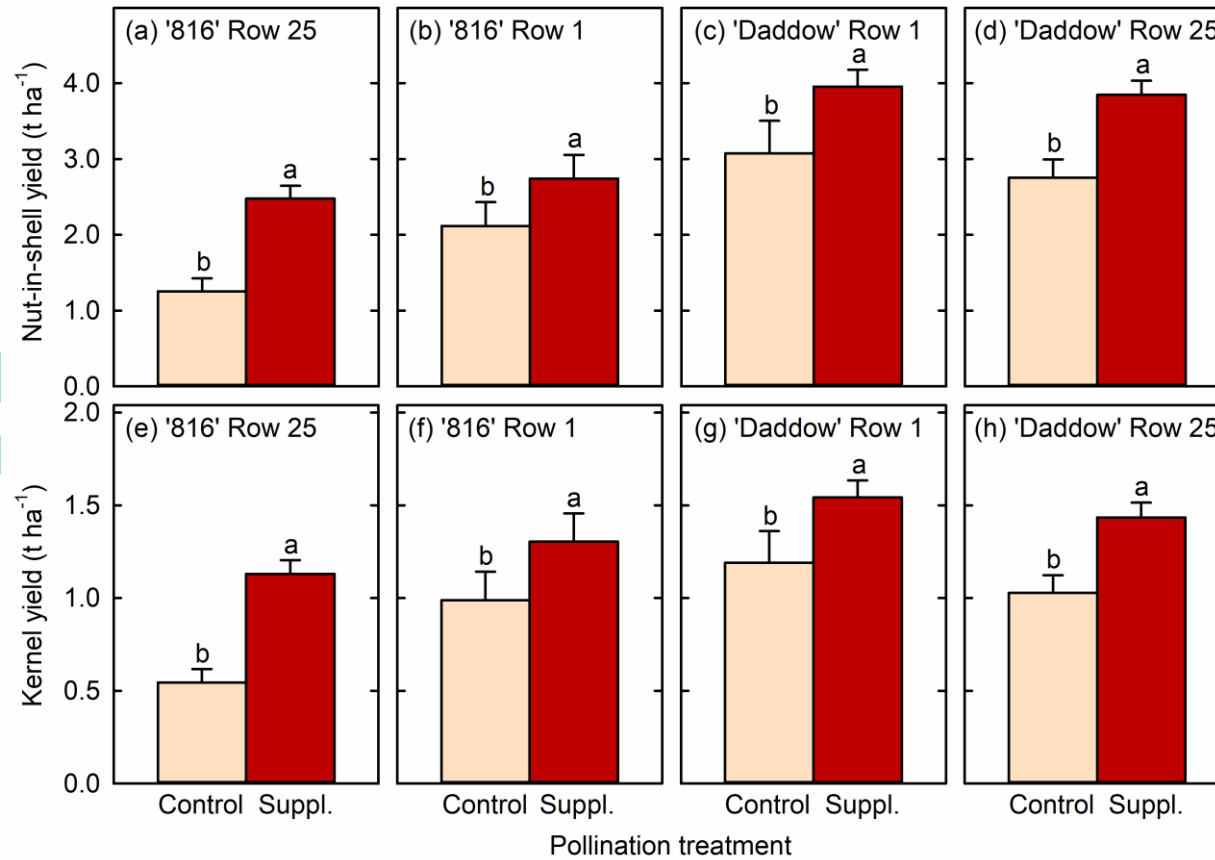
Figure 1

Figure 2

Figure 3

(a) '816' Row 25　(b) '816' Row 1　(c) 'Daddow' Row 1　(d) 'Daddow' Row 25

HB: Honey bee
SB: Stingless bee
SF: Syrphid fly
OI: Other insect
OA: Other animal

No. of flower visitors m^{-3} 15 min^{-1}

Type of flower visitor

HB SB SF OI OA

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Figure 4

Figure 5