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Planting density and fertilisation independently affect seed and oil yields in *Plukenetia volubilis* L. plants

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SUMMARY
To investigate the effects of planting density and fertiliser level on some important agronomic traits of *Plukenetia volubilis*, a perennial oilseed species, a field experiment with four replications in a completely randomised block design was conducted in a tropical region of China in the 2012-2013 growing season. Planting density (1,666, 2,500, 4,444, 5,000, or 10,000 plants ha^{-1}) was assigned to the main plots and a 1:1:1 (w/w/w) NPK fertiliser at 0, 50 100, 150, or 200 kg ha^{-1} was assigned factorially to the sub-plots. The results showed that neither planting density nor the rate of fertilisation affected seed size (dry matter) and the phenological development of *P. volubilis* plants, including the times of initial flowering and maturity, or the pattern of fruit ripening. Planting density, rather than fertiliser level, significantly affected seed oil content, but a non-linear effect was found. Across the different treatments and sampling times, seed size and oil content had relatively high and constant heritabilities indicated by low coefficients of variation. Total seed yields and total oil yields per plot over the growing season ranged from 1,340 - 2,486 kg ha^{-1}, and from 501 - 899 kg ha^{-1}, respectively, with a quadratic response to planting density at all fertiliser levels. Total seed and oil yields increased continuously with the increasing levels of fertiliser used. The absence of any planting density × fertiliser level interaction on total seed and oil yields suggested that planting density and fertilisation had independent effects, and that seed or oil yield responses to planting density were not significantly affected by fertiliser level. We concluded that oil production in *P. volubilis* plants required high levels of fertiliser and, regardless of fertiliser level, a planting density of approx. 4,444 plants ha^{-1} was required to ensure maximum yield in the field.

Planting density has important effects on the vegetative and reproductive development of crops (Ciampitti and Vyn, 2011; Dong *et al.*, 2012). A high planting density is commonly used to increase crop yields. However, if the planting density is too high, this reduces the availability of resources (i.e., incident radiation, water, and nutrients) per plant in the growing season and causes a decline in yield per plant which may not be offset by the increase in planting density (Andrade *et al.*, 1999). A high planting density in maize increased total dry matter production and decreased the harvest index (HI). The optimum planting density reflected a balance between these two effects (Tollenaar, 1989). Although the effects of planting density on crop yield and on various yield components have been studied in many horticultural crops, the results have varied greatly between species. In many species, increased planting densities, up to an optimum level, resulted in increased yields (Brahim *et al.*, 1998). In some species, a lower planting density prolonged the seed-filling period, and thus increased seed size (Rogers and Lomman, 1988). The seed yield per unit area, in response to planting density, depends not only on the species and environmental conditions, but also on the level of fertiliser applied and other agronomic practices (Ciampitti and Vyn, 2011). Limited amounts of available soil nutrients lead to weak plant development, premature senescence, and decreased fruiting, thus limiting the yield potential (Zhang *et al.*, 2012). Although it has been widely reported that the application of fertiliser (especially N) increased plant growth and crop yields in the field (Jackson, 2000; Rotundo and Westgate, 2009), an over-dose of fertiliser can promote excessive vegetative development at the expense of fruit yield, and probably also delays maturity (Cheema *et al.*, 2001). The independent effects of planting density or level of fertilisation on crop yield and yield components have been well-documented (Zhang *et al.*, 2012; Cai *et al.*, 2013), but their interactions have been relatively less well-studied, especially in the field.

Plukenetia volubilis L. is native to South America and is a promising new oilseed crop in the family Euphorbiaceae. *P. volubilis* is a perennial woody vine that produces seeds with a high oil content [40 – 60% (w/w)] which exceeds the quality characteristics of existing oils used for human consumption worldwide. *P. volubilis* seed oil is one of the richest sources of unsaturated fatty acids and its domestic, industrial, medicinal, and cosmetic industry uses have increased the global value of this crop (Cai *et al.*, 2011). *P. volubilis* plants do not exhibit winter dormancy. They grow continuously in tropical regions and therefore flower and fruit almost continuously throughout the year. Each fruit is a capsule (*ca.* 4 – 7 cm in diameter), consisting of four-to-seven pods, with one seed per pod.

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The processes determining the quantity and quality of oil in *P. volubilis* seed are variable and depend on several environmental conditions and agronomic factors (Cai, 2011; Jiao *et al.*, 2012). In addition, *P. volubilis* plants have the capacity to develop new reproductive structures in response to an increase in available resources (Cai, 2011; Jiao *et al.*, 2012; Cai *et al.*, 2012; 2013). Therefore, we predict that there is potential to increase seed yields in *P. volubilis* plants by suitable agronomic management practices.

In a preliminary study, we found that a relatively high planting density was required to optimise seed yields in this new oilseed crop (Cai *et al.*, 2013). To date, no optimum planting density or rate of fertiliser application has been established to achieve the maximum levels of seed or oil production. Therefore, in the present study, a field experiment was conducted to investigate the combined effects of planting density and rate of fertiliser application on the reproductive traits, total seed and oil yields, yield components, and seed quality during a single growing season at Xishuangbanna, a tropical region of China. The overall goal was to provide a better understanding of planting density and fertiliser management for this species at both the local and regional levels, and thus to increase seed and oil yields for commercial-scale oil production.

MATERIALS AND METHODS

Experimental site, plant material, and experimental treatments

Seeds of *P. volubilis* were sown in a nursery in February 2012. When the seedlings were approx. 20 cm tall, in late April 2012, uniformly-sized seedlings were selected and cultivated at four open sites at the Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Sciences (21°56'N, 101°15'E; 560 m asl). The soil was a red–brown type and soil samples were collected in October 2011. The characteristics of the top (0 – 20 cm) layer of soil were: pH 5.42; organic carbon 5.65% (w/v); total nitrogen 0.34 g kg^{-1}; available N 46.0 mg kg^{-1}; available P 14.1 mg kg^{-1}; and available K 22.0 mg kg^{-1}. The climate at Xishuangbanna is dominated by the Southwest monsoon with two distinct seasons (a wet season from May to October, and a dry season from November to April; Cao *et al.* 2006). The average annual temperature is 22.9ºC and the mean annual precipitation is 1,500 mm, of which approx. 85% occurs in the wet season.

The field experiment was conducted using a randomised complete block design with four replicates of each treatment. Planting density was assigned to the main plots with five different intra- and inter-row spacings: 3.0 m × 2.0 m; 2.0 m × 2.0 m; 1.5 m × 1.5 m; 2.0 m × 1.0 m; or 1.0 m × 1.0 m, resulting in planting densities of 1,666, 2,500, 4,444, 5,000, or 10,000 plants ha^{-1}, respectively. Fertilisation rates were assigned to the sub-plots and consisted of five levels (0, 50, 100, 150, or 200 kg ha^{-1}) of a 1:1:1 (w/w/w) mix of NPK spread in an approx. 1.0 m-wide zone in June 2012 (the wet season). In total, there were 100 sub-plots, each approx. 40 m in length.

Since *P. volubilis* is a liana species, all plants were supported to a height of 1.6 m using steel wires.

Measurements

Twenty small fruit (diameter < 0.5 cm) in each sub-plot were tagged at random on 19 November and on 12 December 2012 in only the highest (200 kg ha^{-1}) and lowest (0 kg ha^{-1}) fertiliser level sub-plots at each planting density. Approx. 1.5 months later, the numbers of mature fruit were counted and the "fruit abortion percentage" was calculated as the proportion of empty fruit compared to the total number of fruit tagged.

The stem diameter of each plant was measured 2 cm above soil level, using calipers, to measure plant growth at the end of April 2013. Five-to-six plants were selected at random in each sub-plot, and the dates of first bloom and mature fruit set were recorded for each plant.

Mature fruit from all *P. volubilis* plants were harvested seven-times, by hand, in each replicate sub-plot, throughout the 5-month period of fruit ripening. The total dry mass (DM) of fruit per plot was measured at each harvest. Also at each harvest, sub-samples of mature fruit were peeled and the DM (size) of all seeds per plot was recorded.

Seed oil contents were determined using a Minispec mq-one Seed Analyser (Bruker Optik GmbH, Ettlingen, Germany). A calibration curve was obtained using known reference samples of the oil extracted from *P. volubilis* seeds. As the average DM of seeds accounted for 53.4% of the total fruit DM, and did not differ across all sampling dates and treatments (n = 150), oil yield at each harvest was estimated as follows:

$$\text{Oil yield (kg ha}^{-1}) = \text{Mature fruit DM (kg ha}^{-1}) \times 0.534 \times \text{Seed oil content.}$$

The total oil yield (ha^{-1}) throughout the growing season was then calculated by adding the values from each harvest.

Statistical analysis

All data are presented as mean values ± standard deviations (SD). Seed size (DM), oil content, and seed and oil yields between the different sampling times were analysed by one-way ANOVA and means were compared by Tukey test at $P \leq 0.05$. We used two-way ANOVA to compare fruit set percentages, seed traits, and total seed and oil yields between the different planting densities, fertiliser levels, and their interactions across the different sampling times. We then used LSD contrasts to examine whether each trait differed between planting densities within or between fertiliser levels. The data were tested for normality and homogeneity of variance and, when necessary, were $\log_{10}$-transformed before analysis. The variability of each trait for each factor (planting density or fertiliser level) was quantified using coefficients of variation [CV; i.e., the standard deviation (SD) divided by the mean of the characteristic]. All statistical analyses were conducted using SPSS software Version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Plant reproductive pattern

P. volubilis plants in all treatments started flowering between 18 – 20 June 2013. Neither planting density nor rate of fertilisation affected the time of initial flowering or the first harvest date, which was contrary to previous

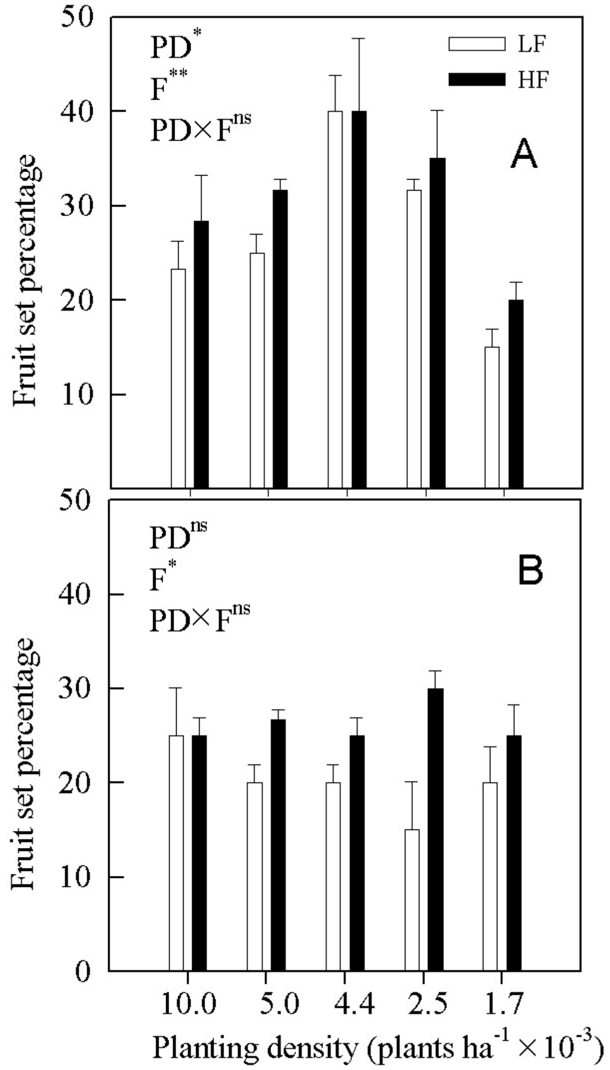

FIG. 1
Fruit set percentages in *P. volubilis* plants cultivated at five different planting densities and two rates of fertilisation (LF or HF) in November (Panel A) and December (Panel B) 2012. PD, planting density; F, fertiliser. LF, low fertilisation rate (0 kg ha^{-1}); HF, high fertilisation rate (200 kg ha^{-1}). Significant at *, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant.

results that long-term exposure to shade delayed the initial date of flowering in *P. volubilis* plants (Cai, 2011). The same initial flowering times with increases in plant density and rate of fertilisation would not necessarily lead to a delay in the time of harvest. Neither planting density nor rate of fertilisation affected the phenological development of *P. volubilis* plants. This was due to the fact that the time from sowing to flowering (or seed maturity) within one season in plants with no requirement for vernalisation is largely determined by their responses to temperature and photoperiod (Olesena *et al.*, 2012).

As a wind-dispersed species with well-developed reproductive organs (i.e., flower and fruit numbers; Jiao *et al.*, 2012), seed production in *P. volubilis* depends mainly on the availability of resources to make seeds (Zorn-Arnold and Howe, 2007). There were no planting density × fertiliser interactions on fruit set in *P. volubilis* plants at the early (November) or late (December) reproductive stages (Figure 1 A,B). Planting density

TABLE I
Summary of two-way ANOVA to evaluate the effects of planting density, rate of fertilisation, and their interaction on seed and oil traits and mean values of each trait under different treatments

Factor	Seed yield (kg ha^{-1})	Seed oil yield (kg ha^{-1})	Seed oil content [%(w/w)]	Seed size (g seed^{-1})
Two-way ANOVA (significance level)[§]				
PD[‡]	***	***	***	ns
F	**	*	ns	ns
PD × F	ns	ns	ns	ns
Treatment means				
PD (plants ha^{-1})				
10,000	1,799.0[†] bc	666.2 b	37.4 a	1.32 a
5,000	1,986.6 ab	725.9 ab	36.6 b	1.35 a
4,444	2,154.6 a	786.1 a	36.5 b	1.36 a
2,500	1,549.2 d	572.5 c	37.1 a	1.37 a
1,666	1,584.8 cd	564.7 c	35.6 c	1.38 a
CV[‡] (%)	14.3	14.5	1.9	1.70
Fertilisation (kg ha^{-1})				
0	1,626.6[†]c	598.8 c	36.7 a	1.35 a
50	1,745.8 c	640.5 c	36.7 a	1.34 a
100	1,812.7 ab	663.6 ab	36.5 a	1.36 a
150	1,860.4 ab	673.1 ab	36.6 a	1.35 a
200	2,028.6 a	739.4 a	36.6 a	1.36 a
CV (%)	8.2	7.8	0.23	0.62

[†]Values are means (n = 4). Values followed by different lower-case letters in each column indicate significant difference at $P \leq 0.05$ by the LSD test.
[‡]PD, planting density; F, fertilisation; CV, coefficient of variation.
[§]ns, not significant ($P > 0.05$); or significant at *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

affected fruit set in *P. volubilis* plants only at the early reproductive stage (November) with the highest fruit set values observed at medium planting densities (Figure 1 A). At higher plant densities, increased crowding could reduce plant growth around the critical seed-filling period, resulting in fruit abortion (Andrade *et al.*, 1999), and thus reduced kernel numbers and seed yields per plant. There are reports that plant density affects fruit set in a large number of wild species in a wide variety of habitats (Stephenson, 1981; Zorn-Arnold and Howe, 2007). Fertiliser application generally increased the fruit set in *P. volubilis* plants at both the early and late reproductive stages (Figure 1 A,B), which agreed with the findings of our previous study (Jiao *et al.*, 2012). A high fruit set percentage in *P. volubilis* plants under high fertiliser levels required an increased photosynthetic input and thus reduced carbohydrate availability (Stephenson, 1981).

Over the 5-month period of production, the patterns of both mature seed and oil yields at the sub-plot level were similar between the different treatments, with the highest values observed in the middle of March (Figure 2 C,D). Seeds that matured later (i.e., after March) had a relatively large size, whereas their seed oil content was not affected by sampling date (Figure 2 A,B).

Seed oil content and seed size (DM)

The influence of planting density on the oil content of oilseed crops has been controversial. For example, there have been reports of an increase (Zhang *et al.*, 2012), a decrease (Momoh and Zhou, 2001), or no effect (Leach *et al.*, 1999) on the oil contents of winter oilseed rape seed (*Brassica napus* L.) with increasing planting density. There have also been reports that the oil contents of castor bean, (*Ricinus communis*; Soratto *et al.*, 2012) and sunflower (*Helianthus annuus*; Narwal and Malik, 1985) seeds were not affected by planting density. In the

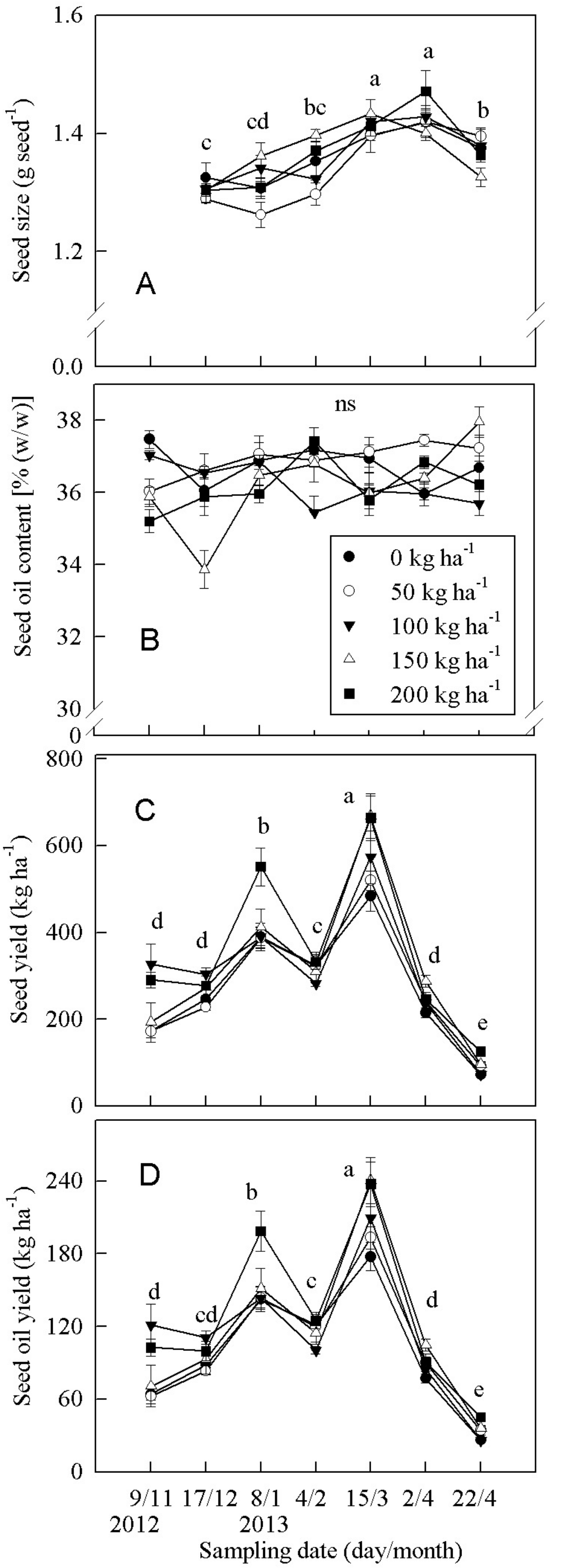

FIG. 2

Mature seed size (Panel A), seed oil content (Panel B), seed yield (Panel C), and seed oil yield (Panel D) in *P. volubilis* plants during one growing season cultivated at different levels of fertilisation combined with a planting density of 4,444 plants ha^{-1} as an example. Mean values (n = 4) with different lower-case letters in each Panel indicate significant differences between fertilisation levels for this planting density at $P \leq 0.05$. ns, not significant.

present study, planting density had a significant, but non-linear affect on the oil content of *P. volubilis* seeds (Table I). Across the different treatments and sampling times, seed oil contents ranged from 33.9 – 37.4% (w/w), which was comparable to previous reports (Cai *et al.*, 2012; Jiao *et al.*, 2012).

It is likely that increased nutrient stress during the seed-filling period resulted in seeds with a reduced oil content (Rotundo and Westgate, 2009). However, seed oil contents decreased with increasing levels of fertilisation in many studies (Jackson, 2000; Cheema *et al.*, 2001; Rathke *et al.*, 2005). This might be due to the reduced availability of carbohydrates for the synthesis of oils under high N supply. However, oil contents did not necessarily correlate inversely with protein contents across different environments (Cai *et al.*, 2012) because the biosynthesis of storage proteins and oils in developing oil seeds are two independent processes, and the genes that control oil and protein synthesis are expressed differently (Votlker and Kinney, 2001). The rates of fertilisation applied here did not affect the oil contents of *P. volubilis* seed across all sampling dates (Table I), which agreed with results from canola and soybean (Hocking *et al.* 1997; Rotundo and Westgate, 2009).

A lower planting density would result in a prolonged seeding-filling period, thus increasing seed size (Leach *et al.*, 1999). However, neither planting density nor rate of fertilisation affected seed size (i.e., seed DM) in *P. volubilis* plants across the different sampling dates (Figure 1 A), confirming results in canola (Angadi *et al.*, 2003). In addition, similar-sized seeds across the different treatments revealed that an increased number of seed (fruit) ha^{-1}, rather than individual seed size, was mainly responsible for the total seed yield throughout the growing season (Cai *et al.*, 2013). The coefficients of variation in oil content and seed size in response to planting density and rate of fertilisation were small (0.23 – 1.90%), indicating that these traits had a relatively high and constant heritability in *P. volubilis* plants (Soratto *et al.*, 2012). Moreover, the low variation in seed oil content indicated that seed quality did not vary greatly across the different treatments throughout the 5-month fruit-ripening period.

Total seed and oil yields

There were no planting density × fertiliser interactions on total seed or oil yields. Thus, planting density and rate of fertilisation were assumed to have independent effects. *P. volubilis* plants showed a similar slight increase in total seed or total oil yields in response to fertiliser level with increased planting density, while the response to planting density was not greatly affected by fertiliser level. This contradicted observations in maize (Asim *et al.*, 2013) and corn (Dong *et al.*, 2012), in which the interaction of plant density and nitrogen level had significant effects on crop yield. Both planting density and fertiliser level significantly affected total seed and oil yields (Table I; Figure 2; Figure 3). Moreover, the coefficients of variation were higher in response to planting density than to fertiliser level, indicating that planting density may be more important as a limiting factor than fertiliser level during the production of seeds and oil in *P. volubilis* plants (Diepenbrock, 2000).

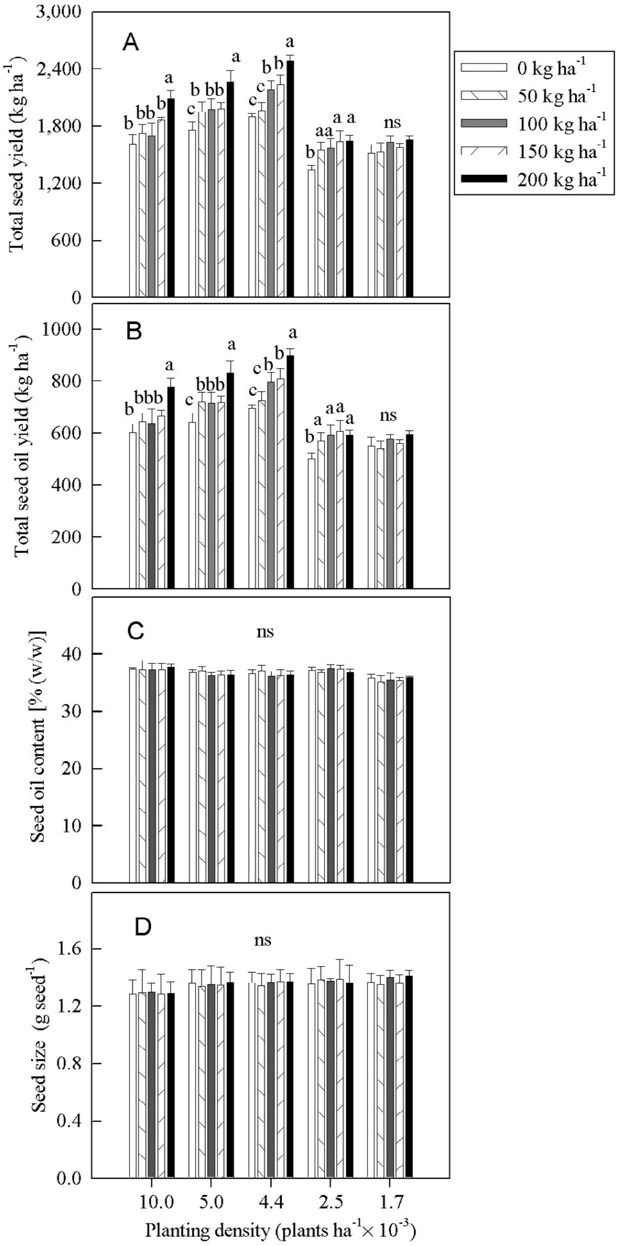

FIG. 3
Total seed yield (Panel A), total seed oil yield (Panel B), seed oil content (Panel C), and mature seed size (Panel D) in *P. volubilis* plants cultivated at five different planting densities and five rates of fertilisation pooled across the different sampling times. Mean values (n = 4) with a different lower-case letter in each Panel indicate significant differences between fertilisation levels within each planting density at $P \leq 0.05$. ns, not significant.

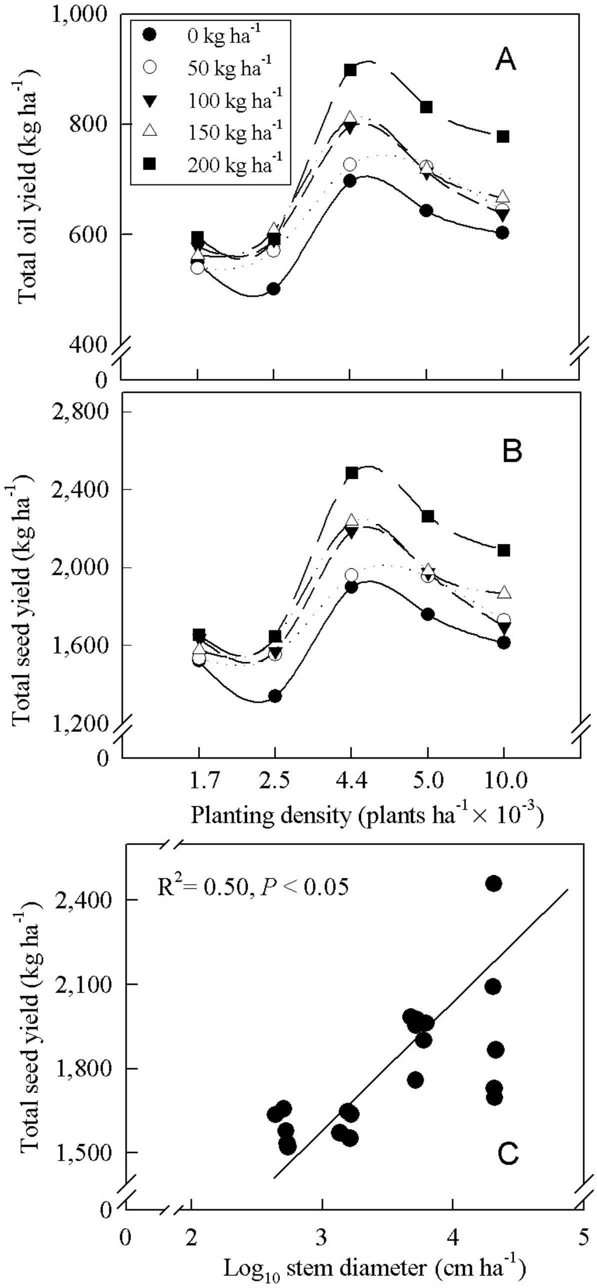

FIG. 4
Relationships between planting density and total oil yield (Panel A) or total seed yield (Panel B) at five different rates of fertilisation throughout a growing season, and between stem diameter and total seed yield (Panel C) in *P. volubilis* plants across five different fertilisation treatments.

Total seed and oil yields over the growing season at the sub-plot level ranged from 1,340 – 2,486 kg ha^{-1}, and from 501 – 899 kg ha^{-1}, respectively, among the different treatments. The increase in total seed yield was accompanied by an increase in plant growth (i.e., stem diameter; Figure 4 C), indicating that the resources demanded by the seed are controlled mainly by the amount of stored resources (i.e., carbohydrates) in the maternal plant during the reproductive stage. The highest yields of both seed and oil occurred at the medium planting density combined with the highest rate of fertilisation (Table I; Figure 3; Figure 4). An increase in planting density to 4,444 plant ha^{-1} was an effective means to increase seed and oil yields in *P. volubilis* plants, after which point both total seed and oil yields declined significantly (Table I; Figure 4 A,B). Regression analysis indicated that the relationships between planting density and total seed or oil yields at any fertilisation level were best expressed by quadratic equations (R^2 = 0.76 – 0.89; all $P \leq 0.01$; Figure 4 A,B), namely:

$$Y = a + b\,D + c\,D^2$$

where Y was the total seed yield or the total oil yield (kg ha^{-1}), D was the planting density (plants ha^{-1}), and a (intercept), b (linear coefficient), and c (quadratic coefficient) were parameters.

Based on these quadratic equations, the estimated optimum planting density for the maximum total seed or oil yields did not vary greatly at all fertiliser levels. Therefore, a planting density of approx. 4,444 plants ha^{-1} is required to ensure the optimum yield of *P. volubilis* plants, regardless of fertiliser level. Seed and oil yields commonly increased curvi-linearly with an increase in planting density in other oilseed crops such as winter oilseed rape (Leach *et al.*, 1999; Momoh and Zhou, 2001; Sidlauskas and Bernotas, 2003), *Zea mays* L. (Ciampitti and Vyn, 2011), and in food crops such as faba bean (*Vicia faba* L.; López-Bellido *et al.*, 2005), and in cotton (Dong *et al.*, 2012).

As the partitioning of DM to reproductive sinks during flowering, and the DM: total DM ratio of seeds were reduced at harvest by shortages of N (Ciampitti and Vyn, 2011), positive impacts of the application of fertiliser on seed yields in oilseed crops such as winter oilseed rape and soybean have been reported (Jackson, 2000; Rathke *et al.*, 2005). In contrast, some authors have reported a plateau or even a reduction in seed yields at high rates of N-fertiliser (Hocking *et al.*, 1997; Cheema *et al.*, 2001). The optimum seed yield in oilseed crops under a particular fertiliser level depends on environmental conditions (Jackson, 2000). In our study, total seed and oil yields increased continuously with increasing levels of fertilisation (Table I; Figure 2), suggesting that *P. volubilis* plants require relatively high fertiliser levels to achieve maximum yields in the field. A strong positive correlation was found between total seed and oil yields across all sampling dates (r = 0.99; $P \leq$ 0.001). Thus, oil production in *P. volubilis* plants is determined primarily by the maximum attainable seed yield, but not by seed oil content (Sidlauskas and Bernotas, 2003; Rathke *et al.*, 2005).

In conclusion, neither planting density nor rate of fertilisation affected the phenological development of *P. volubilis* plants. Planting density and fertilisation affected seed and oil yields in *P. volubilis* plants independently. A planting density of approx. 4,444 plants ha^{-1} was required to ensure maximum seed and oil yields, regardless of fertiliser level. *P. volubilis* plants require relatively high rates of fertilisation application to achieve their maximum seed and oil yields. Future research should focus on a detailed analysis of the optimum rate of application of fertiliser to achieve the highest yield of *P. volubilis* plants and to mitigate losses of nutrients in the field.

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