

Demand and supply of N in seed production of soybean (*Glycine max*) at different N fertilization levels after flowering

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Abstract Nitrogen (N) has been suggested as a determinant of seed production especially in species with high seed N content. Assuming that seed yield was determined as the balance between N demand and supply for seed production, we studied the effect of N fertilization after flowering on soybean (*Glycine max* L. Merr.) yield. Seed N concentration was nearly constant irrespective of N fertilization, indicating that seed production was proportional to the amount of N available for seed growth. N demand for seed production was analyzed as the product of seed number, the rate of N filling in individual seeds, and the length of the reproductive period. N fertilization increased seed number and the reproductive period, but did not influence the N filling rate. Seed number was positively correlated with dry mass productivity after flowering. Three N sources were distinguished: mineral N uptake, symbiotic N₂ fixation and N remobilization from vegetative body. N fertilization increased N uptake and N remobilization, but lowered N₂ fixation. We concluded that N availability in the reproductive period determined seed yield directly through increasing N supply for seed growth and indirectly through increasing seed N demand with enhanced plant dry mass productivity.

Keywords *Glycine max* · N demand and supply · N filling rate · N₂ fixation · Remobilization · Seed yield

Introduction

Seed production is closely related to plant ecological fitness and crop productivity. It is crucial for success of the species in the natural environment and for food production in agricultural systems. Nitrogen (N) has been suggested as a determinant of seed production especially in species with high seed N content (Sinclair and de Wit 1975; Henry et al. 1992; Lawlor 2002; Sinclair and Vadez 2002; Hirose et al. 2005). This is because N is more limiting than carbon in producing seeds with high N content. Kinugasa et al. (2003) grew an annual herb *Xanthium canadense* at elevated CO₂ and found no increase in seed yield despite the increase in carbon availability with elevated CO₂. Seed production in annual species was enhanced by elevated CO₂ only when N allocation to seeds increased (Miyagi et al. 2007). Thus seed yield is restricted by the amount of N allocated to seed, with seed N concentration being more or less constant within species.

Soybean (*Glycine max* L. Merr.) requires a large amount of N for seed production (Sinclair and de Wit 1975), and hence its yield may be sensitive to N fertilization after flowering. However, positive effect of N fertilization after flowering on soybean yield was not always found in published studies (Salvagiotti et al. 2008). This may imply that factors other than N supply contribute to the determination of seed N and dry mass yield. Hirose et al. (2005) presented a simple model that described seed yield as a function of N demand and supply in seed production. The model, assuming constant seed N concentration, determined seed yield by the N when seed N demand exceeds the plant

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potential to supply N for seed, and predicted changes in seed yield in response to the environment. Here, we hypothesize that seed yield is constrained by factors that determine N demand as well as N supply for seed production.

N demand for seed may be determined by the product of three factors: seed number, the rate of N filling in individual seeds, and the length of the reproductive period. N fertilization may not stimulate the rate of N filling in individual seeds when sink activities are limited (Lhuillier-Soundele et al. 1999a). However, it may increase the number of pods and seeds due to enhanced availability of photosynthate (Egli and Zen-wen 1991; Board and Tan 1995; Jiang and Egli 1995) and extend the reproductive period due to delay in leaf senescence (Sinclair and de Wit 1975, 1976). There are three N sources for seed production in soybean: mineral N uptake from soil, symbiotic fixation of atmospheric N₂ and N remobilization from vegetative body. N fertilization may increase mineral N uptake but lower symbiotic N₂ fixation because high soil nitrate levels inhibit N₂ fixation (Hunt and Layzell 1993; Voisin et al. 2002; Fujikake et al. 2002; McKenzie et al. 2003). It may also suppress N remobilization from vegetative body if external N supply meets N demand for seed production (Sunarpi and Anderson 1997; Imsande and Schmidt 1998).

In this paper, we address the factors that restrict seed yield. Assuming constant seed N concentration, we expect that seed production is determined by the amount of N allocated to seeds (Sinclair and Vadez 2002; Hirose et al. 2005). We analyze the response of soybean yield to N fertilization after flowering as a consequence of changes in N demand and supply in seed production. If N fertilization do not increase seed yield, it will result from reduction in N₂ fixation and/or N remobilization from vegetative body. If N fertilization increase seed yield, it will result from increased mineral N uptake that outweighs reduction in N₂ fixation. Enhanced N supply will be associated with the increase in either seed number, N filling rate, or length of the reproductive period.

Materials and methods

Experimental design

Nodulating soybean (*Glycine max* L. Merr. cv. Enrei) and its non-nodulating mutant (cv. En1282) were employed for the experiment (Francisco and Akao 1993). The latter was used as a control to estimate N₂ fixation in the former. Both have a determinate flowering habit (Oikawa et al. 2010). Seeds of both lines were sown on 8 July 2005 on moistened paper in plastic dishes. Radicles emerged in a day after

sowing. Germinated seeds were transplanted each in a pot (4.0 L volume) filled with well-washed Tottori dune sand inoculated with *Bradyrhizobium japonicum*. The number of plants prepared for the experiment was 113 and 92 for cv. Enrei and cv. En1282, respectively. Pots were placed in a greenhouse in the Arid Land Research Center, Tottori University, Japan (35°32'N and 134°13'E). Mean monthly temperatures in the greenhouse were 28.7°C in July, 29.4°C in August, 25.7°C in September and 19.4°C in October.

Plants were grown with weekly N additions. Plants received uniform N fertilization until flowering (2.94 mgN week⁻¹). After flowering, plants were divided into three groups with different N fertilization rates: one with N equal to that before flowering (MN, 2.94 mgN week⁻¹), one with N ten times of MN (HN, 29.4 mgN week⁻¹) and one with one tenth of MN (LN, 0.294 mgN week⁻¹). Standard nutrient solution followed Epstein (1972): N 16 (NO₃⁻: NH₄⁺ = 7:1), P 2, K 6, Ca 4, S 1, and Mg 1 mmol L⁻¹. N in the solution had a mean δ¹⁵N value of 12.0 ± 0.5‰ in excess against atmospheric air. 15/8 strength of this solution was prepared for HN. Solutions for MN and LN were prepared from the HN solution replacing NH₄⁺ with Na⁺ and NO₃⁻ with Cl⁻. Pots received 70 ml of the solution every week from 1 week after germination. Plants received tap water as needed during the experiment. Pots were rotated periodically to minimize differences in growth conditions among plants.

Measurements

The date of flowering was recorded when the first flower appeared and the date of death when all leaves dropped and the stem turned brown. Plants were harvested at about 3-week intervals, and at seed maturation (plant death). Harvests after flowering corresponded to R1 (beginning of blooming), R4 (full pod), R6 (full seed) and R8 (full maturity). Four plants were sampled per treatment at each harvest. Harvested plants were separated into leaves, stems, roots, dead leaves, dead petioles, nodules, and reproductive structures (pods and seeds). The number of nodules was counted. Leaf area was determined with a leaf area meter (AAL-410, Hayashi Denko, Japan). Dry mass of plant parts was determined after oven drying at 80°C for more than 3 days. Samples were ground in a mill and their N content and δ¹⁵N values were measured by an elemental analyzer (Flash EA1112, Thermo Electron, Milan, Italy) coupled to an isotope ratio mass spectrometry (IRMS) via ConFlo III interface (Delta Plus XP, Thermo Fisher Scientific, Bremen, Germany).

The percentage N derived from symbiotic N₂ fixation (N_{dfr}) in the whole plant of nodulating soybean (Enrei) was calculated every harvest with the following equation:

$$N_{\text{dfa}} = (\delta^{15}\text{N}_a - \delta^{15}\text{N}_b) / (\delta^{15}\text{N}_a - \delta^{15}\text{N}_c) \times 100 \quad (1)$$

where $\delta^{15}\text{N}_a$, $\delta^{15}\text{N}_b$ and $\delta^{15}\text{N}_c$ represent $\delta^{15}\text{N}$ value in non-nodulating soybean (cv. En1282), nodulating cv. Enrei and soybean grown with N only from symbiotic fixation. In this study, -1.7‰ was used for $\delta^{15}\text{N}_c$ value in soybean (Yoneyama et al. 1986, 2000).

The model

If seed yield (Y) is limited by the amount of N allocated to seeds, it is determined as the seed N (Y_N) divided by seed N concentration ($[N_{\text{se}}]$):

$$Y = Y_N / [N_{\text{se}}] \quad (2)$$

Considering N sources for soybean, the following equation can be obtained:

$$Y_N = U_N + F_N + R_N \quad (3)$$

where U_N , F_N and R_N represent seed N supplied from mineral N uptake, N_2 fixation and N remobilization from vegetative body, respectively. Assuming leaves as the main source of N remobilization and all remobilized N is allocated to seed (Egli et al. 1978; Zeiher et al. 1982), R_N was calculated as the reduction in leaf N (the sum of N in living and dead leaves) during the reproductive period. Efficiency of N remobilization was calculated as a quotient of remobilized N to the foliage N at the first harvest after flowering (R4) when foliage N reached the maximum. The sum of U_N and F_N , i.e. newly acquired N, was obtained by subtracting R_N from Y_N . We assumed that the ratio of U_N to F_N was the same as that of mineral N uptake to N fixation in plant N acquisition. N demand in seed production was analyzed as:

$$Y_N = S \times A_N \times D \quad (4)$$

where S , A_N and D represent seed number, the rate of N filling in individual seeds, and the length of the reproductive period, respectively.

We calculated model variables with the assumption that the rank order of individuals in terms of total dry mass did not change with plant growth. Statistical analyses were conducted with JMP software (JMP 7, SAS Institute Inc., USA). To test the effects of N treatments on variables, a post hoc test (Tukey–Kramer method) was used after applying ANOVA.

Results

N fertilization did not influence seed N concentration ($[N_{\text{se}}]$, Fig. 1a), supporting the assumption that seed has a constant N concentration. N fertilization, however,

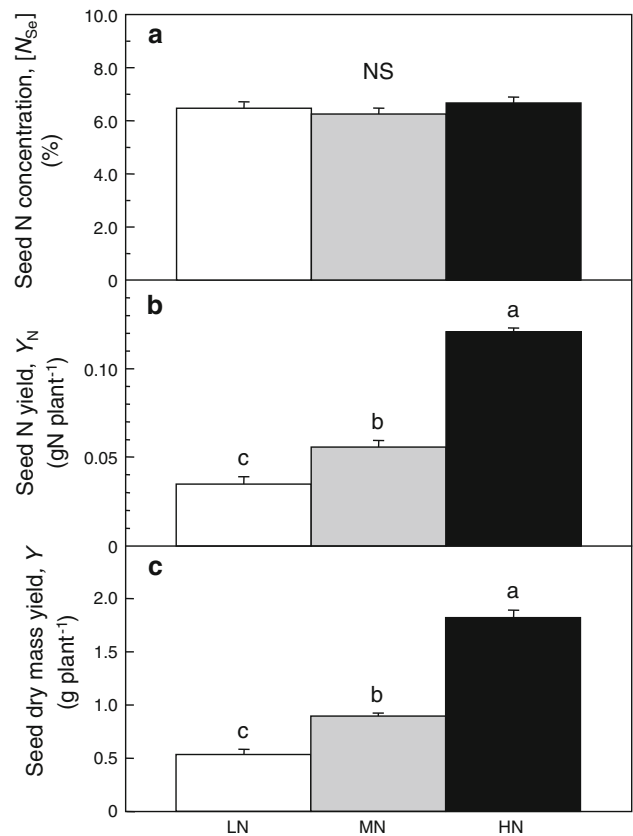


Fig. 1 a Seed N concentration ($[N_{\text{se}}]$), b seed N (Y_N) and c seed dry mass yield (Y) of soybean grown with different N fertilization after flowering: LN ($0.294 \text{ mgN week}^{-1}$), MN ($2.94 \text{ mgN week}^{-1}$) and HN ($29.4 \text{ mgN week}^{-1}$). Different alphabets above columns indicate statistically significant difference (Tukey–Kramer method, $P < 0.05$). Error bars indicate ± 1 SE ($n = 4$)

significantly increased seed N yield (the amount of N in seed at the end of the growth period, Y_N , Fig. 1b). Seed dry mass yield (Y) thus increased with N fertilization (Eq. 2, Fig. 1c).

Figure 2 shows contributions of three N sources to Y_N (Eq. 3). The amount of mineral N taken up from soil (U_N) increased with increasing N levels, although the difference between LN and MN was not significant (Fig. 2a). U_N accounted for Y_N by 4.3, 17.4 and 77.7% at LN, MN and HN, respectively. N_2 fixation (F_N) was significantly smaller at HN than the other two N treatments (Fig. 2b). The fraction of Y_N derived from F_N was 87.9, 74.5 and 5.8% at LN, MN and HN, respectively. Remobilized N from vegetative body (R_N) was largest at HN (Fig. 2c). R_N accounted for 7.7, 8.1 and 16.5% of Y_N at LN, MN and HN, respectively.

The rate of N_2 fixation per plant in the reproductive period was significantly larger in LN and MN than HN plants (Table 1). The number of nodules averaged over the reproductive period was not different between N treatments. The rate of N_2 fixation per nodule, i.e. nodule

activity, decreased at high N fertilization. Efficiency of N remobilization was not significantly different between N treatments, although the maximum foliage N was significantly larger at higher N fertilization (Table 2).

N demand in seed production was analyzed with Eq. 4. Seed number (S) was larger in HN than the other two treatments (Fig. 3a). S was positively correlated with the plant dry mass production in the early reproductive period (R1–R4) ($P < 0.05$, $r^2 = 0.66$, Fig. 4). N filling rates in individual seeds (A_N) were not significantly different

between N levels (Fig. 3b). The reproductive period (D) was 16 days longer in HN than the other two treatments (Fig. 3c). N in individual seeds ($A_N \times D$) increased with N fertilization, implying that dry mass of individual seeds increased as well.

Discussion

N fertilization after flowering significantly increased plant growth and seed yield (Y) (Fig. 1). The positive response of Y to N fertilization after flowering has been reported in many studies (Afza et al. 1987; Wood et al. 1993; Hayati 1995; Wesley et al. 1998; Gan et al. 2002), though no response was also reported (Schmitt et al. 2001; Gutierrez-Boem et al. 2004; Barker and Sawyer 2005; Salvagiotti et al. 2008). The increase in Y was caused by the increase in seed N (Y_N) with constant N concentration of seed ($[N_{sc}]$) (Fig. 1). A meta-analysis of 61 soybean studies showed that the variation of $[N_{sc}]$ was considerably narrow: an average 6.34% with an interquartile range of 6–6.75% (Salvagiotti et al. 2008). Values of $[N_{sc}]$ in the present study were within this interquartile range. Constant $[N_{sc}]$ implies that seed production is proportional to N allocation to seeds (Sinclair and de Wit 1975; Sinclair and Vadez 2002; Kinugasa et al. 2003; Hirose et al. 2005; Miyagi et al. 2007).

N fertilization increased N uptake (U_N) and remobilization (R_N), but decreased N_2 fixation (F_N) (Fig. 2). Reduction in N_2 fixation with N fertilization has been ascribed to inhibition of nodule formation and/or nitrogenase activity (Sprent et al. 1988; Lucinski et al. 2002) by high soil nitrate levels (Hunt and Layzell 1993; Voisin et al. 2002; Fujikake et al. 2002; McKenzie et al. 2003). As the number of nodules did not differ between N treatments (Table 1), the decrease in F_N at HN should have been caused by the reduction in nitrogenase activity.

In contrast to our expectation, N fertilization increased the amount of N supplied from remobilization from vegetative body (R_N) in both absolute and relative terms (Fig. 2c), though its contribution to Y_N was relatively small. The increase in R_N with N fertilization resulted from

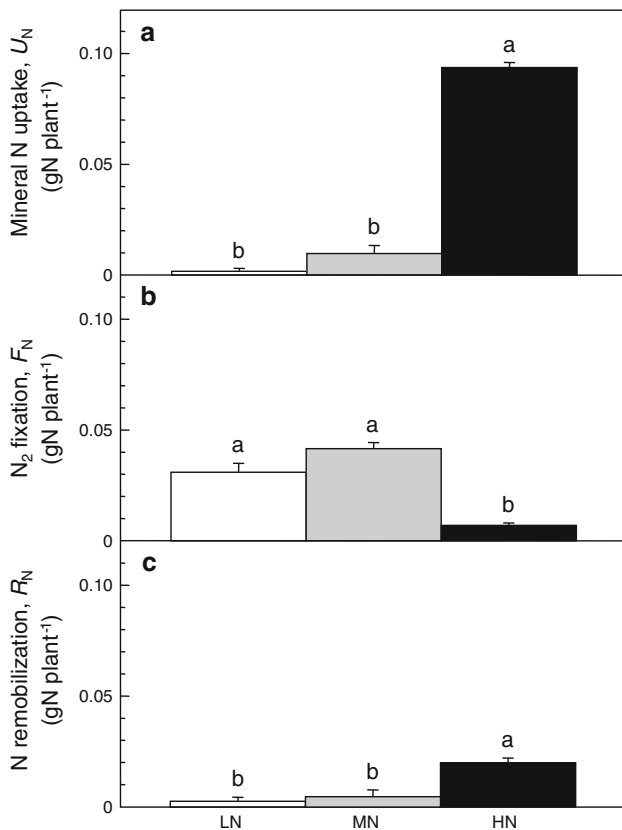


Fig. 2 Sources of seed N (Y_N): **a** seed N supplied from mineral N uptake (U_N), **b** symbiotic N_2 fixation (F_N) and **c** remobilization from vegetative body (R_N). Open, grey and black columns represent LN, MN and HN treatments, respectively. Different alphabets above columns indicate statistically significant difference (Tukey–Kramer method, $P < 0.05$). Error bars indicate ± 1 SE ($n = 4$)

Table 1 The rate of N_2 fixation and nodule number averaged across the reproductive period

	N treatment		
	LN	MN	HN
N_2 fixation rate (mgN plant ⁻¹ day ⁻¹)	0.764 ^a (0.112)	0.965 ^a (0.145)	0.152 ^b (0.025)
Number of nodules	75.3 ^a (2.17)	78.3 ^a (4.65)	65.1 ^a (6.36)
N_2 fixation per nodule (mgN nod ⁻¹ day ⁻¹)	0.0103 ^a (0.0017)	0.0127 ^a (0.0026)	0.0024 ^b (0.0005)

Different alphabets in the same row indicate significant difference between N treatments (Tukey–Kramer method, $P < 0.05$). Values in parentheses indicate SE ($n = 4$)

Table 2 Maximum foliage N in the growing period (at R4 stage) and N remobilization efficiency from leaves

	N treatment		
	LN	MN	HN
Maximum foliage N (mgN plant ⁻¹)	8.10 ^a (1.31)	13.13 ^b (1.11)	41.65 ^c (0.89)
Leaf N remobilization efficiency (%)	38.9 ^a (21.5)	29.8 ^a (19.3)	48.0 ^a (6.3)

Different alphabets in the same row indicate significant difference between N treatments (Tukey–Kramer method, $P < 0.05$). Values in parentheses indicate SE ($n = 4$)

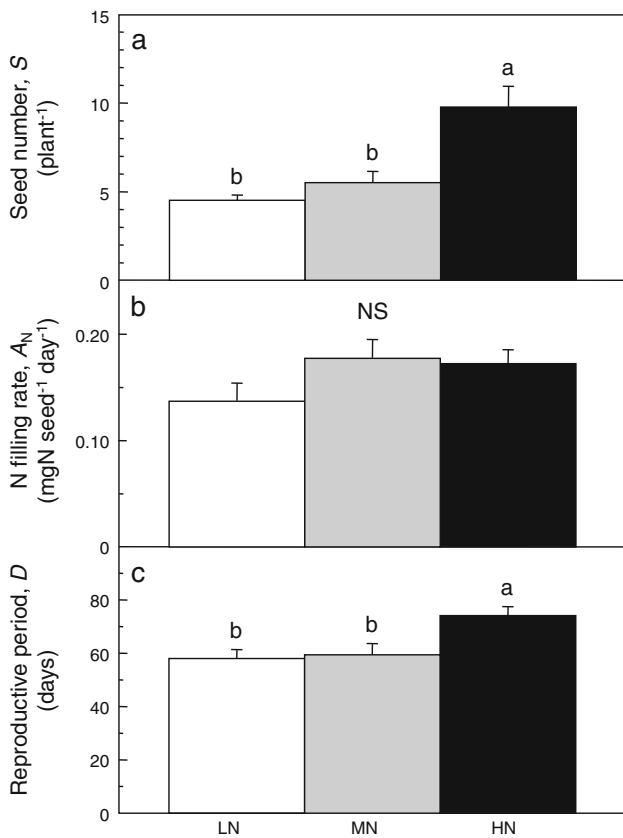


Fig. 3 **a** Seed number (S), **b** the rate of N filling in individual seeds (A_N), and **c** the length of the reproductive period (D). *Open, grey and black columns* represent LN, MN and HN treatments, respectively. *Different alphabets above columns* indicate statistically significant difference (Tukey–Kramer method, $P < 0.05$). *Error bars* indicate ± 1 SE ($n = 4$)

the increase in foliage N pool available for remobilization, because N remobilization efficiency did not change with N fertilization (Table 2). N allocation to vegetative structures continued after flowering in soybean (Pahalwan and Tripathi 1984; Warembourg and Fernandez 1985; Vessey et al. 1990). No reduction in the efficiency of leaf N remobilization with N fertilization has been reported in many studies (e.g. Huang et al. 2008; Bancel 2009; Norris and Reich 2009). A survey of more than 30 studies covering 60 species showed no response of leaf N

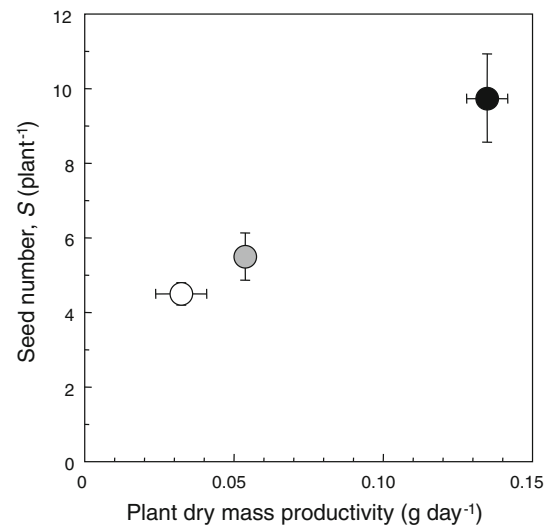


Fig. 4 Relationship between plant dry mass productivity in the early reproductive period (R1–R4) and seed number per plant (S). *Open, grey and black symbols* represent LN, MN and HN treatments, respectively. *Error bars* indicate ± 1 SE ($n = 4$)

remobilization efficiency to increased nutrient availability in about two-thirds of experiments (Aerts 1996).

The increase in Y_N with N fertilization was caused mainly by the increase in seed number (S) (Fig. 3a). Soybean aborts 20–80% of flowers and immature pods (Van Schaik and Probst 1958; Wiebold et al. 1981; Heitholt et al. 1986). If the number of flowers was determined before different fertilization treatments, different number of seeds might reflect the number of aborted flowers and pods. The final pod and seed number was determined by the availability of photosynthates in the early reproductive period (from flowering to pod formation, R1–R5) (Board and Tan 1995, Jiang and Egli 1995). In the present study, S was positively correlated with dry mass productivity in the early reproductive period (R1–R4, Fig. 4). Larger dry mass productivity in HN plants is attributable to their larger canopy leaf area and higher leaf N (both more than 1.5 times larger than those in the other treatments).

It was suggested that the reproductive period (D) would extend with N fertilization due to delay in leaf senescence (Sinclair and de Wit 1975, 1976; Hirose et al. 2005).

Several studies reported lengthening of D with N fertilization (Egli et al. 1985; Munier-Jolani and Duthion 1996; Egli 2004). In the present study, however, D lengthened at HN only, with no shortening at LN (Fig. 3c). Its contribution to the increase in Y_N was relatively small when compared with that of S .

To our knowledge, changes in seed N filling rate (A_N) with N fertilization have not been reported in soybean. In pea, however, A_N increased with the amount of N available for seed growth until a maximum A_N was reached due to source limitation (Lhuillier-Soundele et al. 1999a, b). A_N of soybean did not significantly respond to N fertilization (Fig. 3b), indicating that N availability was large enough for seed production, where seed production might have been sink- rather than source-limited.

Seed N in individual seeds ($A_N \times D$) and consequently seed size increased significantly with increasing N fertilization. Actually seed size increased by 57% (LN vs. HN), while seed number by 117%. Soybean responded to different N availability by changing seed number more than seed size, which was in line with the general trend (Bradshaw 1965; Harper et al. 1970; Borrás et al. 2004). High plasticity in seed number may allow for variable response to resource availability. Less variable seed size may also have been reinforced by agronomic selection (Sadras 2007).

Conclusions

Seed N concentration was nearly constant irrespective of N fertilization after flowering, and therefore seed production was proportional to the amount of N available for seed growth. N fertilization increased seed N and thus seed dry mass yield. The increase in seed N and dry mass yield was associated with the increase in seed number with a less variable seed size. The number of seeds was determined by dry mass productivity at the early stage in the reproductive period. N availability in the reproductive period determined seed yield directly through increasing N supply for seed growth and indirectly through increasing seed N demand with enhanced plant dry mass productivity.

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