Seed reserve dependency of *Leucaena leucocephala* seedling growth for nitrogen and phosphorus

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**Abstract.** Mineral elements stored in seed reserves meet the nutrient demands of seedlings during their initial development and growth. We experimentally examined when seed reserves become insufficient to meet demands for nitrogen (N) and phosphorus (P) of seedlings of *Leucaena leucocephala* (Lam.) de Wit, a tropical woody legume. Seedlings were grown from seeds with four nutrient treatments: receiving all nutrients; all nutrients except N, all nutrients except P or deionised water. Growth curves were compared to quantify the time course of the onset of N and P deficiency during 8 weeks. N deficiency became significant for leaf area and biomass growth after 11 and 16 days, respectively, whereas P deficiency became significant after 31 days for both leaf area and biomass growth. Thus, seed reserves alone could support the P demands of seedlings for more than twice as long as N demands. As nutrient deficiency developed, seedlings adjusted increased relative biomass allocation to roots, diluted organ N and P concentrations but conserved 100% of the initial nutrient pool derived from the seed.

**Additional keywords:** growth analysis, *Leucaena leucocephala*, nitrogen limitation, nitrogen deficiency, phosphorus nutrition, seed reserves, seedling growth.

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**Introduction**

Nitrogen (N) and phosphorus (P) are two macronutrients that commonly limit plant growth and productivity in many ecosystems (Lambers et al. 2008). Leaf N concentration strongly correlates with the concentration of photosynthetic enzymes (Evans 1989) and plants adjust their N allocation patterns to maintain optimal N concentrations in response to availability of N and light (Hikosaka and Terashima 1996). Phosphorus is also required in relatively large quantities for sugar-phosphate intermediates of photosynthesis and respiration; in plant membrane phospholipids; in energy metabolism (ADP, ATP); and in nucleic acids (Taiz and Zeiger 2010). Uptake of these nutrients is facilitated by various morphological and physiological adaptations in roots, including symbiotic relationships with microbes such as nitrogen fixing bacteria and mycorrhizal fungi (Bolan 1991; Heath and Tiffin 2007; Parniske 2008; Lambers et al. 2009). However, in young seedlings, before sufficient root development, N and P demands must be met by supply from seed reserves (Allsopp and Stock 1995).

Compared with most vegetative organs, seeds of most species contain elevated concentrations of N and P to meet demands of developing seedlings. Experiments in which individual mineral elements are omitted from the external medium allow assessments of how long seed reserves alone meet the seedling demands for these elements in response to availability of N and light (Hikosaka and Terashima 1996). Phosphorus is also required in relatively large quantities for sugar-phosphate intermediates of photosynthesis and respiration; in plant membrane phospholipids; in energy metabolism (ADP, ATP); and in nucleic acids (Taiz and Zeiger 2010). Uptake of these nutrients is facilitated by various morphological and physiological adaptations in roots, including symbiotic relationships with microbes such as nitrogen fixing bacteria and mycorrhizal fungi (Bolan 1991; Heath and Tiffin 2007; Parniske 2008; Lambers et al. 2009). However, in young seedlings, before sufficient root development, N and P demands must be met by supply from seed reserves (Allsopp and Stock 1995).

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In the present study, we selected *Leucaena leucocephala* (Lam.) de Wit (Fabaceae), a common small tree native to southern Mexico and northern Central America, to experimentally quantify how long seed reserves support initial seedling growth and development. This species is planted throughout the tropics for fodder, firewood and ‘green manure’, as it can thrive on relatively infertile soils, though higher yields are achieved in fertile soils (Mullen et al. 2003).
Outside its native range, however, *L. leucocephala* may become invasive (Lowe et al. 2000), for example, on the Hawaiian Islands (Baruch and Goldstein 1999) and in the southern United States (PLANTS Database, United States Department of Agriculture 2011). Like many legumes, *L. leucocephala* forms symbioses with both nitrogen fixing bacteria and mycorrhizal fungi (Manjunath et al. 1984; Barea et al. 1990; Puthur et al. 1998; Mahmood et al. 2004). It is unknown whether *L. leucocephala* seeds contain a balanced storage of N and P such that the developing seedling exhausts them simultaneously. A better understanding of the initial nutrient requirement for seedlings in relation to nutrients provided by the seed would be useful for growing *L. leucocephala* as an economically useful species.

We followed an experimental design of comparative growth analysis to evaluate the duration of seed reserve dependency for N and P (cf. Kitajima 2002) and conducted greenhouse experiments to evaluate the time course of seedlings meeting N and P requirements from seed reserves versus the external medium. Because nutrient demands depend on light availability (e.g. Hikosaka and Terashima 1996; Kitajima 2002), the effects of nutrient treatments were examined under two light levels. Seedlings were grown in nutrient-free sterile medium to which nutrient solutions with or without N and P were applied to assess the point in time whether N or P from seed reserves alone could no longer meet the seedlings’ demand. In nature, this timing would be the time by which the seedlings would have to have initiated uptake of nutrients from the soil to maintain positive growth. In the case of *L. leucocephala*, uptake of N and P from infertile soils is likely to involve symbiotic association with rhizobia for N and mycorrhizal fungi for P. We also examined concentrations and pool sizes of N and P at critical developmental stages to explore how seedlings adjust allocation patterns of N and P when these elements are limited in supply from the external environment. If the N in seed reserves becomes exhausted before all other nutrients, as has been shown for pasture grasses and herbs (Fenner 1986; Fenner and Lee 1989; Hanley and Fenner 1997), then we predict that (1) growth differences due to no-N treatment and no-nutrient (deionised water) treatments would appear at the same time; and (2) the effect of no-N would occur earlier than the effect of no-P. Conversely, if P in seed reserves becomes exhausted before all other nutrients, no-P plants would lag behind in growth earlier than no-N plants, around the same time that no-nutrient plants show a growth difference with plants receiving nutrients.

**Materials and methods**

**Plant material and growth conditions**

Seeds of *Leucaena leucocephala* (Lam.) de Wit were collected from five naturally established trees on the campus of the University of Florida in Gainesville (29°60’95”N 82°34’57”W, 52 m above sea level), weighed and germinated in Petri dishes lined with phosphorus-free filter paper, moistened with deionised water. We repeated the greenhouse experiments twice, first to describe the growth-curves with frequent harvests (Exp. 1), and second for analysis of concentrations of N and P in leaves, stems and roots at critical timings identified in the first experiment, obtaining enough material for chemical analysis by combining multiple plants per treatment at each harvest (Exp. 2). Plotting the data of seedling biomass against time for the two experiments together confirmed similarity of growth patterns (data not shown).

In Exp. 1, the seedlings were grown in nutrient-free sterile growth medium (1 : 1 ratio of perlite and washed, coarse quartz sand) in germination flats until their first true leaf was fully expanded ~7 days after germination. They were then transplanted into individual plastic nursery bags (~900 mL). A total of 400 seedlings were randomly assigned to one of four nutrient treatments (modified Johnson solution, Epstein 1972) in which all nutrients (‘control’), no N (‘no-N’), no P (‘no-P’) or no nutrients (deionised water, ‘DF’) were supplied three times per week. Seedlings were placed in a split-block design in 10 blocks on two greenhouse benches (10 seedlings per treatment per block), split to five blocks per bench. Each bench was assigned to either a high- or low-light treatment; the high-light bench received natural light within the greenhouse without artificial light with mid-day maximum PPFD ~600 μmol m⁻² s⁻¹. The remaining five blocks were under frames covered with 50% woven shade cloth. The block locations were rotated within each bench biweekly to minimise the effects of spatial heterogeneity. One randomly selected plant per block was harvested every other week between September and December 2008 (i.e. five plants per treatment combination per harvest) for leaf area and biomass allocation measurements as described below.

The second experiment (Exp. 2), designed for nutrient analysis, was similar in set-up, except we used plastic pots (~500 mL) under a single light treatment (mid-day maximum PPFD ~800 μmol m⁻² s⁻¹) because the growth difference between the two light treatments in the first experiment was minimal. In order to obtain sufficient material for nutrient analysis at the organ level, 7–10 plants were grouped as single replicates, and assigned to a combination of four nutrient treatments and eight blocks. The location of replicate groups within each block was rotated as in Exp 1. Three harvests were conducted between February and April 2010: pre-treatment, intermediate and final. According to the results of Exp. 1, the intermediate harvest was conducted when signs of yellowing of leaves started to become visible in no-N treatments. The final harvest was done when large size differences among the treatments were visible, corresponding to the timing when large biomass and leaf area differences had developed in Exp. 1.

**Chlorophyll concentrations**

Leaf chlorophyll content was determined for the most recently expanded leaf with a SPAD-502 chlorophyll meter (Minolta, Osaka, Japan) calibrated against *L. leucocephala* leaf chlorophyll standards obtained by chlorophyll extraction in N, N-dimethylformamide and spectrophotometry according to Wellburn (1994). For Exp. 2, SPAD values were recorded for 5–10 individual plants per group immediately before each harvest.

**Harvest and nutrient analysis**

After removing them carefully from their pot, each plant was separated into leaves, stems, cotyledons and roots, and leaf area was determined with a LI-3000 leaf area meter (Li-Cor Inc.,
Lincoln, NE, USA). Dry mass of each organ type was determined for each individual plant (Exp. 1) or per block of plants (Exp. 2) after 72 h at 60°C. Finely ground leaf, stem and root samples from Exp. 2 were analysed for N concentration with an elemental analyser (Costech Analytical, Los Angeles, CA, USA) and for P concentration following Jones and Case (1996). In brief, P was extracted from ashed samples in hydrochloric acid, and P was measured colourimetrically in a microplate reader (BioTek Instruments Inc., Winooski, VT, USA) following an ascorbate assay (Jones and Case 1996). Analyses were made on samples pooled per block because analysis of leaf P requires a minimum of 200 mg of dry plant material. Similarly, samples from the pre-harvest were pooled across groups to have sufficient material for analyses.

**Data analyses**

Third-order polynomial functions were fitted to biomass and leaf area plotted against time for Exp. 1, because this function is flexible for description of dynamic growth patterns without unverified assumptions, and calculation of confidence intervals is straightforward (Kitajima 2002). The third-order polynomials were simplified to second order or linear fits if higher-order terms were not significant. Significant separation of growth curves among treatments was identified when the 95% confidence intervals of the estimated curves do not overlap any longer. The first date at which this separation became significant between the ‘all’ nutrient treatment and the no-N or no-P treatment was identified as the time when N or P supply from seed reserve alone was insufficient to allow optimal seedling growth. The difference of nutrient concentrations and pools were tested with ANOVA, and Tukey’s post-hoc comparisons were made when appropriate. All statistical analyses were performed in JMP ver. 8.0 (SAS institute, Cary, NC, USA).

**Results**

*Seedling growth curves*

The growth curves for biomass and leaf area plotted against time characterised with third-order polynomials had significant fits (mean $R^2 = 0.87$, with range 0.74–0.98, $P<0.01$ for all terms). Biomass and leaf area of control plants receiving all nutrients continued to increase over the duration of the experiment (Fig. 1). Growth under no-N treatment lagged behind the all-nutrient treatment after 16 and 26 days in high- and low-light treatments respectively. In contrast, the effect of the no-P treatment did not become significant until after 31 and 35 days in high- and low-light treatments respectively. The effects of the no-N treatment on leaf area became significant after 13 and 11 days in high- and low-light treatments and the effect of no-P treatment on leaf area became significant after 31 and 26 days in high- and low-light treatments respectively (Fig. 1c, d). Accumulation of leaf area was also affected by N deprivation earlier than by P deprivation (Fig. 1c, d). The effects of the no-N treatment on leaf area became significant after 13 and 11 days in high- and low-light treatments and the effect of no-P treatment on leaf area became significant after 31 and 26 days in high- and low-light treatments respectively (Fig. 1c, d). The downward trajectory of leaf area at the very end of the experiment occurred

![Fig. 1. Total seedling dry mass (a, b) and total leaf area (c, d) of developing *Leucaena leucocephala* seedlings under ambient greenhouse conditions (‘high light’) (a, c) and under artificial shade (‘low light’) (b, d) in Exp. 1. The abscissa represents days since initiation of treatment, which was ~7 days after radicle emergence. Third-order polynomials were fit through the data. Vertical arrows indicate the time when the growth curves of plants under no-nitrogen and no-phosphorus treatments became significantly different from that of the all-nutrient treatment. Error bars represent s.e. ($n=8–10$).](https://example.com/fig1.jpg)
under no-N, no-P and DI-water treatments because plants started to drop older leaves under these treatments.

The time-course of root: shoot ratio (R:S) also indicates the earlier onset of N deficiency than P-deficiency under both light conditions (Fig. 2a). The R:S increased steadily for both no-N and DI, but it did not start to increase under no-P treatment until ~28 days under high light. With all-nutrient treatment, R:S increased slowly with time under high-light, but not at all under low-light.

**N and P concentrations and pool sizes**

Concentrations of N and P, as well as N:P ratio, differed among harvests in the expected directions (Table 1). The initial nutrient concentrations in leaves and stems were very high (e.g. [N] = 81 mg g⁻¹, [P] = 5.5 mg g⁻¹ for leaves, Table 1), as expected from high availability of seed-derived N and P. In no-N and DI plant organs, [N] decreased markedly by the intermediate harvest. Concentrations further dropped by the final harvest for all treatments. Similarly, the no-P treatments resulted in the lowest P concentration (down to 0.3 mg g⁻¹ for leaves). Consequently, N:P ratios were the highest in no-P plants (e.g. the leaf value as high as 54.6) and lowest in the no-N plants (e.g. as low as 15.5 for leaves). The nutrient pool size per seed and seedling was estimated by multiplying organ mass with N and P concentrations following by totalling them per plant (see Table S1, available as Supplementary Material to this paper). The whole seedling pool size of N and P increased when these nutrients could be obtained from the soil medium, but remained at the initial size (seed and pre-harvest) when they were not supplied. In plants receiving N and P, pool sizes of N and P increased over time (Fig. 3), even though N and P concentrations decreased with growth (Table 1).

At the final harvest nutrient deficiencies had significantly affected leaf chlorophyll content: plants receiving all nutrients had the highest chlorophyll content per unit leaf area, no-N and DI plants had the lowest, with no-P plants having intermediate values (Table 1). Specific leaf area (leaf area per unit leaf dry mass) did not differ among treatments at the final harvest (data not shown), so chlorophyll content per unit leaf mass exhibited similar differences among the treatments at the final harvest.

**Discussion**

**Importance of seed reserve N and P for initial growth and development**

High concentrations of mineral nutrients in seed reserves ‘buy time’ to enable optimal seedling growth until roots develop sufficiently (reviewed by Kitajima 2007). The main objective...
of our experiment was to quantify whether the duration of exclusive dependency on seed reserves differed for N and P. The results indicate that N and P stored in seeds at high concentrations support optimal seedling growth of *L. leucocephala* for 3–5 weeks following germination even when these nutrients were not available from the soil (Fig. 1). However, concentrations of essential minerals in seeds are not balanced, resulting in some mineral reserves being exhausted before others. Single nutrient elimination of nutrients such as N, P and K from the growth medium results in different concentrations support optimal seedling growth of *L. leucocephala*. Single nutrient elimination of nutrients such as N, P and K from the growth medium results in different concentrations support optimal seedling growth of *L. leucocephala*.

These results are relevant for understanding nutrient-acquisition strategies during seedling establishment of *L. leucocephala*, which is common on soils low in P (Sanginga *et al.* 1988; Radrizzani *et al.* 2010). *L. leucocephala* roots have virtually no root hairs (Munns and Mosse 1980) and under natural conditions arbuscular mycorrhizal fungi associations are formed to increase P uptake (Manjunath *et al.* 1984; Muthukumar and Udaian 2000; Mahmood *et al.* 2004). P is required for nodule formation (Israel 1987) and low P availability results in a reduction of rhizobial nodule number, N-fixation and growth of seedlings (Sanginga *et al.* 1991; Sanginga 1992; Radrizzani *et al.* 2010). Thus, under the field conditions likely to be encountered by germinating *L. leucocephala*, seed-derived P contributes to establishment of N-symbiosis until mycorrhizal P uptake becomes sufficiently established. Conversely, in the absence of nodulation, mycorrhizal infection of *L. leucocephala* roots and consequently, P uptake is lower than in plants with rhizobial nodules (Manjunath *et al.* 1984). As N from seed reserves becomes insufficient within 2–3 weeks after germination, it is advantageous for *L. leucocephala* seedlings to initiate nodule formation within this period. However, in areas far away from source populations, lack of adequate bacterial inocula prevents nodulation and thus, establishment on poor soils (Halliday and Somasegaran 1982).

**Plastic responses to external resource availability**

The duration of seed reserve dependency for a particular resource is not independent of availability of other resources. For example, seed-reserve N can meet seedling N demand for a longer time when growth is suppressed by shade (Kitajima 2002). Greenhouse light environment in our study was only 20–50%
of full sun and shading by 50% had relatively minor effects. The effect of no-N and no-P on biomass appeared earlier in high light than in low light, but the effect of no-N and no-P on leaf area appeared earlier in low light than in high light (Fig. 1). This was likely the result of adaptive plasticity to put priority in leaf area expansion and light energy harvest in shadier environment. Faster growth and accordingly greater nutrient demands in high light may be responsible for clearer effects of N and P limitation on R : S in high light than in low light (Fig. 2).

Plants may continue to grow by diluting nutrient concentration when nutrient availability is limited but only to an extent such that optimal photosynthetic N use efficiency is maintained (Hirose and Kitajima 1986). The minimum leaf N and chlorophyll concentrations were 14 mg N g⁻¹ and 7.3 μg cm⁻² (Table 1), which were similar to 15.5 mg N g⁻¹ and 7.7 μg cm⁻² measured for the seedlings of a tropical tree, *Tabebuia rosea*, grown without N supply for 50 days (Kitajima and Hogan 2003). Such severely N-stressed plants show adjustment in N-allocation between chlorophyll and stromal enzymes (especially Rubisco), as well as chlorophyll a : b ratios (Kitajima and Hogan 2003) to maintain optimal N-use efficiency. It is reasonable to expect that N-limited seedlings of *L. leucocephala* in the current experiment had similar plastic responses to maintain a certain level of photosynthetic nitrogen-use efficiency. Maintenance of sufficient N and P use efficiency may be a likely criterion for plants to stop growing all together to avoid further dilution of available nutrient pools.

**Conclusion and implications**

Our results indicate that seedlings of *L. leucocephala* exhaust seed-derived nitrogen within a few weeks of germination, by which time they must establish access to external supply of N, and P availability is crucial for this to occur. Hence, invasiveness may be constrained in habitats with low N availability away from sources of appropriate rhizobium strains. If successful N symbiosis is established, seed-derived P may support seedlings for a few additional weeks, until P-acquisition by roots or via mycorrhiza becomes necessary to maintain growth. These were apparently aspects of the seedling regeneration strategy of *L. leucocephala* that allow seedlings to hold onto the initial nutrient capital endowed by the mother plant and survive even in severely nutrient limited soils.

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**References**


