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Original article

Differential effects of N, P and K on photosynthesis and partitioning of N in *Pinus pinaster* needles

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Abstract – We investigated the response of one-year-old *Pinus pinaster* Ait. growing on a sandy, nutrient-poor soil to the factorial addition of N, P and K. We tested two hypotheses: (1) fertiliser application increases rates of light-saturated photosynthesis (A_{max}) and leaf-level water-use efficiency (WUE, as indicated by carbon isotope composition), and (2) greater concentrations of N and Rubisco explain fertiliser-promoted increases in A_{max} and WUE. Two years after fertiliser application, the height of *P. pinaster* was significantly greater in plots to which P was added (127 ± 7 cm; mean ± SE of three replicate plots) compared to those without added P (70 ± 10 cm), but was unaffected by addition of N or K. The fraction of N present as Rubisco varied between 4 and 18% and was unrelated to N concentrations, but positively related to P and K concentrations. A_{max} and $\delta^{13}C$ did not vary significantly among treatments and were unrelated to concentrations of Rubisco, N, P or K. We conclude that fertiliser application two years previously has little effect on current A_{max} and $\delta^{13}C$ of *P. pinaster*, and thus the previously reported effects of fertiliser on gas exchange are likely ephemeral.

nitrogen / phosphorus / potassium / photosynthesis / rubisco

Résumé – Effets différentiels de N, P et K sur la photosynthèse et le fractionnement de N dans les aiguilles de *Pinus pinaster*. La réponse de plant de *Pinus pinaster* Ait., poussant sur un sol sableux et pauvre, a été étudiée en fonction de l'addition de N, P et K. Deux hypothèses ont été testées : (1) la fertilisation accroît le taux de saturation lumineuse de la photosynthèse (A_{max}) et le niveau d'efficience de l'eau (WUE, évalué par la composition isotropique du carbone), (2) une plus grande concentration de N et de rubisco explique que la fertilisation accroît A_{max} et WUE. Deux années après l'application de la fertilisation, la hauteur de *P. pinaster* était significativement plus grande dans les placeaux où P avait été ajouté (127 ± 7 cm, moyenne ± SE de 3 répétitions), comparativement à ceux sans addition de P (70 ± 10 cm) mais n'était pas affectée par l'addition de N et K. La fraction de N présente sous la forme de rubisco variait entre 4 et 18 % et n'était pas expliquée par les concentrations en N, mais était positivement expliqué par les concentrations en P et K. A_{max} et δ^{13} ne variaient pas significativement avec le traitement et n'était pas reliés aux concentrations en rubisco, N, P ou K. On conclue que l'application d'un fertilisant deux ans auparavant a peu d'effet sur les valeurs actuelles de A_{max} et $\delta^{13}C$ de *P. pinaster* et alors les effets rapportés auparavant des fertilisants sur les échanges gazeux sont probablement éphémères.

azote / phosphore / potassium / photosynthèse / rubisco

Abbreviations: A_{max}, maximum rate of light-saturated photosynthesis at ambient CO_2; CE, capillary electrophoresis; C_i, intercellular CO_2 concentration; Chl, chlorophyll; g_s, stomatal conductance; LAI, leaf area index; PPFD, photosynthetic photon flux density; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; WUE, water-use efficiency; $\delta^{13}C$, stable carbon isotope composition.

1. INTRODUCTION

Application of fertiliser often increases the growth of *Pinus pinaster* Ait. and other conifers [24, 38]. The mass and area of foliage increases following fertiliser application, and this partially explains the growth response [9, 46]. Fertiliser application also increases rates of photosynthesis in some cases [9, 47, 48], but not all [10, 51]. Water-use efficiency (WUE) can be increased by fertiliser addition [18, 21, 47] and on water-limited sites may be a further explanation of the growth response. However, there are too few studies to unequivocally identify either increased rates of photosynthesis and/or greater WUE as primary causes of the growth response to fertiliser application for field-grown conifers.

Studies on plant nutrition often focus on nitrogen (N) since it is the nutrient required in the largest quantity and is generally the nutrient most likely to limit carbon gain [14]. Among species there is often a strong positive correlation between maximum rates of photosynthesis (A_{max}) and N [16, 19, 56]. This relationship owes much to the large proportion of N (up to 75%)

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present in the chloroplasts, much of it in thylakoid membranes and soluble proteins of the Calvin cycle, particularly the enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39) [16]. Within conifers, N and photosynthesis are positively reported for some species [35, 39]. More generally, however, relationships between N and photosynthesis are less consistent for conifers than for non-conifers, and may be weak or even negative [33, 34, 41, 43, 45, 47, 52, 53].

Strong relationships between phosphorus (P) and A_{max} have been observed for conifers, for example in *Pinus radiata* [15, 47], *P. pinaster* [6, 30] and *Pinus strobus* [43]. A partial explanation may lie in a positive relationship between the concentration of P and amount of Rubisco, as observed in *P. pinaster* [55] and in herbaceous species [11, 23, 26, 42]. In *Picea sitchensis*, Rubisco activity per unit leaf mass was increased by P addition [13]. Similarly, positive relationships between maximum rates of carboxylation (V_{cmax}) and P were reported for *P. pinaster* [30] and *Pinus taeda* [28].

Concentrations of other nutrients, such as potassium (K), may also be related to A_{max} [4, 5, 22]. Growth is expected to improve in response to added K on some, especially sandy, soils owing to the high mobility of K in both soil and plant and generally large plant requirements. K is the most abundant univalent cation in plant cells and plays a significant part in regulating stomatal function [32]. Hence, one means by which K deficiency reduces photosynthesis is by decreasing stomatal conductance [40, 50]. More generally, transpiration increases (and WUE decreases) if K is in poor supply [7, 29]. In these cases, changes in stomatal conductance are not responsible for the decline in photosynthesis; instead the large K requirement for protein synthesis [27] is probably responsible for strong correlations between concentrations of K and proteins such as Rubisco [20].

It is possible that the responses of conifer growth to N, P and K fertiliser share a mechanistic basis in their effects on photosynthesis and Rubisco concentration. Most studies relating N, P and K nutrition to photosynthesis and Rubisco have focussed on a handful of well-studied herbaceous species. Of the few studies on conifers, none have included direct quantification of Rubisco. Previously we reported relationships among addition of P fertiliser, growth, and photosynthesis of *P. pinaster* [55]. Here we extend those observations using a factorial N, P, K fertiliser trial at the same site and with one-year-old *P. pinaster*. We measured the concentration of Rubisco and other major N fractions in seeking to confirm the putative common basis for relationships between N, P, K, A_{max} and WUE (as estimated by stable carbon isotope composition [17]).

2. MATERIALS AND METHODS

2.1. Field site

The field study was conducted in south-west Western Australia, approximately 20 km north of Bullsbrook (latitude 31° 67' S, longitude 116° 02' E, 40 m above sea level) at a site we have described previously [55]. The region has a Mediterranean climate with cool, wet winters and hot, dry summers, in July (mid-winter), the mean daily temperature is 8.7 °C and the maximum is 17.6 °C. In January (mid-summer), the mean daily minimum temperature is 17.0 °C and the maximum is 33.1 °C. Mean annual rainfall at Bullsbrook is 692 mm, with 84% of the total annual rainfall between May and October. Potential annual evaporation [8, 36, 44] is around 1700 mm. The soil at the site is a P-deficient yellow siliceous sand to a depth of at least 3 m. Prior to being planted with *P. pinaster*, the site was occupied by *Banksia* woodland typical of the many seasonally dry and nutrient-poor sites in this area.

In 1995 the site was cleared and planted with seedlings of *P. pinaster* at 1500 stems ha^{-1}. In August 1996, a fertiliser trial was established in a randomised complete block design with eight factorial combinations of N, P and K replicated in three blocks. Plots were around 0.06 ha. Fertiliser was applied by hand to the soil surface. Elemental rates of fertiliser application were: N 275 kg ha^{-1} as urea, P at 140 kg ha^{-1} as double superphosphate, and K at 248 kg ha^{-1} as muriate of potash. All plots received Cu (2 kg ha^{-1}), Zn (4 kg ha^{-1}), Mn (2 kg ha^{-1}).

2.2. Height and photosynthesis measurements

In winter 1998, the height of all trees was measured to the nearest 5 cm. The maximum rate of light-saturated photosynthesis (A_{max}) was measured on five trees per plot several weeks after the first substantial (> 50 mm) winter rainfall on a warm (c. 20 °C maximum) cloudless day. This period was chosen because photosynthesis was unlikely to be limited by either soil or atmospheric water deficits, and foliage nutrient concentrations are more stable than during the spring growth flush. Measurements were made with an open, infra-red gas analyser (LCA-4, Analytical Development Co, Hoddesdon, England) at 340–370 μmol mol^{-1} CO_2, 20–25 °C leaf temperature and >1500 μmol m^{-2} s^{-1} PPFD. Two one-year-old fascicles (four needles) were laid across the leaf chamber. Photosynthesis, transpiration and C_i were allowed to stabilise before measurements were taken, this generally required about five minutes. Immediately following measurement of photosynthesis, foliage was dissected out of the leaf chamber for measurement of area and mass. Additional samples were collected from the five trees per plot used for photosynthesis measurement. One sample was promptly frozen and stored at –20 °C, another sample was dried and ground to a fine powder in a ball mill.

2.3. N and stable carbon isotope composition ($\delta^{13}C$)

Dried samples were analysed for N and $\delta^{13}C$ by ANCA-MS. Samples (5 to 6 mg dry mass) were combusted to CO_2 and N_2 in the presence of O_2 (Roboprep-CN, Europa Scientific, Crewe, UK) before passing into a mass spectrometer (Tracermass, Europa Scientific). $\delta^{13}C$ (in ‰ units) was calculated with respect to the PDB standard: $\{=[^{13}C/^{12}C\text{sample})/(^{13}C/^{12}C\text{standard}) - 1] \times 1000\}$.

2.4. P and K

Dried foliage was digested in sulphuric acid/hydrogen peroxide as described previously [2]. P was analysed colorimetrically by the ascorbic-reduced vanado-molybdophosphoric acid method [37]. K was determined by flame photometry.

2.5. Chlorophylls

Chlorophylls were extracted from frozen samples with *n, n*-dimethylformamide and analysed colourimetrically using the extinction coefficients of Wellburn [58].

2.6. Rubisco

Proteins were extracted from frozen samples, as described by Warren et al. [56]. Needles were ground to a fine powder in a slurry of acid-washed sand, insoluble polyvinylpolypyrrolidone (15% w/w) and extraction buffer (10 mL g^{-1} FW) (80 mM Tris-HCl, 0.1 M β-mercaptoethanol, 2% (w/v) SDS, and 15% (v/v) glycerol). The extract was centrifuged for five minutes in a microfuge and the supernatant used for protein analysis. Proteins were denatured by heating at 95–100 °C for 10 min in a water bath. Benzoic acid was used as an internal reference. Capillary electrophoresis (CE) was performed with a Bio-Rad

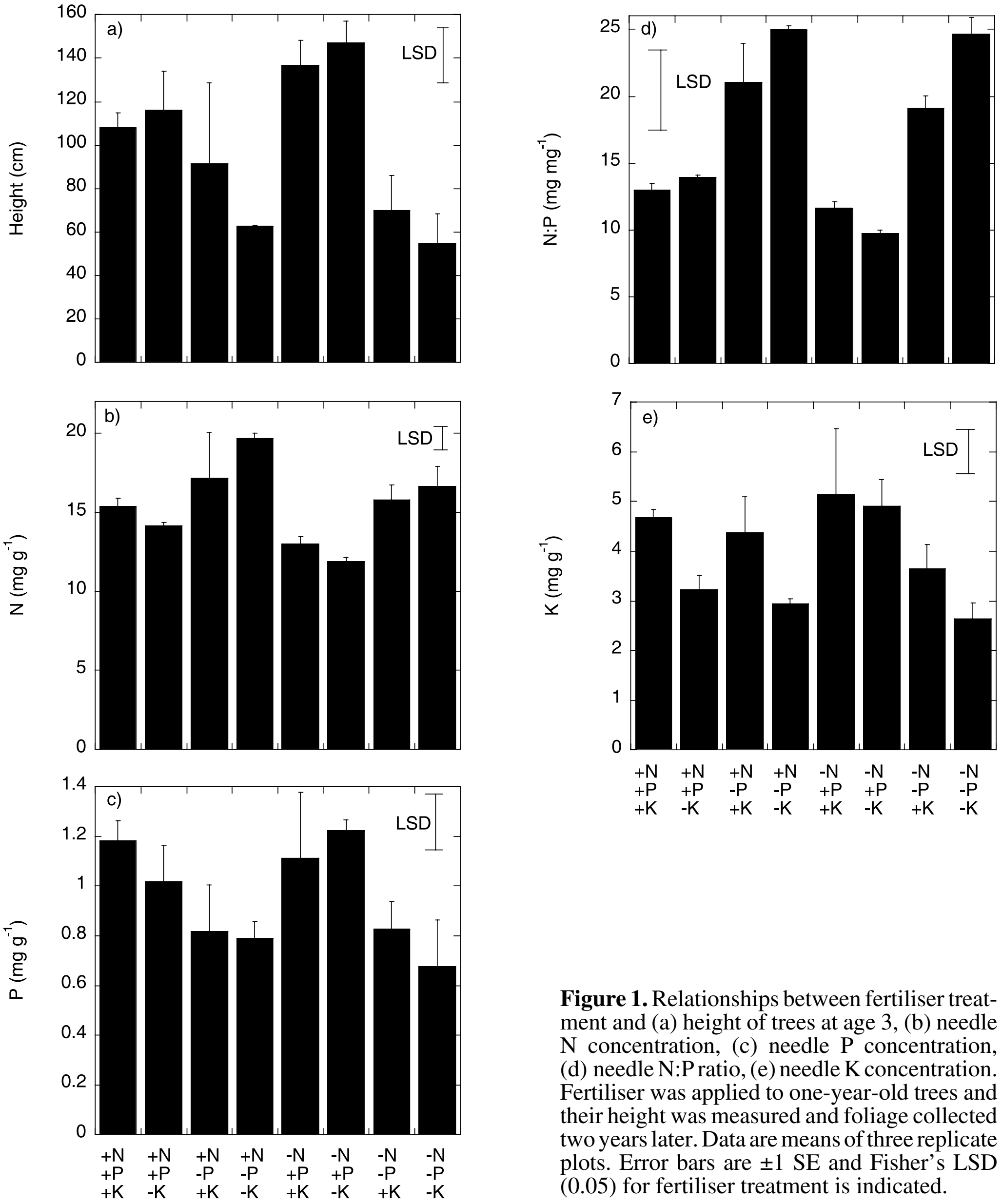

Figure 1. Relationships between fertiliser treatment and (a) height of trees at age 3, (b) needle N concentration, (c) needle P concentration, (d) needle N:P ratio, (e) needle K concentration. Fertiliser was applied to one-year-old trees and their height was measured and foliage collected two years later. Data are means of three replicate plots. Error bars are ±1 SE and Fisher's LSD (0.05) for fertiliser treatment is indicated.

3000 system (Bio-Rad, Hercules, CA, USA) controlled by a computer equipped with CE-3000 software (Bio-Rad). The separation of proteins was performed in CE-SDS protein run buffer (Bio-Rad) in an uncoated fused-silica capillary (50 µm i.d. × 24 cm long, 19.4 cm effective length). Electrophoresis was conducted at 20 °C and a constant voltage of 15 kV. The detector was set at 220 nm and sample was injected electrophoretically at 10 kV for 5 s. The capillary was rinsed sequentially between successive electrophoretic runs with 0.1 M NaOH (90 s), 0.1 M HCl (60 s), CE-SDS protein run buffer (120 s). Dry weight fractions were measured on a separate subsample of needles and the concentration of Rubisco was calculated on a dry weight basis.

2.7. Statistical analysis

Characteristics were compared between nutrient treatments by three-way fully factorial ANOVA. Where treatment means are reported, they are the mean of three replicate plots ± 1 SE.

3. RESULTS

3.1. Growth

Two years after fertiliser was applied, the height of *P. pinaster* varied between 55 and 137 cm and was significantly greater (P = 0.0003) in plots receiving P (127 ± 7 cm; mean ± SE of three replicate plots) than in those without added P (70 ± 10 cm) (Fig. 1a). Addition of N and/or K fertiliser did not affect height ($P > 0.05$).

3.2. Needle concentrations of N, P and K

Needle concentrations of N, P and K were significantly greater in plots to which that element was added compared to those not receiving fertiliser ($P < 0.05$) (Fig. 1). Addition of P fertiliser decreased concentrations of N (P = 0.0005), but increased concentrations of K (P = 0.02). Ratios of N:P were

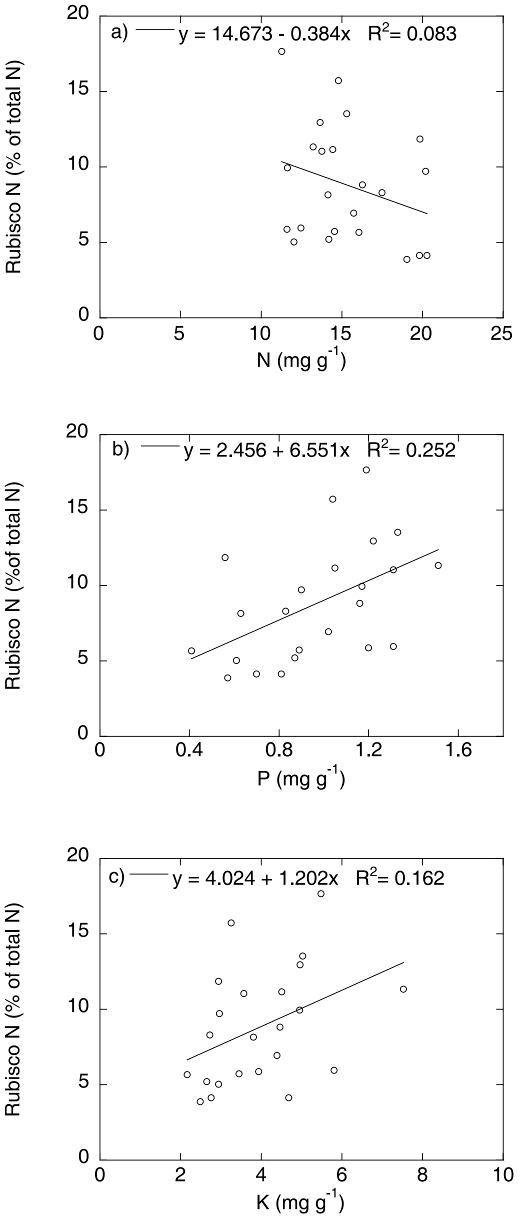

Figure 2. The relationship between the percentage of N present as Rubisco and (a) needle N concentration, (b) needle P concentration, (c) needle K concentration. Fertiliser was applied to one-year-old trees and foliage was collected two years later. Each point is a single measurement.

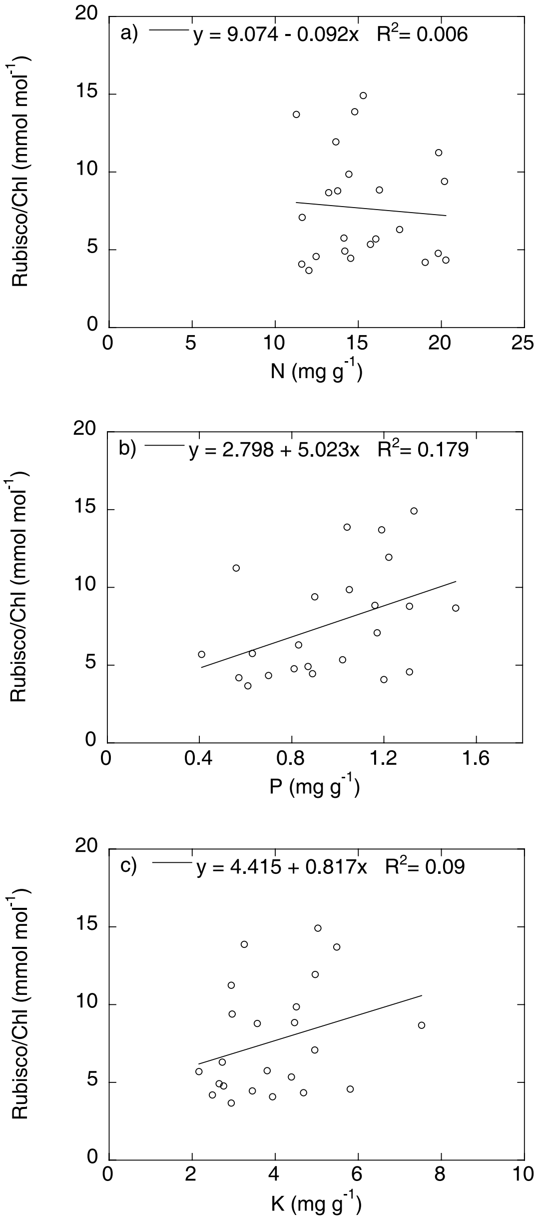

Figure 3. The relationship between the ratio of Rubisco to chlorophyll and (a) needle N concentration, (b) needle P concentration, (c) needle K concentration. Fertiliser was applied to one-year-old trees and foliage was collected two years later. Each point is a single measurement.

significantly affected by addition of P fertiliser (P = 0.0009) (Fig. 1). In plots without added P, the N:P ratio was 19 or greater, whereas it was 14 or less in plots to which P was added. On the other hand, addition of neither N nor K affected N:P ratios ($P > 0.05$).

3.3. Needle concentrations of Rubisco and chlorophyll

The fraction of total N present as Rubisco varied between 4 and 18% and was unrelated to needle concentrations of N, but weakly and positively related to P ($P = 0.015$, $R^2 = 0.25$) and K ($P = 0.05$, $R^2 = 0.16$, Fig. 2). Similarly, concentrations of Rubisco per unit chlorophyll varied between 4 and 14 mmol mol^{-1} and were unrelated to needle concentrations of N or K, and weakly related to P ($P = 0.04$, $R^2 = 0.18$, Fig. 3).

3.4. δ^{13}C and $A_{\max}$

The δ^{13}C signature of *P. pinaster* needles did not vary between treatments ($P > 0.05$), and was unrelated to needle concentrations of N, P, K or Rubisco ($P > 0.05$, Fig. 4). There was no significant difference in specific leaf area (SLA, leaf area per unit dry mass) among treatments ($P > 0.05$; SLA = 2.94 ± 0.03 m^2 kg^{-1}, mean ± SE), and thus trends in $A_{\max}$ were the

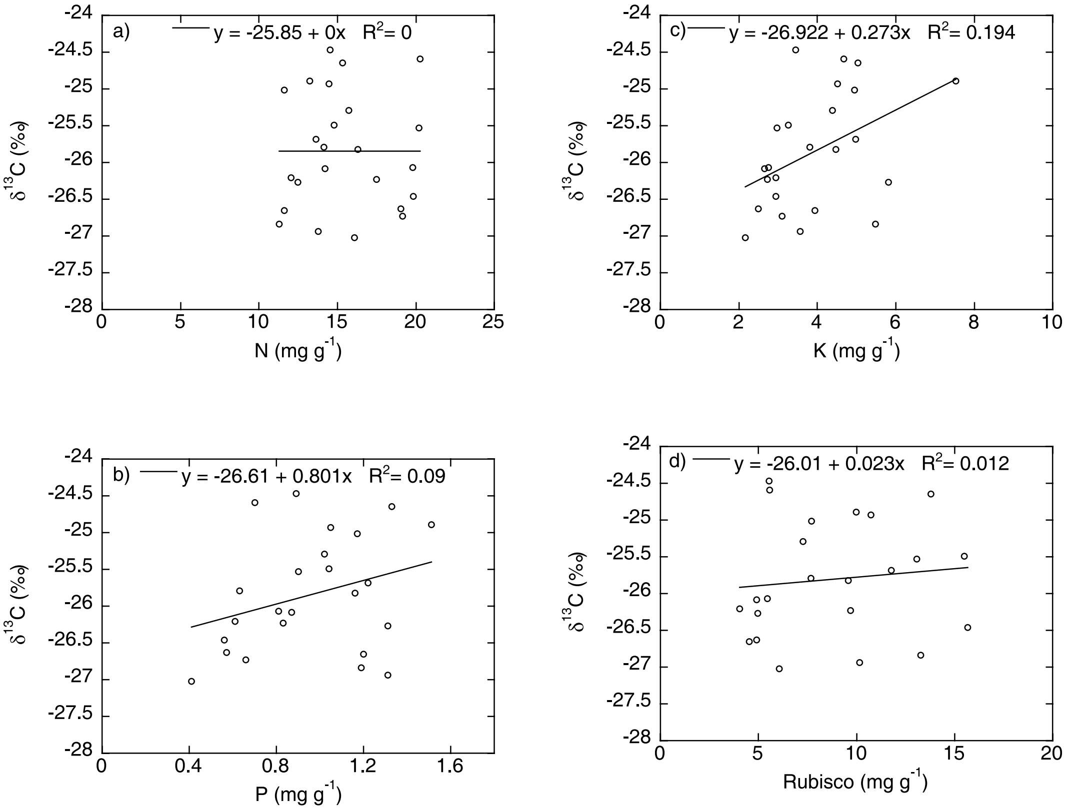

Figure 4. Relationship between stable carbon isotope composition ($\delta^{13}C$) and (a) needle N concentration, (b) needle P concentration, (c) needle K concentration, and (d) Rubisco concentration. Fertiliser was applied to one-year-old trees and foliage was collected two years later. Each point is a single measurement.

same on mass and area bases. Data are presented solely on a mass basis for ease of comparison with nutrient concentration data. A_{max} varied between 33 and 74 nmol g^{-1} s^{-1}, and did not vary significantly among treatments ($P > 0.05$). A_{max} was unrelated to needle concentrations of N, P, K or Rubisco (Fig. 5).

4. DISCUSSION

Height growth of *P. pinaster* was severely limited by the supply of P, but not by N or K (Fig. 1a). That growth was limited by P is supported by the very high N:P ratio of 19 or greater in plots without added P [1, 25]. The absence of a significant N limitation finds further support in our observation that N:P ratios were unaffected by addition of N. Addition of P fertiliser increased height by almost 50%, whereas A_{max} was unaffected – an argument against any lasting role of A_{max} in the growth response. Concentrations of N, P, and K were maintained within a relatively small range, and there was no correlation between A_{max} and concentrations of N, P, or K (Fig. 5). These findings suggest that "fine-tuning" of growth with nutrient supply ensured that all needles produced were photosynthetically competent. A similar lack of photosynthetic response to nutrient addition has been reported in *Pseudotsuga menziesii* [10], *Pinus strobus* [43], *Pinus taeda* [51] and *Pinus pinaster* [55]. Together these findings cast doubt on whether photosynthesis plays a general and/or lasting role in the response of growth to fertiliser addition. The absence of differences in WUE (as indicated by $\delta^{13}C$) is almost certainly a function of the similarity of A_{max} among treatments. We cannot exclude the possibility that small yet significant increases in WUE and/or A_{max} were not detected owing to the inherently high variability of A_{max} and WUE. Nevertheless, modelling studies with *P. radiata* suggest that increased rates of photosynthesis due to fertiliser explain only 10% of the increase of net photosynthesis within the canopy [31]. Increases in leaf area and changes in biomass partitioning probably explain the bulk of the growth response [9, 46].

Two years had elapsed since fertiliser was applied in the present study, and it may well be the case that A_{max} and WUE were greater immediately following fertiliser application, but in the two subsequent years declined to control levels. Our results are further evidence that fertiliser-promoted increases in A_{max} and WUE are often transient, lasting for only the first season after fertiliser application [57]. Increases in A_{max} and WUE beyond a year or so are unlikely unless increased growth of needles and other tissues are matched by continuing fertiliser inputs [12].

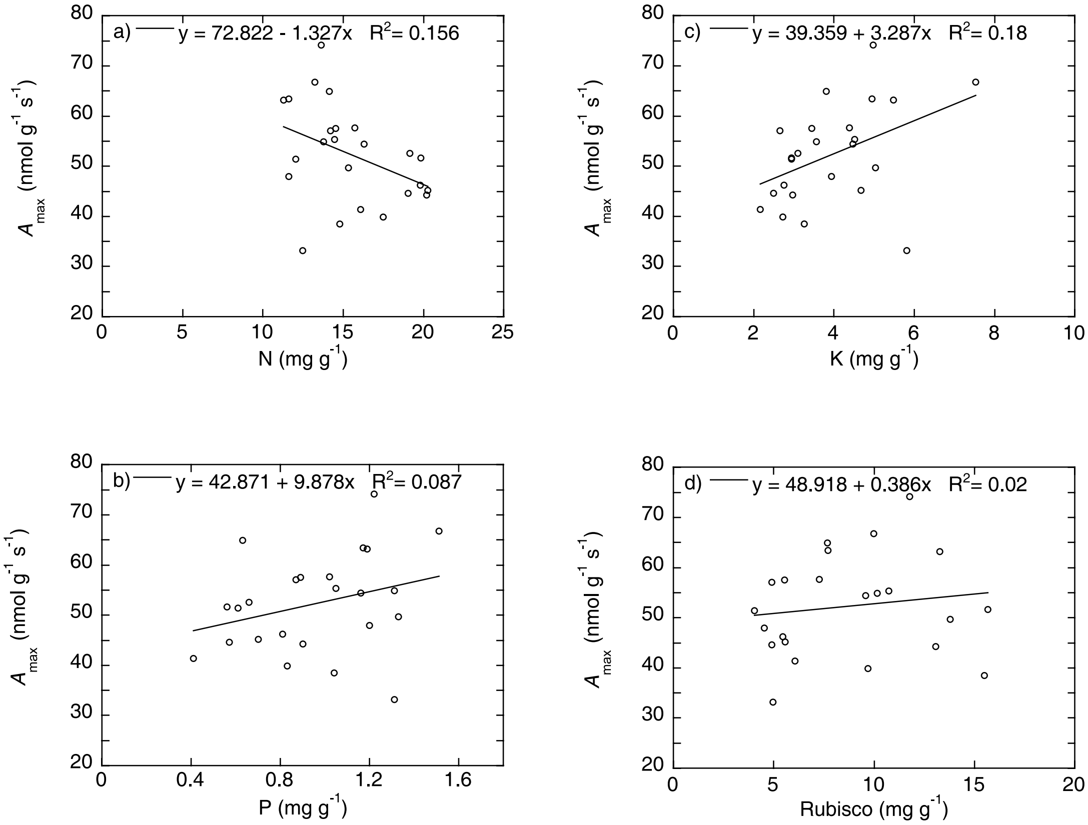

Figure 5. Relationship between the maximum rate of photosynthesis (A_{max}) and (a) needle N concentration, (b) needle P concentration, (c) needle K concentration, and (d) Rubisco concentration. Fertiliser was applied to one-year-old trees and A_{max} was measured two years later. Each point is a single measurement.

Contrary to our hypothesis, there was no evidence that differences in either relative or absolute concentrations of Rubisco could explain differences in A_{max} and growth. Strong relationships among Rubisco, A_{max} and growth are expected where N is a limiting element, whereas no relationship is expected in studies such as this where there is little evidence for a significant N limitation (Fig. 1a; see also N:P ratios, Fig. 1d). Trends in the allocation of N to Rubisco perhaps reflect the dual role of the enzyme in storage and photosynthesis [49]. For example, Rubisco concentration varied three-fold but this did not translate into differences in A_{max} (Fig. 5), suggesting that Rubisco specific activity decreased with increasing Rubisco concentration. Positive relationships between K or P and allocation of N to Rubisco (Figs. 2 and 3) are consistent with recent suggestions that Rubisco accumulates in parallel with storage of P and K owing to the relatively fixed stoichiometry of these elements in plant matter and consequent "futility" of storing one without the other [55]. Alternatively one might argue that this positive relationship is simply a consequence of the high K (and to a lesser extent P) requirement of protein synthesis [20, 27]. Irrespective of its cause, we note that increased allocation of N to Rubisco was associated with low N:P and N:K ratios, which we might contrast with the increased allocation of N to amino acids commonly observed when N is in excess relative to elements such as K or P [3, 54].

5. CONCLUSIONS

Our results with *P. pinaster*, and those from other conifers, cast doubt on whether the growth response to fertiliser is a function of greater A_{max} and/or WUE. By co-ordinating growth with nutrient supply, *P. pinaster* rarely produces needles that are nutrient deficient (with respect to photosynthesis). Allocation of N to Rubisco is plastic and modified by the supply of P and K, in partial support of our hypothesis. However, despite differences in allocation of N to Rubisco, there were no differences in A_{max}. These findings argue against limitation of photosynthesis by Rubisco, and provide tentative support for the hypothesised role of Rubisco as a store of N.

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