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An exponential fertilization dose–response model to promote restoration of the Mediterranean oak *Quercus ilex*

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Abstract Nursery nitrogen (N) fertilization influences seedling N reserves, morphology, photosynthesis rate and stress tolerance and frequently enhances outplanting performance. Although mineral nutrition is a critical aspect of seedling quality, fertility targets of Mediterranean sclerophylous species have not been thoroughly quantified. We sought to define those fertility targets for seedlings of *Quercus ilex*, a key species in Mediterranean areas. Nine fertility treatments, ranging from 0 to 200 mg N seedling^{-1} applied under an exponential regime were tested in a greenhouse dose response trial in which phosphorus (P) and potassium (K) were increased in the same proportion as N (15N:5P:15K). Height and diameter growth were measured weekly, and biomass and nutritional status were analyzed at the end of culture (24 week). Plant growth and nutritional response to increased fertilization followed a curvilinear pattern depicting phases that ranged from deficiency to luxury consumption. Seedling dry mass production was maximized at 125 mg N seedling^{-1} (sufficiency level). N content and concentration increased with fertilization, reaching a maximum at 200 mg N seedling^{-1} (luxury consumption). P and K concentrations peaked at 75 and 25 mg N, respectively, suggesting a dilution effect of these nutrients. Root volume increased linearly up to 100 mg N and declined thereafter. The sufficiency level for *Q. ilex* (125 mg of applied N seedling^{-1}) is notably higher than for other *Quercus* species from other biomes but intermediate to other Mediterranean *Quercus* species. No toxicity was observed at the highest treatment rate (200 mg N) suggesting that increased exponential N rates along with greater P and K proportions than those used in our experiment may further maximize nutrient storage.

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Introduction

Forest restoration plantations in the northern Mediterranean basin have emphasized soil protection; however in the last 20 years there is also an increased interest in promoting biodiversity (Oliet and Jacobs 2012). This has strongly increased the demand for more forest species, particularly of evergreen oaks (MAPA 2006; Oliet and Jacobs 2012). Holm oak (*Quercus ilex* L.) is an evergreen slow growing sclerophyllous tree species that dominates many forest communities in the Mediterranean Basin (IFN3). It plays a basic role in ecosystem function in many Mediterranean woodlands (Blanco et al. 1998; Rodá et al. 1999) and shelters a great diversity of endangered species, such as the Spanish imperial eagle or Iberian lynx. The Iberian Peninsula and Morocco constitute 90 % of its world distribution (Pemán García et al. 2014). Thus, this species has been widely used for forest restoration in Mediterranean ecosystems (Rodá et al. 1999; Rey Benayas and Camacho-Cruz 2004). Consequently, its presence has increased over 71 % in the last years (IFN3). However, slow growth and/or high mortality of planted seedlings are common compared with other Mediterranean woody species (Siles et al. 2010; Cuesta et al. 2010a; Trubat et al. 2011). Poor seedling quality (Villar-Salvador et al. 2004a), post-transplanting stress (Rodá et al. 1999) and low site fertility (Pardos et al. 2005; Valdecantos et al. 2006; Sanz-Pérez et al. 2007) are causes of plantation failures.

One critical aspect to improve seedling quality and thus planting success of this species is mineral nutrition. Nutrient status of seedlings, mainly of nitrogen (N), determines out-planting performance as indicated by the frequent positive relation of seedling trans-planting survival and growth with nutrient concentration and content in this species (Villar-Salvador et al. 2004a; Oliet et al. 2009; Villar-Salvador et al. 2012). In addition, other essential nutrients like P and K can play important roles in postplanting performance of *Q. ilex* (del Campo et al. 2010; Oliet et al. 2011; Andivia et al. 2012a). However, evergreen oaks often are poorly fertilized in nurseries because plants can easily reach the minimum European plant quality standards with low nutrient supply (Peñuelas Rubira and Ocaña Bueno 2000). Additionally, seedlings cultivated with low fertilizer inputs are often thought to be more resistant to stress conditions than high-fertilized plants, especially to drought stress (Tan and Hogan 1997; Trubat et al. 2011; Cortina et al. 2013). Nevertheless, studies conducted with this species show that low N fertilization impairs survival and growth (Villar-Salvador et al. 2004a; Cuesta et al. 2010b). Alternatively, excess fertilization may induce toxicity, often indicated by decreased plant growth and N content but elevated tissue N concentration, sometimes leading to mortality in the nursery (Uscola et al. 2014).

Thus, an ideal fertilization regime should promote nutrient loading and growth of seedlings, while avoiding toxicity effects. However, all plant species do not have the same nutrient demand and specific fertility targets must be defined for each species (Salifu and Timmer 2003; Salifu and Jacobs 2006). Timmer (1997) proposed a conceptual model that can be used to quantify and characterize fertility targets, distinguishing between points of nutrient deficiency, sufficiency, luxury consumption, and toxicity in plants associated with fertilization and considering biomass and nutritional status (Salifu and Jacobs 2006). The model has been validated using a conifer (Salifu and Timmer 2003) and temperate oaks

(Salifu and Jacobs 2006; Birge et al. 2006) across a broad spectrum of N application rates in the nursery. Recommended guidelines that include optimal N application rates and adequate P and K proportions are needed for quantifying and characterizing fertility targets of Mediterranean sclerophyllous oaks such as *Q. ilex*.

Our objectives in this study were to test application of the dose response model over a broad range of N supply from deficiency to toxicity to quantify and characterize fertility targets for growing *Q. ilex* seedlings. We expect that this data will provide preliminary insight to begin formulating recommendations to optimize nursery fertilization systems for *Q. ilex*, which should lead to improved plantation establishment and enhanced restoration success.

Materials and methods

Plant material and growing conditions

Pre-germinated holm oak acorns from Región Alcarria-ES07, Spain seed source, were sown into 656-cm^3 volume (D40 Deepot, Stuewe and Sons, Corvallis, OR, USA) filled with Scotts Metro-Mix$^®$ 560 growing medium (The Scotts Company, Marysville, OH, USA). This medium is comprised of 35–54 % composted pine bark, 20–30 % processed coconut coir pith, 10–20 % sphagnum peat moss, 5–15 % processed bark ash and 5–15 % horticultural perlite. Seedling trays were arranged in a greenhouse (mean day/night temperature of 24/20 °C) under ambient greenhouse light conditions at the Department of Horticulture and Landscape Architecture Plant Growth Facility at Purdue University, West Lafayette IN, USA (40°25′N, 86°55′W). Each pot was irrigated twice a week to container capacity determined gravimetrically at sowing without any fertilizer added until fertilizer treatments started (see below).

Fertilization commenced at week two (28 May) and continued for 20 weeks (20 September). Seasonal doses ranged from 0 to 200 mg N per plant, applied at exponentially increasing rates. Weekly applications were based on exponential functions previously detailed in (Salifu and Jacobs 2006), designed to synchronize fertilizer supply with exponential growth and nutrient uptake of seedlings. Exponential fertilization delivered nutrients at exponentially increasing addition rates (Ingestad and Lund 1986; Timmer and Aidelbaum 1996) according to Eq. (1):

$$N_T = N_S(e^{rt} - 1) \tag{1}$$

where r is the relative addition rate required to increase N_S (initial N content in seed) to a final N content ($N_T + N_S$), and N_T (ranges from 25 to 200) was the desired amount to be added over the number of fertilizer applications (t = 18 week). N_S was determined to be 22 ± 1 mg N seed^{-1} from 15 seeds at planting. The quantity of fertilizer to apply on a specific day (N_t) was computed using Eq. (2):

$$N_t = N_S(e^{rt} - 1) - N_{t-1} \tag{2}$$

where N_{t-1} is the cumulative amount of N added up to and including the previous application.

A commercial water-soluble fertilizer [Miracle Gro$^®$ Excel$^®$ NPK 15-5-15 + microelements (The Scotts Company, Marysville, OH, USA)] was applied in solution. Phosphorous was applied as P_2O_5 while K was as K_2O, and thus, the abundance of

elemental P and K was 5 and 15 %, respectively, in the fertilizer. Total 15 % N consisted of NH_4-N (1.20 %), NO_3-N (11.75 %) and urea-N (2.05 %). Supplemental irrigation was supplied twice weekly at similar rates by periodic weighing of pots to determine amount of water to be added to return pots to container capacity to avoid leaching and potential confounding effects of irrigation on treatment responses. The nine fertilizer treatments (0, 25, 50, 75, 100, 125, 150, 175, and 200 mg N per plant) were randomly assigned to each seedling and arranged in a randomized complete block design with three replicate blocks. Each fertilizer treatment had four seedlings per block, and thus a total of 12 seedlings per fertilizer treatment were used in the experiment. The seedlings were placed on raised benches and rotated bi-weekly within blocks to minimize edge effects.

Plant sampling, morphology and nutrient concentration and content

Height and root collar diameter was measured in all the seedlings from 27 June (7th week) to 20 September weekly (20th week). Stem volume was calculated as the volume of a cone. Height and root collar diameter data of each individual seedling during the growing period were used to estimate the number of shoot growth flushes. Each interval between two consecutive measurements, with height and diameter growth >5 and 0.5 mm, respectively, was considered a flush period. Growth and nutritional response data were sampled at the end of the growing season (26 October; 25 weeks). Three seedlings per block and per treatment were destructively sampled and separated into leaves, stems and roots, measured individually but averaged within block for morphological and nutritional assessment. Root plugs were carefully washed to eliminate growing media. First, root volume (Rv) was measured using the water displacement method (Burdett 1979). Then, all seedling fractions were gently washed with tap water, rinsed in deionized water, oven-dried for 72 h at 68 °C, weighed and ground. Shoot to root ratio was estimated by dividing the mass of leaves and stems by roots.

Chemical analyses on plant samples were conducted by A&L Great Lakes Laboratories (Fort Wayne IN, USA) based on the Association of Official Analytical Chemist (AOAC) methods. Total N concentration was determined by combustion ("Dumas") procedure (AOAC 968.06) using a LECO nitrogen analyzer (LECO Corporation, St. Joseph, MI, USA). Additionally, plant samples were digested in nitric + perchloric acids (AOAC 935.13), and P and K concentration determined using inductively coupled argon plasma (ICAP) analysis (AOAC 985.01). Nutrient content (N, P, K) was calculated by multiplying the nutrient concentration with plant organ mass. Nutrient (N, P, and K) uptake was calculated as nutrient content in each fertilized seedling minus the nutrients from the acorn. Nutrients from the acorn were considered as the average nutrient content in non-fertilized seedlings (0 = control seedlings). Fertilizer nutrient efficiency was calculated as the percentage of nutrients taken up from the total nutrient applied. Nutrient losses were dismissed as leaching was null or minimal during culture. Nutrient uptake efficiency was computed according to the formula Eq. (3):

$$Nutrient\ uptake\ efficiency$$
$$= \frac{Plant\ nutrient\ content - Plant\ nutrient\ content\ unfertilized\ seedlings}{nutrient\ fertilization\ rate} \times 100 \quad (3)$$

Chemical composition of growing media extracts

The growing media of each seedling used for morphology and nutrients analysis was collected before washing the roots. One composite sample was formed by pooling the substrate of three seedlings from each block per treatment. Chemical analyses of growing media extracts (pH, conductivity, NO_3^-, P, K and total N concentration) were conducted by A&L Great Lakes Laboratories (Fort Wayne IN, USA) on dissolved mixture extracts based on recommended chemical soil test procedures for the North Central Region (Warncke 1998).

Vector diagnosis

Vector diagnosis allows for simultaneous comparison of plant dry mass and nutrient status of plants or plant components contrasting in growth in an integrated graphic format known as a vector nomogram (Timmer 1991; Haase and Rose 1995; Salifu and Timmer 2003). The approach offers comprehensive and accurate diagnostic information and facilitates detection of nutritional effects of growth dilution, deficiency, luxury uptake, toxicity and nutrient interactions that tend to complicate conventional diagnostic techniques (Timmer and Armstrong 1987; Imo and Timmer 1997). Plant growth and nutritional response data for vector analysis can be manipulated in two modes: (1) an instantaneous mode that compares plant samples taken at one point in time to identify different nutritional states (Salifu and Timmer 2003), and (2) a dynamic mode that compares treatments over time to identify steady-state nutrition (Imo and Timmer 1992, 1997), and retranslocation processes (Salifu and Timmer 2001). Instantaneous vector diagnosis was employed here to facilitate interpretation of multi-element interactions on seedling growth in response to increased fertilization.

Data analysis

Preliminary analyses showed that block was not statistically significant in any case so for simplicity it was excluded from subsequent analyses. The effect of fertilizer treatment on diameter and height throughout culture was assessed by repeated measures ANOVA for a fully randomized design. The effect of fertilizer treatment on N efficiency, number of flush growth, biomass and N, P, K content and concentration and on growing media (pH, conductivity, NO_3^-, P, K and N concentration) was assessed by one-way ANOVA for a fully randomized design. Data homocedasticity was checked according to Levenne's test. When ANOVA assumptions were not met, data was transformed using logarithm, inverse or square root functions. When significant effects of factors were detected, Tukey's Significant Difference test was used to identify differences between treatment means at $\alpha = 0.05$. Statistical analyses were conducted with R version 3.1.0 (Spring Dance) released on 2014-04-10.

Results

Growth during culture

Both diameter and height increased over time ($F_{12,1287} = 45.27$; $P < 0.001$ and $F_{12,1287} = 54.76$; $P < 0.001$, respectively) and with growing fertilization rates

($F_{8,1287} = 9.02$; $P < 0.001$ and $F_{8,1287} = 86.62$; $P < 0.001$). At the initial dates there were no differences among fertilization treatments. Thereafter, diameter growth was progressive over time and with increasing N fertilization (data not shown). The increase in height was linear but the slope increase with the N fertilization dose (interaction date × treatment $F_{96,1287} = 2.04$; $P < 0.001$; data not shown). Similarly, stem volume also increased over time ($F_{12,1287} = 6.9$; $P < 0.001$; Fig. 1) and with higher fertilization rates ($F_{8,1287} = 323$; $P < 0.001$). At the initial dates there were no differences among fertilization treatments in stem volume. The increase in stem volume was small for low fertilization rates (<75 mg N seedling^{-1}) at final dates but with the higher fertilization applications growth rates increased notably (interaction date × treatment $F_{96,1287} = 6.3$; $P < 0.001$). Finally, the number of growth flushes increased at high fertilization treatments (>175 mg N seedling^{-1}), with 4 flushes in high fertilization rates and only 3 or less in the lower ones (Table 1).

Seedling growth and nutrition at the end of culture

Nutrient uptake efficiency was highest between 50 and 100 and decreased thereafter (Table 1). Fertilization increased plant dry mass ($F_{8,18} = 37.9$; $P < 0.001$). Total mass increased up to 150 mg N almost linearly until reaching a plateau corresponding with maximum rates of 150, 175 and 200 mg N (Fig. 2a). Leaf and stem dry mass increased with fertilization rate to a maximum at 125 mg N and plateaued (Table 1). In contrast, only unfertilized seedlings showed smaller root dry mass than the other treatments. Root volume increased linearly up to 100 mg N and then was constant or slightly declined thereafter. Shoot to root ratio also increased with fertilization rate, reaching a maximum at 125 mg N and then was stable or slightly decreased (Table 1).
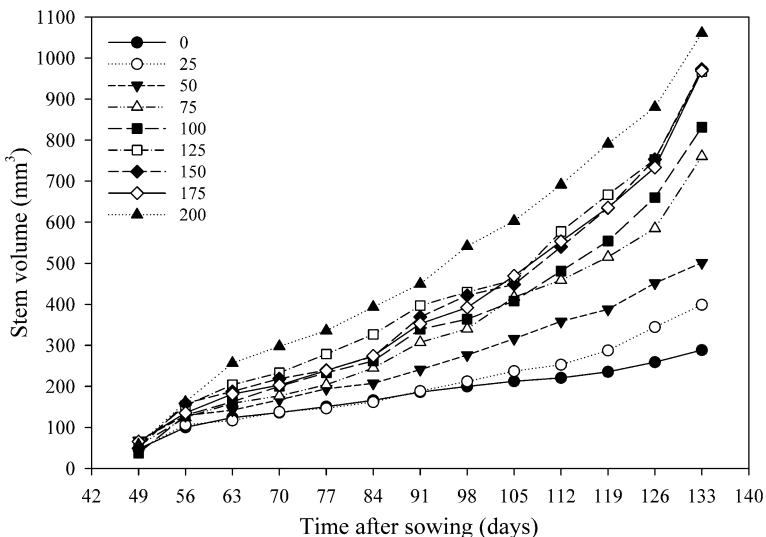

Fig. 1 Stem volume responses over the course of nursery cultivation for *Q. ilex* seedlings (mean) subjected to fertilizer treatments ranging from 0 to 200 mg N per plant for one growing season in the greenhouse. Fertilization followed exponential addition schedules (n = 12)

Table 1 Nutrient uptake efficiency, number of growth flushes, organ mass, shoot:root ratio and root volume (±SE) in *Q. ilex* seedlings cultivated with different N doses under exponential fertilization (ranging from 0 to 200 mg N seedling^{-1}) for one growing season in the greenhouse

	N uptake efficiency (%)	P uptake efficiency (%)	K uptake efficiency (%)	Growth flushes	Leaves (g)	Stem (g)	Root (g)	Shoot:root ratio	Root volume (cm^3)
0				2.7 ± 0.1e	0.49 ± 0.15f	0.12 ± 0.02e	1.50 ± 0.45b	0.41 ± 0.06e	5.7 ± 0.5e
25	58 ± 1c	38 ± 4a	43 ± 9a	3.2 ± 0.2c	1.00 ± 0.16e	0.26 ± 0.03d	1.83 ± 0.49ab	0.73 ± 0.25d	8.0 ± 0.9d
50	63 ± 1a	34 ± 4ab	37 ± 5a	3.0 ± 0.1d	1.64 ± 0.05d	0.54 ± 0.03c	1.76 ± 0.36ab	1.27 ± 0.25bc	9.3 ± 1.0 cd
75	61 ± 2ab	34 ± 5ab	29 ± 3b	3.3 ± 0.1bc	1.87 ± 0.24c	0.55 ± 0.07c	2.16 ± 0.17a	1.13 ± 0.18c	9.7 ± 0.5c
100	62 ± 1a	30 ± 2b	27 ± 4b	3.4 ± 0.1bc	2.19 ± 0.11b	0.76 ± 0.13b	2.32 ± 0.29a	1.28 ± 0.12bc	14.0 ± 0.6a
125	60 ± 1b	24 ± 1c	26 ± 1b	3.2 ± 0.1c	2.78 ± 0.22a	0.97 ± 0.18a	2.19 ± 0.14a	1.72 ± 0.14a	12.0 ± 0.9b
150	58 ± 1c	23 ± 2c	26 ± 3b	3.4 ± 0.1bc	2.68 ± 0.28a	0.85 ± 0.20a	2.89 ± 0.69a	1.24 ± 0.13c	10.3 ± 1.3bc
175	50 ± 1d	18 ± 3d	20 ± 2c	3.5 ± 0.1b	2.85 ± 0.33a	0.99 ± 0.07a	2.38 ± 0.39a	1.64 ± 0.25ab	10.0 ± 0.9c
200	47 ± 1e	16 ± 1d	20 ± 2c	4.0 ± 0.1a	2.90 ± 0.44a	1.11 ± 0.24a	2.46 ± 0.43a	1.65 ± 0.19ab	10.3 ± 1.3c
	$F_{7,16} = 16.79$; $P < 0.001$	$F_{7,16} = 6.6$; $P < 0.001$	$F_{7,16} = 3.5$; $P = 0.02$	$F_{8,107} = 8.3$; $P < 0.001$	$F_{8,18} = 37.8$; $P < 0.001$	$F_{8,18} = 19.9$; $P < 0.001$	$F_{8,18} = 3.12$; $P = 0.02$	$F_{8,18} = 16.1$; $P < 0.001$	$F_{8,18} = 3.26$; $P = 0.017$

The last row corresponds to F and P values of one-way ANOVA (n = 3). For each variable, means followed by the same letter are not statistically different according to Tukey's significant difference test at α = 0.05. Fertilization rates followed exponential addition schedules
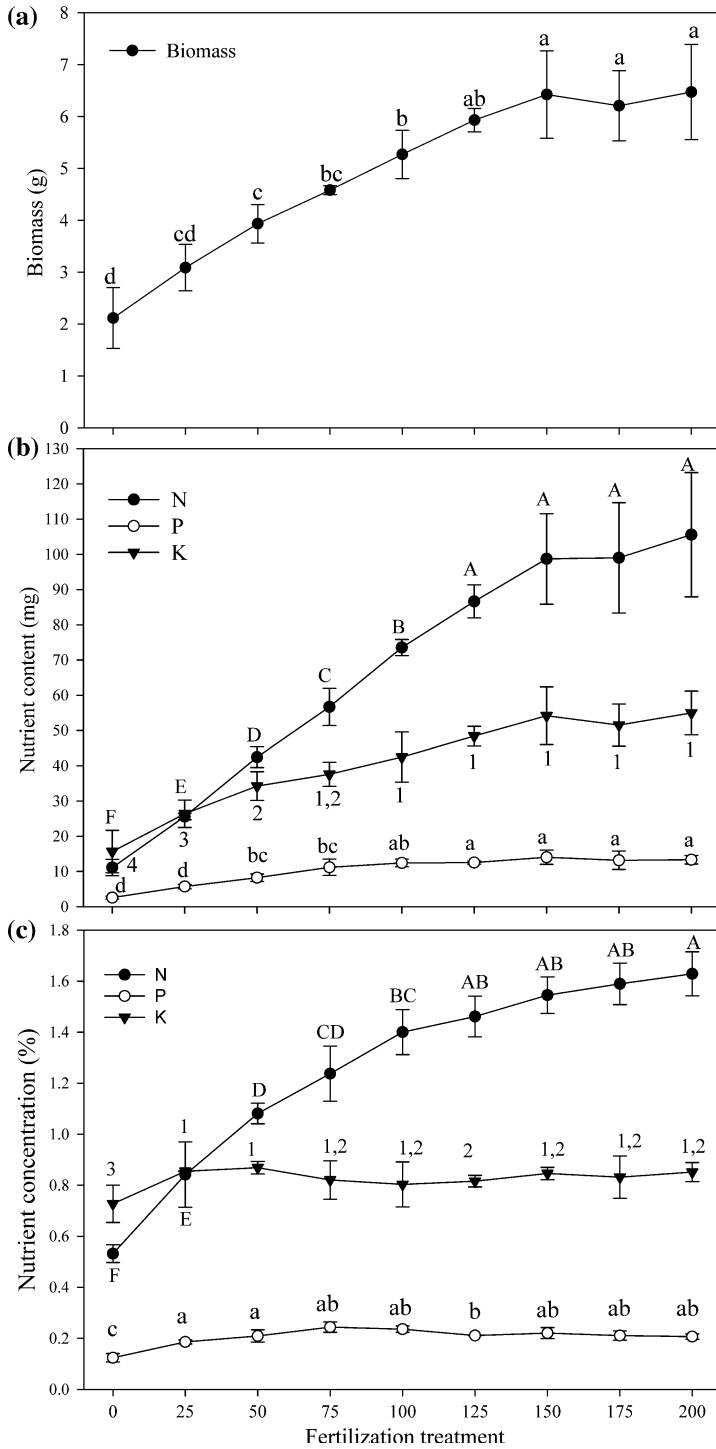
◀ **Fig. 2** Seedling plant dry mass (**a**), N, P and K content (**b**) and N, P and K concentration (**c**) of *Q. ilex* seedlings in response to increasing N supply (ranging from 0 to 200 mg N seedling^{-1}) for one growing season in the greenhouse. For each variable means (±SE) followed by the same capital letter (*N*), lower letter (*P*) or number (*K*) are not statistically different according to Tukey (n = 3). Significant difference test at α = 0.05. Fertilization followed exponential addition schedules

As per biomass, both plant N concentration ($F_{8,18} = 58.9$; $P < 0.001$; Fig. 2b) and content ($F_{8,18} = 40.4$; $P < 0.001$; Fig. 2c) increased with fertilization up to 125 mg N, and differences among the 150, 175 and 200 mg N treatments were small and not statistically significant. *Q. ilex* seedlings that were fertilized with 125 mg N were 2.8 times larger and had 7.8 times more N content and 2.7 times more N concentration than unfertilized plants.

Plant P content ($F_{8,18} = 7.36$; $P < 0.001$; Fig. 2b) and concentration ($F_{8,18} = 20.6$; $P < 0.001$; Fig. 2c) also varied with fertilization and plant P concentration peaked at 75 mg N. Maximum P content was reached at 75 mg N and thereafter there were no differences among fertilization treatments, but P concentration decreased at the highest N fertilization levels (>175 mg N seedling^{-1}). P concentration and content in unfertilized seedlings were 2 and 4.3 times lower, respectively than the ones in the treatments with the highest P levels. K concentration and content also varied significantly with fertilization rate ($F_{8,18} = 2.7$; $P = 0.051$ and $F_{8,18} = 17.5$; $P < 0.001$, Fig. 2c, b, respectively). K concentration was highest at 25 mg N and remained constant or even decreased for the highest N fertilization rates. K content in unfertilized seedlings was 2.7 times lower than the K content in the treatments with the highest K content, while K concentration was only 1.2 times higher.

Nitrogen concentration in the different organs also increased with fertilization rates (Fig. 3). In leaves, N concentration was higher than in other organs and reached the maximum at lower fertilization rates (100 mg N seedling^{-1}), especially compared to roots (175 mg N seedling^{-1}). Phosphorous concentration was higher in the woody organs. In leaves, P concentration reached a maximum at 25 mg N seedling^{-1}, and decreased at the highest fertilization rate. Only P concentration of stems in unfertilized seedlings was lower than the remaining treatments. In roots, the maximum P concentration was reached at 75 mg N seedling^{-1}, with no differences among the remaining treatments. K concentration was higher in stems and roots than in leaves with no differences among fertilization treatments.

Treatment effects on seedling dry mass and nutrient status are expressed on a relative basis when 125 mg N treatment is normalized to 100, taken as the reference for comparison with the other treatments and are represented using the vector nomogram in Fig. 4. Nitrogen was the most responsive nutrient, while K was the lowest, as shown by their vector magnitude. Initial nutrient deficiency associated with increased dry mass, N content and concentration was observed in 0–100 mg N treatments (shift a, Fig. 4a), but subsequently seedlings fertilized at ≥175 mg N attained steady state conditions in terms of biomass but had increased N content and concentration (accumulation phase; shift b). Plant P and K status showed variable trends. There was an initial phase of deficiency, with P and K content and concentration from the lowest N fertilization treatments (shift a in Fig. 4b, c). Thereafter, seedlings attained steady state conditions with respect to K and for fertilization <125 mg N, whereby K concentration remained stable but content increased with N fertilization (shift b and c in Fig. 4c). In the case of P, however, for N fertilization <125 mg N, a marked accumulation of nutrients was associated with lower growth and nutrient content than for 125 mg seedlings but increasing nutrient concentration was observed in 75
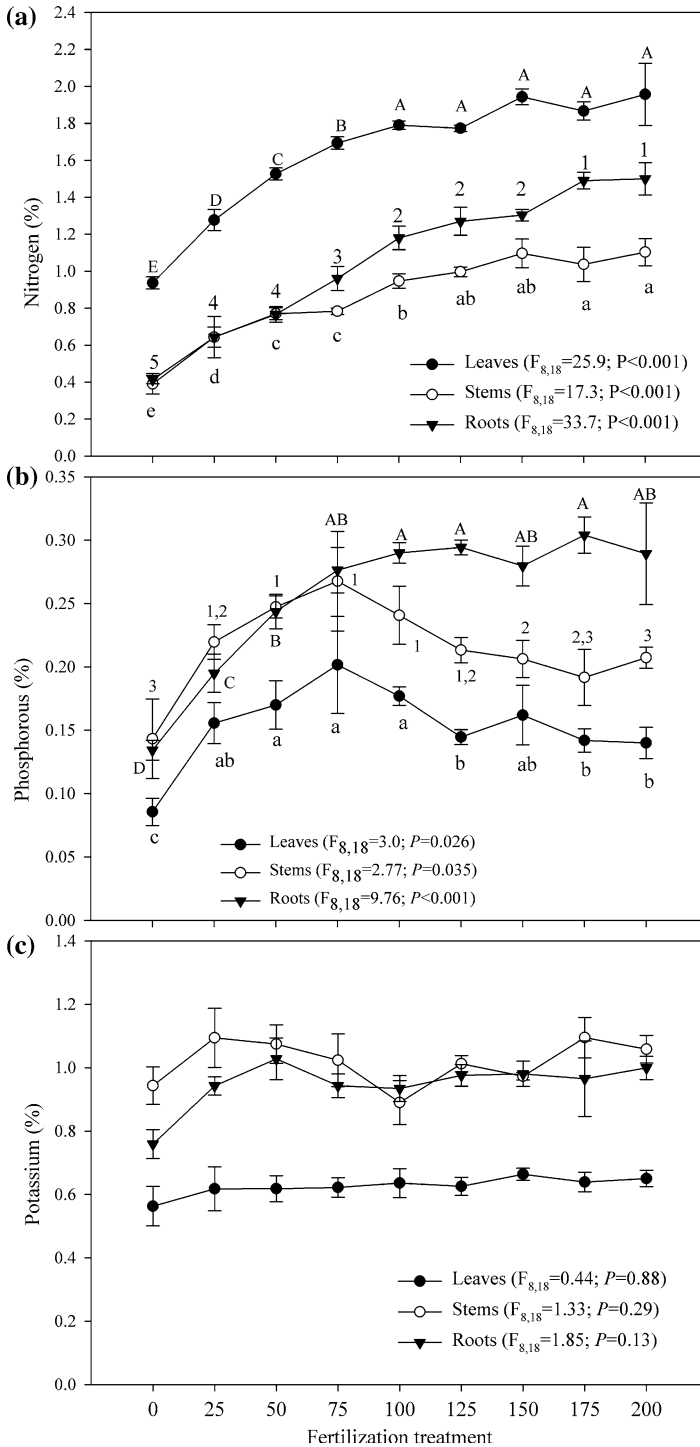
◄ **Fig. 3** Nitrogen, phosphorous and potassium concentration (±SE) in different organs (leaves, stems, and roots) of *Q. ilex* seedlings cultivated with different N dose (ranging from 0 to 200 mg N seedling^{-1}) for one growing season in the greenhouse (n = 3). Legend includes the F and *P* values of one-way ANOVA. For each variable means followed by same lower (leaves) or capital (root) letter or number (stem) are not statistically different according to Tukey's significant difference test at α = 0.05. Fertilization followed exponential addition schedules

and 100 mg N treatments (shift c in Fig. 4b) until reaching the steady state phase in the highest N fertilization treatments (150–200 mg N; shift d in Fig. 4b).

Substrate characteristics

Substrate pH values decreased, while electric conductivity increased with N fertilization (Table 2). Compared to non-fertilized treatments, only the two highest N fertilization treatments (175 and 200 mg N) showed significantly lower pH values.

NO_3^--N, P and K concentration in the substrate had very similar patterns across treatments (Table 2). Unfertilized treatment had the lowest values and nutrient concentration increased under increasing fertilization, with the highest nutrient concentration values at the highest fertilization rates.

Discussion

The response of holm oak to fertilization is time dependent, with null or low response at the initial phase of seedling development (around 77 days after sowing) and a more intense response as developmental stages progress. *Q. ilex* has large seeds, which allows for a prolonged reliance on seed N reserves during seedling development (Villar-Salvador et al. 2010), making oak seedlings relatively independent of soil N at the initial phase of growth, especially during the first flush of growth. Also, exponential fertilization implies low doses at the beginning of culture and increasing N doses as the growing season advances.

Our study results suggest that the sufficiency stage for nursery production of *Q. ilex* using an exponential regime is reached at around 125 mg N applied per seedling, as seedling dry mass production was maximized at this level and N fertilization efficiency was close to the highest values. Relative to unfertilized plants, this rate increased whole plant responses for dry mass (280 %), root volume (211 %), N content (780 %), P content (487 %), and K content (309 %). This sufficiency level of *Q. ilex* it is still relatively high compared to reports of *Quercus* spp. from other biomes, i.e. 25 mg N applied per seedling in container *Quercus rubra* L. (Salifu and Jacobs 2006) or 84 mg N applied per seedling in bareroot *Quercus alba* L. (Birge et al. 2006), which may be associated with the low plasticity of holm oak seedlings to resource availability (Sardans et al. 2006b; Uscola et al. 2014). Nevertheless, *Q. ilex* had intermediate sufficiency levels compared to other Mediterranean oak species; for instance, *Q. faginea* reached the sufficiency level at 150 mg N seedling^{-1}, and *Q. coccifera* had a lower sufficiency level than *Q. ilex*, 100 mg N applied per seedling (Villar-Salvador et al. 2013). While these differences in N sufficiency levels are probably at least partly associated with variation in cultural conditions across experiments, they may also reflect intrinsic ecological differences among species. For example, *Q. coccifera* is a slow growing species found on harsh, poor soil sites and has low plasticity in response to resource variation. In contrast, *Q. faginea* grows faster and occurs on less stressful sites with deeper soils than *Q. coccifera* (Castro-Díez et al. 1997;
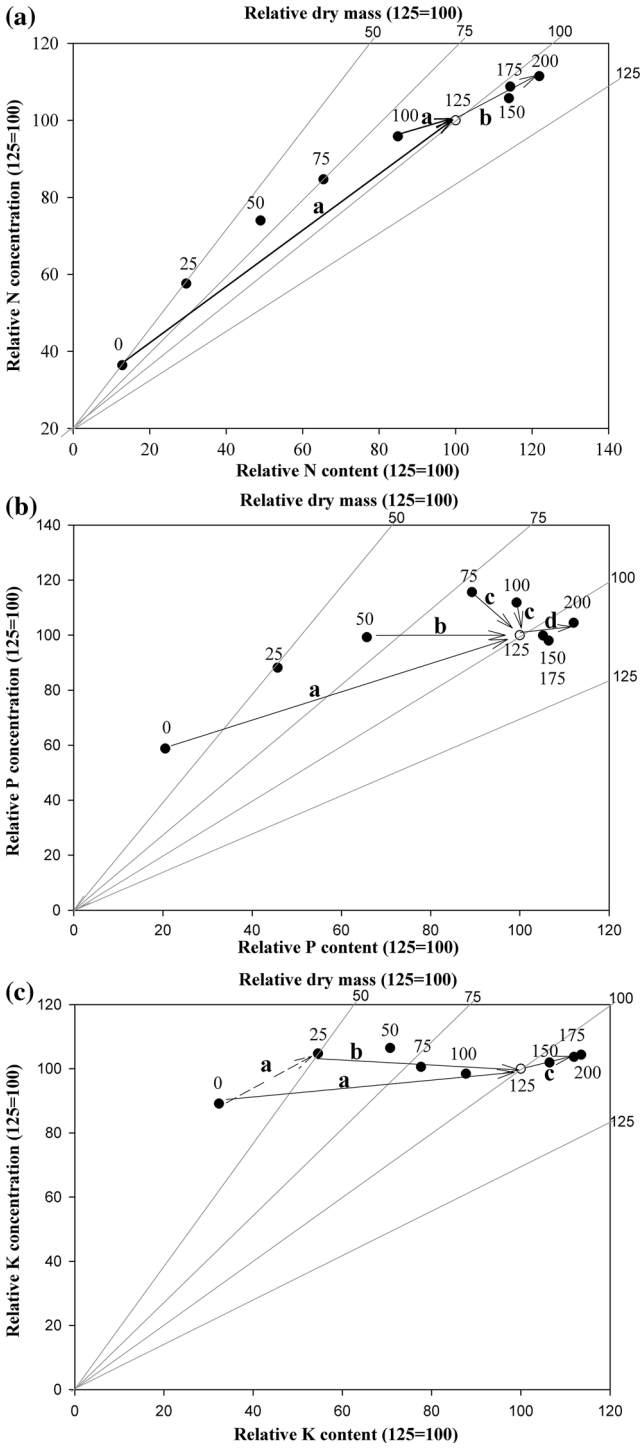
Table 2 pH, electric conductivity (EC), NO_3^- concentration, P and K concentration of the growing media in dissolved mixture extracts (±SE) in *Q. ilex* seedlings cultivated with different N doses (ranging from 0 to 200 mg N seedling^{-1}) for one growing season in the greenhouse (n = 3)

	pH	EC (dS m^{-2})	N-NO$_3^-$ (mg L^{-1})	P (mg L^{-1})	K (mg L^{-1})
0	5.5 ± 0.3a	0.5 ± 0.1c	1.0 ± 0.0d	2.3 ± 0.8f	23.2 ± 5.8f
25	5.4 ± 0.3a	0.6 ± 0.1bc	1.0 ± 0.0d	6.2 ± 1.8e	28.2 ± 4.9e
50	5.3 ± 0.3ab	0.7 ± 0.1b	1.7 ± 1.0d	10.6 ± 1.3d	36.3 ± 5.1d
75	5.4 ± 0.3ab	0.8 ± 0.3ab	5.3 ± 2.7c	14.4 ± 3.2 cd	43.5 ± 9.4d
100	5.0 ± 0.1ab	1.0 ± 0.2ab	10.2 ± 5.9bc	19.7 ± 3.3c	64.2 ± 11.3c
125	5.1 ± 0.2ab	1.1 ± 0.3ab	24.0 ± 18.1b	24.0 ± 6.6bc	68.7 ± 14.5bc
150	5.1 ± 0.2ab	1.3 ± 0.2a	43.0 ± 19.9ab	33.4 ± 7.7ab	88.2 ± 10.4ab
175	4.8 ± 0.2b	1.4 ± 0.3a	61.5 ± 29.0a	39.5 ± 6.4a	102.8 ± 12.9a
200	4.9 ± 0.2b	1.5 ± 0.4a	86.7 ± 32.2a	43.6 ± 9.3a	116.3 ± 23.6a
	$F_{8,45} = 3.03$; $P = 0.0082$	$F_{8,45} = 13.2$; $P < 0.001$	$F_{8,45} = 85.6$; $P < 0.001$	$F_{8,45} = 28.6$; $P < 0.001$	$F_{8,45} = 45.2$; $P < 0.001$

The last row is the F and *P* values of one-way ANOVA. For each variable means followed by same letter are not statistically different according to Tukey significant difference test at α = 0.05

Valladares et al. 2000; Villar et al. 2004). *Q. ilex* is found on intermediate conditions to these species and correspondingly showed an intermediate N sufficiency level.

Fertilization beyond 125 mg N seedling^{-1} promotes N luxury consumption, as indicated by shift b in the vector diagnosis (Fig. 4a). The 200 mg N seedling^{-1} rate (or even higher exponential fertilization rates), may represent the optimum target index to N load for *Q. ilex* seedlings as biomass and N content were maximized without observed toxicity. Thus, higher rates of fertilization could be used to further maximize nutrient storage in this species. Interestingly, the higher fertilization rates increased the number of flush growth phases. Knowledge of seed size and ontogeny of the species in the nursery is crucial to help maximize fertilization efficiency.

However, nutrients other than N should also be considered. The increasing dose response pattern in N concentration was not found for the other nutrients analyzed. At the whole plant level, both P and K showed a plateau at relatively low fertilizer rates, followed by descending concentration values but a continued increase in P and K content with relative dry mass, indicating sufficient supply. Probably at low fertilization rates, N levels are limiting to growth but P and K are sufficient to meet seedling requirements. Above 125 mg N, there was a slight increase in relative dry mass and P and K content, but little change in concentrations, again indicating sufficient P and K supply. P and K concentration

in the substrate increased with increase in fertilization rate and at higher rates than for N. This result would indicate that P and K availability is not limiting, but plant uptake of P and K is not increasing at the same rate as N uptake. The same dilution effect on K concentration was found for *Q. rubra* at application rates of 25 mg of N in the same proportions of macronutrients as the used in this experiment (Salifu and Jacobs 2006). These results indicate that higher P and K amounts should be considered when fertilizing at high N rates, to prevent these imbalances. By fractions, this dilution effect did not occur for root P, yet root P concentration significantly increased with fertilizer rate, suggesting P accumulation in roots.

In this study no toxicity was observed, as no growth reduction occurred at the highest fertilization rates (Timmer 1991). Past experiments have identified toxicity in container seedling production beyond 64 mg N seedling^{-1} in *Picea mariana* (Salifu and Timmer 2003) and 100 mg N seedling^{-1} for *Q. rubra* (Salifu and Jacobs 2006). Also, Villar-Salvador et al. (2013) did not observe toxicity at 200 mg N seedling^{-1} in either *Q. coccifera* or *Q. faginea*. It is difficult to clearly identify mechanisms as to why *Q. ilex* and other Mediterranean *Quercus* species appear to be more tolerant to higher fertilizer doses than other species. Direct ion toxicity is a factor influencing negative growth response at high fertilizer rates.

Similar values of substrate NO_3^--N concentration and pH without observed toxicity in *Q. ilex* were also reported by Uscola et al. (2014). While seedlings of many forest tree species suffer damage beyond an EC of 2.5 dS m^{-2} (Jacobs and Timmer 2005), *Q. ilex* seedlings can tolerate salinity levels up to 3.0 dS m^{-2} (Miyamoto et al. 2004; Uscola et al. 2014), which is notably higher than the values obtained in this experiment. Physiological drought associated with high electrical conductivity is often responsible for toxicity responses in fertilization and it is relatively difficult to separate these causal mechanisms (Jacobs and Timmer 2005). The adaptive drought tolerance of *Q. ilex* (Romane and Terradas 1992; Terradas and Savé 1992) suggests that this species may be relatively tolerant to high fertilization rates associated with nutrient loading under nursery culture, as was observed for other Mediterranean species with toxicity beyond 250 mg N (Oliet et al. 2013). However, *Q. ilex* has shown toxicity at 200 mg N seedling^{-1} but only when the N form applied was ammonium (Uscola et al. 2014). In our experiment, the main N source was nitrate, which also did not show toxicity at 200 mg N seedling^{-1} in Uscola et al.(2014). These results emphasize the importance of considering N form in studies of fertilization and plant nutrition.

N efficiency was similar among the 50–100 mg N fertilization treatments but decreased thereafter, explaining the greater NO_3^--N concentration in the growing media at higher fertilization rates. Increasing nutrient dosage usually leads to an increase in nutrient uptake but also leaching, thus inducing a lower efficiency (Oliet et al. 2004; Del Campo et al. 2011). However in this study leaching was minimized, thus allowing higher N concentration in the substrate. This could explain why N efficiency was much higher compared to other fertilization studies with holm oak (Villar-Salvador et al. 2004a). Some other studies with different species also showed lower N and K efficiencies than those obtained in this experiment (Oliet et al. 2004; Del Campo et al. 2011).

Seedling size and tissue nutrient concentration are positively related to survival and outplanting performance (Oliet et al. 2009; Villar-Salvador et al. 2012; Grossnickle 2012). Thus, N loading seedlings to optimal levels might improve field performance after transplanting (Oliet et al. 2009; Salifu et al. 2009b; Andivia et al. 2012a), as well as drought and cold stress resistance. Andivia et al. (2012a, b) reported that late-season fertilization increased seedling size, nutrient content, root growth capacity, and tolerance to

frosts, but had no effect on survival and growth after planting. For instance, unfertilized *Q. ilex* seedlings had 20 % lower survival than seedlings fertilized with 154 mg N seedling^{-1} (Villar-Salvador et al. 2004a). Andivia et al. (2012a) found that a threshold supply of nutrients of 140 mg N seedling^{-1} is needed to promote frost tolerance in *Q. ilex*. The recommended optimum leaf N concentration range for *Q. ilex* is between 1.2 and 2.0 % (Villar-Salvador et al. 2004a; Oliet et al. 2011). In our experiment all fertilized treatments were within this range, but the higher fertilization treatments reached the upper range limit. Also, the recommended values for N concentration in shoots for *Q. ilex*, which is higher than 1.3 % (Pemán García et al. 2014), was reached in our study after applying over 50 mg N seedling^{-1} (data not shown), and N concentration in leaves reached the recommended values for other *Quercus* species, i.e. higher than 1.4 % for *Q. coccifera* and *Q. suber* or higher than 1.6 % for *Q. faginea* (Pemán García et al. 2014). Furthermore, N concentration in leaves at the sufficiency level is higher than that observed in other nursery studies with *Q. ilex* (Villar-Salvador et al. 2004a; Oliet et al. 2009). However, use of a high N exponential fertilization regime was shown to hinder frost and drought tolerance of *Q. ilex* when grown in a nursery that experienced mild fall conditions, while fall loading and constant low N fertilization did not affect hardening (Heredia-Guerrero et al. 2014). Thus, importance of stored nutrients toward promoting field performance and stress resistance needs to be further assessed for *Q. ilex*.

Phosphorus promotes new root growth after field transplanting for Mediterranean species and is usually one of the limiting factors in Mediterranean soils, especially in alkaline soils (Sardans and Peñuelas 2004; Sardans et al. 2006a; Pascual et al. 2012). Thus, P reserves are an important consideration in seedling post-transplant performance (Villar-Salvador et al. 2004a; Oliet et al. 2011). A significant relationship between root P concentration and root growth potential has been found for *Q. ilex* (Oliet et al. 2011). Root P concentration higher than 0.3 % in *Q. ilex* is associated with increased survival and growth (Villar-Salvador et al. 2004a). Only the highest fertilization regimens had values close to this, indicating that higher proportions of P should be applied to attain nutrient loading of this element (Oliet et al. 2013). K concentration in leaves was above minimum quality standards for this species (Del Campo et al. 2010). Except for unfertilized seedlings, all seedlings reached the recommended values for leaf and root P and K concentration reported for other Mediterranean sclerophylous *Quercus* species, i.e. higher than 0.4, 0.4 and 0.1 % for P; 0.38, 0.35 and 0.4 % for K in *Q. coccifera*, *Q. faginea* and *Q. suber* respectively (Pemán García et al. 2014).

The plateau of root biomass at relatively low rates of N, and no reduction in this trait at the highest N doses indicates a lack of root toxicity. Several hypotheses might explain this plateau. First, increasing N availability shifts allocation patterns of current photosynthetic products to aboveground organs (Hernández et al. 2009). This pattern has been observed in bareroot experiments with other *Quercus* species, leading to a plateau of root biomass in the absence of toxicity (Salifu et al. 2009a; Schmal et al. 2010). Second, the confinement effect of container size precludes the potential expression of root growth. Root confinement has been suggested in some other fertilization studies with this species (Oliet et al. 2009), although container size was less than half the volume used in the current experiment for similar growth duration. Therefore, although it is difficult to disentangle this effect from others, we believe that the plateau observed is probably not due to confinement. The allocation hypothesis also matches the fact that *Q. ilex* shows a conservative pattern of root mass growth in response to nutrient availability (Heredia-Guerrero et al. 2014). Concurrently, reductions in root volume beyond 100 mg of N also suggest changes in the growth patterns of root systems, with more biomass allocated to coarse roots as N rate increases.

Fine root formation of this species is inhibited under high N concentration in the rhizosphere (Uscola et al. 2014). This depletion of root volume may have implications regarding drought avoidance of holm oak planted in the field (Villar-Salvador et al. 2004a, b, 2012). Further testing will be necessary to validate this hypothesis to further refine application of nutrient loading to *Q. ilex*.

Conclusions

Our experiment provides data to better quantify target rates for nursery fertilization in holm oak that will help refine fertilization protocols for production of quality seedlings with high tissue nutrient concentration. Maximum N loading of *Q. ilex* seedlings can be attained with rates over 200 mg of N applied in the nursery. However, concerns about imbalanced morphology (high shoot:root and lower root volume), potential for low frost resistance, or P and K dilution effects under such high N rates must be addressed by testing new formulations along with different fertilization delivery regimes and schedules that foster N, P and K loading.

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