



Nutritional status and genetic variation in the response to nutrient availability in *Pinus pinaster*. A multisite field study in Northwest Spain

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ABSTRACT

The low nutrient availability of the acidic and sandy soils of Galicia (Northwest Spain) is probably the main environmental factor limiting forest primary productivity in the area. These particular edaphic conditions could have imposed selective pressures on maritime pine populations leading to specific local adaptations.

We first assessed the nutritional status of 22 young contemporary *Pinus pinaster* plantations in Northwest Spain, and then analysed the response to fertilization in three family × fertilization trials, and how this response varied across sites and genotypes.

Growth of *P. pinaster* in Northwest Spain appeared to be largely limited by nutrient availability, where most of the plantations showed severe nutrient deficiencies, especially in P and Mg. According to these deficiencies, a strong positive response to nutrient additions was observed in the three trials, with height increments of up to 30% compared with the unfertilized control. However, the response to fertilizers was very variable from site to site, and in some cases did not agree with the foliar nutritional diagnosis. The response to fertilization was also significantly affected by pine genotype, suggesting that the plastic response to nutrient additions within each environment was under genetic control. However, the family response to nutrient availability was not consistent across sites, and no significant differences among families were observed for the RDPI plasticity index – a single index that summarizes the phenotypic change in multiple environments – when analysed across environments.

The strong environmental component modulating phenotypic responses to fertilization could impose an important obstacle to evolve specific adaptations to the local edaphic conditions, as well as to artificially select genotypes adapted to different environments and silviculture regimes.

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1. Introduction

Within its natural range of distribution, maritime pine (*Pinus pinaster* Ait.) populations show high geographical structuration of intraspecific genetic variation (Gonzalez Martinez et al., 2004; Bucci et al., 2007). The extremely variable ecological conditions where this species grows and the isolation of populations have led to several adaptations to local environmental conditions, such as specific parent materials, drought regimes, or fire frequency (e.g. Alia et al., 1997; Tapias et al., 2004; Correia et al., 2008). Within population genetic variation is also high, favouring the ability to evolve in response to local selective pressures (Gonzalez Martinez et al., 2005). In addition, *P. pinaster* is recognized to show a high

phenotypic plasticity, being able to modulate the phenotype expression according to the particular environmental conditions where it grows (Chambel et al., 2007).

Forest soils in Northwest Spain typically show low nutrient availability and large spatial heterogeneity in chemical soil properties, both at small and large scales (Paz-Gonzalez et al., 2000; Gallardo and Covelo, 2005). They are acidic (as results from humid climate, subtractive systems, and acid parent materials) and they have high organic matter content as a consequence of the slow mineralization rates due to aluminum binding (Calvo and Díaz-Fierros, 1982). The high precipitation rates and intensities in the area and the sandy textures of the soils facilitate infiltration and cation leaching, which, together with the low pH, results in low nutrient availabilities. However, soil fertility largely varies across the diverse geological materials found in the region (Macías et al., 1982), and diverse ecological processes at the individual or ecosystem scale also generate small-scale spatial heterogeneity in chemical soil properties (Gallardo and Covelo, 2005).

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Due to high mean annual temperatures and rainfall, and favourable light availabilities, the net primary forest productivity in this region is relatively high. The poor quality of the soils, however, is commonly displayed in important nutrient deficiencies in forest stands that limit their potential growth (e.g. Merino et al., 2003; Zas and Serrada, 2003). P, K and Mg are the main factors limiting tree growth in this region. Soil N content is usually sufficient for forest production, and may even be excessive, resulting in an imbalance with other macronutrients (Zas and Serrada, 2003). Reflecting these nutrient deficiencies, large responses of forest plantations to nutrient additions have been widely reported in the area, and forest fertilization has been strongly recommended (Solla-Gullón et al., 2004, 2006; Zas et al., 2006a).

Almost all work analysing the nutritional status and fertilization needs of forest plantations in Northwest Spain has been carried out on fast-growing exotic species such as *Pinus radiata* (Sánchez-Rodríguez et al., 2002; Zas and Serrada, 2003), *Pseudotsuga menziesii* (Zas, 2003; Solla-Gullón et al., 2006) or *Eucalyptus globulus* (Merino et al., 2003). The nutritional disorders commonly observed in these studies have been attributed to the high specific nutrient demand of these highly productive species. However, no information on nutritional aspects is available for the maritime pine (*Pinus pinaster* Ait.), a fast growing native colonizer, widely spread in SW Europe.

Maritime pine populations from Northwest Spain are included within the Atlantic group, which also includes the well known Landas French provenances and Leiría Portuguese provenances, broadly used for forestry purposes. However, several studies using molecular markers (Bucci et al., 2007), or analysing quantitative variation in provenance trials (Molina, 1965; Alía et al., 1997) have revealed the singularity of the Galician maritime pine origin within the Iberian Peninsula context, and even within the Atlantic group. The particular edaphic conditions in this area may have contributed to the differentiation of these populations through local adaptations to low and heterogeneous nutrient availability. In concordance with this hypothesis, it is axiomatic among local foresters that *P. pinaster* is much less nutrient-demanding and responds much less to fertilization than other fast-growing species (Bará, 1990).

The aims of the present study were (i) to check if *P. pinaster* plantations in Northwest Spain show nutritional disorders as has been demonstrated for other fast growing tree species in the region, (ii) to determine the response to fertilization with different nutrients of *P. pinaster* and how this response varied across different test sites, i.e. how the response to nutrient availability is environmentally modulated, and (iii) to quantify the genetic control of the phenotypic responses to nutrient availability in the north west maritime pine population.

2. Materials and methods

2.1. Experimental approach

In order to answer to the first question, 22 *P. pinaster* reforestations, planted throughout Galicia (Northwest Spain) in 1996 and representative of the maritime pine stands in the region, were selected and sampled for nutritional diagnosis (Fig. 1).

In addition, three family × fertilization trials were established in 2003 (sites B and C) and in 2004 (site A) to analyse the response of *P. pinaster* to establishment fertilization and to quantify the variation in the response to nutrient availability in the NW Coastal *P. pinaster* population (Fig. 1).

Climate throughout the area is temperate humid Atlantic, with high annual precipitation and relatively short summer drought (Table 1). The trial at site A has a slight Mediterranean and

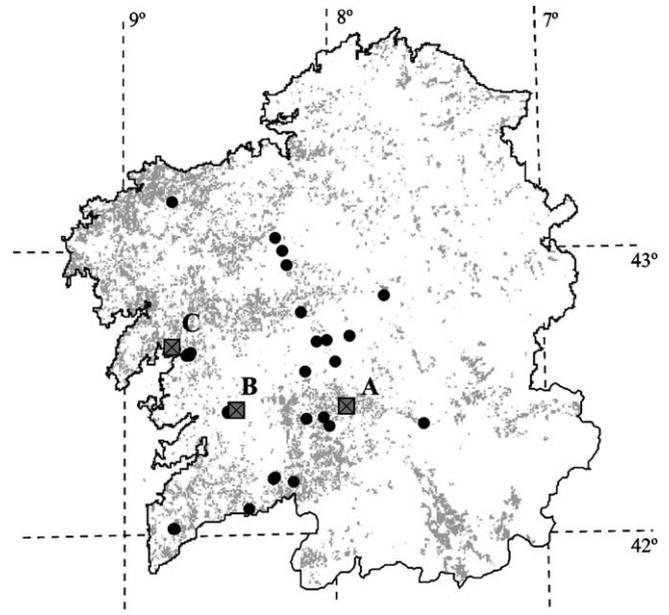


Fig. 1. Location of the 22 reforestation plots (black dots) and the three family × fertilization trials (grey squares, A–C). The shaded area represents the current distribution of *P. pinaster* in Galicia derived from the Third National Forestry Inventory (DGCN, 2002).

Table 1

Mean climatic characteristics during the 5-year study period (2003–2007) in the three family × fertilization *P. pinaster* trials.

	Site A	Site B	Site C
Altitude (m)	520	530	90
Mean annual temperature (°C)	11.7 ± 0.2	12.2 ± 0.2	14.5 ± 0.1
Annual precipitation (mm)	1039 ± 132.5	1976 ± 235.9	1511 ± 166.1
Summer precipitation (mm) ^a	109 ± 13.5	244 ± 22.2	204 ± 30.9
Annual thermal oscillation (°C) ^b	15.6 ± 1.1	12.1 ± 0.2	12.6 ± 0.6

^a Mean precipitation from July to September.

^b Mean of the differences between the mean temperature of the coldest and the warmest month at each year.

Continental influences, with lower annual and summer precipitation and higher temperature fluctuation than sites B and C.

2.2. Plant material

All the *P. pinaster* reforestation stands were planted in 1996 with genetic material from the Region of Provenance '1a Coastal Galicia' (Alía et al., 1996).

The plant material of the family × fertilization trials consisted of open-pollinated families from 28 maternal plus trees selected for superior growth, stem form and branch characteristics in mature plantations or natural stands of *P. pinaster* in Galicia. All seeds were collected in a clonal seed orchard (Sergude, 42.82°N, 8.45°W) in the same year. We also included three unimproved commercial *P. pinaster* seed sources as controls, two from Galicia and one from France (Landas origin). The experimental units consisted of 32 plants with one *P. radiata* extra seed source, not included in the analysis.

2.3. Experimental layout

Reforestation stands were sampled when they were around 2 years old. Within each site, one rectangular sample plot of 50 forested plants was established for each hectare planted. Sample

Table 2

Fertilization treatments applied to *P. pinaster* seedlings after planting. Each code (F₀–F₈) indicates a fertilizer treatment. Presence and absence of nutrients in each fertilizer treatment are indicated by + and –, respectively.

Nutrient	Fertilizer	Dose (plant ⁻¹)	Treatment code								
			F ₀	F ₁	F ₂	F ₃	F ₄	F ₅	F ₆	F ₇	F ₈
N	Ammonium nitrate	5 g N/plant	–	+	–	+	+	+	–	–	–
P-Ca	Calcium phosphate	10 g N/plant	–	+	+	–	+	+	–	+	+
K	Potassium sulphate	15 g N/plant	–	+	+	+	–	+	+	–	+
Mg	Magnesium sulphate	5 g N/plant	–	+	+	+	+	–	+	+	–

plot size was variable depending on the planting density, with a minimum area of 300 m².

The experimental layout in the three family × fertilization trials was a split-plot design replicated in 10 blocks, with 9 fertilization treatments acting as the main factor and the genetic entries as the split factor. In total, we planted 2880 seedlings in each trial, corresponding to 10 blocks × 9 fertilization treatments × 32 genetic entries (28 families of half-sibs + 3 control seed lots + 1 *P. radiata*). Spacing was 3 m × 2 m. Fertilization treatments were an unfertilized control and eight treatments built upon combinations of four commercial fertilizers (Table 2). Plants were fertilized by hand just after planting.

2.4. Sampling and field assessments

Total height and root-collar diameter were measured in all living plants in the sample plots of the 22 reforestation stands at age 2. In the family × fertilization trials, total height in cm was measured in all living plants yearly during the first 5 years after planting.

For foliar nutritional diagnosis of all stands (reforestations and family × fertilization trials), current season needle samples were collected from three branch-ends per tree in the upper third of the crowns of the trees following Ballard and Carter (1986). In the 22 reforestation stands, two composite samples (from a minimum of 10 trees each) were collected in each sampling plot at the end of the second growing season.

Foliar nutrient diagnosis in the three family × fertilization trials was carried out on the unfertilized 'control' plants 5 years after planting. In these trials a more extensive sampling was carried out, with the collection of five composite samples in each trial following the same procedure as above. Samples were placed in plastic bags and transported in ice-cooled containers to the lab. Needle samples were oven-dried at 65 °C to constant weight, finely grounded and preserved for chemical analysis.

Composite soil samples were also taken in all stands for chemical analysis. In the reforestation stands, five soil samples (0–20 cm depth, 8 cm diameter soil corers) were taken from the four corners and centre of each sampling plot, mixed, and homogenized to form one composite sample per plot. In the progeny × fertilization trials, three composite soil samples (composed of five samples each as above) were taken per trial. Soil samples were sieved through a 2 mm screen, oven-dried at 60 °C and stored until chemical analysis.

2.5. Plant and soil analyses

The foliar contents of total P, K, Ca and Mg were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Perking-Elmer Optima 4300DV (MA, USA) after microwave assisted digestion of foliar samples. Total N concentrations were determined by dry combustion using a LECO CN-2000 elemental analyzer (LECO Corporation, St. Joseph, MI). The foliar nutrient critical levels reported by Bonneau (1995) for N, P, K, and Ca nutrition in *P. pinaster*, and by Will (1985) for Mg nutrition in *P. radiata* were used for nutritional diagnosis of all stands.

For soil analyses, total N and total organic C were determined using a LECO CN-2000 elemental analyzer (LECO Corporation, St. Joseph, MI); total K, Ca and Mg were determined by ICP-OES using a Perking-Elmer Optima 4300DV (Massachusetts, USA) after microwave assisted wet digestion as above; and available P was evaluated by the Bray-II method (Bray and Kurtz, 1945) (UV-VIS Beckman). The particle-size distribution in soil samples was determined following the pipette method (Gee and Bauder, 1996), and the pH was measured in deionized H₂O (1: 2.5 d.w.:vol).

Chemical analyses were performed in the central laboratory facilities (C.A.C.T.I.) at Universidade de Vigo (<http://webs.uvigo.es/cactiweb/index.htm>).

2.6. Adjusting spatial autocorrelation

Growth traits assessed in field trials frequently show non-random spatial structures that may affect the efficiency of standard statistical analyses (e.g. Fu et al., 1999; Dutkowski et al., 2006). When spatial heterogeneity is present, near neighbours are more similar than plants further away, i.e. data are autocorrelated, and the requirement of data independence in standard parametric statistics is violated (Legendre, 1993). Although block designs improve the statistical efficiency by controlling some of the spatial variation, there are many situations where block designs are not able to account for all the spatial heterogeneity. In such cases, spatial analysis techniques become essential to correctly analyse spatial autocorrelated data (Dutkowski et al., 2006; Zas, 2006).

We determined the spatial position of each tree using a total station (Pentax R-315) and then we prospected the spatial structure of the dependent variable at each site by constructing the empiric semivariogram for the residuals adjusted for main effects (fertilization, family, and family × fertilization) with the SAS VARIOGRAM procedure (SAS-Institute, 1999). Those variables that were spatially dependent were corrected using the Iterative Spatial Analysis (ISA) method (Zas, 2006). Briefly, the spatial variation of the dependent variable was modeled by the kriging method using a theoretical model fitted to the observed semivariogram and the KRIG2D procedure of SAS. We then adjusted the original variable for its spatial autocorrelation, subtracting the kriging estimate in each position. The new corrected variable was then reanalysed and a new estimate of the main effects was obtained, and used to generate new residuals. The process was repeated iteratively, until convergence of the estimates of main effects (usually five steps are enough). A detailed description of the method can be consulted in Zas (2006). The spatial adjustments were carried out for each site (A–C) and age (1–5) independently.

2.7. Statistical analyses

Spatially adjusted height data at each age in the three family × fertilization trials were analysed by means of a split-split model, in which the site (S) was assigned to the main plot, the fertilization treatments (F) to the subplot, and the genotypes (open-pollinated families, G) to the sub-subplot. The data were

Table 3
Summary of the topsoil (0–20 cm) properties of the 22 reforestation stands and the three family × fertilization trials.

	Reforestation stands (n = 22)				Family × Fertilization trials		
	Mean	Median	Range	CV (%)	Site A	Site B	Site C
pH	4.8	4.8	4.0–5.5	6.8	4.3	4.0	4.3
Organic C (g kg ⁻¹)	78.5	81.4	22.6–134.3	34.3	72.1	105.2	98.8
C:N ratio	16.9	17.8	10.7–22.4	18.9	20.3	14.8	16.1
Total N (g kg ⁻¹)	4.7	4.9	2.1–8.1	34.2	3.6	7.1	6.3
P Bray-II (mg kg ⁻¹)	20.6	6.9	2.4–158.4	174.1	5.9	4.7	4.2
Total K ⁺ (mg kg ⁻¹)	96.8	81.9	40.2–252.5	56.9	61.1	94.9	117.0
Total Ca ²⁺ (mg kg ⁻¹)	97.1	55.6	7.4–394.2	113.9	47.3	68.7	52.7
Total Mg ²⁺ (mg kg ⁻¹)	34.6	23.3	6.3–143.8	102.6	9.6	25.6	24.4

analysed with a mixed model using the SAS MIXED procedure (Littell et al., 2006).

To test for significant effects of including a specific nutrient in the fertilization treatment, specific comparisons between those treatments that only differ in the inclusion of that nutrient were analysed using the CONTRAST statement of the MIXED procedure (Littell et al., 2006). The effect of including N was tested contrasting treatments (F₁, F₃, F₅, F₇) with treatments (F₂, F₄, F₆, F₈) (see Table 2 for treatment description). The effects of adding P-Ca, K, and Mg were tested with the following specific contrasts: (F₁, F₂) versus (F₃, F₆) for P, (F₁, F₂) versus (F₄, F₇) for K, and (F₁, F₂) versus (F₅, F₈) for Mg.

2.8. Variation in the response to nutrient availability

The Simplified Relative Distances Plasticity Index (RDPI_s) proposed by Valladares et al. (2006) was used to estimate, within each site and age, the response of each *P. pinaster* family to variation in nutrient availability. This index is particularly suited for quantifying the phenotypic change of a given genetic entry along more than three environments that do not follow a specific environmental gradient. The index was calculated based on phenotypic distances between the least square means for each family-treatment combination derived from the mixed models. For each age and site, the RDPI_s index for genotype *i* was calculated as

$$RDPI_s(i) = \frac{\sum_{j \neq k} |LSM_{ij} - LSM_{ik}| / (LSM_{ij} + LSM_{ik})}{n}$$

where LSM_{ij} and LSM_{ik} are the least squares for height growth of genotype *i* in treatments *j* and *k*, respectively (being *j* and *k* always different fertilization treatments), and *n* is the total number of distances, i.e. the number of possible pairs of different treatments. In our case, with nine fertilization treatments, $n = 36$.

The RDPI_s indices were calculated for each site independently, resulting in three estimations of the plastic responses for each family and age. For each age, a two way ANOVA was applied to the RDPI_s data to test for the environmental (site) and genetic (family) control of the response to variation in nutrient availability.

3. Results

3.1. Soil nutrient content and nutritional status of *P. pinaster* stands in Northwest Spain

The *P. pinaster* reforestation stands were established on typical Galician forest soils, characterized by a sandy texture, acidic pH, high contents of organic matter, and low levels of nutrients, especially of available P (Table 3). Total N contents were high in almost all sites, showing a more reduced variation than the other macronutrients. On the contrary, available P, and exchangeable Ca and Mg were extremely variable, with coefficients of variation over 100%. The discrepancy between the mean and the median for these three nutrients indicates skewed distributions, with most of the

sites having low nutrient concentrations, and only a few having extreme high values. For instance, in the case of P, despite a mean value of 20.6 mg kg⁻¹, 16 out of the 22 stands had P values below 10 mg kg⁻¹. These results reflect the diversity of previous land uses on the studied sites. Most of the sites were typical forest sites or marginal agricultural lands where agriculture was abandoned many years ago, but some sites were used for crop production until very recently, with organic and inorganic fertilization and intensive tillage favouring higher nutrient contents. Soil characteristics in the three family × fertilization trials were within the range of the reforestation plots (Table 3).

None of the 22 reforestation stands and the 3 family × fertilization trials showed foliar concentrations above the respective critical levels for all macronutrients, indicating that all plots suffer some degree of nutrient deficiencies (Fig. 2). The most frequent deficiencies were in P and Mg, with 16 and 18 out of the 22 stands showing severe deficiencies, respectively. K and Ca foliar concentrations were below the critical level in 11 and 7 cases, respectively. On the other hand, all reforestation stands showed satisfactory foliar N levels except three sites. These high foliar N concentrations were reflected in a large imbalance between N and P nutrition with 20 out of the 22 stands with N:P ratios above the critical threshold of 12.5.

The three family × fertilization trials showed similar patterns of foliar nutrient concentrations, with satisfactory levels of N but marginal or critical levels of the other four macronutrients (Fig. 2).

Phosphorus was the only nutrient for which foliar concentrations were significantly correlated with soil nutrient levels

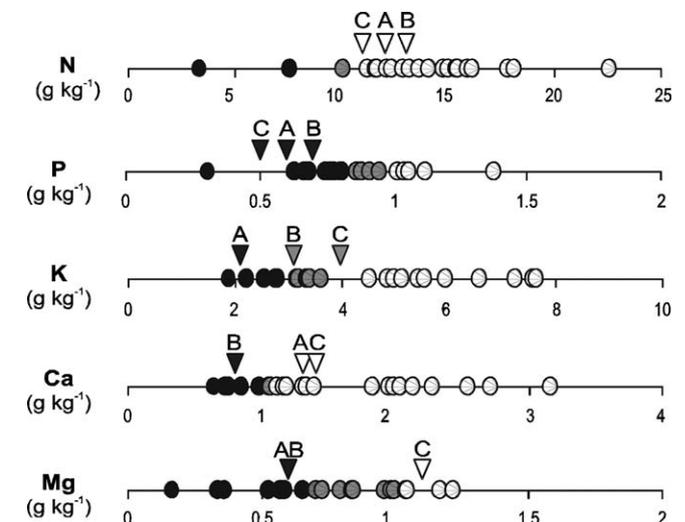


Fig. 2. Foliar nutrient concentration in the 22 *P. pinaster* reforestation stands (dots) and in the three family × fertilization trials (triangles A–C). Each data point represents the mean of 2–3 composite foliar samples of 5–10 trees each. Dot and triangle colours indicate the nutrient diagnosis according to the critical values reported by Bonneau (1995): white = satisfactory levels, grey = marginal levels, and black = critical levels.

Table 4

Summary of the mixed model for height growth of *P. pinaster* half sibs planted under different establishment fertilization treatments in three fertilization × family trials, during 5 years (H1–H5) after establishment and fertilization.

Effect	DF	H1		H2		H3		H4		H5	
		F	P > F	F	P > F	F	P > F	F	P > F	F	P > F
Site	2	1130.6	<0.001	4001.6	<0.001	3285.2	<0.001	3739.8	<0.001	4529.0	<0.001
Fertilization	8	25	<0.001	38.86	<0.001	32.21	<0.001	23.28	<0.001	18.97	<0.001
Site × Fert	16	16.94	<0.001	18.11	<0.001	15.41	<0.001	15.29	<0.001	14.94	<0.001
Family	30	9.4	<0.001	20.47	<0.001	17.84	<0.001	11.98	<0.001	13.97	<0.001
Fam × Fert	240	1.24	0.009	1.31	0.001	1.27	0.003	1.39	<0.001	1.54	<0.001
Site × Fam	60	6.04	<0.001	5.94	<0.001	4.71	<0.001	4.65	<0.001	5.12	<0.001
Site × Fam × Fert	480	1.32	<0.001	1.48	<0.001	1.47	<0.001	1.38	<0.001	1.4	<0.001

DF = degrees of freedom.

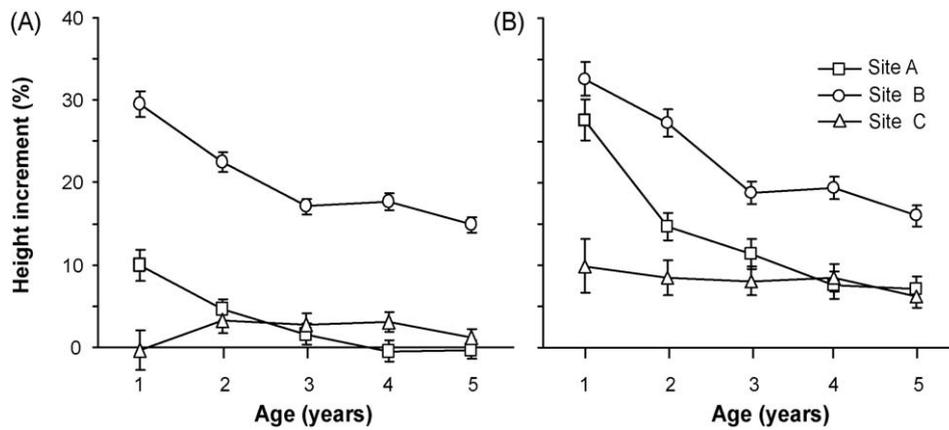


Fig. 3. Effect of fertilization on height growth in three *P. pinaster* family × fertilization trials during the first 5 years after establishment and fertilization application. Overall mean relative response (panel A) of eight different fertilization treatments (see Table 2) relative to the unfertilized control, and maximum relative response to the best fertilization treatment observed at each site (panel B).

($r = 0.45$, $p < 0.05$, $N = 22$). In addition, pine growth in the reforestation stands was significantly correlated with foliar concentrations of P ($r = 0.42$, $p < 0.05$) and Ca ($r = 0.47$, $p < 0.05$).

3.2. Fertilization response in family × fertilization trials

Fertilization treatments significantly increased pine height growth in the three family × fertilization trials (Table 4), but this positive effect decreased with time (Fig. 3). The fertilization × site interaction was strong and significant in all the years (Table 4), indicating that the fertilization treatments had different effects on height growth depending on the trial. Site B showed the strongest response to the fertilization treatments, with an average response ranging from 30% one year after the application of treatments to 15% five years later (Fig. 3A). The average response to the fertilization treatments at the other two sites was much lower and became not significant with time (Fig. 3A).

The specific responses to the fertilization treatments in site B were similar between treatments, and the response to the best treatment (Fig. 3B) was not very different to the average response to all the fertilization treatments (Fig. 3A). Accordingly, specific contrasts revealed no significant effects of single nutrient additions in this site (Fig. 4). At the other two sites, the effect of some specific nutrients on pine growth was especially strong. The addition of P-Ca, K, or Mg significantly increased height growth in site A, as did K in site C, whereas the inclusion of N reduced growth in both sites, and adding P significantly reduced growth in site C. These variable effects of adding specific nutrients resulted in large differences at these two test sites between the maximum response to the best

treatment (Fig. 3B) and the average response to all the fertilization treatments (Fig. 3A). The maximum response was obtained for the F₂ treatment (P-Ca + K + Mg) at sites A and B, and for F₃ (N + K + Mg) at site C.

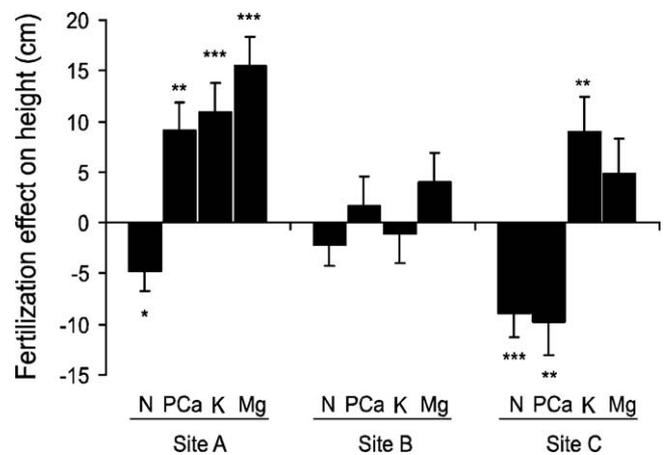


Fig. 4. Estimates of the effect of single nutrients (N, P-Ca, K, and Mg) on *P. pinaster* height in three family × fertilization trials 5 years after establishment and treatment applications. Each bar represents the estimate of the effect of including every specific nutrient in the fertilization treatment obtained from specific contrasts in the mixed model. The linear functions for these contrasts were as follows (see Table 2 for treatment description): (F₁, F₃, F₅, F₇) versus (F₂, F₄, F₆, F₈) for estimating the effect of N addition, (F₁, F₂) versus (F₃, F₆) for P, (F₁, F₂) versus (F₄, F₇) for K, and (F₁, F₂) versus (F₅, F₇) for Mg. Significant effects are denoted by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

Table 5

Results of the ANOVA of plasticity to nutrient availability estimated using the $RDPI_s$ index for *P. pinaster* half-sib families in the different test sites.

Age (years)	Site		Family	
	F	P > F	F	P > F
1	14.37	<0.0001	0.72	0.8396
2	2.29	0.1102	1.45	0.1105
3	23.75	<0.0001	1.06	0.4101
4	14.20	<0.0001	1.55	0.0761
5	20.44	<0.0001	1.06	0.4182

3.3. Genetic variation in the response to fertilization

Differences among families were significant for height growth over the 5 years after planting with no clear trend over time (Table 4). Families also differed significantly in their response to the fertilization treatments, as revealed by the significant family \times fertilization interaction (Table 4). Furthermore, the family \times site interaction and the family \times fertilization \times site were significant too, indicating that both the family growth and the family response to fertilizers varied between sites.

The significant family \times fertilization interaction suggests a genetic variation in the response to nutrient availability within the studied population. To test for the genetic control of this response we analysed the variation of the $RDPI_s$ plasticity index estimated for each genotype and test site by means of ANOVA. With our experimental layout we were unable to detect genetic differences in the response to nutrient availability, but we found a strong environmental effect (Table 5).

According to this weak genetic control of the response to fertilization, the family \times fertilization interaction for height growth was relatively small when compared to the family \times site and the family \times site \times fertilization interactions. Considering the family factor and all the interactions involving this factor as random effects in the general mixed model, the estimate of the family \times fertilization variance component was around 80 times less than the other two interaction components, and 30 times less than the family variance component (data not shown).

4. Discussion

The results of the present paper indicate that growth of *P. pinaster* in North West Spain, although benefiting from favourable temperature and rainfall patterns, is limited by nutrient availability. Soil nutrient concentrations were relatively low in many of the study sites. Foliar nutrient concentrations were below the critical levels in most of the cases, and below the values commonly observed in other *P. pinaster* stands in France and Australia that are known to be nutrient limited (Warren et al., 2005; Trichet et al., 2008). As in other forest tree species in the area, the most common deficiencies were in P and Mg, and to a lesser extent in K and Ca, while N was usually well supplied. Sánchez-Rodríguez et al. (2002) and Zas and Serrada (2003) reported that most of the *P. radiata* plantations studied in Northwest Spain were deficient in P, Mg and, in some cases, in Ca, while they were satisfactory in N. Similarly, high and satisfactory N foliar concentrations and low and frequently critical P, K, Ca and Mg concentrations were reported for *P. menziesii* (Zas, 2003; Solla-Gullón et al., 2006) and *E. globulus* (Merino et al., 2003) in the area. In addition, P was the only nutrient for which foliar concentrations significantly correlated with soil contents, as commonly occurs for nutrients with limited availability (e.g. Gallardo and Coveló, 2005). In agreement with this nutritional diagnosis, pine growth was significantly correlated with foliar P and Ca concentrations. Similarly, early growth and/or site index has been shown to be significantly correlated with foliar

and soil P in *P. radiata* (Sánchez-Rodríguez et al., 2002; Zas and Serrada, 2003) and with foliar P and Ca in *P. menziesii* (Zas, 2003) in Northwest Spain.

According to the deficiencies detected through foliar nutrient analysis, a significant positive response to nutrient additions was observed in the three family \times fertilization trials, although these responses varied from site to site. *P. pinaster* response to fertilization has been reported before (e.g. Warren et al., 2005; Trichet et al., 2008) but little information was available for Northwest Spain, where it is generally accepted that this species responds little to nutrient additions (Bará, 1990). In sandy acidic soils in France (Saur, 1990; Trichet et al., 2008) and Australia (Warren et al., 2005), large growth increments, comparable to those found here, have been reported in response to nutrient additions, especially of P, and superphosphate fertilization is now routinely used in those areas.

Response to fertilization has been also documented in Northwest Spain for other fast-growing species such *P. radiata* (Solla-Gullón et al., 2004; Zas et al., 2006a). In a similar family \times fertilization trial series as the one studied here, *P. radiata* showed large responses to P and Mg additions, with height increments larger than those observed here for *P. pinaster* (up to 170% over the unfertilized control 1 year after fertilization, Zas et al., 2006a). In addition, the response of *P. radiata* to different nutrient combinations was very similar across sites (Zas et al., 2006a), whereas here the response of *P. pinaster* was very variable, despite similar foliar nutritional diagnosis in the three trials. Thus, the response to fertilization agreed with the soil nutrient levels and foliar nutrient diagnosis in some cases but not in others. At site B all nutrients except N were below the critical levels. This was the site where the overall response to fertilizers was higher and no significant differences were detected for specific nutrient additions. At site A, the significant response to P-Ca, K, and Mg, and the negative effect of N agreed with the foliar nutrient diagnosis for these nutrients. Similarly, N additions have been shown to have a negative effect in *P. radiata* fertilization trials (Zas et al., 2006a), where a large imbalance between N and P and between N and K has been reported (Zas and Serrada, 2003). The negative effect of N fertilization was also observed at site C, where N foliar concentrations were again above the critical value. However, at this site, the negative effect of P fertilization was unexpected given the low foliar P concentrations and the high foliar N:P ratio at this site. The negative effect of P at this site can be explained by the incidence of a *Hylobius abietis* (Curculionidae, Coleoptera) attack during the first 2 years after planting that disturbed the normal response to fertilization. The pine weevil preferentially attacked the plants fertilized with P-Ca, causing large height reductions that outweighed the growth gains generated by fertilization (Zas et al., 2006b).

The response to fertilization not only varied among sites, but also among genetic entries. The significant family \times fertilization interaction suggests that the ability to respond to fertilization varied among families, i.e. the phenotypic response or family plasticity to nutrient availability was under genetic control. Theoretically, phenotypic plasticity should be measured at the genotypic level, so the effects of genetic variability can be distinguished from variation in phenotypic expression (Pigliucci, 1996). Because families are not single genotypes, but a mixture of related genotypes with the same mother tree, the value of a given family in a given environment can incorporate some uncontrolled genotypic effects as well as some genotype \times environment interaction. These uncontrolled effects may affect the estimation of actual phenotypic plasticity, so the variation in the family response to nutrient addition is not, strictly speaking, a direct measure of variation in phenotypic plasticity. Ideally, to estimate the genetic control of phenotypic plasticity, individual trees

belonging to different families should have been clonally replicated in the different fertilization treatments. Then the family structure of the sample could be used to estimate the genetic control of plasticity. Although it is well known that there is abundant genetic variation for plastic responses (Pigliucci, 2005), there is very little information based on proper empirical approaches with clonally replicated genotypes allowing the quantification of this variation and the estimation of heritability of plastic responses in strict terms, especially for forest trees (Chambel et al., 2005). With our experimental design, however, we can have several estimates of the sensibility of each family to nutrient availability variation, allowing us to statistically quantify the genetic control of the family responsiveness to fertilization. We found little or no genetic variation for the plastic responses at the family level to nutrient additions across environments. The response to fertilization seemed to be strongly influenced by other environmental factors that differentially modulate the plastic response of each family in each environment. This could be easily due to non-linear reaction norms and different nutritional soil conditions among the test sites. Adding the same amount of nutrients to soils differing in nutrient availability and fertilization efficiency, may have led to variations in the family response to fertilizers (Namkoong et al., 1992). In accordance with this, although soil and foliar diagnosis of the three test sites were quite similar, response to fertilization greatly varied among sites, and this variation altered the family response to the fertilization treatments, as revealed by the significant family \times fertilization \times site interaction term. In addition, the genetic response to the fertilization treatments could also vary among sites because of the influence of other unknown biotic and/or abiotic environmental factors that varied among the test sites. For instance, different symbiotic fungal communities among sites may differentially alter the family responses to nutrient additions in different sites (Mari et al., 2003). Moreover, the ability to get infected by specific mycorrhizal species may differ between pine families, as observed in other conifer species (Korkama et al., 2006). Besides, interactions with other environmental factors are known to alter the plastic response to a specific environmental factor, such is the case with the plastic responses to shade depending on the water availability (Climent et al., 2006; Sánchez-Gómez et al., 2007).

Our results suggest that, although there was genetic variation for the response to nutrient availability, this variation was strongly environmentally modulated, hampering both the evolution of specific responses as adaptations to local environmental conditions, and the possibility of artificially selecting, within breeding programs, genotypes suited to different soil conditions and/or silviculture regimes. Further research, comparing the plasticity of *P. pinaster* populations coming from different soil conditions, would help to understand the hypothesized local adaptations to low and heterogeneous nutrient availability of the NW maritime pine population (Bradshaw, 2006). The large population divergence in the phenotypic response of *P. pinaster* to water availability, with the Atlantic provenance of Leira (Portugal) being the most plastic origin (Chambel et al., 2007), is an attractive start point for this investigation.

In summary, nutritional disorders of *P. pinaster* in Northwest Spain were frequent and similar to those found in other fast growing species, but the qualitative response to fertilization was not as clear as that found for other species, specifically in radiata pine (Zas et al., 2006a). Judging from the results presented here, P and Mg fertilization should be recommended to increase early growth and attain crown closure earlier, but the lack of a consistent response among sites, and the possible undesired effects of fertilization, such as the increase in pest incidences (Zas et al., 2006b) or the increase in susceptibility to storm damage (Cocchi and Bert, 2003; Trichet et al., 2008), suggest that the convenience

of fertilization cannot be generalized and it should be specifically determined in each case. The results also indicated that families differed in the response to the fertilization treatments but this family response was environmentally dependent. The strong environmental component modulating the phenotypic responses to nutrient availability in nutrient-lacking soils could impose an important obstacle for the selection towards local adaptations to low and heterogeneous nutrient availability. Analogously, it hinders the artificial selection of genotypes adapted to produce high productivity phenotypes under different soils and/or fertilization regimes.

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References

- Alía, R., Martín, S., De Miguel, J., Galera, R., Agúndez, D., Gordo, J., Catalán, G., Gil, L., 1996. Las regiones de procedencia de *Pinus pinaster* Ait. OA de Parques Nacionales. DGCONA, Madrid.
- Alía, R., Moro, J., Denis, J.B., 1997. Performance of *Pinus pinaster* provenances in Spain: interpretation of the genotype by environment interaction. Canadian Journal of Forest Research 27, 1548–1559.
- Ballard, T.M., Carter, R.E., 1986. Evaluating forest stand nutrient status. In: Ministry of Forests, British Columbia. Land Management Report, Ministry of Forests, Victoria, British Columbia, p. 60.
- Bará, T.S., 1990. Fertilización Forestal. Consellería de Agricultura, Gandería e Montes. Xunta de Galicia, Santiago.
- Bonneau, M., 1995. Fertilisation des forêts dans les pays tempérés: théorie, bases du diagnostic, conseils pratiques, réalisations expérimentales. ENGREF, Nancy, France.
- Bradshaw, A.D., 2006. Unravelling phenotypic plasticity—why should we bother? New Phytologist 170, 644–648.
- Bray, B.M., Kurtz, L.T., 1945. Determination of total, organic and available forms of phosphorus in soils. Soil Science 59, 39–45.
- Bucci, G., Gonzalez Martinez, S.C., Le Provost, G., Plomion, C., Ribeiro, M.M., Sebastiani, F., Alía, R., Vendramin, G.G., 2007. Range-wide phylogeography and gene zones in *Pinus pinaster* Ait. revealed by chloroplast microsatellite markers. Molecular Ecology 16, 2137–2153.
- Calvo, A.R.M., Díaz-Fierros, F., 1982. Mineralización del carbono y del nitrógeno en suelos forestales de Galicia. Anales de Edafología y Agrobiología 41, 1–10.
- Climent, J., Aranda, I., Alonso, J., Pardos, J.A., Gil, L., 2006. Developmental constraints limit the response of Canary Island pine seedlings to combined shade and drought. Forest Ecology and Management 231, 164–168.
- Correia, I., Almeida, M.H., Aguiar, A., Alía, R., David, T.S., Pereira, J.S., 2008. Variations in growth, survival and carbon isotope composition ($\delta^{13}C$) among *Pinus pinaster* populations of different geographic origins. Tree Physiology 28, 1545–1552.
- Cocchi, V., Bert, D., 2003. Wind-firmness in *Pinus pinaster* Ait. stands in Southwest France: influence of stand density, fertilisation and breeding in two experimental stands damaged during the 1999 storm. Annals of Forest Science 60, 209–226.
- Chambel, M.R., Climent, J., Alía, R., 2007. Divergence among species and populations of Mediterranean pines in biomass allocation of seedlings grown under two watering regimes. Annals of Forest Science 64, 87–97.
- Chambel, M.R., Climent, J., Alía, R., Valladares, F., 2005. Phenotypic plasticity: a useful framework for understanding adaptation in forest species. Investigación Agraria. Sistemas y Recursos Forestales 14, 334–344.
- DGCN, 2002. Tercer Inventario Forestal Nacional 1997–2006: Galicia. Ministerio de Medio Ambiente, Dirección General de Conservación de la Naturaleza, Madrid, Spain.
- Dutkowsky, G.W., Costa-e-Silva, J., Gilmour, A.R., Wallendorf, H., Aguiar, A., 2006. Spatial analysis enhances modelling of a wide variety of traits in forest genetic trials. Canadian Journal of Forest Research 36, 1851–1870.
- Fu, Y.B., Yanchuk, A.D., Namkoong, G., 1999. Spatial patterns of tree height variations in a series of Douglas-fir progeny trials: implications for genetic testing. Canadian Journal of Forest Research 29, 714–723.
- Gallardo, A., Covelo, F., 2005. Spatial pattern and scale of leaf N and P concentration in a *Quercus robur* population. Plant and Soil 273, 269–277.
- Gee, G.W., Bauder, J.W., 1996. Particle size analysis. In: Klute, A. (Ed.), Methods of Soil Analyses. Part I. American Society of Agronomy, Madison, pp. 383–441.
- Gonzalez Martinez, S.C., Gil, L., Alía, R., 2005. Genetic diversity estimates of *Pinus pinaster* in the Iberian Peninsula: a comparison of allozymes and quantitative traits. Investigación Agraria. Sistemas y Recursos Forestales 14, 3–12.
- Gonzalez Martinez, S.C., Mariette, S., Ribeiro, M.M., Burban, C., Raffin, A., Chambel, M.R., Ribeiro, C.A.M., Aguiar, A., Plomion, C., Alía, R., Gil, L., Vendramin, G.G.,

- Kremer, A., Reviron, M.P., 2004. Genetic resources in maritime pine (*Pinus pinaster* Aiton): molecular and quantitative measures of genetic variation and differentiation among maternal lineages. *Forest Ecology and Management* 197, 103–115.
- Korkama, T., Pakkanen, A., Pennanen, T., 2006. Ectomycorrhizal community structure varies among Norway spruce (*Picea abies*) clones. *New Phytologist* 171, 815–824.
- Legendre, P., 1993. Spatial autocorrelation: a trouble or new paradigm? *Ecology* 74, 1659–1673.
- Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D., Schabenberger, O., 2006. SAS System for Mixed Models, second edition. SAS Institute, Cary, NC.
- Macías, F., Calvo, R.M., García, C., García-Rodeja, E., Silva, B., 1982. El material original: su formación e influencia en las propiedades de los suelos de Galicia. *Anales de Edafología y Agrobiología* 42, 1746–1768.
- Mari, S., Jonsson, A., Finlay, R., Ericsson, T., Kähr, M., Eriksson, G., 2003. Genetic variation in nitrogen uptake and growth in mycorrhizal and nonmycorrhizal *Picea abies* (L.) Karst. seedlings. *Forest Science* 49, 258–267.
- Merino, A., Rodríguez-López, A., Brañas, J., Rodríguez-Soalleiro, R., 2003. Nutrition and growth in newly established plantations of *Eucalyptus globulus* in north-western Spain. *Annals of Forest Science* 60, 509–517.
- Molina, F., 1965. Comportamiento racial de *Pinus pinaster* en el noroeste de España. *Anales IFIE* 2, 221–238.
- Namkoong, G., Jonsson, A., Eriksson, G., 1992. Genetic variation in nutrient response functions. *Theoretical and Applied Genetics* 85, 165–172.
- Paz-Gonzalez, A., Vieira, S.R., Castro, M.T.T., 2000. The effect of cultivation on the spatial variability of selected properties of an umbric horizon. *Geoderma* 97, 273–292.
- Pigliucci, M., 1996. How organisms respond to environmental changes: from phenotypes to molecules (and vice versa). *Trends in Ecology and Evolution* 11, 168–173.
- Pigliucci, M., 2005. Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology and Evolution* 20, 481–485.
- Sánchez-Gómez, D., Zavala, M.A., Valladares, F., 2007. Functional traits and plasticity linked to seedlings' performance under shade and drought in Mediterranean woody species. *Annals of Forest Science* 65, 311.
- Sánchez-Rodríguez, F., Rodríguez-Soalleiro, R., Español, E., López, C.A., Merino, A., 2002. Influence of edaphic factors and tree nutritive status on the productivity of *Pinus radiata* D. Don plantations in northwest Spain. *Forest Ecology and Management* 171, 181–189.
- SAS-Institute, 1999. SAS/STAT User's guide. Version 8. SAS Institute Inc., Cary, NC.
- Saur, E., 1990. Influence of copper and phosphorus fertilization on growth and mineral nutrition of maritime pine (*Pinus pinaster*) grown in a sandy soil rich in organic matter. *Annales des Sciences Forestières* 47, 67–74.
- Solla-Gullón, F., Santalla, M., Rodríguez-Soalleiro, R.J., Merino, A., 2006. Nutritional status and growth of a young *Pseudotsuga menziesii* plantation in a temperate region after application of wood-bark ash. *Forest Ecology and Management* 237, 312–321.
- Solla-Gullón, F., Taboada, M.P., Rodríguez-Soalleiro, R., Merino, A., 2004. Respuesta inicial del aporte de cenizas de biomasa arbórea en el estado nutricional de una plantación joven de *Pinus radiata* D. Don. *Investigación Agraria. Serie de Recursos forestales* 13, 281–294.
- Tapias, R., Climent, J., Pardos, J.A., Gil, L., 2004. Life history of Mediterranean pines. *Plant Ecology* 171, 53–68.
- Trichet, P., Loustau, D., Lambrot, C., Linder, S., 2008. Manipulating nutrient and water availability in a maritime pine plantation: effects on growth, production, and biomass allocation at canopy closure. *Annals of Forest Science* 65, 814.
- Valladares, F., Sánchez-Gómez, D., Zavala, M.A., 2006. Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. *Journal of Ecology* 94, 1103–1116.
- Warren, C.R., McGrath, J.F., Adams, M.A., 2005. Differential effects of N, P and K on photosynthesis and partitioning of N in *Pinus pinaster* needles. *Annals of Forest Science* 62, 1–8.
- Will, G.M., 1985. Nutrient Deficiencies and Fertiliser Use in New Zealand Exotic Forests. FRI Bulletin no 97. Forest Research Institute, Rotorua, New Zealand.
- Zas, R., 2003. Foliar nutrient status and tree growth response of young *Pseudotsuga menziesii* Mirb. (Franco) to nitrogen, phosphorus and potassium fertilization in Galicia (Northwest Spain). *Investigación Agraria. Sistemas y Recursos Forestales* 12, 75–85.
- Zas, R., 2006. Iterative kriging for removing spatial autocorrelation in analysis of forest genetic trials. *Tree Genetics & Genomics* 2, 177–186.
- Zas, R., Pichel, F., Martínez, P., Fernández-López, J., 2006a. Fertilization × genotype interaction in *Pinus radiata* open pollinated seedlings planted in three locations in Galicia (Northwest Spain). *New Forest* 32, 253–263.
- Zas, R., Sampedro, L., Prada, E., Lombardero, M.J., Fernández-López, J., 2006b. Fertilization increases *Hylobius abietis* L. damage in *Pinus pinaster* Ait. seedlings. *Forest Ecology and Management* 222, 137–144.
- Zas, R., Serrada, R., 2003. Foliar nutrient status and nutritional relationships of young *Pinus radiata* D. Don plantations in north-west Spain. *Forest Ecology and Management* 174, 167–176.