Genetic variation and phenotypic plasticity of nutrient re-allocation and increased fine root production as putative tolerance mechanisms inducible by methyl jasmonate in pine trees

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Summary

1. Plant plastic responses to herbivore damage may include rapid, active reallocation of plant resources to reduce the impact of herbivory on future plant fitness. However, whether these inducible tolerance responses can be extended to pine trees and how these responses could be modulated by genetic and environmental factors remains unclear.

2. Biomass allocation and phosphorus (P) and nitrogen (N) concentrations in above- and below-ground tissues were measured in *Pinus pinaster* juveniles belonging to 33 open-pollinated families grown under two P availabilities (P-deficient and complete fertilization). Measurements were taken 15 days after half of the plants received a foliar spray treatment of 22 mmol L^{-1} methyl jasmonate (MJ) to simulate above-ground herbivore attack.

3. Simulated above-ground herbivory promoted a strong preferential allocation of biomass below ground in the form of fine roots, leading to an almost two-fold increase in fine root biomass in MJ-treated plants and a significant reduction in above-ground tissues and coarse roots. In addition, MJ signalling increased P and N concentrations in the shoots while reducing (P) or maintaining (N) concentrations in the roots. These results suggest that induced resource sequestration is not a generalized strategy in this pine species. Fine root biomass and concentration of N and P in plant tissues showed additive genetic variation, but responses to MJ signalling did not vary among families. Allocation of biomass to fine roots was not affected by P availability, whereas allocation of P to the shoot was more intense under complete fertilization.

4. *Synthesis:* Two new putative tolerance mechanisms inducible by MJ signalling may help to minimize the impact of above-ground herbivore damage on the future fitness of young pine trees by (i) allocation of carbon to fine roots, this appeared to be a generalized strategy with weak environmental modulation and (ii) reallocation of P and N from roots to shoots, which was largely affected by P availability, and thus susceptible to greater phenotypic variation in heterogeneous environments. We provide evidence that changes in tolerance-related traits are rapidly inducible by herbivory cues in this pine species. These results should be integrated with induced resistance responses to fully understand the costs and benefits associated with induced responses to herbivory.

Key-words: biomass allocation, conifers, herbivory, nitrogen allocation, phosphorus allocation, phosphorus availability, *Pinus pinaster*, plant–herbivore interactions

Introduction

Plant phenotypes can rapidly change following herbivore attack with a display of plastic responses known as induced responses (Heil 2010). Induced resistance, which aims to deter, reduce or delay current and subsequent attacks, has been extensively studied (e.g. Cipollini & Heil 2010; Eyles *et al.* 2010). Tolerance to herbivory, defined as the capacity to maintain plant fitness irrespective of the damage inflicted by the herbivores (Heil 2010), is another plant strategy for overcoming herbivory (Fornoni 2011). Little is known, however, about the mechanisms involved in conferring high levels of tolerance.

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Evidence is emerging that plant tolerance may rely on plastic changes in tolerance-related traits that occur in the immediate aftermath of an attack, forming part of the integrated plant responses to herbivory rather than compensatory secondary responses to tissue loss (Schwachtje *et al.* 2006; Erb *et al.* 2009; Kerchev *et al.* 2011). A few recent studies using short-lived radioisotopes have reported rapid changes in carbon and nutrient allocation in response to real or simulated herbivory in annual (Schwachtje & Baldwin 2008; Gómez *et al.* 2010; Hanik *et al.* 2010) and woody plants (Babst *et al.* 2005, 2008; Frost & Hunter 2008). The most commonly reported pattern seems to be induced resource sequestration, that is, removing nutrients and carbon resources away from the site of damage into storage tissues shortly after the attack (reviewed by Orians, Thorn & Gómez 2011). This strategy may prevent the loss of new photosynthates, and the stored resources may be crucial for sustaining regrowth and reproduction once the herbivory threat has passed (Gómez *et al.* 2010), thus diminishing the impact of herbivores on plant fitness (Babst *et al.* 2005). Additionally, reduced nutritional quality of target host tissues may reduce insect performance, thereby improving plant resistance (Mattson 1980).

Reallocation of biomass and nutrients in response to herbivory might be a particularly relevant tolerance strategy in woody plants because their longevity and large size make them more exposed to insect herbivory (Haukioja & Koricheva 2001), and they may have a greater potential for storage of biomass and nutrients (Stevens, Kruger & Lindroth 2008). Indeed, significant shifts in biomass and nutrient partitioning after real or simulated herbivory have been reported in several angiosperm trees (Babst *et al.* 2005, 2008; Stevens, Waller & Lindroth 2007; Frost & Hunter 2008; Stevens, Kruger & Lindroth 2008; Eyles, Pinkard & Mohammed 2009). Surprisingly, despite their ecological and economic importance, very little information is available in the case of conifers (Ayres *et al.* 2004). Conifers may substantially differ from angiosperm trees in their responses due to different anatomy, life-history traits and ecophysiology (e.g. Hoch, Richter & Körner 2003).

Although it is widely accepted that the effect of herbivore damage on future plant fitness depends on resource availability (Hawkes & Sullivan 2001) and that we know that resistance responses are genetically variable and environmentally dependent (Ballhorn *et al.* 2011; Sampedro, Moreira & Zas 2011), little is known about the genetic variation of the tolerance-related plastic responses to herbivory within populations and how they are linked to resource availability (Heil 2010; Fornoni 2011). Unravelling these questions is essential to fully understand the ecological and adaptive relevance of induced tolerance and its integration with other herbivore-induced plant responses (Núñez-Farfán, Fornoni & Valverde 2007; Fornoni 2011).

The aim of this article was to study the inducibility, additive genetic variation and plasticity to nutrient availability of biomass partitioning and nutrient reallocation: two traits putatively related to tolerance and potentially responsive to herbivory damage in *Pinus pinaster* juveniles. We mimicked above-ground herbivory with the application of methyl jasmonate (MJ), a plant hormone central to the responses elicited by wounding damage (e.g. Heidel & Baldwin 2004), thus avoiding the side effects of using experimental tissue removal. We manipulated growth of pine seedlings through P availability, as this nutrient is considered to be the main limiting factor for pine growth in our study area (Martíns *et al.* 2009 and references therein). Phosphorus availability increased the incidence of insect herbivory in this pine population (Zas *et al.* 2006; Sampedro *et al.* 2009), which shows significant genetic variation in tolerance (Zas, Moreira & Sampedro 2011). In a previous article with the same plant material, we found that P availability determined the allocation to constitutive chemical defences and their inducibility (Sampedro, Moreira & Zas 2011). Here, we extend that study to determine whether induced responses in pines might include changes in traits putatively related to tolerance to herbivore damage. We hypothesize that pine responses to above-ground herbivory may include major plastic responses other than the induction of chemical defences, as moving nutrient and carbon resources to below-ground compartments. We suggest that, as previously observed for inducibility of chemical defences, these responses could be genetically variable and modulated by soil P availability.

Materials and methods

EXPERIMENTAL DESIGN

We carried out a greenhouse experiment following a randomized split–split design replicated in four blocks, with P availability (two levels: complete fertilization and P-deficient fertilization) as the whole factor, MJ induction of defensive responses (two levels: MJ treatment and control) as the split factor and 33 genetic entries (open-pollinated half-sib families, known mother trees) as the split–split factor. In total, there were 528 pine juveniles, corresponding to four blocks × two P availabilities × two MJ treatments × 33 genetic entries.

PLANT MATERIAL, GREENHOUSE CONDITIONS, FERTILIZATION AND MJ INDUCTION

Pinus pinaster half-sib families were randomly selected from a broader experimental collection of mother trees representative from the Atlantic coast population of Galicia (NW Spain). The climate in this area is temperate humid Atlantic, with annual precipitation of about 1500 mm and mean annual temperatures of 11 °C, typically ranging between 25 °C (maximum daily mean) and 4 °C (minimum daily mean). Soils in this region are typically thin, sandy and acidic, with high organic matter content, high total nitrogen content and very low concentration of available P, although soil fertility has a marked spatial heterogeneity. *Pinus pinaster* is a fast-growing sun-demanding pioneer pine species native from western Mediterranean basin that occupies large extensions in this region. Early growth of this species is critical for future fitness, and insect herbivory is a major cause of pine seedling mortality (see Appendix S1 in Sampedro, Moreira & Zas 2011).

\Seeds from those mother trees were individually weighed and sown on 7 February 2006 in sterilized 2-L pots containing sterilized perlite and cultured in an isolated glass greenhouse with controlled light (minimum 12 h per day) and temperature (10 °C

night, 25 °C day), and daily watering by subirrigation. To avoid interference from soil microbes such as pathogens and mycorrhizal colonization, seeds were preventively treated with a fungicide (Fernide®, Syngenta Agro, Spain) that was also applied every 2 months during pine growth. No mycorrhizal colonization was detected in the whole experiment.

One month after sowing, we began applying the fertilizer treatments (complete and P-limited fertilizer) by subirrigation every 2 days. The complete fertilizer was a balanced solution containing 100 : 20 : 70 : 7 : 9 mg L^{-1} of N : P : K : Ca : Mg, respectively and the necessary amounts of micronutrients and trace elements (see detailed chemical composition in Table S1). This solution was a modification of that used by local nurseries for optimum seedling growth of this pine species. The P-limited fertilizer solution contained the recommended levels of N, K, Ca and Mg, as described previously, but the availability of P was reduced 10-fold to 2 mg P L^{-1} (Table S1). In previous trials, we found that this P-deficient treatment led to P concentration in plant tissues similar to the lowest values observed in the field (Martíns *et al.* 2009). Fertilizer solutions were freshly prepared every 2 weeks, and pH was adjusted to pH 6.5 in both treatments.

On 2 August 2006, when average plant heights in P-deficient and complete fertilization treatments were 21.9 ± 0.7 and 44.3 ± 1.3 cm, respectively, half of the plants were treated with a solution of 22 mmol L^{-1} MJ (#39270-7; Sigma-Aldrich, St Louis, MO, USA) suspended in deionized water with ethanol 2.5% (v:v). The rest of the plants were treated only with the carrier solution (2.5% ethanol) and acted as control. Treatments were sprayed evenly over the foliage to run off with a handheld sprayer. Each individual plant, weighed before and after treatment application, received 2.6 ± 0.2 or 3.7 ± 0.3 mL of solution (P-deficient and complete fertilization plants, respectively; mean $\pm$ SE). MJ dose and concentration were previously determined by means of some trials performed with the same plant material and according to previous research (Moreira, Sampedro & Zas 2009). To avoid cross-contamination, the two treatments were applied in two different rooms, and juveniles remained in separate rooms for 24 h to allow drying.

SAMPLING AND MEASUREMENTS

Two weeks after MJ application, we measured plant height and stem-basal diameter, and all pine juveniles were harvested for biomass determination and further chemical analyses. Plants were carefully cleaned of foreign matter and perlite, separated into coarse and fine roots (these latter defined as those of diameter < 2 mm), shoots, adult needles (secondary needles bundled in dwarf shoots) and juvenile (primary) needles. Plant material was then oven-dried for 72 h at 65 °C to constant weight, weighed to the nearest 0.001 g, grounded with liquid nitrogen and stored for further nutrient analyses. Total biomass was determined as the sum of root, shoot, and adult and juvenile needle biomass. The total number of adult and juvenile needles was also counted. The density of needles in the main stem was calculated as the number of total needles (adult plus juvenile) per cm of the main-stem length.

NUTRIENT ANALYSES

To reduce the analytical effort to reasonable levels, nutrient concentration was analysed in a subsample of 11 randomly selected pine families. Sample size for nutrient concentrations was thus $N = 176$ plants. For chemical analyses, 0.3 g of grounded plant material (juvenile needles, adult needles, shoots or coarse roots) was digested in a mixture of selenous sulphuric acid and hydrogen peroxide (Martíns

et al. 2009). Nitrogen was determined colorimetrically in diluted aliquots of this digestion using a BioRad 680 microplate reader (Hercules, CA, USA) at 650 nm (Sims *et al.* 1995). Phosphorus was analysed in the same diluted aliquots by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Perkin-Elmer Optima 4300DV (Shelton, MA, USA) at the central laboratory facilities of Universidade de Vigo – CACTI (http://webs.uvigo.es/cactiweb/). Pine needle standards (NIST#1575) were used for checking the correct quantification. Nitrogen and P concentrations were expressed in milligram per gram tissue on a dry weight basis.

STATISTICAL ANALYSES

Analyses were carried out with the proper mixed model to solve split–split designs (Littell *et al.* 2006) using the Proc Mixed procedure of SAS System. The main effects of P availability (P), MJ induction (MJ) and the P × MJ interaction were treated as fixed factors. The Block (B) effect, B × P and B × P × MJ interactions were considered random to analyse the main factors P and MJ with the appropriate error terms (B × P and B × P × MJ, respectively). Family (G) and its interactions with main effects (P, MJ and P × MJ) were considered random, and associated variance components were estimated by restricted maximum likelihood. The statistical significance of the variance components for each random factor was assessed using likelihood ratio tests, where the differences in two times the log-likelihood of the models including and excluding that random factor are distributed as one-tailed chi-square, with one degree of freedom (Littell *et al.* 2006). Residuals of seed weight within families were used as a covariate for analysing the variables of growth to remove this relevant maternal effect affecting early pine performance and thus reduce the error term. When needed, normality was achieved by log-transforming the original variables. Equality of residual variance across MJ and P treatments was tested in all cases, and residual heterogeneity variance models were used when significant deviations were found (Littell *et al.* 2006).

Results

PLANT GROWTH AND BIOMASS ALLOCATION

Application of MJ significantly reduced the growth of pine juveniles after just 15 days, where total height, basal diameter and total biomass of MJ-induced plants (37.2 ± 0.6 cm; 4.3 ± 0.1 mm; 24.1 ± 1.1 g) were significantly smaller than control plants (44.9 ± 0.6 cm; 4.6 ± 0.1 mm; 29.7 ± 1.1 g; $P < 0.05$ in all cases). Application of MJ stopped or reduced shoot and adult needle growth (Table 1, Fig. 1) and also the development of new adult needles ($F_{1,6} = 8.4$; $P = 0.028$). All these variables were affected by soil P availability and largely varied among pine families (Table 1). We only observed a significant effect of P × MJ interaction in the biomass of adult needles (Table 1), which was originated by a larger reduction in the adult needle biomass under well-fertilized conditions than in P-deficient plants (Fig. 1). All these changes led to significant and relevant changes in the plant morphology above ground after 15 days. Application of MJ significantly reduced the height/diameter ratio ($F_{1,6} = 29.7$; $P = 0.002$), because it reduced primary growth rate (height) more than secondary growth rate (diameter). Application of MJ also promoted a more packed distribution of needles in the main stem (37 ± 1.9

Table 1. Summary of the mixed model for biomass of adult needles, juvenile needles, shoots and coarse and fine roots of 33 *Pinus pinaster* open-pollinated families under P-deficient and complete fertilization treatments in constitutive (control) and MJ-induced conditions 15 days after MJ application. The family effect (F) and derived interactions are random effects, and the corresponding likelihood ratio significance tests (χ^2) are shown. Phosphorus availability (P) and methyl jasmonate (MJ) induction of defences are fixed effects, and the *F* values and corresponding d.f. are shown

	d.f. (*F*)	Adult needles		Juvenile needles		Shoot		Coarse roots		Fine roots	
		F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value
Family (F)		51.2	**0.000**	18.0	**0.000**	27.6	**0.000**	54.9	**0.000**	33.3	**0.000**
F × P		4.6	**0.016**	0.7	0.201	1.0	0.159	2.0	0.079	0.4	0.263
F × MJ		0.0		0.5	0.240	1.0	0.159	2.0	0.079	0.0	
F × P × MJ		0.2	0.327	0.5	0.240	0.0		0.0		0.0	
Block	3,3	5.3	0.103	6.9	0.074	1.9	0.310	2.8	0.213	5.3	0.101
P	1,3	97.2	**0.002**	189.2	**0.001**	55.7	**0.005**	38.9	**0.008**	59.2	**0.005**
MJ	1,6	45.7	**0.001**	0.3	0.622	7.2	**0.037**	72.6	**0.000**	284.6	**0.000**
P × MJ	1,6	9.8	**0.020**	4.0	0.091	1.8	0.234	1.7	0.241	2.3	0.178
SWr	1,383	46.5	**0.000**	17.3	**0.001**	44.2	**0.000**	22.8	**0.000**	54.3	**0.000**

Significant *P* values ($P < 0.05$) are in boldface.

and 45 ± 2 needles cm^{-1} in control and treated plants, respectively; $F_{1,6} = 25.8$; $P = 0.002$) because MJ stopped the apical growth but not the development of new needles in the main stem.

Application of MJ also modified allocation of biomass to roots, strongly reducing the growth of coarse roots but promoting a marked boost in fine root biomass (Table 1, Fig. 1). Biomass of fine roots in the MJ-treated plants was almost 90% and 40% (in low and high P availability, respectively) greater than those in the control plants. These changes led to altered plant morphology below ground just 15 days after MJ application (Supporting Information Fig. S1). Simulated herbivory increased the root/shoot ratio, with a significant effect of P × MJ interaction, because the increase was more marked when P availability was low (Fig. 2a). Application of MJ also drastically increased the fine-to-coarse root ratio (Fig. 2b), irrespective of the P availability. We found significant differences among pine families for fine-to-coarse root ratio, but did not for root/shoot ratio (Fig. 2). Above- and below-ground allocation responses were similar in all families as revealed by the non-significant MJ × F interaction (Fig. 2).

PHOSPHORUS AND NITROGEN CONCENTRATION IN PLANT TISSUES

Manipulation of P availability significantly affected P concentration in adult and juvenile needles, shoots and roots of *P. pinaster* juveniles (Table 2). Phosphorus concentration in all plant compartments was significantly greater in plants growing under complete fertilization (Fig. 3). Simulated herbivory with MJ significantly affected P concentration in shoots and roots of *P. pinaster* juveniles 15 days after MJ application (Table 2), increasing P concentration in shoots and reducing it in roots (Fig. 3). The effect of simulated herbivory on P concentration in shoots was much greater under complete P nutrition than under P-deficient conditions, and in the case of roots, it was only significant in plants growing under complete P

nutrition (significant P × MJ interaction in both cases, Fig. 3). MJ did not affect P concentration of adult and juvenile needles (Table 2). Differences in P concentration among pine families were observed in needles and roots, but not in shoots (Table 2). Pine families did not differ in their response to MJ treatment for P concentration in shoots and roots (not significant F × MJ interaction, Table 2).

Manipulation of P availability significantly affected N concentration in roots and needles and also marginally in shoots; however, the effect was small with only slightly greater N concentration in plants growing under complete P fertilization (Table 3, Fig. 4). Concentration of N in the shoots, but not in the other compartments, was significantly affected by MJ signalling (Table 3). Induced plants showed 25% greater N concentration in their shoots than control plants (Fig. 4). The P × MJ interaction was significant for N concentration in adult needles and roots and marginally significant for shoots (Table 3). Application of MJ reduced the N concentration in adult needles of P-deficient plants, whereas no significant effect was observed in complete fertilized plants (Fig. 4). On the other hand, MJ reduced N concentration in roots of plants growing in complete fertilization and increased it in plants growing in P-deficient conditions (Fig. 4). Differences among pine families in N concentration were observed in all plant compartments (Table 3). However, pine families did not differ in their response to MJ treatment, as revealed by the non-significant Family × MJ interaction (Table 3).

When analysing the absolute nutrient content in each plant tissue (i.e. the corresponding nutrient concentration × biomass), we found that the content of both N and P in the shoots was significantly greater in MJ-treated plants than in control plants ($F_{1,6} = 27.7$, $P = 0.002$ for P content and $F_{1,6} = 8.4$, $P = 0.028$ for N content). This indicates that the observed changes in the nutrient concentration in the shoots of MJ-treated plants were not side effects derived from differential above-ground growth rates after MJ application.
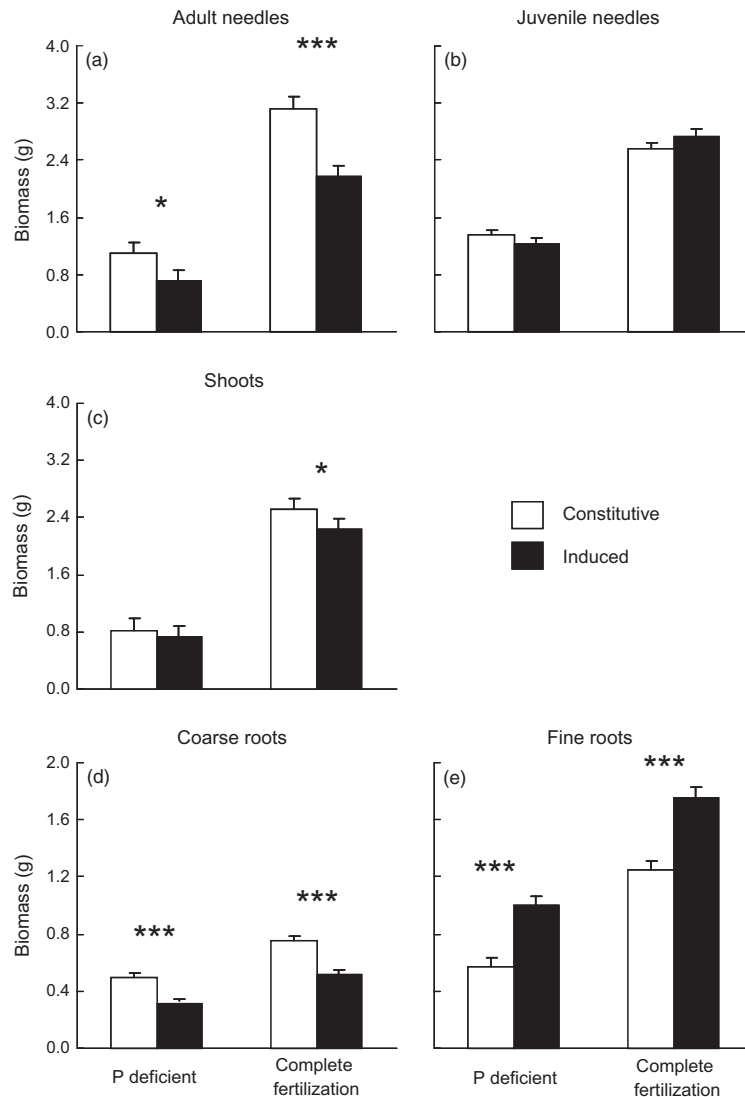
Fig. 1. Biomass of (a) adult needles, (b) juvenile needles, (c) shoot, (d) coarse and (e) fine roots of methyl jasmonate (MJ)-induced (black bars) and control (constitutive, white bars) *Pinus pinaster* juveniles belonging to 33 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a P-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are means $\pm$ SEM ($N = 132$). Results of the mixed model are presented in Table 1. Asterisks indicate significant differences within each fertilization treatment due to simulated herbivory at $*P < 0.05$ and $***P < 0.001$.

Discussion

INDUCED ALLOCATION OF BIOMASS TO FINE ROOTS

Simulated herbivory through MJ signalling reduced growth of above-ground tissues (adult needles and shoots) and also of coarse roots, but induced a strong boosting of the fine root system. Specifically, fine root biomass of MJ-induced plants increased nearly two-fold in 2 weeks, resulting in a three-fold greater fine-to-coarse root ratio. The reduction observed in the starch pool after MJ application (Sampedro, Moreira & Zas 2011) was very small in comparison to the huge increase in fine root biomass, suggesting that current photosynthates were likely a major source for the new fine root biomass, in accordance with recent observations using short-lived radioisotopes (Hanik *et al.* 2010).

We found that above-ground herbivore signalling induced allocation of biomass to below-ground tissues, not to storage tissues (we observed induced reduction in biomass in coarse roots), but to fine roots. Fine roots are sinks in terms of carbon balance, and allocation of carbon resources to those tissues cannot be further reallocated to other functions. Thus, our results do not support the hypothesis of induced sequestration of carbon resources in young pines (Orians, Thorn & Gómez 2011). Alternatively, it seems that young pines show a preferential investment in absorptive tissues. Juvenile plants of fast-growing colonizer tree species, for which a robust growth potential is vital for future fitness (Haukioja & Koricheva 2001), could obtain long-term benefits from this kind of induced tolerance strategy. Induced allocation of carbon resources to fine roots could directly improve the ability of plants for water and nutrient acquisition that will be essential
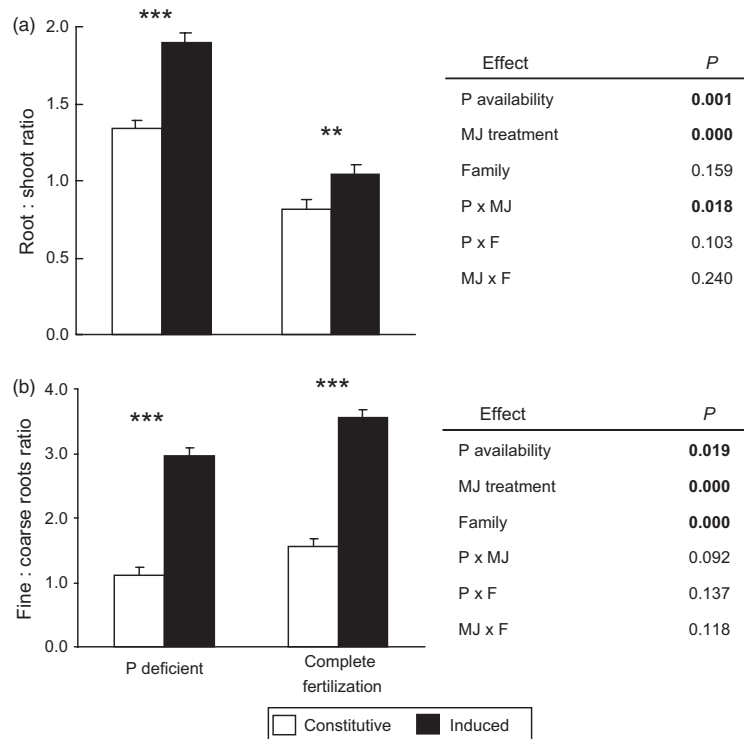
Fig. 2. (a) Root/shoot biomass ratio and (b) fine root/coarse root biomass ratio of methyl jasmonate (MJ)-induced (black bars) and control (constitutive, white bars) *Pinus pinaster* juveniles belonging to 33 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a P-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are means ± SEM (N = 132). P values in the tables indicate the results of the mixed models. Significant P values (P < 0.05) are in boldface. Asterisks indicate significant differences within each fertilization treatment due to simulated herbivory at **P < 0.01 and ***P < 0.001.

Table 2. Summary of the mixed model for P concentration in adult needles, juvenile needles, shoots and roots of 11 *Pinus pinaster* open-pollinated families under P-deficient and complete fertilization treatments in constitutive and MJ-induced conditions 15 days after MJ application. The family effect (F) and derived interactions are random effects, and the corresponding likelihood ratio significance tests (χ^2) are shown. Phosphorus availability (P) and methyl jasmonate (MJ) induction of defences are fixed effects, and the F values and corresponding d.f. are shown

| | | P concentration | | | | | | | |
| | | Adult needles | | Juvenile needles | | Shoots | | Roots | |
	d.f. (F)	F/χ^2	P value	F/χ^2	P value	F/χ^2	P value	F/χ^2	P value
Family (F)		0.1	0.376	7.9	**0.002**	1.3	0.127	5.9	**0.008**
F × P		1.7	0.096	7.2	**0.004**	0		6.4	**0.006**
F × MJ		0.0		0.0		1.0	0.159	3.0	**0.042**
F × P × MJ		1.5	0.110	0.0		0.3	0.292	4.9	**0.013**
Block	3,3	12.5	**0.034**	4.7	0.119	3.2	0.185	2.9	0.204
P	1,3	355.9	**0.000**	394.7	**0.000**	456.5	**0.000**	2673.0	**0.000**
MJ	1,6	2.0	0.209	4.2	0.087	83.5	**0.000**	16.0	**0.007**
P × MJ	1,6	4.9	0.069	1.4	0.286	39.4	**0.001**	16.8	**0.006**

Significant P values (P < 0.05) are in boldface.

for above-ground regrowth (Ayres *et al.* 2004; Erb *et al.* 2009), but also indirectly through facilitation of mycorrhizal colonization (Regvar, Gogala & Žnidaršič 1997) and/or increased mineralization in the rhizhosphere.

Although preferential induced allocation of biomass to below-ground structures has been reported in annual plants

(Schwachtje *et al.* 2006; Henkes *et al.* 2008; Gómez *et al.* 2010) and woody plants (Babst *et al.* 2005, 2008), this type of response is not ubiquitous. In the case of woody plants, the opposite, that is defoliation-induced shifts in biomass partitioning from fine and coarse roots to above-ground tissues, has also been reported in *Eucalyptus* (Eyles, Pinkard &
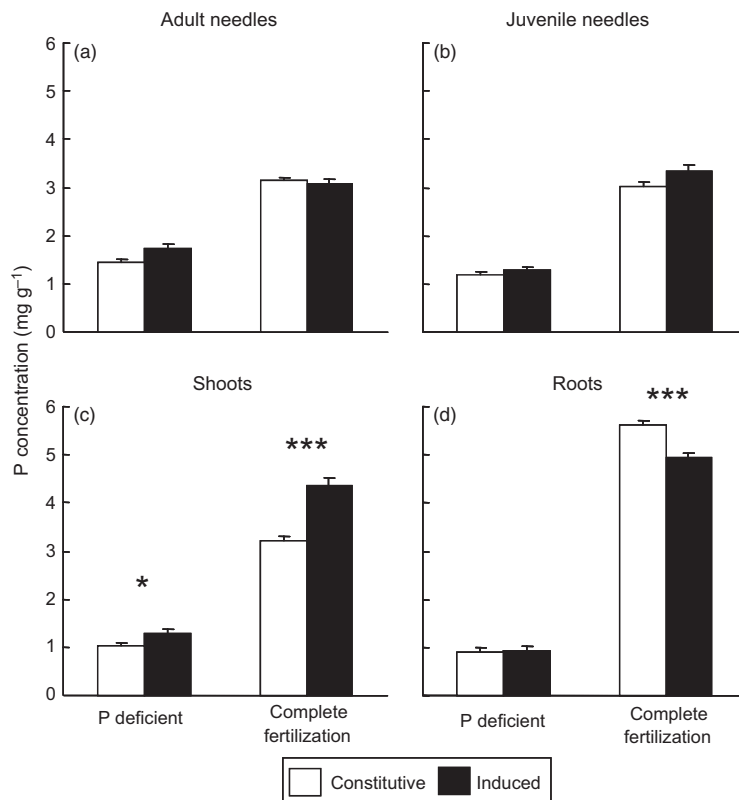
Fig. 3. Phosphorus concentration in (a) adult needles, (b) juvenile needles, (c) shoot and (d) roots of methyl jasmonate (MJ)-induced (black bars) and control (constitutive, white bars) *Pinus pinaster* juveniles belonging to 11 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a P-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are means ± SEM ($N = 44$). Results of the mixed model are presented in Table 2. Asterisks indicate significant differences within each fertilization treatment due to simulated herbivory *$P < 0.05$ and ***$P < 0.001$.

Table 3. Summary of the mixed model for N concentration in adult needles, juvenile needles, shoots and roots of 11 *Pinus pinaster* open-pollinated families under P-deficient and complete fertilization treatments in constitutive and MJ-induced conditions 15 days after MJ application. The family effect (F) and derived interactions are random effects, and the corresponding likelihood ratio significance tests (χ^2) are shown. Phosphorus availability (P) and methyl jasmonate (MJ) induction of defences are fixed effects, and the F values and corresponding d.f. are shown

		N concentration							
		Adult needles		Juvenile needles		Shoots		Roots	
	d.f. (F)	F/χ^2	P value	F/χ^2	P value	F/χ^2	P value	F/χ^2	P value
Family (F)		17.2	**0.000**	11.7	**0.000**	4.9	**0.013**	10.4	**0.001**
F × P		0.1	0.376	0.3	0.292	0.8	0.186	0.9	0.171
F × MJ		0		0		0.4	0.264	0.8	0.186
F × P × MJ		0		0		0.1	0.376	0	
Block	3,3	9.0	0.052	0.9	0.518	0.6	0.644	1.0	0.479
P	1,3	42.0	**0.007**	22.6	**0.018**	7.0	0.077	55.5	**0.005**
MJ	1,6	2.6	0.158	0.4	0.536	153.2	**0.000**	1.2	0.323
P × MJ	1,6	6.2	**0.046**	0.1	0.960	3.9	0.094	26.2	**0.002**

Significant P values ($P < 0.05$) are in boldface.

Mohammed 2009), *Quercus* (Frost & Hunter 2008) and *Populus* (Stevens, Kruger & Lindroth 2008). Discrepancies between studies could be explained in terms of different response dynamics, variation in the time elapsed since damage (Eyles, Pinkard & Mohammed 2009; Metlen, Aschehoug & Callaway 2009; Gomez, van Dijk & Stuefer 2010) and damage severity
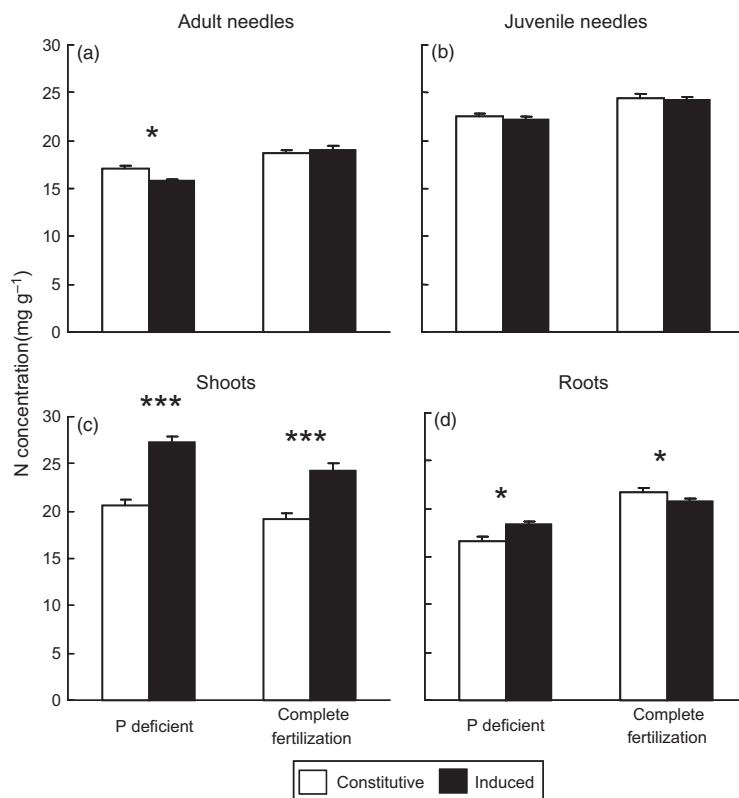
Fig. 4. Nitrogen concentration in (a) adult needles, (b) juvenile needles, (c) shoot and (d) roots of methyl jasmonate (MJ)-induced (black bars) and control (constitutive, white bars) *Pinus pinaster* juveniles belonging to 11 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a P-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are means ± SEM ($N = 44$). Results of the mixed model are presented in Table 3. Asterisks indicate significant differences due to simulated herbivory within tissues at *$P < 0.05$ and ***$P < 0.001$.

(Frost & Hunter 2008). But intrinsic differences among taxa, such as carbon storage patterns (e.g. Hoch, Richter & Körner 2003), and even differences in terms of life history within related species could explain different strategies.

INDUCED ALLOCATION OF N AND P TO SHOOTS

Above-ground simulated herbivory in young pines rapidly increased the concentration of N and P in the shoots while maintaining (N) or reducing (P) concentrations in the roots. The lack of an effect on nutrient concentration of juvenile and adult needles and absence of major changes in the whole plant pool of nutrients suggest an induced mobilization of mineral resources from roots to shoots. Again, these results disagree with the trend of hiding nitrogen away from herbivores commonly reported in herbs and annual plants (Newingham, Callaway & BassiriRad 2007; Gómez *et al.* 2010). Our results also contrast with those observed in oak seedlings, beech and fir saplings in which above-ground herbivory or leaf clipping stimulated the storage of N into roots (Ayres *et al.* 2004; Frost & Hunter 2008). Alternatively, increasing nutrient concentration around damaged tissues may be important to boost the *de novo* synthesis of induced resistance mechanism (Babst *et al.* 2005; Gómez *et al.* 2010). Accordingly, we found stem diterpenes increased 15–20% after MJ induction in these plants

(Sampedro, Moreira & Zas 2011). Nitrogen, in particular, would be required for the production of large amounts of terpenoid synthases and for the differentiation of new xylem resin canals, while phosphate is required for the intense phosphorylation processes leading to the biosynthesis of terpenoids induced by MJ signalling in conifer tissues (e.g. Miller *et al.* 2005). We would suggest that induced nutrient sequestration could be not an effective strategy when N and P are required for massive *in situ* synthesis of defensive chemical defences, as in the case of the stem of pine trees.

On the other hand, increasing the concentration of induced defences in the stem could help to alleviate the negative side effects caused by moving nutrients to stems after above-ground herbivory. Increased insect performance may be expected when feeding on nitrogen-rich tissues, which is likely to lead to greater susceptibility (Mattson 1980; Ayres *et al.* 2000). In the case of young pines, the greater nitrogen concentration in target tissues could be shielded or counterbalanced by the simultaneous increase in stem oleoresin and phenolics observed in those plants (Sampedro, Moreira & Zas 2011), leading to similar or even poor tissue quality for herbivores.

Taken together, our results provide little support for induced resource sequestration as generally favoured strategy in this species. Although it seems accepted that re-allocation of

nutrients and carbon resources can be a common herbivore-induced plant response, it is difficult to identify a general pattern in the direction that resources could be moved across plant taxa (Orians, Thorn & Gómez 2011). Further research should address whether the pattern of moving carbon resources to absorptive tissues and nutrients to the stem observed in *P. pinaster* is a common response within the *Pinus* phylogeny, according to their specific anatomical and evolutionary constraints. Alternatively, we could speculate that this pattern could depend on the particular life histories of the species considered. In a recent meta-analysis, Endara & Coley (2011) found that species adapted to resource-poor environments grow inherently more slowly, investing more in constitutive defences and supporting lower herbivory than species from productive habitats. Thus, habitat quality affiliation and subsequent intrinsic growth rates could shape the strategies of defensive investment, and likely those of tolerance responses too (Coley 1987; Agrawal 2011; Endara & Coley 2011). The latter could explain the differences between our results (in a fast-growing colonizer pine) and those reported for other long-lived species, such as those for oak seedlings (Frost & Hunter 2008), which likely have a slow-growing/low-resource availability strategy.

GENETIC VARIATION AND ENVIRONMENTAL MODULATION OF BIOMASS AND NUTRIENT REALLOCATION

Biomass of fine roots and P and N concentrations showed significant overall additive genetic variation and, although inducible, did show no significant genetic variation in the inducibility, that is, responses were consistent among families. Contrastingly, we found genetic variation in inducibility of stem oleoresin and needle total phenolics and tannins in these same plants (Sampedro, Moreira & Zas 2011). These results are not striking, as tolerance traits are expected to be less variable within a population than resistance traits (Roy & Kirchner 2000). Rising herbivore incidence is expected when a host population is more tolerant, when greater would be the fitness advantage of being a tolerant genotype. The opposite is predicted, however, for resistance (Roy & Kirchner 2000).

Our results provide evidence that changes in tolerance-related traits are inducible by herbivore cues, as induced resistance, and are likely to be part of the integrated plant responses to herbivory in this species. Although resistance and tolerance have sometimes been thought of as alternative strategies (e.g. Van der Meijden, Wijn & Verkaar 1988; Fineblum & Rausher 1995), they could be two complementary adaptive responses to herbivory (e.g. Agrawal, Strauss & Stout 1999; Stevens, Waller & Lindroth 2007). We found that MJ signalling is implicated in both the induction of chemical defences (Sampedro, Moreira & Zas 2011) and in changes in putative traits involved with tolerance responses (the present article). By joining together databases of family means, we have explored possible genetic correlations among inducibility (defined as the difference between the family mean in MJ-induced plants and the family mean in control plants, for a given family) of traits of quantitative resistance (resin and total phenolics) and the inducibility of putative tolerance traits (fine root biomass and nutrient reallocation). We only found a significant negative genetic correlation ($r = -0.61$; $P < 0.001$; $N = 33$) between the inducibility of phenolics and the increase in fine root biomass, but only under P-limited conditions. In other words, under scarce P availability and reduced growth rates, pine families that showed greater induction of phenolic compounds after MJ-simulated herbivory were those with the smaller induced allocation of biomass to fine roots. All the other family correlations were not significant ($P > 0.1$). This provides evidence for the existence of a context-dependent conflict where synthesis of phenolic compounds, but not resin defences, is trading off with fine-root-induced biomass allocation. This result is consistent with previous observations that vegetative costs (in terms of reduced total plant biomass, height and diameter) of induced defences were found for leaf phenolics (Sampedro, Moreira & Zas 2011) but not for resin-based defences. This result also suggests that a greater proportion of the vegetative costs of herbivore-induced responses arise from the construction costs of induced tolerance traits, rather than the synthesis of induced chemical defences. Further research should address this question.

Patterns of environmental modulation on biomass reallocation markedly differed from those observed for nutrients. The increase in fine root biomass after MJ signalling was unaffected by the actual P availability. However, the induced allocation of P from roots to shoots was greater under complete fertilization than in P-impoverished conditions (significant MJ × P interaction), and we also found an interactive effect of P availability on N reallocation. Thus, although no genetic variation in nutrient reallocation responses was found, the strong environmental modulation of those responses, together with the high spatial heterogeneity in P availability in the study area (Martíns *et al.* 2009), could lead to large phenotypic variation across spatial scales in these mechanisms.

Conclusions

In response to above-ground MJ signalling, juveniles of this pine species (i) prioritized the allocation of biomass to below-ground absorptive structures, leading to a strong boosting of the fine root system, while reducing growth of coarse roots and above-ground structures and (ii) increased the allocation of N and P to the shoots, likely diverting these nutrients from the roots. These mechanisms inducible by herbivory signalling are putatively related to tolerance to herbivory and are a new result for pine trees. Biomass of fine roots and P and N concentrations in plant tissues showed significant overall additive genetic variation and, although inducible, did not show significant genetic variation in the inducibility, that is, responses were consistent among families. Boosting of fine roots appeared to be a generalized strategy with weak environmental modulation, whereas induced shifts in N and P to the shoots were

strongly affected by P availability. Thus, spatial variation in P availability at field could contribute to phenotypic variation in induced reallocation of nutrients. Our results indicate that induced resource sequestration is not likely a generalized herbivore-induced response in young pine trees, and evidence that herbivore-induced changes in tolerance-related traits are part of the integrated plant responses to herbivory in this species.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Chemical composition of the solutions used in both fertilization treatments. In both solutions pH was adjusted to 6.5 with diluted HCl.

Figure S1. Example picture of the root systems of *P. pinaster* juveniles in control (a) and MJ-induced (22 mmol L^{-1} MJ application to the foliage) (b) 20 days after treatment application. Please note the differences in the structure of the root system after foliar MJ application.

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