Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability

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Summary

1. Production of antiherbivore chemical defences is generally assumed to be costly in terms of fitness, although some studies have failed to detect such costs. A convincing explanation is that the expression of fitness costs depends on environmental conditions such as nutrient availability.

2. We performed a greenhouse experiment with 33 half-sib families in order to study the phenotypic plasticity of constitutive and methyl jasmonate-induced chemical defences to soil phosphorus (P) availability, the existence of genetic trade-offs (costs) between growth and the production of those defences and the extent to which P availability may modulate the expression of those costs.

3. We measured some proxies of vegetative fitness (primary growth, secondary growth and total biomass), plant reserves (soluble sugars and starch) and the concentration of quantitative chemical defences (diterpene content in the stem, total polyphenolics and condensed tannins in the needles).

4. Phosphorus availability had a considerable effect, both on the allocation of resources to constitutive and induced defences and on the expression of vegetative costs associated with those chemical defences. Constitutive investment in chemical defences was greater under P-limited conditions for all studied traits. Inducibility of foliar phenolic compounds was greater under P-limited conditions, and it was strongly constrained under high P availability. Availability of P did not affect the inducibility of stem diterpenes.

5. All defensive traits showed significant genetic variation, with different levels of genetic control in constitutive and induced modes, and genetic variation in their inducibility. We found significant negative genetic correlations (i.e. trade-offs) between growth and defensive investment, but costs of chemical defences emerged only in P-limited conditions. Vegetative costs of constitutive defences were detected for stem diterpenes but not for needle phenolics, while costs of induced defences were found for leaf phenolics but not for stem diterpenes.

6. *Synthesis.* Our results indicate that P availability controls the production of chemical defences in this pine species, influencing the resource allocation to constitutive defences, the inducibility of those defences and the emergence of related vegetative costs. Phosphorus availability thus appears as a major driver in the evolution of pine resistance to insects and a potential factor in maintaining genetic variation in defences.

Key-words: conifers, diterpenes, fitness costs, genetic variation, induced resistance, methyl jasmonate, phenolic compounds, phenotypic plasticity, phosphorus availability, plant–herbivore interactions

Introduction

Conifers include some of the tallest and longest-living trees in the world and they constitute apparent targets for a wide variety of pests and pathogens. To defend themselves, conifers produce and store a number of secondary metabolites that are present in large amounts in their tissues (Mumm & Hilker 2006). In particular, terpenoid oleoresin and phenolics are known to be effective quantitative defences, with higher concentrations commonly associated with increased direct resistance against a diverse array of insect herbivores such as

bark beetles (Franceschi, Krokene & Krekling 2005), defoliators (Mumm & Hilker 2006) or phloem feeders (e.g. Wainhouse *et al.* 2008).

The expression of plant defences is known to respond plastically to the biotic environment and new mechanisms and/or greater concentrations of chemical defences are quickly activated after herbivore damage to increase resistance (Eyles *et al.* 2010; Heil 2010). The main induced direct defences in conifers include the formation of traumatic resin canals in the xylem (e.g. Krokene, Nagy & Solheim 2008), changes in the composition of resin and increased resin flow (see review by Bohlmann 2008) and the accumulation of phenolic compounds (e.g. Franceschi, Krekling & Christiansen 2002).

Chemical defences are also plastic to abiotic environmental factors such as nutrient availability. Several models have been formulated to explain the patterns of phenotypic variation usually found in plant secondary chemistry and also the effect of environmental factors on the relative investment in primary and secondary metabolism within and among species (reviewed by Stamp 2003). Founded on the existence of within-plant physiological trade-offs, the growth–differentiation balance hypothesis (GDBH, as unified by Herms & Mattson 1992) assumes that chemical defences must, to some extent, come at a price in terms of a reduction in the growth rate because their synthesis diverts carbon from other plant functions. Since growth appears to be more sensitive to resource limitation than carbon fixation, GDBH predicts that moderate growth limitation imposed by external factors such as low nutrient availability will result in the accumulation of carbohydrates and, subsequently, in increased concentrations of constitutive carbon-based secondary compounds (Herms & Mattson 1992). From a more evolutionary point of view, other models such as the optimal defence theory (ODT, McKey 1974, 1979; Zangerl & Bazzaz 1992) also predict that plants growing in resource-limited environments should be constitutively well protected, since costs for replacing the tissues damaged by herbivores would be greater in nutrient-limited environments.

That reduced soil nutrient availability is associated with increased defensive mechanisms is well documented, but the response could vary depending on the defensive compound considered (e.g. Björkman *et al.* 1998) and the particular nutrient considered (Wright *et al.* 2010). In a convincing meta-analysis, Koricheva *et al.* (1998) found carbon-based secondary compounds to be strongly affected by N nutrition, but weakly affected or unaffected by P availability. These discrepancies may arise because of the different roles of N and P in primary and secondary cellular metabolism. For instance, the protein competition model (PCM, Jones & Hartley 1999) states that the synthesis of proteins and phenolic compounds are trading off because their biosynthetic pathways share the amino acid phenylalanine as a common resource. The PCM thus predicts that P limitation will have a smaller influence than N availability on the concentration of phenolic defensive compounds.

Like constitutive defences, induced defences can be also modulated by the environment and are assumed to be costly to produce (e.g. Van Dam & Baldwin 1998; Agrawal, Strauss & Stout 1999; Cipollini & Heil 2010). However, the environmental modulation of the relative investment in induced defences has been poorly studied, especially in woody plants. Based on the same arguments as those for constitutive defences, the GDBH proposes that induced defences may also be greater under low nutrient availability, although the response may be nonlinear (Herms & Mattson 1992). In one of the few published studies relating to pine trees, the inducible resin flow, however, was reported to be greatest when individual tree growth was greatest, i.e. when conditions were favourable (Lombardero *et al.* 2000).

Despite the important and prolonged directional selection imposed by herbivores in the evolution of resistance mechanisms, genetic variation in resistance traits remains widespread within the plant kingdom (Zangerl & Bazzaz 1992). The persistence of genetic variation in resistance traits has been explained in terms of the costs of chemical defences and the temporal and spatial heterogeneity in the balance of costs and benefits of resistance traits (Núñez-Farfán, Fornoni & Valverde 2007 and references therein). In particular, it is widely accepted that strategies based on induced defences are considered as cost-saving because their associated costs materialize only when functionally necessary (e.g. Baldwin 1998).

The existence of costs associated with the expression of constitutive defences has been well documented in several herbaceous species in recent decades (reviewed by Koricheva 2002) and more recently also in woody plants (e.g. Donaldson, Kruger & Lindroth 2006; Osier & Lindroth 2006). Although more difficult to study and detect, the existence of costs of induced defences has been also reported in the last 10 years (see reviews by Heil & Baldwin 2002; Cipollini, Purrington & Bergelson 2003; Walters & Heil 2007; Cipollini & Heil 2010). Several authors have also found that the emergence and the extent of the costs of induced defences depend on environmental conditions (Van Dam & Baldwin 1998, 2001; Dietrich, Ploss & Heil 2005; Cipollini 2010). Most of those studies, however, had been performed on annual and herbaceous plants, and thus information on the environmental modulation of costs of induced defences in long-lived woody plants, with life-history determinants greatly different to those of annual and herbaceous plants, is still scarce.

In this research, we studied the independent and interactive effects of plant genotype and P availability on constitutive and induced defences of juvenile Maritime pine (*Pinus pinaster* Ait). We tried to identify potential genetic trade-offs between growth and quantitative allocation to constitutive and induced defences and to determine whether phosphorus availability mediates the realized costs associated with chemical defences. We hypothesized that the concentration of chemical defences would be greater in conditions of P limitation, reduced plant growth and carbon excess. Moreover, P limitation could affect the inducibility of those defences and also determine the expression of underlying trade-offs between growth and defences. We performed a greenhouse experiment with 33 half-sib families, manipulating plant growth by controlling P availability (complete and P-deficient fertilization) and mimicking herbivore-induced responses using methyl jasmonate (MJ),

a phytohormone that elicits defensive responses similar to those induced by herbivore attacks in pine trees (Martin *et al.* 2002; Miller *et al.* 2005; Ralph *et al.* 2006). We measured carbon reserves in the stem, primary growth, secondary growth and total biomass as proxies of vegetative fitness, and three secondary metabolites (diterpene content in the stem and total polyphenolics and condensed tannins in the needles) as quantitative defensive traits. As in other regions, P is the main limiting resource for the studied Maritime pine population, where soil fertility shows a high spatial heterogeneity (Martíns *et al.* 2009). Early growth of this sun-demanding pioneer pine species is critical for future fitness, but early resistance to herbivory is also extremely important, because insects are a major cause of pine seedling mortality (see Appendix S1 in Supporting Information).

Material and methods

EXPERIMENTAL DESIGN

We carried out a controlled greenhouse experiment with pine genetic entries, P availability and induction of defences with MJ as the main factors. The experiment followed a randomized split–split design replicated in four blocks, with P availability (two levels: complete fertilization and P-limited fertilization) as the whole factor; MJ-induction of defensive responses (two levels: control and MJ-induced plants) 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 4 blocks × 2 P availabilities × 2 MJ treatments × 33 genetic entries.

PLANT MATERIAL, GREENHOUSE CONDITIONS, FERTILIZATION AND MJ-INDUCTION

Pinus pinaster half-sib families were randomly selected from a broader collection of mother trees belonging to the Atlantic coast population of Galicia (NW Spain). A description of climate, soil characteristics, genetic variation in resistance and other characteristics of the study area and pine population can be consulted in Appendix S1.

To avoid interference from soil microbes such as pathogens and mycorrhiza colonization, seeds were preventively treated with a fungicide (Fernide®; Syngenta Agro, Spain), sown in sterilized 2-L pots containing sterilized perlite in February 2006 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. Fungicide was also applied every 2 months during pine growth.

One month after sowing we began applying the fertilizer treatments (complete and P-limited fertilizer) by subirrigation every 2 days. The complete fertilizer (herein called P20) 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 Appendix S2, Tables S2A, S2B). 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 above, but the availability of P was reduced 10-fold to 2 mg P L^{-1} (treatment P2, Appendix S2). 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 height in P2 and P20 treatments were 21.9 ± 0.7 cm and 44.3 ± 1.3 cm, respectively, half of the plants were treated with a solution of 22 mM MJ (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 with a handheld sprayer, each plant receiving 2.6 ± 0.2 or 3.7 ± 0.3 mL of solution (P2 and P20 plants, respectively; mean ± SE). 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, plant height and stem basal diameter were measured and all pine juveniles were harvested, transported to the laboratory in ice coolers and immediately sampled for chemical analyses and total biomass determination. Roots of all plants were checked to ensure they were free of mycorrhizae. Immediately after harvesting, a fresh 10-cm-long piece of the lowest part of the stem of each plant was sampled, weighed, then frozen and preserved at −30 °C in cryogenic vials for diterpene analysis. A subsample of needles (*c.* 2 g) was also immediately weighed, then oven-dried (45 °C to constant weight) and subsequently manually ground in a mortar with liquid nitrogen for analyses of phenolic compounds. In 11 randomly selected pine half-sib families a subsample (*c.* 1 g) of stem and needles were taken and finely ground to determine starch and soluble sugars, and foliar N and P, respectively.

CHEMICAL ANALYSIS

Leaf phenolics were extracted and analysed as described by Moreira, Sampedro & Zas (2009). Using this method, phenolics were extracted from 300 mg of plant tissue with aqueous methanol (1:1 v:v) in an ultrasonic bath for 15 min, followed by centrifugation and subsequent dilution of the methanolic extract. Total phenolic content was determined colorimetrically by the Folin-Ciocalteu method in a Bio-rad 650 microplate reader (Bio-Rad Laboratories, Philadelphia, PA, USA) at 740 nm, using tannic acid as standard. Condensed tannins in the aqueous methanol extracts were determined by the procyanidine method as in Baraza *et al.* (2004). The methanolic extract was mixed with acidified butanol and a ferric ammonium sulphate solution, allowed to react in a boiling water bath for 50 min and then cooled rapidly on ice. The concentration of condensed tannins in this solution was determined colorimetrically with the same equipment at 550 nm, using as standard purified condensed tannins of quebracho (*Schinopsis balansae* Engl.; Droguería Moderna, Vigo, Spain).

Concentration of diterpenoid resin in the stem diterpenes was determined as previously described in Moreira, Sampedro & Zas (2009). Briefly, about 5 g fresh weight of stem material was transferred into pre-weighed test tubes, resin compounds were extracted with hexane in an ultrasonic bath, the extract was filtered (Whatman GF/D, Whatman Int. Ltd, Maidstone, Kent, UK) into new test tubes, and the whole extraction step repeated again. The solvent in the tubes was evaporated to dryness and the mass of the non-volatile resin residue was determined at the nearest 0.0001 g. This gravimetric determination of non-volatile resin was well correlated ($r = 0.9214$; $P = 0.00002$) with the concentration of the diterpenoid fraction as quantified by gas chromatography in previous trials (Sampedro, Moreira & Zas 2011).

The concentrations of soluble sugars and non-structural carbohydrate reserves (starch) in the stem were analysed by the anthrone

method (Hansen & Møller 1975). Soluble sugars were extracted from finely grounded stem with aqueous ethanol (80% v/v). Starch was extracted with 1.1% hydrochloric acid in a water bath at 100 °C for 30 min, followed by centrifugation and subsequent dilution of the extract. Soluble sugars and starch concentration were determined colorimetrically as above at 630 nm, using glucose and potato starch, respectively, as standards.

Total N in the needles was determined with a CN-2000 macro elemental analyser (LECO Corporation, St. Joseph, MI, USA) and total P in the needles by ICP-OES (Perkin-Elmer Optima 4300DV; Waltham, MA, USA) after wet digestion (Walinga, Van Der Lee & Houba 1995) at the central facilities of Universidade de Vigo, Spain (http://webs.uvigo.es/cactiweb/).

STATISTICAL ANALYSES

The effects of design factors were analysed using the PROC-MIXED procedure of the SAS System with the proper mixed model to solve a split–split design. Phosphorus treatment (P), MJ-induction, family (G), block (B), and the interactions between P, MJ and G were considered fixed factors. The $B \times P$ and $B \times P \times MJ$ interaction were considered random factors in order to analyse the main factors P and MJ with the appropriate error terms ($B \times P$ and $B \times P \times MJ$, respectively) (Littell *et al.* 2006). 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). Data are shown as means ± SE.

The correlation between pine growth and chemical defences in constitutive mode was examined across families and phosphorus treatments, in order to evaluate allocation costs to constitutive defences. To quantify the costs of allocation to induced responses in terms of the growth loss associated with the MJ-induced responses, we studied the family relationships within each P availability treatment between inducibility of phytochemical traits (diterpenes and phenolic compounds) and costs of MJ-induction in terms of growth (total height, basal diameter and total biomass). Inducibility of a given defensive chemical for the pine family f was defined as the difference $MJ_f - CTR_f$ between the family mean concentration in induced (MJ_f) and control (constitutive, CTR_f) plants. Similarly, vegetative fitness costs of inducibility for the pine family f were defined as the difference in height, diameter or biomass between induced and control plants ($MJ_f - CTR_f$). A trade-off is denoted by a significant negative family relationship between inducibility and vegetative costs (the greater the induction of defences, the greater the cost in terms of growth).

Results

PINE GROWTH AND RESERVES

Manipulation of phosphorus availability led to marked differences in pine growth (Fig. 1; Appendix S3 – Table S3a in Supporting information). Total height, basal stem diameter and total biomass of the juveniles that grew under the P-limited treatment were 40%, 20% and 60% lower, respectively, than those that received complete fertilizer. Pine families differed significantly in primary and secondary growth and biomass (Fig. 1). Total height, basal stem diameter and total biomass varied among pine families from 36.1 to 44.1 cm, from 4.0 to 4.9 mm and from 20.8 to 33.6 g, respectively. However, we
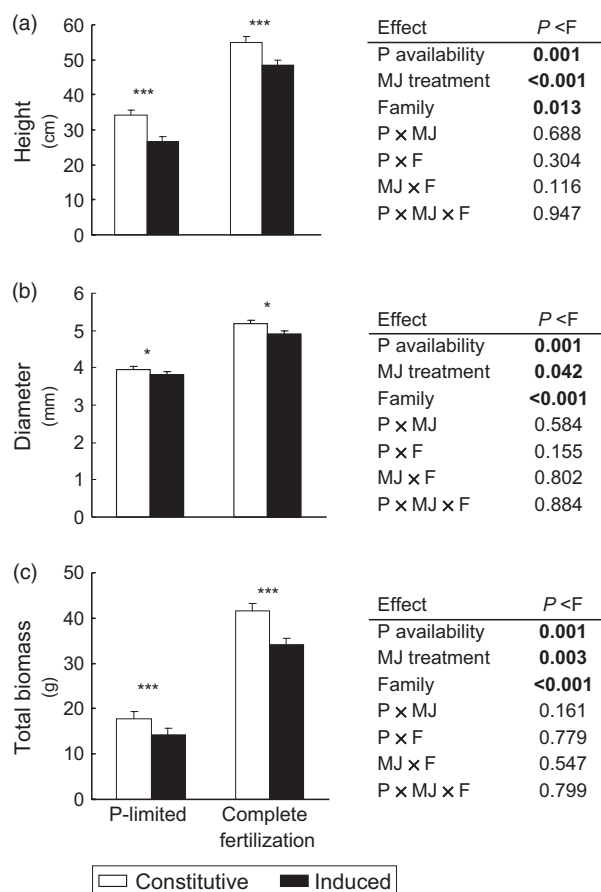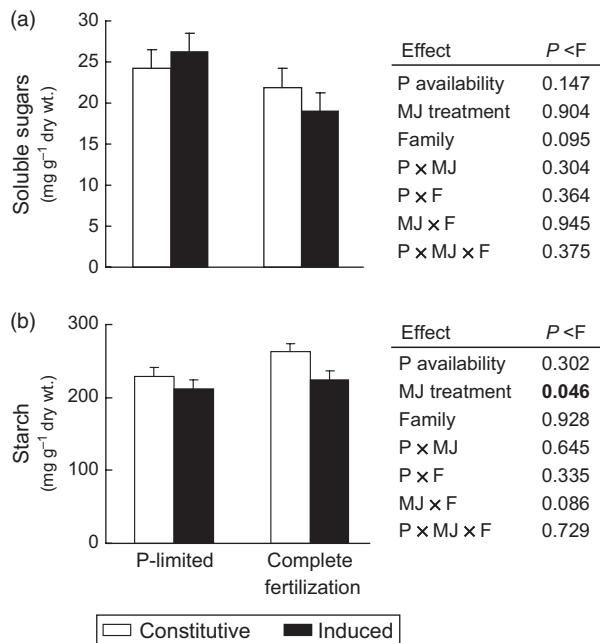

Fig. 1. Plant height (a), stem base diameter (b), and total biomass (c) of methyl jasmonate-induced (MJ) and control (constitutive) *Pinus pinaster* juveniles belonging to 33 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a phosphorus-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are LS means ± SE ($N = 132$). *P* values in the tables indicate the results of the mixed model, where significant *P* values ($P < 0.05$) are typed in bold. Asterisks above the bars indicate significant *P* values of specific comparisons between control and induced plants (*$P < 0.05$; ***$P < 0.001$).

did not detect significant genetic variation in the growth response to P availability (Family × P interaction; Fig. 1; Table S3a).

Phosphorus availability strongly determined leaf P concentration ($F_{1,3} = 440$; $P < 0.001$). Foliar P in P-limited plants was 1.24 ± 0.03 mg P g^{-1}, while plants with complete P fertilizer had 3.17 ± 0.14 mg P g^{-1}. Foliar N concentration was also significantly affected ($F_{1,3} = 24.6$; $P = 0.016$) but differences in foliar N concentration were small (22.3 ± 0.30 mg g^{-1} and 24.3 ± 0.27 mg g^{-1} in P-limited and complete fertilizer plants respectively).

The concentrations of soluble sugars and non-structural carbohydrate reserves in the stems were not affected by P availability, nor did they differ among pine families (Fig. 2; Table S3b) suggesting equivalent levels of carbon reserves.

Application of MJ significantly depressed primary and secondary growth, total biomass and starch reserves (Figs. 1 and 2b). Total height, basal stem diameter, total biomass and starch content in the juveniles treated with MJ were 15%, 5%,

Fig. 2. Concentration of soluble sugars (a) and non-structural storage carbohydrates (starch, b) in the stem of methyl jasmonate-induced (MJ) and control (constitutive) *Pinus pinaster* juveniles belonging to 11 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a phosphorus-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are LS means ± SE. ($N = 44$). *P* values in the tables indicate the results of the mixed model. Significant *P* values ($P < 0.05$) are typed in bold.

20% and 10% lower, respectively, than in control juveniles. However, exogenous application of MJ did not affect concentration of soluble sugars in the stems (Fig. 2a). We did not find significant $P \times MJ$ interactive effects (Figs 1 and 2), suggesting that P availability did not affect the growth reduction due to MJ-induction.

PINE CHEMICAL DEFENCES

Phosphorus availability had substantial and significant effects on plant defensive chemistry (Fig. 3; Table S3c). Concentration of plant defences increased under P-limited conditions, with similar responses among all pine families (non significant Family × P interaction). Concentrations of stem diterpenes, total phenolics and condensed tannins in the P-deficient juveniles were 40%, 40% and 75% greater, respectively, than those in juveniles with complete fertilizer.

Concentration of secondary chemicals was enhanced significantly by MJ application (Fig. 3). Induced concentration of stem diterpenes, total phenolics and condensed tannins were 15%, 15% and 30% greater, respectively, than those in control plants. The induction of foliar phenolic compounds (both total phenolics and condensed tannins) was significantly affected by the P availability (Figs 3b,c), where inducibility was significantly greater under P-limited conditions and constrained in the complete fertilizer treatments. This pattern was not observed for stem diterpenes (Fig. 3a), for which MJ was found to elicit similar responses in both P treatments.
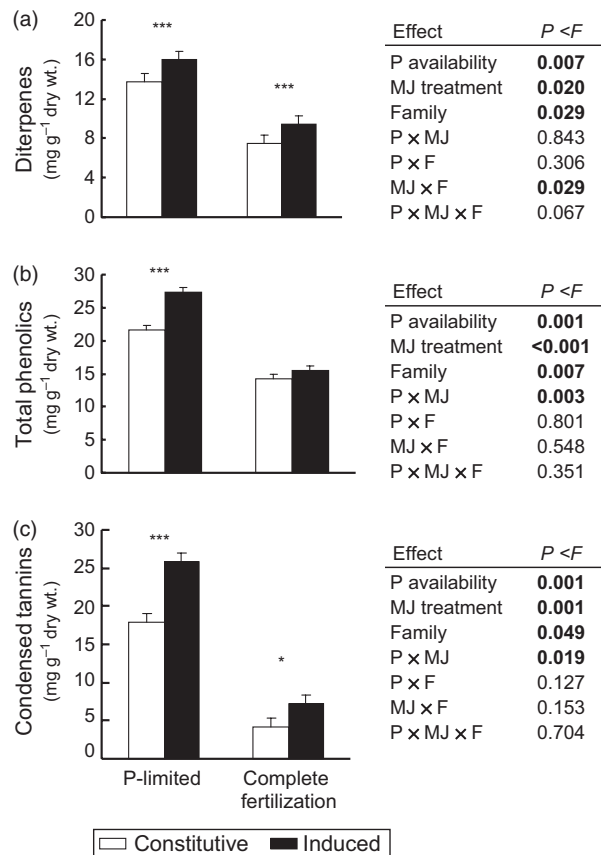
Fig. 3. Quantitative carbon-based chemical defences in methyl jasmonate-induced (MJ) and control (constitutive) *Pinus pinaster* juveniles belonging to 33 open-pollinated families growing in a nutrient-rich (complete fertilization) or in a phosphorus-limited medium, showing concentration of (a) stem diterpenes, (b) leaf total polyphenolics, expressed as tannic acid equivalents and (c) leaf condensed tannins, expressed as quebracho condensed tannin equivalents. Plants were destructively sampled 15 days after application of MJ. Bars are LS means ± SE. ($N = 132$). *P* values in the tables indicate the results of the mixed model. Significant *P* values ($P < 0.05$) are typed in bold. Asterisks above the bars indicate significant *P* values of specific comparisons between control and induced plants (*$P < 0.05$; ***$P < 0.001$).

All defensive traits showed significant genetic variation (Fig. 3), with different levels of genetic control in constitutive and induced modes, and genetic variation in their inducibility. The constitutive concentration of stem diterpenes, total phenolics and condensed tannins varied *c.* 1.8-fold, 1.5-fold and 2.5-fold, respectively, among pine families. Independent analyses within each MJ treatment revealed a strong genetic control of the constitutive concentration of stem diterpenes ($F_{32, 177} = 2.34$; $P = 0.0002$) but no significant genetic control of MJ-induced diterpene content ($F_{32, 165} = 1.15$; $P = 0.2822$). In contrast, no genetic variation was found for the constitutive phenolic content ($F_{32, 190} = 0.810$; $P = 0.7535$) but the induced concentration of phenolic compounds did vary significantly across families ($F_{32, 187} = 1.58$; $P = 0.0335$). Accordingly, we found significant genetic variation in inducibility of stem diterpenes (Family × MJ interaction, Fig. 3a) but not of phenolic compounds (Figs 3b,c).

GENETIC CORRELATION BETWEEN GROWTH AND
DEFENCES

We found significant negative family relationships between the
concentration of constitutive stem diterpenes and height
growth and biomass in P-limited conditions, but not in the
complete fertilizer treatment (Table 1). Family relationships
between growth traits and the constitutive concentration of
total phenolics or condensed tannins were not significant in
either P-limited or complete fertilizer treatments. No genetic
correlation was observed between diterpenes and phenolic
compounds, but a positive genetic correlation between total
phenolics and condensed tannins was found ($R = 0.63$;
$P < 0.001$).

On the other hand, we found that P availability strongly
modulated the expression of realized vegetative costs associ-
ated with the MJ-induced responses. We found significant
negative family correlations between inducibility of phenolic
compounds and the vegetative costs of induced responses
(Figs 4b,e,h), but only when plants were grown in the P-lim-
ited condition. This relationship was especially strong for
diameter and biomass, where the increase of total polypheno-
lics explained up to 47% of the variance of growth loss
among families. We also found a significant negative genetic
relationship between inducibility of condensed tannins
and costs for height growth, but again only when P was
limited (Fig. 4c). We did not detect significant relationships
between inducibility of stem diterpenes and vegetative costs
(Figs 4a,d,g).

Discussion

Our results showed that investment in growth and in constitu-
tive and induced carbon-based defences were strongly deter-
mined by the P availability in the early stages of pine life.
Compared to those that were grown with complete fertiliza-
tion, pine juveniles growing with limited P availability showed
(i) reduced growth rates, (ii) the same concentration of carbon
reserves, (iii) lower foliar P concentration but similar foliar N
concentration, (iv) higher concentration of constitutive and
induced defences, and (vi) higher inducibility of phenolic com-
pounds but (vii) unaffected stem diterpene inducibility. These
results agree with several physiological and evolutionary mod-
els of plant defence such as the GDBH and the ODT (Rhoades

1979; Herms & Mattson 1992), which predict that plants grow-
ing in resource-limited environments should be better pro-
tected by chemical defences. However, GDBH does not
explain why carbon reserves and inducibility of diterpenoid
resin were unaffected by P availability.

Our findings illustrate the importance of P availability for
pine tree defence, which differs from the general observation
that carbon-based secondary compounds are strongly affected
by N nutrition, but weakly affected or unaffected by P avail-
ability, noted in the meta-analysis of Koricheva *et al.* (1998).
Accordingly, our results also disagree with those reported by
Wright *et al.* (2010), who extended the predictions of the PCM
(Jones & Hartley 1999) by testing the phenolic concentration
in foliage of plants with variable P availability and constant N
availability in two independent field studies involving up to
110 plant species (including trees). They found no effect of P
availability on the concentration of constitutive phenolics in
leaves. However, our results show that, in conifers at least, var-
iation of soil P availability may indeed determine the concen-
tration of leaf phenolics.

Both diterpenes in the stem and phenolics in the needles
were plastic to P, and their reaction norms were homogeneous
across families (no significant Family × P interaction). How-
ever, although it is generally recognized that resin-based
defences in the stem are greater when resources are scarce (e.g.
Lombardero *et al.* 2000), plasticity to nutrient availability can-
not be extended to all other constitutive conifer defences or tis-
sues. For instance, increased nutrient availability has been
found to increase the activity of defensive proteins and resin
acids in the needles (Björkman *et al.* 1998; Barto *et al.* 2008)
and phenolic compounds in the phloem (Wallis *et al.* 2011),
but to reduce the density of resin canals in the phloem (More-
ira *et al.* 2008), phenolics in the needles (Björkman *et al.*
1998) and resin acids and phenolics in the shoots (Holopainen
et al. 1995). Manipulation of soil fertility did not, however,
significantly affect the concentration of leaf volatile terpenes
(Holopainen *et al.* 1995; Sampedro *et al.* 2010), sesqui- and
mono-terpenes in the phloem (Wallis *et al.* 2011) and resin
canals in the xylem (Moreira *et al.* 2008). In particular, the
considerable effect of P availability on constitutive and
induced chemical defences observed in this greenhouse experi-
ment were consistent with extensive field studies showing
reduced resistance of P-fertilized juvenile pine trees to a
phloem insect herbivore (Zas *et al.* 2006, 2008).

Table 1. Family relationships between growth traits and concentration of constitutive chemical defences of *Pinus pinaster* juveniles growing in a phosphorus-limited or in a well-fertilized (complete fertilization) medium

	P-limited			Complete fertilization		
	Height	Diameter	Biomass	Height	Diameter	Biomass
Stem diterpenes	**−0.408 (0.018)**	−0.182 (0.310)	**−0.392 (0.024)**	−0.137 (0.448)	0.080 (0.658)	−0.068 (0.707)
Leaf total phenolics	0.043 (0.814)	0.047 (0.793)	−0.129 (0.475)	0.118 (0.512)	0.267 (0.133)	0.146 (0.418)
Leaf condensed tannins	0.007 (0.969)	−0.287 (0.106)	−0.323 (0.067)	−0.099 (0.585)	0.078 (0.665)	0.012 (0.948)

Pearson correlation coefficients and associated significance levels (within brackets) are shown. Significant Pearson's *r* correlation coeffi-
cients ($P < 0.05$) are given in boldface. $N = 33$ open-pollinated families.
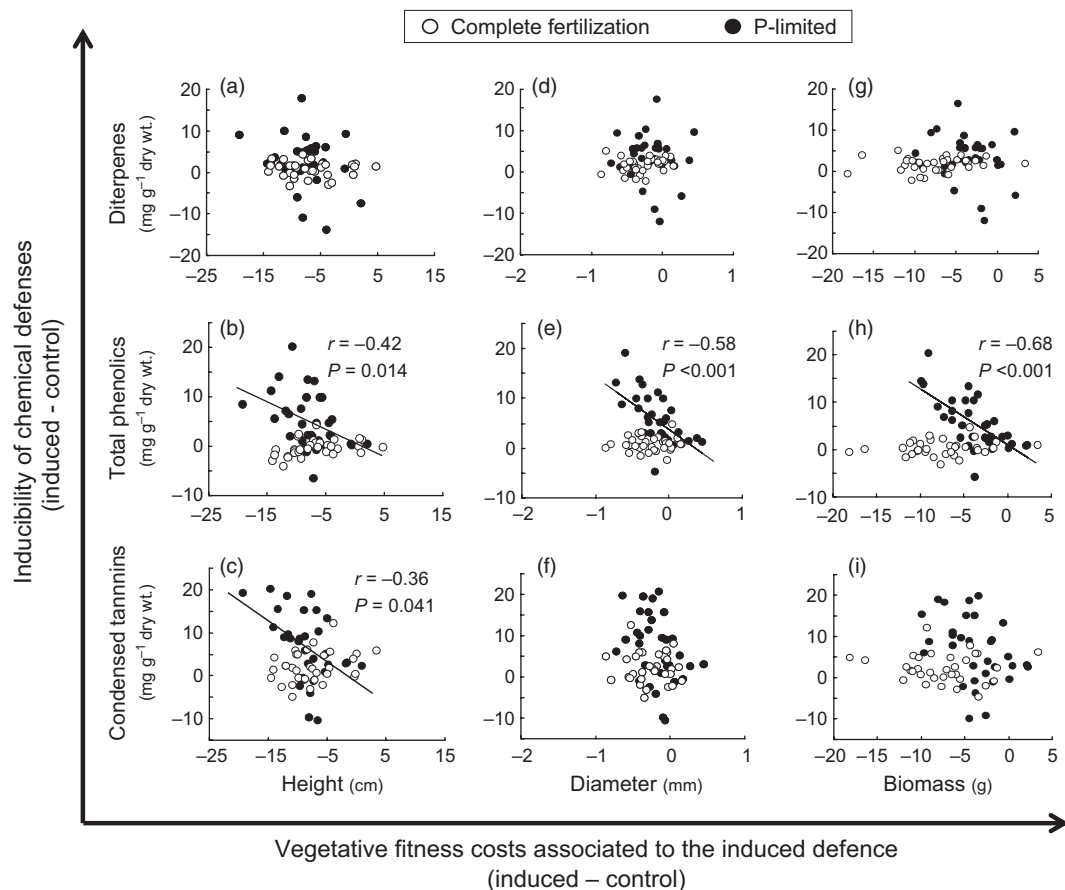
Fig. 4. Family relationships between inducibility of quantitative chemical defences (stem diterpenes, leaf total polyphenolics and leaf condensed tannins) and vegetative costs associated to the production of methyl jasmonate (MJ) induced responses in *Pinus pinaster* juveniles growing in a nutrient-rich (complete fertilization, open dots) or in a phosphorus-limited medium (filled dots). Inducibility was calculated as the concentration of a given chemical defence in the MJ-treated plants (family mean value) minus that in control plants. Vegetative costs were measured in the same way in terms of plant height (a, b, c), diameter (d, e, f) and total biomass (g, h, i). With this metric, negative significant relationships denote an evolutionary trade-off, as the greater inducibility in a given chemical defence, the greater vegetative cost. Regression lines, Pearson *r* coefficients and corresponding *P* values are shown for the significant relationships, which were found only in the P-limited treatment. Dots are family means. *N* = 33 open-pollinated families in all cases.

We found no negative genetic correlations between defensive traits, suggesting no constraints on the independent evolution of stem diterpenes and leaf phenolics. Information regarding the relative importance of these defences against the broad number of biotic challenges that a pine must face during its life is inconclusive. Although it is commonly assumed that leaf phenolics are effective against defoliators and that resin compounds are key defences against phloem feeders and stem borers, it should be noted that phenolic compounds in the phloem are also implicated in resistance against the latter and leaf terpenoids could deter the former (Mumm & Hilker 2006). Our results, providing evidence that main pine chemical defences are not trading off, are consistent with the idea that pine resistance depends on the proper combination of defensive chemical traits (resin, phenolics and other N based defences) and strategies (constitutive-induced, resistance–tolerance, direct–indirect resistance) adequate to each particular environmental conditions as proposed by Agrawal & Fishbein (2006).

We found large genetic variation in all growth and defensive traits studied, but more interestingly we also found additive genetic variation in the inducibility of the stem diterpenes (significant Family × MJ interaction). Besides, although Family × MJ interaction was not significant for phenolics, the different levels of genetic variation observed in control and MJ-induced plants, with significant differences among families found only in the MJ-induced treatment, does show the existence of genetic variation in the inducibility in this trait, too (Agrawal *et al.* 2002). Thus, our results indicate the existence of additive genetic variation for both constitutive concentration and inducibility of the three studied defensive traits. Genetic variation in secondary chemistry has been reported for several tree species, including conifers (e.g. Orians *et al.* 2003; Roberds *et al.* 2003; Osier & Lindroth 2006; Donaldson & Lindroth 2007). To our knowledge, however, this is the first work reporting additive genetic variation in inducibility of defences in pine trees. This prerequisite allows the continued evolution of defensive strategies in response to the herbivore

pressure under the constraints imposed by the environment on the cost–benefit balance.

In this study, P availability not only affected the allocation to defensive chemicals, but also modulated the emergence of vegetative costs of constitutive defences. Under P-limited conditions, growth rates were lower in those families that showed the higher concentrations of constitutive content of stem diterpenes. This genetic constraint means that, at least in the P-limited environments like the native habitat of this species, selection for increased constitutive diterpene concentration would result in reduced growth rates. Costs of constitutive defences are more likely expressed under resource-limiting conditions, because allocation conflicts would be more evident in such conditions (Bergelson & Purrington 1996). The influence of resource availability on the costs of constitutive defences in trees, expressed as negative correlations between growth and defensive traits, was covered only recently by a number of studies on willow, quaking aspen and trembling aspen. Most of them agree that costs of constitutive resistance were greater in resource-limiting environments (Lindroth, Roth & Nordheim 2001; Donaldson, Kruger & Lindroth 2006; Osier & Lindroth 2006; Donaldson & Lindroth 2007), although absence of costs in terms of growth (Orians *et al.* 2003) and even higher costs under higher nutrient availability were also found (Stevens, Waller & Lindroth 2007).

Similarly, the results presented here confirm that the induction of chemical defences in juvenile pines is costly and that the expression of those costs depends on nutrient availability. Under P-limited conditions, growth was strongly reduced in those families in which the induction of phenolic compounds was higher, while no relation was found in the well-fertilized environment. The existence of costs associated with the production of induced defences has been well documented for annual plants during recent years (see the exhaustive review by Cipollini & Heil 2010), but scant information is available for woody plants and trees. A few studies found that the production of induced defences was associated with reduced growth rates (Björkman *et al.* 1998; Heijari *et al.* 2005; Gould *et al.* 2008; Sampedro, Moreira & Zas 2011), but failed to elucidate whether this association was genetically determined, and could thus have evolutionary consequences. The environmental modulation of the costs of induced defences was also found in annual plants (e.g. Cipollini, Purrington & Bergelson 2003), although with contrasting results ranging from the magnification of costs under resource deprivation (e. g. Heil & Baldwin 2002) to larger costs in high-resource environments (Cipollini 2010). The life history of the species, the type of defensive mechanism and its pleiotropic implications in other physiological processes, the environmental factor considered and/or the plant ontogenetic development may all condition the emergence of costs of induced defences and contribute to the lack of empirical consensus (Cipollini, Purrington & Bergelson 2003). Some of these factors could explain why costs of constitutive defences were detected for stem diterpenes but not for phenolic compounds in the leaves, whereas the opposite situation occurred with the expression of costs of induced defences. As mentioned above, that resource-limiting environments would favour the expression of costs in any resistance trait seems, thus, to be hard to generalize.

Vegetative costs associated with the MJ-induction were fairly high, with reductions of 20% in total biomass and 15% in height compared to control plants just within 15 days, and could compromise future fitness of juvenile pines. Although it is not well known how relative investments in defences changes along the ontogeny in conifers, Barton & Koricheva (2010) found in their meta-analysis that chemical defences in woody plants were generally maximized during the seedling and juvenile stages. Vegetative costs associated with chemical defences have also been shown to be greatest in early stages, as this is when root growth is prioritized and structural defences such as resin ducts must be constructed (Boege & Marquis 2005; Orians *et al.* 2010). However, in light-demanding tree species such as *P. pinaster*, in which seedling establishment is a key stage in determining future fitness (see Appendix S1), early vegetative costs of chemical defences are likely to be translated into relevant opportunity costs later in the plant's development. As suggested by Heil (2010), long-term field studies are necessary to address the ecological relevance of these early costs in relation to the fitness benefits of the induced defences.

In our experiment we deliberately excluded the likely interference of soil microbes, particularly those from mycorrhizal fungi. It should be noted, however, that under field conditions pine trees usually are associated with mycorrhizas, which in many cases compensate for deficient P availability. In addition, as mycorrhizal fungi derive recently fixed carbon resources from their host, they could also potentially alter the expression of costs of defences. On the other hand, it is known that jasmonate can affect mycorrhizal colonization (e.g. Regvar, Gogala & Žnidaršič 1997), and thus under field conditions wound-induced responses could lead to ecological costs, including either positive/negative effects on mycorrhizas (Hartley & Gange 2009). The role of rhizosphere microbes on plant immunocompetence is a new frontier in understanding plant defensive responses (Pineda *et al.* 2010) and further research is needed to evaluate whether mycorrhizal fungi directly modulate pine induced responses (e.g. through priming) and thus the extent of any associated costs.

In summary we showed P availability had large and relevant effects on both the allocation to carbon-based constitutive and induced defences, and on the expression of vegetative fitness costs associated with those chemical defences in juvenile Maritime pine. Due to the relevance of early growth and resistance for these light-demanding pioneer trees, which come from a habitat with high spatial variability in soil fertility, P availability appears to be a primary driver of the evolution of pine defensive strategies against herbivores and a potential factor in maintaining genetic variation in pine quantitative defences.

Acknowledgements

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Study system.

Appendix S2. Composition of the fertilization solutions used for pine growth.

Appendix S3. Summary of the mixed models for growth, carbohydrate reserves and chemical defensive traits.

Table S2A. Chemical composition of the solutions used in both fertilization treatments.

Table S2B. Actual concentration of N and P in the fertilizer solutions used for both treatments.

Table S3A. Results of the mixed model for pine juvenile height, stem base diameter, and total biomass for the main fixed effects (Block, Phosphorus availability, Methyl jasmonate induction and Family) and their interactions.

Table S3B. Results of the mixed model for soluble sugars and starch for the main fixed effects (Block, Phosphorus availability, Methyl jasmonate induction and Family) and their interactions.

Table S3C. Results of the mixed model for stem diterpenes, leaf total phenolics and leaf condensed tannins for the main fixed effects (Block, Phosphorus availability, Methyl jasmonate induction and Family) and their interactions.