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

Running title: **Costs of constitutive and induced pine tree defences**

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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 on 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 ten 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; Van Dam & Baldwin 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 (Miller *et al.* 2005; Martin *et al.* 2002; 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 ºC night, 25 ºC day) and daily watering by subirrigation. Fungicide was also applied every two months during pine growth.

One month after sowing we began applying the fertilizer treatments (complete and P-limited fertilizer) by subirrigation every two 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). 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 two 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, #39270-7) 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 lab 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 ºC in cryogenic vials for diterpene analysis. A subsample of needles (c. 2 g) was also immediately weighed, then oven-dried (45 ºC 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 vol:vol) 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 Biorad 650 microplate reader (Bio-Rad Laboratories, 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 in a

Biorad 650 microplate reader 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) 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 0.00001 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 2010).

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 ºC for 30 min, followed by centrifugation and subsequent dilution of the extract. Soluble sugars and starch concentration were determined colorimetrically in a Biorad 650 microplate reader at 630 nm, using glucose and potato starch, respectively, as standards.

Total N was determined with a CN-2000 macro elemental analyser (LECO Corporation, St. Joseph, MI, USA) and total P 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×P and B×P×MJ interaction were considered random factors in order to analyse the main factors P and MJ with the appropriate error terms (B×P and B×P×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). 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 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, 2b). Total height, basal stem diameter, total biomass and starch content in the juveniles treated with MJ were 15%, 5%, 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 × MJ interactive effects (Figs. 1, 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, 3c), 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.

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, 3c).

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

305 constitutive concentration of total phenolics or condensed tannins were not significant in either
306 P-limited or complete fertilizer treatments. No genetic correlation was observed between
307 diterpenes and phenolic compounds, but a positive genetic correlation between total phenolics
308 and condensed tannins was found ($R = 0.63$; $P < 0.001$).

309 On the other hand, we found that P availability strongly modulated the expression of
310 realized vegetative costs associated with the MJ-induced responses. We found significant
311 negative family correlations between inducibility of phenolic compounds and the vegetative
312 costs of induced responses (Figs 4b, 4e, 4h), but only when plants were grown in the P-limited
313 condition. This relationship was especially strong for diameter and biomass, where the increase
314 of total polyphenolics explained up to 47% of the variance of growth loss among families. We
315 also found a significant negative genetic relationship between inducibility of condensed tannins
316 and costs for height growth, but again only when P was limited (Fig. 4c). We did not detect
317 significant relationships between inducibility of stem diterpenes and vegetative costs (Figs 4a,
318 4d, 4g).

319

320 **Discussion**

321 Our results showed that investment in growth and in constitutive and induced carbon-based
322 defences were strongly determined by the P availability in the early stages of pine life. Compared
323 to those that were grown with complete fertilization, pine juveniles growing with limited P
324 availability showed (i) reduced growth rates, (ii) the same concentration of carbon reserves, (iii)
325 lower foliar P concentration but similar foliar N concentration, (iv) higher concentration of
326 constitutive and induced defences and (vi) higher inducibility of phenolic compounds but (vii)
327 unaffected stem diterpene inducibility. These results agree with several physiological and
328 evolutionary models of plant defence such as the GDBH and the ODT (Rhoades 1979; Herms &
329 Mattson 1992), which predict that plants growing in resource-limited environments should be

330 better protected by chemical defences. However, GDBH does not explain why carbon reserves
331 and inducibility of diterpenoid resin were unaffected by P availability.
332 Our findings illustrate the importance of P availability for pine tree defence, which differs
333 from the general observation that carbon-based secondary compounds are strongly affected by N
334 nutrition, but weakly affected or unaffected by P availability, noted in the meta-analysis of
335 Koricheva *et al.* (1998). Accordingly, our results also disagree with those reported by Wright *et*
336 *al.* (2010), who extended the predictions of the PCM (Jones & Hartley 1999) by testing the
337 phenolic concentration in foliage of plants with variable P availability and constant N availability
338 in two independent field studies involving up to 110 plant species (including trees). They found
339 no effect of P availability on the concentration of constitutive phenolics in leaves. However, our
340 results show that, in conifers at least, variation of soil P availability may indeed determine the
341 concentration of leaf phenolics.
342 Both diterpenes in the stem and phenolics in the needles were plastic to P, and their
343 reaction norms were homogeneous across families (no significant Family × P interaction).
344 However, although it is generally recognized that resin-based defences in the stem are greater
345 when resources are scarce (e.g. Lombardero *et al.* 2000), plasticity to nutrient availability cannot
346 be extended to all other constitutive conifer defences or tissues. For instance, increased nutrient
347 availability has been found to increase the activity of defensive proteins and resin acids in the
348 needles (Björkman *et al.* 1998; Barto *et al.* 2008) and phenolic compounds in the phloem (Wallis
349 *et al.* 2010), but to reduce the density of resin canals in the phloem (Moreira *et al.* 2008),
350 phenolics in the needles (Björkman *et al.* 1998) and resin acids and phenolics in the shoots
351 (Holopainen *et al.* 1995). Manipulation of soil fertility did not, however, significantly affect the
352 concentration of leaf volatile terpenes (Holopainen *et al.* 1995; Sampedro *et al.* 2010), sesqui-
353 and mono-terpenes in the phloem (Wallis *et al.* 2010) and resin canals in the xylem (Moreira *et*
354 *al.* 2008). In particular, the considerable effect of P availability on constitutive and induced
355 chemical defences observed in this greenhouse experiment were consistent with extensive field

356 studies showing reduced resistance of P-fertilized juvenile pine trees to a phloem insect
357 herbivore (Zas *et al.* 2006; Zas *et al.* 2008).

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

370 We found large genetic variation in all growth and defensive traits studied, but more
371 interestingly we also found additive genetic variation in the inducibility of the stem diterpenes
372 (significant Family × MJ interaction). Besides, although Family × MJ interaction was not
373 significant for phenolics, the different levels of genetic variation observed in control and MJ-
374 induced plants, with significant differences among families found only in the MJ-induced
375 treatment, does show the existence of genetic variation in the inducibility in this trait, too
376 (Agrawal *et al.* 2002). Thus, our results indicate the existence of additive genetic variation for
377 both constitutive concentration and inducibility of the three studied defensive traits. Genetic
378 variation in secondary chemistry has been reported for several tree species, including conifers
379 (e.g. Orians *et al.* 2003; Roberds *et al.* 2003; Osier & Lindroth 2006; Donaldson & Lindroth
380 2007). To our knowledge, however, this is the first work reporting additive genetic variation in
381 inducibility of defences in pine trees. This prerequisite allows the continued evolution of

382 defensive strategies in response to the herbivore pressure under the constraints imposed by the
383 environment on the cost–benefit balance.

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

399 Similarly, the results presented here confirm that the induction of chemical defences in
400 juvenile pines is costly and that the expression of those costs depends on nutrient availability.
401 Under P-limited conditions, growth was strongly reduced in those families in which the
402 induction of phenolic compounds was higher, while no relation was found in the well-fertilized
403 environment. The existence of costs associated with the production of induced defences has been
404 well documented for annual plants during recent years (see the exhaustive review by Cipollini &
405 Heil 2010), but scant information is available for woody plants and trees. A few studies found
406 that the production of induced defences was associated with reduced growth rates (Björkman *et*
407 *al.* 1998; Heijari *et al.* 2005; Gould *et al.* 2008; Sampedro, Moreira & Zas 2010), 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 mycorrhizae, 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 mycorrhizae (Hartley & Gange 2009). The role of rhizosphere microbes on plant inmunocompetence 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.

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644

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.*

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.*

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

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.*

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669

670 **Table 1**. Family relationships between growth traits and concentration of constitutive chemical
671 defences of *Pinus pinaster* juveniles growing in a phosphorus-limited or in a well-fertilized
672 (complete fertilization) medium. Pearson correlation coefficients and associated significance
673 levels (within brackets) are shown. Significant Pearson's r correlation coefficients ($P < 0.05$) are
674 given in boldface. N = 33 open-pollinated families

675

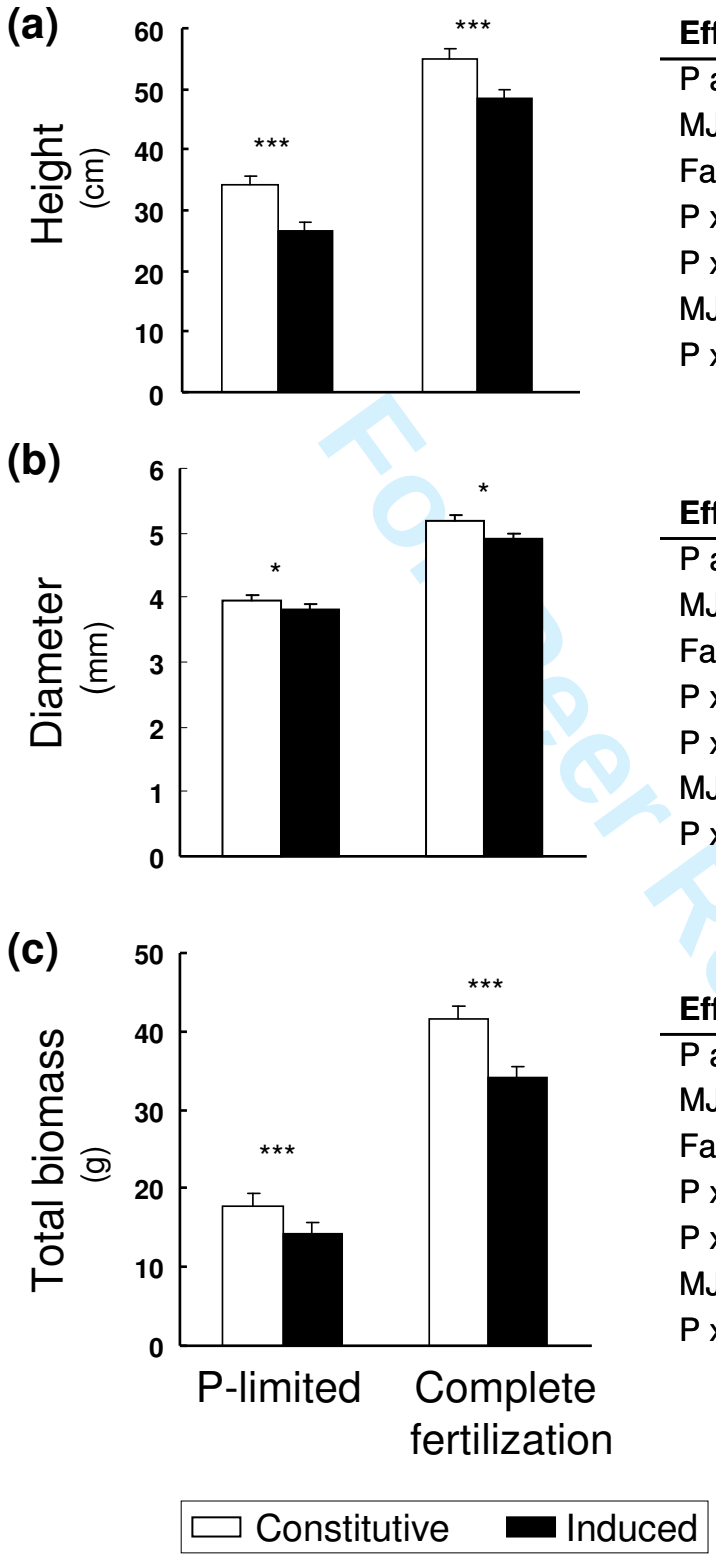
	P-limited			Complete fertilization		
	Height	Diameter	Biomass	Height	Diameter	Biomass
Stem diterpenes	**-0.408**	-0.182	**-0.392**	-0.137	0.080	-0.068
	(0.018)	(0.310)	**(0.024)**	(0.448)	(0.658)	(0.707)
Leaf total phenolics	0.043	0.047	-0.129	0.118	0.267	0.146
	(0.814)	(0.793)	(0.475)	(0.512)	(0.133)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

676

677 **FIGURE LEGENDS**

678 **Fig. 1.** Plant height (a), stem base diameter (b), and total biomass (c) of methyl-jasmonate
679 induced (MJ) and control (constitutive) *Pinus pinaster* juveniles belonging to 33 open-pollinated
680 families growing in a nutrient-rich (complete fertilization) and in a phosphorus-limited media.
681 Plants were destructively sampled 15 days after application of MJ. Bars are LS means ± SE (N
682 = 132). *P* values in the table indicate the results of the mixed model, where significant *P* values
683 ($P < 0.05$) are typed in bold. Asterisks above the bars indicate significant *P* values of specific
684 comparisons between control and induced plants (*, $P < 0.05$; ***, $P < 0.001$).
685
686 **Fig. 2.** Concentration of soluble sugars (a) and non-structural storage carbohydrates (starch, b)
687 in the stem of methyl-jasmonate induced (MJ)and control (constitutive) *Pinus pinaster* juveniles
688 belonging to 11 open-pollinated families growing in a nutrient-rich (complete fertilization) and
689 in a phosphorus-limited media. Plants were destructively sampled 15 days after application of
690 MJ. Bars are LS means ± SE. (N = 44). *P* values in the table indicate the results of the mixed
691 model. Significant *P* values ($P <0.05$) are typed in bold.
692
693 **Fig. 3.** Quantitative carbon-based chemical defences in methyl-jasmonate induced (MJ)and
694 control (constitutive) *Pinus pinaster* juveniles belonging to 33 open-pollinated families growing
695 in a nutrient-rich (complete fertilization) or in a phosphorus-limited medium, showing
696 concentration of (a) stem diterpenes, (b) leaf total polyphenolics, expressed as tannic acid
697 equivalents and (c) leaf condensed tannins, expressed as quebracho condensed tannin
698 equivalents. Plants were destructively sampled 15 days after application of MJ. Bars are LS
699 means ± SE. (N = 132). *P* values in the table indicate the results of the mixed model. Significant
700 *P* values ($P < 0.05$) are typed in bold. Asterisks above the bars indicate significant *P* values of
701 specific comparisons between control and induced plants (*, $P < 0.05$; ***, $P < 0.001$).
702

703 **Fig. 4.** Family relationships between inducibility of quantitative chemical defences (diterpenes
704 in the stem, needle total polyphenolics and needle condensed tannins) and vegetative costs
705 associated to the production of methyl-jasmonate (MJ) induced responses in *Pinus pinaster*
706 juveniles growing in a nutrient-rich (complete fertilization, open dots) or in a phosphorus-
707 limited medium (filled dots). Inducibility was calculated as the concentration of a given
708 chemical defence in the MJ-treated plants (family mean value) minus that in control plants.
709 Vegetative costs were measured in the same way in terms of plant height (a, b, c), diameter (e, f,
710 g) and total biomass (h, i, j). With this metric, negative significant relationships denote an
711 evolutionary trade-off, as the greater inducibility in a given chemical defence, the greater
712 vegetative cost. Regression lines, Pearson r coefficients and corresponding *P* values are shown
713 for the significant relationships, which were found only in the P-limited treatment. Dots are
714 family means. N = 33 open-pollinated families in all cases.

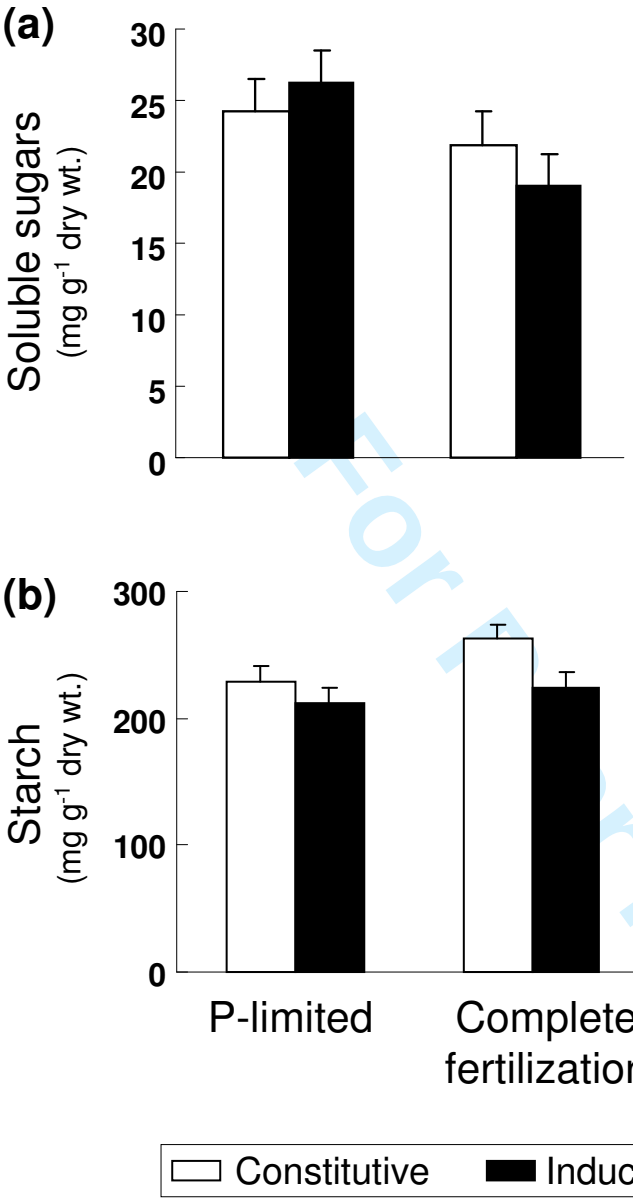


Effect	*P* <F
P availability	**0.001**
MJ treatment	**<0.001**
Family	**0.013**
P x MJ	0.688
P x F	0.304
MJ x F	0.116
P x MJ x F	0.947

Effect	*P* <F
P availability	**0.001**
MJ treatment	**0.042**
Family	**<0.001**
P x MJ	0.584
P x F	0.155
MJ x F	0.802
P x MJ x F	0.884

Effect	*P* <F
P availability	**0.001**
MJ treatment	**0.003**
Family	**<0.001**
P x MJ	0.161
P x F	0.779
MJ x F	0.547
P x MJ x F	0.799

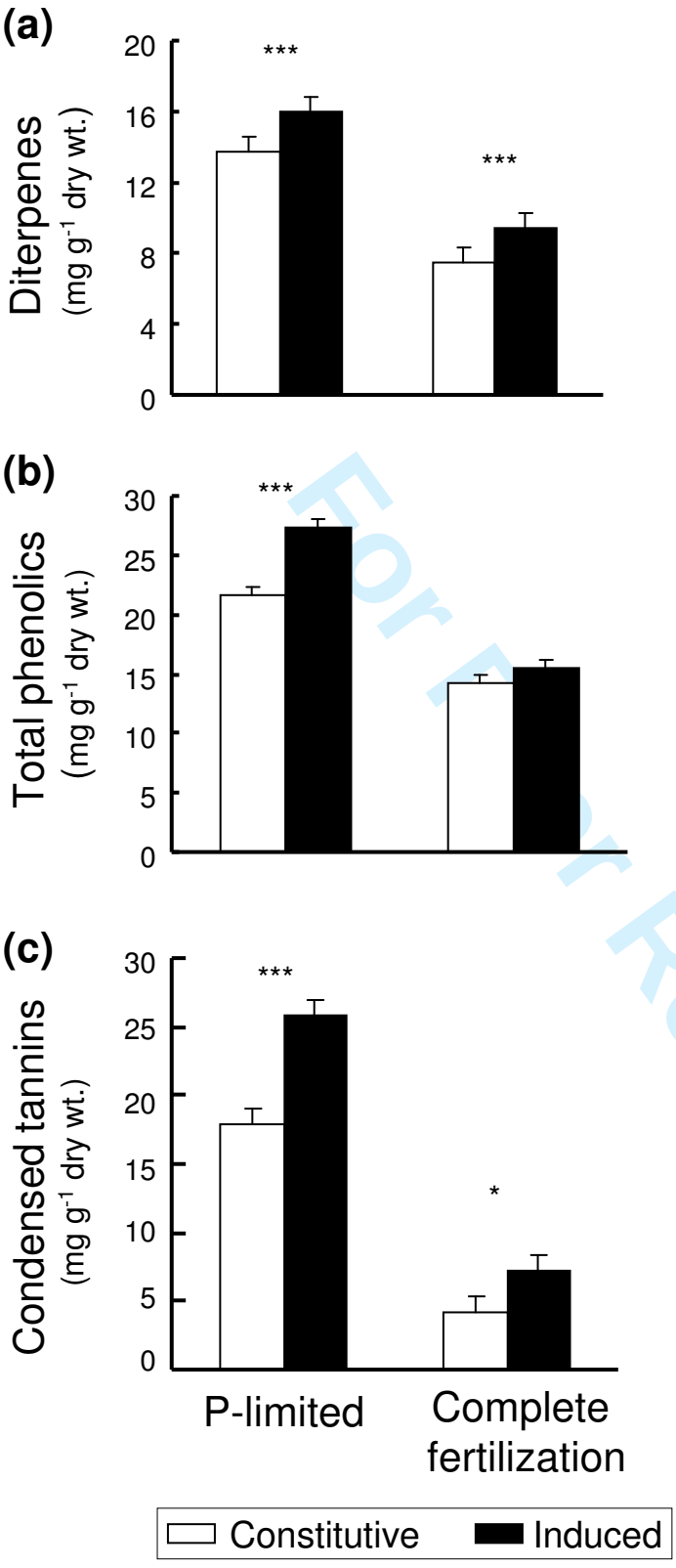
Figure 1



Effect	*P* <F
P availability	0.147
MJ treatment	0.904
Family	0.095
P x MJ	0.304
P x F	0.364
MJ x F	0.945
P x MJ x F	0.375

Effect	*P* <F
P availability	0.302
MJ treatment	**0.046**
Family	0.928
P x MJ	0.645
P x F	0.335
MJ x F	0.086
P x MJ x F	0.729

Figure 2



Effect	*P <F*
P availability	**0.007**
MJ treatment	**0.020**
Family	**0.029**
P x MJ	0.843
P x F	0.306
MJ x F	**0.029**
P x MJ x F	0.067

Effect	*P <F*
P availability	**0.001**
MJ treatment	**<0.001**
Family	**0.007**
P x MJ	**0.003**
P x F	0.801
MJ x F	0.548
P x MJ x F	0.351

Effect	*P <F*
P availability	**0.001**
MJ treatment	**0.001**
Family	**0.049**
P x MJ	**0.019**
P x F	0.127
MJ x F	0.153
P x MJ x F	0.704

Figure 3

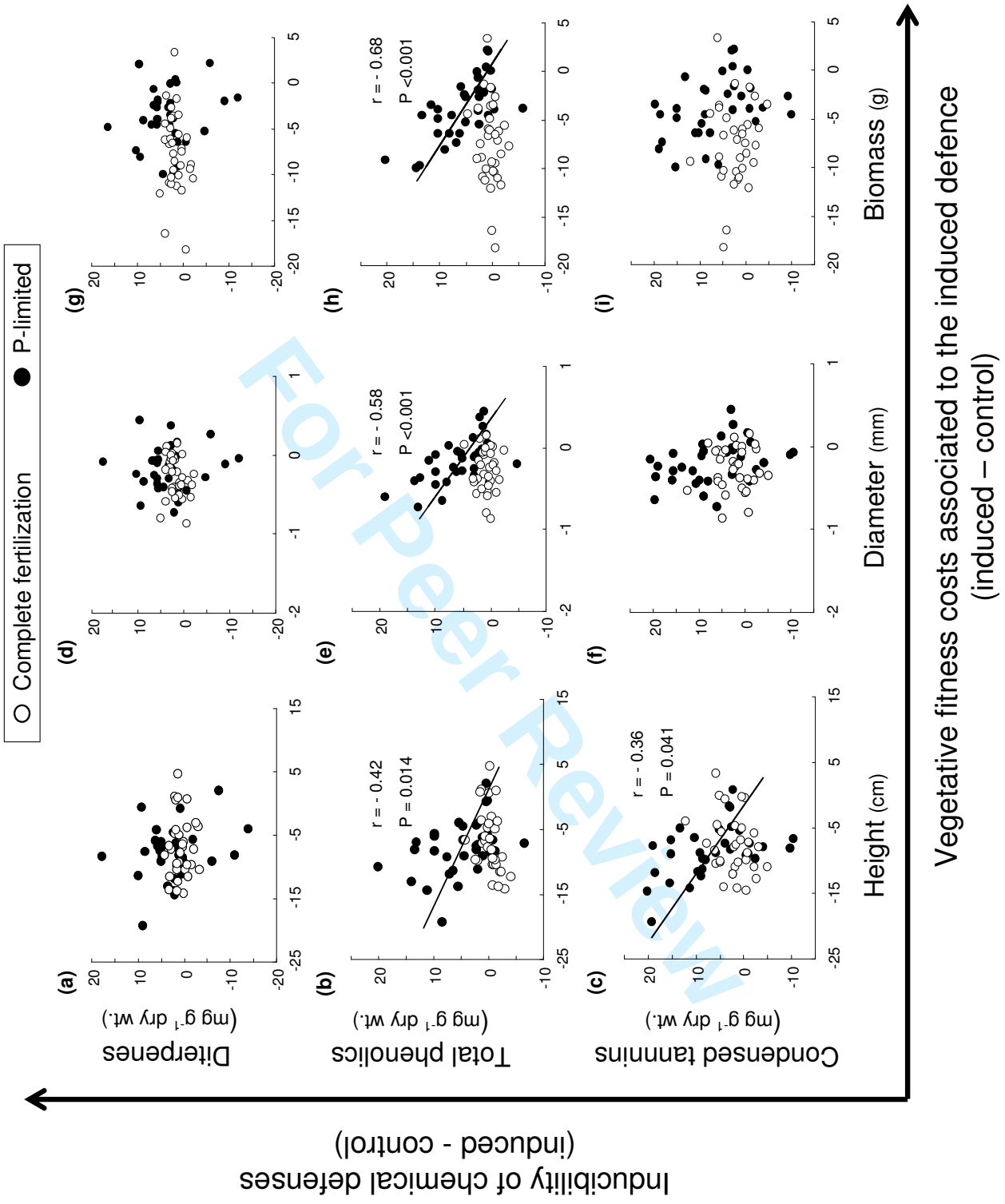

Complete fertilization
P-limited
Diterpenes (mg g⁻¹ dry wt.)
Total phenolics (mg g⁻¹ dry wt.)
Condensed tannins (mg g⁻¹ dry wt.)
Height (cm)
Diameter (mm)
Biomass (g)
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
r = - 0.42
P = 0.014
r = - 0.58
P <0.001
r = - 0.36
P = 0.041
r = - 0.68
P <0.001
Inducibility of chemical defenses
(induced - control)
Vegetative fitness costs associated to the induced defence
(induced – control)

Figure 4

Appendix S1. Study system

Pinus pinaster is a pioneer pine species originally from the western Mediterranean Basin. The plant material used in this study belongs to the coastal population of Galicia (NW Spain), at the northwestern range of its natural distribution. The climate in this area is temperate humid Atlantic, with annual precipitation usually over 1500 mm and mean annual temperatures of 11ºC, ranging typically between 25ºC and 4ºC maximum and minimum daily means, respectively. Soils are thin, sandy and acidic, with high organic matter content, high total N content and very low concentration of available P. Soil fertility in this region typically has a marked spatial heterogeneity, and P is the main limiting nutrient in Maritime pine forest stands (Martíns *et al.* 2009) and other forest species (Merino *et al.* 2003; Zas & Serrada 2003).

As with other pioneer light-demanding trees, early growth rate in *P. pinaster* is essential for future fitness, and thus reductions in early height growth and vigour could reduce future competitive ability, causing relevant opportunity fitness costs. On the other hand, early resistance to herbivory during the first stages of life is also extremely important because insects are a major cause of early pine seedling mortality. In instance, pine weevils such as *Hylobius abietis* can produce intense juvenile mortalities reaching up 60–80% in conifer forest regenerations (Orlander & Nordlander 2003). Previous studies have shown genetic variation in resistance to this weevil within the studied population of *P. pinaster* (Zas *et al.* 2005), where wounding intensity in forestry genetic trials showed a negative genetic correlation with survival ($R^2 = 0.55$; $P < 0.001$) and growth. Additionally, the damage by the pine weevil was significantly greater in fertilized plants, specially in those fertilized with P (Zas *et al.* 2006), that showed reduced density of resin canals (Moreira *et al.* 2008). Further studies have found that the concentration of diterpenes in the stem was positively related with

resistance to this weevil in *in vivo* and *in vitro* bioassays (Moreira, Sampedro & Zas 2009; Sampedro *et al.* 2009).

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

Table S2A. Chemical composition of the solutions used in both fertilization treatments. In both solutions pH was adjusted to 6.5 with diluted HCl.

Substance in the fertilizer solution		Fertilization Treatment	
		Complete fertilization (P20) mg L^{-1}	Phosphorus limited (P2) mg L^{-1}
Magnesium nitrate	$Mg(NO_3)_2 \cdot 6H_2O$	94.95	94.95
Calcium Sulfate dihydrate	$CaSO_4 \cdot 2H_2O$	30.07	30.07
Potassium sulfate	K_2SO_4	13.05	13.05
Potassium nitrate	KNO_3	165.88	165.88
Ammonium dihydrogen phosphate	$(NH_4)H_2PO_4$	85.27	8.53
Ammonium nitrate	NH_4NO_3	138.86	185.26
Ammonium iron (II) sulfate hexahydrate	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	2.8087	2.8087
Copper (II) sulfate anhydrous	$CuSO_4$	0.0753	0.0753
Zinc sulfate heptahidrate	$ZnSO_4 \cdot 7H_2O$	0.2639	0.2639
Manganese (II) sulfate monohydrate	$MnSO_4 \cdot H_2O$	1.2306	1.2306
Boric Acid	H_3BO_3	1.1439	1.1439
Molybdenum (IV) sulfide	MoS_2	0.0013	0.0013

Table S2B. Actual concentration of nitrogen and phosphorus in the tanks with the fertilizer solutions of both treatments. Concentration of P was close to those expected of 20 and 2 mg L^{-1}. Concentration of N was similar between treatments. P was measured by ICP-OES; N was analyzed with a continuous flow nutrient analyzer.

Nutrient in the solution	**Treatment**	
	Complete fertilization (P20)	Phosphorus limited (P2)
$P\text{-}PO_4$ $(mg\ P \cdot L^{-1})$	18.43	2.05
$N\text{-}NO_3$ $(mg\ N \cdot L^{-1})$	67.0	79.4
$N\text{-}NH_4$ $(mg\ N \cdot L^{-1})$	45.4	35.9
$N\text{-}NO_x$ $(mg\ N \cdot L^{-1})$	0.13	0.66

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

Table S3A. Results of the mixed model for seedling height, stem base diameter, and total biomass, showing the degrees of freedom (DF), error term, F values (F) and associated significance levels (p) for the main fixed effects (Block, Phosphorus availability, Methyl-jasmonate induction and Family) and their interactions. Significant *P* values ($P<0.05$) are typed in bold.

			Height		Diameter		Biomass	
	DF	error term	F	p > F	F	p > F	F	p > F
Block (B)	3	B×P	1.26	0.428	4.25	0.133	3.31	0.176
Phosphorus (P)	1	B×P	142.52	**0.001**	165.98	**0.001**	151.44	**0.001**
Methyl jasmonate (MJ)	1	B×P×MJ	49.16	**<0.001**	6.66	**0.042**	22.29	**0.003**
P × MJ	1	B×P×MJ	0.18	0.688	0.33	0.584	2.55	0.161
Family (F)	32	error	1.69	**0.013**	3.06	**<0.001**	3.91	**<0.001**
P × F	32	error	1.12	0.304	1.27	0.155	0.80	0.779
MJ × F	32	error	1.33	0.116	0.78	0.802	0.95	0.547
P × MJ × F	32	error	0.62	0.947	0.71	0.884	0.78	0.799

Table S3B. Results of the mixed model for soluble sugars and starch showing the degrees of freedom (DF), error term, F values (F) and associated significance levels (p) for the main fixed effects (Block, Phosphorus availability, Methyl-jasmonate induction and Family) and their interactions. Significant *P* values ($P<0.05$) are typed in bold.

			Soluble sugars		Starch	
	DF	error term	F	p > F	F	p > F
Block (B)	3	B×P	0.36	0.791	0.98	0.506
Phosphorus (P)	1	B×P	3.79	0.147	1.55	0.302
Methyl jasmonate (MJ)	1	B×P×MJ	0.02	0.905	5.86	**0.046**
P × MJ	1	B×P×MJ	1.27	0.304	0.23	0.645
Family (F)	32	error	1.67	0.095	0.43	0.928
P × F	32	error	1.11	0.364	1.15	0.335
MJ × F	32	error	0.40	0.945	1.71	0.086
P × MJ × F	32	error	1.09	0.375	0.69	0.729

Table S3C. Results of the mixed model for stem diterpenes, leaf total phenolics and leaf condensed tannins showing degrees of freedom (DF), error term, F values (F) and associated significance levels (p) for the main fixed effects (Block, Phosphorus availability, Methyl-jasmonate induction and Family) and their interactions. Significant *P* values ($P<0.05$) are typed in bold.

			Diterpenes		Total phenolics		Condensed tannins	
	DF	error term	F	p > F	F	p > F	F	p > F
Block (B)	3	B×P	2.14	0.274	33.80	**0.008**	16.65	**0.022**
Phosphorus (P)	1	B×P	43.78	**0.007**	144.30	**0.001**	165.71	**0.001**
Methyl jasmonate (MJ)	1	B×P×MJ	9.96	**0.020**	47.44	**<0.001**	33.16	**0.001**
P × MJ	1	B×P×MJ	0.04	0.843	21.52	**0.003**	10.12	**0.019**
Family (F)	32	error	1.56	**0.029**	1.78	**0.007**	1.47	**0.049**
P × F	32	error	1.12	0.306	0.78	0.801	1.31	0.127
MJ × F	32	error	1.56	**0.029**	0.95	0.548	1.27	0.153
P × MJ × F	32	error	1.43	0.067	1.08	0.351	0.85	0.704