

RESEARCH PAPER

Genetics, phosphorus availability, and herbivore-derived induction as sources of phenotypic variation of leaf volatile terpenes in a pine species

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Abstract

Oleoresin produced and stored in pine tree leaves provides direct resistance to herbivores, while leaf volatile terpenes (LVT) in the resin are also powerful airborne infochemicals. Resin concentration and profile show considerable spatial and temporal phenotypic variation within and among pine populations. LVT biochemistry is known to be under genetic control, and although LVT should be plastic to diverse abiotic and biotic environmental factors such as nutrient availability and herbivore attack, little is known about their relative contributions and interactive effects. The aim of this paper was to clarify whether reduced phosphorus availability could increase the LVT concentration and affect the expression of herbivore-derived induced defences, and how plasticity would contribute to the phenotypic variation of LVT. The constitutive and methyl-jasmonate (MeJa) induced LVT concentration and profile were analysed in 17 half-sib *Pinus pinaster* families growing under two levels of P-availability (complete and P-limited fertilization). Individual terpene concentrations showed large additive genetic variation, which was more pronounced in the control than in MeJa-induced pines. MeJa application did not affect the LVT concentration, but significantly modified the LVT profile by depleting the α -pinene content and reducing the sesquiterpene fraction. Low P-availability strongly reduced plant growth and foliar nutrient concentrations, but did not affect LVT concentration and profile, and did not interact with MeJa-induction. Results indicate a strong homeostasis of LVT concentration to P-availability, and minor changes in the LVT profile due to MeJa-induction. Genetic variation appears to be the main source of phenotypic variation affecting the LVT concentration in this pine species.

Key words: Chemical defence, conifer, constitutive defences, genetic variation, induced defences, leaf volatile terpenes, methyl jasmonate, phenotypic plasticity, *Pinus pinaster*.

Introduction

Many of the defensive traits and strategies that conifers have evolved against insect herbivores rely on oleoresin, a complex mixture of volatile (monoterpenes, -C₁₀- and sesquiterpenes, -C₁₅-) and non-volatile compounds (mainly diterpene acids, -C₂₀-) (Bohlmann, 2008; Phillips and Croteau, 1999). As with phenolic compounds, resin

terpenoids are quantitative chemical defences, with higher concentrations commonly associated with increased resistance (Bauce *et al.*, 1994; Wainhouse *et al.*, 2008). Oleoresin flows out from damaged or injured phloem, xylem, and needles when their resin ducts are exposed, thus providing direct resistance against a diverse array of herbivores and

pathogens (Jactel *et al.*, 1996; Heijari *et al.*, 2005; Mumm and Hilker, 2006; Zeneli *et al.*, 2006).

In addition to their role in direct resistance, monoterpenes (MT) and sesquiterpenes (SQT) volatilized from injured tissues are airborne molecular messengers involved in plant–animal, animal–animal, and plant–plant signalling (Peñuelas *et al.*, 1995; Phillips and Croteau, 1999; Peñuelas and Llusia, 2004). These compounds can simultaneously act as herbivore repellent and as olfactory clues for herbivore host selection (Pureswaran *et al.*, 2004), as well as attracting herbivore predators and parasitoids (Llusia and Peñuelas, 2001). Terpenes of vegetal origin also work in animal–animal signalling, for example, as co-factors for bark beetle aggregation (Erbilgin *et al.*, 2003). There is also evidence that terpenes could play a role in within-plant and between-plant defensive signalling (Peñuelas *et al.*, 1995). Undamaged conifer needles have a large potential for emitting volatile organic compounds through the cuticle and stomata (Peñuelas and Llusia, 2003, 2004). The emission rates of individual volatile terpenes from the foliage have been found to be dependent on their relative concentration within the plant tissue and on their physico-chemical characteristics, for example, their vapour pressure (Peñuelas and Llusia, 2001; Schurgers *et al.*, 2009). Any change in foliar volatile terpene chemistry would, thus, inevitably be translated into new airborne messages that could indirectly affect plant performance and fitness. Therefore, relevant trade-offs between foliar terpene chemistry and their associated ecological costs can occur (Mumm and Hilker, 2006).

Resin terpenoid synthesis and accumulation in conifers are known to be under genetic control with variation occurring within and between populations (Fady *et al.*, 1992; Trapp and Croteau, 2001; O'Reilly-Wapstra *et al.*, 2006). But chemical defences are also plastic traits, and their concentration can be modulated by the environment and by the interactive response of genotypes to environmental conditions such as resource availability. These effects can all contribute to the large phenotypic variation in resin content usually found in pine stands in the field.

Resin compounds in pine trees are inducible by biotic stimuli such as herbivore attack and fungal infestation, although other stimuli can elicit a similar response, including mechanical wounding, debarking, and chemical elicitors such as methyl jasmonate (MeJa) and other plant hormones (Bohmann, 2008; Zeneli *et al.*, 2006; Moreira *et al.*, 2009). Herbivore-induced defences in pine trees usually lead to quantitative changes in the terpenoid content of the stems and increased resin flux (Franceschi *et al.*, 2002; Martin *et al.*, 2002; Zeneli *et al.*, 2006). Qualitative changes in the terpene profile and altered patterns of terpene emission following wounding and after MeJa-induction have also been reported (Gijzen *et al.*, 1993; Litvak and Monson, 1998; Hilker *et al.*, 2002; Huber *et al.*, 2004; Mumm *et al.*, 2004).

Several ecological models formulated to explain the phenotypic variation in plant defences (reviewed by Stamp, 2003) agree that increasing plant resource availability is

associated with relaxed defensive mechanisms through lower concentration of the secondary metabolites implicated in direct resistance (Coley *et al.*, 1985; Koricheva *et al.*, 1998; Peñuelas and Estiarte, 1998). This has been widely reported for broadleaf trees (Mutikainen *et al.*, 2000; Donaldson and Lindroth, 2007), but information for conifers is much more limited (Kainulainen *et al.*, 1996; Lombardero *et al.*, 2000). Deficient N or P nutrition, for instance, could determine plastic responses in the allocation of carbon to growth and defence, and similarly modulate the ability for expressing induced responses to herbivory.

Knowledge of the responses of terpenoid chemistry in conifers to abiotic challenges comes mainly from studies focused on stem resin content and composition (Viiri *et al.*, 2001; Turtola *et al.*, 2002; Zeneli *et al.*, 2006). Information on how pine foliar terpenoid chemistry is affected by induced responses to herbivory, and how environmental factors can modulate this response is, however, much more limited. As the fitness value of leaves differ from those of stems, several ecological models predict that defensive patterns in leaves could differ from those observed in stems or other plant tissues (Zangerl and Bazzaz, 1992). Moreover, induced responses involving terpenoid chemistry in leaves may differ from those reported for stems and roots owing to the greater emitting potential of leaves and the dual function of LVT in direct and indirect resistance through airborne signalling (Huber *et al.*, 2004).

The aim of this paper is to clarify whether the concentration and profile of LVT reflect genetic variation, nutrient limitation, herbivore-induced responses or interactive effects among those factors. According to the predictions of current ecological models of plant defence, it was hypothesized that LVT concentration would be greater in conditions of limited nutrient availability, reduced plant growth, and greater carbon availability, as has been shown to occur in other conifer tissues (Huber *et al.*, 2005). Besides, nutrient limitation could compromise the ability of pine trees to express resin-based herbivore-induced defences in their leaves. A greenhouse factorial experiment with 17 half-sib Maritime pine (*Pinus pinaster* Aiton) families, belonging to the Atlantic Coast population of Galicia (NW Spain), was performed by manipulating plant growth through controlling P availability (two levels, complete fertilization and P-limited), and mimicking herbivore-induced responses by applying MeJa. As with other large ecological regions, phosphorus is the main limiting resource for the studied Maritime pine population, that typically grows in extremely nutrient-poor soils with high spatial heterogeneity in soil fertility (Martins *et al.*, 2009).

Materials and methods

Experimental design

The experimental layout was a randomized split–split design replicated in four blocks, with two fertilizer treatments (complete fertilization and P-limited fertilization) as the main factor, two MeJa treatments (control, representing constitutive defences, and

MeJa-induced) as the split factor, and 17 *P. pinaster* genetic entries as the split-split factor. In total, 272 pine seedlings were analysed, corresponding to 4 blocks×2 P fertilizer treatments×2 MeJa treatments×17 genetic entries.

Plant material, greenhouse conditions, fertilization, and induction with MeJa

On 7 February 2006, open-pollinated seeds from 17 *P. pinaster* mother trees from the coastal region of Galicia (NW Spain) were sown in 2.0 l pots containing sterilized perlite in a greenhouse with controlled light (12/12 h light/dark), (10/25 °C day/night temperature), and daily watering by subirrigation. Starting on 10 March 2006, the fertilizer treatments (complete and P-limited) were applied by sub-irrigation every 2 d. The complete fertilizer treatment (herein called P20, due to 20 mg P l⁻¹ in the fertilizer solution) was a balanced solution containing 100:20:70:7:9 mg l⁻¹ of N:P:K:Ca:Mg, respectively. This solution was a modification of that used by local nurseries for optimum seedling growth of this pine species. P-limited fertilizer solution (P2) contained the recommended levels of N, K, Ca, and Mg, as described above, but the P input was reduced 10-fold to 2 mg P l⁻¹. Fertilizer solutions were freshly prepared every 2 weeks, and pH was adjusted to pH 6.5 in both treatments. The chemical composition of the fertilizer solutions are given in Supplementary Table S1 at *JXB* online.

On 2 August 2006, when average plant heights in the P2 and P20 treatments were 22.6±0.7 cm and 47.8±1.2 cm, respectively, half of the seedlings were treated with a solution of 22 mM MeJa (Sigma-Aldrich, no. 39270-7) suspended in deionized water with 2.5% ethanol (v:v). The rest of the seedlings were treated with 2.5% ethanol as a control. Treatments were sprayed evenly over the foliage with a handheld sprayer, each seedling 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 seedlings remained in separate rooms for 24 h to allow drying.

Sampling and chemical analysis

On 17 August 2006, plant height and diameter were measured again. All plants were then harvested, transported to the laboratory in ice coolers and immediately processed for biomass determination and needle sampling. All primary needles of each tree were carefully separated from stem material and roots, and thoroughly mixed. A subsample of the needles was immediately frozen with liquid nitrogen and preserved at -80 °C in screw-capped cryogenic vials (VWR ref. no. 479-3223) for further terpenoid extraction, and another subsample was oven-dried at 65 °C for nutrient analysis. Total N was determined with a Carlo Erba CN-Macro elemental analyser and total P by ICP-OES (Perkin-Elmer Optima 4300DV, Ma., USA) after wet digestion (Walinga *et al.*, 1995) at the central facilities of Universidade de Vigo, Spain (<http://webs.uvigo.es/cactiweb/>).

Extraction and analysis of terpenoids were performed using the same methodology as Blanch *et al.* (2009). Briefly, needle samples were ground under liquid nitrogen in Teflon tubes and terpenes were extracted with ultrapure n-pentane in an ultrasonic bath at 25 °C using dodecane (Merck, no. 1.09658.0005) as the internal standard. The MT and SQT in the extract were analysed by gas chromatography using a HP59822B GC-MS (Agilent, Ca., USA) in a HP-5MS capillary column (30 m×0.25 mm×0.25 µm), with He as the carrier gas. Identification of peaks was performed by a comparison of the mass spectra in the single ion 93 m/z with the Wiley 275L library and with known standards (seven MT and three SQT, all from Fluka Chemie AG, Buchs, Switzerland). Calibration curves for quantification were prepared with commercial standards of the most abundant compounds in the samples. Individual terpene concentration was expressed in mg g⁻¹ leaf dry weight (DW).

Statistical analyses

Analyses were carried out with the appropriate mixed model for solving split-split designs (Littell *et al.*, 2006) using the Proc Mixed procedure of the SAS System. The main effects of P availability, MeJa induction, and the P×MeJa interaction were treated as fixed factors. The Block (B) effect, B×P and B×P×MeJa interactions were considered random in order to analyse the main factors P and MeJa with the appropriate error terms (B×P and B×P×MeJa, respectively). Family (G) and its interactions with main effects (P, MeJa, and P×MeJa) were considered random, and associated variance components were estimated by restricted maximum likelihood (REML). The statistical significance of the variance components for each random factor was assessed using likelihood ratio tests (Littell *et al.*, 2006), where the differences in two times the log-likelihood of the models including and excluding that random factor are distributed as one tailed χ^2 , with one degree of freedom. When genetic variance was significant, the corresponding narrow sense heritability (h^2) was calculated. The pine families were considered true half-sibs (same mother but different father), and thus additive genetic variance was estimated as four times the family variance. Heritability was calculated as the ratio of additive genetic variance to total phenotypic variance (Falconer and Mackay, 1996). Equality of residual variance across treatments was tested in all cases, but significant deviations were not found. When necessary, normality was achieved by log transforming the original variables. Multivariate analysis of variance of terpene profiles was performed by using the four most abundant MT and the four most abundant SQT. Differences in slopes and intercepts describing family relationships between MT and SQT, and α - and β -pinene concentrations were analysed by means of analysis of covariance using the Proc Mixed procedure (Littell *et al.*, 2006). Data are shown as mean ±SE.

Results

Both P limitation and MeJa affected growth and performance of pine seedlings. Limiting P availability seriously compromised primary and secondary growth of pine seedlings, where height and total biomass of P-limited plants (30.54±1.46 cm; 15.97±1.32 g) were significantly lower than those with adequate P nutrition (51.30±1.46 cm; 37.52±1.31 g; both $P < 0.001$). Leaf P concentration was also affected by P availability in the substrate ($F_{1,3}=593$; $P < 0.001$); P-limited plants contained 0.98±0.102 mg P g⁻¹ while plants with adequate P fertilization had 2.08±0.105 mg P g⁻¹. Nitrogen concentration was slightly but significantly lower ($F_{1,3}=30$; $P=0.012$) in the foliage of P-limited plants than in adequately fertilized plants (24.8±0.79 mg g⁻¹ and 28.9±0.80 mg g⁻¹, respectively). In addition, MeJa induction strongly depressed primary shoot growth ($F_{1,6}=111$; $P < 0.001$). The growth rate of shoots during the 25 d after application of induction treatments was 3.1±0.12 mm d⁻¹ in control plants but only 1.11±0.02 mm d⁻¹ in MeJa-induced seedlings.

Total LVT concentration in pine seedlings ranged from 1.7 to 66 mg g⁻¹ leaf DW. Twelve MT and 13 SQT were identified in the foliage of our *P. pinaster* seedlings (Table 1). The major MT identified were α -pinene, camphene, β -pinene, and β -myrcene, jointly accounting for approximately 90% of total MT. The most abundant SQT were *trans*-caryophyllene, α -humulene, and germacrene-D, accounting for approximately 75% of total SQT (Table 1).

Table 1. Concentration and molar fraction of individual volatile terpene species found in the primary needles of constitutive (control) and MeJa-induced *P. pinaster* seedlings belonging to 17 open-pollinated families

Significance tests (*P* value) of the MeJa induction effect in the mixed model for each individual compound are shown. Bold *P* values were significant at $\alpha < 0.05$. Concentrations are expressed on a dry weight basis.

	Terpene concentration ($\mu\text{g g}^{-1}$)			Terpene molar fraction (%)		
	Constitutive	MeJa induced	<i>F</i> > <i>P</i>	Constitutive	MeJa induced	<i>F</i> > <i>P</i>
Monoterpenes						
α -Pinene	4357.5	2755.5	0.003	29.17	20.48	0.000
Camphene	1349.0	1474.2	0.140	9.00	10.05	0.045
β -Pinene	6187.2	7363.3	0.038	37.45	49.88	0.000
β -Myrcene	1377.8	931.8	0.140	8.38	6.13	0.033
α -Phellandrene	47.3	6.3	0.626	0.19	0.04	0.217
Δ -3-Carene	325.3	108.8	0.182	2.03	0.85	0.077
α -Terpinene	3.4	2.0	0.372	0.03	0.01	0.235
Limonene	463.2	463.9	0.891	3.38	3.63	0.225
(+)-3-Carene	7.1	1.2	0.080	0.04	0.01	0.043
α -Terpinolene	44.9	10.9	0.221	0.28	0.08	0.085
Linalyl acetate	29.1	143.2	0.003	0.13	0.69	0.001
Bornyl acetate	118.9	161.0	0.258	0.50	0.69	0.018
Total monoterpenes	14310.3	13422.0	0.578	90.57	92.54	0.012
Sesquiterpenes						
α -Copaene	54.7	54.8	0.278	0.27	0.31	0.311
Jupinene	38.2	25.2	0.481	0.17	0.15	0.592
Trans-Caryophyllene	745.2	515.8	0.002	3.46	2.38	0.002
α -Humulene	285.0	210.6	0.002	1.51	1.19	0.019
α -Amorphene	17.4	20.5	0.070	0.08	0.14	0.068
Germacrene D	248.3	198.2	0.653	1.30	1.08	0.220
α -Muurolole	117.1	93.8	0.938	0.58	0.52	0.688
Δ -Cadinene	78.8	73.7	0.813	0.43	0.41	0.818
Caryophyllene oxide	29.2	19.1	0.356	0.13	0.07	0.216
Geranyl acetate	51.1	31.6	0.361	0.31	0.19	0.341
Farnesyl acetate	146.6	109.2	0.445	0.59	0.51	0.588
Unknown	57.2	35.4	0.408	0.22	0.15	0.317
Farnesyl acetone	21.7	16.7	0.593	0.11	0.09	0.772
α -Bisabolene	51.8	41.8	0.749	0.27	0.27	0.678
Total sesquiterpenes	1942.3	1446.1	0.006	9.43	7.46	0.012

Response of LVT concentration to P availability

Experimental manipulation of P availability did not affect the concentration of total LVT, total MT or total SQT (Table 2), evidencing a lack of plasticity related to nutrition in these traits. Similarly, the concentration of individual terpenoids was not affected by P availability. Accordingly, multivariate analysis did not detect a significant effect of P availability on the LVT profiles (MANOVA, Wilk's $\lambda_8, 241=0.962$; $P=0.3064$; see Supplementary Table S2 at *JXB* online). In addition, the concentration of LVT was not affected by the interaction of P availability and MJ-induction treatments (MJ \times P interaction in Table 2 and Fig. 1).

Genetic variation and heritability of LVT concentration

Pine families included in this study had terpene concentrations from 6 mg to 27 mg g^{-1} leaf DW (see Supplementary Fig. S1 at *JXB* online). Both total MT and total SQT showed large genetic variance (Table 2). However, the lack

of plasticity of foliar terpenoid concentration to P availability was uniform for all the studied families, as revealed by the non-significant G \times P interaction (Table 2), i.e. there was no genetic variation in plasticity.

Heritability estimates for the constitutive concentration of total MT and SQT were fairly high (1.11 and 0.83, respectively; Table 3). Strong genetic control of the constitutive concentration of most individual MT and SQT compounds was observed, with heritability estimates ranging from 0.49 to 1.30. However, additive genetic variation of terpene concentration in MeJa-induced plants was notably weaker, and only significant for α -pinene, Δ^3 -carene, (+)-3-carene, jupinene, and α -muurolole, with heritabilities between 0.39 and 1.02 (Table 3).

MeJa-induced responses in the LVT concentration and profile

Total LVT and total MT concentrations did not differ between induced and control plants (Table 2; Fig. 1A). The

Table 2. Summary of the mixed model for volatile terpene content found in the primary needles of *P. pinaster* seedlings belonging to 17 open-pollinated families cultivated at two levels of phosphorus availability, and induced by application of MeJa

The family effect (G) and derived interactions are random effects, and the variance components (VC, %) and corresponding likelihood ratio significance tests (χ^2) are shown. Phosphorus availability (P) and methyl jasmonate (MeJa) induction of defences are fixed effects, and the F values and corresponding df are shown. Bold values are significant at $\alpha < 0.05$.

	Monoterpenes			Sesquiterpenes			Total volatile terpenes			
	df (F)	% VC	F/ χ^2	P value	% VC	F/ χ^2	P value	% VC	F/ χ^2	P value
Family (G)		20.5	32.5	0.000	12.6	15.2	0.000	20.8	33.3	0.000
G×P		0.0	0.0		0.0	0.0		0.0	0.0	
G×MeJa		0.0	0.0		0.0	0.0		0.0	0.0	
G×P×MeJa		0.0	0.0		0.0	0.0		0.0	0.0	
Residual		79.5			87.4			79.2		
Block	3, 3		0.7	0.601		3.9	0.146		0.9	0.524
P	1, 3		1.3	0.345		2.2	0.232		1.7	0.286
MeJa	1, 6		0.4	0.578		17.4	0.006		1.2	0.323
P×MeJa	1, 6		0.2	0.698		0.0	0.999		0.2	0.670

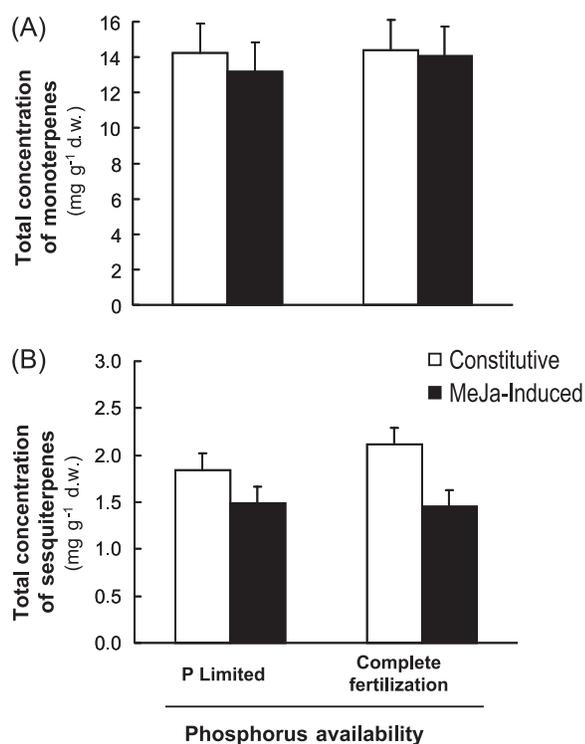


Fig. 1. Constitutive (white bars) and MeJa-induced (black bars) concentration of monoterpenes (a) and sesquiterpenes (b) in the needles of *P. pinaster* seedlings grown in two different phosphorus availabilities. Fertilization had no significant effect, and chemical induction with MeJa significantly reduced sesquiterpene concentration in the two P treatments. Error bar is s.e., $n=72$. Concentrations are expressed on a dry weight basis.

total SQT concentration was reduced significantly in induced plants by 26% (Table 2; Fig. 1B). A significant positive genetic relationship between the MT and SQT concentrations was found, leading to similar ratios across families, but this relationship was altered after MeJa induction (Fig. 2). The intercept of the relationship between

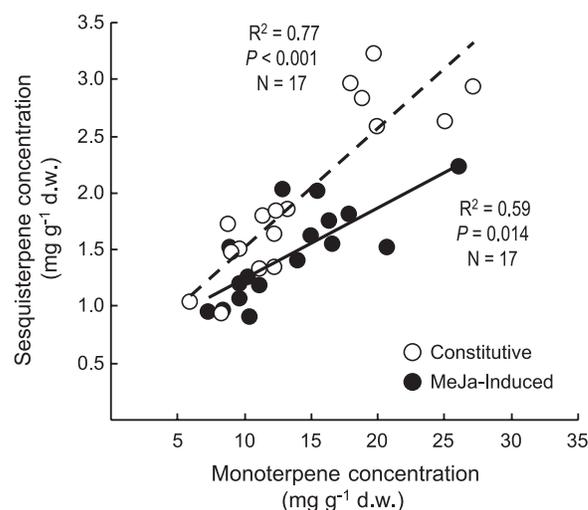


Fig. 2. Family relationship between total concentration of sesquiterpenes and monoterpenes in the needles of *P. pinaster* seedlings under constitutive conditions and after defensive induction with MeJa. The relative contribution of mono- and sesqui-terpenes was similar among families presenting a broad range of terpene concentrations. MeJa induced a shift to lower sesquiterpene concentration, which was consistent among families. Analysis of covariance showed that intercepts were significantly different ($F_{1,31}=15.1$; $P < 0.001$), and slopes were marginally different ($F_{1,30}=4.17$; $P=0.050$). Each point represents an open pollinated family ($n=8$ replicates). Concentrations are expressed on a dry weight basis.

SQT and MT (Fig. 2) was significantly lower for induced plants than for control plants (ANCOVA $F_{1,31}=15.1$; $P < 0.001$) indicating that foliar oleoresin was depleted in SQT following MeJa induction. Slopes of both relationships were only marginally different (ANCOVA $F_{1,30}=4.17$; $P=0.050$). This trend was consistent across families.

Although the same compounds were found in control and in MeJa-induced plants, induced plants had an altered

Table 3. Significance tests of the family effect and corresponding narrow sense heritability estimates for the constitutive (control plants) and induced (MeJa treated plants) concentration of individual volatile terpenes in the needles of 17 *P. pinaster* open-pollinated families. Most of the more abundant terpenes showed high heritabilities. Heritability was calculated only when family effect was significant as the ratio of additive genetic variance ($\sigma_A^2=4\cdot\sigma_F^2$, where σ_F^2 is the family variance) to total phenotypic variance. Bold values are significant at $\alpha < 0.05$.

	Constitutive mode			MeJa-induced mode		
	χ^2	<i>P</i> value	h_i^2	χ^2	<i>P</i> value	h_i^2
Monoterpenes						
α -Pinene	12.7	0.000	0.87	5.2	0.011	0.45
Camphene	0.6	0.213		1.3	0.126	
β -Pinene	16.3	0.000	1.00	1.9	0.085	
β -Myrcene	0.0	0.500		0.0	0.500	
α -Phellandrene	1.8	0.093		0.1	0.407	
Δ -3-Carene	9.1	0.001	0.64	6.6	0.005	0.60
α -Terpinene	0.7	0.199		0.0	0.478	
Limonene	11.8	0.000	0.83	2.6	0.053	
(\pm)-3-Carene	2.2	0.067		3.1	0.038	0.39
α -Terpinolene	8.2	0.002	0.67	1.0	0.154	
Linalyl acetate	4.7	0.015	0.49	0.8	0.180	
Bornyl acetate	7.5	0.003	0.64	0.2	0.349	
Total monoterpenes	17.8	0.000	1.06	3.2	0.037	0.38
Sesquiterpenes						
α -Copaene	12.8	0.000	0.87	0.0	0.500	
Jupinene	26.3	0.000	1.30	16.7	0.000	1.02
<i>Trans</i> -Caryophyllene	14.7	0.000	0.94	1.1	0.145	
α -Humulene	14.9	0.000	0.95	0.4	0.276	
α -Amorphene	0.0	0.500		0.0	0.500	
Germacrene D	6.1	0.007	0.56	0.0	0.500	
α -Muurolole	2.1	0.074		7.8	0.003	0.65
Δ -Cadinene	0.1	0.396		0.2	0.337	
Caryophyllene oxide	0.0	0.500		0.0	0.500	
Geranyl acetate	0.0	0.500		0.2	0.326	
Farnesyl acetate	0.0	0.500		0.7	0.194	
Unknown	0.0	0.447		0.0	0.500	
Farnesyl acetone	0.0	0.500		0.0	0.500	
α -Bisabolene	5.6	0.009	0.54	0.0	0.500	
Total sesquiterpenes	12.2	0.000	0.83	0.1	0.385	
Overall volatile terpenes	19.3	0.000	1.11	3.1	0.039	0.37

terpene profile, as shown by multivariate analysis. Both MT and SQT profiles were affected significantly by MeJa induction (MANOVA; Wilk's $\lambda=0.492$, $P < 0.001$ and Wilk's $\lambda=0.808$, $P < 0.001$ for MT and SQT, respectively).

The MeJa-induced response involved a marked decrease in the absolute concentration of some compounds (mainly α -pinene, but also Δ^3 -carene and β -myrcene; Table 1), that was counterbalanced by a significant increase in the absolute concentration of other compounds (predominately β -pinene, the major volatile terpene identified in our samples, but also linalyl and bornyl acetate; Table 1).

Changes in the concentration of major compounds affected the relative contribution (molar fractions) of individual terpenes to the complex blend of foliar volatiles (Table 1). The relative amounts (molar fractions, Table 1) of α -pinene, β -myrcene, and (+)-3-carene, *trans*-caryophyllene, and α -humulene were reduced significantly after MeJa application. By contrast, the molar fraction of other compounds such as β -pinene, linalyl acetate, and bornyl

acetate were significantly ($P < 0.05$) greater in induced plants than in the constitutive profiles of control plants (Table 1).

MeJa-induced alteration of the isomeric ratio of α - to β -pinene

The application of MeJa had contradictory effects on the biochemistry of the two major constituents of leaf MT pool, the isomers α - and β -pinene (Table 1). Although the joint contribution of both isomers was similar in induced and control plants ($F_{1,6}=0.087$; $P=0.783$), the ratio of α - and β -pinene was altered significantly following the application of MeJa ($F_{1,6}=64.9$; $P < 0.001$). MeJa application favoured the accumulation of β -pinene at the expense of α -pinene. Moreover, strong positive family relationships between the concentrations of α - and β -pinene (Fig. 3), both in constitutive and in induced mode were found (Fig. 3). Slopes of the α -/ β - pinene in control and MeJa-induced

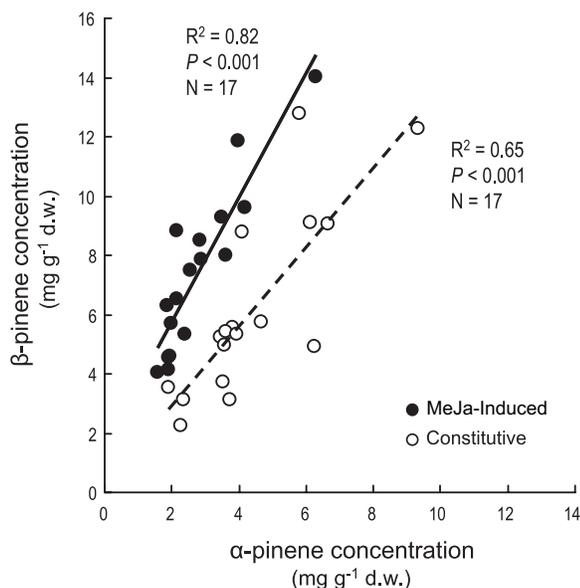


Fig. 3. Family relationships between concentration of β -pinene and α -pinene in the needles of *P. pinaster* seedlings under constitutive conditions and after defensive induction with MeJa. Both relationships were strong, positive, and consistent among families. Induction with MeJa shifted upwards the relationships. Analysis of covariance showed that intercepts were significantly different ($F_{1,31}=33.87$; $P < 0.0001$) but slopes were not different ($F_{1,30}=3.56$; $P=0.069$). Each point represents an open pollinated family, $n=8$. Concentrations are expressed on a dry weight basis.

plants did not differ but intercepts were significantly different (Fig. 3), indicating that the relationship between both isomers at the family level significantly shifted upwards after the induction with MeJa (Fig. 3).

Discussion

Lack of response of LVT to P availability

Phosphorus limitation reduced seedling growth by 57% and leaf P concentration by 52% in our study. Leaf P concentrations were similar to those found in an extensive field survey of 25 *P. pinaster* stands in Galicia, that recorded leaf P concentrations from 0.62 g to 1.88 g mg^{-1} (Martins *et al.*, 2009). Leaf P concentrations in the P2 treatment were within the levels considered 'marginally deficient' in field evaluations, and those in the high P treatment were within the 'satisfactory' levels suggested by Bonneau (1995).

Although P limitation increased the concentration of resin in the stem and phenolics in the foliage of the pine seedlings from this study (X Moreira *et al.*, unpublished results), P availability had no apparent effect on LVT concentration or the LVT profile. This trend was consistent for all families, regardless of the defensive mode (constitutive or MeJa-induced). Therefore, these results contradict our initial hypothesis that LVT concentrations would be

plastic to resource availability, with higher concentrations produced under nutrient limitation due to greater investment in plant defences.

From a physiological point of view, it is commonly accepted that increased growth rates due to fertilization can trade-off with carbon allocation to secondary metabolites, leading to reduced concentration of chemical defences in tissues of fast growing genotypes or plant species (Bryant *et al.*, 1983; Coley *et al.*, 1985; Peñuelas and Estiarte, 1998). Growth is predominant over defensive investments when resources are abundant, and the production of secondary metabolites reaches a maximum when resource availability is suboptimal for primary growth (Koricheva *et al.*, 1998). In a broader evolutionary context, optimal allocation models predict decreasing investment in defences with increasing resource availability, because the reduced cost of tissue production could compensate the risks of tissue lost due to herbivore predation (Zangerl and Bazzaz, 1992; Van Dam *et al.*, 1996; Zangerl and Rutledge, 1996).

In conifers, these theoretical models are a good explanation for the allocation to secondary metabolites in xylem and phloem. Reduced levels of stem resin have been found to be associated with greater growth rates due to fertilization (Wainhouse *et al.*, 1998). Lower densities of phloem resin canals and reduced resistance against herbivores in fertilized and fast-growing plants were also observed in *P. pinaster* and *P. radiata* seedlings at field (Zas *et al.*, 2006, 2008; Moreira *et al.*, 2008). However, secondary chemistry is not necessarily consistent throughout a plant (Van Dam *et al.*, 1996; Kainulainen *et al.*, 2000) and leaf terpene response to environmental variation could differ from those in other tissues, exhibiting positive, neutral, and even negative responses to increased nutrient availability (Peñuelas and Staudt, 2010). Our results are in agreement with those from a few controlled experiments that found N and P availability to have no effect on LVT in *Pinus sylvestris*, *Picea abies*, and *Pinus halepensis* (Heyworth *et al.*, 1998; Barnola and Cedeño, 2000; Ormeño *et al.*, 2008), or to cause a minor reduction in monoterpene content in adult leaves, but not in growing leaves of fertilized trees (Kainulainen *et al.*, 1996; Blanch *et al.*, 2009).

The lack of influence of P availability on LVT concentration could be due to the fact that LVT are not only direct chemical defences, but also indirect defences through their emission as relevant infochemicals during regular stomatic functioning. Genotypes with greater LVT concentration would be better defended, but could also emit more LVT into the environment, with possible undesired ecological consequences such as enemy attraction. Thus pine seedlings that do not spread airborne information about their nutritional or immune status that might attract their enemies could be favoured in determinate environments, something that could be achieved through the active maintenance of the homeostasis in LVT concentration.

An alternative explanation for these results, according to models of optimal allocation of defences, could be that leaves in young pine seedlings have to be extremely well defended due to their great construction costs and major

relevance for seedling growth, performance, and fitness (Zangerl and Berenbaum, 1987; Zangerl and Rutledge, 1996; Ohnmeiss and Baldwin, 2000). Thus, the LVT content in primary needles of pine seedlings would be maximized, regardless of their nutritional status.

In conclusion, leaves of P-limited pine seedlings were not better defended with terpenes, in terms of LVT concentration or profile, than those receiving adequate P fertilization, and the responses were consistent among the 17 pine families. Further experiments should confirm these results in mature trees and determine whether terpene emission patterns are affected by nutrient availability.

Quantitative MeJa-induced responses in the LVT

A minor reduction in the content of SQT in induced plants and no quantitative changes in the content of MT after MeJa-induction were found. These results agree with other studies that report minor or no alterations in LVT in induced Douglas-fir and spruce seedlings, even when major quantitative or qualitative changes were found in other tissues of those plants such as stem wood and roots (Viiri *et al.*, 2001; Turtola *et al.*, 2002; Huber *et al.*, 2005; Zeneli *et al.*, 2006). By contrast, significant accumulation of volatile terpenes in the leaves (although still small relative to those found in other tissues) but with no changes in the terpene pattern were reported in spruce and Scots pine 20 weeks after MeJa application (Martin *et al.*, 2003; Holopainen *et al.*, 2009).

The minor effects of MeJa found on LVT do not imply methodological problems, as MeJa was effective at increasing the content of resin in the stem and of phenolics in the foliage, and also in reducing the growth of pine seedlings from this study (Moreira *et al.*, unpublished data). Large increases in xylem and phloem oleoresin have usually been reported by other authors after MeJa application (reviewed by Bohlmann, 2008). Thus, our results suggest that the responses of secondary chemistry to herbivory in leaves differs from that in stems (Van Dam *et al.*, 1996; Kainulainen *et al.*, 2000) and it is possible that constitutive protection of leaves is close to the maximum possible, leaving only a small margin for induction.

Increased emission of terpenes has also been suggested as an explanation for the lack of terpene accumulation in the leaves of induced conifers (Litvak and Monson, 1998). Another possibility is that pine leaves are structurally limited in their capacity for greater terpene foliar concentrations because of the absence of induced traumatic resin canals within the needles (Huber *et al.*, 2005; Krokene *et al.*, 2008). Further research using isotope labelling could address the former possibility.

MeJa-induced responses in the profile of LVT

MeJa-induced response in LVT 25 d after MeJa application was more qualitative than quantitative. An effective herbivore-induced defensive strategy could be to produce more toxic terpenoids, but in this study no changes were observed

in known toxic compounds such as limonene, camphene, and α -pinene (Zou and Cate, 1995; Lindgren *et al.*, 1996; Thoss and Byers, 2006). However, changes were observed in the relative contribution (molar fractions) of major individual terpenes such as *trans*-caryophyllene, α -humulene, linalyl and bornyl acetate, β -myrcene, and (+)-3-carene, which can be ecologically important since their relative contribution determines the terpene fingerprint and thus the biological meaning of the airborne message when LVT are released into the environment. The main effect induced by MeJa was the depletion of α -pinene and the relative enrichment of β -pinene, without alteration of the total needle MT concentration. Reducing the relative contribution of known insect attractants (Tomlin *et al.*, 1997; Pureswaran *et al.*, 2004) in the blend of foliar terpenes could be a beneficial induced response, because it would reduce the risk of further herbivory whilst maintaining the levels of chemical defences within the leaves.

The significant induced responses to the application of MeJa (changes in the ratio of mono/sesquiterpenes and ratio of α/β pinene) were consistent across families, with all families sharing the same directionality and proportionality of the response. Further research would address the possible benefits of these observed responses for direct and indirect resistance of pine seedlings. If the observed changes in the ratio of various terpenes are adaptive, it is hypothesized that the ecological cost of airborne signalling, considering the antagonistic and synergistic ecological effects derived from the same airborne message (Raffa, 2001; Heil, 2008) is more important than the direct antiherbivory benefits of increasing LVT concentrations.

Genetic additive variation and control of LVT

This study demonstrates that large additive genetic variation, with high narrow sense heritabilities, for both total terpene concentration in foliage and concentrations of several individual terpenes exists in Maritime pine. The existence of resin-producer varieties of Maritime pines and of geographical differentiation in the terpene profiles of the stem resin flow has been reported before (Baradat *et al.*, 1972; Arrabal *et al.*, 2005). Concentration of individual terpenes seems to be regulated by only a few genes (Phillips and Croteau, 1999) with an additive inheritance model (Jactel *et al.*, 1996; Nault *et al.*, 1999; O'Reilly-Wapstra *et al.*, 2006).

The not significant Family \times MeJa interaction for total, MT and SQT seems to suggest that plasticity to biotic factors is not under genetic control. However, considering that terpene concentrations in induced plants represent the sum of the pre-existing constitutive response plus that resulting from the induced response, the reduction in genetic control over the constitutive concentration of several major terpenes (such as β -pinene) after MeJa induction suggests the existence of genetic variation in the inducibility, that would be counteracting the genetic variation in the constitutive levels of LVT (Agrawal *et al.*, 2002).

Our results indicate that predictions of environmental modulation of plant defence in conifers are not appropriate in describing volatile terpene production and accumulation in the foliage of Maritime pine seedlings. MeJa-induced responses in the foliage of Maritime pine seedlings differed from those reported for other plant parts such as the stem and roots. Benefits for direct anti-herbivore resistance due to increasing LVT concentration could be counterbalanced by ecological costs resulting from greater terpene emission from the foliage. It is likely that these costs are constraining the plasticity of volatile terpenes in leaves, but not in stems and other tissues with lower emitting potential.

In summary, phosphorus availability affected growth and foliar P concentration but not the concentration of LVT. MeJa application induced changes in the terpene profile (mainly by depletion of α -pinene) but had a negligible effect on the concentration of LVT (by reducing the amount of SQT). Genetic variation appears to be the main source of phenotypic variation in leaf volatile terpenes of this pine species.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Chemical composition of the solutions used as fertilizers.

Supplementary Table S2. Multivariate analysis (MANOVA) of leaf volatile terpene profiles as affected by P availability and MeJa-induction.

Supplementary Fig. S1. Genetic variation in the concentration of leaf volatile terpenes in 17 *P. pinaster* open-pollinated families.

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