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RESEARCH PAPER

Effects of phosphorus availability and genetic variation of leaf terpene content and emission rate in *Pinus pinaster* seedlings susceptible and resistant to the pine weevil, *Hylobius abietis*

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Keywords

Galicia; herbivory; maritime pine; nutrient stress; plant resistance to insects; plant–insect interactions.

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ABSTRACT

We studied the effects of phosphorus fertilisation on foliar terpene concentrations and foliar volatile terpene emission rates in six half-sib families of *Pinus pinaster* Ait. seedlings. Half of the seedlings were resistant to attack of the pine weevil *Hylobius abietis* L., a generalist phloem feeder, and the remaining seedlings were susceptible to this insect. We hypothesised that P stress could modify the terpene concentration in the needles and thus lead to altered terpene emission patterns relevant to plant–insect signalling. The total concentration and emission rate ranged between 5732 and 13,995 $\mu g \cdot g^{-1}$ DW and between 2 and 22 $\mu g \cdot g^{-1}$ DW$\cdot h^{-1}$, respectively. Storage and emission were dominated by the isomers α- and β-pinene (77.2% and 84.2% of the total terpene amount amassed and released, respectively). In both resistant and susceptible families, P stress caused an increase of 31% in foliar terpene concentration with an associated 5-fold decrease in terpene emission rates. A higher terpene content in the leaves implies that the 'excess carbon', available under limiting growth conditions (P scarcity), is allocated to terpene production. Sensitive families showed a greater increase in terpene emission rates with increasing P concentrations, which could explain their susceptibility to *H. abietis*.

INTRODUCTION

Phosphorus (P) has many roles in plant growth and metabolism. One of the principal functions of P is energy transfer through the action of adenosine triphosphate (ATP). ATP and its derivatives ADP and AMP are involved in different aspects of energy transfer in all growing plant tissues. Apart from this global function, P is also necessary for assembling nucleic acids (DNA and RNA), proteins, enzymes and carbohydrates. It plays an essential role in photosynthesis and is involved in the formation of sugars and starch. The various roles of P denote its relevance in many vital processes, such as the formation of seeds or the development of roots. It also speeds up plant maturation and helps the plant to resist stress (Urbano 1999).

Fertilisation of young pine seedlings and the subsequent boosting of primary growth rates could, however, lead to increased susceptibility to pests and diseases by altering patterns of energy allocation to growth and defence and/or improving tissue quality for herbivorous insects. In this vein, Zas *et al.* (2006a, 2008), in a field study of seedlings of *Pinus pinaster* Ait and *Pinus radiata* D. Don, found that traditional silvicultural practices such as P fertilisation could lead to seedlings having greater susceptibility to the pine weevil *Hylobius abietis*. This could at least be partially explained by a reduction in seedling resistance (Moreira *et al.* 2008). The pine weevil *H. abietis* is a generalist phloem feeder and is a major pest in conifer plantations throughout Europe, where it causes significant regeneration problems by feeding on the bark of young pine seedlings (Leather *et al.* 1999; Conord *et al.* 2006). The susceptibility of *P. pinaster* to this insect has been found to be under strong genetic control, with some pine families consistently more damaged than others (Zas *et al.* 2005). Moreover, it has been shown that *H. abietis* might be attracted by some monoterpenes, such as α-pinene (Moreira *et al.* 2008).

Increased nutrient availability could directly increase the nutritional value of plant tissues and thus increase the insects' preference for them (Ayres & Lombardero 2000; Moreira *et al.* 2009). Phosphorus fertilisation of P-stressed pine seedlings may also diminish the allocation of energy to constitutive and induced defences by favouring growth. Several models of plant resistance suggest altered patterns of allocation to chemical defences in environments with increased nutrient availability. The carbon nutrient balance (Bryant *et al.* 1983) states that when growth is limited by nutrients, plants allocate the 'excess carbon' to the production of secondary metabolites. The growth differentiation

balance (Lorio 1986) recognises that all secondary metabolites have an ontogenetically determined phenology and their synthesis is enhanced during periods of plant differentiation. Growth dominates under favourable conditions, and differentiation is at a maximum only when conditions are suboptimal for growth. The optimal allocation model (Tuomi *et al.* 1991) predicts a decreased investment in defence with increasing resource availability, because the reduced cost of tissue production could compensate for the higher risk of herbivore predation. High P availability could also lead to an enhancement of the appearance of the fertilised plants to the insect. Furthermore, changes in leaf-contained organic compounds due to fertilisation can be translated into changes in the amount of carbon-based secondary volatile compounds emitted, as stated in the 'excess carbon' hypotheses (Peñuelas & Estiarte 1998), thus altering plant–animal interactions.

Maritime pine (*P. pinaster*) has been widely chosen for forestation in Galicia (NW Spain) since the 18th century. Despite being partly replaced in the last decades by species with higher production, such as *P. radiata* and *Eucalyptus globulus*, *P. pinaster* is still the most important forest tree species in Galicia. According to the last forest survey (DGCN 2000), Galicia contains more than 500,000 ha of pure and mixed *P. pinaster* stands, which represents around 44% of the total Galician wooded area. The intensive silviculture applied to *P. pinaster* stands in Galicia entails short rotations (15–45 years), during which there is a considerable extraction of nutrients from the system (Merino *et al.* 2003).

Maritime pine plantations in Galicia commonly suffer substantial nutrient deficiencies (Martins *et al.* 2009). These plantations are usually located on acid and sandy soils with low amounts of available nutrients, especially P. Moreover, the loss of nutrients through harvesting can lead to decreased reserves of available nutrients in soil (Dambrine *et al.* 2000; Merino *et al.* 2003). Under these conditions, P is one of the most limiting factors for growth in *P. pinaster* stands in NW Spain (Martins *et al.* 2009).

Previous studies suggest that when P is readily available there could be an alteration in the concentration (Kainulainen *et al.* 1996; Powell & Raffa 1999) and emission rate (Fares *et al.* 2007) of defensive secondary products such as terpenes due to different allocation to secondary metabolites, depending on the developmental state of the plant, the biosynthesis pathway, cost of synthesis and storage of compounds.

The main objective of the present study was to determine the effect of P availability on the content and emission rate of leaf volatile terpenes. We hypothesised that P availability could modify the terpene concentration in needles and the photosynthetic activity of *P. pinaster* thus leading to altered terpene emission patterns relevant to plant–insect signalling. Additionally, we aimed to study the role of genetic variation in resistance to pests. To these ends, we analysed the effect of P fertilisation on terpene concentrations and terpene emission rates in half-sib families of *P. pinaster* seedlings cultivated under controlled conditions. These seedling families had previously been found to be resistant or susceptible to the large pine weevil under field conditions in Galician forests.

MATERIAL AND METHODS

Experimental design and plant material

We performed a two factorial experiment with different pine genetic entities and P fertilisation treatments under controlled conditions. The experimental layout was a randomised split-plot design replicated in three blocks, with four P fertilisation treatments acting as the whole factor and six genetic entities as the split factor. In total, we sampled 72 pine seedlings, corresponding to three blocks ×4 P fertilisation treatments ×6 genetic entities nested into two groups according to their susceptibility to attack by the pine weevil (*H. abietis*), namely, 'susceptible' and 'resistant' families.

The *P. pinaster* families belonged to six half-sib families (open-pollinated, known mother trees), all of which were native to the coastal region of Galicia (NW Spain). Three families were previously recognised to be susceptible to attack by pine weevil (*H. abietis*) in an extensive field study, while the other three families appeared to be more resistant to this pest (Zas *et al.* 2005). Damage (debarked area by the pine weevil) to the susceptible families in that field study was more than double that suffered by the resistant families (Zas *et al.* 2005).

Greenhouse conditions and experimental fertilisation

On 7 February 2006, *P. pinaster* seeds were individually sown in 2-l pots containing perlite in a glass greenhouse (36.5-m long and 15-m wide) with controlled temperature (10 and 22 °C, night and day, respectively) and daily water irrigation.

On 15 March 2006, we started to apply the fertilisation treatments using sub-irrigation (every 2 days) with four different fertilisation treatments. The complete balanced fertiliser (P20) was prepared according optimum requirements for maritime pine growth, containing 100 ppm N, 20 ppm P, 40 ppm K, 10 ppm Ca, 20 ppm Mg, and the necessary amounts of micronutrients and trace elements. The other fertiliser solutions (P10, P5 and P2) differed only in the concentration of P, which was reduced to 10, 5 and 2 ppm, respectively, in order to promote growth restriction by increasing P limitation. The pH values were adjusted to 6.5 in all solutions. Fertiliser solutions were replaced every 2 weeks. The experiment was carried out in the facilities of CIF Lourizán (Pontevedra, NW Spain, UTM coordinates 29T, 42°24′33′ N, 8°39′47′ W).

Photosynthetic activity and terpene emission collection

Photosynthetic activity and terpene emissions were measured as described in Blanch *et al.* (2009). On 24–27 July 2006, measurements of net photosynthetic rates, stomatal conductance and terpene emissions were conducted. These measurements were done under controlled standard conditions (30 °C and 1000 $\mu M \cdot m^{-2} \cdot h^{-1}$ PAR). The CO_2 exchange was measured using a non-dispersive infrared gas analyser (IRGA), model ADC-LCPro+ (ADC Inc., Hoddesdon, Hertfordshire, UK) connected to a conifer leaf chamber (ADC). CO_2 uptake (A) and stomatal conductance (g_s) were measured in lateral shoots of *P. pinaster*. A and g_s values were expressed on a projected leaf area basis as measured with a Li-Cor 3100 Area Meter (Li-Cor Inc., Lincoln, NE, USA).

In order to sample terpene emissions, a T-system was installed outside the cuvette of the IRGA-porometer. We used a calibrated air sampling pump at constant flow (Q_{max}; Supelco, Bellefonte, PA, USA) to trap isoprenoids by passing part of the air through cartridges (8-cm long and 0.3-cm internal diameter) filled with terpene adsorbents Carbopack B, Carboxen 1003 and Carbopack Y (Supelco) separated by plugs of quartz wool. The sampling time was 5 min, and the flow varied between 470 and 500 ml·min^{-1}, depending on the tubes' adsorbent, quartz wool packing. The hydrophobic properties of the tubes were designed to minimise sample displacement by water. In these tubes, terpenes did not suffer any chemical transformation, as checked with standards (α-pinene, β-pinene, camphene, myrcene, p-cymene, limonene, sabinene, camphor and dodecane). Prior to use, these tubes were conditioned for 10 min at 350 °C with a stream of purified helium. The trapping and desorption efficiency of liquid and volatilised standards such as α-pinene, β-pinene or limonene was practically 100%. In order to eliminate the problem of a 'memory' effect of previous samples, blanks of 5-min air sampling without plants were carried out immediately before and after each measurement. The glass tubes were stored in a portable fridge at 4 °C and taken to the laboratory, where they were stored at −28 °C until analysis (within 24–48 h). There were no observable changes in terpene concentrations after storage of the tubes, as checked by analysing replicate samples immediately and after 48-h storage. Emission rate calculations were made on a mass balance basis and by subtracting the control values (without plants) from the values of samples with plants.

Seedling harvest and nutrient analyses

On 1 August 2006, we measured height and basal diameter (mean of two measures). A composite sample of primary needles from different parts of each tree was collected, frozen and preserved at −80 °C in tightly closed glass vials for the analysis of foliar terpene content. Then, the pines were destructively sampled, and roots, stems and mature and young needles from each seedling were carefully separated, dried for 72 h at 65 °C and weighed to the nearest 0.001 g. The needle samples were finely ground, labelled and preserved for nutrient analysis.

For the analysis of N and P content, 0.3 g of needles was digested in a mixture of selenous sulphuric acid and hydrogen peroxide (Walinga *et al.* 1995). Nitrogen was colorimetrically analysed in diluted aliquots of this digestion using a BioRad 680 microplate reader (BioRad, Hercules, CA, USA) at λ = 650 nm, according the method proposed in Sims *et al.* (1995). Phosphorus was analysed in the same diluted aliquots using inductively coupled plasma optical emission spectroscopy (ICP-OES) with a Perkin-Elmer Optima 4300DV (Perkin-Elmer, MA, USA) in the central laboratory facilities at Universidade de Vigo – CACTI (http://www.webs.uvigo.es/cactiweb/). Nitrogen and P concentrations were expressed in milligram per gram dry weight (DW) of tissue.

Terpene analysis

Terpene analyses were conducted as described in Blanch *et al.* (2009): tubes with trapped emitted monoterpenes were inserted in an OPTIC3 injector (ATAS GL International BV 5500 AA, Veldhoven, The Netherlands) connected to a Hewlett Packard HP59822B GC-MS (Hewlett Packard, Palo Alto, CA, USA), where they were desorbed at 250 °C for 3 min. Terpenes were separated using a TRB-5 fused silica capillary column, 30 m × 0.25 mm × 0.25 μm film thickness (Teknokroma, Barcelona, Spain). After sample injection, the initial temperature (40 °C) was increased at 30 °C·min^{-1} up to 60 °C, and thereafter at 10 °C·min^{-1} up to 150 °C maintained for 3 min, and thereafter at 70 °C·min^{-1} up to 250 °C, which was maintained for another 5 min. Helium flow was 1 ml·min^{-1}. The identification of terpenes was conducted using GC-MS and compared with standards from Fluka (Buchs, Switzerland), literature spectra and GCD Chemstation G1074A HP with the Wiley275 library. Terpene calibration curves (for four different terpene concentrations) were always significant ($r^2 > 0.99$). The most abundant terpenes had very similar sensitivity (differences were <5%). The terpene concentration was referred to the needle DW.

For extraction of resin terpenoids in the needles, three to four needles were ground under liquid nitrogen in Teflon tubes with a Teflon embolus. Then, we added 1 ml pentane as extractant and 0.1 μl dodecane, a non-terpenoid internal standard. Teflon tubes with pentane samples were centrifuged in an ultrasonic bath for 5 min at 5000 *g* and 5–10 °C to separate the liquid and solid phases. Pentane extracts were immediately recovered and transferred to chromatography glass vials. After recovering the pentane extract, the mass of the needle pellet was determined by oven-drying at 65 °C for 4 days. Terpenes in the extract were analysed using a GC-MS (Hewlett Packard) with a robotic sample processor (FOCUS) (ATAS GL International, Veldhoven, The Netherlands). Separation, quantification and identification were performed as described above.

Statistical analyses

All traits were analysed with the following model: $Y_{ijkl} = \mu + B + P + R + G(R) + P \times G(R) + P \times R + B \times R + B \times P + \varepsilon_{ijk,}$ where Y_{ijkl} is the variable of the trait, μ is the overall mean, B, P, R and G are the main fixed effects of block, P fertilisation, resistance group and genotype, and ε_{ijk} is the experimental error. Genotype was nested within resistance types G(R). The B × P interaction was considered a random factor to accurately analyse the split-plot design (Littell *et al.* 2006). The MIXED procedure of SAS was used. When main effects were significant, differences among treatment means were tested for significance using the LSMEAN statement. The PROC GLM procedure of SAS was used for the MANOVA analyses; Wilk's lambda statistics were used.

RESULTS

Plant growth and needle nutrient concentrations

Fertiliser treatments strongly affected plant growth ($F = 20.82$, $P < 0.001$) and P concentration in plant tissues ($F = 141.39$, $P < 0.001$) (Table 1). Plants with complete fertilisation (P20) produced around 2.5-fold more biomass than plants with lower P fertilisation (Fig. 1). Phosphorus concentration in needles was strongly influenced by fertilisation,

Table 1. Summary of the split-plot model for P and N concentration in needles, total biomass, net photosynthetic rates, stomatal conductance, transpiration rates, total terpene content and total terpene emission rates of *P. pinaster*. B, P, R and G are the main effects of block, fertilisation, resistance and genotype. Genotype was nested in resistance G(R). Bold values indicate significant statistical differences.

	DF	DF	P needles		N needles		total biomass		net photo-synthetic rate		stomatal conductance		transpiration rate		total terpene concentration		terpene emission rate	
	num	den	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
B	3	9	1.19	0.3661	0.89	0.4841	1.81	0.2151	0.01	0.9981	0.07	0.9730	0.17	0.9151	0.48	0.7038	1.61	0.2544
P	3	9	141.39	**<0.0001**	5.97	**0.0160**	20.82	**0.0002**	4.48	**0.0347**	2.58	0.1179	6.12	**0.0148**	4.25	**0.0396**	9.76	**0.0034**
R	1	33	7.79	**0.0087**	3.16	0.0846	2.73	0.1081	1.30	0.2623	0.28	0.5977	0.19	0.6685	0.22	0.6456	19.48	**0.0001**
G(R)	4	33	1.15	0.3526	0.78	0.5483	2.95	**0.0345**	2.72	**0.0460**	10.23	**<0.0001**	1.76	0.1603	4.16	**0.0078**	16.78	**<0.0001**
P × G(R)	12	33	1.85	0.0793	0.89	0.5632	1.94	0.0659	2.03	0.0539	2.70	**0.0118**	1.49	0.1788	1.99	0.0584	3.56	**0.0028**
P × R	3	33	3.75	**0.0201**	1.45	0.2462	4.53	**0.0091**	7.35	**0.0007**	6.17	**0.0019**	5.08	**0.0053**	0.94	0.4329	5.32	**0.0046**
B × R	3	33	0.21	0.8917	0.68	0.5697	1.52	0.2271	0.79	0.5106	0.88	0.4595	0.38	0.7658	1.05	0.3823	2.47	0.0813

showing increasing values in accordance with the P fertiliser level. Plants under balanced fertilisation had P concentrations around 3-fold higher than P-stressed plants (Fig. 1). The only treatment that drove the P concentration in needles below critical levels was P2. This treatment was therefore the one that generated the clearest P deficiency.

Nitrogen concentration in needles was only slightly higher, but significantly ($F = 5.97$, $P < 0.05$), in complete fertilisation than in P-stressed plants (Table 1, Fig. 1).

Those pine families that were resistant to insect attack in the field contained slightly higher concentrations of P ($F = 7.79$, $P < 0.01$) in leaf tissues than the susceptible families, but there were no differences in terms of N concentration ($F = 3.16$, $P > 0.05$) and total biomass ($F = 2.73$, $P > 0.05$) (Table 1, Fig. 1).

Photosynthesis, stomatal conductance and transpiration rates

Fertiliser treatments decreased photosynthesis ($F = 4.48$, $P < 0.05$) and transpiration ($F = 6.12$, $P < 0.05$) (Table 1, Fig. 2): complete fertilisation (P20) produced lower A and E values than the lowest fertiliser treatment P2 (Fig. 2). However, these effects differed between resistant and sensitive families, as revealed by the strong interaction P × R (Table 1) for A, g_s and E. Sensitive families had the lowest values of A and E in the P10 treatment, and resistant families had the lowest values of A and E in the P20 treatment. There were significant differences between families in photosynthesis ($F = 2.72$, $P < 0.05$) and stomatal conductance ($F = 10.23$, $P < 0.001$) (Table 1).

Volatile terpenes

Several mono- and sesquiterpenes were found in both leaves and terpene emissions. The relative amounts of the different compounds as a percentage of the total amount are shown in Table 2. The isomers α- and β-pinene dominate both leaf accumulation (77.2%) and emission (84.2%) of the total terpene amount. Δ^3-carene is also present at high amounts, accounting for 14.3% of the leaf concentration and 5% of the emission rate. The remaining compounds appeared in smaller percentages (Table 2). The mean leaf terpene concentrations ranged from an average of 7.9 $mg \cdot g^{-1}$ in P20 to an average of 12.6 $mg \cdot g^{-1}$ in P2 (Fig. 3). The mean emission rates were, however, fairly high, ranging from 2.5 $\mu g \cdot g^{-1} \cdot h^{-1}$ (P2) to 16 $\mu g \cdot g^{-1} \cdot h^{-1}$ (P20) (Fig. 3).

MANOVA analysis for content of the individual compounds showed significant differences among P treatments ($\lambda = 0.15$, $P < 0.01$), resistance types ($\lambda = 0.44$, $P < 0.01$) and genotypes ($\lambda = 0.03$, $P < 0.0001$), but there was no significant effect of P × R. Thus, the different P treatments influenced not only the individual compound concentrations but also the whole terpene profile of our samples (Table 3).

Total leaf terpene concentration significantly increased with P deficiency ($F = 4.25$, $P < 0.05$) (Table 1, Fig. 3). In contrast, total terpene emission rates significantly decreased with P deficiency ($F = 9.76$, $P < 0.01$) (Table 1, Fig. 3). This effect was much higher in the sensitive than in the resistant families (P × R interaction $F = 5.32$; $P = 0.0046$; Fig. 3). There was a strong effect of family within resistance ($F = 26.78$, $P < 0.0001$) and of the interaction P × G(R) ($F = 3.56$, $P = 0.0028$) for terpene emission rates, demonstrating that different families show different behaviours. Not all sensitive families increased their emission significantly under high P availability.

DISCUSSION

Terpene content and emission

The observed mean terpene concentrations in *P. pinaster* needles were lower than those reported in other studies for the same species (Arrabal *et al.* 2005) or in other pine species (*e.g.*, Blanch *et al.* 2009). In contrast, the mean terpene emission rates were higher than values found in the literature (*e.g.*, 0.2 $\mu g \cdot g^{-1} \cdot h^{-1}$ reported by Simon *et al.* 1994). These differences could be due to the distinct environmental conditions during the measurements, which were relatively warmer in our experiment compared with those in other studies (Kesselmeier & Staudt 1999). The low concentration in needles and the relatively high emission rates observed in the present study suggest that *P. pinaster* tends to emit monoterpenes instead of keeping them within leaf terpene pools.

Our results, where α- and β-pinene were 77.2% and 84.2% of the total emissions and leaf concentrations, respectively (Table 2), are consistent with previous studies that have shown that α- and β-pinene are the principal terpenes emitted (Simon *et al.* 1994) and accumulated (Arrabal *et al.* 2005; Ormeño *et al.* 2009) by *P. pinaster*. Apart from being the terpenes with the highest concentrations, α- and β-pinene vapour pressure is two to three times higher than the other emitted terpenes, and their reaction rate constants with O_3,

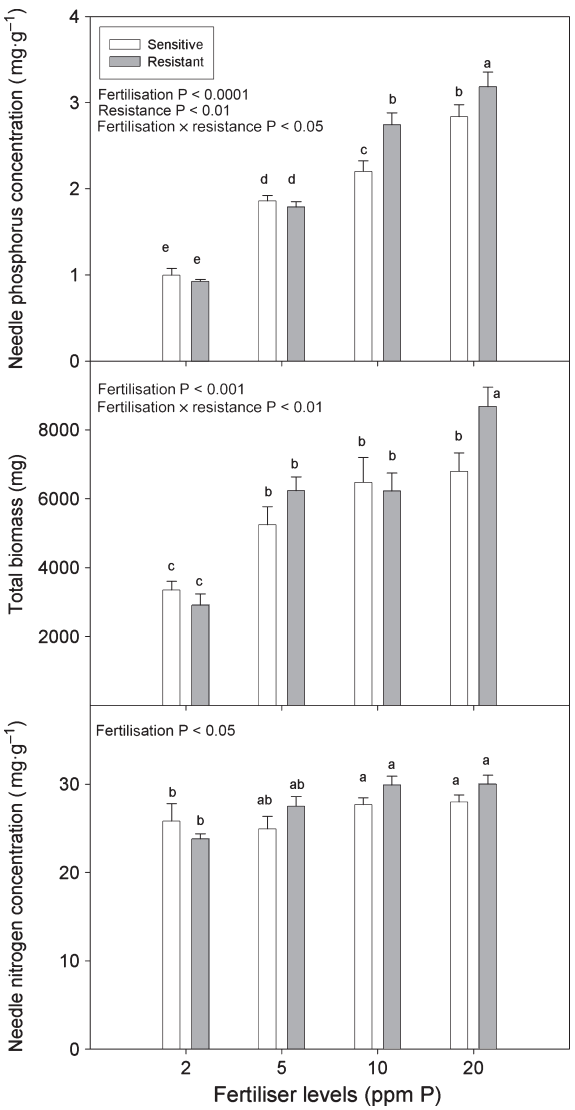

Fig. 1. Nitrogen and phosphorus concentrations in needles and total biomass for different P fertiliser treatments and resistance family groups. Vertical bars indicate standard error of the mean (n = 9). Different letters indicate significant statistical differences among fertiliser levels.

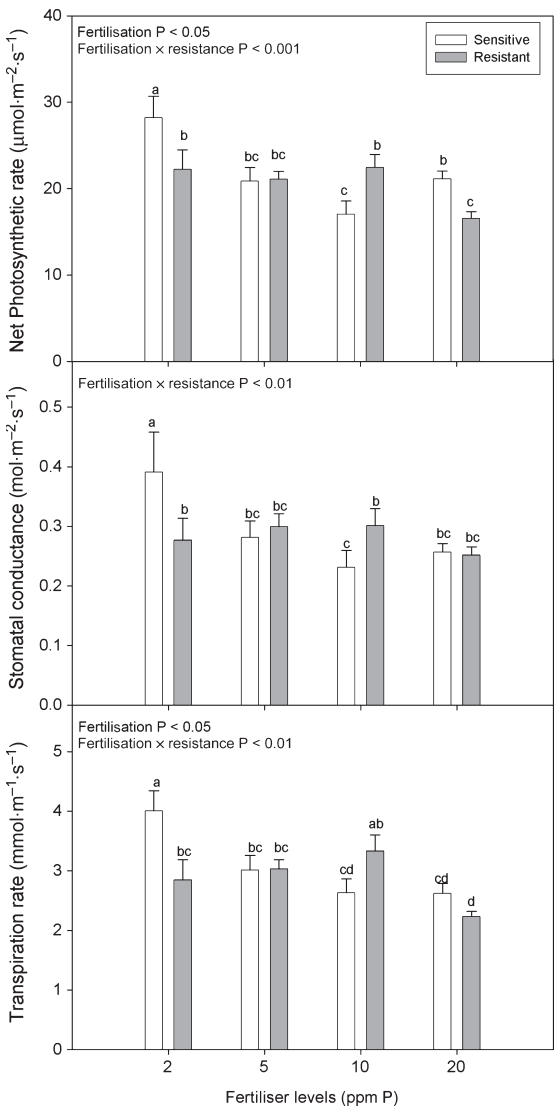

Fig. 2. Net photosynthetic rates, stomatal conductance and transpiration rates for different P fertiliser treatments and resistance family groups. Vertical bars indicate standard error of the mean (n = 9). Different letters indicate significant statistical differences among fertiliser levels.

OH^- and NO_3^- are lower (Atkinson 1990). These results together this demonstrate their key role in emissions and their presence in the environment.

Phosphorus and genetic effects on photosynthesis, terpene content and emission

Phosphorus concentration in needles was above the P deficiency level proposed from field studies (Bonneau 1995) in plants, with P5, P10 and P20 fertiliser levels. Hence, our fertilisation levels ranged from high to low, but were always within the normal physiological margin. The fertilisation treatment was significantly effective: the higher the fertiliser dose, the higher the concentration of P in the plant, as previously also reported (Keay *et al.* 1968). Moreover, P fertilisation also increased the biomass of fertilised plants (Fig. 1), in accordance with the growth response to P fertilisation on P impoverished soils observed in the field (Martins *et al.* 2009). Curiously, photosynthesis rates decreased significantly with higher P doses ($P < 0.01$) and stomatal conductance and transpiration showed a non-significant tendency ($P < 0.1$) to decrease with higher P doses (Fig. 2). Despite the fact that P plays an essential role in photosynthesis, and is involved in the formation of sugars and starch (Urbano 1999), previous authors have also reported negative correlations between P fertilisation and photosynthesis (Cheaib *et al.* 2005). Warren & Adams (2002) suggested that the lack of photosynthetic response to P supply is the result of a deficiency in N, induced by the high P supply.

Resistant and non-resistant pine families showed opposite responses to initial P deficiency. This contrast may arise from different nutrient use efficiency of the two groups. This accords with reports in many tree species showing genetic differences in nutrient use efficiency in response to fertilisation (*e.g.*, Baligar *et al.* 2001; Zas *et al.* 2006b, 2008).

Table 2. Total and percentage terpene concentration (n = 68) and emission rate (n = 70) for all families and all treatments.

	terpene concentration		terpene emission	
	$\mu g \cdot g^{-1}$	%	$\mu g \cdot g^{-1} \cdot h^{-1}$	%
cis-ocimene	14.66	0.16		
α-pinene	4203.48	46.65	4.33	46.80
camphene	63.36	0.70	0.34	3.70
β-pinene	2757.90	30.60	3.46	37.45
myrcene	133.80	1.48	0.05	0.57
Δ^3-carene	1288.85	14.30	0.47	5.05
sabinene	296.44	3.29	0.06	0.67
β-phellandrene			0.25	2.69
terpinolene	30.39	0.34	0.04	0.39
α-fenchene	27.68	0.31		
trans-caryophyllene	65.16	0.72		
α-humulene	29.49	0.33		
germacrene	50.92	0.57		
limonene+β-phellandrene			0.17	1.80
other compounds	47.59	0.53	0.08	0.89

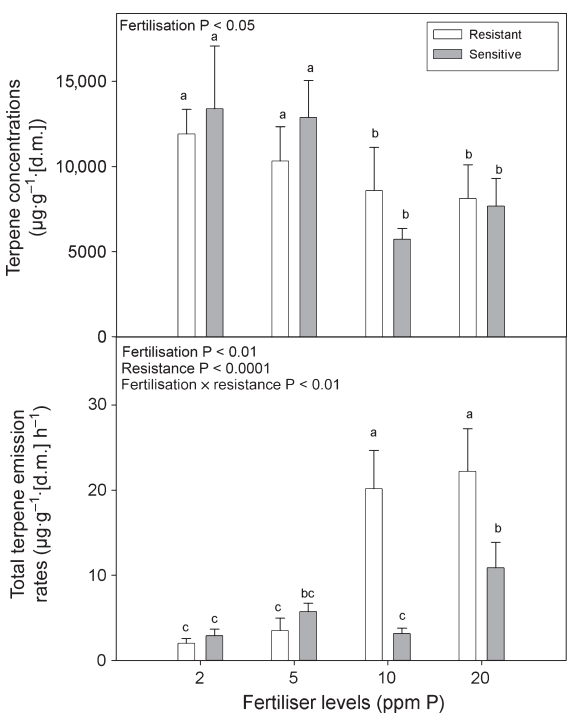

Fig. 3. Total terpene content and total terpene emission rates for different fertiliser treatments and resistance family groups. Vertical bars indicate standard error of the mean (n = 9). Different letters indicate significant statistical differences among fertiliser levels.

Regarding the production and emission rates of terpenes, the most P-stressed conditions (doses P2 and P5) led to higher leaf terpene concentrations and lower terpene emission rates (Fig. 3). Higher amounts of terpenes were emitted under less stressed conditions (doses P10 and P20) in comparison with the most stressed conditions, especially in the sensitive families (Fig. 3). These higher emission rates can be explained by the fact that P is a basic component in terpene formation, which presents, in the methylerythritol pyrophosphate pathway (MEP), phosphorylated precursors such as isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP). Moreover, it has been estimated that to form isoprene, 20 ATP and 14 NADPH are required (Sharkey & Yeh 2001).

Table 3. Summary of the multivariate analysis for total terpene content for *P. pinaster*. B, P, R and G are the main effects of block, fertilisation, resistance and genotype. Genotype was nested in resistance G(R). Bold values indicate significant statistical differences.

MANOVA hypothesis	Wilk's Lambda	P-value
non-general P effects	0.15194675	**0.0036**
non-general R effects	0.44274740	**0.0060**
non-general G(R) effects	0.03232137	**<.0001**
multivariance analysis	0.28185726	0.3472

An increase in P could increase the risk of attack by *H. abietis* in the field due to the rise in the amount of α-pinene emitted, since this terpene attracts *H. abietis* (Moreira *et al.* 2008). In fact, the amount of debarked area in young seedlings in the field has been found to increase with higher P availability (Zas *et al.* 2006b). The preference of pine weevil for sensitive families could be explained in part by the higher α-pinene emission rates of those families under the most fertilised conditions (P10 and P20), compared to resistant families (Fig. 3).

In conclusion, a higher P availability altered plant physiology (higher biomass, higher nutrient concentrations), decreased the leaf accumulation of leaf terpenes and increased the emission rates of terpenes in *Pinus pinaster*, a terpene-storing species. There was a genetic effect, with different responses in physiology and terpene production and emission of pine families dependent on their susceptibility to weevil damage in the field. The higher terpene emission rates of susceptible families under high nutrient availability could explain the pattern of weevil damage observed in the field (Zas *et al.* 2008).

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