Title

Effects of phosphorus deficiency and genetic variation on leaf terpene contents and emission rates in *Pinus pinaster* seedlings susceptible and resistant to the attack of the pine weevil *Hylobius abietis* L.

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Short title

Phosphorus deficiency and genetic variation on *Pinus pinaster* terpene emissions and contents

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Abstract

We studied the effects of phosphorus fertilization on foliar terpene concentrations and on foliar volatile terpene emission rates in different half-sib families of *Pinus pinaster* Ait. seedlings. Half of them appeared to be resistant to the attack of the pine weevil *Hylobius abietis* L., a generalist phloem feeder, and the other half appeared to be susceptible to this insect. We hypothesized that P stress could modify the terpene concentration in the needles and thus derive to altered terpene emission patterns relevant to plant-insect signalling. The total concentrations and emission rates ranged between 5732 and 13995 $\mu g\ g^{-1}$ d.m. and between 2 and 22 $\mu g\ g^{-1}$ d.m. h^{-1} respectively. The storage and emission were dominated by the isomers α and β-pinene (77.2 % and 84.2 % of the total terpene amount respectively). P stress caused in both resistant and susceptible families an increase of 31% of the foliar terpene concentrations with an associated 5-fold decrease of the terpene emission rates. Those higher contents would indicate an allocation of the "excess of carbon" generated due to growth being limited because of P scarcity, to terpene emissions. Sensible families showed a higher increase of terpene emission rates, which could be related to plant-animal communication.

Introduction

Phosphorus has many roles in plant growth and metabolism. One of the principal functions of phosphorus is energy transfer: through the action of adenosine triphosphate (ATP). ATP and its derivatives, ADP and AMP, are involved in all aspects of energy transfer in every part of plant growth. Apart from this global function, phosphorus 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 phosphorus denote the fact that it is important in the formation of seeds and the development of roots. It also speeds plant maturity and helps the plant resist stresses (Urbano, 1999).

However, fertilization of young pine seedlings and the subsequent boosting of primary growth rates could lead to increased susceptibility to pests and diseases due to altered allocation patterns of energy to growth and defence and/or improved tissue quality for the insects. In this sense, in a field study Zas *et al.* (2006a; 2008) found that traditional silvicultural practices such as phosphorus fertilization could lead to

66 greater susceptibility to the attack by the pine weevil *Hylobius abietis* L. in seedlings
67 of *P. pinaster* and *P. radiata*, which may be at least partially explained by a reduction
68 in resistance (Moreira *et al.* 2008). The pine weevil *Hylobius abietis* L. is a generalist
69 phloem-feeder that constitute a major pest in conifer plantations in all Europe, where
70 causes important regeneration problems due to the fact that adults feed the bark of
71 young seedlings (Conord *et al.* 2006; Leather *et al.* 1999). The susceptibility of *P.*
72 *pinaster* to *H. abietis* attack has been found to present an intense genetic variation,
73 where some families were consistently more damaged than others (Zas *et al.* 2005).
74 Greater nutrient availability could directly increase the nutritional value of the
75 plant tissues and thus increase the preference by the insects (Ayres & Lombardero
76 2000; Moreira *et al.* 2009). Phosphorus fertilization on P stressed pine seedlings may
77 diminish the allocation of energy to constitutive and induced defences by favouring
78 the growth rates. Several models of plant defence suggest altered patterns of
79 allocation to chemical defences in environments with increased nutrient availability.
80 The Carbon nutrient balance (Bryant *et al.* 1983) stated that when growth is limited
81 by nutrients, plants allocate the "excess carbon" to the production of secondary
82 metabolites. The Growth differentiation balance (Lorio 1986) recognizes that all
83 secondary metabolites have an ontogenetically determined phenology and that their
84 synthesis is emphasized during periods of plant differentiation. Growth dominates
85 during favourable conditions, and differentiation is at a maximum only when
86 conditions are suboptimal for growth. This could be more evident in tree species with
87 predeterminated growth such as pine trees. The Optimal allocation model (Tuomi *et*
88 *al.* 1991) predicts decreasing investment in defence with increasing resource
89 availability, because reduced costs of tissue production could compensate higher risks
90 of herbivore predation. Higher phosphorus availability could also lead to a higher
91 appearance of the fertilized plants to the insect. Fertilization could change the amount
92 of emitted and leaf-contained volatile organic compounds as it may affect the
93 secondary metabolism as stated by "excess carbon" hypotheses (Peñuelas & Estiarte
94 1998).
95 Maritime pine (*Pinus pinaster* Ait.) has been widely chosen as forestation
96 species in Galicia (NW Spain) since the XVIIIth century. Despite being partly
97 replaced in the last decades by species with higher productions like *Pinus radiata* and
98 *Eucalyptus globulus*, *P. pinaster* is still the most important forest tree species in
99 Galicia. According to the last forest survey (DGCN 2000), Galicia has 389,489 ha of

monospecific stands and 243,735 ha of mixed stands with eucalyptus or broad-leaved species. Thus, *P. pinaster* is present in 44% of the total Galician wooded area. The intensive silviculture applied to *P. pinaster* entails short rotations (15 to 45 years), in which there is an important extraction of nutrients of the system (Merino *et al.* 2003).

Conifer plantations in Galicia commonly suffer important nutrient deficiencies (Martins *et al.* 2009). These plantations are usually located on acid soils with few nutrients, especially phosphorus. Moreover, the loss of nutrients due to harvesting can lead to decreased reserves of soil available limiting nutrients (Dambrine *et al.* 2000; Merino *et al.* 2005). Under those conditions, phosphorus stress is commonly found in conifer and especifically in *P. pinaster* stands in NW Spain (Martins *et al.* 2009).

We hypothesized that P stress could modify the terpene concentration in the needles and the photosynthetic activity of *P. pinaster* thus leading to altered terpene emission patterns relevant to plant-insect signalling. The objective of the present study was to test this hypothesis. With this aim and the additional aims of studying the effect of genetic variation and the relationships with the resistance to pests, we analyzed the effect of P fertilization on terpene concentrations and on terpene emission rates in half-sib families of *P. pinaster* seedlings cultivated under controlled conditions, previously found to be resistant or susceptible to the large pine weevil in field conditions in Galicia forests.

Material and Methods

Experimental design and plant material

We performed a two factorial experiment with different pine genetic entries and phosphorus fertilization treatments under controlled conditions. The experimental layout was a randomized split-plot design replicated in four blocks, with four phosphorus fertilization treatments acting as the whole factor and six genetic entries as the split factor. In total, we sampled 72 pine seedlings, corresponding to 3 blocks (randomly selected within the 4 blocks) × 4 phosphorus fertilization treatments × 6 genetic entries nested into two susceptibility groups, ‘susceptible’ and ‘resistant’ families.

P. pinaster families belonged to six different open-pollinated families with common mother tree (half-sibs). Three families were previously recognized to be susceptible to the attack by the pine weevil (*Hylobius abietis*) in an extensive field study, while the other three families appeared to be more resistant to this plague (Zas *et al*. 2005).

Damage (debarked area by the pine weevil) to the susceptible families in that field study was more than two-fold greater than that suffered by the resistant families (Zas *et al.* 2005). All progenies are native from the coastal region of Galicia (NW Spain).

Plant material, greenhouse conditions and experimental fertilization

On 7 February 2006, *P. pinaster* seeds were individually sown in 2 L. pots containing perlite in a greenhouse with controlled temperature (10-22 ºC at night and day, respectively), low photosynthetic photon flux density and daily water irrigation.

On 15 March 2006, we started to apply the fertilization treatments by sub-irrigation (every two days) with four different fertilization treatments. The complete balanced fertilization ("P20") was prepared according optimum requirements for maritime pine tree growth, containing 100 ppm of N, 20 ppm of P, 40 ppm of K, 10 ppm of Ca, 20 ppm of Mg, and the necessary amounts of micronutrients and trace elements. The other phosphorus fertilization ("P10", "P5" and "P2") differed only in the concentration of phosphorus, which were unbalanced to limit the growth promoting a P limitation, with a concentration of 10, 5 and 2 ppm of phosphorus in the fertilizer solution, respectively. The pH values were adjusted to 6.5 in all the solutions. Fertilizer solutions were replaced every two weeks. The experiment was carried out in a glass greenhouse (36.5 m long and 15 m wide) belonging to the facilities of CIF Lourizán (Pontevedra, NW Spain, UTM coordinates 29T 42º24'33'' N 8º39'47''W).

Photosyntetic activity and terpene emission collection

On 24-27 July 2006, measurements of net photosyntetic rates, stomatal conductance and terpene emissions were conducted. These measurements were done at controlled standard conditions (30ºC and 1000 μmol m^{-2} h^{-1} PAR). CO_2 exchange was measured using a non-dispersive infra-red gas analyzer (IRGA), model ADC-LCPro+ (ADC Inc. Hoddesdon, Hertfordshire, England) connected to a conifer leaf chamber (ADC Inc. Hoddesdon, Hertfordshire, England). CO_2 uptake (A) and stomatal conductance (g_s) were measured in lateral shoots on *P. pinaster*. A and g_s values were expressed on a projected leaf area basis measured with Li-Cor 3100 Area Meter (Li-Cor Inc., Nebraska, USA).

In order to sample terpene emissions, a T-system was installed outside the cuvette of the IRGA-porometer. Part of the air passed through cartridges (8 cm long

and 0.3 cm internal diameter) manually filled with terpene adsorbents Carbopack B, Carboxen 1003, and Carbopack Y (Supelco, Bellefonte, Pennsylvania) separated by plugs of quartz wool by using a Q_{max} air sampling pump (Supelco, Bellefonte, Pennsylvania) at constant flow. The hydrophobic properties of the tubes were supposed to minimize sample displacement by water. In these tubes, terpenes did not suffer chemical transformations 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 ºC with a stream of purified helium. The sampling time was 5 min, and the flow varied between 470 and 500 ml min^{-1} depending on the tubes' adsorbent and quartz wool packing. A calibrated air sampling pump was used to trap isoprenoids. The trapping and desorption efficiency of liquid and volatilized standards such as α-pinene, β-pinene or limonene was practically 100%. In order to eliminate the problem of 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 ºC and taken to the laboratory where they were stored at -28 ºC until analysis (within 24-48 h). There were no observable changes in terpene concentrations after storage of the tubes as checked by analyzing replicate samples immediately and after 48-h storage. Emission rate calculations were made on mass balance basis and by subtracting the control values (without plants) from the values of samples with plants.

Seedling harvesting and nutrient analyses

On 1 August 2006, we measured height and basal diameter (mean of two measures) and then we destructively sampled all the pine seedlings. For the analysis of foliar terpene content, a composite sample of primary needles from different parts of each tree was collected, deep frozen and preserved at -80 ºC into close-tight glass vials. To estimate final seedling biomass we separated into roots, stems, braquiblasts and needles and immediately put in a drying oven for 72 h at 65 °C. After being removed from the oven, we weighed samples to the nearest 0.001 g. All the samples were finely grounded, labelled and preserved for nutrient analysis.

For the analysis of nitrogen and phosphorus content, 0.3 g of phloem, roots, braquiblasts and needles were 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 (California,

202 USA) at λ = 650 nm according the method proposed by Sims *et al.* (1995).
203 Phosphorus was analysed in the same diluted aliquots by inductively coupled plasma
204 optical emission spectroscopy (ICP-OES) using a Perkin-Elmer Optima 4300DV
205 (Massachusetts, USA) in the central laboratory facilities at Universidade de Vigo –
206 CACTI (www.uvigo.es/webs/cactiweb/). Nitrogen and phosphorus concentration
207 were expressed in mg g^{-1} dried weight of tissue.
208
209 *Terpene analysis*
210 Tubes with trapped emitted monoterpenes were inserted in an OPTIC3 injector
211 (ATAS GL International BV 5500 AA Veldhoven, The Netherlands) where they were
212 desorbed at 250 ^{o}C during 3 min. Terpenes were separated using a 30m x 0.25mm x
213 0.25μm film thickness capillary column (SPB TM-5 Fused Silica Capillary column ,
214 Supelco Inc., Bellefonte, PE, USA). After sample injection, the initial temperature
215 (40°C) was increased at 30 ºC min^{-1} up to 60 ºC, and thereafter at 10 ºC min^{-1} up to
216 150 ºC maintained for 3 min, and thereafter at 70 ºC min^{-1} up to 250 ºC, which was
217 maintained for another 5 min. Helium flow was 1 mL min^{-1}. The identification of
218 terpenes was conducted by GC-MS and comparison with standards from Fluka
219 (Buchs, Switzerland), literature spectra and GCD Chemstation G1074A HP with the
220 Wiley275 library. Terpene calibration curves (for 4 different terpene concentrations)
221 were always significant ($r^2 > 0.99$). The most abundant terpenes had very similar
222 sensitivity (differences were less than 5%). Terpene concentration was referred to
223 needle dried weight (d.w.).
224 For extraction of resin terpenoids in the needles, three-four needles were
225 grounded under liquid nitrogen in Teflon tubes with a Teflon embolus. Then, we
226 added 1 mL of pentane as extractant and 0.1 μl of dodecane, a non-terpenoid internal
227 standard. Teflon tubes with pentane samples were centrifuged in an ultrasonic bath for
228 5 minutes at 5000 rpm and 5-10 ºC to separate the liquid and solid phases. Pentane
229 extracts were immediately recovered and transferred to chromatography glass vials.
230 After recovering the pentane extract, the mass of the needle pellet was determined by
231 oven-drying at 65 ºC for 4 days. Terpenes in the extract were analyzed using a
232 Hewlett Packard HP59822B GC-MS (Palo Alto, CA, USA) with a robotic sample
233 processor (FOCUS) (ATAS GL International BV 5500 AA Veldhoven, The

234 Netherlands). Separation, quantification and identification were performed as
235 described above.
236
237 *Statistical analyses*
238 All traits were analyzed by the following model: $H_{ijk} = \mu + B + P + R + G(R) +$
239 $P*G(R) + P*R + B*R + B*P + \varepsilon_{ijk}$, where H_{ijk} is the variable of the trait, μ is the
240 overall mean, B, P, R and G are the main effects of block, phosphorus fertilization,
241 resistance and genotype, and ε_{ijk} is the experimental error. Genotype was nested in
242 resistance G(R). B*P was considered a random factor for properly analyze the split
243 plot design (Littell *et al.* 2006). A MIXED procedure of SAS was used. When main
244 effects were significant, differences among treatment means were tested for
245 significance using the LSMEAN statement.
246 A PROC GLM procedure of SAS (Littell *et al.* 2006) was used for the
247 MANOVA analyses; Wilk's Lambda statistics were used.
248
249 **Results**
250 *Plant growth and needle nutrient concentrations*
251 Fertilization treatments strongly affected plant growth ($F = 20.82$, $P < 0.001$) and
252 phosphorus concentration in plant tissues ($F = 141.39$, $P < 0.0001$) (Table 1). Plants
253 with complete fertilization (20 ppm) produced 2.5-fold greater biomass than plants
254 with lower fertilization (Fig. 1). P concentration in needles was strongly influenced by
255 fertilization, showing increasing values accordingly to the P fertilization, where plants
256 under balanced fertilization exhibited P concentrations 3-fold greater than P stressed
257 plants (Fig. 1). The only treatment that drove P concentration in needles under critical
258 levels was 2 ppm; therefore, this treatment was the one that generated the clearest P
259 deficiency.
260 Nitrogen concentration in needles was only slightly greater, but significant (F
261 $= 5.97$, $P < 0.05$) in complete fertilization than in P stressed plants (Table 1, Fig.1).
262 Those families with a resistant behaviour at field showed slightly higher
263 concentrations of P ($F = 7.79$, $P < 0.01$) in leaf tissues than susceptible families, but
264 no differences in terms of N concentrations ($F = 3.16$, $P < 0.1$) and total biomass ($F=$
265 2.73, $P = 0.1$) were detected (Table 1, Fig.1).
266

Photosynthesis (A), stomatal conductance (g_s) and transpiration rates (E)

Fertilization treatments decreased photosynthesis ($F = 4.48$, $P < 0.05$) and transpiration ($F = 6.12$, $P < 0.05$) (Table 1, Fig. 2): complete fertilization (20 ppm) produced lower *A* and *E* than the lowest fertilization treatment (2 ppm) (Fig. 2). However, these effects were different in resistant families than in sensible families: there was a strong interaction *P*R* (Table 1) for *A*, g_s and *E*: sensible families showed the lowest values of *A* and *E* at 10 ppm of fertilization, and resistant families showed the lowest values of *A* and *E* at 20 ppm of fertilization.

Different families had significant differences in photosynthesis ($F = 2.72$, $P < 0.05$) and stomatal conductance ($F = 10.23$, $P < 0.0001$) (Table 1).

Volatile terpenes

Several mono- and sesquiterpenes were found in both leaf concentrations and in terpene emissions. The relative percentages in the total amount is shown in Table 2. The isomers α and β-pinene dominate the production (77.2 %) and emission (84.2 %) of the total terpene amount. Δ^3-carene is also present with high percentage: 14.3% of the concentrations, and 5% of the emission rates. The rest of the compounds appeared in smaller percentages.

MANOVA analysis for the individual compounds showed significant differences for phosphorus deficiency ($\lambda = 0.15$, $P<0.01$), resistance ($\lambda = 0.44$, $P<0.01$) and genotype ($\lambda = 0.03$, $P<0.0001$), but there was not significant effect for P*res, which indicates that the different treatments influenced not the individual but the whole terpene profile of our samples (Table 3).

Terpene concentrations significantly increased with phosphorus deficiency ($F= 4.25$, $P < 0.05$) (Table 1, Fig. 3). On the contrary, total terpene emission rates significantly decreased with phosphorus fertilization ($F= 9.76$, $P < 0.01$) (Table 1, Fig. 3). This increase was much higher in sensible species than in resistant families (Fig. 3).

There was a high effect of Genotype ($F = 26.78$, $P < 0.0001$) in terpene emission rates: different families showed different behaviours. Thus, total terpene emission rates significantly increased ($F = 19.48$, $P<0.0001$) in sensible families at higher P doses (10 and 20 ppm).

Discussion

Terpene compounds

The mean concentration values ranged from an average of 7.9 mg g^{-1} (with 20 ppm of P addition) to an average of 12.6 mg g^{-1} (with 2 ppm of P addition) (Fig. 3), which are lower than other literature values for the same species (Arrabal *et al.* 2005) or in other pine species (e.g. Blanch *et al.* 2009).

P. pinaster is not considered a big isoprenoid emitter (Kesselmeier & Staudt 1999). However, the mean emission rates values ranged from an average of 2.5 μg g^{-1} h^{-1} (with 2 ppm of P addition) to an average of 16 μg g^{-1} h^{-1} (with 20 ppm of P addition) (Fig. 3) which is much higher than literature values: Simon *et al.* (1994) found emission rates of 0.2 μg g^{-1} h^{-1} in *P. pinaster*.

The difference of values could be due to the differences in climate during the measures, which were warmer in our location. Moreover, emission rates depend on several other factors such as ontogeny, the qualitative and quantitative composition of the terpenes, the terpene pool position, the pathway of diffusion of the terpene within the plant, the morphology of the vegetal surface, the vapour pressure of terpenes, the temperature, the relative humidity, the water stress, and mechanical as well as chemical injuries of the plant. These lower concentration values and the higher emission rate suggest that in the site of measurement (Galicia) and the season of the year considered (summer), *P. pinaster* tends to emit the monoterpenes instead of keeping them in the terpene pools.

Our results where α- and β-pinene were the 77.2% and 84.2% of the total emission rates and concentrations respectively (Table 2) agree with previous studies that have shown that α- and β-pinene are the principal terpenes emitted (Simon *et al.* 1994) and contained (Arrabal *et al.* 2005; Ormeño *et al.* 2009) by *P. pinaster* . The vapour pressure of these two compounds is two to three times higher that those of other terpenes, whereas the rate constants of their reactions with O_3, OH^- and NO_3^- are lower (Atkinson 1990).

Phosphorus and genetic effects

P concentration in leaves was above the P deficiency levels proposed for field studies (Bonneau 1995) in all cases. That is, our fertilization ranged from high levels to low levels, but always within the regular physiological margin. According to Fig. 1, the

fertilization stress treatment was significantly effective: the higher the fertilization dose, the higher the concentration of P in the plant. The increase of nitrogen and phosphorus leaf concentrations with P fertilization has been previously reported (Keay *et al.* 1968). Moreover in agreement with previous authors that have reported the effect that P fertilization increases the growth of *P. pinaster* (Zas *et al.* 2006b), P fertilization increased the amount of biomass in fertilized plants (Fig. 1).

There was a general effect of P deficiency on photosynthesis rates, stomatal conductance and transpiration which showed certain tendency to increase with lower P doses (Fig. 3). The most fertilized seedlings showed a decrease of A, g_s and E in comparison with the least fertilized seedlings. Previous authors have also reported negative correlations between P fertilization and A (Cheaib *et al.* 2005; Loustau *et al.* 1999). Warren & Adams (2002) suggested that the lack of photosynthetic response to P supply was the result of a deficiency of N induced by high P supply.

Resistant and non-resistant species showed contrary responses to initial P deficiency. Genetic differences in nutrient use efficiency in response to fertilization in many tree species have been previously reported (i.e. Zas *et al.* 2006b; 2008).

Regarding the production and emission rates of terpenes, the most P-stressed conditions (doses 1 and 2 ppm) showed higher leaf terpene concentrations and lower terpene emission rates (Fig. 3). P deficient plants seem to store the produced terpenes in storing pools instead of emitting them. Higher amounts of terpenes were emitted in the less stressed conditions (doses 10 and 20 ppm) in comparison with the most stressed conditions, especially in sensible species (Fig. 3). These higher emission rates can be explained by many of the theories based on the "excess carbon" hypothesis (Peñuelas & Estiarte 1998) such as the Carbon-Nutrient Balance theory (Bryant *et al.* 1983) and the Growth Differentiation theory (Lorio 1986), which state that when the resources are higher than the needs for growth, plants use those resources to produce carbon based secondary metabolites (Peñuelas & Estiarte 1998).

An increase of P would also increase the attack of *H. abietis* due to the fact that fertilization increases the amount of α-pinene emitted and that *H. abietis* is attracted by the monoterpene α-pinene (Moreira *et al.* 2008). As a result, the amount of debarked area in young seedlings would increase with higher P availability (Zas *et al.* 2006b). The fact that sensible species emit more terpenes with higher P concentrations than resistant species could be explained as a defence of these plants to

H. Abietis: sensible plants emit more terpenes under fertilized conditions (*Fig.3*) in order to attract natural predators of the weevil that could attack them under those high fertilized conditions. Degenhardt (2008) showed that terpene emissions can attract nematodes to the plant that emits those terpenes, and nematodes are natural predators of *H. abietis* (Dillon *et al.* 2006).

In conclusion, higher phosphorus availability altered the plant physiology (higher biomass, higher nutrient concentrations), increased the production of monoterpenes, and decreased the of *P. pinaster* (storing species). There was a genetic effect: different families showed different responses in physiology and in terpene production and emission. The higher terpene emission rates of sinsible families could explain a greater attraction of predators of the pine weevil.

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Tables

Table 1. Summary of the split-plot model for P and N concentration in needles, total biomass, net photosynthetic rates (μg m^{-2} s^{-1}), stomatal conductance (mol m^{-2} s^{-1}), transpiration rates (μmol m^{-2} s^{-1}), Total Terpene Contents (μg g^{-1}[d.m.]) and Total Terpene Emission Rates (μg g^{-1}[d.m.] h^{-1}) of *P. pinaster*. B P R and G are the main effects of block, fertilization, resistance and genotype. Genotype was nested in resistance G(R).

			P needles		N needles		Total biomass	
	DF num	DF den	F	p	F	p	F	p
B	3	9	1,19	0,3661	0,89	0,4841	1,81	0,2151
P	3	9	141,39	**<.0001**	5,97	**0,0160**	20,82	**0,0002**
R	1	33	7,79	**0,0087**	3,16	0,0846	2,73	0,1081
G(R)	4	33	1,15	0,3526	0,78	0,5483	2,95	**0,0345**
P*G(R)	12	33	1,85	0,0793	0,89	0,5632	1,94	0,0659
P*R	3	33	3,75	**0,0201**	1,45	0,2462	4,53	**0,0091**
B*R	3	33	0,21	0,8917	0,68	0,5697	1,52	0,2271

			Net photosynthetic rate		Stomatal conductance		Transpiration Rate	
	DF num	DF den	F	p	F	p	F	P
B	3	9	0,01	0,9981	0,07	0,9730	0,17	0,9151
P	3	9	4,48	**0,0347**	2,58	0,1179	6,12	**0,0148**
R	1	33	1,30	0,2623	0,28	0,5977	0,19	0,6685
G(R)	4	33	2,72	**0,0460**	10,23	**<.0001**	1,76	0,1603
P*G(R)	12	33	2,03	0,0539	2,70	**0,0118**	1,49	0,1788
P*R	3	33	7,35	**0,0007**	6,17	**0,0019**	5,08	**0,0053**
B*R	3	33	0,79	0,5106	0,88	0,4595	0,38	0,7658

			Total terpene concentration		Terpene emission rate	
	DF num	DF den	F	p	F	p
B	3	9	0,48	0,7038	1,61	0,2544
P	3	9	4,25	**0,0396**	9,76	**0,0034**
R	1	33	0,22	0,6456	19,48	**0,0001**
G(R)	4	33	4,16	**0,0078**	16,78	**<.0001**
P*G(R)	12	33	1,99	0,0584	3,56	**0,0028**
P*R	3	33	0,94	0,4329	5,32	**0,0046**
B*R	3	33	1,05	0,3823	2,47	0,0813

507 Table 2. Individual and total terpene concentrations (n=68) and emission rates (n=70)
508 for all families and all treatments.
509

	Terpene concentration		Terpene emission	
	μg g^{-1}	%	μg $g^{-1}h^{-1}$	%
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+b-phellandrene			0,17	1,80
other compounds	47,59	0,53	0,08	0,89

510
511
512 Table 3. Summary of the Multivariance Analysis for Total Terpene Contents (μg g^{-1}d.m.) and Total Terpene Emission Rates (μg g^{-1}d.m. h^{-1}) for *P. pinaster*. B P R and G
514 are the main effects of block, fertilization, resistance and genotype. Genotype was
515 nested in resistance G(R).
516

Manova hipótesis	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

517
518

519 **Figure legends**

520

521 Fig.1 Nitrogen and Phosphours concentrations (mg g^{-1} d.m.) in needles and total
522 biomass (mg), for fertilization and resistance treatments. Vertical bars indicate
523 standard error of the mean (n=12 in the two upper panels, n=24 in the lower panel).
524 Different letters indicate significant statistical differences among fertilization levels.

525

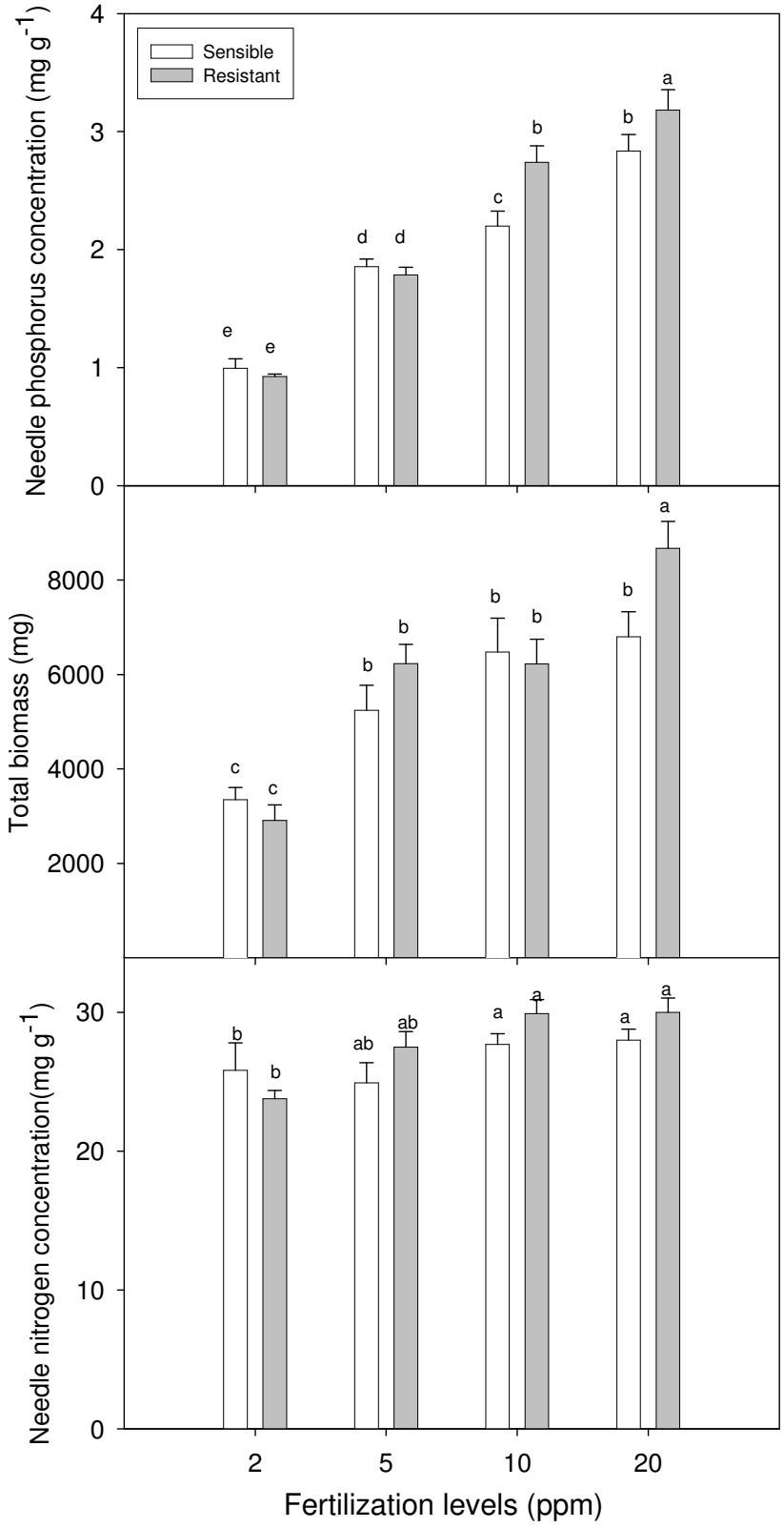
526 Fig.2 Net photosynthetic rates (µg m^{-2} s^{-1}), stomatal conductance (mol m^{-2} s^{-1}) and
527 transpiration rates (mmol m^{-2} s^{-1}) for fertilization and resistance treatments. Vertical
528 bars indicate standard error of the mean (n=12). Different letters indicate significant
529 statistical differences among fertilization levels.

530

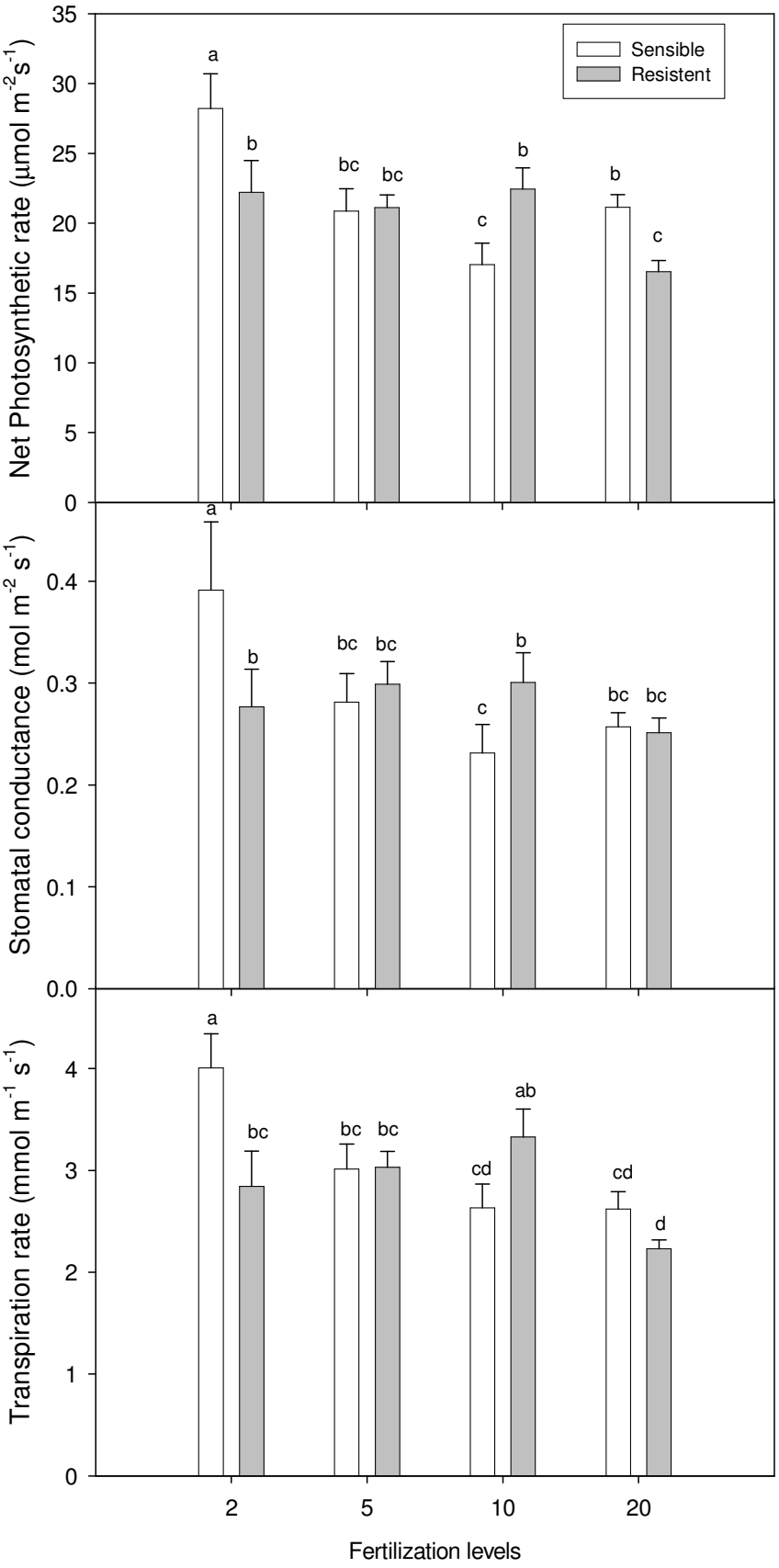
531 Fig 3. Total Terpene Contents (µg g^{-1}[d.m.]) and Total Terpene Emission Rates (µg g^{-1}d.m. h^{-1}) for fertilization and resistance treatments. Vertical bars indicate standard
532
533 error of the mean (n=12). Different letters indicate significant statistical differences
534 among fertilization levels.

535

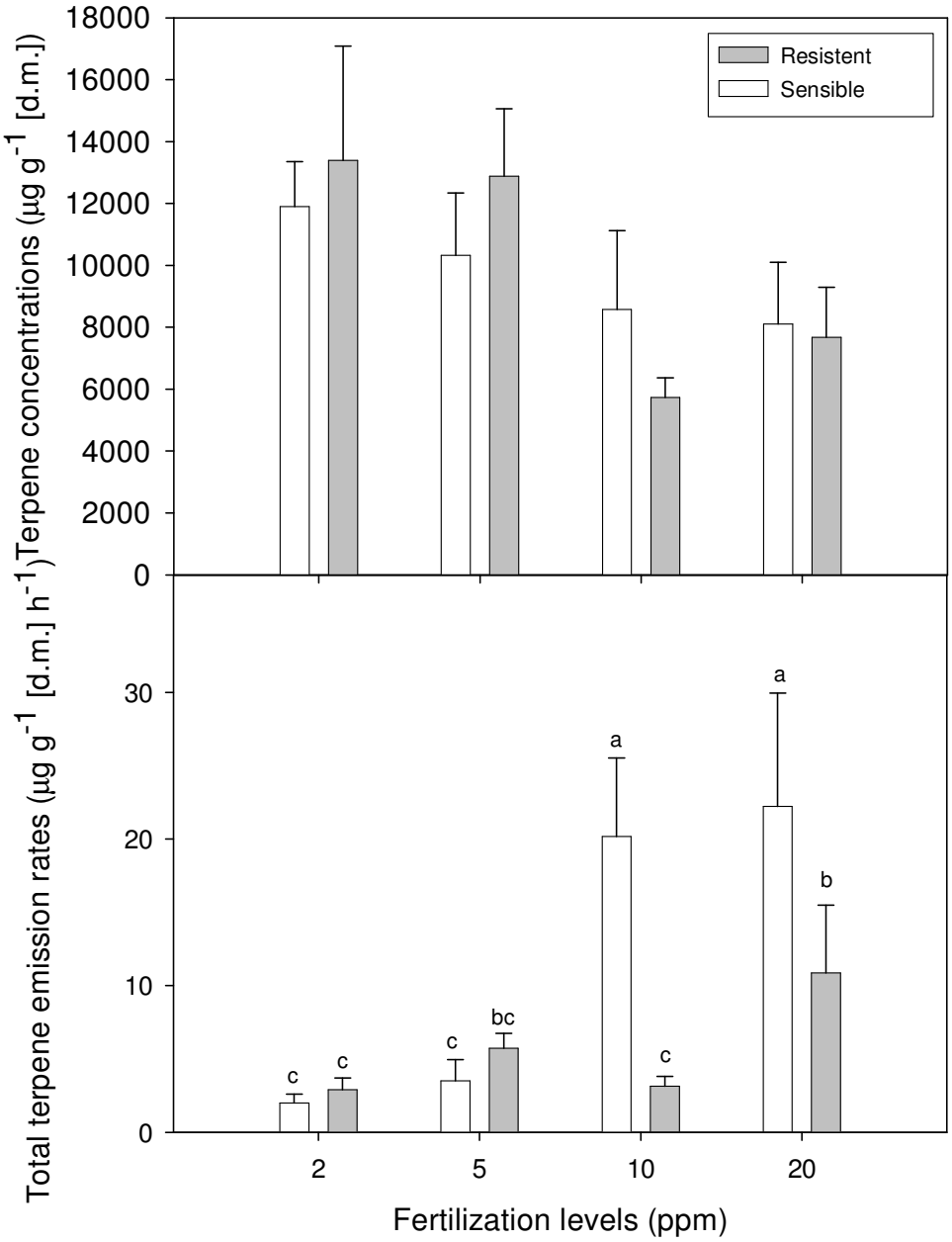
536 **Illustrations**



537
538 Fig. 1


Sensible
Resistent
Net Photosynthetic rate (μmol m-2s-1)
Stomatal conductance (mol m-2 s-1)
Transpiration rate (mmol m-1 s-1)
Fertilization levels
2
5
10
20

539
540 Fig. 2.


Resistent
Sensible
Terpene concentrations (µg g-1 [d.m.])
Total terpene emission rates (µg g-1 [d.m.] h-1)
Fertilization levels (ppm)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
30
20
10
0
2
5
10
20
c
c
c
bc
a
c
a
b

541
542 Fig. 3