

Alterations of the resin canal system of *Pinus pinaster* seedlings after fertilization of a healthy and of a *Hylobius abietis* attacked stand

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Abstract Changes in resource availability and biotic and abiotic stress may alter the defensive mechanisms of pine trees. The effect of fertilisation on the resin canal structure of *Pinus pinaster* seedlings established in two trials in NW Spain, one attacked by *Hylobius abietis* and the other non-attacked, was studied. The leaders of 50 plants were destructively sampled and the resin canal density, the canal area and its relative conductive area in the phloem and xylem were assessed. Experimentally increased nutrient availability significantly decreased resin canal density in the phloem of the seedlings in the two analysed trials, where unfertilised seedlings presented up to 30% more resin canal density than the fertilised seedlings (mean value \pm SEM = 0.32 ± 0.02 resin canals mm^{-2} in the fertilised plants versus 0.45 ± 0.04 resin canals mm^{-2} in the control plants). Fertilisation had no effect on the resin canal system in the xylem, but significantly increased tracheid size. Significant differences of resin canals among sites were observed mainly in the xylem; the resin canal density was 1.7-fold greater in the attacked site than in the non-attacked site. The similar structure of phloem resin canals in both sites supports that phloem resin canals are constitutive mechanisms of defence in *P. pinaster*, whereas xylem resin

canals would be constitutive mechanisms but also inducible mechanisms of resistance following the attack of pine weevils or bark beetles.

Keywords Herbivory · Constitutive defence · Induced defence · Phloem · Xylem · Large pine weevil

Introduction

Conifers have colonised diverse habitats to a greater extent than any group of plants, and this success is partially a result of the highly evolved defence systems against invading organisms (Schulman 1954; Phillips and Croteau 1999). Plant defence mechanisms have been classified according to their permanence in time into constitutive and inducible defences. Constitutive defences are permanent structures or chemical compounds that occur regardless of the presence of damage. The preformed resin canal system present in the cortex of many conifer species is considered as one of the initial and most important defence mechanisms against stem-invading insects. The resin of conifers is a complex mixture of plant secondary compounds, which may function as herbivore defences increasing larval mortality and altering weevils and bark beetle feeding behaviour (Alfaro 1995; Alfaro et al. 2002). Constitutive resin ducts are longitudinally present in the xylem and may contribute to resin flow when they are connected to radial resin ducts, which crosses between xylem and phloem (Wu and Hu 1997), existing a positive correlation between the amount of resin flow and axial resin duct density (Blanche et al. 1992). Several authors have found that number, size and density of resin canals in the phloem or xylem determine whether a tree could be successfully attacked by debarking weevils. For example, Alfaro et al. (1997) found

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that *Picea sitchensis* resistant trees to *Pissodes strobi* had significantly greater resin canal density than *P. sitchensis* susceptible trees.

Inducible defences are activated in response to tissue attack or wounding, and contribute to reduce the performance or preference of herbivores. Inducible defences include the increasing synthesis of antifeedant secondary compounds, such as some monoterpenes and phenolics (Klepzig et al. 1995), the formation of traumatic resin ducts in the xylem (Alfaro 1995; Tomlin et al. 1998; Solla et al. 2006), swelling and proliferation of polyphenolic parenchyma cells in the bark (Krokene et al. 2003; Franceschi et al. 2000), and the formation of a wound reaction zone (Oven and Torelli 1994; Solla et al. 2002). The intensity of both constitutive and induced defence mechanisms in Pinaceae depends on tree age, genotype, species, and can be also affected by environmental factors such as nutrient availability (Rosner and Hannrup 2004; van Akker et al. 2004).

Fertilisation has been observed to decrease secondary metabolite concentration in plants and both constitutive and induced resistances against herbivores (Mattson 1980; Kyto et al. 1996; Koricheva et al. 1998). Several ecological models such as the optimal defense, the growth–differentiation balance or the growth rate hypotheses predict that plant response to nutrient supply could result in a decrease of the investment of C-based secondary compounds for the synthesis of defensive compounds such as terpenes and resin acids (see Stamp 2003). The effects of fertilisation on the incidence of herbivory, insect preference and performance, plant growth and resin flux in Pinaceae have been previously studied (Ayres et al. 2000; Lombardero et al. 2000; Zas et al. 2006). However, we lack information about environmental effects on the resin canal system in maritime pine (*Pinus pinaster* Ait.). This species is of vital importance to the forest economy of Galicia (NW Spain), where it occupies 400,000 ha, nearly 40% of the productive forest area (Xunta de Galicia 2001). In this region, soil fertility is a main factor controlling productivity (Zas 2003; Zas and Serrada 2003). Given the importance of *P. pinaster* and fertilisation in this region, several progeny \times fertilisation trials with identical experimental design were established in 2003. The massive attack of *Hylobius abietis* L. to one of these trials just after planting gave us an excellent opportunity to study how the genotype and the fertilisation treatment affected plant growth and weevil damage (Zas et al. 2005, 2006). *H. abietis* is a devastating silvicultural pest among coniferous seedlings throughout Europe (Orlander and Nordlander 2003). Adults feed on the stem bark and phloem of young seedlings causing great economic losses due to high mortality, growth reduction, and stem deformities (Orlander and Nordlander 2003; Zas et al. 2006). Increased plant vulnerability to *H. abietis* due to the

fertilisation could be derived from a greater growth rate leading to more phloem availability, an increase of plant tissue quality or an alteration of the defensive status. The aim of this study was to determine in two fertilisation trials (attacked by *H. abietis* and non-attacked) if an increase in nutrient availability due to fertilisation could alter the resin canal system of *P. pinaster* seedlings.

Materials and methods

Study area

The study area is located on the west coast of Galicia (NW Spain). It included two twin *P. pinaster* experimental field trials belonging to the Galician breeding program of maritime pine, both having similar soil and climate, and distanced 30 km. The first site is located at Rebordelo (42.46°N, 8.48°W, 530 m a.s.l., 18 km from sea), and the second trial is located at Rianxo (42.36°N, 8.46°W, 90 m a.s.l., 6 km from the sea). The climate is temperate humid Atlantic, with annual precipitation of about 2,200 mm and mean annual temperatures of 12.2 and 14.5°C in Rebordelo and Rianxo, respectively. Soils in both sites derived from the parent granite; they are thin, sandy and acidic (pH in H₂O Rebordelo \sim 4.0, Rianxo \sim 4.3), with high organic matter content (Rebordelo \sim 180 g kg⁻¹ ash-free dry weight, Rianxo \sim 170 g kg⁻¹ ash-free dry weight), high total nitrogen content (Rebordelo \sim 7.1 g N kg⁻¹, Rianxo \sim 6.3 g N kg⁻¹), and presenting very low concentrations of other nutrients, especially of available phosphorus (Rebordelo Olsen P \sim 4.2 mg kg⁻¹, Rianxo Olsen P \sim 4.7 mg kg⁻¹). Before plant establishment, Rebordelo site was covered by *Ulex europaeus* L., and Rianxo site was covered by a 35-year-old *P. pinaster* stand, clear-cutted on January 2002.

Plant material and experimental design

Fifty 2-year-old *P. pinaster* seedlings of two twin fertilisation trials were submitted to five fertilisation treatments. Treatments were an unfertilised control, and the others had a common base of potassium and magnesium sulphate but they were differentiated by the absence or presence of ammonium nitrate and calcium phosphate (Table 1). Plants were fertilised by hand just after planting. Seedlings originated from an improved seedlot produced in a first generation Sergude clonal seed orchard (Sergude 42.82°N, 8.45°W). The experimental design consisted of five completely randomised blocks per trial. The plant material was previously studied in two companion papers (Zas et al. 2005, 2006). Trials were established on March 2003 with 6-month-old containerised *P. pinaster* seedlings. During

Table 1 Fertilisation treatments applied to *P. pinaster* seedlings during plantation

Nutrient	Fertilizer	Dose (g plant ⁻¹) ^a	Control	+N+P	-N+P	+N-P	-N-P
N	Ammonium nitrate	5	–	+	–	+	–
P-Ca	Calcium phosphate	10	–	+	+	–	–
K	Potassium sulphate	15	–	+	+	+	+
Mg	Magnesium sulphate	5	–	+	+	+	+

Presence and absence of nutrients within the fertilizer mixture is indicated by + and – respectively

^a Dose are expressed in g of each principal nutrient

the summer of 2003, the seedlings at Rianxo were attacked by *H. abietis*, and the infestation was intense and spatially uniform in the entire plantation (Zas et al. 2006).

Field assessments and histology

The damage caused by *H. abietis* at Rianxo was scored on February 2004. The intensity of the wounds in each of tenth parts of the stem was evaluated by means of an elastic rule, following a 4-level-variable (0 = undamaged, 1 = some wounds, 2 = many wounds, and 3 = death due to girdling). The phloem wounding intensity for each tree was the sum of the ten parts of the stem (Zas et al. 2006). Plant height was measured in October 2004.

From each plant, a 15-cm long segment from the apical stem (2004 growth), and a 5-cm long segment from a lateral branch (2004 growth) were destructively sampled. Segments were immediately fixed in formalin acetic acid (FAA), and then transferred to 70% EtOH for storage until sectioning and staining. Two 90- μ m thick cross sections from each segment were cut using a sliding microtome. Sections were stained with 0.1% aqueous Safranin according standard procedures during 12 h (Ruzin 1999). Photographs were taken with a Nikon Digital Sight DS-U1, mounted on a Nikon SMZ-U binocular microscope at 20 \times magnification. Image analysis was performed on two quadrants per section, covering about 75% of the total transectional area. The Image-Pro Plus v.4.5 software was used.

The resin canal system of leaders and lateral branches in the phloem and in the xylem was characterised through (1) the resin canal density, the number of resin canals per unit area, (2) the mean area of the resin canals in the quadrant, and (3) the relative conductive area, obtained by dividing the area occupied by the canals in a quadrant by the total area of the quadrant. The number of radial resin ducts in the xylem was also assessed. All variables for each tree were the average of the two quadrants. In order to check general cell enlargement arising as a consequence of fertilisation, tracheid size was estimated on both unfertilised and fertilised trees of Rebordelo. In six square sectors (~ 0.5 mm \times 0.5 mm) per section, the number of tracheids was counted using the population density procedure of the

software. Tracheid size (apoplast included) was obtained by dividing the area of a given sector by the number of tracheids counted in that sector.

Statistical analyses

The effects of site and fertilisation on total height and on the canal traits were analysed by means of ANOVA using the general linear model (GLM) $Y_{ijk} = \mu + S_i + F_j + B(S)_{ik} + SF_{ij} + \varepsilon_{ijk}$, where μ is the general mean, S_i and F_j are the main effects of site i ($i = 1$ to 2) and fertilisation treatment j ($j = 1$ to 5), $B(S)_{ik}$ is the effect of the k th block nested within the i th site, SF_{ij} is the interaction between the site and the fertilisation treatment, and ε_{ijk} is the experimental error.

Pine weevil damage was analysed in the attacked trial using the model $Y_{jk} = \mu + F_j + B_k + \varepsilon_{jk}$, where μ is the general mean, F_j is the main effect of fertilisation treatment j ($j = 1$ to 5), B_k is the effect of the block and ε_{jk} is the experimental error.

The data were checked for normality and homogeneity of variances. When main effects were significant, differences among means were tested for significance using the Tukey test. Pearson correlation was used to evaluate the relationships among all traits measured in the phloem and the xylem between the stems and the lateral branches. Correlations were carried out both for the whole data set ($n = 50$), and separately for each site ($n = 25$). All the analyses were performed using Statistica 6.0.

Results

Effects of fertilisation on the resin canal structure

The intensity of the attack by *H. abietis* at the Rianxo site was significantly greater on fertilised plants, where the mean phloem wounding intensity of the phosphorus fertilised plants (+N+P and -N+P) was nearly 3-fold greater than the phloem wounding intensity of the control plants (Fig. 1). Plant growth in both trials was significantly enhanced by fertilisation ($F_{4,31} = 6.27$; $P = 0.0008$) (Fig. 2).

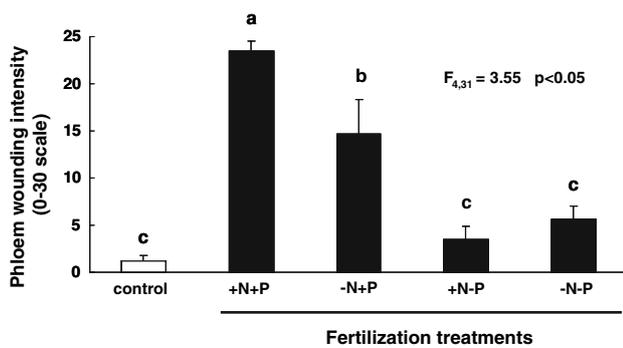


Fig. 1 Effects of fertilisation on the phloem wounding intensity caused by *H. abietis* in 2-year-old *P. pinaster* seedlings at Rianxo (infested site). LS means \pm SEM. Different letters indicate significant differences at $P < 0.05$

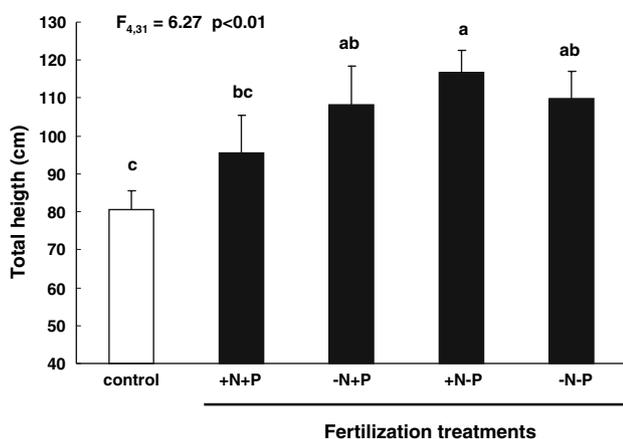


Fig. 2 Effects of fertilisation on plant height of 2-year-old *P. pinaster* seedlings at Rianxo and Rebordelo. LS means \pm SEM. Different letters indicate significant differences at $P < 0.05$

The resin canal system in the phloem was significantly affected by fertilisation (Table 2). The non-fertilised control trees showed a phloem resin canal density 30% greater than the fertilised seedlings (Fig. 3a). Transverse resin duct area was not affected by fertilisation (Table 2) and ranged from $14.3 \times 10^3 \pm 1.9 \times 10^3 \mu\text{m}^2$ (mean values \pm SEM) in the control seedlings to $20.5 \times 10^3 \pm 4.3 \times 10^3 \mu\text{m}^2$ in the $-N-P$ fertilised trees. The relative conductive area of resin ducts in the phloem was not affected by fertilisation (Table 2).

The resin canal system in the xylem was not affected by fertilisation (Table 2). Resin canal density (Fig. 3b), individual transverse canal area (mean value = $124.4 \pm 17.4 \mu\text{m}^2$) and relative conductive area did not differ among trees submitted to different fertilisation treatments (mean values of control and $-N-P$ trees of $26.4 \times 10^3 \pm 3.0 \times 10^3$ and $32.3 \times 10^3 \pm 3.4 \times 10^3 \mu\text{m}^2 \text{mm}^{-2}$, respectively).

Tracheid density was significantly affected by fertilisation ($P = 0.0004$, using the stem diameter as covariate), and

Table 2 F -ratio and signification values of the GLM showing the effect of site (S) and fertilisation (F) on the resin canal traits measured in the phloem and in the xylem of *P. pinaster* seedlings

Trait	Effect			
	Site (S) $F_{(1,31)}$	Fertilisation (F) $F_{(4,31)}$	Block (S) $F_{(9,31)}$	S \times F $F_{(4,31)}$
Resin canals of phloem				
Density	0.3	2.8*	3.8**	1.8
Mean canal area	2.0	2.3	0.8	1.1
Relative conductive area	7.5*	0.3	0.5	0.3
Resin canals of xylem				
Density (axial)	29.8***	0.8	0.7	1.7
Mean canal area	1.2	1.5	1.4	0.8
Relative conductive area	18.8***	0.8	0.5	1.3
Density (radial)	4.3*	1.3	2.9*	0.4

Significance is indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

ranged from 545 ± 45 tracheids mm^{-2} , for the unfertilised trees, to 253 ± 56 tracheids mm^{-2} for the $+N+P$ fertilised trees. In consequence, the mean areas occupied transversally by single tracheids (apoplast included) of unfertilised and $+N+P$ fertilised trees were 1.8×10^3 and $3.9 \times 10^3 \mu\text{m}^2$, respectively.

Site differences

Site quality of twin trials appeared to be similar, since the total height values of the unfertilised control plants (79.0 ± 8.7 and 78.2 ± 8.4 cm for Rebordelo and Rianxo, respectively) did not significantly differ ($F_{1,31} = 0.004$, $P = 0.946$). Height of control plants in both sites could be comparable since Rianxo control plants registered almost no damage by *H. abietis*. Considering all trees, however, plant growth was significantly different between the two twin trials ($F_{1,31} = 30.6$, $P < 0.00001$), with mean values (\pm SEM) of 116.8 ± 3.9 and 86.9 ± 3.8 cm for Rebordelo and Rianxo plants, respectively. Site \times fertilisation interaction was not significant ($F_{4,31} = 2.4$, $P = 0.07$).

Differences among sites were mainly observed for the structure of resin canals in the xylem (Table 2). Xylem resin canal density was 1.7-fold greater in Rianxo trees, wounded by *H. abietis*, than in Rebordelo trees (Fig. 4b). Similar results were obtained for the relative conductive area, which was 1.5-fold greater in Rianxo than in Rebordelo ($35.7 \times 10^3 \pm 2.5 \times 10^3$ vs. $23.4 \times 10^3 \pm 0.9 \times 10^3 \mu\text{m}^2 \text{mm}^{-2}$). However, no significant differences of the individual area of resin canals in the xylem among sites were detected (Table 2). Radial resin ducts density was also significantly greater in Rianxo plants than in Rebordelo plants (2.76 ± 0.36 vs. 1.68 ± 0.37 radial resin

Fig. 3 Effect of fertilisation on the resin canal density in the phloem (a) and in the xylem (b) of 2-year-old *P. pinaster* seedlings. LS means \pm SEM. Different letters indicate significant differences at $P < 0.05$

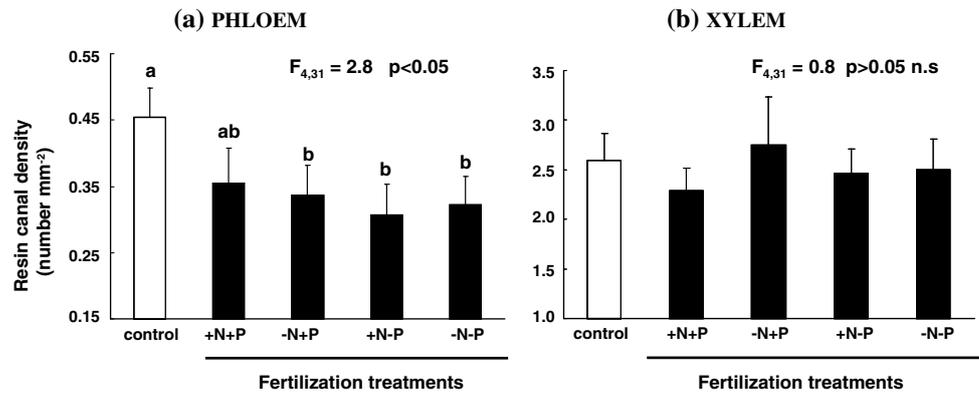
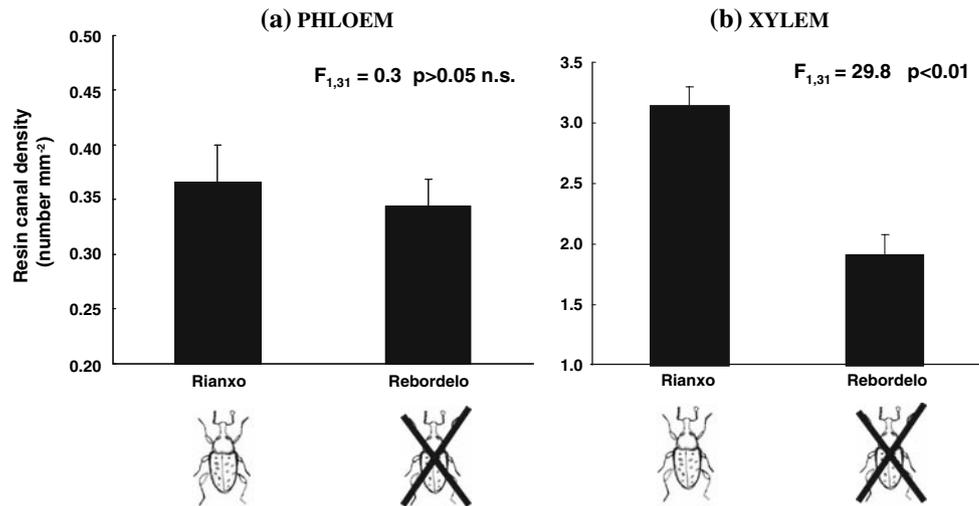


Fig. 4 Resin canal density in the phloem (a) and in the xylem (b) of *P. pinaster* seedlings at Rianxo (attacked by *H. abietis*) and Rebordelo (non-attacked). LS means \pm SEM



ducts mm⁻²; Table 2), indicating greater potential flux of resin in the attacked trial.

In the phloem, resin canal density and the area per canal were similar in plants at both sites (Table 2, Fig. 4a), but the relative conductive area was significantly lower in Rianxo than in Rebordelo plants (Table 2) ($10.0 \times 10^3 \pm 1.4 \times 10^3$ and $15.7 \times 10^3 \pm 1.4 \times 10^3 \mu\text{m}^2 \text{mm}^{-2}$, respectively), the opposite trend than in the xylem. We suspect that this result responds to the different phloem thickness of stems among sites, 8% significantly thicker in the plants at Rianxo than in the plants at Rebordelo ($F_{1,30} = 5.22$; $P = 0.029$; using the stem diameter as covariate). Fertilisation did not affect the thickness of the phloem.

Relationships between resin canal traits of stems and branches

A positive linear relationship among the resin canal density in the xylem of stems and branches was obtained for both sites (Rianxo $N = 25$, $r = 0.57$, $P < 0.01$; Rebordelo $N = 25$, $r = 0.47$, $P < 0.01$) and also after merging both data sets

($N = 50$, $r = 0.53$, $P < 0.01$). The positive linear relationship among the resin canal density in the xylem of stems and branches was also significant for Rebordelo ($N = 25$, $r = 0.60$, $P < 0.01$) and for all plants analysed together ($N = 50$, $r = 0.38$, $P < 0.01$), but not for Rianxo plants ($N = 25$, $r = 0.23$, ns), indicating that the resin canal density in the xylem of stems was modified by the attack of *H. abietis*, since this insect mainly feeds on the stem. In the xylem, no relationship among the relative conductive areas of the stems and branches was obtained. In the phloem, no relationship among any of the traits measured on stems and branches was obtained.

Discussion

Fertilisation significantly reduced the resin canal density in the phloem, as it was previously reported for 12-year-old *Picea glauca* \times *P. engelmannii* (van Akker et al. 2004). Our results give support to several ecological models predicting the response of plants to increased nutrient

availability. The synthesis and storage of carbon-based defensive compounds could be limited by growth, and the amount of these compounds would decrease when trees obtain extra nutrients (Coley et al. 1985). Similarly, growth and defence have been proposed as competing sinks for energy and assimilates, thus increased growth could result in decreased defence (Herms and Mattson 1992). Optimal defence hypothesis also predicts that at increased nutrient availability, a given plant tissue could appear less defended, as the value in terms of fitness of that tissue would be lower due to greater re-growth capability in case of loss (Hamilton et al. 2001).

This evidence may explain the higher feeding preference of *H. abietis* on fertilised plants. However, herbivore insects could also prefer fertilised plants due to higher plant tissue quality in terms of greater nitrogen content (Ayres et al. 2000), which is being studied in a companion paper. Furthermore, since it is known that nutrient availability could affect terpene profile emission (Lerdau et al. 1994; Petterson 2007), and the plant chemical emission affects host selection by the large pine weevil (Norlander 1991), an indirect modulation of weevil behaviour by nutrient availability could also explain the observed differential preference.

Decline in the observed resin canal density could be explained well by the differentiation of fewer resin canals as well by a bigger cell size as a consequence of fertilisation, or by a combination of those two possibilities. Our results suggest that a general tracheid enlargement, pushing the resin canals farther apart, is likely explaining at some extent the observed decline in density besides the production of fewer canals. Whatever the cause, the fact is that the defensive function, i.e. the probability of encountering a resin duct when wounding the phloem, causing resin flows out, was significantly decreased at the fertilised plots.

Meanwhile, fertilisation did not affect the xylem resin canal density, and possibly a greater sample size for each fertilisation treatment would have been necessary. Differences among sites were observed for the relative conductive area, and for the axial and radial resin canal density in the xylem, with values significantly greater in the Rianxo-attacked stand than in Rebordelo. In spite of the inconveniences of rigour derived from analysing two different sites, the same quality index inferred from similar climate and edaphic properties, and from the same plant growth in the control plants, suggests that this effect is likely due to induced responses in the xylem caused by the insect attack. In the leaders of *Picea glauca*, Alfaro (1995) observed the formation of traumatic resin ducts in the xylem in response to the attack by *P. strobi*. After weevil attack, plants produced traumatic resin canals arranged in a ring fashion in the developing xylem. In the same way, the fungi *Cronartium ribicola* and *Melampsorella caryophyllacearum* were

reported to cause changes in the cambial zone of *Pinus monticola* and *Abies alba*, respectively, including the development of cambium-derived xylem traumatic resin ducts (Hudgins et al. 2005; Solla et al. 2006). We were unable to distinguish among constitutive and traumatic resin canals in the xylem of the attacked *P. pinaster* seedlings. Although some sections of wounded pines showed rings of resin ducts similar to those reported for *Picea*, *Abies* or *Pinus*, in most of the sections we did not find marked rings, probably because the attack by *H. abietis* was continuous from spring to autumn. Further effort should be done by means of induction experiments to confirm the traumatic character of the observed rings of xylem canals in *P. pinaster* and their relation to large pine weevil attack.

Histology of resin canals appears a good procedure to assess the defensive levels of *P. pinaster* seedlings, although it has the disadvantage of being destructive. Unfortunately, the relationships among traits measured in the stems and in the lateral branches were not consistent enough to allow field sampling without destroying the plant leaders.

Our results agree with previous findings proposing that resin canals as part of the *Pinaceae* constitutive defence system and being involved in the induced defensive responses of trees following the attack of bark and phloem insect feeders. The effect of fertilisation observed on the phloem resin canal density, together with the similar structure of phloem resin canals observed in both sites, strongly support the fact that the phloem resin canals are constitutive mechanisms of defence, being mainly affected by genotype and environmental factors and not by insect attack (Franceschi et al. 2005). Whereas xylem resin canals would be constitutive mechanisms but also inducible mechanisms of resistance following the attack of pine weevils or bark beetles.

This is the first work reporting the effect of N and P fertilisation on the resin canal system of *P. pinaster*. Despite the reduced sample size used, fertilisation significantly decreased the phloem resin canal density, reducing the defensive capacity of the seedlings. This result may have relevance to current forest management practices, especially in those sites potentially affected by *H. abietis* attack. Forthcoming studies would include genetic variation of *P. pinaster* canal defensive systems to differing nutrient availability, environmental conditions, and *H. abietis* damage.

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