Growth and nutritional response of *Pinus pinaster* after a large pine weevil (*Hylobius abietis*) attack

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Abstract

Hylobius abietis is an important pest of coniferous plantations in Europe, to which high mortality, stem deformities and growth loss are typically attributed. In pine trees, as in other long-lived organisms, the costs of short term resistance against invading organisms could be shown in the long term. We examined the nutritional status of *Pinus pinaster* after a two-years long *H. abietis* attack, measuring needle and phloem N and P concentrations, and the impact of the damage on subsequent growth, survival and stem deformities over a period of five years. The study sites were a *P. pinaster* family x fertilization trial, and a neighbouring twin trial not attacked with similar climate and soil characteristics. Growth losses after the *H. abietis* attack were important (up to 40%) but restricted to the first years after the attack. Five years after the attack the annual height increment of pines in the attacked stand was not related to the initial damage suffered, and plants showed regular stems, normal leader dominance and regular height after 5 yr. These findings, consistent with the comparison between stands, suggest a strong compensatory growth and that *P. pinaster* is a tolerant species to the large pine weevil. Needle nutrient concentrations in the healthy stand were, as expected, significantly greater in experimentally fertilized plants, and they were linearly related to those in phloem showing equilibrated stoichiometry both for nitrogen ($r = 0.86$; $p < 0.01$; $N = 25$) and phosphorus ($r = 0.84$; $p < 0.01$; $N = 25$). However, at the attacked stand, nutrient concentrations in the needles did not follow the experimentally manipulated nutrient availability in soils; and phosphorus concentration in the needles was unexpectedly not related to those in the phloem. The pine seedlings attacked by *H. abietis* showed altered potential of allocating nutrients to their tissues according to the nutrient availability existing in the soil, and also altered stoichiometry in N and P concentration among phloem and leaves. Maritime pine seem to be tolerant to the pine weevil attack, at least in the conditions of this study, where pine weevil damage caused a deep alteration of nutrient allocation and nutritional status. Further research is needed to elucidate to what extent altered nutrient

allocation may be part of an induced response to the attack or just derived from the vascular injury caused by the weevil wounding in the phloem.

Keywords: Herbivory · Fertilization · Forest Pest · Resistance · Tolerance · Nutrient allocation

Introduction

Damage by the large pine weevil, *Hylobius abietis* L. (Coleoptera: Curculionidae) is the most severe threat to newly planted conifer seedlings after clearcutting in Europe (Nordlander et al. 2003). Adults feed on the stem bark and phloem of young seedlings, causing great economic losses if no protection measures are applied (Orlander and Nordlander 2003; Petersson and Orlander 2003; Nordlander et al. 2008). The pine weevil has been estimated to cause the death of up to 80% of coniferous seedlings planted following clear-cutting (von Sydow and Birgersson 1997; Orlander and Nilsson 1999; Nordlander et al. 2008), and severe growth losses in the surviving seedlings (Orlander and Nilsson 1999; Zas et al. 2006). Besides mortality and growth reductions, *H. abietis* attack can cause the leader loss by stem girdling, which can lead to stem form defects in those plants where some lateral branches emerge to recover apical dominance. Although there is a clear consensus that *H. abietis* cause important growth reduction and stem deformities, experimental data supporting those effects are scarce. Furthermore, most of the papers reporting growth losses are usually limited to one or two years after damage.

Besides growth loss and stem deformities, the nutritional status and nutrient allocation of plants could also be influenced by insect grazing. Plants subjected to insect attack have been found to present greater or lower nutrient concentrations in their plant tissues, and even altered within plant variability in nutrient concentrations, than their uninfested counterparts (Polley and Detling 1989; Ayres et al. 2000). In a revealing

experimental study, Newingham et al. (2007) recently reported a significant shift in nitrogen allocation to the shoot in the flowering plant *Centaurea maculosa* (Asteraceae) when infested with a belowground herbivore. However, little is known about the existence of herbivore induced responses involving altered resource allocation patterns in conifers or other long-lived plants.

Most of *H. abietis* damage has been reported in spruce and Scots pine (Orlander and Nilsson 1999; Orlander et al. 2000), but *Pinus radiata* and other European southern pines such as maritime pine (*Pinus pinaster* Ait.) are also a target species of this generalist phloem herbivore. Maritime pine is the most important forest tree species in Galicia (NW Spain), occupying nearly 400,000 ha (~27% of the Galician wooded area). In 2003 several *P. pinaster* family $\times$ fertilization trials were established to test the plasticity of 28 half sib families of the actual Galician maritime pine breeding population in relation to soil fertility, one of the main factors determining site index of coniferous stands in Galicia (e.g. Sánchez-Rodríguez et al. 2002). One of those trials suffered a massive attack of *H. abietis* just after planting. We studied the differential preference of the weevil due to the fertilization and the genetic material in two companion papers (Zas et al. 2005; 2006). Briefly, we found that wounding intensity by the pine weevil in fertilized trees was up to 2.9-fold greater than in unfertilized control plants during the two consecutive years of attack; families markedly differed in their susceptibility to the insect; and damage by the weevil produced deep impact on fitness, where mortality was closely correlated to wounding intensity, and more damaged plants showed reduced growth rates in the following growing season.

In this paper we describe how two consecutive years of large pine weevil attack in this *P. pinaster* family $\times$ fertilization trial affected the nutrient allocation and the subsequent growth recovery in the surviving seedlings. We studied the nutrient concentration in needles and phloem immediately after the attack, and we measured the plant growth during five years after planting. As we lack reference nutrient values from the seedlings before the attack,

because weevil damage began too early after planting, we also studied the same properties in a neighbouring twin family $\times$ fertilization trial free of *H. abietis* damage with similar climate and soil properties, and identical design and genetic material.

Material and methods

Location and description of the genetic trials

We studied two twin *P. pinaster* experimental field trials located in the west coast of Galicia (NW Spain) separated by 30 km. The trial attacked by the pine weevil, previously described in companion papers (Zas et al. 2005; 2006), is located at Rianxo (42.36° N; 8.46° W; altitude 90 m a.s.l.; 6 km from the sea). The healthy stand used as reference is located at Rebordelo (42.46° N; 8.48° W; altitude 530 m a.s.l.; 18 km from sea). The climate in both sites is temperate humid Atlantic. Annual precipitation during the study period (2003 – 2007) was 1511 $\pm$ 195 mm and 1976 $\pm$ 221 mm, and the mean annual temperatures were 14.8 $\pm$ 1.9 °C and 12.1 $\pm$ 0.6 °C in Rebordelo and Rianxo, respectively. Monthly precipitation and mean monthly temperature strongly correlated between the two sites during those years ($R^2 = 0.900$ and $R^2 = 0.989$ respectively, $N = 60$, $p < 0.001$).

Soils in both sites derived from granite and they are thin, sandy and acidic. Main characteristics just before plantation were low pH (pH in H₂O Rebordelo $\sim$ 4.0; Rianxo $\sim$ 4.3), high organic matter content ($\sim$ 180 and 170 g kg⁻¹ soil ash-free dry weight in Rebordelo and Rianxo, respectively), high total nitrogen content (Rebordelo $\sim$ 7.1 g N kg⁻¹ soil; Rianxo $\sim$ 6.3 g N kg⁻¹ soil), and very low concentrations of other nutrients, especially of available phosphorus (Rebordelo Olsen P $\sim$ 4.2 mg kg⁻¹ soil; Rianxo Olsen P $\sim$ 4.7 mg kg⁻¹ soil). Before trial establishment, Rebordelo stand was covered mainly by *Ulex europaeus* L., and Rianxo stand was covered by a 35 year-old *P. pinaster* stand. The clear-felling of the latter stand in January 2002 motivated the subsequent weevil infestation.

The experimental design was identical in both family $\times$ fertilization trials. It was a split-plot block design replicated in 10 blocks, with the fertilization treatments acting as the main factor, and the pine families as the split factor. Whole plots were around 12 x 18 m in size. In total, we planted 2790 seedlings in each trial, corresponding to 10 blocks $\times$ 9 fertilization treatments $\times$ 31 pine genetic entries. A more detailed description of the trials is in Zas et al. (2006).

Six-month-old containerized *P. pinaster* seedlings were planted at beginning of March 2003. Immediately after planting, nine fertilization treatments (with different combinations of four commercial fertilizers) were randomly assigned to the whole plots within each block, and manually distributed 30 cm around the seedlings. Plant material in both trials consisted of 28 open-pollinated families from plus trees randomly selected in a first generation seed orchard (Sergude, 42.82° N, 8.45° W), and three commercial seed sources as controls. Seedlings were grown together at the same nursery for both sites, and were about 15 cm tall at planting. Seedlings at Rianxo were attacked by *H. abietis* during 18 months, since 3 months after planting (June 2003) until the end of autumn of the second year (December 2004), with peaks of damage at late spring and early autumn. The spatial analysis of the infestation revealed that the damage was uniformly spread throughout the experimental area (Zas et al. 2006).

Sampling and field assessments

In order to reduce the analytical effort to reasonable levels, for the purpose of the present paper we studied a randomly selected subsample of the whole trials at each site. We sampled eight families under five fertilization treatments in five blocks; and additionally three of these families were sampled in all the 10 blocks of the trials in order to account for the spatial variability. The sample size was 275 trees for each trial.

The four fertilization treatments selected for tree sampling within the trials had a common base of potassium sulphate (applied at 15 g K plant⁻¹) and magnesium sulphate (at 5 g Mg plant⁻¹), plus an alternative combination of presence or absence of ammonium nitrate (5 g N plant⁻¹) and calcium phosphate (10 g P plant⁻¹). In summary, four fertilization treatments (+N+PCa; -N+PCa; +N-PCa; -N-PCa) plus an unfertilized control were selected for this study.

Plant growth was measured yearly (well in November or December) from 2003 to 2007 in both trials. Growth variables measured were total height in cm and stem-base diameter in mm. Stem deformities (bottom stem bifurcations and strong bottom stem curvatures) were evaluated in the fifth year as a binomial variable (presence-absence) in both trials.

The damage by *H. abietis* in the attacked stand was assessed during the first (February 2004) and the second year (December 2004) after planting. The intensity of wounding was evaluated by estimating the relative debarked area along the stem caused by the weevil using a four-level scale (0 = undamaged, 1 = some wounds, 2 = many wounds, and 3 = death due to girdling). To minimize subjectivity, the pine stem was divided in ten parts using an elastic ruler, recording the weevil wounding score in each part, and summing the ten values up, resulting in a 0-30 scoring for each tree. For the second year evaluation we followed the same method and scale of damage, but the stem height was only divided in five parts. As wounding preferences were extensively studied in a former paper for the whole trial (N = 2790 trees; Zas et al. 2006), for exploring the relationship with the nutritional status and subsequent growth increments in the present study we exclusively considered the wounding score in the 275-trees subsample.

The nutritional status was studied two years after planting (December 2004), immediately after the insect attack at Rianxo. We evaluated nitrogen and phosphorus concentration in the needles of all the 275 plants of each trial. In each tree, a group of about

100 needles from the apical stem and orientated towards the West was collected in December 2004, when nutrient concentrations are relatively stable (Will 1971), and transported to the lab in ice coolers. Needle samples were oven dried at 65 °C to constant weight, finely ground, labelled and preserved for chemical analysis.

To study the nutrient allocation, 25 seedlings were destructively sampled in the same five fertilization treatments, and in five randomly selected blocks in each site. These seedlings belonged to the mixed commercial seedlot produced in the same seed orchard as the studied families, which is formed by a random representation of the seeds produced by all the 116 plus trees in this seed orchard. A section 15 cm long of the apical stem (2004 growth) was sampled and transported in ice coolers to the lab, where the phloem was immediately separated by hand using a surgical knife, oven-dried and processed as above. Needles of these trees were sampled and processed as previously described.

Chemical analysis

For nitrogen and phosphorus content, 0.3 g of phloem 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, USA) at $\lambda = 650$ nm (Sims et al. 1995). Phosphorus was analysed in the same diluted aliquots by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Perkin-Elmer Optima 4300DV (Massachusetts, USA) in the central laboratory facilities at Universidade de Vigo – CACTI (www.uvigo.es/webs/cactiweb/). Nitrogen and phosphorus concentration were expressed in mg g^{-1} dried weight of tissue. Sample size for foliar nutrient content was $N = 275$ in each stand; and for phloem-needle relationships $N = 25$ in each stand.

Statistical analyses

Within each site, nutrient contents and final tree growth were analyzed using the PROC-MIXED procedure of the SAS System (Littell et al. 2006) and the following within site mixed model $Y_{ijk} = \mu + Fer_i + Fam_j + B_k + Fer \times Fam_{ij} + Fer \times B_{ik} + \varepsilon_{ijk}$, where μ is the general mean, Fer_i , Fam_j and B_k are the main effects of fertilization treatment i ($i = 1$ to 5), family j ($j = 1$ to 8) and block k ($k = 1$ to 10), $Fer \times Fam_{ij}$ and $Fer \times B_{ik}$ are the corresponding interactions, and ε_{ijk} is the experimental error. To analyze the whole plot factor (i.e. fertilization) with the appropriate error term, the $Fer \times B$ interaction was considered a random effect (Littell et al. 2006). For the purpose of the present paper, the main factor of study was fertilization and their interactions, and we do not present results regarding genetic effects. Family, as block, was anyway included in the model for reducing the residual variance to improve the power of the analyses. The genetic variation in pine susceptibility to the insect for the first and second year assessments were extensively studied in a companion paper (Zas et al., 2005). When main effects were significant, differences among treatment means were tested for significance using the LSMEAN statement (SAS-Institute 1999). Data are shown as least square means $\pm$ standard error (LS means $\pm$ s.e.).

To compare height and diameter growth over the five years between the two test sites we performed a repeated measures mixed model across sites using the PROC-MIXED procedure of the SAS System (Littell et al. 2006). The model included site (main factor), block (nested in site), fertilization (whole plot factor), family (split factor), time (repeated measures), and the corresponding interactions. A first order autoregressive model was assumed for the covariance structure of the repeated measures. For adequately account for the multisite split-plot design, the model included four different error terms.

Differences in binomial variables between stands were analyzed with a chi-square test. Linear regressions and Pearson correlations were used to evaluate the relationships between weevil damage, pine growth and nutrient contents in plant tissues. These analyses were carried out on family means ($N = 8$) and on raw data ($N = 275$ for the subsample of

families used in foliar nutrient status; and $N = 25$ for the destructive sample of the commercial seedlot used in needle-phloem correlations).

Results

Growth loss

Repeated measures analysis of tree height performed on the 5 years series in the two sites revealed a significant site $\times$ time interaction ($F_{4,1847} = 67.5$, $p < 0.001$). No significant differences between the attacked and the not attacked stands were found in height of the sampled trees at the first year after planting (Fig. 1a), suggesting similar forest site-quality for both stands. The intense pine weevil attack promoted significant growth losses during the following years in the infested stand, as revealed by a growth reduction of 40%, 15% and 5% in the second, third and fourth year, respectively, comparing to the growth observed in the stand with no attacks (Fig. 1a). However, plants in the attacked stand had apparently compensated for earlier growth losses by the 5th year, and overcome in size those in the not attacked stand.

Five years after the attack, the total height increment was not significantly related to the intensity of damage suffered ($r = -0.10$, $p = 0.12$, $N = 275$).

A similar trend was observed for diameter growth, with a significant site $\times$ time interaction ($F_{2,1137} = 21.20$, $p < 0.001$; Fig. 1b). During the second and the third year, diameter of plants in the infested stand was 35% and 25% lower than in the not attacked stand, respectively (Fig. 1b). However, no significant differences between stands were observed in diameter growth in the fifth year, indicating complete compensation of diameter growth, as with height growth (Fig. 1b).

The early growth response to the fertilization treatments was significant in both trials (Fertilization $F_{4,36} = 2.78$, $p < 0.05$ at Rianxo, and $F_{4,36} = 10.1$, $p < 0.001$ at Rebordele). The observed responses to fertilization during the first year were similar between sites, where the

pine height produced by the best fertilizer treatments was 53 ± 3 cm at Rianxo and 61 ± 3 cm at Rebordelo; while those achieved in unfertilized controls were respectively 48 ± 3 cm and 44 ± 2 cm. However, after five years, height of fertilized trees (overall mean height of fertilized trees = 362 ± 3 cm) was significantly greater than the control (315 ± 3 cm) in the healthy stand (Table 1; Fig. 2e), whereas the effect of fertilization on final height and diameter at age five was lost in the attacked stand (Table 1; Fig 2f).

Mortality and stem deformities

Although 94% of the seedlings in the attacked stand (Rianxo) were damaged by the insect, mortality was only 12% after one year and 17% after two years (% of planted seedlings). These values were only slightly higher than the mortality registered in the healthy stand (3% and 8% after the first and second year, respectively).

In the infested stand, damage by the pine weevil caused the leader loss of 35% and 48% of the seedlings one and two years after the attack, respectively (Zas et al., 2006). However, although bottom stem deformities were significantly more frequent in the infested stand ($\chi^2_1 = 17.3$, $p = 0.0003$), only 4.6% of the surviving seedlings showed severe stem deformities five years after the attack, indicating a good recovery of apical dominance by lateral branches.

Nutritional status

Increasing experimentally the nutrient availability in the not attacked stand significantly affected, as expected, the phosphorus and nitrogen concentration in the needles (Table 1, Fig. 2a, 2c). Phosphorus needle concentration was significantly greater in the treatments with phosphorus addition than in the unfertilized control (Fig. 2a). Nitrogen content, which is considered the superfluous nutrient in Galician soils, was generally greater in the unfertilized control than in the fertilized plants (Fig. 2c). Conversely, no significant

effect of the fertilization treatments was detected on needle nutrient concentrations in the attacked stand (Table 1; Fig. 2b, 2d).

Phloem and needle nutrient concentrations showed a strong and positive linear relationship in the not attacked stand for nitrogen (Fig. 3a) and phosphorus (Fig. 3b). However, an unexpected lack of relationship was observed for phloem-needle phosphorus in the infested stand (Fig. 3b), while only a weak relationship appeared for nitrogen concentration between those tissues (Fig. 3a), evidencing altered stoichiometry in plant tissues.

Wound intensity in the second year, i.e. just before needle sampling, was significantly and negatively correlated with N and P needle concentration when analysed on a raw data basis ($r = -0.28$; $N = 275$; $p < 0.001$ for N, and $r = -0.13$, $N = 275$, $p < 0.05$ for P), and with P concentration when analysed on a family mean basis ($r = -0.64$, $N = 8$, $p < 0.05$).

Discussion

Impact of weevil attack on pine growth, stem deformities and mortality

The large pine weevil, *H. abietis*, is an important insect pest of coniferous reforestation in Europe, to which important growth losses are commonly attributed. Our results showed a significant plant growth reduction in the infested stand during the following three years after the attack. During those years, plants showed an abnormally low interannual growth rate that suggests an important energy investment in repairing the damage suffered. Growth losses due to attacks by other weevil species have been previously reported, but little information is available in the case of *H. abietis*. White pine weevil (*Pissodes strobi*) attack reduced *Pinus strobus* height growth by 40 to 60% in a year (Hamid et al., 1995), and reduced significantly the total height in spruce plantations 10 years after planting (Kiss and Yanchuk 1991; King et al., 1997). Spruce growth remained negatively correlated with the initial *P. strobi* damage up to six years after the attack (vanAkker et al., 2004). However, *P.*

pinaster seedlings in our study were able to compensate for their early growth reduction, both in height and diameter, by the fifth year. To our knowledge this is the first work reporting subsequent growth patterns suggesting compensatory growth in a conifer plantation attacked by *H. abietis*. Plant growth compensation for insect damage has been commonly reported in long-lived woody plants (Trumble et al. 1993; Edenius et al. 1993; Bast and Reader 2003), and can be interpreted as a tolerance mechanism by which trees can reduce the impact of herbivores in plant fitness (Strauss and Agrawal 1999), allocating more resources to vegetative growth than undamaged trees. In this sense, *P. pinaster* seems to be highly tolerant to *H. abietis* at least on these sites.

The inference of compensatory growth was strongly supported by the fact that pine height growth in the attacked stand was unrelated to initial weevil damage five years after the infestation, while that relationship was strongly negative (the most wounding the less subsequent annual growth) in 2 and 3 yrs old pine seedlings (Zas et al., 2006). Thus, pines were able to overcome the initial growth reductions caused by the insect.

The similar pine heights at the 5th year in the attacked and in the not attacked stand also support this compensatory growth investment. However, as in other “natural experiments” this conclusion relies on the assumption that site quality was similar in both sites, and that the two sites would have produced equivalent growth in the absence of insect attacks. We assumed same site quality based on (i) the similar soil properties before planting; (ii) similar climate, based on last 20 yrs precipitation and temperature mean values, the specific values observed during the experiment, and the close correlations between both sites for precipitation and temperature regimes (see Methods section); (iii), the same early growth response to the fertilization treatments; (iv) the same early growth of unfertilized seedlings in both stands; and our personal experience with this species in the area. However, we cannot ensure exactly the same forest-site index for both stands, neither the absence of unaccounted site effects. Thus, the observed growth patterns could be alternatively explained simply by

better site quality in the attacked stand, promoting greater annual growth rates after the insect attack. Further manipulative experiments with protected control plants would definitely demonstrate the compensatory growth investment suggested by the comparison between stands.

The intensity of the attack was fairly high in the studied trial, with near all plants attacked at some level, and near half of the seedlings losing their leaders. However, five years after the attack the surviving trees showed very few stem deformities (less than 4% of trees), and a generalized recovering of the apical dominance. These results also suggest high tolerance of *P. pinaster* to the pine weevil in the study area.

In this study we observed much lower mortality due to *H. abietis* than those reported for northern European coniferous forests, where mortality commonly reach up to 80% of the seedlings planted following a clear-cutting (von Sydow and Birgersson 1997; Orlander and Nilsson 1999). Some hypotheses could contribute to explain these findings. In one hand, the suggested tolerance of this pine species, discussed above, could favour a lower mortality with the same level of damage. In agreement with this idea, Zas et al. (2008) found that mortality of *Pinus radiata* seedlings due to weevil damage was nearly twice that observed in *P. pinaster* adjacently planted for the same lever of insect damage (Zas et al. 2008). On the other hand, a lower mortality of *P. pinaster* after *H. abietis* attack could simply result from a lower intensity of damage to each individual tree. Pine weevil populations in higher latitudes seem to be denser than those observed in the study area (Moreira et al., 2008). Besides, weevil biology could be constrained by biogeographical considerations, because the studied area represents its southern limit of the distribution (Orlander and Nilsson 1999). Furthermore, reduced availability of oviposition sites in Galicia could lead to weaker booms of emerging insects. The pine plantations after clear-cutting in NW Spain are clearly fewer and smaller than those observed in northern forests. Unfavourable conditions for the aggregation of *H. abietis* after clear-cutting are also probable because Galician landscape is

fragmented and composed by a mosaic of small size properties of broadleaves and coniferous stands, interspersed with grasslands, cropland and scrub, lacking of large continuous extensions of coniferous forest.

In summary, all these findings consistently support that *P. pinaster* is a highly tolerant species to this pine weevil, at least in the area of this study. Lombardero et al. (2008) have also found evidences of high tolerance of *P. pinaster* to other insect herbivores at the same area. These authors reported that the impact of bark beetle attacks on the growth of *P. pinaster* was much lower than on other pine species, although the intensity of insect attacks were more than twice greater in *P. pinaster*. In agree with these observations, reduced costs of plant tissue reconstruction after herbivore grazing in favourable environments for high primary production (as the warm temperature and high precipitation in the NW Spain), could favour tolerance mechanisms against herbivory instead of investments in resistance (Strauss and Agrawal, 1999; Fine et al., 2004).

Impact on nutritional status

The response to fertilization treatments in the healthy stand agrees with the common deficiencies typically found in Galician forest soils. In this region P is a clear limiting nutrient while N is well supplied or even in excess (Sánchez-Rodríguez et al. 2002; Zas and Serrada 2003). And so, the regular trend observed in pine trees in these soils is an increase of P needle concentration due to P fertilization; and a lack of a response to N additions (e.g. Zas 2003). The higher N content in the unfertilized controls can be explained by a dilution effect in the fertilized trees because of their higher growth promoted by the base fertilization of potassium and magnesium sulphate. However, and despite the early response in growth to the fertilization treatments, we did not detect significant effects of fertilization on needle phosphorus and nitrogen concentration in the attacked stand. In concordance with this findings, lower concentrations in needle nutrient appeared inversely related to the previous

level of damage suffered. These results suggest that the attack by the pine weevil strongly modified the potential of the pine seedlings to allocate their resources to the needles according to the nutrient availability existing in the soil.

Another main finding of this study is that the seedlings attacked by *H. abietis* showed altered stoichiometry in N and P concentration among phloem and needles. Strong positive correlations among needles, stem, and root nutrient contents are commonly found in forest plantations, and are the regular trend in coniferous seedlings (Parks et al. 2000). We found strong positive relationships between the content of nutrients in phloem and needles in the not attacked stand. Nutrient analysis of greenhouse-grown *P. pinaster* seedlings of the same families also revealed a strong correspondence between nutrients in different tissues (Moreira et al., unpublished data). Thus, the lack of the corresponding relationship in the infested stand suggests that weevil damage is modifying the regular nutrient allocation patterns.

The results observed in the attacked stand are clearly anomalous and the most plausible explanation for these anomalies is precisely the damage caused by the pine weevil, which definitely characterize this site. Moreover, the fact that the relationship between weevil damage and needle nutrient concentrations is significant and negative despite the positive effect of fertilization on weevil damage also supports the hypothesis that the pine weevil damage is the cause of the altered nutrient allocation pattern. However, due to the experimental design, we cannot reject that site differences could also contribute to explain the unexpected observed patterns.

It has been suggested that reduced or increased levels of nutrients in target plant tissues could be part of induced responses to herbivory (Karban and Baldwin 1997; Newingham et al. 2007). Furthermore, Newingham et al. (2007) documented not simply altered nitrogen concentrations in target tissues of infested knapweeds but even allocation of nitrogen away from the target tissues. Karban and Baldwin (1997) highlighted the importance of such responses, not only because alterations of primary metabolites can

directly modify the tissue nutrient quality, but also because changes in primary metabolites may also change the efficacy of secondary compounds.

The disruption of the normal nutrient allocation pattern following the insect attack found in the present study could be part of this type of induced responses to herbivory. This study would be the first time such a response is documented in pine trees. Alternatively to this hypothesis, feeding on the phloem by the pine weevil may also directly interrupt the nutrient transport along the stem due to the vascular injury caused by the weevil wounding activity in the phloem. Manipulative experiments using chemical elicitors of induced responses, such as methyl jasmonate, that does not involve physical damage in the vascular tissues will definitively help to prove or reject the existence of induced changes in the nutrient allocation patterns in long-lived plants such as pine trees. Disregarding whether the altered nutrient allocation within plant tissues is part of an induced response mechanism to the weevil attack, or it was derived directly from the vascular damage after insect wounding on the phloem, our results indicate that plant nutritional status was largely altered by the insect damage, which may have further important consequences on other plant vital processes.

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Table 1. Summary of the mixed model for phosphorus (P) and (N) and concentration in the needles, and height in the fifth year (H₅) of *P. pinaster* seedlings at two twin family × fertilization trials, one of them attacked by *H. abietis* (Rianxo) and another not attacked (Rebordelo).

			P		N		H5	
	¹ DF _{num}	DF _{denom}	F	p>F	F	p>F	F	p>F
Rianxo (attacked)								
Block	9	36	2.23	0.043	0.74	0.668	0.50	0.867
<u>Fertilization</u>	4	36	1.41	0.251	0.96	0.441	0.80	0.531
<u>Family</u>	7	153	0.81	0.578	1.09	0.374	1.21	0.301
Fam × Fert	28	153	1.21	0.231	1.10	0.340	1.17	0.273
Rebordelo (not attacked)								
Block	9	36	2.23	0.043	1.27	0.288	1.09	0.396
<u>Fertilization</u>	4	36	3.87	0.010	4.27	0.006	2.97	0.032
<u>Family</u>	7	177	2.38	0.024	2.29	0.029	0.87	0.528
Fam × Fert	28	177	0.75	0.813	0.75	0.810	0.96	0.531

¹ DF = degrees of freedom.

FIGURE CAPTIONS

Figure 1. Height (1a) and stem base diameter (1b) of *P. pinaster* seedlings during five consecutive years after planting in two twin family $\times$ fertilization trials, one of them attacked by *H. abietis* (Rianxo, black bars) and another not attacked (Rebordelo, white bars). Overall LS means across all fertilization and treatments $\pm$ S.E. are presented, according to repeated measures ANOVA. N = 275 per trial. Asterisks indicate significant differences at $p < 0.01$ (**) and $p < 0.001$ (***); n.s.: not significant.

Figure 2. Effects of experimental fertilization on the concentration of phosphorus (2a, 2b) and nitrogen (2c, 2d) in the needles, and on the height at year 5 (2e, 2f) of *P. pinaster* seedlings in two twin family $\times$ fertilization trials, one of them attacked by *H. abietis* (Rianxo, right panels, black bars) and another not attacked (Rebordelo, left panels, white bars). Samples for foliar analyses were taken immediately after the second year attack, two years after planting. LS means $\pm$ S.E. N = 55 per treatment and trial. Different letters indicate significant differences at $p < 0.05$.

Figure 3. Nitrogen (a) and phosphorus (b) nutrient concentrations in needles vs phloem in 2 years-old *P. pinaster* seedlings in two twin family $\times$ fertilization trials, one of them attacked by *H. abietis* (Rianxo, solid dots and lines) and another not attacked (Rebordelo, open dots, dashed lines). Each point represents a randomly-selected destructively sampled tree belonging to the same seed source at both stands. N = 25 for each site.

Figure 1R

Figure 1. Sampedro et al., TREES-D-08-0211 revised

Figure 2. Sampedro et al., TREES-D-08-0211 revised

Figure 3. Sampedro et al., TREES-D-08-0211 revised