

1 **Genetic variation and phenotypic plasticity of nutrient re-allocation and increased**
2 **fine root production as putative tolerance mechanisms inducible by methyl-jasmonate**
3 **in pine trees**

4

5 Running title: **Putative induced tolerance responses in pine juveniles**

6

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23 **Supplementary Material**

24 Table S1. Chemical composition of the solutions used in both fertilization treatments. In

25 both solutions pH was adjusted to 6.5 with diluted HCl.

26

27 Figure S1. Example picture of the root systems of *P. pinaster* juveniles in control (a) and

28 MJ-induced (22 mM MJ application to the foliage) (b), 20 days after treatment application.

29 Please note the differences in the fine root system due MJ application.

30

31 **Abstract**

32 1.- Plant plastic responses to herbivore damage may include rapid, active reallocation of
33 plant resources in order to reduce the impact of herbivory on future plant fitness. However,
34 whether these inducible tolerance responses can be extended to pine trees and how these
35 responses could be modulated by genetic and environmental factors remains unclear.

36 2.- Biomass allocation and phosphorus (P) and nitrogen (N) concentration in above- and
37 below-ground tissues were measured in *Pinus pinaster* juveniles belonging to 33 open-
38 pollinated families grown under two P availabilities (P-deficient and complete
39 fertilization). Measurements were taken 15 days after half of the plants received a foliar
40 spray treatment of 22 mM methyl jasmonate (MJ) to simulate above-ground herbivore
41 attack.

42 3.- Simulated above-ground herbivory promoted a strong preferential allocation of biomass
43 below ground in the form of fine roots, leading to an almost 2-fold increase in fine root
44 biomass in MJ-treated plants, and a significant reduction in above-ground tissues and
45 coarse roots. In addition, MJ-signalling increased P and N concentrations in the shoots
46 while reducing (P) or maintaining (N) concentrations in the roots. These results suggest
47 that induced resource sequestration is not a generalised strategy in this pine species. Fine
48 root biomass and concentration of N and P in plant tissues showed additive genetic
49 variation, but responses to MJ-signalling did not vary among families. Allocation of
50 biomass to fine roots was not affected by P availability, whereas allocation of P to the
51 shoot was more intense under complete fertilization.

52 4.- *Synthesis*: Two new putative tolerance mechanisms inducible by MJ-signalling may
53 help to minimize the impact of above-ground herbivore damage on the future fitness of
54 young pine trees by: (i) allocation of carbon to fine roots, this appeared to be a generalised
55 strategy with weak environmental modulation; and (ii) reallocation of P and N from roots

56 to shoots, this result was largely affected by P availability, and thus susceptible to greater
57 phenotypic variation in heterogeneous environments. We provide evidence that changes in
58 tolerance-related traits are rapidly inducible by herbivory cues in this pine species. These
59 results should be integrated with induced resistance responses in order to fully understand
60 the costs and benefits associated with induced responses to herbivory.

61

62 **Keywords**

63 Biomass allocation; conifers; herbivory; nitrogen allocation; phosphorus allocation;
64 phosphorus availability; *Pinus pinaster*; plant-herbivore interactions

65

66 **Introduction**

67 Plants phenotypes can rapidly change following herbivore attack, these effects are known
68 as induced responses (Heil 2010). Induced resistance, which aims to deter, reduce or delay
69 current and subsequent attacks, has been extensively studied (e.g. Cipollini & Heil 2010;
70 Eyles *et al.* 2010). Tolerance to herbivory, defined as the capacity to maintain plant fitness
71 irrespective of the damage inflicted by the herbivores (Heil 2010), is another plant strategy
72 for overcoming herbivory (Fornoni 2011). Little is known, however, about the
73 mechanisms involved in conferring high levels of tolerance. Evidence is emerging that
74 plant tolerance may rely on plastic changes in tolerance-related traits which occur in the
75 immediate aftermath of an attack, forming part of the integrated plant responses to
76 herbivory rather than compensatory secondary responses to tissue loss (Schwachtje *et al.*
77 2006; Erb *et al.* 2009; Kerchev *et al.* 2011;). A few recent studies using short-lived
78 radioisotopes have reported rapid changes in carbon and nutrient allocation in response to
79 real or simulated herbivory in annual (Schwachtje & Baldwin 2008; Gómez *et al.* 2010;
80 Hanik *et al.* 2010) and woody plants (Babst *et al.* 2005, 2008; Frost & Hunter 2008). The

81 most commonly reported pattern seems to be induced resource sequestration, that is,
82 removing nutrients and carbon resources away from the site of damage and into storage
83 tissues shortly after the attack (reviewed by Orians, Thorn & Gómez 2011). This strategy
84 may prevent the loss of new photosynthates and the stored resources may be crucial for
85 sustaining regrowth and reproduction once the herbivory threat has passed (Gómez *et al.*
86 2010), thus diminishing the impact of herbivores on plant fitness (Babst *et al.* 2005).
87 Additionally, reduced nutritional quality of target host tissues may reduce insect
88 performance, thereby improving plant resistance (Mattson 1980).

89 Reallocation of biomass and nutrients in response to herbivory might be a
90 particularly relevant tolerance strategy in woody plants because their longevity and large
91 size make them more exposed to insect herbivory (Haukioja & Koricheva 2001), and they
92 may have a greater potential for storage of biomass and nutrients (Stevens, Kruger &
93 Lindroth 2008). Indeed, significant shifts in biomass and nutrient partitioning after real or
94 simulated herbivory have been reported in several angiosperm trees (Babst *et al.* 2005;
95 2008; Stevens, Waller & Lindroth 2007; Frost & Hunter 2008; Stevens, Kruger &
96 Lindroth 2008; Eyles, Pinkard & Mohammed 2009). Surprisingly, despite their ecological
97 and economic importance, very little information is available in the case of conifers (Ayres
98 *et al.* 2004). Conifers may substantially differ from angiosperm trees in their responses
99 due to different anatomy, life history traits and ecophysiology (e.g. Hoch, Richter &
100 Körner 2003).

101 Although it is widely accepted that the effect of herbivore damage on future plant
102 fitness depends on resource availability (Hawkes & Sullivan 2001) and that we know that
103 resistance responses are genetically variable and environmentally dependent (Ballhorn *et*
104 *al.* 2011; Sampedro, Moreira & Zas 2011), little is known about the genetic variation of
105 the tolerance-related plastic responses to herbivory within populations and how they are

106 linked to resource availability (Heil 2010; Fornoni 2011). Unravelling these questions is
107 essential to fully understand the ecological and adaptive relevance of induced tolerance
108 and its integration with other herbivore-induced plant responses (Núñez-Farfán, Fornoni &
109 Valverde 2007; Fornoni 2011).

110 The aim of this paper was to study the inducibility, additive genetic variation and
111 plasticity to nutrient availability of biomass partitioning and nutrient reallocation: two
112 traits putatively related to tolerance and potentially responsive to herbivory damage in
113 *Pinus pinaster* juveniles. We mimicked above-ground herbivory with the application of
114 methyl jasmonate (MJ), a plant hormone central to the responses elicited by wounding
115 damage (e.g. Heidel & Baldwin 2004), thus avoiding the side effects of using experimental
116 tissue removal. We manipulated growth of pine seedlings through P availability, as this
117 nutrient is considered to be the main limiting factor for pine growth in our study area
118 (Martíns *et al.* 2009 and references therein). Phosphorus availability increased the
119 incidence of insect herbivory in this pine population (Zas *et al.* 2006; Sampedro *et al.*
120 2009), which shows significant genetic variation in tolerance (Zas, Moreira & Sampedro
121 2011). In a previous paper with the same plant material, we found that P availability
122 determined the allocation to constitutive chemical defences and their inducibility
123 (Sampedro, Moreira & Zas 2011). Here, we extend that study to determine whether
124 induced responses in pines might include changes in traits putatively related to tolerance to
125 herbivore damage. We hypothesize that pine responses to above-ground herbivory may
126 include major plastic responses other than the induction of chemical defences, as moving
127 nutrient and carbon resources to below-ground compartments. We suggest that, as
128 previously observed for inducibility of chemical defences, these responses could be
129 genetically variable and modulated by soil P availability.

130

131 **Material and methods**

132 **Experimental design**

133 We carried out a greenhouse experiment following a randomized split-split design
134 replicated in four blocks, with P availability (two levels: complete fertilization and P-
135 deficient fertilization) as the whole factor, MJ-induction of defensive responses (two
136 levels: MJ-treatment and control) as the split factor; and thirty-three genetic entries (open-
137 pollinated half-sib families, known mother trees) as the split-split factor. In total, there
138 were 528 pine juveniles, corresponding to 4 blocks \times 2 P availabilities \times 2 MJ treatments \times
139 33 genetic entries.

140

141 **Plant material, greenhouse conditions, fertilization and MJ-induction**

142 *Pinus pinaster* half-sib families were randomly selected from a broader experimental
143 collection of mother trees representative from the Atlantic coast population of Galicia (NW
144 Spain). The climate in this area is temperate humid Atlantic, with annual precipitation of
145 about 1500 mm and mean annual temperatures of 11 °C, typically ranging between 25 °C
146 (maximum daily mean) and 4 °C (minimum daily mean). Soils in this region are typically
147 thin, sandy and acidic, with high organic matter content, high total nitrogen content and
148 very low concentration of available P, although soil fertility has a marked spatial
149 heterogeneity. *P. pinaster* is a fast-growing sun-demanding pioneer pine species native
150 from western Mediterranean basin that occupies large extensions in this region. Early
151 growth of this species is critical for future fitness, and insect herbivory is a major cause of
152 pine seedling mortality (see Appendix S1 in Sampedro, Moreira & Zas 2011).

153 Seeds from those mother trees were individually weighed and sown on 7 February
154 2006 in sterilized 2-l pots containing sterilized perlite, and cultured in an isolated glass
155 greenhouse with controlled light (minimum 12 h per day) and temperature (10°C night,

156 25°C day), and daily watering by subirrigation. To avoid interference from soil microbes
157 such as pathogens and mycorrhizal colonization, seeds were preventively treated with a
158 fungicide (Fernide®, Syngenta Agro, Spain), that was also applied every two months
159 during pine growth. No mycorrhizal colonization was detected in the whole experiment.

160 One month after sowing we began applying the fertilizer treatments (complete and
161 P-limited fertilizer) by subirrigation every two days. The complete fertilizer was a
162 balanced solution containing 100:20:70:7:9 mg l⁻¹ of N:P:K:Ca:Mg, respectively and the
163 necessary amounts of micronutrients and trace elements (see detailed chemical
164 composition in Supplementary Material, Table S1). This solution was a modification of
165 that used by local nurseries for optimum seedling growth of this pine species. The P-
166 limited fertilizer solution contained the recommended levels of N, K, Ca and Mg, as
167 described above, but the availability of P was reduced 10-fold to 2 mg P l⁻¹ (Supplementary
168 Material, Table S1). In previous trials we found that this P-deficient treatment led to P
169 concentration in plant tissues similar to the lowest values observed in the field (Martíns *et*
170 *al.* 2009). Fertilizer solutions were freshly prepared every two weeks, and pH was adjusted
171 to pH 6.5 in both treatments.

172 On 2 August 2006, when average plant heights in P-deficient and complete
173 fertilization treatments were 21.9 ± 0.7 cm and 44.3 ± 1.3 cm respectively, half of the
174 plants were treated with a solution of 22 mM MJ (Sigma-Aldrich, #39270-7) suspended in
175 deionized water with ethanol 2.5% (v:v). The rest of the plants were treated only with the
176 carrier solution (2.5% ethanol) and acted as control. Treatments were sprayed evenly over
177 the foliage to runoff with a handheld sprayer. Each individual plant, weighed before and
178 after treatment application, received 2.6 ± 0.2 or 3.7 ± 0.3 ml of solution (P-deficient and
179 complete fertilization plants, respectively; mean ± s.e.). MJ dose and concentration were
180 previously determined by means of some trials performed with the same plant material and

181 according to previous research (Moreira, Sampedro & Zas 2009). To avoid cross-
182 contamination, the two treatments were applied in two different rooms, and juveniles
183 remained in separate rooms for 24 h to allow drying.

184

185 **Sampling and measurements**

186 Two weeks after MJ application, we measured plant height and stem basal diameter, and
187 all pine juveniles were harvested for biomass determination and further chemical analyses.
188 Plants were carefully cleaned of foreign matter and perlite, separated into coarse and fine
189 roots (these latter defined as those of diameter < 2 mm), shoots, adult needles (secondary
190 needles bundled in dwarf shoots), and juvenile (primary) needles. Plant material was then
191 oven dried for 72 h at 65°C to constant weight, weighed to the nearest 0.001 g, grounded
192 with liquid nitrogen and stored for further nutrient analyses. Total biomass was determined
193 as the sum of root, shoot, and adult and juvenile needle biomass. The total number of adult
194 and juvenile needles was also counted. The density of needles in the main stem was
195 calculated as the number of total needles (adult plus juvenile) per cm of main stem length.

196

197 **Nutrient analyses**

198 To reduce the analytical effort to reasonable levels, nutrient concentration was analyzed in
199 a subsample of 11 randomly selected pine families. Sample size for nutrient concentrations
200 was thus N = 176 plants. For chemical analyses, 0.3 g of grounded plant material (juvenile
201 needles, adult needles, shoots or coarse roots) was digested in a mixture of selenous
202 sulphuric acid and hydrogen peroxide (Martíns *et al.* 2009). Nitrogen was determined
203 colorimetrically in diluted aliquots of this digestion using a BioRad 680 microplate reader
204 (California, USA) at 650 nm (Sims *et al.* 1995). Phosphorus was analyzed in the same
205 diluted aliquots by inductively coupled plasma optical emission spectroscopy (ICP-OES)

206 using a Perkin-Elmer Optima 4300DV (Massachusetts, USA) at the central laboratory
207 facilities of Universidade de Vigo – CACTI (www.uvigo.es/webs/cactiweb/). Pine needle
208 standards (NIST#1575) were used for checking the correct quantification. Nitrogen and P
209 concentration were expressed in mg g⁻¹ tissue on a dry weight basis.

210

211 **Statistical analyses**

212 Analyses were carried out with the proper mixed model to solve split-split designs (Littell
213 *et al.* 2006) using the Proc Mixed procedure of SAS System. The main effects of P
214 availability (P), MJ induction (MJ) and the P × MJ interaction were treated as fixed factors.
215 The Block (B) effect, B × P and B × P × MJ interactions were considered random in order
216 to analyse the main factors P and MeJa with the appropriate error terms (B × P and B × P ×
217 MJ, respectively). Family (G) and its interactions with main effects (P, MJ and P × MJ)
218 were considered random, and associated variance components were estimated by restricted
219 maximum likelihood. The statistical significance of the variance components for each
220 random factor was assessed using likelihood ratio tests, where the differences in two times
221 the log-likelihood of the models including and excluding that random factor are distributed
222 as one tailed χ^2 , with one degree of freedom (Littell *et al.* 2006). Residuals of seed weight
223 within families were used as a covariate for analyzing the variables of growth in order to
224 remove this relevant maternal effect affecting early pine performance and thus reduce the
225 error term. When needed, normality was achieved by log-transforming the original
226 variables. Equality of residual variance across MJ and P treatments was tested in all cases,
227 and residual heterogeneity variance models were used when significant deviations were
228 found (Littell *et al.* 2006).

229

230 **Results**

231 **Plant growth and biomass allocation**

232 Application of MJ significantly reduced growth of pine juveniles after just 15 days, where
233 total height, basal diameter and total biomass of MJ-induced plants (37.2 ± 0.6 cm; $4.3 \pm$
234 0.1 mm; 24.1 ± 1.1 g) were significantly smaller than control plants (44.9 ± 0.6 cm; $4.6 \pm$
235 0.1 mm; 29.7 ± 1.1 g; $P < 0.05$ in all cases). Application of MJ stopped or reduced shoot
236 and adult needle growth (Table 1, Figure 1) and also the development of new adult needles
237 ($F_{1,6} = 8.4$; $P = 0.028$). All these variables were affected by soil P availability and largely
238 varied among pine families (Table 1). We only observed a significant effect of P×MJ
239 interaction in the biomass of adult needles (Table 1), which was originated by a larger
240 reduction of the adult needle-biomass under well-fertilized conditions than in P-deficient
241 plants (Figure 1). All these changes led to significant and relevant changes in the plant
242 morphology above ground after 15 days. Application of MJ significantly reduced the
243 height: diameter ratio ($F_{1,6} = 29.7$; $P = 0.002$), because it reduced primary growth rate
244 (height) more than secondary growth rate (diameter). Application of MJ also promoted a
245 more packed distribution of needles in the main stem (37 ± 1.9 and 45 ± 2 needles cm^{-1} in
246 control and treated plants, respectively; $F_{1,6} = 25.8$; $P = 0.002$) because MJ stopped the
247 apical growth but not the development of new needles in the main stem.

248 Application of MJ also modified allocation of biomass to roots, strongly reducing
249 the growth of coarse roots but promoting a marked boost in fine root biomass (Table 1,
250 Figure 1). Biomass of fine roots in MJ-treated plants was almost 90% and 40% (in low and
251 high P availability, respectively) greater than those in control plants. These changes led to
252 altered plant morphology below ground just 15 days after MJ application (Supplementary
253 Material, Figure S1). Simulated herbivory increased the root: shoot ratio, with a significant
254 effect of P×MJ interaction, because the increase was more marked when P availability was
255 low (Figure 2a). Application of MJ also drastically increased the fine-to-coarse root ratio

256 (Figure 2b), irrespective of the P availability. We found significant differences among pine
257 families for fine-to-coarse root ratio, but did not for root: shoot ratio (Figure 2). Above-
258 and below-ground allocation responses were similar in all families as revealed by the non-
259 significant MJ \times F interaction (Figure 2).

260

261 **Phosphorus and nitrogen concentration in plant tissues**

262 Manipulation of P availability significantly affected P concentration in adult and juvenile
263 needles, shoots and roots of *P. pinaster* juveniles (Table 2). Phosphorus concentration in
264 all plant compartments was significantly greater in plants growing under complete
265 fertilization (Figure 3). Simulated herbivory with MJ significantly affected P concentration
266 in shoots and roots of *P. pinaster* juveniles 15 days after MJ application (Table 2),
267 increasing P concentration in shoots and reducing it in roots (Figure 3). The effect of
268 simulated herbivory on P concentration in shoots was much greater under complete P
269 nutrition than under P-deficient conditions, and in the case of roots it was only significant
270 in plants growing under complete P nutrition (significant P \times MJ interaction in both cases,
271 Figure 3). MJ did not affect P concentration of adult and juvenile needles (Table 2).
272 Differences in P concentration among pine families were observed in needles and roots,
273 but not in shoots (Table 2). Pine families did not differ in their response to MJ treatment
274 for P concentration in shoots and roots (not significant F \times MJ interaction, Table 2).

275 Manipulation of P availability significantly affected N concentration in roots and
276 needles and also marginally in shoots, however the effect was small with only slightly
277 greater N concentration in plants growing under complete P fertilization (Table 3, Figure
278 4). Concentration of N in the shoots, but not in the other compartments, was significantly
279 affected by MJ signalling (Table 3). Induced plants showed 25% greater N concentration
280 in their shoots than control plants (Figure 4). The P \times MJ interaction was significant for N

281 concentration in adult needles and roots, and marginally significant for shoot (Table 3).
282 Application of MJ reduced the N concentration in adult needles of P-deficient plants,
283 whereas no significant effect was observed in complete fertilized plants (Figure 4). On the
284 other hand, MJ reduced N concentration in roots of plants growing in complete
285 fertilization and increased it in plants growing in P-deficient conditions (Figure 4).
286 Differences among pine families in N concentration were observed in all plant
287 compartments (Table 3). However, pine families did not differ in their response to MJ
288 treatment, as revealed by the non-significant Family \times MJ interaction (Table 3).

289 When analyzing the absolute nutrient content in each plant tissue (i.e. the
290 corresponding nutrient concentration \times biomass), we found that the content of both N and
291 P in the shoots was significantly greater in MJ-treated plants than in control plants ($F_{1,6} =$
292 $27.7, P = 0.002$ for P content and $F_{1,6} = 8.4, P = 0.028$ for N content). This indicate that
293 the observed changes in nutrient concentration in the shoots of MJ-treated plants were not
294 side effects derived from differential aboveground growth rates after MJ application.

295

296 **Discussion**

297 **Induced allocation of biomass to fine roots**

298 Simulated herbivory through MJ-signalling reduced growth of above-ground tissues (adult
299 needles and shoots) and also of coarse roots, but induced a strong boosting of the fine root
300 system. Specifically, fine root biomass of MJ-induced plants increased nearly 2-fold in 2
301 weeks, resulting in a 3-fold greater fine-to-coarse root ratio. The reduction observed in the
302 starch pool after MJ application (Sampedro, Moreira & Zas 2011) was very small in
303 comparison to the huge increase in fine root biomass, suggesting that current
304 photosynthates were likely a major source for the new fine root biomass, in accordance
305 with recent observations using short-lived radioisotopes (Hanik *et al.* 2010).

306 We found that above-ground herbivore signalling induced allocation of biomass to
307 below-ground tissues, not to storage tissues (we observed induced reduction in biomass in
308 coarse roots), but to fine roots. Fine roots are sinks in terms of carbon balance and
309 allocation of carbon resources to those tissues cannot be further reallocated to other
310 functions. Thus, our results do not support the hypothesis of induced sequestration of
311 carbon resources in young pines (Orians, Thorn & Gómez 2011). Alternatively, it seems
312 that young pines show a preferential investment in absorptive tissues. Juvenile plants of
313 fast growing colonizer tree species, for which a robust growth potential is vital for future
314 fitness (Haukioja & Koricheva 2001), could obtain long-term benefits from this kind of
315 induced tolerance strategy. Induced allocation of carbon resources to fine roots could
316 directly improve the ability of plants for water and nutrient acquisition that will be
317 essential for above-ground regrowth (Ayres *et al.* 2004; Erb *et al.* 2009), but also indirectly
318 through facilitation of mycorrhizal colonization (Regvar, Gogala & Žnidaršič 1997) and/or
319 increased mineralization in the rhizosphere.

320 Although preferential induced allocation of biomass to below-ground structures has
321 been reported in annual plants (Schwachtje *et al.* 2006; Henkes *et al.* 2008; Gómez *et al.*
322 2010) and woody plants (Babst *et al.* 2005; 2008), this type of response is not ubiquitous.
323 In the case of woody plants the opposite, i.e. defoliation-induced shifts in biomass
324 partitioning from fine and coarse roots to above-ground tissues, has also been reported in
325 *Eucalyptus* (Eyles, Pinkard & Mohammed 2009), *Quercus* (Frost & Hunter 2008) and
326 *Populus* (Stevens, Kruger & Lindroth 2008). Discrepancies between studies could be
327 explained in terms of different response dynamics, variation in the time elapsed since
328 damage (Eyles, Pinkard & Mohammed 2009; Metlen, Aschehoug & Callaway 2009;
329 Gomez, van Dijk & Stuefer 2010) and damage severity (Frost & Hunter 2008). But
330 intrinsic differences among taxa, such as carbon storage patterns (e. g. Hoch, Richter &

331 Körner 2003), and even differences in terms of life history within related species could
332 explain different strategies.

333

334 **Induced allocation of N and P to shoots**

335 Above-ground simulated herbivory in young pines rapidly increased the concentration of N
336 and P in the shoots while maintaining (N) or reducing (P) concentrations in the roots. The
337 lack of an effect on nutrient concentration of juvenile and adult needles and absence of
338 major changes in the whole plant pool of nutrients suggest an induced mobilization of
339 mineral resources from roots to shoots. Again these results disagree with the trend of
340 hiding nitrogen away from herbivores commonly reported in herbs and annual plants
341 (Newingham, Callaway & BassiriRad 2007; Gómez *et al.* 2010). Our results also contrast
342 with those observed in oak seedlings, beech and fir saplings in which above-ground
343 herbivory or leaf clipping stimulated the storage of N into roots (Ayres *et al.* 2004; Frost &
344 Hunter 2008). Alternatively, increasing nutrient concentration around damaged tissues may
345 be important to boost the *de-novo* synthesis of induced resistance mechanism (Babst *et al.*
346 2005; Gómez *et al.* 2010). Accordingly, we found stem diterpenes increased 15–20% after
347 MJ induction in these plants (Sampedro, Moreira & Zas 2011). Nitrogen, in particular,
348 would be required for the production of large amounts of terpenoid synthases and for the
349 differentiation of new xylem resin canals, while phosphate is required for the intense
350 phosphorylation processes leading to the biosynthesis of terpenoids induced by MJ-
351 signalling in conifer tissues (e. g. Miller *et al.* 2005). We would suggest that induced
352 nutrient sequestration could be not an effective strategy when N and P are required for
353 massive *in situ* synthesis of defensive chemical defences, as in the case of the stem of pine
354 trees.

355 On the other hand, increasing the concentration of induced defences in the stem
356 could help to alleviate the negative side effects caused by moving nutrients to stems after
357 above-ground herbivory. Increased insect performance may be expected when feeding on
358 nitrogen rich tissues, which is likely to lead to greater susceptibility (Mattson 1980, Ayres
359 *et al.* 2000). In the case of young pines, the greater nitrogen concentration in target tissues
360 could be shielded or counterbalanced by the simultaneous increase in stem oleoresin and
361 phenolics observed in those plants (Sampedro, Moreira & Zas 2011), leading to similar or
362 even poor tissue quality for herbivores.

363 Taken together, our results provide little support for induced resource sequestration
364 as generally favoured strategy in this species. Although it seems accepted that re-allocation
365 of nutrients and carbon resources can be a common herbivore-induced plant response, it is
366 difficult to identify a general pattern in the direction that resources could be moved across
367 plant taxa (Orians, Thorn & Gómez 2011). Further research should address whether the
368 pattern of moving carbon resources to absorptive tissues and nutrients to the stem observed
369 in *P. pinaster* is a common response within the *Pinus* phylogeny, according to their
370 specific anatomical and evolutionary constraints. Alternatively, we could speculate that this
371 pattern could depend on the particular life histories of the species considered. In a recent
372 meta-analysis Endara & Coley (2011) found that species adapted to resource-poor
373 environments grow inherently more slowly, investing more in constitutive defences and
374 supporting lower herbivory than species from productive habitats. Thus, habitat quality
375 affiliation and subsequent intrinsic growth rates could shape the strategies of defensive
376 investment, and likely those of tolerance responses too (Coley 1987; Agrawal 2011;
377 Endara & Coley 2011). The latter could explain the differences between our results (in a
378 fast-growing colonizer pine) and those reported for other long-lived species, such as those

379 for oak seedlings (Frost & Hunter 2008) which have a slow-growing/low-resource
380 availability strategy.

381

382 **Genetic variation and environmental modulation of biomass and nutrient reallocation**

383 Biomass of fine roots and P and N concentration showed significant overall additive
384 genetic variation, and though inducible, did show no significant genetic variation in the
385 inducibility, that is, responses were consistent among families. Contrastingly, we found
386 genetic variation in inducibility of stem oleoresin and needle total phenolics and tannins in
387 these same plants (Sampedro, Moreira & Zas 2011). These results are not striking, as
388 tolerance traits are expected to be less variable within a population than resistance traits
389 (Roy & Kirchner 2000). Rising herbivore incidence is expected when a host population is
390 more tolerant, when greater would be the fitness advantage of being a tolerant genotype.
391 The opposite is predicted, however, for resistance (Roy & Kirchner 2000).

392 Our results provide evidence that changes in tolerance-related traits are inducible
393 by herbivore cues, as induced resistance, and are likely to be part of the integrated plant
394 responses to herbivory in this species. Although resistance and tolerance have sometimes
395 been thought of as alternative strategies (e.g. Van der Meijden, Wijn & Verkaar 1988;
396 Fineblum & Rausher 1995), they could be two complementary adaptive responses to
397 herbivory (e.g. Agrawal, Strauss & Stout 1999; Stevens, Waller & Lindroth 2007). We
398 found that MJ-signalling is implicated in both the induction of chemical defences
399 (Sampedro, Moreira & Zas 2011) and in changes in putative traits involved with tolerance
400 responses (the present paper). By joining together databases of family means, we have
401 explored possible genetic correlations among inducibility (defined as the difference
402 between the family mean in MJ-induced plants and the family mean in control plants, for a
403 given family) of traits of quantitative resistance (resin and total phenolics) and the

404 inducibility of putative tolerance traits (fine root biomass and nutrient reallocation). We
405 only found a significant negative genetic correlation ($r = - 0.61$; $P < 0.001$; $N = 33$)
406 between the inducibility of phenolics and the increase of fine root biomass, but only under
407 P-limited conditions. In other words, under scarce P availability and reduced growth rates,
408 pine families that showed greater induction of phenolic compounds after MJ-simulated
409 herbivory were those with the smaller induced allocation of biomass to fine roots. All the
410 other family correlations were not significant ($P > 0.1$). This provides evidence for the
411 existence of a context-dependent conflict where synthesis of phenolic compounds, but not
412 resin defences, is trading-off with fine root induced biomass allocation. This result is
413 consistent with previous observations that vegetative costs (in terms of reduced total plant
414 biomass, height and diameter) of induced defences were found for leaf phenolics
415 (Sampedro, Moreira & Zas 2011) but not for resin-based defences. This result also
416 suggests that a greater proportion of the vegetative costs of herbivore-induced responses
417 arises from the construction of induced tolerance traits, rather than the synthesis of induced
418 chemical defences. Further research should address this question.

419 Patterns of environmental modulation on biomass reallocation markedly differed
420 from those observed for nutrients. The increase in fine-root biomass after MJ signalling
421 was unaffected by the actual P availability. However, the induced allocation of P from
422 roots to shoots was greater under complete fertilization than in P-impooverished conditions
423 (significant MJ \times P interaction), and we also found an interactive effect of P availability on
424 N reallocation. Thus, although no genetic variation in nutrient reallocation responses was
425 found, the strong environmental modulation of those responses, together with the high
426 spatial heterogeneity in P availability in the study area (Martins *et al.* 2009) could lead to
427 large phenotypic variation across spatial scales in these mechanisms.

428

429 **Conclusions**

430 In response to above-ground MJ-signalling, juveniles of this pine species (i) prioritized the
431 allocation of biomass to below-ground absorptive structures leading to a strong boosting
432 of the fine root system, while reducing growth of coarse roots and above-ground
433 structures; and (ii) increased the allocation of N and P to the shoots, probably diverting
434 these nutrients from the roots. These herbivore-induced mechanisms are putatively related
435 to tolerance to herbivory and are a new result for pine trees. Biomass of fine roots and P
436 and N concentration in plant tissues showed significant overall additive genetic variation,
437 and though inducible, did not show significant genetic variation in the inducibility, that is,
438 responses were consistent among families. Boosting of fine roots appeared to be a
439 generalized strategy with weak environmental modulation, whereas induced shifts in N
440 and P to the shoots were strongly affected by P availability. Thus, spatial variation in P
441 availability at field could contribute to phenotypic variation in induced reallocation of
442 nutrients. Our results indicate that induced resource sequestration is not likely a
443 generalized herbivore-induced response in young pine trees, and evidence that herbivore-
444 induced changes in tolerance-related traits are part of the integrated plant responses to
445 herbivory in this species.

446

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599 **Table 1.** Summary of the mixed model for biomass of adult needles, juvenile needles,
600 shoots and coarse and fine roots of thirty-three *P. pinaster* open-pollinated families under P
601 deficient and complete fertilization treatments in constitutive (control) and MJ-induced
602 conditions 15 days after MJ application. The family effect (F) and derived interactions are
603 random effects, and the corresponding likelihood ratio significance tests (χ^2) are shown.
604 Phosphorus availability (P) and methyl jasmonate (MJ) induction of defences are fixed
605 effects, and the F values and corresponding *df* are shown. Significant *P* values ($P < 0.05$) are
606 typed in bold.

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	Adult needles			Juvenile needles		Shoot		Coarse roots		Fine roots	
	<i>df</i> (F)	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value
Family (F)		51.2	0.000	18.0	0.000	27.6	0.000	54.9	0.000	33.3	0.000
F×P		4.6	0.016	0.7	0.201	1.0	0.159	2.0	0.079	0.4	0.263
F×MJ		0.0		0.5	0.240	1.0	0.159	2.0	0.079	0.0	
F×P×MJ		0.2	0.327	0.5	0.240	0.0		0.0		0.0	
Block	3, 3	5.3	0.103	6.9	0.074	1.9	0.310	2.8	0.213	5.3	0.101
P	1, 3	97.2	0.002	189.2	0.001	55.7	0.005	38.9	0.008	59.2	0.005
MJ	1, 6	45.7	0.001	0.3	0.622	7.2	0.037	72.6	0.000	284.6	0.000
P×MJ	1, 6	9.8	0.020	4.0	0.091	1.8	0.234	1.7	0.241	2.3	0.178
SWr	1, 383	46.5	0.000	17.3	0.001	44.2	0.000	22.8	0.000	54.3	0.000

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613 **Table 2.** Summary of the mixed model for P concentration in adult needles, juvenile
614 needles, shoots and roots of eleven *P. pinaster* open-pollinated families under P deficient
615 and complete fertilization treatments in constitutive and MJ-induced conditions 15 days
616 after MJ application. The family effect (F) and derived interactions are random effects, and
617 the corresponding likelihood ratio significance tests (χ^2) are shown. Phosphorus
618 availability (P) and methyl jasmonate (MJ) induction of defences are fixed effects, and the
619 F values and corresponding *df* are shown. Significant *P* values ($P < 0.05$) are typed in bold.
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	P concentration								
	Adult needles			Juvenile needles		Shoots		Roots	
	<i>df</i> (F)	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value
Family (F)		0.1	0.376	7.9	0.002	1.3	0.127	5.9	0.008
F×P		1.7	0.096	7.2	0.004	0		6.4	0.006
F×MJ		0.0		0.0		1.0	0.159	3.0	0.042
F×P×MJ		1.5	0.110	0.0		0.3	0.292	4.9	0.013
Block	3, 3	12.5	0.034	4.7	0.119	3.2	0.185	2.9	0.204
P	1, 3	355.9	0.000	394.7	0.000	456.5	0.000	2673.0	0.000
MJ	1, 6	2.0	0.209	4.2	0.087	83.5	0.000	16.0	0.007
P×MJ	1, 6	4.9	0.069	1.4	0.286	39.4	0.001	16.8	0.006

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626 **Table 3.** Summary of the mixed model for N concentration in adult needles, juvenile
627 needles, shoots and roots of eleven *P. pinaster* open-pollinated families under P deficient
628 and complete fertilization treatments in constitutive and MJ-induced conditions 15 days
629 after MJ application. The family effect (F) and derived interactions are random effects, and
630 the corresponding likelihood ratio significance tests (χ^2) are shown. Phosphorus
631 availability (P) and methyl jasmonate (MJ) induction of defences are fixed effects, and the
632 F values and corresponding *df* are shown. Significant *P* values ($P < 0.05$) are typed in bold.

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	N concentration								
	Adult needles			Juvenile needles		Shoots		Roots	
	<i>df</i> (F)	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value	<i>F</i> / χ^2	<i>P</i> value
Family (F)		17.2	0.000	11.7	0.000	4.9	0.013	10.4	0.001
F×P		0.1	0.376	0.3	0.292	0.8	0.186	0.9	0.171
F×MJ		0		0		0.4	0.264	0.8	0.186
F×P×MJ		0		0		0.1	0.376	0	
Block	3, 3	9.0	0.052	0.9	0.518	0.6	0.644	1.0	0.479
P	1, 3	42.0	0.007	22.6	0.018	7.0	0.077	55.5	0.005
MJ	1, 6	2.6	0.158	0.4	0.536	153.2	0.000	1.2	0.323
P×MJ	1, 6	6.2	0.046	0.1	0.960	3.9	0.094	26.2	0.002

636 **FIGURE CAPTIONS**

637 **Figure 1.** Biomass of (a) adult needles, (b) juvenile needles, (c) shoot and (d) coarse and
638 (e) fine roots of MJ-induced (black bars) and control (constitutive, white bars) *P. pinaster*
639 juveniles belonging to 33 open-pollinated families growing in a nutrient-rich (complete
640 fertilization) and in a P-limited media. Plants were destructively sampled 15 days after
641 application of MJ. Bars are means \pm s.e.m. (N = 132). Results of the mixed model are
642 presented in Table 1. Asterisks indicate significant differences within each fertilization
643 treatment due to simulated herbivory at $P < 0.05$ (*) and $P < 0.001$ (***)).

644

645 **Figure 2.** (a) Root: shoot biomass ratio and (b) fine root: coarse root biomass ratio of MJ-
646 induced (black bars) and control (constitutive, white bars) *P. pinaster* juveniles belonging
647 to 33 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a
648 P-limited media. Plants were destructively sampled 15 days after application of MJ. Bars
649 are means \pm s.e.m. (N = 132). *P* values in the tables indicate the results of the mixed
650 models. Significant *P* values ($P < 0.05$) are typed in bold. Asterisks indicate significant
651 differences within each fertilization treatment due to simulated herbivory at $P < 0.01$ (**)
652 and $P < 0.001$ (***)).

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654 **Figure 3.** Phosphorus concentration in (a) adult needles, (b) juvenile needles, (c) shoot and
655 (d) roots of MJ-induced (black bars) and control (constitutive, white bars) *P. pinaster*
656 juveniles belonging to 11 open-pollinated families growing in a nutrient-rich (complete
657 fertilization) and in a P-limited media. Plants were destructively sampled 15 days after
658 application of MJ. Bars are means \pm s.e.m. (N = 44). Results of the mixed model are
659 presented in Table 2. Asterisks indicate significant differences within each fertilization
660 treatment due to simulated herbivory $P < 0.05$ (*) and $P < 0.001$ (***)).

661 **Figure 4.** Nitrogen concentration in (a) adult needles, (b) juvenile needles, (c) shoot and
662 (d) roots of MJ-induced (black bars) and control (constitutive, white bars) *P. pinaster*
663 juveniles belonging to 11 open-pollinated families growing in a nutrient-rich (complete
664 fertilization) and in a P-limited media. Plants were destructively sampled 15 days after
665 application of MJ. Bars are means \pm s.e.m. (N = 44). Results of the mixed model are
666 presented in Table 3. Asterisks indicate significant differences due to simulated herbivory
667 within tissues at $P < 0.05$ (*) and $P < 0.001$ (***)).

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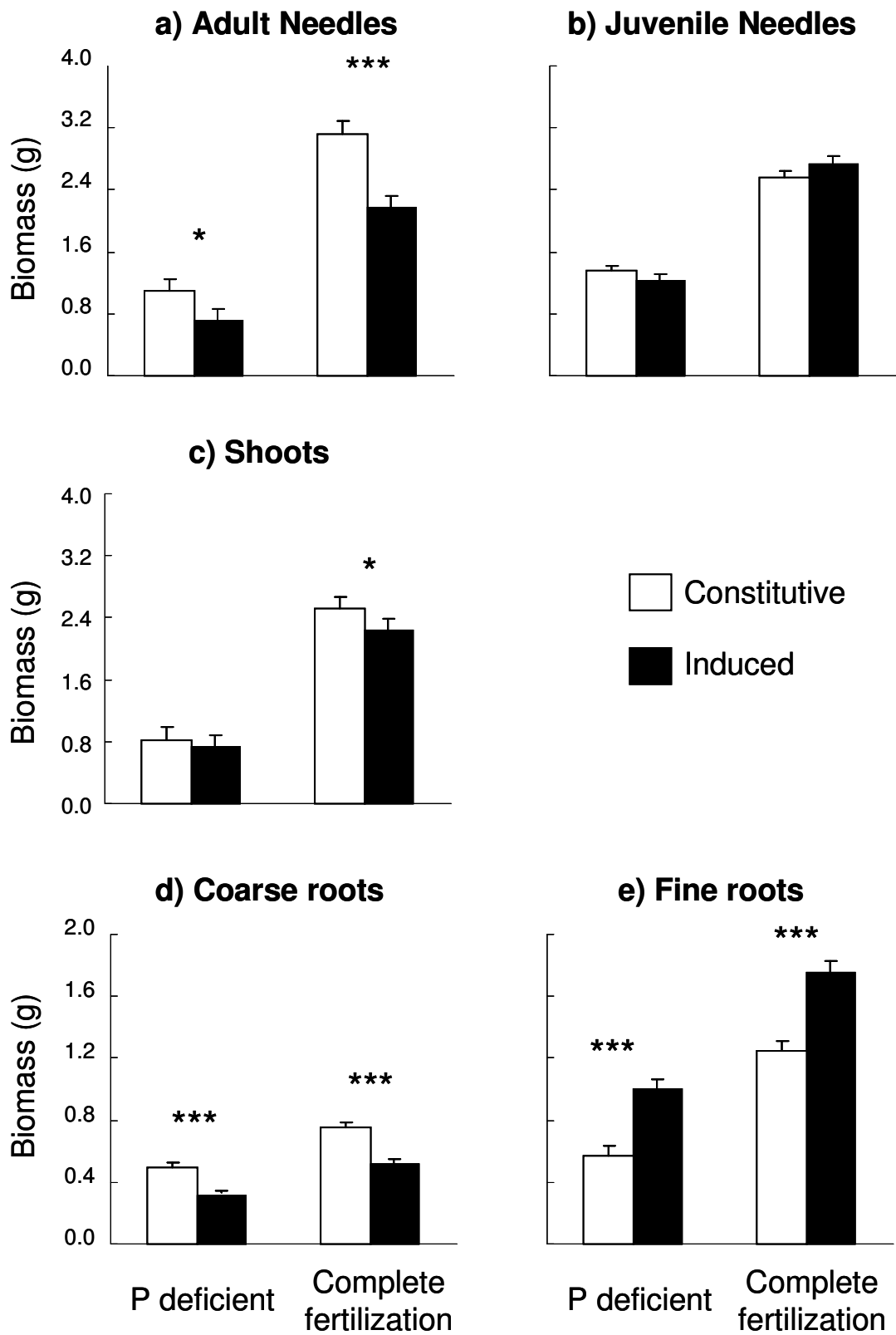
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709 Figure 1. Moreira et al.
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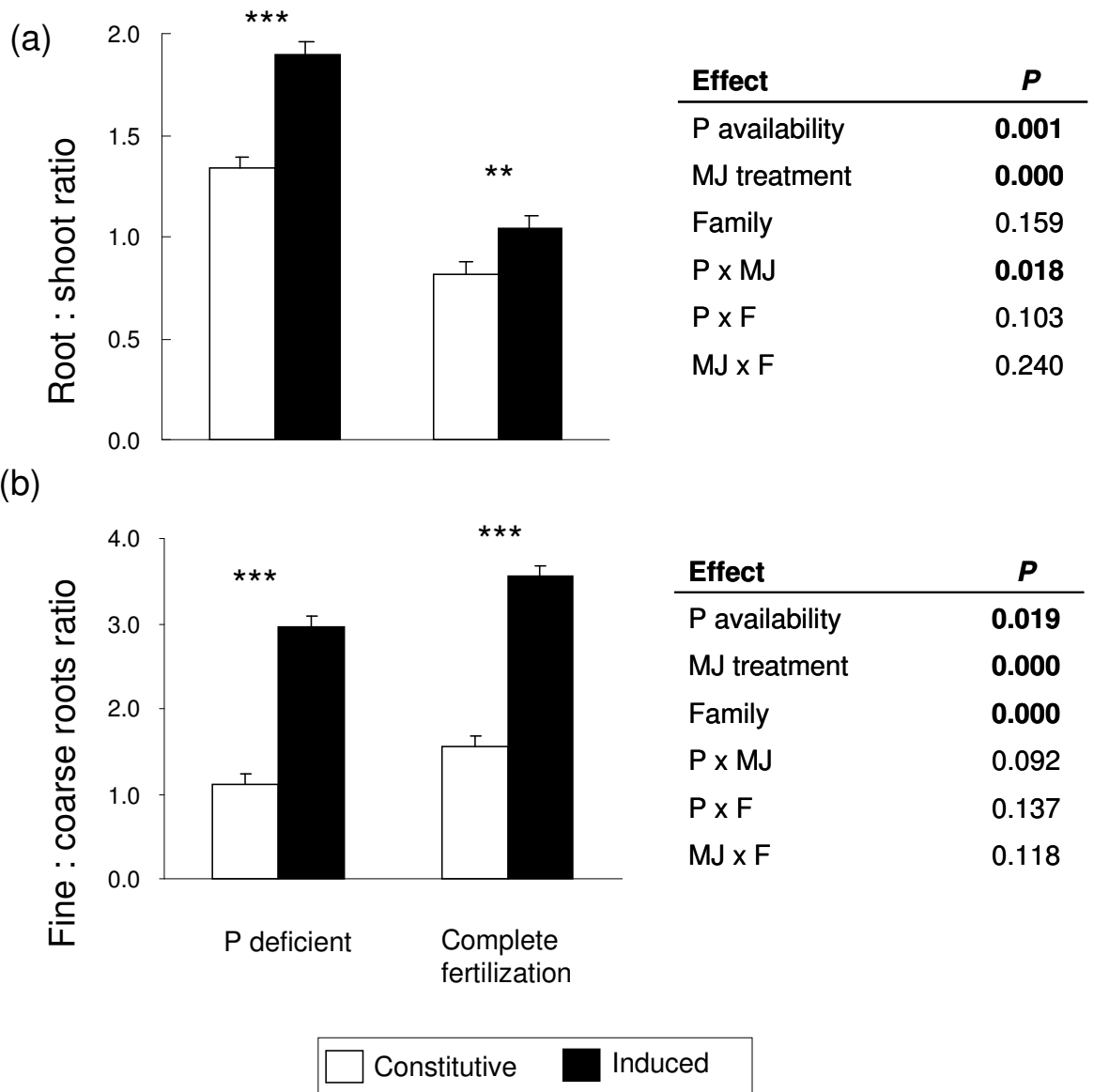


Figure 2. Moreira et al

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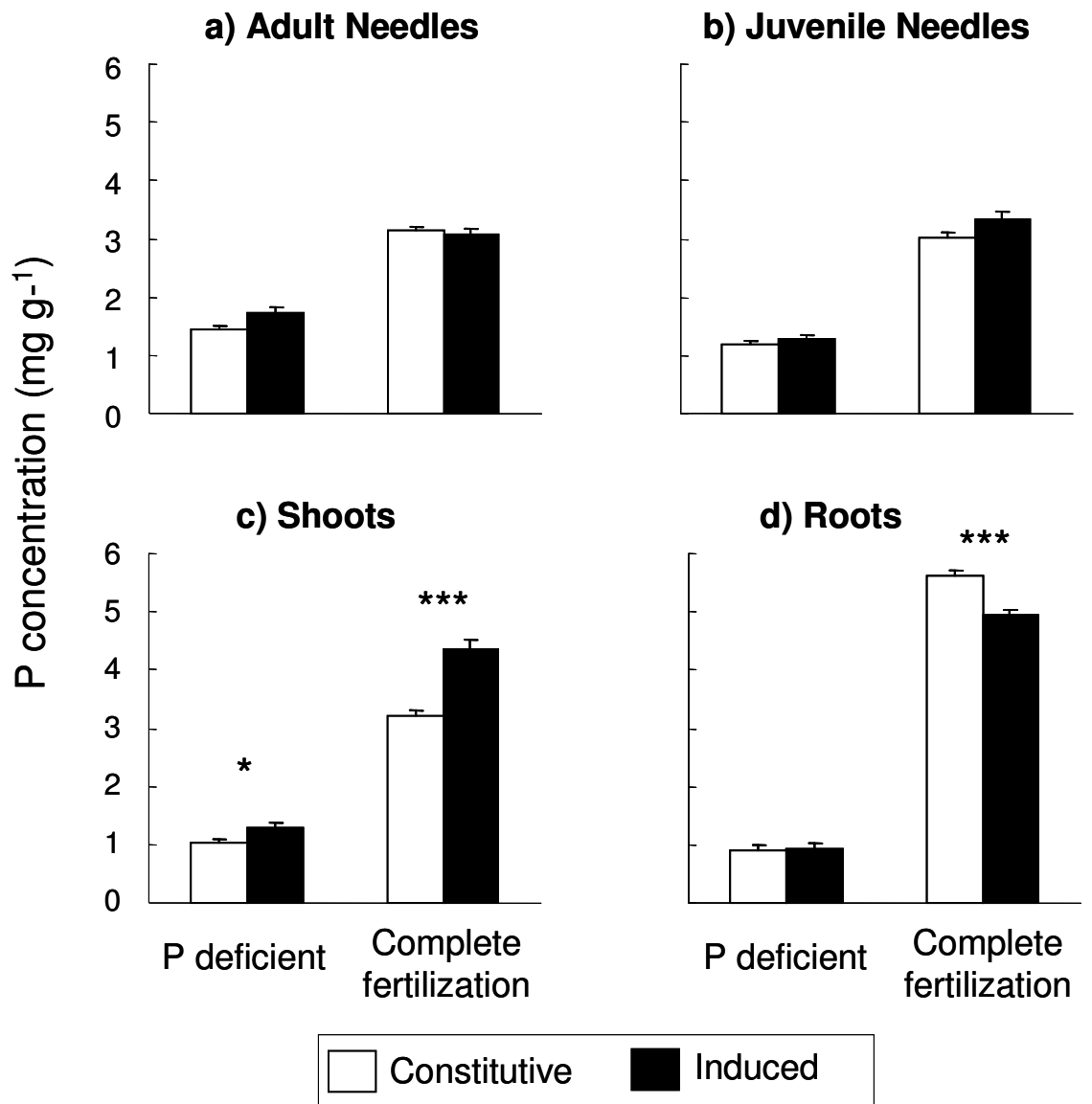


Figure 3. Moreira et al.

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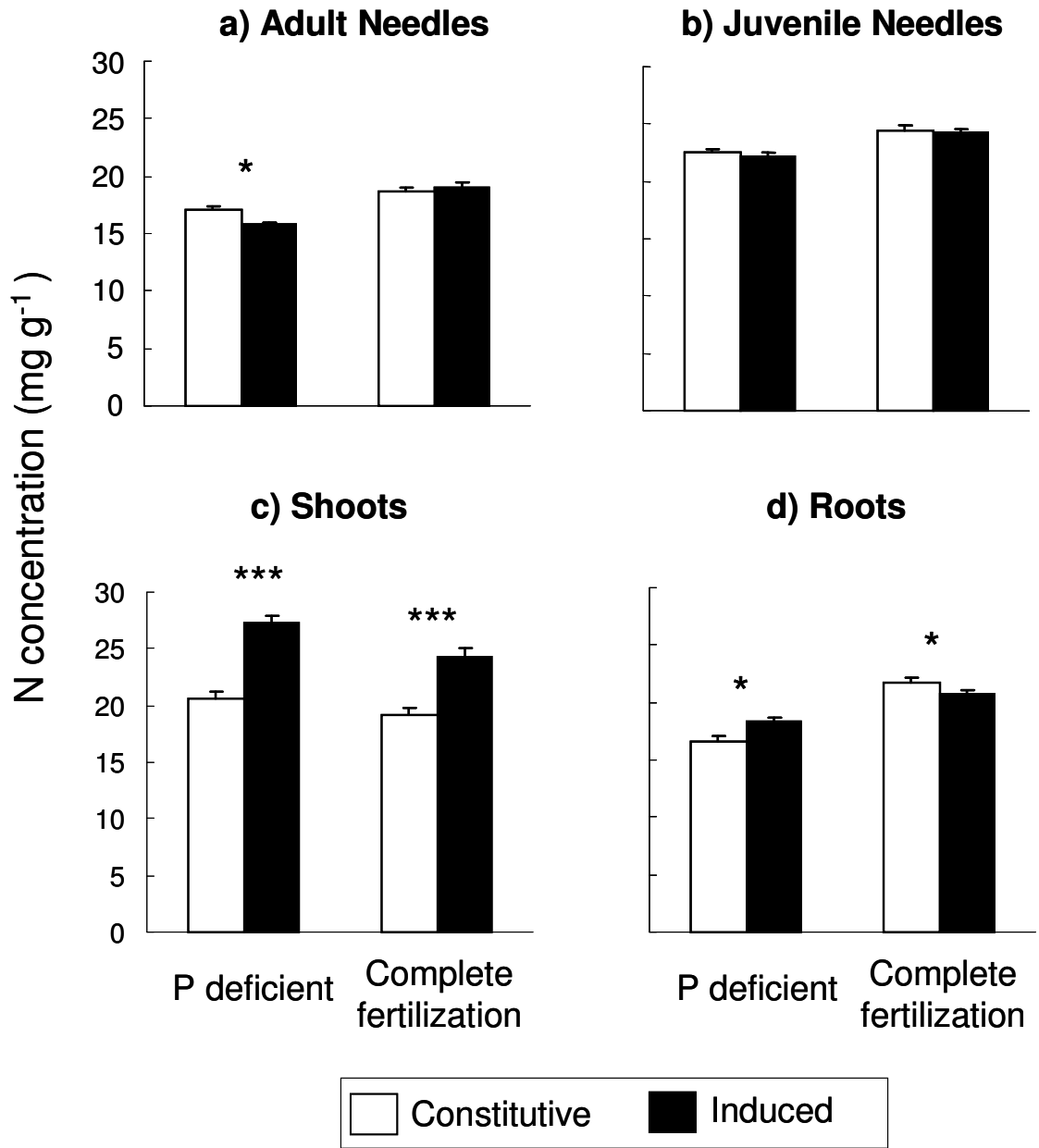


Figure 4. Moreira et al.