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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22

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 × 2 P availabilities × 2 MJ treatments ×

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 ºC, typically ranging between 25 ºC

146   (maximum daily mean) and 4 ºC (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 (10ºC night,

156 25ºC 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**

9

Plant growth and biomass allocation

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

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

10

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 × 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×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 × 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 × 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 × MJ interaction (Table 3).

289 When analyzing the absolute nutrient content in each plant tissue (i.e. the

290 corresponding nutrient concentration × 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 rhizhosphere.

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 constrains. 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-impoverished conditions

423   (significant MJ × 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 (Martíns *et al.* 2009) could lead to

427   large phenotypic variation across spatial scales in these mechanisms.

428

Conclusions

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

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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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	df (F)	Adult needles		Juvenile needles		Shoot		Coarse roots		Fine roots	
		F / χ^2	*P* value	F / χ^2	*P* value	F / χ^2	*P* value	F / χ^2	*P* value	F / χ^2	*P* 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	
	df (F)	F / χ^2	*P* value	F / χ^2	*P* value	F / χ^2	*P* value	F / χ^2	*P* 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	
	df (F)	F / χ^2	*P* value	F / χ^2	*P* value	F / χ^2	*P* value	F / χ^2	*P* 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**

FIGURE CAPTIONS

Figure 1. Biomass of (a) adult needles, (b) juvenile needles, (c) shoot and (d) coarse and (e) fine roots of MJ-induced (black bars) and control (constitutive, white bars) *P. pinaster* juveniles belonging to 33 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a P-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are means ± s.e.m. (N = 132). Results of the mixed model are presented in Table 1. Asterisks indicate significant differences within each fertilization treatment due to simulated herbivory at $P < 0.05$ (*) and $P < 0.001$ (***).
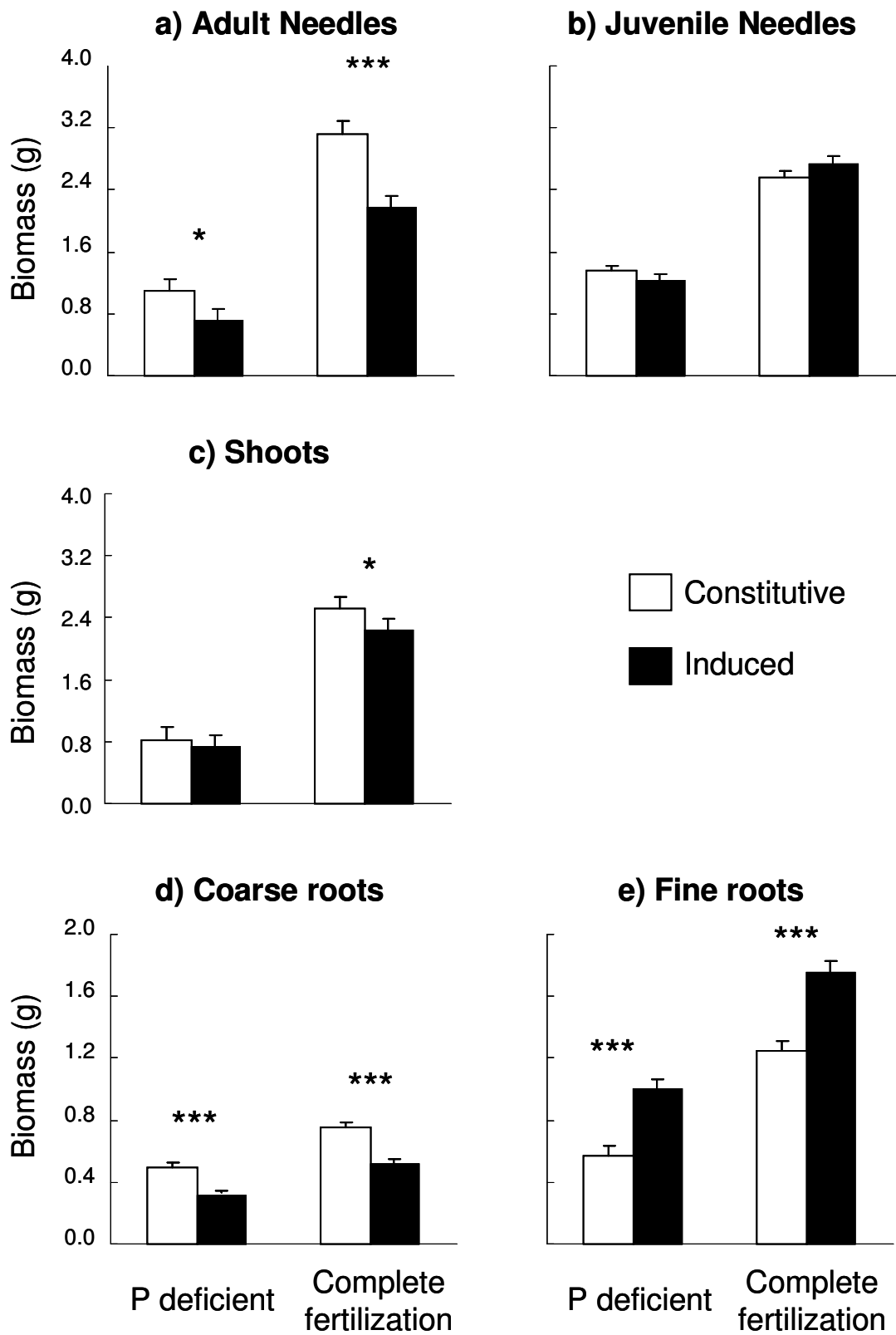
Figure 2. (a) Root: shoot biomass ratio and (b) fine root: coarse root biomass ratio of MJ-induced (black bars) and control (constitutive, white bars) *P. pinaster* juveniles belonging to 33 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a P-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are means ± s.e.m. (N = 132). P values in the tables indicate the results of the mixed models. Significant P values ($P < 0.05$) are typed in bold. Asterisks indicate significant differences within each fertilization treatment due to simulated herbivory at $P < 0.01$ (**) and $P < 0.001$ (***).

Figure 3. Phosphorus concentration in (a) adult needles, (b) juvenile needles, (c) shoot and (d) roots of MJ-induced (black bars) and control (constitutive, white bars) *P. pinaster* juveniles belonging to 11 open-pollinated families growing in a nutrient-rich (complete fertilization) and in a P-limited media. Plants were destructively sampled 15 days after application of MJ. Bars are means ± s.e.m. (N = 44). Results of the mixed model are presented in Table 2. Asterisks indicate significant differences within each fertilization 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 ± 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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Figure 1. Moreira et al.

(a)

Effect	P
P availability	**0.001**
MJ treatment	**0.000**
Family	0.159
P x MJ	**0.018**
P x F	0.103
MJ x F	0.240

(b)

Effect	P
P availability	**0.019**
MJ treatment	**0.000**
Family	**0.000**
P x MJ	0.092
P x F	0.137
MJ x F	0.118
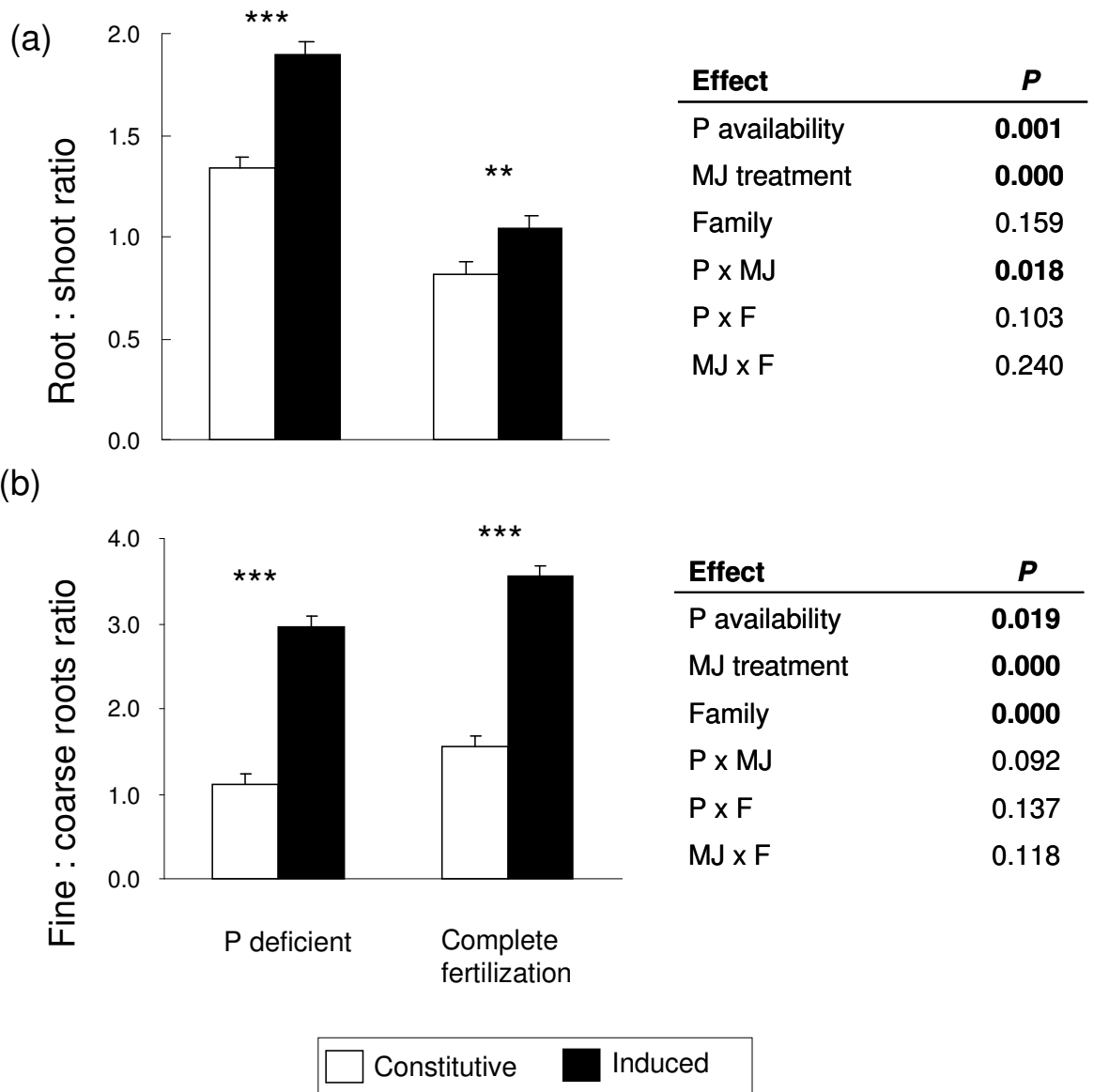
735   Figure 2. Moreira et al

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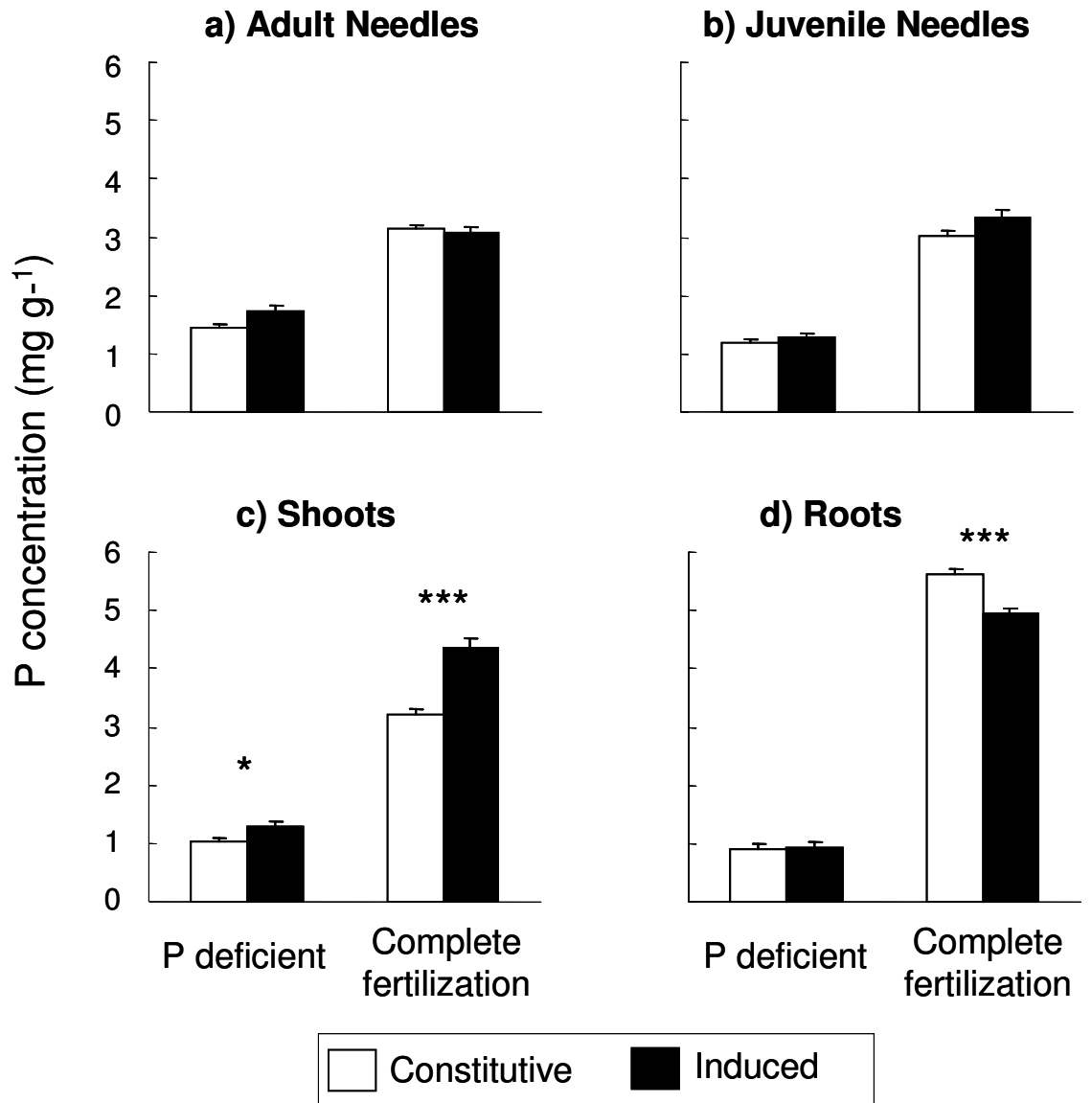746
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Figure 3. Moreira et al.

a) Adult Needles

N concentration (mg g^{-1})

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b) Juvenile Needles

c) Shoots

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P deficient   Complete fertilization

d) Roots

*   *

P deficient   Complete fertilization

□ Constitutive   ■ Induced

Figure 4. Moreira et al.