

VARIATION ACROSS POPULATIONS IN RESISTANCE TO A KEY HERBIVORE AND ITS RELATIONSHIP WITH CONSTITUTIVE AND INDUCED SECONDARY METABOLITES IN A MEDITERRANEAN PINE TREE

Xosé López-Goldar^{1,2,4,5}, Caterina Villari^{2,3}, Pierluigi Bonello², Anna-Karin Borg-Karlson⁴, Delphine Grivet^{5,6}, Rafael Zas¹, and Luis Sampedro²

Resistance to herbivores and pathogens has been recognized as an adaptive key plant trait (Futuyma and Agrawal 2009), particularly at early stages of plant development (Goodger et al. 2013). In long-lived organisms like trees, resistance against enemies usually relies on high concentrations of a diverse array of plant secondary metabolites (PSM) (Wiggins et al. 2016). These compounds reduce enemy performance and/or plant damage in a dose-dependent manner (Zhao and others 2011). PSM production is highly plastic, involving substantial changes in the profile and concentrations of PSM upon enemy attack (Heil 2009, Karban et al. 1999). This biochemical plasticity, referred to as inducibility, is a source of functional phenotypic variation in resistance and is commonly considered a trait under selection (Agrawal et al. 2015). However, very few studies have addressed the relationship between inducibility of defences with effective resistance against herbivores (Bingham and Agrawal 2010, Rasmann et al. 2015), and much less in long-lived plants (Moreira et al. 2014, Ward et al. 2012). One of the main reasons for this scarcity in the literature is that exploring relationships between inducibility of PSM and the effective resistance against herbivory is challenging. First, it requires knowing the concentration of PSM before and after exposure to herbivory when measuring plant damage or insect performance. Moreover, the mere fact of sampling plant tissues also induces plant defences, biasing inducibility estimation (Moreira et al. 2012). For small plants that require destructive sampling to evaluate defensive traits, this issue can only be solved using clonal replicates of the same genotype. Second, most studies on this topic have reported positive

associations between few defensive traits and resistance (Pratt and Mooney 2013, Stevens et al. 2007), while plant resistance are expected to be multivariate (Agrawal 2005, Lason et al. 2011, O'Reilly-Wapstra et al. 2014).

In this study, we aimed at investigating whether intraspecific variation in effective resistance of maritime pine (*Pinus pinaster*) to the pine weevil *Hylobius abietis* (a harmful insect herbivore) may be predicted by variation in inducibility of PSM. To do so, we used clonally replicated genotypes with known family structure from 10 populations representative of the main distribution range of maritime pine, and that belonged to the clonal collection 'CLONAPIN Bank 1' [10 populations × 5 families per population × 5 genotypes per family = 250 genotypes in total] (López-Goldar et al. 2018). We performed a greenhouse experiment where 2-years old pines were distributed in a split-plot design replicated in 5 blocks, with population as the whole plot factor and the factorial combination of family (3–5 families per population) and the induction treatment [2 levels: control and induced] as the split factor. For the induced treatment, we exogenously applied 25 mM methyl jasmonate (MJ) to half of the plants, where the other half were left untreated. MJ is a plant hormone involved in herbivore damage signalling that has been widely accepted as a chemical elicitor (Sampedro et al. 2011, Zas et al. 2014). One month after induction, two clonal replicates not subjected to herbivory (one control and one MJ-induced) were used to measure the concentration of constitutive terpenes and phenolics in the stem phloem, as well as their inducibility, by gas chromatography-mass

¹ Misión Biológica de Galicia, Consejo Superior de Investigaciones Científicas. Apdo. 28, 36080, Pontevedra, Spain, (xlgoldar@mbg.csic.es).

² Department of Plant Pathology, The Ohio State University, Columbus, OH 43210.

³ Daniel B. Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA 30602.

⁴ Ecological Chemistry Group, Department of Chemistry, Royal Institute of Technology, 100 44 Stockholm, Sweden.

⁵ Department of Forest Ecology & Genetics, Forest Research Centre, INIA-CIFOR, Carretera de la Coruña km 7.5, 28040 Madrid, Spain.

⁶ Sustainable Forest Management Research Institute, INIA-University of Valladolid, Avda. Madrid 44, 34004 Palencia, Spain.

spectrometry (GC-MS) and GC-flame ionization detection (GC-FID), and by ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) and UHPLC-diode array detection (UHPLC-DAD). We identified and quantified (in $\text{mg}\cdot\text{g}^{-1}$ dry weight of stem tissue) 98 chemical compounds that were classified into eight chemical groups [monoterpenes, sesquiterpenes, diterpenes, flavonoids, hydroxycinnamic acids (HCAs), lignans, eugenols, and fatty acids]. Another control and MJ-induced clonal plants were exposed to a couple of insects for 48 hours to measure plant damage (in mm^2) as a proxy of resistance (i.e., the greater the damage, the lower the resistance). Prior to herbivory treatment, insects were starved for 48 hours and then weighed, and plant diameter was measured.

Variation in *H. abietis* damage was analyzed by fitting a mixed model using the PROC MIXED procedure in SAS v9.4 (Littell et al. 2006). For the statistical analyses, the induction treatment (MJ), pine population (P), pine family within population [F(P)] and MJ×P and MJ×F(P) interactions were considered as fixed factors. Block (B) and the B×P interaction were considered as random factors to analyze the main effect of P with the appropriate error term (Littell et al. 2006). The genotype was also included as a random factor to account for the dependence between the two clonal replicates subjected to herbivory within each block. Weevil weight and stem diameter of the plant were included as covariates. Because structured genetic variation among populations was previously found for this species (Jaramillo-Correa et al. 2015, Serra-Varela et al. 2015), the same model was fit to explore the genetic variation among genetic clusters of populations (Atlantic France, Atlantic Spain, Central Spain, South Spain, and Morocco) and among families within clusters. In order to estimate the variance contribution of each factor to the genetic variation among populations and families within populations in resistance to the pine weevil, the previous mixed model was run again assuming all factors as random factors.

To explore the relationships between plant resistance and PSM in the stem for the constitutive and inducibility states, we performed pairwise correlations and stepwise multiple regression analyses between the weevil damage and PSM

concentration at the genotypic level. Prior the analyses, we accounted for the non-independence among genotypes within families and populations by incorporating the population structure (Q) and kinship (K) matrices in mixed models (López-Goldar et al. 2018). We fitted a mixed model for constitutive (C) and for the inducibility (MJ – C) of each total and individual PSM and weevil damage. For each defensive state, population (P) and family within population [F(P)] were considered as fixed factors. Block (B) and the B×P interaction were considered random factors as above. The predicted values of PSM variables and weevil damage from each plant were extracted from each mixed model and used for the analyses.

Plant resistance to weevil herbivory in the stem significantly varied among plant populations (fig. 1) and genetic groups of populations ($F_{4,140} = 4.73$, $P = 0.001$), but not between families within populations (fig. 1) or within genetic groups of populations ($F_{43,140} = 1.36$, $P = 0.096$). Spanish Atlantic populations showed the greatest constitutive resistance ($61.5 \pm 7.3 \text{ mm}^2$ of damage; figure 2), whereas the French Atlantic populations were the most susceptible ($94.9 \pm 12.3 \text{ mm}^2$ of damage; fig. 2). The MJ-induction treatment significantly increased plant resistance to weevil herbivory in the stem (figs. 1 and 2). The inducibility of resistance was similar for all plant populations and families [no significant MJ×P and MJ×F(P) interactions, fig. 1], but differed among genetic groups (MJ×GP interaction, $F_{4,140} = 3.36$, $P = 0.012$). In fact, greater inducibility of resistance after induction was found in both Atlantic genetic groups (71 and 67 percent in Atlantic France and Atlantic Spain, respectively) than in the other genetic groups (Central Spain, 37 percent; South Spain, 34 percent; and Morocco, 45 percent). Among the main effects of our study, that accumulated an explained variance of 36 percent of the total variance in resistance, MJ-induction effect accounted for > 80 percent of that explained variance in resistance, with the contribution of all remaining factors being comparatively much lower (fig. 1).

When exploring pairwise relationships at the genotypic level, we found that neither total nor individual PSM at the constitutive level

Fixed effect	DF (n, d)	F	P-value
MJ-Induction (MJ)	1, 140	104	<0.0001
Population (P)	9, 36	2.32	0.0353
Family [F(P)]	38, 140	1.33	0.1205
MJ×P	9, 140	1.62	0.1159
MJ×F(P)	38, 140	0.92	0.6032
Weevil weight	1, 140	18.5	<0.0001
Plant diameter	1, 140	4.66	0.0326

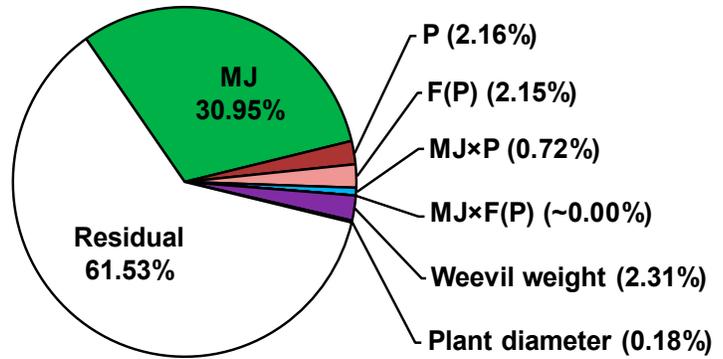


Figure 1—Summary of the mixed model testing the effects of the induction treatment with methyl-jasmonate (MJ), population (P), family within population [F (P)] and the MJ×P and MJ×F(P) interactions on the early resistance to herbivory by the pine weevil on plants from 10 maritime pine populations. Variance components for each effect and the residual are shown in the companion pie chart. Weevil weight and plant diameter were included as covariates. Significant p -values ($p < 0.05$) are highlighted in bold.

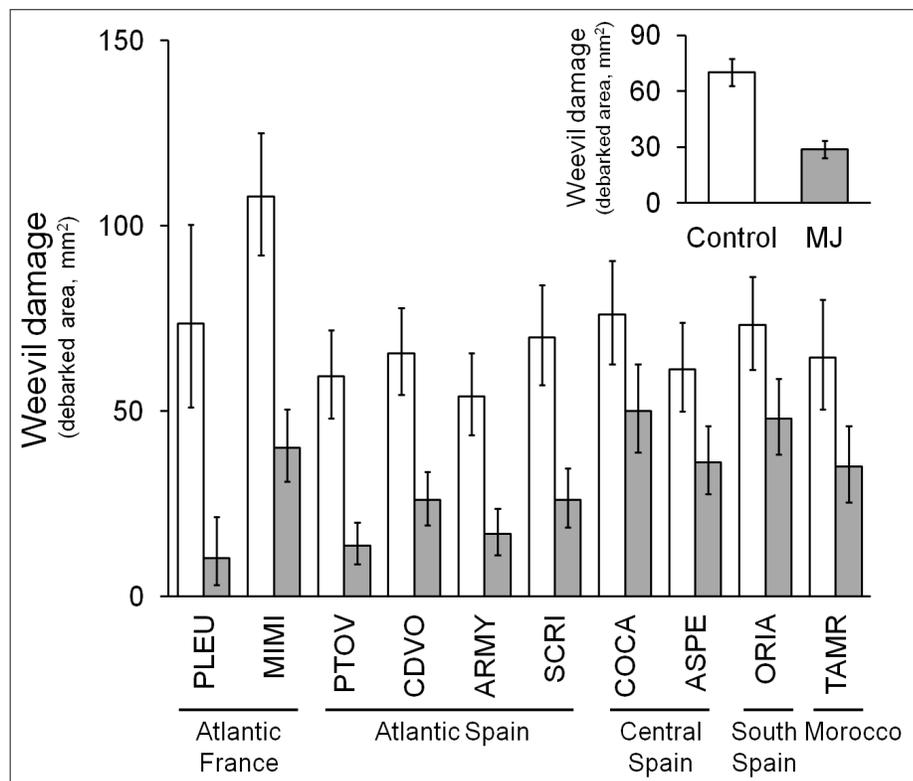


Figure 2—Intraspecific genetic variation in constitutive resistance (white bars) and MJ-induced resistance (grey bars) to the pine weevil for 10 maritime pine populations along its main distribution range, grouped by the main genetic groups in the species. The inset panel shows the main effect of the MJ-induction on the resistance to weevil herbivory. Bars represent the least square mean \pm SE (N = 8 to 25 plants for each population; N = 197 to 205 plants for each induction treatment in the small panel).

were significantly associated with weevil damage. Contrarily, we found significant negative relationships between inducibility of total flavonoids and total lignans with weevil damage ($r = -0.33$, $P = 0.001$ and $r = -0.36$, $P < 0.001$, respectively). When exploring individual PSMs, we found significant negative relationships between the inducibility of lignan

hexoside derivative 1 and 2 with weevil damage ($r = -0.30$, $P = 0.003$; $r = -0.33$, $P = 0.001$, respectively). In the multiple regression model, concentration of constitutive PSM significantly predicted 70 percent of the total variation in weevil damage, but only 6 PSM were negatively related to weevil damage (table 1). Inducibility of PSM significantly predicted 68 percent of the

total variation of weevil damage, and 8 PSM were negatively related to weevil damage after MJ-induction (table 1).

This study represents a good example of the relevance of using clonal replicates of genotypes, belonging to families within populations along the distribution range of a single conifer species, to evaluate the role of inducibility of PSM in

resistance. Moreover, our findings are based on a rigorous framework by accounting for spurious associations due to genetic relatedness, often overlooked in intraspecific studies. Here we provide evidences that multivariate analyses of PSM, rather than bivariate correlations, provide more realistic information about the potentially causal relationships between PSM and resistance to herbivory in pine trees.

Table 1—Summary of the multiple regression analysis explaining the resistance to the pine weevil using the concentration of individual PSM as predictor variables in constitutive (upper part of the table) and in inducibility (lower part of the table) defensive states in 102 genotypes from 10 maritime pine populations. The regression coefficients (β) and partial R^2 of PSM included in the model after stepwise selection method are shown

Defensive mode	Plant secondary chemicals	β	Partial R^2
Constitutive PSM ($N = 87$) Intercept = 7.908 Model adjusted $R^2 = 0.70$ $F_{18,62} = 18.97$ $P < 0.001$	β-Phellandrene + Limonene	-0.98	0.150
	Lignan hexoside derivative 1	-1.58	0.077
	Ferulic acid	-0.33	0.028
	α-Copaene	-1.89	0.025
	Unk P11	-0.35	0.024
	α-Cubebene + α-Longipinene	-0.76	0.016
	Coumaric acid hexoside	0.60	0.079
	Methyl thymyl ether	0.57	0.054
	α -Phellandrene	0.52	0.040
	Methyl eugenol	0.87	0.035
	Elemol	0.75	0.031
	Eugenol	0.36	0.029
	Bicyclosesquiphellandrene	1.31	0.028
	Myrcene	0.56	0.022
	Lignan xyloside derivative 2	0.40	0.021
	Citronellyl propionate	0.46	0.018
Sabinene	0.53	0.015	
Unk P6	0.39	0.011	
Inducibility of PSM ($N = 93$) Intercept = -36.03 Model adjusted $R^2 = 0.68$ $F_{12,78} = 17.32$ $P < 0.001$	Unk P5	-22.46	0.234
	Lignan hexoside derivative 2	-19.17	0.060
	Isopimaric acid	-10.05	0.060
	Elemol	-12.11	0.058
	<i>cis</i>-β-Ocimene	-9.04	0.048
	Methyl thymyl ether	-10.36	0.040
	Pimaric acid	-11.76	0.029
	<i>trans</i>-Pinocampnone	-10.15	0.022
	Oleic acid C18:1	10.03	0.048
	Abietic acid	31.24	0.039
	Ferulic acid hexoside	8.03	0.024
	<i>trans</i> - β -Ocimene	4.78	0.019

PSM associated with resistance to weevil damage (negative β) are typed in bold. Linear regression model at each defensive mode is significant at $p < 0.05$. Genotypic sample size of each defensive state is indicated in parentheses. Given that some clonal replicates (control or MJ-induced) were not available for several genotypes, sample size for the inducibility dataset was slightly lower than the original number of genotypes used. Unk P# = unidentified phenolic compound.

LITERATURE CITED

- Agrawal, A.A. 2005. Natural selection on common milkweed by a community of specialized insect herbivores. *Evolutionary Ecological Research*. 7: 17.
- Agrawal, A.A.; Hastings, A.P.; Bradburd, G.S. [et al.]. 2015. Evolution of plant growth and defense in a continental introduction. *The American Naturalist*. 186(1): E1–E15.
- Bingham, R.A.; Agrawal, A.A. 2010. Specificity and trade-offs in the induced plant defence of common milkweed *Asclepias syriaca* to two lepidopteran herbivores. *Journal of Ecology*. 98(5): 1014–1022.
- Futuyma, D.J.; Agrawal, A.A. 2009. Macroevolution and the biological diversity of plants and herbivores. *Proceedings of the National Academy of Sciences U S A*. 106(43): 18054–18061.
- Goodger, J.Q.; Heskes, A.M.; Woodrow, I.E. 2013. Contrasting ontogenetic trajectories for phenolic and terpenoid defences in *Eucalyptus froggattii*. *Annals of Botany*. 112(4): 651–659.
- Heil, M. 2009. Plastic defence expression in plants. *Evolutionary Ecology Research*. 24(3): 555–569.
- Iason, G.R.; O'Reilly-Wapstra, J.M.; Brewer, M.J. [et al.]. 2011. Do multiple herbivores maintain chemical diversity of Scots pine monoterpenes? *Philosophical Transactions of the Royal Society :B: Biological Sciences*. 366(1569): 1337–1345.
- Jaramillo-Correa, J.P.; Rodriguez-Quilon, I.; Grivet, D. [et al.]. 2015. Molecular proxies for climate maladaptation in a long-lived tree (*Pinus pinaster* Aiton, Pinaceae). *Genetics*. 199(3): 793–807.
- Karban, R.; Agrawal, A.A.; Thaler, J.S.; Adler, L.S. 1999. Induced plant responses and information content about risk of herbivory. *Trends in Ecology & Evolution*. 14(11): 443–447.
- Littell, R.C.; Milliken, G.A.; Stroup, W.W. [et al.]. 2006. *SAS System for Mixed Models*. 2nd ed. Cary, NC.: SAS Institute Inc.
- López-Goldar, X.; Villari, C.; Bonello, P. [et al.]. 2018. Inducibility of plant secondary metabolites in the stem predicts genetic variation in resistance against a key insect herbivore in maritime pine. *Frontiers in Plant Science*. 9: 1651.
- Moreira, X.; Mooney, K.A.; Rasmann, S. [et al.]. 2014. Trade-offs between constitutive and induced defences drive geographical and climatic clines in pine chemical defences. *Ecology Letters*. 17(5): 537–546.
- Moreira, X.; Zas, R.; Sampedro, L. 2012. Quantitative comparison of chemical, biological and mechanical induction of secondary compounds in *Pinus pinaster* seedlings. *Trees*. 26(2): 677–683.
- O'Reilly-Wapstra, J.M.; Hamilton, M.; Gosney, B. [et al.]. 2014. Genetic correlations in multi-species plant/herbivore interactions at multiple genetic scales. *Advances in Ecological Research*. 50: 267–295.
- Pratt, J.D.; Mooney, K.A. 2013. Clinal adaptation and adaptive plasticity in *Artemisia californica*: implications for the response of a foundation species to predicted climate change. *Global Change Biology*. 19(8): 2454–2466.
- Rasmann, S.; Chassin, E.; Bilat, J. [et al.]. 2015. Trade-off between constitutive and inducible resistance against herbivores is only partially explained by gene expression and glucosinolate production. *Journal of Experimental Botany*. 66(9): 2527–2534.
- Sampedro, L.; Moreira, X.; Zas, R. 2011. Resistance and response of *Pinus pinaster* seedlings to *Hylobius abietis* after induction with methyl jasmonate. *Plant Ecology*. 212(3): 397–401.
- Serra-Varela, M.J.; Grivet, D.; Vincenot, L. [et al.]. 2015. Does phylogeographical structure relate to climatic niche divergence? A test using maritime pine (*Pinus pinaster* Ait.). *Global Ecology and Biogeography*. 24(11): 1302–1313.
- Stevens, M.T.; Waller, D.M.; Lindroth, R.L. 2007. Resistance and tolerance in *Populus tremuloides*: genetic variation, costs, and environmental dependency. *Evolutionary Ecology Research*. 21(6): 829–847.
- Ward, D.; Shrestha, M.K.; Golan-Goldhirsh, A. 2012. Evolution and ecology meet molecular genetics: adaptive phenotypic plasticity in two isolated Negev desert populations of *Acacia raddiana* at either end of a rainfall gradient. *Annals of Botany*. 109(1): 247–255.
- Wiggins, N.L.; Forrister, D.L.; Endara, M.J. [et al.]. 2016. Quantitative and qualitative shifts in defensive metabolites define chemical defense investment during leaf development in *Inga*, a genus of tropical trees. *Ecology and Evolution*. 6(2): 478–492.
- Zas, R.; Bjorklund, N.; Nordlander, G. [et al.]. 2014. Exploiting jasmonate-induced responses for field protection of conifer seedlings against a major forest pest, *Hylobius abietis*. *Forest Ecology and Management*. 313: 212–223.
- Zhao, T.; Krokene, P.; Hu, J. [et al.]. 2011. Induced terpene accumulation in Norway spruce inhibits bark beetle colonization in a dose-dependent manner. *PLoS One*. 6(10): e26649.