View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Edinburgh Research Explorer

THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Drought-induced mortality in Scots pine

Citation for published version:
MacAllister, S, Mencuccini, M, Sommer, U, Engel, J, Hudson, A, Salmon, Y & Dexter, KG 2019, 'Drought-induced mortality in Scots pine: Opening the metabolic black box', *Tree physiology*, vol. 39, no. 8, tpz049, pp. 1358-1370. https://doi.org/10.1093/treephys/tpz049

Digital Object Identifier (DOI):
10.1093/treephys/tpz049

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Tree physiology

Publisher Rights Statement:
This is a pre-copyedited, author-produced version of an article accepted for publication in Tree physiology following peer review. The version of record Sarah MacAllister, Maurizio Mencuccini, Ulf Sommer, Jasper Engel, Andrew Hudson, Yann Salmon, Kyle G Dexter, Drought-induced mortality in Scots pine: opening the metabolic black box, Tree Physiology, , tpz049, https://doi.org/10.1093/treephys/tpz049 is available online at: https://academic.oup.com/treephys/advance-article/doi/10.1093/treephys/tpz049/5481586

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 03. Jul. 2020

1 **Drought-induced mortality in Scots pine: opening the metabolic black box**

2

3 Sarah MacAllister[1],

4 Maurizio Mencuccini[2,3],

5 Ulf Sommer[4],

6 Jasper Engel[4],

7 Andrew Hudson[5],

8 Yann Salmon[6],

9 Kyle G. Dexter[1,7]

10

11 [1]School of GeoSciences, University of Edinburgh, UK

12 [2]CREAF, Barcelona, Spain

13 [3]ICREA, Barcelona, Spain

14 [4]NBAF-Birmingham, School of Biosciences, University of Birmingham, UK

15 [5]School of Biological Sciences, University of Edinburgh, UK

16 [6]University of Helsinki, Finland

17 [7]Royal Botanic Garden Edinburgh, UK

18

19 Corresponding Author: Sarah MacAllister

20 S.l.wilkinson2@gmail.com

21

22 Word counts for abstract (200) and main text (6463)

23 Number of tables (3)

24 Number of figures (3)

25 Number of references (69)

26

27

28

29

30

31

ABSTRACT

- Forests are sensitive to droughts, which increase the mortality rate of tree species. Various processes have been proposed to underlie drought-induced tree mortality, including hydraulic failure, carbon starvation, and increased susceptibility to natural enemies. To give insights into these processes, we assessed the metabolic effects of a mortality-inducing drought on seedlings of *Pinus sylvestris* (Scots Pine), a widespread and important Eurasian species.
- In testing seedlings from provenances with varying water availability, we found divergence over time in the foliar metabolic composition of droughted vs. well-watered individuals, with the former showing increased abundance of aromatic amino acids and decreases in secondary metabolism associated with defence.
- We observed no significant differences amongst provenances in these effects: seedlings from drought-prone areas showed the same foliar metabolic changes under drought as seedlings from moist environments, although morphological effects of drought varied by provenance.
- Overall, our results demonstrate how severe drought may lead to decreases in compounds derived from aromatic amino acids and compromise secondary metabolic pathways related to defences against natural enemies, thereby contributing to the risk of drought-induced mortality in *Pinus sylvestris*.

Keywords (6-10): drought, *Pinus sylvestris*, metabolomics, plant defence, seedling, provenance effects, genotype by environment, carbon starvation, hydraulic failure

Running header (short title): Drought effects on growth and metabolism of *Pinus sylvestris* seedlings

64 INTRODUCTION

65 Fluctuations in environmental conditions necessitate appropriate plant responses. Despite
66 documentation of widespread forest dieback triggered by drought in all major forested biomes
67 (Allen et al. 2010; Allen et al. 2015), and of elevated mortality under drought in various tree
68 species (Mueller et al. 2005; Breshears et al. 2009; Martinez-Vilalta et al. 2010; Rigling et al.
69 2013), the underlying mechanisms of drought-induced mortality, species-specific vulnerability
70 and population level resilience are still poorly known. The theoretical framework of
71 physiological mechanisms of tree mortality currently focuses on hydraulic failure, where fluid
72 transport breaks down, and decreasing carbon availability, where metabolic demands may be
73 unmet owing to depletion of non-structural carbohydrates (e.g., McDowell et al. 2008; Galiano
74 et al. 2011; Körner 2015; Adams et al. 2017). Depending on the tree species, these mechanisms
75 need not be mutually exclusive (McDowell et al. 2008; Salmon et al. 2015; Mencuccini et al.
76 2015). For example, mortality of *Pinus edulis* was found to occur through both hydraulic failure
77 and carbon starvation (Sevanto et al. 2014). Further to these hypotheses, owing to the coupling
78 of xylem and phloem fluid transport, phloem transport limitations under drought have been
79 suggested to contribute to failure to supply the non-structural carbohydrates (NSC) essential to
80 plant metabolism (McDowell & Sevanto 2010; Sevanto et al. 2014). Defining carbon
81 limitations and carbon starvation prior to death has proven hard (e.g., Sala et al. 2012; Sevanto
82 2014), because little is known about the multiple metabolic pathways via which energy flows
83 to cover the metabolic needs of plants and because of the many ways by which carbon and
84 water limitations can interact in plants.

85

86 Owing to high mortality levels during seedling establishment, this stage represents a major
87 bottleneck to recruitment into a population. Seedling establishment has been used as an
88 indicator of the effects of climate change on species assembly in plant communities (Sternberg
89 et al. 1999; Kullman 2002; Lloret et al. 2009). However, despite the heightened mortality rates
90 and increased sensitivity to climate change of this demographic, the mechanism of drought-
91 induced mortality at the stage of seedling establishment is understudied (Lloret et al. 2004,
92 2009).

93

94 Scots pine is widespread across Eurasia, serving as a key timber species and abundant and
95 ecologically important in natural forest stands. Scots pine populations are known to vary
96 ecotypically (Rehfeldt et al. 2002). Latitudinal and longitudinal clines of phenotypic variability
97 in physiological traits have been observed, as well as differences in adaptive plasticity across

98 provenances (summarised by Semerci et al. 2017). Both phenotypic plasticity and local
99 adaptation could have a role in enabling resilience to drought. Relative to structural and
100 morphological plasticity, metabolism changes over short time-scales and is a more immediate
101 reflection of a plant's response to environmental stressors, including drought. Therefore, a
102 dynamic picture of a plant's response to drought over time can be obtained in metabolic studies,
103 both within and across provenances, which may shed light on how drought-induced mortality
104 is related to particular metabolic pathways. Drought stress is discernible at the level of plant
105 metabolic phenotypes. For example, shifts in carbon metabolism and secondary metabolite
106 synthesis related to water deficit and oxidative stress have been detected in multiple plant
107 species (Zhao et al. 2015, Bowne et al. 2012, Ings et al. 2013, Gargallo-Garriga et al. 2014).
108 Oxidative stress caused by the accumulation of cytotoxic reactive oxygen species (ROS) may
109 be of particular relevance to metabolic activity during mortality-inducing drought, since the
110 extent of oxidative damage is governed by the activity of particular metabolic pathways and
111 the capacity of antioxidant defences to avert an imbalance of ROS (Cruz de Carvalho et al.
112 2008).
113
114 Although the effects of drought on the metabolome of woody plants have been investigated
115 before (e.g., Gargallo-Garriga et al. 2014, 2015; Hamanishi et al. 2015, de Simón et al. 2017),
116 investigation of metabolic changes during drought to the point of mortality has not been carried
117 out. Because the levels of carbon reserves may change and even increase during drought,
118 investigation of the metabolic responses just prior to death are required to determine the
119 sequence of metabolic events leading to mortality (Ryan 2011). Non-targeted metabolomics
120 offers the possibility of capturing a global picture of metabolism, rather than just that related
121 to known metabolic pathways, and it can aid the discovery of novel pathways and interactions
122 amongst pathways (Hall 2006). Both phenotypic plasticity and local adaptation could have a
123 role in enabling resilience to drought and the seedling response is of particular importance,
124 since it represents a bottleneck in terms of higher mortality rates (Castro et al. 2005; Matias et
125 al. 2011; Semerci et al. 2016). Despite increasing knowledge on plasticity and genetic
126 variability of morphological and physiological traits, very little is known about plasticity at the
127 metabolic level. Insights into metabolic changes during seedling drought-induced mortality
128 will help to address this knowledge gap.
129
130 Here, we conduct an in-depth comparison of the foliar metabolome of *P. sylvestris* seedlings
131 that were droughted to the point of death versus well-watered, control seedlings. Further, we

132 compared seedlings from provenances that differ in natural water availability, in order to assess
133 if there is provenance-specific variation in metabolic responses to drought. Additional
134 measurements of biomass and functional traits were taken to determine if changes at the whole-
135 plant level correspond to those observed at the metabolic level. We hypothesise that drought
136 will have major effects on plant metabolism when photosynthesis becomes compromised and
137 carbohydrate availability for plant defence pathways is restricted. We also hypothesise that
138 seedlings from provenances that do not regularly experience intense droughts will show greater
139 metabolic and whole-plant changes under the drought treatment.
140
141 MATERIALS AND METHODS

142 *Experimental conditions and sampling*
143 The experiment was carried out over 5 weeks in July and August of 2015 in a controlled growth
144 chamber at the University of Edinburgh (UK), under constant conditions with diurnal cycles
145 of 16 h light at 100 µmol m^{-2} s^{-1} and 8 h darkness. The light level was chosen to reduce the rate
146 of water use and consequent dehydration of the plants during the drought, as potted plants
147 would otherwise rapidly undergo hydraulic failure in a manner not reflective of field conditions
148 (Adams et al. 2017). Relative humidity was 65% (day) and 50% (night), with a constant
149 day/night temperature of 21˚C. This is the current mean temperature at the southern range limit
150 of this species during July and August (Matías and Jump 2014). The experiment was carried
151 out on 10 month old seedlings, germinated from seeds originating from four provenances
152 spanning a gradient of water availability from wet (Scotland) to intermediate (Austria, Poland)
153 to dry (Spain). Climatic characteristics of the four populations from which seeds were collected
154 are presented in Table 1. Mean annual precipitation was obtained from 1901 to 2015 from the
155 CRU TS3.10 Dataset (Harris et al. 2014). Maximum climatic water deficit values for these
156 sites, which correspond to the summed difference between evapotranspiration and precipitation
157 over the dry season, were obtained from Chave et al. (2014). To give an indication of the degree
158 of water stress expected at the various seed-collection sites, mean soil pF values in the summer
159 month with the maximum mean pF value (July/August) from 1990 to 2014 were obtained from
160 the European Drought Observatory (edo.jrc.ec.europa.eu/ on February 2nd, 2015). pF expresses
161 the force with which different quantities of water are retained in the soil (Woodruff 1940), with
162 lower values indicating higher water availability for plants. The seeds were collected from
163 open-pollinated trees, with at least 5 maternal parent trees sampled per provenance site. A total

164 of 1200 seedlings were included in the experiment, with 600 per treatment and 300 per
165 provenance.
166
167 Two months prior to instigating drought and control treatments, the seedlings were re-potted
168 into 7 x 7 x 8 cm pots with Levingtons M3 pot and bedding high nutrient mix (Everris, Ipswich,
169 UK). The drought treatment consisted of complete withdrawal of irrigation that resulted in a
170 steep decline in soil water content (Fig. S1). A set of 10 seedlings were droughted one week
171 before the others and were used to indicate when a final sampling point prior to mortality should
172 be carried out. By the end of the first week after the final harvest, 80-90% of seedlings in the
173 drought treatment had died, while mortality had not yet started by the time the final samples
174 had been collected. A one-week, re-watering period was used to confirm that the seedlings
175 were indeed dead. During the experiment, 40 pots across treatments and provenance (5 pots
176 per provenance and treatment) were weighed at 09:00 on days 0, 14, 29 and 36. At the end of
177 the experiment following plant harvesting, the pots were oven dried at 70°C for 48 hours to
178 obtain the dry weight. The gravimetric soil water content (θ_d) (grams of water per gram of
179 oven-dried soil) was calculated as (wet soil weight - dry soil weight) / dry soil weight. Then
180 volumetric water content (θ_{vd}) was calculated as gravimetric soil water content (θ_d) x (bulk
181 density (d_b) / density of water (d_w)).
182
183 *Ecophysiological measurements*
184 To assess pre-experiment morphological variation amongst provenances, height and crown
185 depth were measured for 15 seedlings per provenance at the start of the experiment. To assess
186 morphological impacts of drought, at each sample point including the start of the experiment
187 (t1=0, t2=11, t3=29), 5 individuals were sampled per provenance per treatment for trait
188 analyses. Fresh leaf weight (FW, g) was measured by separating all needles from the shoot and
189 weighing them. After this, total leaf area (TLA, cm^2) was obtained using scanned images of
190 all leaves and the ImageJ software (Image-J 136b; NIH, Bethesda, Maryland, USA). All
191 needles were saturated in vials of water for 24 hours in order to obtain the turgid weight (TW,
192 g). Dry weights (DW, g) of total needles and stem tissue were obtained after oven-drying for
193 48 hours at 70 °C. The percent relative water content was then calculated as: RWC = (FW-
194 DW)/ (TW-DW) x 100. Water deficit was calculated as WD = (TW-FW)/ (TW-DW). Specific
195 leaf area was calculated as SLA = TLA/ DW.

196 Entire root systems were washed and arranged so as to avoid overlapping lateral roots on a
197 clear plastic tray with a white background. Images taken with a digital camera were converted
198 to binary and analysed to obtain total root length using RootReader2D plugin (Clark *et*
199 *al.,*2013) in ImageJ. Roots were oven dried at 70°C for 48 hours and weighed to measure root
200 biomass (g). Specific root length (SRL, m g^{-1}), the ratio of root length to dry mass of roots, was
201 also calculated.

202 To determine photosynthetic responses to drought, chlorophyll fluorescence from needles was
203 measured by means of a Plant Efficiency Analyzer (Hansatech Instruments Ltd., Norfolk,
204 England). The ratio of variable (F_v) to maximum fluorescence (F_m) was taken, since this value
205 has been widely used for assessing plant physiological status and the state of Photosystem II
206 (PSII) (Murchie & Lawson, 2013). F_v/F_m was measured for 5 seedlings per provenance at four
207 time points (0, 12, 26 and 33 days). The middle portion of one needle per seedling was placed
208 in the centre of the leaf clip measuring area. Needles were detached and dark adapted in leaf
209 clips supplied with the analyser for 30 min at room temperature. Then the minimum
210 fluorescence (F_o), maximum fluorescence (F_m), variable fluorescence ($F_v = F_m - F_o$), and the
211 ratio F_v/F_m were recorded using a saturating intensity pulse for 0.7 s at 80% intensity level of
212 photon flux density (4620 µmol m^{-2} s^{-1}). The maximal quantum yield at PSII was calculated as
213 $F_v/F_m = (F_m – F_o)/F_m$.
214
215 *Metabolomics sampling, extraction and analysis*
216 To determine metabolic responses to drought, the foliar tissues of five randomly selected
217 individuals per provenance per treatment were sampled at midday at three time points (0, 11,
218 and 29 days). As the sampling was destructive, these were different individuals than those
219 sampled for trait measurements. Entire shoots were immediately frozen in liquid N_2 to ensure
220 no wounding response was elicited by removing tissue from the plant, and then stored
221 temporarily in frozen aluminium foil on dry ice. Samples were stored in a -80°C freezer until
222 metabolite extraction.
223
224 A monophasic extraction method was used to extract metabolites. The solvent comprised
225 acetonitrile, methanol, and HPLC-grade water (2:2:1) and was kept on wet ice to avoid
226 evaporation. Frozen needle tissue was weighed into MK-28 Precellys homogenisation tubes
227 (Stretton Scientific, Stretton, Derbyshire, UK) containing steel beads. The volume of solvent
228 to be added was adjusted according to the fresh tissue weight and 5 % extra acetonitrile and

229 methanol were added to compensate for estimated differences in relative water content between
230 drought and control. Following the addition of solvent, samples were vortexed and
231 homogenised in a Precellys-24 bead-based homogenizer (Stretton Scientific) at room
232 temperature with two 3 minute pulses of 6800 rpm. The homogenate was transferred to a 1.5
233 ml microtube (Sarstedt, Nümbrecht, Germany) and half the volume of solvent was used to wash
234 the Precellys tube to ensure that any residual homogenate was transferred. Samples were placed
235 on ice and vortexed for 10 s before the mixture was centrifuged at 19 °C, 14000 rpm (19064
236 rcf, 10 min). Finally, 400 µl of supernatant were transferred to a new microtube, which was
237 stored at -80°C prior to speed vacuum drying (Wu *et al.,*2008). After centrifugation, equal
238 volumes of the supernatant were transferred into a new plastic tube and dried in a SpeedVac
239 before storage at -80°C. Taking up the samples in 100 µl water / methanol 1:1 with 0.1 %
240 formic acid could not be achieved by vortexing alone and required 20 min of sonication. A
241 quality control (QC) sample was pooled from 10 µl each.
242
243 After centrifugation at 15000 rpm (21885 rcf) for 10 min at 4°C (Biofuge), 20 µl per sample
244 were pipetted into a 96-well plate in a controlled randomised order, with QC samples placed
245 equidistantly amongst them. The samples were analysed by UHPLC-MS on a Thermo
246 Scientific Q Exactive mass spectrometer attached to a Thermo Dionex Ultimate 3000 RS
247 system, equipped with a Thermo Hypersil Gold column (100 x 2.1 mm, 1.9 µm particles) (Clark
248 et al. 2017). Solvent A was 0.1 % formic acid in water and Solvent B was 0.1 % formic acid
249 in methanol. Liquid chromatography was performed over 14 min at a flow rate of 400 µl/min
250 containing a gradient from A to B from 1 to 8 min. Following analysis of preliminary test
251 samples of aqueous and organic phases, data were collected in positive ion and profile mode,
252 *m/z* (mass to charge ratio) 100-1000 Da at 70,000 resolution. Three additional runs of the
253 pooled QC sample were performed, one in positive ion mode at higher resolution (140,000),
254 and two runs using data dependent acquisition; MS/MS fragmentation dependent on the highest
255 5 signals per MS scan, in positive and negative ion mode. MS data were converted into mzML
256 format using MS Convert, and an R based XCMS/Camera script was used to obtain a first raw
257 intensity matrix, which was imported into MatLab (SimStitch 3.1). Blank filtering was applied
258 using a two-fold sample over blank threshold and peak signal filtering used an 80 % sample
259 filter, applied per group to generate a Sample Filtered Matrix. The dataset was normalised using
260 the probabilistic quotient normalisation (PQN) algorithm to correct for peak intensity
261 differences. Missing values were imputed using a K Nearest Neighbour algorithm (k=5). All

262 values were then transformed using a generalised logarithm (g-log) to minimise
263 heteroscedasticity in downstream statistical analyses (Parsons et al. 2007; Di Guida et al. 2016).
264
265 *Statistical and bioinformatics analyses*
266 In order to assess the effect of drought treatment, provenance and their interaction on
267 ecophysiological traits, we conducted 2-way analyses of variance (ANOVAs). Initial analyses
268 using all time points in the model and time as factor showed that most of the variation in
269 response variables occurred at the third time point and we thus focused on contrasting results
270 for the second and third time points. Where necessary, variables were log or arcsine
271 transformed prior to analysis to improve the normality of model residuals. This applied to:
272 crown percentage of shoot, maximum root length percentage of total root length, leaf RWC
273 and F_v/F_m data.
274
275 Metabolomics data were analysed using a combination of multivariate and univariate statistics.
276 A visual comparison of the QC samples in multivariate space generated by a principal
277 component analysis (PCA) assured us of the technical quality of metabolomics profiles (Fig.
278 S2). The QC samples were then removed from the data for further analyses. We next conducted
279 an additional exploratory PCA to visualise how the overall metabolome varied among
280 provenances, experimental treatments and time points.
281
282 To test for the overall response of the metabolome during the experiment, we used regularised
283 multiple analyses of variance (rMANOVA). The combined implementation of multiple
284 analytical approaches has been strongly recommended when analysing metabolomics datasets
285 (Karp et al. 2005; Goodacre et al. 2007; Vinaixa et al. 2012). Multivariate analysis of variance
286 (MANOVA) cannot be used for analysis of high-dimensional data where the number of
287 observations is (much) less than the number of variables. This issue is avoided by using
288 regularized MANOVA (rMANOVA), a multivariate data analysis method that has been
289 specifically developed for analysis of multi-factor untargeted metabolomics data (Engel et al.
290 2015). rMANOVA can be considered as a MANOVA where a regularized (shrinkage)
291 estimator of the within-group variation is used rather than the sample estimator. Because of
292 this, the method is applicable to data where the number of observations is much smaller than
293 the number of variables. This method is closely related to ANOVA simultaneous component
294 analysis (ASCA), which is a well-known method for analysis of such data. The difference
295 between rMANOVA and ASCA is that rMANOVA tries to better take the correlations between

296 the observed peak intensities into account, which often makes it a more sensitive method to
297 detect significant differences among groups (Engel *et al.,*2015). We constructed a full model
298 with factors drought, time point, and provenance, including all possible two-way and three-
299 way interactions. A permutation test with 1000 permutations was used to assess the
300 significance of each factor and interaction in the rMANOVA model (Matlab, R2014b).
301
302 ANOVAs, in combination with Benjamini–Hochberg false discovery correction at $\alpha = 0.05$
303 (Benjamini & Hochberg 1995), were used for univariate analyses of the data. As provenance
304 did not generally show any significant results on its own or in interaction with other factors,
305 we focused on the effects of sampling time point and drought treatment on metabolite
306 composition of individuals. Specifically, we conducted pairwise comparisons that contrasted
307 different time points and experimental treatments. We first determined the number of
308 metabolites that showed significant differences in abundance between groups, as measured by
309 metabolite peak height.
310
311 For metabolite identifications, we focused on metabolites that showed significant differences
312 in abundance between drought and control at time point 3 and had an average absolute $\log_2$
313 fold change value greater than 1 in this comparison (i.e. were twice as abundant or twice as
314 rare in droughted versus control seedlings). We focused on this comparison because
315 multivariate and univariate analyses indicated that the greatest differences in metabolic
316 composition were between individuals in drought versus control at time point 3 and because
317 this represents the time point at which individuals in the drought treatment were closest to
318 drought-induced mortality.
319
320 Putative metabolite annotations were carried out by matching *m/z* values to the Kyoto
321 Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al*. 2016), LipidMaps (Fahy et al.
322 2007) and BioCyc (Caspi et al. 2016) databases. These annotations were carried out with the
323 software MI-PACK using a 2 ppm (KEGG with molecular formula search up to *m/z* 620),
324 3 ppm (BioCyc) or 4 ppm (LipidMaps) error margin, thereby also defining a range of
325 reasonable molecular formulae (Weber & Viant 2010). Since isomers and adducts are included
326 and not always distinguishable, and the number of possible compounds increases over the *m/z*
327 range, not all peaks could be assigned with putative metabolite identification. The LC-MS/MS
328 data collected assisted the annotation (level 2 identification; Sumner *et al.* 2007).
329

330 To aid in identification of metabolic activity of specific pathways, given incomplete knowledge
331 of individual metabolite identities, the library *mummichog* (Li et al., 2013) from the
332 *MetaboAnalystR* package (Xia et al., 2015) was used. *Mummichog* employs a probabilistic
333 framework to bypass the need for complete prior metabolite identification, by combining
334 knowledge of metabolite spectral features with knowledge of linkages among known
335 metabolites within curated metabolic pathways (based on putative annotations). Given a list of
336 *m/z* ratios, values of log-fold change from control and univariate tests of significance for
337 individual compounds, *mummichog* computes a list of all possible candidate metabolites,
338 including common isotopic derivatives and adducts. If the list of significant features reflects
339 true metabolic activity, the true metabolites should show 'enrichment' (i.e., a significant
340 increase in the treatment relative to the control, or vice versa) for particular pathways, while
341 the falsely matched compounds should be distributed more randomly. In practice, *mummichog*
342 calculates a contingency table of enriched metabolites for every known pathway relative to the
343 total number of metabolites in that pathway; it adjusts the Fisher t-test by incorporating the
344 EASE measure of pathway size to increase robustness and draws random permutations of all
345 listed metabolites (including falsely matched compounds) across all pathways to derive a
346 gamma null distribution of P values, which is tested against the observed enrichment list. To
347 identify the metabolic pathways most likely altered immediately prior to death, we employed
348 *mummichog* to test for enrichment in the control against the drought treatment for the last time
349 point of the experiment. We ran *mummichog* in positive analytical mode, assuming a mass
350 accuracy of 3ppm and running 1000 permutations for each run. We considered cut-off
351 probability points varying from 0.05 to 0.0001 for the significance of the gamma distribution
352 and examined which pathways were most consistently identified across all cut-off thresholds
353 (fewer pathways are identified with lower thresholds). Software R version 3.2.2 was used for
354 data analyses (R Core Team, 2015).
355
356

357 RESULTS

358 *Seedling biomass and photochemical capacity*

359 At the start of the experiment (day 0, time point 1), there were significant differences amongst
360 provenances in height, with the tallest seedlings being from the Austrian provenance, Pernitz
361 (ANOVA, d.f=3, F=3.09, p=0.03). There were marginal differences in SLA, with the highest
362 SLA in seedlings from the Scottish provenance, Rothiemurchus (ANOVA, d.f=3, F=2.81,

363 p=0.072). Provenances did not differ significantly in any other seedling traits at the start of the
364 experiment ($p > 0.1$). At time point 2 in our study (day 11), most morphological variables were
365 not significantly affected by the drought treatment or provenance. Two exceptions were a
366 significant difference between drought treatment and control for root to shoot ratio (ANOVA,
367 d.f=1, F=11.08, p=0.002) and significant differences among provenances for maximum
368 quantum yield of PSII, Fv/Fm (ANOVA, d.f=3, F=2.9, p=0.04). There were no significant
369 interactions of treatment by provenance at time point 2.

370

371 By time point 3 (day 29), greater morphological differences between drought and control
372 treatments were apparent. There was a significant decrease in total, shoot and root dry weight
373 of seedlings under drought, as well as significant provenance interactions with the drought
374 treatment (Table S1; Fig. 1A-C). Seedlings from Rothiemurchus were most affected under
375 drought and showed reduced total, shoot and root dry weight, while the least reduction in
376 biomass was found for the Spanish provenance. Root to shoot ratios significantly decreased
377 under drought, though no provenance interaction effect was found (Fig. 1D). Drought treatment
378 significantly decreased the specific root length and maximum root length by day 29 of the
379 experiment, while no provenance effects were detected (Table S1; Fig. S3). Drought also
380 significantly increased the maximum root length as a fraction of the total root length by day 29
381 of the experiment (ANOVA, d.f=1, F: 11.1, p=0.002) (Fig. S4). A significant effect of drought
382 treatment on F_v/F_m ratios was found after day 26 (Fig. S5). There was also a provenance
383 interaction with drought on F_v/F_m ratios on day 26. F_v/F_m decreased most in seedlings from
384 Rothiemurchus and Pernitz and was least reduced in seedlings from Sierra Nevada (Fig. S5).

385

386 *Metabolomics*

387 Our UHPLC-MS approach generated peak height data for 4640 distinct peaks that putatively
388 represent distinct metabolites with their adducts and isotopes, with peak height representing
389 relative metabolite abundance. As an initial analysis of the data, PCA scores plots were
390 constructed where the samples were coloured according to the levels of the factors of interest
391 (e.g. drought vs control). No separation between the samples was observed except for the
392 interaction between drought and time. The drought time point 3 samples were clearly separated
393 from the other samples (Fig. S6). A principal component analysis-based reduction of the
394 metabolomics data gave two main components that together explained 20% of the variation in
395 the data, while a scree plot showed a sharp reduction in variation explained by subsequent axes.

396 A visual assessment of these two principal components (Fig. 2A&B) suggests that metabolite
397 composition did not change substantially between time points 1 and 2, but that the leaf
398 metabolome of seedlings in the drought treatment diverged substantially from seedlings in the
399 control treatment at time point 3. For each experimental treatment at each time point, there was
400 substantial overlap amongst provenances in metabolite composition, becoming more
401 pronounced as the experiment progressed (Fig. 2A&B). These visual impressions were
402 confirmed in multivariate analyses of the data by regularised MANOVA (rMANOVA; Engel
403 et al. 2015). This showed a significant effect of drought treatment ($p < 0.001$), time ($p < 0.001$)
404 and their interaction ($p < 0.001$) on leaf metabolic composition of seedlings, while provenance
405 had no significant effects, either on its own ($p = 0.981$) or in interaction with time ($p = 0.787$)
406 or experimental treatment ($p = 0.987$).
407
408 Univariate pairwise comparisons of specific time points and treatments showed that a much
409 greater number of metabolites differed significantly in abundance between the drought and
410 control treatments at time point 3 (1138 signals) than at time point 2 (190 signals) (Fig. 2C).
411 Within the drought treatment, seedlings showed a substantial difference in metabolite
412 abundances between time points 2 and 3 (892 signals). Within the control treatment, fewer
413 metabolites changed significantly in abundance between time points 2 and 3 (125 signals) (Fig.
414 S7). The smallest changes in metabolite abundance were observed between seedlings at the
415 start of the experiment and seedlings at time point 2, both in the drought (14 signals) and control
416 (29 signals) treatments (Fig. 2C; Fig S7).
417
418 For further scrutiny and identification, we selected signals that showed a significant difference
419 in abundance under drought vs. control at time point 3, and that had a $\log_2$ fold difference in
420 abundance greater than one (i.e., were twice as abundant or twice as rare under drought vs.
421 control at time point 3). The selection of these metabolites is visualised in a Volcano plot (Fig.
422 3). Overall, more metabolites experienced a significant and substantial decline under drought
423 ($\log_2$ fold change >1) than a significant and substantial increase (183 vs. 146 signals). Yet,
424 among the metabolites that changed the most under drought vs. control, those that increased
425 showed a greater absolute magnitude of change than those that decreased (Fig. 3). For example,
426 among the top 25 signals in terms of absolute magnitude of change, 23 of those increased under
427 drought vs. control, while only two decreased.
428

429 We were able to obtain putative annotations for a minority of the signals that showed significant
430 decreases or increases in abundance under drought vs. control. Of the 183 peaks that were
431 significantly less abundant under drought and show a log_2 fold change less than -1 (i.e. are at
432 least twice as rare on average in drought vs. control at time point 3), only 29 were matched in
433 the KEGG, LIPDMAPS or BIOCYC databases. Of the 49 signals with the greatest decrease
434 under drought relative to control, only 10 have putative annotations in these databases (Table
435 2; see Table S2 for full list). Of the 146 peaks that are significantly more abundant in drought
436 vs. control and show a log_2 fold change of at least 1 (i.e. are at least twice as common on
437 average in drought vs. control), 98 have no putative annotations in the KEGG, LIPIDMAPS or
438 BIOCYC databases. Of the 22 compounds with the greatest increase under drought relative to
439 control in terms of fold-changes, only 10 have putative annotations (Table 3; see Table S3 for
440 full list). The metabolites that were more abundant under drought were mainly aromatic amino
441 acids, which may signify up-regulation of aromatic amino acid biosynthesis via the shikimate
442 pathway or protein degradation under drought stress. A number of upregulated signals
443 coeluting at 4.08 min are likely breakdown products of only one metabolite, tryptophan
444 (manual interpretation).
445
446 Pathway analysis with *mummichog* identified the following pathways as the primary sites of
447 metabolic enrichment immediately prior to death: aminoacyl-tRNA, phenylalanine (Phe),
448 vitamin B6, phenylpropanoid, isoquinoline alkaloid, arginine and proline (Pro), Phe and
449 Tyrosine (Tyr) and Tryptophan (Trp) biosynthesis (Table S4). Interestingly, the amino acids
450 synthesised via the shikimate pathway (Tyr, Trp and Phe) clearly increased during drought,
451 while several of those in the downstream pathways from shikimate decreased, although the
452 downstream response was compound-specific. Many of the significant decreases during
453 drought were seen for mevalonate and compounds in the mevalonate/MEP-DOXP (or non-
454 mevalonate) pathways, such as carotenoids, sterols, ecdysones, terpenoids and glycosides with
455 a second active group of the same type.
456

457 ## DISCUSSION

458 This study investigated the foliar metabolic responses of *Pinus sylvestris* seedlings to severe
459 drought, which eventually led to drought-induced mortality, and to ascertain whether ecotypic
460 adaptation in the metabolic responses to drought would be discernible amongst provenances.
461 Pronounced metabolic effects of drought were found, potentially related to a decoupling of

462 carbon assimilation and secondary metabolism as photosynthesis became compromised. This
463 manifested through an increase in free amino acids and a decrease in secondary metabolite
464 production via the shikimate and mevalonate/MEP-DOXP pathways. Our second hypothesis
465 was not supported, as we found no significant foliar metabolic differences amongst
466 provenances in the response to drought. The latter is particularly interesting in light of the fact
467 that we found morphological responses to drought to vary by provenance. Seedlings from the
468 driest provenance were least affected morphologically by drought.
469

470 *Seedling foliar metabolome response to drought*
471 There were significant differences in the metabolite composition of the control and drought
472 treatment groups, with a clear divergence in metabolic profiles following four weeks of drought
473 stress after which mortality rapidly ensued (Fig. 2). Free amino acids (Tyr, Trp and Pro; Table
474 3) produced during primary metabolism were identified by ANOVA and rMANOVA. Tyr and
475 Trp increased with the highest fold change under the drought treatment. Pathway analysis with
476 *mummichog* highlighted the aminoacyl t-RNA pathway as enriched, owing to the abundance
477 of free amino acids. However, the inference of increased t-RNA synthesis based solely on the
478 presence of free amino acids seems untenable, especially given that drought stress conditions
479 would presumably have impeded protein synthesis. Besides the already mentioned aromatic
480 amino acids Tyr and Trp, significant increases were also found in several other amino acid
481 metabolic pathways (i.e., phenylalanine, proline, glutamine, valine, leucine, isoleucine,
482 arginine, histidine), leaving only the metabolic pathways of cysteine, methionine, glycine,
483 serine and threonine unaffected. The shikimate pathway involved in aromatic amino acid
484 biosynthesis was identified as a dominant pathway by *mummichog*. Plants synthesise aromatic
485 amino acids, such as Tyr and Trp, via the metabolically costly 7-step shikimate pathway, to
486 which over 30% of photosynthetically derived carbon can be directed (Maeda & Dudareva
487 2012). Secondary compounds involved in plant defence and abiotic stress responses can be
488 derived from shikimate. It is conceivable that, rather than biosynthesis via the shikimate
489 pathway, protein degradation accounts for the increasing levels of free amino acids detected.
490 The higher abundance of aromatic amino acids relative to other amino acids however seems to
491 indicate that the shikimate pathway is involved. Nevertheless, a concomitant increase in
492 shikimate and chorismate intermediates was not found, which has been used to confirm
493 involvement of the shikimate pathway in accumulation of free aromatic amino acids (Tyr, Trp
494 and Phe) in drought sensitive wheat leaves (Michaletti *et al*. 2017). Pathway analysis

495 furthermore identified the mevalonate pathways in secondary metabolism as most affected by
496 drought, with decreases in key metabolites involved with plant defence.
497
498 Tyrosine (Tyr) was the metabolite that showed the strongest increase under the drought
499 treatment. Tyr hyperaccumulation in young shade leaves of *Inga umbellifera* has been linked
500 to decreased insect larval performance, thus representing a rare example of an amino acid
501 functioning as a defensive compound (Lokvam *et al.,*2006). Among the annotated metabolites,
502 tryptophan (Trp) showed the third greatest increase under drought versus control. Trp
503 biosynthetic enzymes have been shown to be up-regulated in response to oxidative stress
504 treatment in *Arabidopsis* (Zhao *et al.,* 1998). The increase in aromatic and non-aromatic amino
505 acids, some of which are key precursors of important defence and antioxidant pathways,
506 occurred while several defence compounds decreased (Table S4). Significant decreases also
507 occurred for phosphomevolanate and other downstream compounds that have similar
508 biological roles.
509
510 For coniferous species, carbon-based secondary metabolites, terpenoids and phenolic
511 compounds are expressed constitutively and are inducible to high concentrations and provide
512 effective defence against many pests and pathogens (Keeling & Bohlmann 2006). Decreases
513 of secondary metabolites were detected at 29 days of drought, by ANOVA and rMANOVA.
514 This decrease in metabolites involved in defence during the last phase of drought may reflect
515 a decreased capacity to employ the mevalonate pathways at a stage during drought where
516 photosynthesis and growth were already strongly impacted, and carbohydrate availability was
517 limited.
518
519 Increased free amino acids under stress may also be the result of protein breakdown, rather
520 than *de novo* biosynthesis of amino acids. For example, branched chain amino acids and
521 aromatic amino acids (Tyr and Phe) have been shown to increase under osmotic stress via
522 protein degradation in *Arabidopsis thaliana*, with a higher fold change than other amino acids;
523 this increase was potentially owing to lower basal levels of branched chain amino acids and
524 aromatic amino acids prior to stress induction, or slower catabolism subsequently (Huang &
525 Jander 2017). In *Arabidopsis*, accumulation of aromatic amino acids occurs more rapidly than
526 branched chain amino acids (Fàbregas and Fernie 2019). It is possible that the accumulation of
527 free aromatic amino acids has a protective role under conditions of elevated oxidative stress,
528 by scavenging free radicals (Stadtman & Levine 2003). Indeed, 2-phenylacetamide was ranked

529 as the second most elevated metabolite under drought and is a product of oxidation of the
530 aromatic amino acid phenylalanine. However, the pool size changes in amino acids may simply
531 represent an artefact of protein breakdown coupled with differential rates of amino acid
532 catabolism. Under conditions of carbon starvation, catabolism of amino acids would provide a
533 source of energy. The degradation pathways (catabolism) of aromatic amino acids Phe and Trp
534 are not well-documented in plants (Hildebrandt et al. 2015). A comprehensive study of amino
535 acid contents that included low abundance amino acids (such as aromatic ones) showed that
536 the amount of aromatic amino acids produced by protein degradation was sufficient for
537 secondary metabolite production, with the exception of Trp under certain stress conditions
538 (Hildebrant et al. 2018). Proteolysis can account for the accumulation of amino acids under
539 osmotic stress and fast catabolism of Lys as well as branched chain amino acids has been shown
540 to be induced to provide alternative respiratory substrate during drought stress (Araujo et al.
541 2011; Batista-Silva et al. 2019; Pires et al. 2016). Thus turnover of amino acids may be
542 explained in terms of proteolysis and tightly regulated amino acid metabolism.
543
544 One of the compounds with the largest increase under drought was proline. Proline is an amino
545 acid that functions as a compatible solute, carrying no net charge at physiological pH, by raising
546 osmotic pressure in the cytoplasm and stabilising proteins and cellular membranes (Szabados
547 & Savoure 2010). Additionally, proline has been indicated to exhibit antioxidant capacity in
548 free radical scavenging (Smirnoff & Cumbes 1989). Whereas aromatic amino acid
549 accumulation under osmotic stress was linked to protein breakdown in *Arabidopsis*, increased
550 biosynthesis accounted for elevated levels of proline (Huang & Jander 2017). Indeed, proline
551 is biosynthesised in a number of plant species under drought and has been shown to exhibit
552 osmoprotective and antioxidant capacities (Hayat *et al.,*2012). Furthermore, it is suggested that
553 proline accumulation under stress may have a role in signalling as well as maintenance of
554 NAD(P)/NAD(P)H ratios that enable metabolic pathways to function in generating secondary
555 metabolites (Hare & Cress 1997). Biosynthesis of proline under water deficit occurs in an
556 abscisic acid-dependent manner and can also be influenced by sugar availability, indicating a
557 regulatory mechanism to restrict proline accumulation when carbohydrate status is low (Rook
558 *et al.,*2001, Verslues & Bray 2006).
559
560 A number of important, significant peaks determined by univariate and multivariate statistics
561 were not identifiable through interrogation of metabolic databases, possibly owing to taxon
562 specificity and the poor development of metabolite databases for non-model plant species. This

563 is in addition to adducts and breakdown products often not covered by common databases. This
564 finding highlights the difficulty of current metabolomics approaches, at least for conifers, and
565 suggests that insufficient understanding of the metabolic pathways affected by drought is
566 currently hindering our understanding of the nature of carbon limitations prior to drought-
567 induced mortality.
568

569 *Provenance variation in the seedling drought response*

570 Total seedling biomass was reduced under drought treatment and this reduction varied
571 significantly across provenances. Seedlings from the wettest provenance, Rothiemurchus
572 (Scotland), were most affected under drought in terms of biomass reduction, while seedlings
573 from the driest provenance, Sierra Nevada (Spain), were least affected. Under drought, total
574 leaf area was significantly reduced, but there was no observed needle abscission. Rather, the
575 drought is thought to have inhibited needle growth. Needles also showed a reduction in the
576 maximal efficiency of photosystem II (F_v/F_m) by day 26 of the drought treatment, indicating
577 that photosynthesis was compromised, with the wettest provenance being most affected. F_v/F_m
578 declines when water stress becomes severe (Epron & Dryer 1992; Iijima *et al.,*2006; Ditmarova
579 *et al.,*2010; Way *et al.,*2013). Potentially this could be a result of decoupling between
580 photosynthesis and secondary metabolism, with C limitation once assimilation is not occurring
581 owing to stomatal closure in this isohydric species.
582

583 A limitation of this study is the lack of information on how mortality rate varies by provenance.
584 Matias and Jump (2014) found that mortality rate of *Pinus sylvestris* under drought is strongly
585 affected by temperature, with the southern Spanish provenance showing higher survival
586 probabilities than the northern Finnish provenance under drought at the current temperature
587 regime of the southern range limit. This effect may not be significant in our experiment owing
588 to the shorter timeframe (5 weeks compared with 19 weeks), since Matias and Jump found no
589 significant effect of temperature on provenances until after 5 weeks of treatment. Survival is
590 vital in determining seedling recruitment at the population level, thus if mortality rate is
591 relatively constant among provenances over short intense drought episodes, then the superior
592 drought response of the Spanish provenance in terms of morphological and physiological traits
593 would not have adaptive significance.
594

595 In contrast, metabolic profiles were not found to differ significantly by provenance, a result
596 robust to changes in the signal filter threshold employed. Previous studies on intraspecific
597 variation in conifers have found evidence for a stronger environmental than genetic signal on
598 metabolomes of developing xylem in *Pseudotsuga menziesii* (Robinson et al. 2007). However,
599 differences in foliar metabolomes of *Pinus pinaster* were found to be strongly related to the
600 aridity of the provenance site of origin, suggesting local adaptation (Meijon et al. 2016). Also,
601 Du et al. (2015) found that *Pseudotsuga menziesii* seedlings showed a provenance specific
602 drought response, with the drier provenance increasing aromatic amino acids. In this study, the
603 metabolic phenotypes of *P. sylvestris* seedlings do not appear to be population specific or to
604 show local adaptation, or at least such differences did not have a sufficiently large effect size
605 to be statistically detectable. *Pinus sylvestris* is a widespread species with high plasticity
606 evident across European populations in traits related to physiology, phenology and morphology
607 (Olekysn et al. 1998, 2000; Semerci et al. 2017), but the findings of this study suggest a
608 limitation of foliar metabolic plasticity to react to extreme mortality-inducing drought episodes.
609 The relatively long mean leaf life span of *Pinus* species (over 3 years) may account for the
610 discrepancy between the drought response in foliar metabolic profiles and other measured leaf
611 traits. In evolutionary terms, leaf longevity has a pivotal role in the leaf economic spectrum,
612 with trade-offs between persistence and productivity constraining both morphological and
613 biochemical leaf traits (Warren and Adams 2000; Onoda et al. 2017). Plasticity in needle
614 longevity is known for *Pinus sylvestris* (Pensa and Jalkanen 2005). Juvenile needles in three
615 Mediterranean pine species were found to exhibit a leaf strategy to maximise carbon gain and
616 the transition to adult needles reflected changes in traits according to the drought stress
617 tolerance of each species (Kuusk et al. 2017). Foliar integration of traits has been found to be
618 a function of ontogeny; canalisation of leaf traits are found in plants of reproductive age,
619 whereas in juvenile plants weaker correlations among functional leaf traits indicate lower foliar
620 integration and higher phenotypic plasticity (Damian et al. 2016). However, the selection of
621 leaf traits in this study did not include biochemical or metabolic functional traits. Metabolically
622 divergent drought responses in mature trees may be more likely than for seedlings with the
623 same leaf age, which all show a propensity to maximise carbon gain to the detriment of
624 environmental stress tolerance; thus, canalisation of leaf traits may have affected metabolic
625 profiles.
626

627 *Conclusions*
628 There was a strong impact of drought at the metabolic level in *P. sylvestris* needles, with the
629 effect becoming very apparent just before seedling mortality. In response to drought, we found
630 increases in many free amino acids and decreased concentrations of secondary metabolites in
631 the mevalonate/MEP-DOXP pathways. The identification of decreases in compounds derived
632 from aromatic amino acids and secondary metabolic pathways related to plant defence shows
633 that these metabolically costly pathways are down-regulated under drought stress, revealing
634 the incapacity of severely droughted seedlings to produce defensive compounds against biotic
635 stress. However, proline, a compound important for osmoregulation, plant signalling and
636 antioxidant defence, was strongly upregulated, suggesting that its high concentration was
637 important in the latest phases of survival prior to death. While seedling biomass and
638 photochemical efficiency were found to be most strongly reduced by drought for
639 Rothiemurchus, the wettest provenance, there was a lack of provenance effects on metabolite
640 abundances. Overall, our findings indicate that important metabolite changes under drought
641 were centred around the shikimate and the mevalonate/MEP-DOXP pathways. It also
642 demonstrates that a large number of unknown compounds were affected by drought and are
643 therefore of interest for future research.
644

645 *Acknowledgements*
646 This research was partly funded via Natural Environment Research Council (NERC) grant
647 NE/I011749/1 to MM and via a NERC PhD studentship to SM. A grant from the NERC
648 Biomolecular Analysis Facility (NBAF868) made the metabolomics study possible.
649

650 *Author Contribution*
651 S.M and K.G.D. designed and executed the experiment; S.M measured plant traits and
652 extracted the metabolites; U.S and J.E led the metabolomics analyses; K.G.D and M.M
653 conducted additional analyses; S.M wrote the manuscript; and all authors contributed to
654 revisions.

655

656 REFERENCES
657 Allen CD, Macalady AK, Chenchouni H, Bachelet D, McDowell N, Vennetier M, Kitzberger
658 T, Rigling A, Breshears DD, Hogg ET, Gonzalez P (2010) A global overview of

659 drought and heat-induced tree mortality reveals emerging climate change risks for
660 forests. Forest Ecology and Management 259: 660-684.
661 Adams H et al. (2017) A multi-species synthesis of physiological mechanisms in drought-
662 induced tree mortality. Nature Ecology and Evolution, 1: 1285-1291.
663 Allen CD, Breshears DD, McDowell NG (2015) On underestimation of global vulnerability to
664 tree mortality and forest die-off from hotter drought in the Anthropocene. Ecosphere 6
665 1-55.
666 Anderegg WR, Hicke JA, Fisher RA, Allen CD, Aukema J, Bentz B, Hood S, Lichstein JW,
667 Macalady AK, McDowell NG and Pan Y (2015) Tree mortality from drought, insects,
668 and their interactions in a changing climate. New Phytologist 208: 674-683.
669 Araújo, WL, Tohge, T, Ishizaki, K, Leaver, CJ, Fernie, AR(2011). Protein degradation—An
670 alternative respiratory substrate for stressed plants. Trends in Plant Science, 16, 489–
671 498. https://doi.org/10.1016/j.tplants.2011.05.008
672 Batista-Silva, W., Heinemann, B., Rugen, N., Nunes-Nesi, A., Araújo, W. L., Braun, H. P., &
673 Hildebrandt, T. M. (2019). The role of amino acid metabolism during abiotic stress
674 release. Plant, cell & environment.
675 Benjamini Y, Hochberg Y. (1995) Controlling the false discovery rate: a practical and powerful
676 approach to multiple testing. J R Stat Soc B 57: 289–300.
677 Bowne JB, Erwin TA, Juttner J, Schnurbusch T, Langridge P, Bacic A, Roessner U (2012)
678 Drought responses of leaf tissues from wheat cultivars of differing drought tolerance at
679 the metabolite level. Molecular Plant 5: 418–429.
680 Breshears DD, Cobb NS, Rich PM, Price KP, Allen CD, Balice RG *et al.* (2005) Regional
681 vegetation die-off in response to global-change-type drought. Proceedings of the
682 National Academy of Sciences, USA 102: 15144–8.
683 Caspi R., Billington R., Ferrer L., Foerster H., Fulcher C.A., Keseler I.M., Kothari A.,
684 Krummenacker M., Latendresse M., Mueller L.A., Ong Q., Paley S., Subhraveti P.,
685 Weaver D.S., Karp P.D(2016) The MetaCyc Database of metabolic pathways and
686 enzymes and the BioCyc collection of Pathway/Genome Databases. Nucleic Acids
687 Research 44(D1):D471-80.
688 Chave J, Réjou-Méchain M, Búrquez A, Chidumayo E, Colgan MS, Delitti WBC *et al.* (2014)
689 Improved pantropical allometric models to estimate the above ground biomass of
690 tropical forests. Global Change Biology, 20:3177-3190.
691 Clark, M.S., Sommer, U., Sihra, J.K., Thorne, M.A., Morley, S.A., King, M., Viant, M.R. and
692 Peck, L.S., (2017) Biodiversity in marine invertebrate responses to acute warming

693 revealed by a comparative multi-omics approach. Global Change Biology, 23(1),
694 pp.318-330.
695 Clark RT, Famoso AN, Zhao K, Shaff JE, Craft EJ, Bustamante CD *et al*. (2013) High-
696 throughput two-dimensional root system phenotyping platform facilitates genetic
697 analysis of root growth and development. Plant Cell & Environment 36: 454–466.
698 Cruz de Carvalho MH. (2008) Drought stress and reactive oxygen species. Plant Signaling &
699 Behavior 3: 156–165.
700 Damián X, Fornoni J, Domínguez CA and Boege K, (2018) Ontogenetic changes in the
701 phenotypic integration and modularity of leaf functional traits. Functional ecology,
702 32:234-246.
703 de Simón, B. F., Sanz, M., Cervera, M. T., Pinto, E., Aranda, I., & Cadahía, E. (2017). Leaf
704 metabolic response to water deficit in *Pinus pinaster* Ait. relies upon ontogeny and
705 genotype. Environmental and experimental botany, 140, 41-55.
706 Di Guida R, Engel J, Allwood JW, Weber RJ, Jones MR, Sommer U, Viant MR, Dunn WB
707 (2016) Non-targeted UHPLC-MS metabolomic data processing methods: A
708 comparative investigation of normalisation, missing value imputation, transformation
709 and scaling. Metabolomics 12: 1-14.
710 Ditmarova L, Kurjak D, Palmroth S, Kmet J, Strelcova K. (2010) Physiological responses of
711 Norway spruce (*Picea abies* (L.) Karst) seedlings to drought stress. Tree Physiology
712 30: 205–213.
713 Dixon RA (2001) Natural products and plant disease resistance. Nature 411: 843-847.
714 Du B, Jansen K, Kleiber A, Eiblmeier M, Kammerer B, Ensminger I, Gessler A, Rennenberg
715 H, Kreuzwieser J. (2015) A coastal and an interior Douglas fir provenance exhibit
716 different metabolic strategies to deal with drought stress. Tree Physiology 36: 148–163.
717 Engel J, Blanchet L, Bloemen B, Van den Heuvel LP, Engelke, UHF, Wevers RA, Buydens
718 LMC(2015) Regularized MANOVA (rMANOVA) in untargeted metabolomics.
719 Analytica Chimica Acta 899: 1-12.
720 Epron D, Dreyer E (1992) Effects of severe dehydration on leaf photosynthesis in *Quercus*
721 *petraea* (Matt) Liebl – photosystem-II efficiency, photochemical and non-
722 photochemical fluorescence quenching and electrolyte leakage. Tree Physiology 10:
723 273–284.
724 Fàbregas N and Fernie AR, (2019). The metabolic response to drought. Journal of
725 Experimental Botany, 70:1077-1085.

726 Facchini PJ (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular
727 regulation, and metabolic engineering applications. Annual Review of Plant Biology
728 52: 29-66.
729 Fahy E, Sud M, Cotter D, Subramaniam S (2007) LIPID MAPS online tools for lipid research.
730 Nucleic Acids Res 35:W606–W612.
731 Galiano L, Martínez-Vilalta J, Lloret F (2011) Carbon reserves and canopy defoliation
732 determine the recovery of Scots pine 4 yr after a drought episode. New Phytologist 190:
733 750-759.
734 Gargallo-Garriga A, Sardans J, Pérez-Trujillo M, Rivas-Ubach A, Oravec M, Vecerova K,
735 Urban O, Jentsch A, Kreyling J, Beierkuhnlein C, Parella T, Peñuelas J (2014)
736 Opposite metabolic responses of shoots and roots to drought. Scientific Reports, 4:
737 6829, 10.1038/srep06829
738 Goodacre R, Broadhurst D, Smilde AK, Kristal BS, Baker DJ, et al. (2007) Proposed minimum
739 reporting standards for data analysis in metabolomics. Metabolomics 3: 231–241.
740 Hall RD (2006) Plant metabolomics: from holistic hope, to hype, to hot topic. New Phytologist
741 169: 453–468.
742 Hamanishi, E. T., Barchet, G. L., Dauwe, R., Mansfield, S. D., & Campbell, M. M. (2015).
743 Poplar trees reconfigure the transcriptome and metabolome in response to drought in a
744 genotype-and time-of-day-dependent manner. BMC genomics, 16(1), 329.
745 Hare PD, Cress WA (1997) Metabolic implications of stress-induced proline accumulation in
746 plants. Plant Growth Regulation 21: 79-102.
747 Harris I, Jones PD, Osborn TJ, Lister DH (2014) Updated high-resolution grids of monthly
748 climatic observations - the CRU TS3.10 Dataset. International Journal of
749 Climatology 34, 623-642.
750 Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A(2012) Role of proline under
751 changing environments: A review. Plant Sign & Behaviour 7: 1456–1466.
752 Hildebrandt TM, Nunes Nesi A, Araujo WL, Braun HP (2015) Amino acid catabolism in
753 plants. Molecular Plant 8: 1563 –1579.
754 Hildebrandt, T. M. (2018). Synthesis versus degradation: directions of amino acid metabolism
755 during Arabidopsis abiotic stress response. Plant molecular biology, 98(1-2), 121-135.
756 Huang T, Jander G(2017) Abscisic acid-regulated protein degradation causes osmotic stress-
757 induced accumulation of branched-chain amino acids in *Arabidopsis thaliana*. Planta
758 246: 737–747.

759 Iijima H, Shibuya M, Saito H, Takahashi K (2006) The water relation of seedlings of *Picea*
760 *jezoensis* on fallen logs. Canadian Journal of Forest Research 36: 664–670.
761 Ings J, Mu LA, Robson PR, Bosch M (2013) Physiological and growth responses to water
762 deficit in the bioenergy crop *Miscanthus x giganteus*. Frontiers in Plant Science 4: 468.
763 Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M (2016) KEGG as a reference
764 resource for gene and protein annotation. Nucleic Acids Research 44: D457-D462.
765 Karp NA et al (2005) Impact of replicate types on proteomic expression analysis. Journal of
766 Proteome Research 4:1867–1871.
767 Keeling CI, Bohlmann J (2006) Genes, enzymes and chemicals of terpenoid diversity in the
768 constitutive and induced defence of conifers against insects and pathogens. New
769 Phytologist 170: 657–675.
770 Körner C (2015) Paradigm shift in plant growth control. Current Opinion in Plant
771 Biology 25: 107–114.
772 Kullman L (2002) Rapid recent range-margin rise of tree and shrub species in the Swedish
773 Scandes. Journal of Ecology. 90: 68-77Li S, Park Y, Duraisingham S, Strobel FH, Khan
774 N, Soltow QA, Jones DP, Pulendran B(2013) Predicting network activity from high
775 throughput metabolomics. PLoS Computational Biology 9: e1003123.
776 Kuusk V, Niinemets Ü. and Valladares F, (2017) A major trade-off between structural and
777 photosynthetic investments operative across plant and needle ages in three
778 Mediterranean pines. Tree physiology, 38:543-557.
779 Lloret F, Peñuelas J, Estiarte M (2004) Experimental evidence of reduced diversity of seedlings
780 due to climate modification in a Mediterranean-type community. Global Change
781 Biology. 10: 248-258.
782 Lloret F, Peñuelas J, Prieto P, Llorens L, Estiarte M (2009) Plant community changes induced
783 by experimental climate change: Seedling and adult species composition. Perspectives
784 in Plant Ecology, Evolution and Systematics. 11: 53-63.
785 Lokvam J, Brenes-Arguedas T, Lee JS, Coley PD, Kursar TA. (2006) Allelochemic function
786 for a primary metabolite: the case of L-tyrosine hyper-production in *Inga umbellifera*
787 (Fabaceae). American Journal of Botany, 93: 1109-1115.
788 Maeda H, Dudareva N (2012) The Shikimate pathway and aromatic amino acid biosynthesis
789 in plants. Annual Review of Plant Biology 63: 73–105.
790 Martinez-Vilalta J, Mencuccini M, Vayreda J, Retana J (2010) Interspecific variation in
791 functional traits, not climatic differences among species ranges, determines

792 demographic rates across 44 temperate and Mediterranean tree species. Journal of
793 Ecology 98: 1462–1475.
794 Matías L, Jump AS (2014) Impacts of predicted climate change on recruitment at the
795 geographical limits of Scots pine. Journal of Experimental Botany 65: 299-310.
796 McDowell N, Pockman WT, Allen CD, Breshears DD, Cobb N, Kolb T *et al.* (2008)
797 Mechanisms of plant survival and mortality during drought: why do some plants
798 survive while others succumb to drought? New Phytologist 178: 719–39.
799 McDowell NG, Sevanto S (2010) The mechanisms of carbon starvation: how, when, or does it
800 even occur at all? New Phytologist 186: 264–266.
801 McDowell NG, Beerling DJ, Breshears DD, Fisher RA, Raffa KF, Stitt M (2011) The
802 interdependence of mechanisms underlying climate-driven vegetation mortality.
803 Trends in Ecology and Evolution 26:523–532.
804 Meijon M, Feito I, Oravec M, Delatorre C, Weckwerth W, Majada J, Valledor L (2016)
805 Exploring natural variation of *Pinus pinaster* Aiton using metabolomics: Is it possible
806 to identify the region of origin of a pine from its metabolites? Molecular Ecology 25:
807 959–976.
808 Mencuccini M, Minunno F, Salmon Y, Martínez-Vilalta J, Hölttä T, (2015) Coordination of
809 physiological traits involved in drought-induced mortality. New Phytologist, 208: 396-
810 409.
811 Michaletti A, Reza Naghavi M, Toorchi M, Zolla L & Rinalducci S (2018) Metabolomics and
812 proteomics reveal drought-stress responses of leaf tissues from spring-wheat. Nature
813 Scientific Reports 8: 5710.
814 Mueller RC, Scudder CM, Porter ME, Trotter RT, Gehring CA, Whitham TG (2005)
815 Differential tree mortality in response to severe drought: evidence for long-term
816 vegetation shifts. Journal of Ecology 93: 1085–1093.
817 Murchie EH, Lawson T (2013) Chlorophyll fluorescence analysis: a guide to good practice and
818 understanding some new applications. Journal of Experimental Botany 64: 3983–3998.
819 Oleksyn J, Zytkowiak R, Karolewski P, Reich PB, Tjoelker MG (2000) Genetic and
820 environmental control of seasonal carbohydrate dynamics in trees of diverse *Pinus*
821 *sylvestris* populations. Tree Physiology 20: 837-847.
822 Oleksyn J, Tjoelker MG, Reich PB (1998) Adaptation to changing environment in Scots pine
823 populations across a latitudinal gradient. Silva Fennica 32: 129–140.

824 Onoda Y, Wright IJ, Evans JR, Hikosaka K, Kitajima K, Niinemets Ü, Poorter H, Tosens T
825 and Westoby M (2017) Physiological and structural tradeoffs underlying the leaf
826 economics spectrum. New Phytologist, 214:1447-1463.
827 Parsons HM, Ludwig C, Günther UL, Viant MR (2007) Improved classification accuracy in 1-
828 and 2-dimensional NMR metabolomics data using the variance stabilising generalised
829 logarithm transformation. BMC Bioinformatics 8: 234.
830 Pensa M and Jalkanen R (2005) Variation in needle longevity is related to needle-fascicle
831 production rate in Pinus sylvestris. Tree physiology, 25:1265-1271.
832 Pires MV, Pereira Júnior AA, Medeiros DB, Daloso DM, Pham PA, Barros KA, Engqvist MK,
833 Florian A, Krahnert I, Maurino VG, et al. The influence of alternative pathways of
834 respiration that utilize branched-chain amino acids following water shortage in
835 Arabidopsis. Plant Cell Environ. 2016, 39, 1304–1319.
836 R Core Team. 2015. *R: A language and environment for statistical computing.* R Foundation
837 for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
838 Rehfeldt GE, Tchebakova NM, Perfenova YI, Wykoff WR, Kuzmina NA & Milyutin LI (2002)
839 Intraspecific responses to climate in *Pinus sylvestris.* Global Change Biology 8: 919-
840 929.
841 Rigling A, Bigler C, Eilmann B, Feldmeyer-Christe E, Gimmi U, Ginzler C *et al.* (2013)
842 Driving factors of a vegetation shift from Scots pine to pubescent oak in dry Alpine
843 forests. Global Change Biology 19: 229–240.
844 Robinson AR, Ukrainetz NK, Kang KY, Mansfield SD (2007) Metabolite profiling of Douglas-
845 fir (*Pseudotsuga menziesii*) field trials reveals strong environmental and weak genetic
846 variation. New Phytologist 174: 762–73.
847 Rook F, Corke F, Card R, Munz G, Smith C, Bevan MW (2001) Impaired sucrose-induction
848 mutants reveal the modulation of sugar-induced starch biosynthetic gene expression by
849 abscisic acid signalling. The Plant Journal 26: 421-433.
850 Ryan MG (2011) Tree responses to drought. Tree Physiology 31, 237–239.
851 Sala A, Woodruff DR, Meinzer FC(2012) Carbon dynamics in trees: feast or famine? Tree
852 Physiology 32:764–75.
853 Salmon Y, Torres-Ruiz JL, Poyatos R, Martinez-Vilalta J, Meir P, Cochard H, Mencuccini M
854 (2015) Balancing the risks of hydraulic failure and carbon starvation: a twig scale
855 analysis in declining Scots pine. Plant, Cell & Environment 38: 2575-2588.

856 Semerci A, Semerci H, Çalişkan B, Çiçek N, Ekmekçi Y, Mencuccini M (2017) Morphological
857 and physiological responses to drought stress of European provenances of Scots pine.
858 European Journal of Forest Research 136: 1-14.
859 Sevanto S, McDowell NG, Dickman LT, Pangle R, Pockman WT (2014) How do trees die? A
860 test of the hydraulic failure and carbon starvation hypotheses. Plant, Cell &
861 Environment 37: 153-161.
862 Smirnoff N, Cumbes J (1989) Hydroxyl radical scavenging activity of compatible solutes.
863 Phytochemistry 28: 1057–1060.
864 Stadtman ER, Levine RL (2003) Free radical-mediated oxidation of free amino acids and
865 amino acid residues in proteins. Amino Acids 25: 207-218.
866 Sternberg, M., Brown, V.K., Masters, G.J. et al. Plant Ecology (1999) 143: 29.
867 Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA *et al.* (2007) Proposed
868 minimum reporting standards for chemical analysis. Metabolomics 3:211–221.
869 Szabados L, Savoure A (2010) Proline: a multifunctional amino acid. Trends Plant Science 15:
870 89-97.
871 Verslues PE, Bray E (2006) Role of abscisic acid (ABA) and Arabidopsis thaliana ABA-
872 insensitive loci in low water potential-induced ABA and proline accumulation. Journal
873 of Experimental Botany 57: 201–12.
874 Vinaixa M, Samino S, Saez I, Duran J, Guinovart JJ, Yanes O (2012) A guideline to univariate
875 statistical analysis for LC/MS-based untargeted metabolomics-derived
876 data. Metabolites. 2:775–95.
877 Warren CR and Adams MA (2000) Trade-offs between the persistence of foliage and
878 productivity in two Pinus species. Oecologia, 124:487-494.
879 Way DA, Crawley C, Sage RF (2013) A hot and dry future: Warming effects on boreal tree
880 drought tolerance. Tree Physiology 33: 1003–1005.
881 Weber, R. J. M., & Viant, M. R. (2010). MI-Pack: Increased confidence of metabolite
882 identification in mass spectra by integrating accurate masses and metabolic pathways.
883 Chemometrics and Intelligent Laboratory Systems, 104: 75–82.
884 Woodruff CM (1940) Soil moisture and plant growth in relation to pF. Soil Science Society
885 America Proceedings 5: 36-41.
886 Wu Q-S, Xia R-X, Zou Y-N (2008) Improved soil structure and citrus growth after inoculation
887 with three arbuscular mycorrhizal fungi under drought stress. European Journal of Soil
888 Biology 44: 122–128.

889 Xia J, Sinelnikov I, Han B, Wishart DS (2015) MetaboAnalyst 3.0 - making metabolomics
890 more meaningful. Nucleic Acids Research 43: W251-257.
891 Zhao J, Last RL, Williams CC(1998) Induction of Arabidopsis tryptophan pathway enzymes
892 and camalexin by amino acid starvation, oxidative stress, and an abiotic elicitor. Plant
893 Cell 10: 359–370.
894 Zhao Y. *et al*. (2015) A metabolomics study delineating geographical location-associated
895 primary metabolic changes in the leaves of growing tobacco plants by GC-MS and
896 CE-MS. Nature 9: 1–11.

897 FIGURE LEGENDS

898 **Figure 1** Total biomass (A), shoot dry weight (B), root dry
899 weight (C), and root to shoot dry weight ratio (D) on day 29 of
900 the experiment (n = 10 for all groups). Provenances are:
901 Rothiemurchus, Scotland (RM), Pernitz, Austria (PER), Jarocin,
902 Poland (JAR), and Sierra Nevada, Spain (SN). Boxplots
903 represent the median of the data and the lower and upper
904 quartiles (25% and 75%). Whiskers represent the most extreme
905 data point that is no more than 1.5 times the interquartile range
906 from the box. Control treatment is grey while drought treatment
907 is black. Different letters indicate significant differences
908 ($p<0.05$).

909 **Figure 2 Figure 2A)** Mean ± standard errors for principle
910 component 1 and **B)** principle component 2 for different
911 provenances at different time points in the drought experiment
912 (t1=0, t2=11 and t3=29 days). These two components together
913 explain 20% of the variation in the metabolomics data. Drought
914 treatment is indicated by the dashed lines. Provenances are
915 colour coded as follows: blue – Rothiemurchus; orange –
916 Pernitz; green – Jarocin; red – Sierra Nevada. **C)** Schematic
917 showing the number of metabolites (out of 4640) that differ
918 significantly in abundance between given time points and
919 treatments. The thickness of the arrows between ellipses reflects
920 the level of the differences in metabolic composition between
921 treatments and time points.

922 **Figure 3** Volcano plot of the metabolite changes at time point 3
923 (day 29) after imposing a severe drought. Metabolites are plotted
924 as a function of the $\log_2$(fold change) from control and as –
925 $\log_{10}$(P value) of the change from control. Thus, positive values
926 for fold change indicate increased abundance under drought and
927 negative values indicated decreased abundance. Metabolites that
928 showed a log2(fold change) smaller than +- 1 and a q-value
929 smaller than 0.05 were selected for further scrutinity and

930 identification (colored in red). Non-selected metabolites are
931 coloured grey. Note that the volcano plot displays the raw,
932 uncorrected, p-values. The horizontal dashed line shows the
933 FDR-corrected critical p-value ($q < 0,05$), i.e. all points with p-
934 values smaller than the critical value (all points above the line)
935 are the features for which the null hypothesis of no difference
936 was rejected. The plot is partially annotated for the compounds
937 showing the most pronounced and most significant changes
938 immediately prior to the drought-induced mortality. Legend:
939 Trp, tryptophan; Tyr, tyrosine; Umb, umbelliferone; Gluc,
940 dihydrozeatin riboside glucoside; Pro, proline; Phe,
941 phenylalanine; Skat, skatole; Ser, serine; Jasm, jasmonate; Leu,
942 leucine; Leu-Gly-Pro, leucine, glycine, proline; Gib,
943 gibberelline; Styr, styrene; Pyr, pyrole; Spong,
944 spongipregnoloside; Orien, iso-orientin; Ecdys,
945 dehydroecdysone; Thap, thapsigorgin; Osc, oscillatoxin; Spin,
946 gentibioside spinosin; Cort, hydrocortisone caproate; Purp,
947 purpurescin; Vit D, vitamine D; Acr, Acronycine.

948

949

950

951

952