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### ► To cite this version:

Alexandru Milcu, Ruben Puga-Freitas, Aaron M Ellison, Manuel Blouin, Stefan Scheu, et al.. Genotypic variability enhances the reproducibility of an ecological study. *Nature Ecology & Evolution*, 2018, 2 (2), pp.279 - 287. 10.1038/s41559-017-0434-x . hal-01749308

**HAL Id: hal-01749308**

**<https://institut-agro-dijon.hal.science/hal-01749308>**

Submitted on 8 Dec 2023

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# *Genotypic variability enhances the reproducibility of an ecological study*

Article

Accepted Version

Milcu, Alexandru, Puga-Freitas, Ruben, Ellison, Aaron M., Blouin, Manuel, Scheu, Stefan, Freschet, Grégoire T., Rose, Laura, Barot, Sebastien, Cesarz, Simone, Eisenhauer, Nico, Girin, Thomas, Assandri, Davide, Bonkowski, Michael, Buchmann, Nina, Butenschoen, Olaf, Devidal, Sebastien, Gleoxner, Gerd, Gessler, Arthur, Gigon, Agnes, Greiner, Anna, Grignani, Carlo, Hansart, Amandine, Kayler, Zachary, Lange, Markus, Lata, Jean Christophe, Le Galliard, Jean Francois, Lukac, Martin ORCID logo ORCID: <https://orcid.org/0000-0002-8535-6334>, Mannerheim, Neringa, Muller, Marina E. H., Pando, Anne, Rotter, Paula, Scherer-Lorenzen, Michael, Seyhun, Rahme, Urban-Maed, Katherine, Weigelt, Alexandra, Zavattaro, Laura and Roy, Jacques (2018) Genotypic variability enhances the reproducibility of an ecological study. *Nature Ecology & Evolution*, 2 (2). pp. 279-287. ISSN 2397-334X doi: <https://doi.org/10.1038/s41559-017-0434-x> Available at <https://centaur.reading.ac.uk/74258/>

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To link to this article DOI: <http://dx.doi.org/10.1038/s41559-017-0434-x>

Publisher: Nature

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1 **Genotypic variability enhances the reproducibility of an ecological study**

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58 **Many scientific disciplines are currently experiencing a “reproducibility crisis” because**  
59 **numerous scientific findings cannot be repeated consistently. A novel but controversial**  
60 **hypothesis postulates that stringent levels of environmental and biotic standardization in**  
61 **experimental studies reduces reproducibility by amplifying impacts of lab-specific**  
62 **environmental factors not accounted for in study designs. A corollary to this hypothesis is**  
63 **that a deliberate introduction of controlled systematic variability (CSV) in experimental**  
64 **designs may lead to increased reproducibility. We tested this hypothesis using a multi-**  
65 **laboratory microcosm study in which the same ecological experiment was repeated in 14**  
66 **laboratories across Europe. Each laboratory introduced environmental and genotypic CSV**  
67 **within and among replicated microcosms established in either growth chambers (with**  
68 **stringent control of environmental conditions) or glasshouses (with more variable**  
69 **environmental conditions). The introduction of genotypic CSV led to lower among-**  
70 **laboratory variability in growth chambers, indicating increased reproducibility, but had no**  
71 **significant effect in glasshouses where reproducibility was generally lower. Environmental**  
72 **CSV had little effect on reproducibility. Although there are multiple causes for the**  
73 **“reproducibility crisis”, deliberately including genetic variation may be a simple solution**  
74 **for increasing the reproducibility of ecological studies performed in controlled**  
75 **environments.**

76

77       Reproducibility—the ability to duplicate a study and its findings—is a defining feature of  
78 scientific research. In ecology, it is often argued that it is virtually impossible to accurately  
79 duplicate any single ecological experiment or observational study. The rationale is that the  
80 complex ecological interactions between the ever-changing environment and the extraordinary

81 diversity of biological systems exhibiting a wide range of plastic responses at different levels of  
82 biological organization make exact duplication unfeasible<sup>1,2</sup>. Although this may be true for  
83 observational and field studies, numerous ecological (and agronomic) studies are carried out with  
84 artificially assembled simplified ecosystems and controlled environmental conditions in  
85 experimental microcosms or mesocosms (henceforth, “microcosms”)<sup>3–5</sup>. Since biotic and  
86 environmental parameters can be tightly controlled in microcosms, results from such studies  
87 should be easier to reproduce. Even though microcosms have frequently been used to address  
88 fundamental ecological questions<sup>4,6,7</sup>, there has been no quantitative assessment of the  
89 reproducibility of any microcosm experiment.

90       Experimental standardization—the implementation of strictly defined and controlled  
91 properties of organisms and their environment—is widely thought to increase both  
92 reproducibility and sensitivity of statistical tests<sup>8,9</sup> because it reduces within-treatment  
93 variability. This paradigm has been recently challenged by several studies on animal behavior,  
94 suggesting that stringent standardization may, counterintuitively, be responsible for generating  
95 non-reproducible results<sup>9–11</sup> and contribute to the actual reproducibility crisis<sup>12–15</sup>; the results  
96 may be valid under given conditions (i.e., they are local “truths”) but are not generalizable<sup>8,16</sup>.  
97 Despite rigorous adherence to experimental protocols, laboratories inherently vary in many  
98 conditions that are not measured and are thus unaccounted for, such as experimenter, micro-scale  
99 environmental heterogeneity, physico-chemical properties of reagents and lab-ware, pre-  
100 experimental conditioning of organisms, and their genetic and epigenetic background. It even has  
101 been suggested that attempts to stringently control all sources of biological and environmental  
102 variation might inadvertently lead to the amplification of the effects of these unmeasured  
103 variations among laboratories, thus reducing reproducibility<sup>9–11</sup>.

104           Some studies have gone even further, hypothesizing that the introduction of controlled  
105 systematic variation (CSV) among the replicates of a treatment (e.g., using different genotypes or  
106 varying the organisms' pre-experimental conditions among the experimental replicates) should  
107 lead to less variable mean response values between the laboratories that duplicate the  
108 experiments<sup>9,11</sup>. In short, it has been argued that reproducibility may be improved by shifting the  
109 variance from among experiments to within them<sup>9</sup>. If true, then introducing CSV will increase  
110 researchers' ability to draw generalizable conclusions about the directions and effect sizes of  
111 experimental treatments and reduce the probability of false positives. The trade-off inherent to  
112 this approach is that increasing within-experiment variability will reduce the sensitivity (i.e. the  
113 probability of detecting true positives) of statistical tests. However, it currently remains unclear  
114 whether introducing CSV increases reproducibility of ecological microcosm experiments, and if  
115 so, at what cost for the sensitivity of statistical tests.

116           To test the hypothesis that introducing CSV enhances reproducibility in an ecological  
117 context, we had 14 European laboratories simultaneously run a simple microcosm experiment  
118 using grass (*Brachypodium distachyon* L.) monocultures and grass and legume (*Medicago*  
119 *truncatula* Gaertn.) mixtures. As part of the reproducibility experiment, the 14 laboratories  
120 independently tested the hypothesis that the presence of the legume species *M. truncatula* in  
121 mixtures would lead to higher total plant productivity in the microcosms and enhanced growth of  
122 the non-legume *B. distachyon* via rhizobia-mediated nitrogen fertilization and/or nitrogen  
123 sparing effects<sup>17-19</sup>.

124           All laboratories were provided with the same experimental protocol, seed stock from the  
125 same batch, and identical containers in which to establish microcosms with grass only and grass-  
126 legume mixtures. Alongside a control (CTR) with no CSV and containing a homogenized soil



127 substrate (mixture of soil and sand) and a single genotype of each plant species, we explored the  
128 effects of five different types of within- and among-microcosm CSV on experimental  
129 reproducibility of the legume effect (Fig. 1): 1) within-microcosm environmental CSV ( $ENV_W$ )  
130 achieved by spatially varying soil resource distribution through the introduction of six sand  
131 patches into the soil; 2) among-microcosm environmental CSV ( $ENV_A$ ), which varied the  
132 number of sand patches (none, three, or six) among replicate microcosms; 3) within-microcosm  
133 genotypic CSV ( $GEN_W$ ) that used three distinct genotypes per species planted in homogenized  
134 soil in each microcosm; 4) among-microcosm genotypic CSV ( $GEN_A$ ) that varied the number of  
135 genotypes (one, two, or three) planted in homogenized soil among replicate microcosms; and 5)  
136 both genotypic and environmental CSV ( $GEN_W+ENV_W$ ) within microcosms that used six sand  
137 patches and three plant genotypes per species in each microcosm. In addition, we tested whether  
138 CSV effects are modified by the level of standardization within laboratories by using two  
139 common experimental approaches ('SETUP' hereafter): growth chambers with tightly controlled  
140 environmental conditions and identical soil (eight laboratories) or glasshouses with more loosely  
141 controlled environmental conditions and different soils (six laboratories; see Supplementary  
142 Table 1 for the physico-chemical properties of the soils).

143 We measured 12 parameters representing a typical ensemble of response variables reported  
144 for plant-soil microcosm experiments. Six of these were measured at the microcosm-level: shoot  
145 biomass, root biomass, total biomass, shoot-to-root ratio, evapotranspiration, and decomposition  
146 of a common substrate using a simplified version of the "teabag litter decomposition method"<sup>20</sup>.  
147 The other six were measured on *B. distachyon* alone: seed biomass, height, and four shoot-tissue  
148 chemical variables; N%, C%,  $\delta^{15}N$ ,  $\delta^{13}C$ . All 12 variables were then used to calculate the effect  
149 of the presence of a nitrogen-fixing legume on ecosystem functions in grass-legume mixtures

150 ('net legume effect' hereafter) (Supplementary Table 2), calculated as the difference between the  
151 values measured in the microcosms with and without legumes, an approach often used in  
152 legume-grass binary cropping systems<sup>19,21</sup> and biodiversity-ecosystem function experiments<sup>17,22</sup>.

153 Statistically significant differences among the 14 laboratories were considered an indication  
154 of irreproducibility. In the first instance, we assessed how our experimental treatments (CSV and  
155 SETUP) affected the number of laboratories that produced results that could be considered to  
156 have reproduced the same finding. We then determined how experimental treatments affected  
157 standard deviation (SD) of the legume effect for each of the 12 variables both within- and  
158 among-laboratories; lower among-laboratory SD implies that the results were more similar,  
159 suggesting increased reproducibility. Lastly, we explored the relationship between within- and  
160 among-laboratory SD, and how the experimental treatments affected the statistical power of  
161 detecting the net legume effect.

162 Although each laboratory followed the same experimental protocol, we found a remarkably  
163 high level of among-laboratory variation for most response variables (Supplementary Fig. 1) and  
164 the net legume effect on those variables (Fig. 2). For example, the net legume effect on mean  
165 total plant biomass varied among laboratories from 1.31 to 6.72 g dry weight (DW) per  
166 microcosm in growth chambers, suggesting that unmeasured laboratory-specific conditions  
167 outweighed effects of experimental standardization. Among glasshouses, differences were even  
168 larger: the net legume effect on mean plant biomass varied by two orders of magnitude, from  
169 0.14 to 14.57g DW per microcosm (Fig. 2). Furthermore, for half of the variables (root biomass,  
170 litter decomposition, grass height, foliar C%,  $\delta^{15}\text{C}$  and  $\delta^{15}\text{N}$ ) the direction of the net legume  
171 effect varied with laboratory.

172 Mixed-effects models were used to test the effect of legume species presence (LEG),  
173 laboratory (LAB), CSV, and their interactions (with experimental block—within-LAB growth  
174 chamber or glasshouse bench—as a random factor) on the 12 response variables. The impact of  
175 the presence of legumes varied significantly with laboratory and CSV for half of the variables, as  
176 indicated by the LEG×LAB×CSV three-way interaction (Table 1, Supplementary Figs 2 and 3).  
177 For the other half, significant two-way interactions between LEG×LAB and CSV×LAB were  
178 found. The same significant interactions were found when analyzing the first (PC1) and second  
179 (PC2) principal components from a principal component analysis (PCA) that included all 12  
180 response variables; PC1 and PC2 together explained 45% of the variation (Table 1;  
181 Supplementary Fig. 4ab). Taken together, these results suggest that the effect size or direction of  
182 the net legume effect was significantly different (i.e. not reproducible) in some laboratories and  
183 that the introduced CSV treatment affected reproducibility. In a complementary analysis  
184 including the SETUP in the model (and accounting for the LAB effect as a random factor), we  
185 found that the impact of the CSV treatment varied significantly with the SETUP (CSV×SETUP  
186 or LEG×CSV×SETUP interactions; Supplementary Table 3), suggesting the reproducibility of  
187 the results differed between glasshouses and growth chambers.

188 To answer the question of how many laboratories produced results that were statistically  
189 indistinguishable from one another (i.e. reproduced the same finding), we used Tukey's post-hoc  
190 Honest Significant Difference (HSD) test for the LAB effect on the first and second principal  
191 components describing the net legume effect, which together explained 49% of the variation  
192 (Supplementary Fig. 4cd). Out of the 14 laboratories, seven (PC1) and 11 (PC2) laboratories  
193 were statistically indistinguishable in controls; this value increased in the treatments with  
194 environmental or genotypic CSV for PC1 but not PC2 (Table 2). When we analyzed responses in

195 growth chambers alone, five of eight laboratories were statistically indistinguishable in controls,  
196 but this increased to six out of eight laboratories when we considered treatments with only  
197 environmental CSV and seven of eight in treatments with genotypic CSV ( $GEN_W$ ,  $GEN_A$  and  
198  $GEN_W+ENV_W$ ). In glasshouses, introducing CSV did not affect the number of statistically  
199 indistinguishable laboratories with respect to PC1 but decreased the number of statistically  
200 indistinguishable laboratories with respect to PC2 (Table 2).

201 We also assessed the impact of the experimental treatments on the among- and within-  
202 laboratory SD. Analysis of the among-laboratory SD of the net legume effect revealed a  
203 significant  $CSV \times SETUP$  interaction ( $F_{5,121}=7.38$ ,  $P < 0.001$ ) (Fig. 3a, b). This interaction  
204 included significantly lower fitted coefficients (i.e., lower among-laboratory SD) in growth  
205 chambers for  $GEN_W$  ( $t_{5,121} = -3.37$ ,  $P = 0.001$ ),  $GEN_A$  ( $t_{5,121} = -2.95$ ,  $P = 0.004$ ) and  
206  $ENV_W+GEN_W$  ( $t_{1,121} = -3.73$ ,  $P < 0.001$ ) treatments relative to CTR (see full model output for  
207 among-laboratory SD in Supplementary Note). For these three treatments, the among-laboratory  
208 SD of the net legume effect was 18% lower with genotypic CSV than without it, indicating  
209 increased reproducibility (Fig. 3a). The same analysis performed on within-laboratory SD of the  
210 net legume effect only found a slight but significant increase of within-laboratory SD in the  
211  $GEN_A$  treatment ( $t_{5,121} = 3.52$ ,  $P < 0.001$ ) (see model output for within-laboratory SD in  
212 Supplementary Note). We then tested whether there was a relationship between within- and  
213 among-laboratory SD with a statistical model for among-laboratory SD as a function of within-  
214 laboratory SD, SETUP, CSV and their interactions. We found a significant within-laboratory  
215  $SD \times SETUP \times CSV$  three-way interaction ( $F_{5,109} = 2.4$ ,  $P < 0.040$ ) affecting among-laboratory SD  
216 (Supplementary Note). This interaction was the result of a more negative relationship between

217 within- and among-laboratory SD in glasshouses relative to growth chambers, but with different  
218 slopes for the different CSV treatments (Fig. 4).

219 Introducing CSV can increase within-laboratory variation, as indicated by the positive  
220 coefficients fitted in some of the CSV treatments in the model output for within-laboratory SD  
221 (see Supplementary Note). Thus, for the three CSV treatments that produced the most consistent  
222 results ( $GEN_W$ ,  $GEN_A$ ,  $ENV_W+GEN_W$ ), we analyzed the statistical power of detecting the net  
223 legume effect within individual laboratories. In growth chambers, adding genotypic CSV led to a  
224 slight reduction in statistical power relative to CTR (57% in CTR vs. 46% in the three treatments  
225 containing genotypic variability) that could have been compensated for by using eleven instead  
226 of six replicated microcosms per treatment. In glasshouses, owing to a higher effect size of  
227 legume presence on the response variables, the statistical power for detecting the legume effect  
228 in CTR was slightly higher (68%) than in growth chambers, but was reduced to 51% on average  
229 for the three treatments containing genotypic CSV, a decrease that could have been compensated  
230 for by using 16 replicated microcosms instead of six.

231 Overall, our study shows that results produced by microcosm experiments can be strongly  
232 biased by lab-specific factors. Based on the principal component explaining most of the variation  
233 in the twelve response variables (PC1), only seven out of the 14 laboratories produced results  
234 that can be considered reproducible (Table 2) with the current standardization procedures. This  
235 result is in line with the only other comparable study<sup>12</sup> (to the best of our knowledge) reporting  
236 that out of ten laboratories, only four generated similar leaf growth phenotypes of *Arabidopsis*  
237 *thaliana* (L). In addition to highlighting that approximately one in two ecological studies  
238 performed in microcosms under controlled environments produce statistically different results,  
239 our study provides supporting evidence for the hypothesis that introducing genotypic CSV can

240 increase reproducibility of ecological studies<sup>9–11</sup>. However, the effectiveness of genotypic CSV  
241 for enhancing reproducibility varied with the setup; it led to lower (–18%) among-laboratory SD  
242 in growth chambers only, with no benefit observed in glasshouses. Lower among-laboratory SD  
243 in growth chambers implies that the microcosms containing genotypic CSV were less strongly  
244 affected by unaccounted-for lab-specific environmental or biotic variables. Analyses performed  
245 at the level of individual variables (Table 1) showed that introducing genotypic CSV affected the  
246 among-laboratory SD in most, but not all variables. This suggests that the relationship between  
247 genotypic CSV and reproducibility is probabilistic and results from the decreased likelihood that  
248 microcosms containing CSV will respond to unaccounted for lab-specific environmental factors  
249 in the same direction and with the same magnitude. The mechanism is likely to be analogous to  
250 the stabilizing effect of biodiversity on ecosystem functions under changing environmental  
251 conditions<sup>23–26</sup>, but additional empirical evidence is needed to confirm this conjecture.

252 Introducing genotypic CSV increased reproducibility in growth chambers (with stringent  
253 control of environmental conditions) but not in glasshouses (with more variable environmental  
254 conditions). Higher among-laboratory SD in glasshouses may indicate the existence therein of  
255 stronger laboratory-specific factors, and our deliberate use of different soils in the glasshouses  
256 presumably contributed to this effect. However, the among-laboratory SD in glasshouses  
257 decreased with increasing within-laboratory SD, irrespective of CSV, an effect that was less  
258 clear in growth chambers (Fig. 4). This observation appears to be in line with the hypothesis put  
259 forward by Richter et al.<sup>9</sup>, who proposed that increasing the variance within experiments can  
260 reduce the among-laboratory variability of the mean effect sizes observed in each laboratory.  
261 Yet, despite the negative correlation between within- and among-laboratory SD observed in  
262 glasshouses, the among-laboratory SD remained higher in glasshouses than in growth chambers.

263 Therefore, we consider that the hypothesized mechanistic link between CSV-induced higher  
264 within-laboratory SD and increased reproducibility is poorly supported by our dataset.  
265 Nevertheless, one possible explanation for the lack of effect on reproducibility in glasshouses is  
266 that our CSV treatments did not introduce a sufficiently high level of within-laboratory  
267 variability to buffer against laboratory-specific factors for all response variables; across the  
268 twelve response variables, the average main effect (i.e., without the interaction terms) of the  
269 CSV treatment contributed to a low percentage ( $2.6\% \pm 1.6$  s.e.m.) of the total sum of squares  
270 relative to the main effects of laboratory ( $43.4\% \pm 5.2$  s.e.m.) and legumes ( $10.9\% \pm 3.1$  s.e.m.).  
271 A similar conjecture was put forward by the other two studies that explored the role of CSV for  
272 reproducibility in animal behavior<sup>9,10</sup>. At present we are unable to conclude that the introduction  
273 of stronger sources of controlled within-laboratory variability can increase reproducibility in  
274 glasshouses with more loosely controlled environmental conditions and different soils.

275 Our results indicate that genotypic CSV is more effective in increasing reproducibility than  
276 environmental CSV, irrespective of whether the CSV was introduced within or among individual  
277 replicates (i.e., microcosms). However, we cannot discount the possibility that we found this  
278 result because our treatments with environmental CSV were less successful in increasing within-  
279 microcosm variability. Additional experiments could test whether other types of environmental  
280 CSV, such as soil nutrients, texture, or water availability, might be more effective at increasing  
281 reproducibility.

282 We expected higher overall productivity (i.e., a net legume effect) in the grass-legume  
283 mixtures and enhanced growth of *B. distachyon* because of the presence of the nitrogen (N)-  
284 fixing *M. truncatula*. However, these species were not selected because of their routine pairings  
285 in agronomic or ecological experiments (they are rarely used that way), but rather because they

286 are frequently present in controlled environment experiments looking at functional genomics.  
287 Contrary to our expectation, and despite the generally lower  $^{15}\text{N}$  signature of *B. distachyon* in the  
288 presence of N-fixing *M. truncatula* (suggesting that some of the N fixed by *M. truncatula* was  
289 taken up by the grass), the biomass of *B. distachyon* was lower in the microcosms containing *M.*  
290 *truncatula*. Seed mass and shoot %N data of *B. distachyon* was lower in mixtures  
291 (Supplementary Fig. 1), suggesting that the two species competed for N. The lack of a significant  
292 N fertilization effect of *M. truncatula* on *B. distachyon* could have resulted from the  
293 asynchronous phenologies of the two species: the 8–10-week life cycle of *B. distachyon* may  
294 have been too short to benefit from the N fixation by *M. truncatula*.

295 Because well-established meta-analytical approaches can account for variation caused by  
296 local factors and still detect the general trends across different types of experimental setups,  
297 environments, and populations, we should ask whether the additional effort required for  
298 introducing CSV in experiments is worthwhile. Considering the current reproducibility crisis in  
299 many fields of science<sup>27</sup>, we suggest that it is, for at least three reasons. First, some studies  
300 become seminal without any attempts to reproduce them. Second, even if a seminal study that is  
301 flawed due to laboratory-specific biases is later proven wrong, it usually takes significant time  
302 and resources before its impact on the field abates. Third, the current rate of reproducibility is  
303 estimated to be as low as one-third<sup>12–14</sup>, implying that most data entering any meta-analysis are  
304 biased by unknown lab-specific factors. Addition of genotypic CSV may enhance the  
305 reproducibility of individual experiments and eliminate potential biases in data used in meta-  
306 analyses. Last, if each individual study is less affected by laboratory-specific unknown  
307 environmental and biotic factors, then we would also need fewer studies to draw solid  
308 conclusions about the generality of phenomena. Therefore, we argue that investing more in



309 making individual studies more reproducible and generalizable will be beneficial in both the  
310 short and long run. At the same time, adding CSV can reduce statistical power to detect  
311 experimental effects, so some additional experimental replicates would be needed when using it.

312 Arguably, our use of statistical significance tests of effects sizes to determine reproducibility  
313 might be viewed as overly restrictive and better suited to assessing reproducibility of parameter  
314 estimates rather than assessing the generality of the hypothesis under test<sup>27</sup>. We used this  
315 approach because no generally accepted alternative framework is available to assess how close  
316 the multivariate results from multiple laboratories need to be to conclude that they reproduced  
317 the same finding. It is worth noting that although the direction of the legume effect was the same  
318 in the majority of laboratories, the differences among laboratories were very large (e.g., up to  
319 two orders of magnitude for shoot biomass) and in 10% of the 168 laboratory  $\times$  variable  
320 combinations (14 laboratories  $\times$  12 response variables) the direction of the legume effect differed  
321 from the among-laboratory consensus (Fig. 2).

322 In conclusion, our study shows that the current standardization procedures used in ecological  
323 microcosm experiments are inadequate in accounting for lab-specific environmental factors and  
324 suggests that introducing controlled variability in experiments may buffer effects of lab-specific  
325 factors. Although there are multiple causes for the reproducibility crisis<sup>15,28,29</sup>, deliberately  
326 including genetic variation in the studied organisms can be a simple solution for increasing the  
327 reproducibility of ecological studies performed in controlled environments. However, as the  
328 introduced genotypic variability only increased reproducibility in experimental setups with  
329 tightly controlled environmental conditions (i.e., in growth chambers using identical soil), our  
330 study indicates that the reproducibility of ecological experiments can be enhanced by a

331 combination of rigorous standardization of environmental variables at the laboratory level as  
332 well as controlled genotypic variability.

333

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## 400 **Acknowledgements**

401 This study benefited from the CNRS human and technical resources allocated to the  
402 ECOTRONS Research Infrastructures and the state allocation 'Investissement d'Avenir' ANR-  
403 11-INBS-0001 and from financial support by the ExpeER (grant no. 262060) consortium funded  
404 under the EU-FP7 research program (FP2007-2013). *Brachypodium* seeds were kindly provided  
405 by Richard Sibout (Observatoire du Végétal, Institut Jean-Pierre Bourgin, F-78026 Versailles  
406 Cedex France) and *Medicago* seeds were supplied by Jean-Marie Prosperi (INRA Biological  
407 Resource Centre, F-34060 Montpellier Cedex 1, France). We further thank Jean Varale, Gesa  
408 Hoffmann, Paul Werthenbach, Oliver Ravel, Clement Piel and Damien Landais, David  
409 Degueldre, Thierry Mathieu, Pierrick Aury, Nicolas Barthès, Bruno Buatois, Raphaëlle Leclerc  
410 for assistance during the study. For additional acknowledgements see Supplementary Information.

## 411 **Author contributions**

412 A.M. and J.R. designed the study with input from M.B, S.B and J-C.L. Substantial methodological  
413 contributions were provided by M.B., S.S., T.G., L.R. and M.S-L. Conceptual feedback on an early  
414 version was provided by G.F., N.E., J.R. and A.M.E. Data were analysed by A.M. with input from  
415 A.M.E. A.M. wrote the manuscript with input from all co-authors. All co-authors were involved  
416 in carrying out the experiments and/or analyses.

## 417 **Author Information**

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421

## 422 **METHODS**

423 All laboratories tried to the best of their abilities to carry out an identical experimental protocol.  
424 Whereas not all laboratories managed to recreate precisely all details of the experimental  
425 protocol, we considered this to be a realistic scenario under which ecological experiments using  
426 microcosms are performed in glasshouses and growth chambers.

### 427 **Germination**

428 The seeds from the three genotypes of *Brachypodium distachyon* (Bd21, Bd21-3 and Bd3-1) and  
429 *Medicago truncatula* (L000738, L000530 and L000174) were first sterilized by soaking 100  
430 seeds in 100 mL of a sodium hypochlorite solution with 2.6% active chlorine, and stirred for 15  
431 min using a magnet. Thereafter, the seeds were rinsed 3 times in 250 mL of sterile water for 10-  
432 20 seconds under shaking. Sterilized seeds were germinated in trays (10 cm deep) filled with  
433 vermiculite. The trays were kept at 4°C in the dark for three days before being moved to light  
434 conditions (300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR) and 20/16°C and 60/70% air RH for day- and night-time,  
435 respectively. When the seedlings of both species reached 1 cm in height above the vermiculite,  
436 they were transplanted into the microcosms.

### 437 **Preparation of microcosms**

438 All laboratories used identical containers (2-liter volume, 14.8-cm diameter, 17.4-cm height).  
439 Sand patches were created using custom-made identical “patch makers” consisting of six rigid  
440 PVC tubes (2.5 cm in diameter and 25 cm long), arranged in a circular pattern with an outer  
441 diameter of 10 cm. A textile mesh was placed at the bottom of the containers to prevent the  
442 spilling of soil through drainage holes. Filling of microcosms containing sand patches started  
443 with the insertion of the empty tubes into the containers. Thereafter, in growth chambers, 2000-g  
444 dry-weight of soil, subtracting the weight of the sand patches, was added into the containers and

445 around the “patch maker” tubes. Because different soils were used in the glasshouses, the dry  
446 weight of the soil differed depending on the soil density and was first estimated individually in  
447 each laboratory as the amount of soil needed to fill the pots up to 2 cm from the top. After the  
448 soil was added to the containers, the tubes were filled with a mixture of 10% soil and 90% sand.  
449 When the microcosms did not contain sand patches, the amount of sand otherwise contained in  
450 the six patches was homogenized with the soil. During the filling of the microcosms, a common  
451 substrate for measuring litter decomposition was inserted at the center of the microcosm at 8 cm  
452 depth. For simplicity as well as for its fast decomposition rate, we used a single batch of  
453 commercially available tetrahedron-shaped synthetic tea bags (mesh size of 0.25 mm) containing  
454 2 g of green tea (Lipton, Unilever), as proposed by the “tea bag index” method<sup>20</sup>. Once filled, the  
455 microcosms were watered until water could be seen pouring out of the pot. The seedlings were  
456 then manually transplanted to predetermined positions (Fig. 1), depending on the genotype and  
457 treatment. Each laboratory established two blocks of 36 microcosms each, resulting in a total of  
458 72 microcosms per laboratory, with blocks representing two distinct chambers in growth  
459 chamber setups or two distinct growth benches in the same glasshouse.

#### 460 **Soils**

461 All laboratories using growth chamber setups used the same soil, whereas the laboratories using  
462 glasshouses used different soils (see Supplementary Table 1 for the physicochemical properties  
463 of the soils). The soil used in growth chambers was classified as a nutrient-poor cambisol and  
464 was collected from the top layer (0–20 cm) of a natural meadow at the Centre de Recherche en  
465 Ecologie Expérimentale et Prédictive—CEREEP (Saint-Pierre-Lès-Nemours, France). Soils used  
466 in glasshouses originated from different locations. The soil used by laboratory L2 was a fluvisol  
467 collected from the top layer (0–40 cm) of a quarry site near Avignon, in the Rhône valley,

468 Southern France. The soil used by laboratory L4 was collected from near the La Cage field  
469 experimental system (Versailles, France) and was classified as a luvisol. The soil used by labs  
470 L11 and L12 was collected from the top layer (0-20cm) within the haugh of the river Dreisam in  
471 the East of Freiburg, Germany. This soil was classified as an umbric gleysol with high organic  
472 carbon content. The soil from laboratory L14 was classified as a eutric fluvisol and was collected  
473 on the field site of the Jena Experiment, Germany. Prior to the establishment of microcosms, all  
474 soils were air-dried at room temperature for several weeks and sieved with a 2-mm mesh sieve.  
475 A common inoculum was provided to all laboratories to assure that rhizobia specific to *M.*  
476 *truncatula* were present in all soils.

#### 477 **Abiotic environmental conditions**

478 The set points for environmental conditions were 16 h light (at  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR) and 8 h  
479 dark, 20/16°C, 60/70% air RH for day- and night-time, respectively. Different soils (for  
480 glasshouses) and treatments with sand patches likely affected water drainage and  
481 evapotranspiration. The watering protocol was thus based on dry weight relative to weight at full  
482 water holding capacity (WHC). The WHC was estimated based on the weight difference between  
483 the dry weight of the containers and the wet weight of the containers 24 h after abundant  
484 watering (until water was flowing out of the drainage holes in the bottom of each container). Soil  
485 moisture was maintained between 60 and 80% of WHC (i.e. the containers were watered when  
486 the soil water dropped below 60% of WHC and water added to reach 80% of WHC) during the  
487 first 3 weeks after seedling transplantation and between 50 and 70% of WHC for the rest of the  
488 experiment. Microcosms were watered twice a week with estimated WHC values from two  
489 microcosms per treatment. To ensure that the patch/heterogeneity treatments did not become a  
490 water availability treatment, all containers were weighed and brought to 70 or 80% of WHC



491 every two weeks. This operation was synchronized with within-block randomization. All 14  
492 experiments were performed between October 2014 and March 2015.

### 493 **Sampling and analytical procedures**

494 After 80 days, all plants were harvested. Plant shoots were cut at the soil surface, separated by  
495 species, and dried at 60°C for three days. Roots and any remaining litter in the tea bags were  
496 washed out of the soil using a 1-mm mesh sieve and dried at 60°C for three days. Microcosm  
497 evapotranspiration rate was measured before the harvesting as the difference in weight changes  
498 from 70% of WHC after 48 h. Shoot C%, N%,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$  were measured on pooled shoot  
499 biomass (including seeds) of *B. distachyon* and analyzed at the Göttingen Centre for Isotope  
500 Research and Analysis using a coupled system consisting of an elemental analyzer (NA 1500,  
501 Carlo Erba, Milan, Italy) and a gas isotope mass spectrometer (MAT 251, Finnigan, Thermo  
502 Electron Corporation, Waltham, Massachusetts, USA).

### 503 **Data analysis and statistics**

504 All analyses were done using R version 3.2.4<sup>29</sup>. Prior to data analyses, each laboratory was  
505 screened individually for outliers. Values that were lower or higher than  $1.5 \times \text{IQR}$  (interquartile  
506 range)<sup>30</sup> within each laboratory, and representing less than 1.7% of the whole dataset, were  
507 considered to be outliers due to measurement errors or typos. These values were removed and  
508 subsequently treated as missing values. We then assessed whether the impact of the presence of  
509 legume (LEG) varied with laboratory (LAB) and the treatment of controlled systematic  
510 variability (CSV). This was tested individually for each response variable (Table 1) with a  
511 mixed-effects model using the “nlme” package<sup>31</sup>. Following the guidelines suggested by Zuur et  
512 al. (2009)<sup>32</sup>, we first identified the most appropriate random structure using a restricted  
513 maximum likelihood (REML) approach and selected the random structure with the lowest

514 Akaike information criterion (AIC). For this model, CSV and LAB were included as fix factors,  
515 experimental block as a random factor, and a “varIdent” weighting function to correct for  
516 heteroscedasticity resulting from more heteroscedastic data at the LAB and LEG level (R syntax:  
517 “model= lme (response variable ~ LEG\*CSV\*LAB, random=~1|block, weights=varIdent (form  
518 = ~1|LAB\*LEG)”) (Table 2). As the LAB and SETUP experimental factors were not fully  
519 crossed (i.e. laboratories performed the experiment only in one type of setup), the two  
520 experimental variables could not be included simultaneously as fixed effects. Therefore, to test  
521 for the SETUP effect, we used an additional complementary model including CSV and SETUP  
522 as fix effects and laboratory as a random factor (R syntax: “model= lme (response variable ~  
523 LEG\*CSV\*SETUP, random=~1|LAB/block, weights=varIdent (form = ~1|LAB\*LEG)”)  
524 (Supplementary Table 3). To test whether the results were affected by the collinearity among the  
525 response variables, the two models also were run on the first (PC1) and second (PC2) principal  
526 components the 12 response variables (Fig. 4ab). PCs were estimated using the “FactoMineR”  
527 package<sup>33</sup>, with missing values replaced using a regularized iterative multiple correspondence  
528 analysis<sup>34</sup> in the “missMDA” package<sup>35</sup>. The same methodology was used to compute a second  
529 PCA derived from the net legume effect on the 12 response variables (Supplementary Fig. 4cd).  
530 To assess how many laboratories produced results that were statistically indistinguishable from  
531 one another, we applied Tukey’s post-hoc HSD test in the “multcomp” package to lab-specific  
532 estimates of PC1 and PC2 (Table 2).

533 To assess how the CSV treatments affected the among- and within-laboratory variability,  
534 we used the standard deviation (SD) instead of the coefficient of variation, because the net  
535 legume effect contained both positive and negative values. To calculate among- and within-  
536 laboratory SDs, we centered and scaled the raw values using the z-score normalization [z-scored

537 variable = (raw value–mean)/SD] individually for each of the 12 response variables. Among-  
538 laboratory SD was computed from the mean of the laboratory z-scores for each response  
539 variable, CSV, and SETUP treatments (n = 144; 6 CSV levels × 2 SETUP levels × 12 response  
540 variables). Within-laboratory SDs were computed from the values measured in the six replicated  
541 microcosms for each CSV and SETUP treatment combination, individually for each response  
542 variable, resulting in a dataset with the same structure as for among-laboratory SDs (n = 144; 6  
543 CSV levels × 2 SETUP levels × 12 response variables). Some of the 12 response variables were  
544 intrinsically correlated, but most had correlation coefficients < 0.5 (Supplementary Fig. 5) and  
545 were therefore treated as independent variables. To analyze and visualize the relationships  
546 between the SDs calculated from variables with different units, before the calculation of the  
547 among- and within-laboratory SD, the raw values of the 12 response variables were centered and  
548 scaled.

549       The impact of experimental treatments on among- and within-laboratory SD was analyzed  
550 using mixed-effect models, following the same procedure described for the individual response  
551 variables. The model with the lowest AIC included a random slope for the SETUP within each  
552 response variable as well as a “varIdent” weighting function to correct for heteroscedasticity at  
553 the variable level (R syntax: “model= lme (SD ~ CSV\*SETUP, random=~SETUP|variable,  
554 weights=varIdent (form = ~1|variable)) (see also Supplementary Notes). The relationship  
555 between within- and among-laboratory SD also was tested with a model with similar random  
556 structure but with among-laboratory SD as a dependent variable and within-laboratory SD, CSV,  
557 and SETUP as predictors.

558       Because the treatments containing genotypic CSV increased reproducibility in growth  
559 chambers, but slightly increased within-laboratory SD, we also examined the effect of adding

560 CSV on the statistical power for detecting the net legume effect in each individual laboratory.  
561 This analysis was done with the “power.anova.test” function in the “base” package. We  
562 computed the statistical power of detecting a significant net legume effect (if one had used a one-  
563 way ANOVA for the legume treatment) for CTR, GEN<sub>W</sub>, GEN<sub>A</sub> and ENV<sub>W</sub>+GEN<sub>W</sub> treatments  
564 for each laboratory and response variable. This allowed us to calculate the average statistical  
565 power for the aforementioned treatments and how many additional replicates would have been  
566 needed to achieve the same statistical power as we had in the CTR.  
567 The data that support the findings of this study are publicly available at  
568 <https://doi.pangaea.de/10.1594/PANGAEA.880980>

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584 **Table 1 | Impact of experimental treatments on response variables.** Mixed-effects model outputs summarizing the F- and P-values  
 585 (as asterisks) for the impacts of the presence of legumes (LEG), controlled systematic variability (CSV) and laboratory (LAB) on the  
 586 12 response variables. We also present the impact of experimental treatments on the first and second principal components (PC1 and  
 587 PC2) of all 12 response variables. The response variables we measured are a typical ensemble of variables measured in plant-soil  
 588 microcosm experiments (BM = biomass). † symbol indicates response variables measured for the grass *B. distachyon* only, whereas  
 589 the rest of the variables were measured at the microcosm level, i.e. including the contribution of both the legume and the grass species.  
 590 Asterisks indicate the significance levels (\*\*\*) for  $P < 0.001$ ; \*\* for  $P < 0.01$ ; \* for  $P < 0.05$ ; + for  $P < 0.1$ ; ns for  $P > 0.1$ ). DF =  
 591 numerator degrees of freedom.  
 592  
 593

	DF	Shoot BM	Root BM	Seed BM†	Total BM	Shoot/Root	Grass height†	Shoot N%†
LEG	1	4602.95 (***)	1131.65 (***)	2186.64 (***)	690.73 (***)	1137.01 (***)	3.33 (+)	449.87 (***)
CSV	5	15.57 (***)	23.93 (***)	58.01 (***)	1.78 (ns.)	23.98 (***)	23.36 (***)	0.78 (ns.)
LAB	13	1088.67 (***)	182.53 (***)	364.57 (***)	1251.96 (***)	183.42 (***)	317.33 (***)	335.18 (***)
LEG×CSV	5	23.64 (***)	4.48 (***)	33.62 (***)	3.49 (**)	4.51 (***)	2.62 (*)	1.34 (ns)
LEG×LAB	13	235.99 (***)	40.58 (***)	78.17 (***)	116.63 (***)	40.38 (***)	49.89 (***)	14.12 (***)
CSV×LAB	65	6.55 (***)	3.15 (***)	6.93 (***)	7.33 (***)	3.17 (***)	10.16 (***)	1.98 (***)
LEG×LAB×CSV	65	2.22 (***)	1.12 (ns.)	2.70 (***)	1.18 (ns.)	1.12 (ns.)	1.45 (*)	1.71 (***)
		n = 1005	n = 989	n = 997	n = 976	n = 987	n = 1008	n = 1008
	DF	Shoot C%†	Shoot $\delta^{15}\text{N}^\dagger$	Shoot $\delta^{13}\text{C}^\dagger$	ET	Litter	PC1	PC2
LEG	1	110.67 (***)	14.43 (***)	26.62 (***)	1269.93 (***)	1.81 (ns.)	1242.53 (***)	988.88 (***)
CSV	5	0.16 (ns.)	8.85 (***)	75.73 (***)	9.37 (***)	1.05 (ns.)	12.87 (***)	22.56 (***)

LAB	13	174.50 (***)	258.30 (***)	888.42 (***)	748.66 (***)	117.34 (***)	920.65 (***)	513.83 (***)
LEG×CSV	5	2.55 (*)	6.48 (***)	5.15 (***)	1.24 (ns.)	1.77 (ns.)	7.08 (***)	11.79 (***)
LEG×LAB	13	11.90 (***)	16.78 (***)	2.52 (**)	172.74 (***)	2.05 (*)	118.12 (***)	28.22 (***)
CSV×LAB	65	1.67 (**)	4.39 (***)	4.97 (***)	21.69 (***)	2.97 (***)	7.22 (***)	2.76 (***)
LEG×LAB×CSV	65	1.33 (*)	1.84 (***)	1.23 (ns.)	1.53 (**)	1.17 (ns.)	0.93 (ns.)	1.65 (**)
		n = 1008	n = 963	n = 973	n = 1002	n = 974	n = 1008	n = 1008

---

595 **Table 2 | Impact of experimental treatments on the number of laboratories that reproduced the**  
 596 **same finding.** Numbers represent the total number of statistically indistinguishable laboratories based  
 597 on a Tukey's post-hoc Honest Significant Difference test of the first (PC1) and second (PC2) principal  
 598 components of the net legume effect of the 12 response variables (see Supplementary Fig. 4cd for the  
 599 PCA results). For a detailed description of experimental treatments and abbreviations, see Fig. 1.  
 600

Source	All laboratories (n = 14)		Glasshouses (n = 6)		Growth chambers (n = 8)	
	PC1	PC2	PC1	PC2	PC1	PC2
CTR	7	11	3	5	5	5
ENV <sub>W</sub>	10	9	3	3	6	6
ENV <sub>A</sub>	8	8	3	4	6	6
GEN <sub>W</sub>	8	10	3	3	6	7
GEN <sub>A</sub>	11	10	3	3	7	8
ENV <sub>W</sub> +GEN <sub>W</sub>	11	10	4	3	7	7

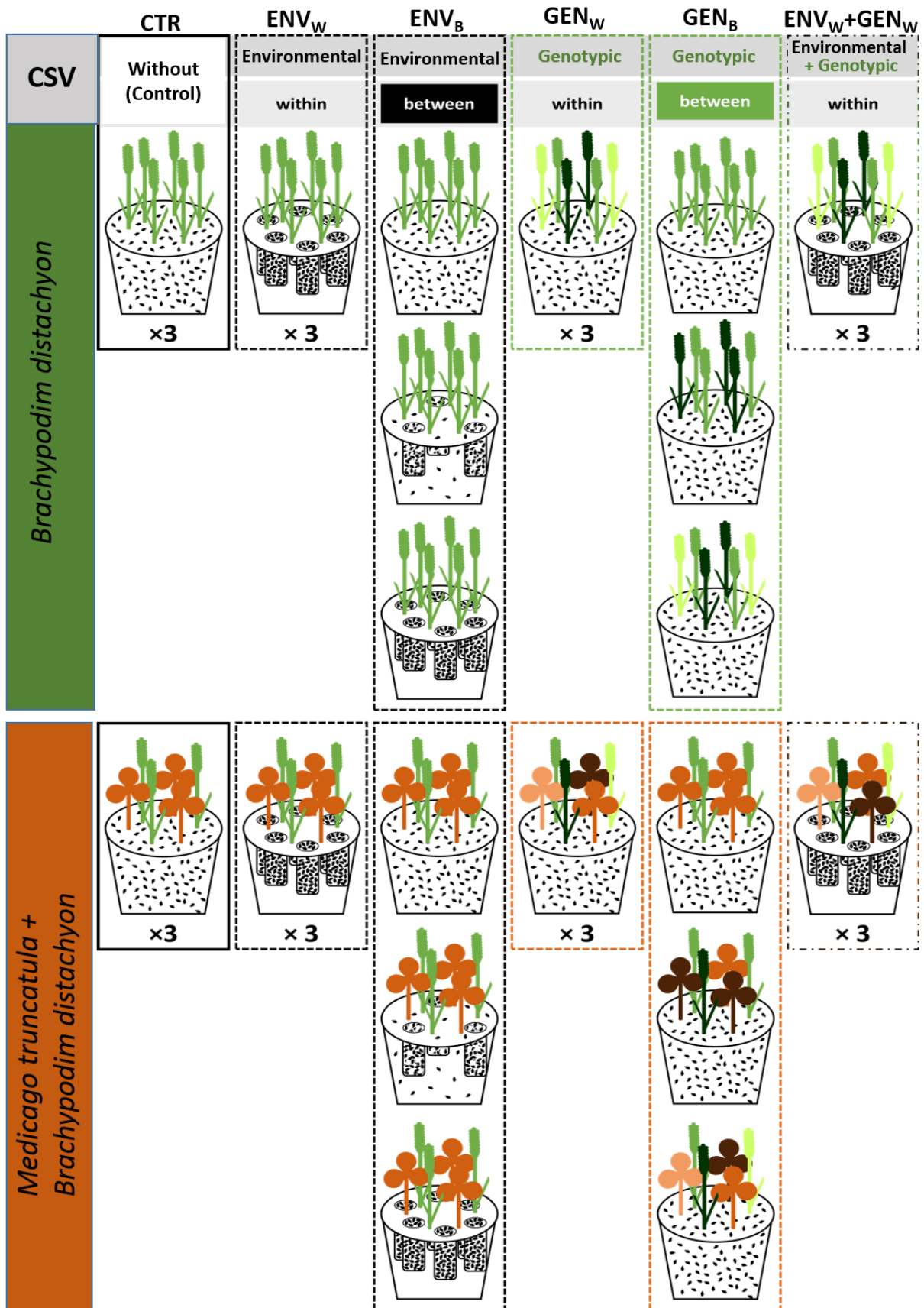
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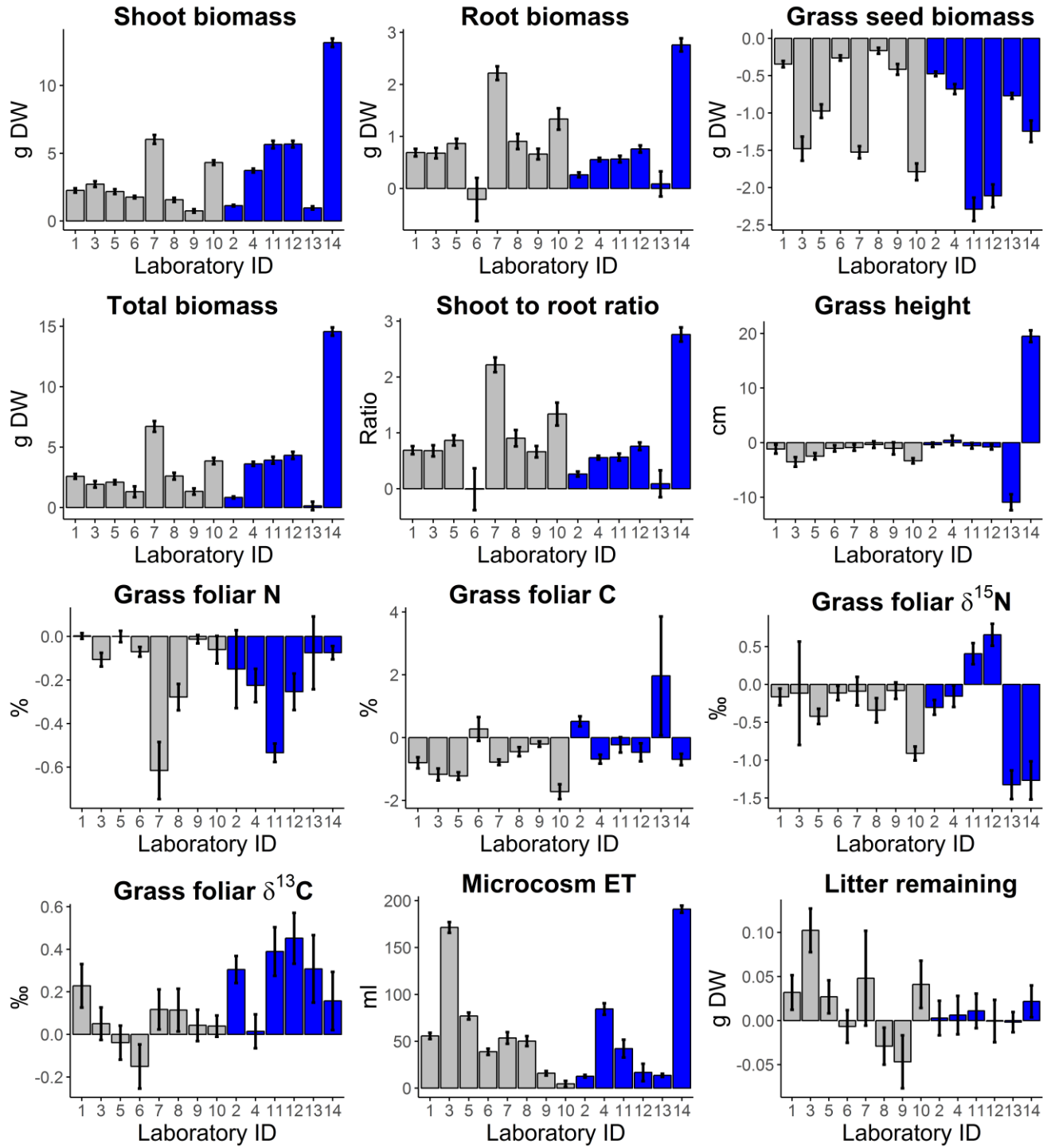
**Figure legends**

603 **Fig. 1 | Experimental design of one block.** Grass monocultures of *Brachypodium distachyon* (green  
604 shades) and grass-legume mixtures with the legume *Medicago truncatula* (orange-brown shades) were  
605 established in 14 laboratories; shades of green and orange-brown represent three distinct genotypes of  
606 *B. distachyon* (Bd21, Bd21-3 and Bd3-1) and *M. truncatula* (L000738, L000530 and L000174). Plants  
607 were established in a substrate with equal proportions of sand (black spots) and soil (white), with the  
608 sand being either mixed with the soil or concentrated in sand patches to induce environmental  
609 controlled systematic variability (CSV). Combinations of three distinct genotypes were used to  
610 establish genotypic CSV. Alongside a control (CTR) with no CSV and containing one genotype  
611 (L000738 and/or Bd21) in a homogenized substrate (soil-sand mixture), five different types of  
612 environmental or genotypic CSV were used as treatments: 1) within-microcosm environmental CSV  
613 (ENV<sub>W</sub>) achieved by spatially varying soil resource distribution through the introduction of six sand  
614 patches into the soil; 2) among-microcosm environmental CSV (ENV<sub>A</sub>), which varied the number of  
615 sand patches (none, three or six) among replicate microcosms; 3) within-microcosm genotypic CSV  
616 (GEN<sub>W</sub>) that used three distinct genotypes per species planted in homogenized soil in each microcosm;  
617 4) among-microcosm genotypic CSV (GEN<sub>A</sub>) that varied the number of genotypes (one, two or three)  
618 planted in homogenized soil among replicate microcosms; and 5) both genotypic and environmental  
619 CSV (GEN<sub>W</sub>+ENV<sub>W</sub>) within microcosms that used six sand patches and three plant genotypes per  
620 species in each microcosm. The “× 3” indicates that the same genotypic and sand composition was  
621 repeated in three microcosms per block. The spatial arrangement of the microcosms in each block was  
622 re-randomized every two weeks. The blocks represent two distinct chambers in growth chamber  
623 setups, whereas in glasshouse setups the blocks represent two distinct growth benches in the same  
624 glasshouse.  
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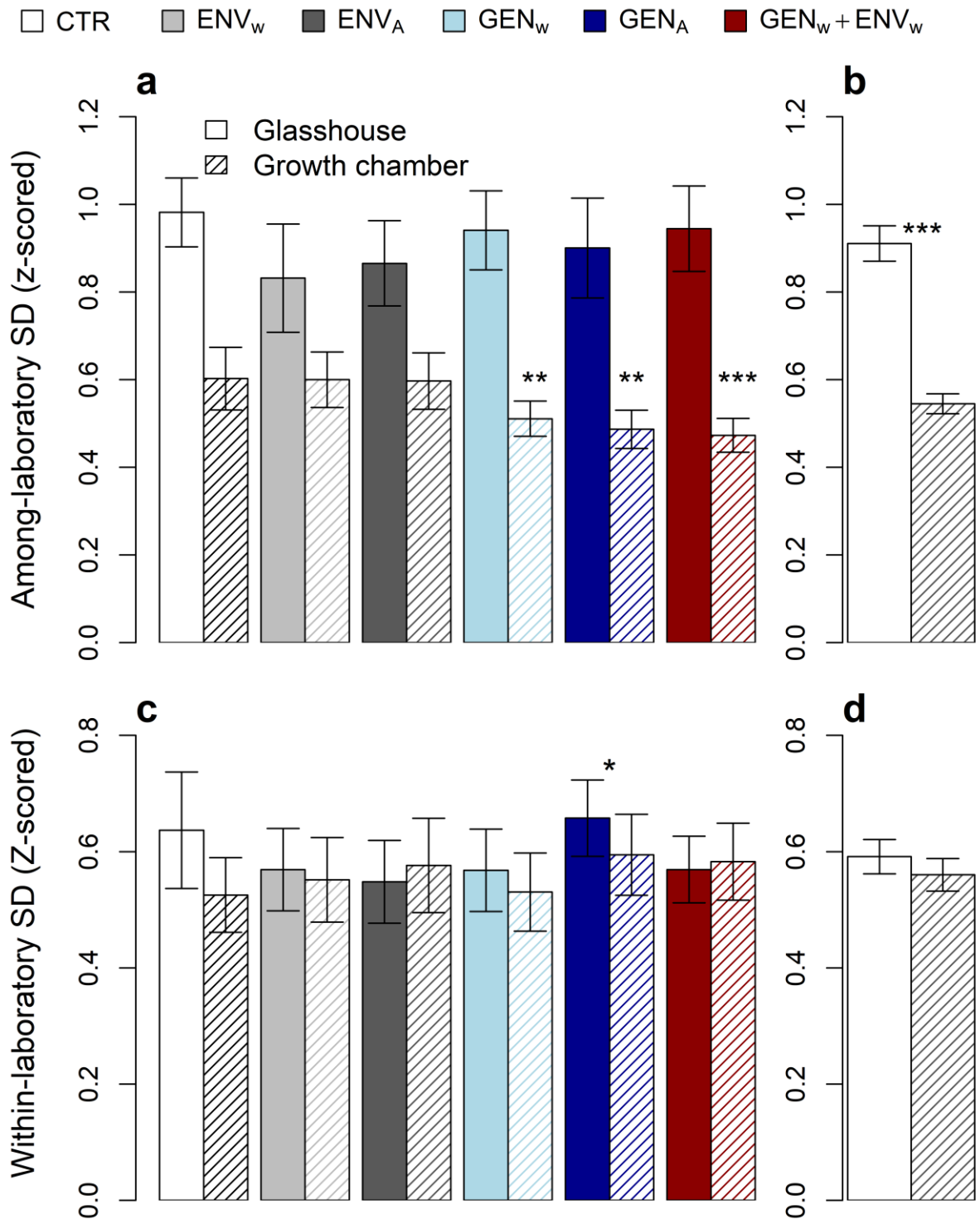
630 **Fig. 2 | Net legume effect for the 12 response variables in 14 laboratories as affected by**  
631 **laboratory and SETUP (growth chamber vs. glasshouse) treatment.** The grey and blue bars  
632 represent laboratories that used growth chamber and glasshouse set-ups, respectively. Bars show  
633 means by laboratory obtained by averaging over all CSV treatments, with error bars indicating  $\pm 1$   
634 s.e.m. (n = 72 microcosms per laboratory).  
635



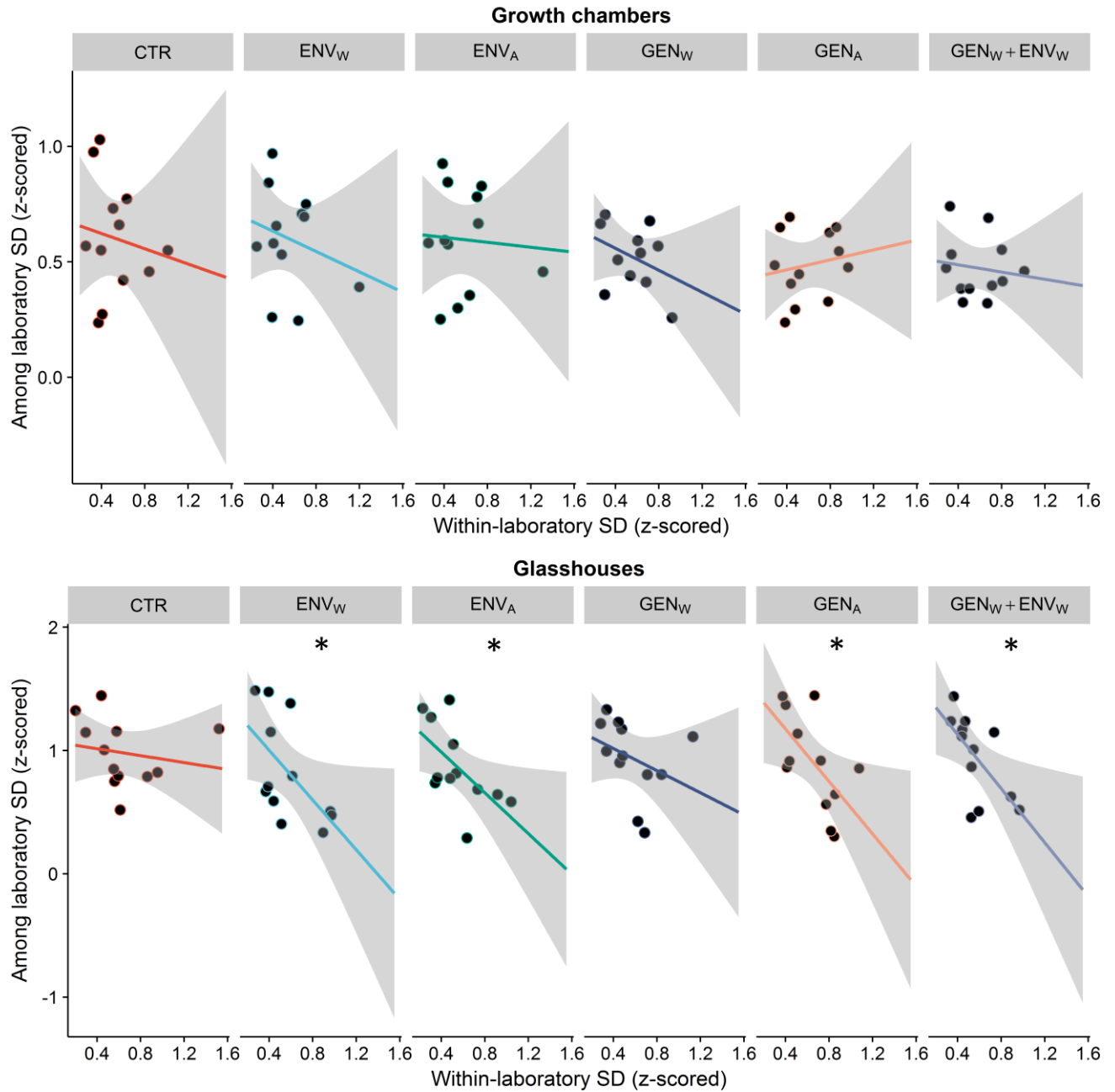
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638  
639 **Fig. 3 | Among- and within-laboratory standard deviation (SD) of the net legume effect as**  
640 **affected by experimental treatments.** Among-laboratory SD as affected by CSV and SETUP (a) and  
641 SETUP only (b). Within-laboratory SD as affected by CSV and SETUP (c) and SETUP only (d).  
642 Lower among-laboratory SD indicates enhanced reproducibility. Solid-filled bars and striped bars  
643 represent glasshouse (n = 6) and growth chamber setups (n = 8), respectively. Asterisks represent *P*-  
644 values (\*\*\* for  $P < 0.001$ , \*\* for  $P < 0.01$ , \* for  $P < 0.05$ ) indicating significantly different fitted  
645 coefficients according to the mixed-effects models (see Supplementary Notes for full model outputs);  
646 in (c) the star indicates the significant difference between GEN<sub>A</sub> and CTR, irrespective of the type of  
647 SETUP. For a detailed description of experimental treatments and abbreviations see Fig. 1.



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650  
 651 **Fig. 4 | Relationship between within-laboratory SD and among-laboratory SD of the net legume**  
 652 **effect as affected by experimental treatments.** The figure illustrates the significant within-laboratory  
 653 SD×SETUP×CSV three-way interaction ( $F_{5,109} = 2.4, P < 0.040$ ) affecting among-laboratory SD  
 654 (Supplementary Note). This interaction is the result of a more negative relationship between within-  
 655 and among-laboratory SD in glasshouses relative to growth chambers, but with different slopes for the  
 656 different CSV treatments. Points represent the 12 response variables. Asterisks represent  $P$  values <

657 0.05 for the individual linear regressions. Note the different scale for the y-axis between growth  
658 chambers and glasshouses. For a detailed description of experimental treatments and abbreviations see