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## Metabolic profiling of *Lolium perenne* shows functional integration of metabolic responses to diverse subtoxic conditions of chemical stress

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1 **Title:**

2 **Metabolic profiling of *Lolium perenne* shows functional integration of metabolic**  
3 **responses to diverse subtoxic conditions of chemical stress**

4  
5 **Running title: Metabolic profiling of chemical stress responses**

6  
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39

39 **SUMMARY**

40

41 Long-term adjustment and survival of perennial ryegrass to subtoxic levels of diverse  
42 xenobiotic and heavy-metal stresses are associated with major flexibility and complex  
43 regulations of central carbon and nitrogen metabolisms.

44

45

46

47 **ABSTRACT**

48

49 Plant communities are confronted with a great variety of environmental chemical stresses.  
50 Characterization of chemical stress in higher plants has often been focused on single or  
51 closely-related stressors under acute exposure, or restricted to a selective number of molecular  
52 targets. In order to understand plant functioning under chemical stress conditions close to  
53 environmental pollution conditions, the C3 grass *Lolium perenne* was subjected to a panel of  
54 different chemical stressors (pesticide, pesticide degradation compound, polycyclic aromatic-  
55 hydrocarbon, heavy metal) under conditions of seed-level or root-level subtoxic exposure.  
56 Physiological and metabolic profiling analysis on roots and shoots revealed that all of these  
57 subtoxic chemical stresses resulted in discrete physiological perturbations and complex  
58 metabolic shifts. These metabolic shifts involved stressor-specific effects, pointing out to  
59 multi-level mechanisms of action, such as the effects of glyphosate and its degradation  
60 product aminomethylphosphonic acid on quinate levels. They also involved major generic  
61 effects that linked all of the subtoxic chemical stresses with major modifications of nitrogen  
62 metabolism, especially affecting asparagine, and of photorespiration, especially affecting  
63 alanine and glycerate. Stress-related physiological effects and metabolic adjustments were  
64 shown to be integrated through a complex network of metabolic correlations converging on  
65 asparagine, leucine, serine, and glucose-6-phosphate, which could be potentially modulated  
66 by differential dynamics and interconversion of soluble sugars (sucrose, trehalose, glucose).  
67 Underlying metabolic, regulatory and signalling mechanisms linking these subtoxic chemical  
68 stresses with a generic impact on nitrogen metabolism and photorespiration are discussed in  
69 relation with carbohydrate and low energy sensing.

70

70 **KEY WORDS**

71

72 AMPA, carbon-nitrogen balance, copper, glyphosate, phytoremediation, polycyclic aromatic  
73 hydrocarbon, residual pollution, perennial ryegrass, tebuconazole, xenobiotics

74

75 **ABBREVIATIONS**

76 AMPA, aminomethylphosphonic acid; BCAA, branched chain amino acids; Cu, copper; F,  
77 fluoranthene; Fru, fructose; Fru-6-P, fructose-6-phosphate; G, glyphosate; Glc, glucose; Glc-  
78 6-P, glucose-6-phosphate; GT, combination of glyphosate and tebuconazole; HCA,  
79 hierarchical cluster analysis; PAH, polycyclic aromatic hydrocarbon; PCA, principal  
80 component analysis; PSII, photosystem II; Suc, sucrose; T, tebuconazole; TCA, tricarboxylic  
81 acids; Tre, trehalose; Tre-6-P, trehalose-6-phosphate.

82

## 82 INTRODUCTION

83

84 Modern agriculture uses large amounts of numerous pesticides in order to control pests and  
85 weeds, and to get around resistance mechanisms of target organisms (Helander et al., 2012).  
86 Various processes (drift, runoff, leaching) lead to contamination of terrestrial and aquatic  
87 environments by mixtures of chemicals, mainly pesticides, pesticide degradation products,  
88 and adjuvants (Dévier et al., 2011; Helander et al., 2012), but also metals (Bidar et al., 2009)  
89 and polycyclic aromatic hydrocarbons (PAH) (Kreslavski et al., 2014). Non-target plants can  
90 suffer direct damages by chemicals because of their sessile lifestyle. However, the overall  
91 effects of these complex, diffuse, and chronic chemical pollutions are difficult to predict  
92 (Dévier et al., 2011). Most studies focus on the effects of a restricted number of pollutants,  
93 mainly heavy metals and plant-targeting chemicals such as herbicides, whereas better  
94 understanding of chemical stress effects requires that a much greater number of chemical  
95 stressors, and their corresponding degradation products, be studied. Moreover, exposure  
96 protocols do not necessarily reflect conditions of environmental pollution. For most  
97 compounds, the mode of action and biological effects of low-level and multiple-stress  
98 exposure may differ from effects of toxic or lethal contamination and remain poorly  
99 understood. Serra et al. (2013) have shown that, under environmentally-relevant  
100 contamination levels, xenobiotics and associated degradation products induced cryptic  
101 metabolic perturbations in *Arabidopsis thaliana*, in relation with non-target and signalling  
102 effects. These perturbations highlighted complex interactive effects, whether positive or  
103 negative, between structurally-different xenobiotics.

104 Chemical stress induces different responses according to the ability of plant species or  
105 ecotypes to integrate stress signals and develop coordinated molecular responses (Ramel et  
106 al., 2012; Couée et al., 2013). Perennial ryegrass, *Lolium perenne*, which is the most widely  
107 grown grass in temperate regions and a primary food source for grazing ruminants  
108 (Barbehenn et al., 2004), has been reported to be tolerant to diverse chemical stressors (Dear  
109 et al., 2006; Bidar et al., 2009; D’Orazio et al., 2013). It also accelerates degradation of  
110 contaminants in soils (Krutz et al., 2005; D’Orazio et al., 2013), and plays important roles in  
111 improving remediation of heavy-metal-contaminated soils (Bidar et al., 2009). *Lolium*  
112 *perenne* has been commonly used in revegetation and phytoremediation projects (Bidar et al.,  
113 2009; Hu et al., 2012), and in vegetative filter strips (Krutz et al., 2005). However,  
114 mechanisms involved in *Lolium* tolerance to chemical stressors have not been elucidated, thus  
115 highlighting the need for without *a priori* approaches such as metabolomics.

116 In order to characterize chemical stress responses, *Lolium perenne* was subjected to subtoxic  
117 levels of contrasted chemical stressors : (i) the widely used broad-spectrum herbicide  
118 glyphosate (Helander et al., 2012), which inhibits aromatic amino acid production through  
119 inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate  
120 pathway, (ii) its degradation product aminomethylphosphonic acid (AMPA), which may  
121 interact with glycine metabolism (Serra et al., 2013), (iii) the triazole fungicide tebuconazole,  
122 whether alone or in association with glyphosate, (iv) the PAH molecule fluoranthene, and (iv)  
123 the heavy metal copper. Exposure of germinating seeds and root systems, and different  
124 modalities of treatment, were used to reflect realistic conditions of environmental exposure  
125 (Serra et al., 2013). Plant responses were dissected by physiological and metabolomic  
126 analysis (Obata and Fernie, 2012) in order to highlight significant stress-induced shifts in  
127 *Lolium* metabolic profiles, and to identify chemical stressor-specific and generic responses to  
128 chemical stress.

129

## 129 MATERIALS AND METHODS

130

### 131 *Plant material and growth conditions*

132 Seeds of *Lolium perenne* (Brio cultivar) were washed in ethanol and surface-sterilized in  
133 bayrochlore (20 g L<sup>-1</sup> in water) containing 0.05% tween (v/v) for 20 min and rinsed five times  
134 in sterilized water. Moistened seeds were placed in Petri dishes in the dark at 4°C for 7 d in  
135 order to break dormancy and homogenize germination. Imbibed seeds were sown on pieces of  
136 gauze and placed at the top of sterile tubes containing liquid growth medium. Gauze pieces  
137 were moistened by soaking gauze edges into culture medium. Germination and hydroponic  
138 growth were carried out under axenic conditions in a control growth chamber at 22°C/20°C  
139 under a 16 h light (6000 lux)/8 h dark regime. Growth solution consisted of Hoagland basal  
140 salt mix (No.2, Caisson Laboratories, North Logan, UT, USA) adjusted to pH 6. Direct  
141 exposure to chemicals was carried out by sowing seeds on chemical-stressor-containing  
142 growth medium. Developmental and physiological parameters were measured after 11 d of  
143 growth. Transfer experiments consisted in root-level shock exposure of young plants at the  
144 same stage of photosynthetic development. After 7 d of growth under control conditions,  
145 gauze pieces supporting seedlings were transferred to fresh growth solution containing  
146 chemical stressors. Developmental and physiological parameters were measured 4 d later,  
147 thus corresponding to 11 d of total growth. Metabolite profiling of seedlings was carried out  
148 after direct and transfer exposures. Different chemical treatments were applied: the broad-  
149 spectrum herbicide glyphosate (G, 1 µM), its degradation product aminomethylphosphonic  
150 acid, AMPA (A, 50 µM), the fungicide tebuconazole (T, 4 µM), the PAH fluoranthene (F,  
151 500 µM), copper (Cu, 6 µM) and a combination of glyphosate and tebuconazole (GT, 1 µM  
152 and 4 µM respectively). In order to characterize long-term effects, developmental and  
153 physiological parameters were also measured after 30 d of growth under direct exposure to  
154 chemical stress. For these experiments, control and contaminated growth solutions were  
155 renewed every 11 d. All of these experiments were carried out with at least 5 independent  
156 replicates, each consisting of at least 10 plantlets.

157

### 158 *Analysis of growth and photosynthetic parameters*

159 Seedlings were aligned on glass plates and photographed. Lengths of main root and  
160 elongating leaf were measured using ImageJ software. Pigment contents (chlorophylls and  
161 total carotenoids) and maximum photosystem II (PSII) efficiency ( $F_v/F_m$ ) were quantified as  
162 described in Serra et al. (2013).

163

#### 164 ***Metabolic profiling***

165 Roots and leaves of seedlings were collected just before start of daylight period, freeze-dried,  
166 ground in liquid nitrogen and stored at -20°C until use. For each sample, five mg of powder  
167 was suspended in 600  $\mu$ L of methanol:chloroform solution (2:1, v/v) and mixed for 1.5 min.  
168 Samples were transferred to -20°C for 10 min before adding 400  $\mu$ L of cold (4°C) water.  
169 Samples were mixed vigorously for 1 min, and centrifuged twice for 5 min at 4,000 g (4°C).  
170 One hundred and twenty  $\mu$ L of upper aqueous phase, containing polar metabolites, were  
171 transferred to chromatographic glass vials and vacuum-dried (MiVac, Genevac Ltd., Ipswich,  
172 England). Derivatization of extracts was conducted as previously described (Serra et al., 2013;  
173 Supplementary Method S1), using a CTC CombiPal autosampler (GERSTEL GmbH and  
174 Co.KG, Mülheim an der Ruhr, Germany), ensuring identical derivatization time and process  
175 for all samples. Extracts were analyzed using gas chromatography mass spectrometry  
176 (GC/MS). GC/MS system consisted of a Trace GC Ultra chromatograph and a Trace DSQII  
177 quadrupole mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA).  
178 Parameters of injection and chromatography were similar to those described in Serra et al.  
179 (2013) (Supplementary Method S1). Detection was achieved using electron impact ionization.  
180 Temperature of the ion source was set at 250°C and MS transfer line at 300°C. Peaks were  
181 accurately annotated using mass spectra (two specific ions) and retention times. Calibration  
182 curves were established with 61 pure reference compounds (Supplementary Table S1) at 1, 2,  
183 5, 10, 20, 50, 100, 200, 500, 750, 1000, 1500 and 2000  $\mu$ M. Each metabolite was quantified  
184 according to its corresponding calibration curve, using XCalibur v2.0.7 software (Thermo  
185 Fisher Scientific Inc, Waltham, MA, USA) and expressed in nmol per mg dry weight (DW).

186

#### 187 ***Statistical analysis***

188 Physiological and metabolic parameters were measured on at least 5 independent replicates of  
189 at least 10 individual plantlets. Statistical analyses were carried out with version 3.0.1 of R  
190 software. Pairwise comparisons of means used the non-parametric Mann-Whitney-Wilcoxon  
191 test. In order to characterize relationships between treatments and responses, principal  
192 component analyses (PCAs), based on correlation matrix of averages (Ramel et al., 2009;  
193 Serra et al., 2013), and hierarchical classifications were carried out using the FactoMineR  
194 package of R. In order to compare variations between treatments, relative levels of responses  
195 against control condition were calculated using  $\log_2(\text{ratio})$ . Metabolic responses between  
196 treatments were compared by performing heatmap and hierarchical clustering with EPCLUST

197 software using correlation measure-based distances and average linkage. Pair-wise  
198 physiological trait-physiological trait, physiological trait-metabolite, and metabolite-  
199 metabolite correlations were determined by Pearson correlation analysis.  
200

## 200    **RESULTS**

201

### 202    *Effects of subtoxic levels of environmental pollutants on growth and development*

203    The experimental set-up involved root application of chemical stressors and parallel analysis  
204    of root growth and leaf growth, which, in *Lolium perenne*, respectively rely on the root apex  
205    (Gonthier and Francis, 1989) and on the base of the growing leaf (Kavanová et al., 2008).  
206    Chemical stressors were applied to germinating seeds and root systems at no-observed-  
207    adverse-effect-levels (NOAEL; Dorato and Engelhardt, 2005) for PSII efficiency, chlorophyll  
208    levels, carotenoid levels, and root growth under conditions of transfer exposure, where  
209    seedlings grown during 7 d in control medium were subjected to root-level application of  
210    chemical stressors during 4 d (Fig. 1A-D). These levels of chemical stressors were at least 20-  
211    fold lower than EC (effect concentration)50 values for these parameters under conditions of  
212    transfer exposure. Such levels also corresponded to no-observed-adverse-effect-levels for  
213    PSII efficiency under direct exposure (Fig. 1H), where germination and seedling growth  
214    occurred in the presence of chemical stressors. However, direct exposure conditions had a  
215    much greater impact on root length (Fig. 1E), leaf length (Fig. 1F), and photosynthetic  
216    pigments (Fig. 1G). Leaf length responses under direct exposure showed the same trend as  
217    those observed under transfer exposure, thus indicating that transfer and direct exposures  
218    reflected different intensities of chemical stress (Fig. 1B, F). However, in a study of long-term  
219    (30 day) growth, *Lolium perenne* seedlings maintained development under these levels of  
220    chemical stress, or even escaped major root growth inhibition in the case of AMPA, thus  
221    confirming that exposures to chemical stress were subtoxic (Fig. 2).

222    The impact of treatments depended on type of pollutant, on physiological trait and on  
223    modality of chemical exposure (Fig. 1). Root length was particularly responsive to chemical  
224    stress under conditions of direct exposure, with all of the chemical treatments inducing  
225    significant decrease of growth, except fluoranthene which induced increase of root length  
226    (Fig. 1E). Differences of root length between controls of direct and transfer exposure  
227    experiments suggested that root growth was sensitive to mechanical processes that may occur  
228    during transfer protocol. However, these effects did not result in greater sensitivity to  
229    chemical stress (Fig. 1A, E). Direct exposure to copper, glyphosate and its degradation  
230    product AMPA showed the same extent of negative impact for root length (Fig. 1E) and  
231    photosynthetic pigment levels (Fig. 1G), without affecting leaf length (Fig. 1F). Leaf length  
232    was slightly decreased by fluoranthene, thus contrasting with its positive effect on root length.

233 Glyphosate-tebuconazole mixture (GT) induced a more negative effect on root length than  
234 observed for each pollutant alone, although the combined effect was not strictly additive (Fig.  
235 1E). In contrast, length of elongating leaves was similarly negatively affected by tebuconazole  
236 and glyphosate-tebuconazole mixture whereas glyphosate, alone or in combination with  
237 tebuconazole, did not have any effect (Fig. 1F). Chlorophyll levels were negatively affected  
238 by glyphosate, whereas tebuconazole showed a positive effect and lifted the negative effect of  
239 glyphosate (Fig. 1G). Such differential responses to these treatments were observed for PSII  
240 efficiency with no effect of glyphosate and glyphosate-tebuconazole mixture (Fig. 1H). PSII  
241 efficiency thus showed no adverse effect of chemical stress, with only AMPA and  
242 tebuconazole inducing a slight increase (Fig. 1H). Maintenance of PSII efficiency at or above  
243 control level indicated that photosynthesis remained active in the presence of the different  
244 chemicals under the conditions of exposure applied. The present range of chemical stresses  
245 therefore seemed to act primarily on growth and development rather than on photosynthesis.  
246 Comparison of effects of direct chemical stress on root length and leaf length indicated that  
247 glyphosate, AMPA and copper had stronger impacts on root growth, whereas tebuconazole  
248 and, to a lesser extent, fluoranthene, had stronger impacts on leaf growth (Fig. 1E, F).

249

#### 250 *Subtoxic levels of chemical stressors cause major metabolic shifts*

251 Metabolomic analysis of a set of 61 metabolites was performed to reveal discriminant  
252 components of metabolic responses to chemical stress. Root and leaf metabolic profiles,  
253 resulting from treatments and exposure modalities, were analysed using PCA and associated  
254 hierarchical classification. Major metabolic variations separated samples into four response  
255 patterns corresponding to each kind of tissues and exposure conditions (Supplementary Figure  
256 S1). Metabolic patterns were determined firstly by the nature of the tissue (root or leaf), then  
257 by the type of chemical stress exposure, and finally by the nature of the chemical stressor  
258 (Supplementary Figure S1), showing that subtoxic chemical stress did not deeply modify  
259 general metabolic signatures of organs and exposure modalities.

260 Metabolic responses were separately analysed (Fig. 3) for each type of exposure and for each  
261 organ. All of the chemical stressors caused major metabolic variations in both roots and  
262 leaves, whatever the type of exposure. Under conditions of transfer exposure, chemical  
263 stressors induced metabolic changes (Fig. 3A, B) in the absence of significant physiological  
264 impact (Fig. 1). Copper, glyphosate or its degradation product AMPA caused, under transfer  
265 exposure, important metabolic changes in leaves (Fig. 3B) without any physiological effect on  
266 leaf growth or photosynthesis (Fig. 1B, D). Under conditions of direct exposure, chemical

267 stressors induced metabolic changes in the absence of significant impact on photosynthesis  
268 (Fig. 1G, H). Some response patterns showed close similarities: in root tissues, transfer and  
269 direct exposures resulted in great proximity for control and AMPA, and for glyphosate and  
270 glyphosate-tebuconazole mixture (Fig. 3A, C). In contrast, the distance between fluoranthene  
271 and tebuconazole greatly differed between transfer and direct exposures in the case of root  
272 tissues. Divergences in metabolic profiles of potentially close chemical treatments were also  
273 found. The glyphosate degradation product AMPA was never associated to its parent  
274 compound glyphosate (Fig. 3). Similarly, tebuconazole was disconnected from glyphosate-  
275 tebuconazole mixture, despite their close physiological impacts (Figs 1, 3). Finally, in some  
276 cases, such as the effects of AMPA on root or of glyphosate-tebuconazole mixture on leaf,  
277 global metabolic patterns were close to the control (Fig. 3C, D) despite major physiological  
278 effects (Fig. 1E, F).

279

### 280 *Coordination of metabolome changes under conditions of subtoxic chemical stress*

281 Among the 61 metabolites that were analysed and quantified, 42 showed significant variations  
282 in relation with at least one of the chemical stress treatments. Variations of these 42  
283 metabolites relatively to control [ $\log_2(\text{ratio})$ ] were classified by hierarchical cluster analysis  
284 (HCA) in order to define for each metabolite a pattern of chemical stress response across the 6  
285 different stressors, the 2 modalities of exposure and the 2 organs (Fig. 4). This HCA of the 24  
286 analyses separated metabolites into distinct clusters and subclusters of chemical stress  
287 responses that were grouped into 8 general clusters (Fig. 4). Several clusters were  
288 characterised by strong relationships between co-metabolites, such as tight clustering of TCA  
289 (citrate, succinate, fumarate; cluster F), of fructose (Fru) and glucose (Glc) (cluster G), or of  
290 Fru-6-P and Glc-6-P (cluster C). On the other hand, clustering of co-metabolites and of  
291 unrelated metabolites, such as Trp and inositol (cluster F), pointed out to potential regulatory  
292 networks (Fig. 4).

293 Highly-contrasted clusters, such as clusters A and B on one hand, and clusters G and H on the  
294 other hand, were characterized, respectively, by general increase and general decrease of  
295 metabolite levels in response to chemical stressors. The N-rich amino acid Asn (cluster A)  
296 showed significant increase across most of the conditions, thus indicating that nitrogen re-  
297 distribution and mobilization were important features of chemical stress response in *L.*  
298 *perenne*. Clustering of Met with Asn could be ascribed to its positive variations, which were  
299 however much more limited than these of Asn, and to the absence of major negative  
300 variations. Cluster B consisted of a set of amino acids including all of the branched-chain

301 amino acids (Leu, Ile, Val), two aromatic amino acids (Tyr, Phe), and Lys, in association with  
302 glycerol and phosphate. It was characterized by common increase in leaf across all chemical  
303 stressors in at least one exposure condition. Moreover, cluster B metabolites increased in  
304 roots across several chemical stressors (G under direct exposure, and GT, T, and F under both  
305 transfer and direct exposures), which may also reflect the importance of root metabolism  
306 perturbations under conditions of subtoxic chemical stress (Fig. 5, Supplementary Figure S2).  
307 In contrast, ribose and glycerate in, respectively, clusters G and H showed significant  
308 decrease across most of the conditions. Moreover, the ribose-associated cluster, as well as the  
309 glycerate-associated cluster, comprised stress-related metabolites [gamma aminobutyric acid  
310 (GABA), Pro, pipercolate] thus suggesting that chemical stress responses involved  
311 remodelling of general abiotic stress responses. Variations of Ala in cluster H may be in line  
312 with remodelling of nitrogen metabolism that was highlighted by clusters A and B.  
313 Metabolites from cluster C to cluster F exhibited varied responses of increase or decrease  
314 depending on treatments. Some compounds showed changes of very low amplitude. This was  
315 especially the case for arabitol and sorbitol (cluster D). In contrast, all of the chemical  
316 stressors increased lactate levels (cluster D) in both roots and leaves, in at least one modality  
317 of exposure, thus pointing out to glycolysis and energy dysfunction. Cluster E comprised a set  
318 of amino acids, including Asn co-metabolite Asp, and other N-rich compounds, such as the  
319 polyamine precursor ornithine and the polyamine putrescine. Interestingly, this cluster of  
320 amino-compounds included the disaccharides Suc and Tre, thus indicating potential  
321 importance of carbon and nitrogen relationships.

322

### 323 *Specific effects of chemical stressors on metabolite levels*

324 Some compounds showed contrasted variations according to the nature of chemical stressor,  
325 thus giving important insights into specific effects of a given chemical (Table 1). In addition  
326 to inducing the highest increase of Asn levels (5.7 fold in leaves, Fig. 5, Supplementary  
327 Figure S2), AMPA led to a strong decrease of quinate, a shikimate pathway component, in  
328 leaves (3.6-fold, Fig. 5, Supplementary Figure S2). In contrast, glyphosate, which targets a  
329 key step of shikimate pathway and aromatic amino acid synthesis, caused significant increase  
330 of quinate levels in roots, but not in leaves, either alone or in association with tebuconazole  
331 (Fig. 5, Supplementary Figure S2). Such specific variations of quinate suggested particular  
332 interference of glyphosate and AMPA with the shikimate pathway, despite the absence of  
333 major effects on levels of aromatic amino acids (Phe, Tyr, Trp) (Fig. 5, Supplementary Figure  
334 S2). Another striking effect of AMPA was a nearly 2-fold decrease of cell wall metabolite

335 arabinose in roots (Fig. 5, Supplementary Figure S2). Significant decrease of citrate and/or  
336 succinate was observed in roots of tebuconazole- and copper-treated plants, thus suggesting  
337 TCA cycle depression and mitochondrial respiration dysfunction (Fig. 5). Fluoranthene  
338 treatment was characterised, under conditions of transfer exposure, by accumulation of Fru,  
339 Glc, and Ser in roots and leaves, confirming major changes in carbon and nitrogen  
340 metabolisms (Table 1, Fig. 4, Supplementary Figure S2). Accumulation of Pro and soluble  
341 sugars in fluoranthene-treated leaves pointed out to possible osmotic stress, although the  
342 levels of other metabolic markers of osmotic stress response, such as polyols, were not  
343 affected (Figs 4, 5). Fluoranthene-enhanced root growth, associated with accumulation of cell  
344 wall metabolite arabinose in roots under transfer exposure, may thus represent increased water  
345 exploration resulting from osmotic stress response (Figs 1, 4, Supplementary Figure S2).  
346 Action of fluoranthene was also characterised by global increase of ethanolamine, potentially  
347 indicating changes in phospholipid metabolism under fluoranthene treatments (Supplementary  
348 Figure S2).

349

#### 350 ***Identification of metabolic drivers of physiological responses to chemical stress***

351 In order to determine whether physiological responses to chemical stress were related to  
352 metabolic changes, organ-specific trait-metabolite Pearson correlations were carried out (Fig.  
353 6, Supplementary Table S2). This correlation approach highlighted global trends in  
354 relationships between metabolites and trait responses, rather than chemical stressor  
355 specificities. Leaf length was significantly negatively correlated to only one metabolite in  
356 leaves, Glc-6-P. In contrast, leaf chlorophyll level was positively correlated to Glc-6-P, and  
357 also positively correlated to several metabolites of cluster B (Fig. 4), mainly represented by  
358 amino acids (Val, Leu, Phe, Lys, Tyr) and glycerol, to putrescine (cluster E), to arabinose  
359 (cluster F), and to pipercolate (cluster H) (Fig. 6, Supplementary Table S2). Putrescine and  
360 pipercolate are metabolites that generally increase under stress (Hummel et al., 2004; Servillo  
361 et al., 2012). However, under chemical treatments inducing significant, but limited, pigment  
362 loss (A, G, Cu; Fig. 1G), their levels were decreased (Supplementary Figure S2). Thus, in  
363 accordance with chlorophyll-amino acid correlations (Fig. 6), responses to chemical stress in  
364 *Lolium perenne* were related to carbon and nitrogen metabolic rearrangement rather than to  
365 induction of stress response metabolic pathways. In contrast, under tebuconazole treatment,  
366 which increases chlorophyll levels (Fig. 1C, G), increase of these amino acids may indicate  
367 metabolic adaptation rather than proteolysis-related sensitivity (Fig. 5, Supplementary Figure  
368 S2).

369 Leaf length was negatively correlated to several metabolites in root, the most highly  
370 correlated being the amino acids Asn (cluster A), Lys, Ile, and Tyr (cluster B) (Fig. 6,  
371 Supplementary Table S2). Although links between metabolite levels in root and leaf growth  
372 were not direct, such strong negative correlations with amino acids, in particular with N-rich  
373 amino acids such as Asn and Lys, highlighted the importance of root-shoot relationships  
374 under conditions of chemical stress. A negative correlation was also found with Ser (cluster  
375 E), which is a central amino acid involved in photorespiration and in synthesis of glutathione  
376 components.

377 No significant correlation was found between root length and metabolite levels in roots.  
378 However, root length exhibited positive correlations with two metabolites quantified in  
379 leaves, Pro (cluster G) and quinate (cluster D) (Fig. 6, Supplementary Table S2). These  
380 positive correlations indicated that negative effects of chemical stress on root length were  
381 associated with decrease of Pro or quinate levels in leaves, although their accumulation has  
382 been reported under abiotic or chemical (herbicide, allelopathic compounds) stresses in  
383 *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Pisum sativum* (Verbruggen and Hermans,  
384 2008; Orcaray et al., 2010). Variations of Pro and quinate levels in leaves thus reflected a  
385 reorientation of leaf metabolic pathways in relation to root growth impacts. Since Pro is one  
386 end-product of amino acid synthesis, and quinate is a reserve compound of the shikimate  
387 pathway for aromatic acid synthesis (Orcaray et al., 2010), these variations may reflect  
388 perturbations of root-shoot nitrogen dynamics.

389

### 390 ***Metabolite-metabolite correlations highlight the importance of carbon-nitrogen regulations***

391 In order to reveal metabolic networks involved in chemical stress responses, a global pair-  
392 wise metabolite-metabolite correlation matrix was calculated (Supplementary Table S3), by  
393 Pearson correlation analysis of the complete set of organ- and modality-related metabolite  
394 data. Most of the physiological trait-correlated metabolites (Fig. 6) were correlated ( $p < 0.05$ ),  
395 mainly positively, with a range of metabolites across the eight clusters (Figs 7, 8). Among  
396 these fifteen metabolites, eleven metabolites (Leu, Lys, Val, Tyr, Phe, Ile, quinate, Ser,  
397 arabinose, Pro, and pipecolate) exhibited similar or close correlation networks  
398 (Supplementary Table S3), mainly integrating amino acids (Met, Ile, Tyr, Leu, Lys, Val, Phe,  
399 Ser, Thr, Gly, Asp, Glu, Trp, Ala), carbohydrate metabolism compounds (Glc, Fru, Tre,  
400 TCA), stress metabolites (arabitol, inositol, Pro, GABA, pipecolate), and compounds such as  
401 arabinose, ethanolamine and quinate. Putrescine exhibited a close, but more restricted,  
402 correlation profile, with only ten correlations involving amino acids, TCA, and stress

403 metabolites. The least correlated metabolite was Asn, which exhibited five correlations,  
404 mainly with metabolically-close amino acids such as its direct precursor Asp and also Ser,  
405 Thr, and Ile. Except for copper and glyphosate under transfer exposure, chemical stressors  
406 induced Asn accumulation, which was almost systematically correlated to increase of these  
407 metabolites (Figs 5, 7, Supplementary Figure S2), thus confirming predominant roles for Asn  
408 and nitrogen metabolism in chemical stress responses of *L. perenne*. This correlation level  
409 observed in the global matrix was extended by considering organ-specific correlation  
410 matrices. Asn was negatively correlated to quinate in leaves, and positively correlated to Glc-  
411 6-P, Fru-6-P and Fru in roots (Supplementary Table S2).

412 In the global correlation matrix, physiological trait-correlated metabolite Glc-6-P exhibited  
413 the most differentiated correlation profile, with no correlation with amino acids and, in  
414 contrast, with numerous correlations with carbohydrates (Fru-6-P, Tre, Suc, Fru) and stress-  
415 related metabolites (lactate, arabitol, sorbitol, ornithine, pipercolate) (Figs 7, 8, Supplementary  
416 Table S3). Nevertheless, considering modality-specific correlation matrices analyzing  
417 separately transfer and direct exposure-related data, Glc-6-P was negatively correlated to key  
418 metabolites previously identified by metabolite-physiological trait correlations (amino acids,  
419 TCA, stress metabolites, arabinose, quinate; Supplementary Table S4). This was also the case  
420 for Fru-6-P, which exhibited, for both transfer and direct exposure modalities, negative  
421 correlations to physiological parameter-correlated metabolites (Supplementary Table S4).  
422 Additionally, Fru was positively correlated to the same correlated metabolites, except Asn, in  
423 the global correlation matrix (Supplementary Table S3), thus highlighting the importance of  
424 soluble and phosphorylated sugars in metabolic responses to chemical stress.

425 The disaccharide Tre, whose levels generally increased in response to all types of chemical  
426 stress (except in the presence of tebuconazole), was characterized, like ribose, by a majority  
427 of negative correlations (25 against 5 positive correlations) (Figs 7, 8). Variations in Tre  
428 levels were closely linked with variations of almost all of previously-cited metabolites (Leu,  
429 Glc, Ser, succinate, Glc-6-P, Ala), thus suggesting a central role of Tre, as represented in  
430 Figure 8, in spite of low concentration and weak variations (Supplementary Figure S2). Tre  
431 was negatively linked to succinate, with depletions or increases of this highly variable TCA  
432 being associated, respectively, with increases or depletions of Tre in most cases (Figs 4, 7).  
433 Tre was negatively correlated to Glc, which could reflect direct transformation of Tre into Glc  
434 by trehalase. In contrast, no correlation was found between Tre and Suc, in contrast with  
435 studies showing parallel variations of Suc and Tre [or trehalose-6-phosphate (Tre-6-P)] (Lunn  
436 et al., 2014). One of the positive correlations exhibited by Tre was with its precursor Glc-6-P,

437 which can generate Tre-6-P and then Tre through combined activities of Trehalose-phosphate-  
438 synthase (TPS) and Trehalose phosphate phosphatase (TPP) (Figs 7, 8). However, comparison  
439 of relative levels of Tre and other correlated metabolites, such as Glc and Glc-6-P, did not  
440 show causal and strict relationships, thus suggesting that these convergent correlations could  
441 be attributed to more central regulatory roles for Tre, or Tre-6-P.

442 Considering correlation networks between soluble sugars (Fig. 8), the highest correlation was  
443 found for Glc-6-P and Fru-6-P, which exhibited similar increases in response to all of the  
444 chemical stressors, except glyphosate. Suc and Glc were positively correlated, their levels  
445 being increased by AMPA, and decreased by glyphosate, tebuconazole, or copper. The  
446 patterns of Glc and Fru, which are directly dependent on Suc metabolism, were similar for  
447 most chemical stress conditions. Nevertheless, correlation between Suc and Fru was not  
448 significant, thus suggesting that Glc and Fru levels could be regulated through stress  
449 mechanisms involving Glc-6-P and Tre pathways. A major feature of chemical stress response  
450 in *Lolium perenne* was therefore the modification of carbohydrate metabolism, leading to  
451 decrease of Suc, Glc (which are among the 13 highly variable metabolites with  $\text{Log}_2(\text{ratio}) > 1$   
452 or  $< -1$ ; Supplementary Figure S2, Fig. 5) or Fru levels, globally correlated with accumulation  
453 or maintenance of Tre, Fru-6-P and Glc-6-P.

454 Carbohydrate metabolism was linked to amino acid metabolism, with Tre being negatively  
455 correlated to Leu and Ser (Fig. 7). Leu decrease in leaves was associated to Tre accumulation  
456 in the presence of AMPA (Fig. 5). Leu belongs to branched-chain amino acids (BCAA; Ile,  
457 Leu, Val), which were grouped in cluster B, and were strongly interconnected. Simultaneous  
458 increase of Leu, Ile and Val levels in response to tebuconazole (Fig. 4), or their concomitant  
459 decrease in leaves under direct exposure in response to copper (Fig. 4), indicated coordinated  
460 changes of BCAA catabolism. Cluster B metabolites showed the greatest number of  
461 correlations with other metabolites. Leu was positively correlated to chlorophyll levels (Fig.  
462 6) and to 27 metabolites, among which Ser and shikimate pathway derivative quinate.

463 Ser (cluster E) was among the most highly variable metabolites [ $\text{Log}_2(\text{ratio}) > 1$  or  $< -1$ ;  
464 Supplementary Figure S2, Fig. 5] and was highly accumulated in response to fluoranthene  
465 and tebuconazole. Ser exhibited 33 significant correlations, among which there were only two  
466 negative cases, with Tre and ribose (Fig. 7), and was strongly positively correlated to Gly and  
467 Glu, which are close photorespiratory intermediates. Indeed, levels of these amino acids  
468 increased in response to most of the chemical stressors under at least one condition, except for  
469 glyphosate treatment and copper treatment where they decreased jointly (Fig. 5,  
470 Supplementary Figure S2). Ser was positively correlated to Asn and Ala, which constitute

471 amino group donors for synthesis of the photorespiratory intermediate Gly (Fig. 7). These  
472 correlations may confirm involvement of the photorespiratory pathway in chemical stress  
473 responses, and highlighted the importance of Ser. Photorespiration and N status, which were  
474 closely connected and regulated, thus seemed to play important roles in responses to chemical  
475 stress in *Lolium*.  
476

## 476 **DISCUSSION**

477

### 478 *Chemical stressors at subtoxic levels have major impacts on growth and metabolic* 479 *composition*

480 Subtoxic levels of chemical stressors induced significant physiological effects in *Lolium*  
481 *perenne*. The extent of these effects depended on type of pollutant, on modality of exposure,  
482 and on physiological trait. Parallel investigation of physiological modifications in roots and  
483 leaves for different chemicals highlighted primary impacts of chemical stressors on roots, the  
484 first organ exposed to stress, across various stressors and across various modes of action.  
485 Leaf-related parameters (leaf length, pigment contents), exhibited intermediary sensitivity to  
486 chemicals. In contrast, PSII efficiency ( $F_v/F_m$ ) remained unaffected by the different  
487 treatments under conditions of transfer or direct exposures (Fig. 1D, H). Although a species-  
488 specific effect of *Lolium perenne* could not be excluded, this result suggests low sensitivity of  
489 PSII efficiency to low or sublethal levels of chemical stressors, contrasting with its wide use  
490 as chemical stress marker under high exposure level (Li et al., 2013; Mateos-Naranjo and  
491 Perez-Martin, 2013; Kreslavski et al., 2014). Low-intensity chemical stress therefore seems  
492 to act primarily on growth and development rather than on photosynthesis, thus suggesting  
493 that developmental processes may be important targets of subtoxic chemical stress.

494 Although transfer and direct exposures led to similar trends across treatments, in terms of  
495 physiological responses, direct exposure induced more pronounced effects than transfer  
496 exposure. These results could be related to intensity of chemical stress, and to levels of  
497 chemical stress sensitivity. Exposure modalities reflected different durations of exposure, with  
498 a nearly three times longer exposure for direct exposure. Exposure modalities differed in the  
499 developmental stage at which *Lolium* was submitted to chemicals, since direct exposure was  
500 applied to seeds, whereas transfer experiments affected 7-d-old plantlets.

501 Subtoxic levels of chemical stressors induced metabolic effects that did not overrun general  
502 metabolic signatures of plant organs and of exposure modalities (Supplementary Figure S1).  
503 Nevertheless, metabolic analysis of roots, which were the primary site of exposure, under  
504 direct exposure (Fig. 3C), which gave the most intense physiological effects (Fig. 1), revealed  
505 different types of chemical stress responses: i) a low-intensity response highlighted by  
506 AMPA, ii) a glyphosate response (G, GT) characterized by quinate accumulation, iii) a heavy-  
507 metal response characterized by citrate and succinate depletion, and iv) a cyclic-compound  
508 response (F, T), characterized by putrescine accumulation. These metabolic changes, not  
509 deeply altering the global plant metabolome, and occurring with maintenance of plant growth

510 (Fig. 2), were likely to reflect chemical stress adjustment rather than deregulation of  
511 homeostasis, leading, under long-term exposure, to avoidance of root growth inhibition for  
512 most of chemical stressors (glyphosate, tebuconazole, copper) (Fig. 2).

513 The effects of chemical stressors, such as those induced by AMPA, the degradation product of  
514 glyphosate, led to important changes of metabolic composition, especially affecting amino  
515 acid and sugar compositions, which are major components of ryegrass nutritional quality for  
516 herbivores (Barbehenn et al., 2004). It was noteworthy that such changes of metabolic  
517 composition could occur in the absence of major physiological impact, as was the case for  
518 transfer exposure and for some of the direct exposure treatments (Figs 1, 3), thus emphasising  
519 that assessment of chemical stress impact in the environment should integrate a wider range  
520 of parameters than PSII efficiency, chlorophylls or plant growth. Perennial ryegrass is a major  
521 component of grazed pastures and of grasslands, which cover a large fraction of Earth's land  
522 surface (Barbehenn et al., 2004). The chemical stressors that were shown in the present study  
523 to affect ryegrass, in terms of growth/biomass and of metabolite contents (i.e. soluble sugars,  
524 amino acids), are part of diffuse pollution problems associated with agriculture (Dévier et al.,  
525 2011). Discrete adjustments of grassland species to diffuse soil pollution may have important  
526 consequences on grassland functions, such as herbivore nutrition (Barbehenn et al., 2004),  
527 carbon sequestration and global change mitigation (Laliberté and Tylianakis, 2012). In this  
528 global context, understanding the mechanisms of ryegrass-xenobiotic interactions is of  
529 primary importance.

530

### 531 ***Comparative analysis reveals novel mechanisms of action of chemical stressors***

532 Metabolomics has been used in pesticide and bioregulator research to study modes of action  
533 of chemicals and associated metabolic responses (Grossmann et al., 2012). Most studies have  
534 been carried out on one chemical or a few close chemicals affecting known cellular targets.  
535 The present work showed that parallel metabolomic investigation of different chemical  
536 stressors was useful to discover common and specific response patterns, including novel  
537 effects that could not be inferred from commonly-known modes of action. General chemical  
538 stress responses consisted in a systematic or frequent increase of key amino acids, among  
539 which N-rich amino acid Asn, branched-chain amino acids and Lys, and in a systematic  
540 decrease of glycerate and Ala (Figs 4, 5). Under chemical stress, *Lolium perenne* underwent  
541 metabolism reorganization rather than stress response induction, commonly characterized by  
542 stress metabolite accumulation (Fig. 6). None of the key metabolites involved in general  
543 chemical stress responses was significantly correlated to root or leaf physiological parameters

544 (Supplementary Table S2), thus suggesting that physiological responses may be more related  
545 to specific actions of each chemical stressor rather than to a general metabolic response.

546 Besides common responses, chemical stressors induced specific metabolite variations (Table  
547 1), thus highlighting potential novel modes of action. Glyphosate, inhibitor of the shikimate  
548 pathway (Gomes et al., 2014), generated large accumulation of quinate in roots, contrasting  
549 with its absence of effects on aromatic amino acid levels (Phe, Tyr). Maintenance of these  
550 EPSPS inhibition markers could be ascribed to low level of glyphosate exposure (Serra et al.,  
551 2013). However, quinate accumulation in glyphosate-treated pea seedlings has been reported  
552 to result from glyphosate-induced modification of carbon flux in the direction of quinate  
553 synthesis (Orcaray et al., 2010). Glyphosate toxicity could originate from high quinate level in  
554 root, associated with shikimate pathway deregulation, leading to shoot and root growth  
555 inhibition (Orcaray et al., 2010; Zulet et al., 2013). This characteristic quinate accumulation  
556 was maintained under glyphosate-tebuconazole mixture, which resulted in root growth  
557 inhibition. Glyphosate also affects cell division in sea urchin by impeding activation of cell  
558 cycle regulator CDK1/cyclin B (Marc et al., 2002). Since CDKs and cell cycle checkpoints  
559 are universal in eukaryotes, root growth inhibition of *Lolium perenne* under glyphosate  
560 treatment may reflect action of glyphosate on root meristem activity and cell division. AMPA  
561 was also found to inhibit root growth (Fig. 1). However, analysis of related metabolic profiles  
562 indicated different modes of action in comparison to its parent compound glyphosate, since  
563 AMPA led to large quinate depletion in leaves (Fig. 5, Supplementary Figure S2). Such  
564 quinate variations could be due to shikimate pathway deregulation, or redistribution of carbon  
565 by this highly translocable carbon form (Orcaray et al., 2010). AMPA also led to arabinose  
566 decrease in roots (Fig. 5), clearly highlighting cell wall metabolism disturbance with potential  
567 significant impacts on root growth (Fig. 1). Given the structural analogy of AMPA with the  
568 amino acid Gly (Serra et al., 2013; Gomes et al., 2014), cell wall disturbance could originate  
569 from AMPA-induced imbalance in glycine-rich proteins (GRPs) in cell walls. GRPs are  
570 induced under stress and are hypothesized to interact with signalling pathways, thus  
571 suggesting a potential role as cell wall structure regulator (Caffall and Mohnen, 2009).

572 Regarding *Lolium* metabolic profiles, AMPA altered Gly metabolism dynamics as previously  
573 reported (Serra et al., 2013). However, whereas it led to Gly depletion in *Arabidopsis thaliana*  
574 (Serra et al., 2013), it globally increased Gly levels in *Lolium perenne* (Fig. 5, Supplementary  
575 Figure S2).

576 Metabolic profiles induced by some chemical stresses confirmed previously reported modes  
577 of action. Copper treatment induced, under direct exposure, TCA depletion in roots, which

578 could originate from increasing energy demand for ATP-synthase and ATPase-dependent  
579 copper exclusion (Li et al., 2013; Lin et al., 2013), as well as from release of citrate- and  
580 succinate-containing root exudates for metal complexation (Meier et al., 2012).  
581 Tebuconazole, which decreases gibberellin levels by interacting with cytochrome P450 in the  
582 phytosterol biosynthesis pathway (Child et al., 1993; Lamb et al., 2001), also affected TCA  
583 levels (Table 1), as described by Ribeiro et al. (2012) in the case of paclobutrazol, a  
584 gibberellin biosynthesis inhibitor. TCA levels however varied differently, increasing in  
585 *Arabidopsis thaliana* leaves under paclobutrazol, being relatively unchanged in *Arabidopsis*  
586 *thaliana* seedlings under tebuconazole (Serra et al., 2013), and decreasing in *Lolium perenne*  
587 root under tebuconazole, thus showing species-specific triazole responses. Triazoles have  
588 been reported to induce abiotic stress tolerance (Horn et al., 2013), triazole-induced inhibition  
589 of gibberellin potentially affecting sugar-related signalling pathways and growth regulation  
590 (Child et al., 1993; Lamb et al., 2001). Tebuconazole was hypothesized to improve mRNA  
591 and protein stability and to induce compatible solute accumulation, leading to stress tolerance  
592 (Horn et al., 2013). Despite root growth inhibition, maintenance of high levels of pigment  
593 contents was found for T and GT treatments in comparison to G treatment (Fig. 1).  
594 Nevertheless, no clear accumulation of compatible solutes was observed (Fig. 5,  
595 Supplementary Figure S2), suggesting that interactions between chemicals may interfere with  
596 expected positive effects of tebuconazole. Indeed, effects of glyphosate-tebuconazole mixture  
597 likely resulted in a mix of deleterious effects of single chemicals, mainly characterized by  
598 inhibition of root and leaf growth associated with quinate and Asn accumulation, and TCA  
599 depletion (Figs 1, 4, 5, Supplementary Figure S2). Finally, increase of Ser levels by  
600 fluoranthene (Fig. 5) was in accordance with increase of glutathione levels reported by  
601 Kummerová et al. (2013) in pea and maize. In fact, fluoranthene, and more largely PAHs,  
602 generate, particularly in the meristem zones, reactive oxygen species, which must be  
603 detoxified (Zezulka et al., 2013; Kreslavski et al., 2014). Oxidation of membrane lipids by  
604 PAHs (Kreslavski et al., 2014) may lead to membrane disturbance that could explain the  
605 osmotic stress response observed in *Lolium* under fluoranthene treatment. This osmotic stress  
606 response was characterized by root growth enhancement (Fig. 1A, E) and leaf Pro  
607 accumulation (Fig. 5), as observed in *Lolium* seedlings under pyrene treatment (Chigbo and  
608 Batty, 2012), and by arabinose variations, which may reflect modifications of cell wall pectic  
609 fraction (Mustard and Renault, 2004). Association of fluoranthene and tebuconazole  
610 treatments into a cyclic-compound response under conditions of direct exposure (Fig. 3C,  
611 most intense physiological effects) may also be related to oxidative stress. Indeed, this

612 association was characterized by accumulation of the antioxidant compound putrescine  
613 (Scandalios, 2005), while fluoranthene generates reactive oxygen species (Kreslavski et al.,  
614 2014) and tebuconazole induces antioxidant systems (Zhang et al., 2010).

615

616 ***Reorientation of carbon and nitrogen metabolism is a major feature of chemical stress***  
617 ***response***

618 Our results showed that modifications in carbon and nitrogen metabolism were key factors in  
619 subtoxic chemical stress responses: (i) N-rich Asn was highly accumulated under most of the  
620 chemical stress conditions (Fig. 5, Supplementary Figure S2), (ii) Asn levels in roots were  
621 negatively correlated with leaf length (Fig. 6), (iii) Asn levels were positively correlated with  
622 levels of other amino acids (Fig. 7). In parallel, levels of either Glc, Fru or Suc decreased in  
623 response to each of the chemical stressors in at least one of the chemical stress conditions  
624 (Figs 4, 5; Supplementary Figure S2). Such differential dynamics of Glc, Fru and Suc could  
625 reflect modifications of carbon balance and carbon utilization, which were also suggested by  
626 effects of chemical stress on growth (Fig. 1) and on energy metabolism (Fig. 4). Further  
627 studies should determine whether changes of carbon balance entail higher synthesis of storage  
628 carbohydrates, as has been shown in *Lolium perenne* under other conditions of abiotic stress  
629 (Amiard et al., 2003).

630 Accumulation of Asn has generally been associated with different conditions of abiotic stress  
631 (mineral deficiencies, drought, salt, toxic metals) where the plant is unable to support normal  
632 protein synthesis (Lea et al., 2007; Maaroufi-Dguimi et al., 2011). Jia et al. (2001) have  
633 demonstrated that metsulfuron-methyl herbicide stress led to impairment of nitrogen  
634 metabolism and increase of Asn levels in soybean. Relationships between nitrogen  
635 metabolism and soluble carbohydrates have been shown in leaves of *Lolium perenne*, where  
636 nitrogen deficiency was associated with lower sucrose content (Lattanzi et al., 2012). *Oryza*  
637 *sativa* genotypes with contrasting tolerance to zinc deficiency and bicarbonate excess also  
638 exhibit decrease of Glc and Fru and increase of Asn, Asp, Gln, Val and Ile (Rose et al., 2012).  
639 The specific effects of chemical stressors therefore occur in association with perturbations of  
640 carbon-nitrogen homeostasis and trans-regulation of global amino acid metabolism, which  
641 may be part of global and common responses to environmental stresses.

642 Asn is important for nitrogen storage and transport from sources to sinks, especially under  
643 stress conditions of carbon limitation (Lam et al., 1998). Asparagine synthetase genes that are  
644 involved in Asn synthesis are regulated by levels of carbohydrates (Lam et al., 1998; Foito et  
645 al., 2013). Jia et al. (2001) suggested that Asn could be a signalling molecule involved in

646 sensing nitrogen status. Moreover, Asn has been characterized as an ammonia detoxification  
647 product (Lam et al., 1998; Lea et al., 2007). It would thus be important to determine whether,  
648 under conditions of subtoxic chemical stress, nitrogen mobilization is associated with  
649 ammonia toxicity and to what extent Asn accumulation plays a signalling role in orchestrating  
650 metabolic adjustments.

651 Asn, which is an amino group donor for synthesis of photorespiratory intermediate Gly, was  
652 positively correlated to Ser (Fig. 7). This was also the case for Ala, another amino group  
653 donor for photorespiration. Asn accumulation under chemical stress and such metabolic and  
654 functional correlations pointed out to involvement of photorespiratory pathway regulation in  
655 chemical stress responses in *Lolium perenne*, in line with previously-described effects of  
656 environmental stresses on other plant species (Ros et al., 2013), and highlighted the  
657 importance of Ser, which was recently described as a metabolic signal for transcriptional  
658 control of photorespiratory pathway genes in *Arabidopsis* (Timm et al., 2013).  
659 Photorespiration metabolism and nitrogen status, which are closely connected and co-  
660 regulated (Florian et al., 2013), are therefore likely to play important roles in responses to  
661 chemical stress in *Lolium perenne*, thus emphasising the need to improve current knowledge  
662 on photorespiratory processes in C3 grasses. Perturbations of nitrogen nutrition affect leaf  
663 growth of *Lolium perenne* (Kavanová et al., 2008). Because of the negative correlations  
664 between leaf growth and Asn and Ser levels in roots (Fig. 6), it would be important to  
665 investigate potential involvement of these two amino acids in root-shoot signalling, especially  
666 under chemical stress conditions primarily affecting root systems. It was noteworthy that a  
667 major common response to all of the chemical stressors was the depletion of two  
668 photorespiration-related compounds, Ala and glycerate (Florian et al., 2013). Given the  
669 importance of photorespiration increase in abiotic stress responses (Voss et al., 2013), this  
670 depletion may indicate potential perturbations of adaptive mechanisms in chemically-stressed  
671 ryegrass.

672

673 ***Coordination of chemical stress responses is associated with complex networks of***  
674 ***correlations between amino acid and soluble sugar dynamics***

675 The important metabolic changes described above were shown to be strongly interconnected  
676 (Figs 7, 8), thus indicating underlying mechanisms of regulation for coordination of amino  
677 acid, photorespiration and carbohydrate dynamics under conditions of subtoxic stress. As  
678 shown in Fig. 7, all of the metabolic response clusters were interconnected through significant  
679 correlations that converged towards cluster E, especially towards Ser, which showed an

680 extended network of positive correlations (Fig. 7), and towards Tre, which showed an  
681 extended network of negative correlations (Figs 7, 8). Ser was significantly correlated  
682 (Supplementary Table S3) with Glc ( $r = 0.76$ ) and with Suc ( $r = 0.74$ ). The dynamics of  
683 soluble sugars, Glc-6-P and Fru-6-P was structured as a Suc/Glc nexus in interaction with a  
684 Fru/Fru-6-P/Glc-6-P/Tre nexus, both nexus being centered on a negative Glc/Tre correlation  
685 (Fig. 8). All of these relationships suggested that stress-induced physiological and metabolic  
686 changes (Figs 6, 7) could be modulated by differential dynamics and interconversion of  
687 soluble sugars. The central position of these amino acid and soluble carbohydrate correlations,  
688 especially those involving Ser and Tre, revealed novel aspects of chemical stress responses  
689 that are seldom taken into account. Finally, the integration of Glc-6-P regulation in the  
690 network of soluble sugar dynamics could be related to the stability and buffering of Glc-6-P  
691 levels under subtoxic chemical stress (Fig. 5). The regulation of this stability may be an  
692 important determinant of the long-term adjustment of *Lolium perenne* to chemical stress (Fig.  
693 2).

694 Such metabolic and physiological correlations can result from combinations of metabolic,  
695 regulatory and signalling relationships (Obata and Fernie, 2012). Glc-6-P is a major metabolic  
696 intermediate (Valluru and Van den Ende, 2011; Schluepmann et al., 2012) and an allosteric  
697 regulator of enzyme activities (Toroser et al., 2000). Glc-6-P can interact with signalling  
698 systems, as substrate for the synthesis of the signalling metabolite Tre-6-P (Lunn et al., 2014;  
699 Yadav et al., 2014) and as potential inhibitor of the energy sensor SNF1 (sucrose non-  
700 fermenting 1)-related kinase 1 (SnRK1) (Toroser et al., 2000; Valluru and Van den Ende,  
701 2011; Nunes et al., 2013). Soluble carbohydrates involved in the central correlations described  
702 above (especially Glc, Suc, Tre) have been associated with signalling networks involving  
703 hexokinases (Granot et al., 2014), SnRK1 (Baena-González and Sheen, 2008; Dietrich et al.,  
704 2011; Lunn et al., 2014) or the target-of-rapamycin (TOR) kinase (Lastdrager et al., 2014;  
705 Xiong and Sheen, 2014). Ser is a metabolic signal for transcriptional control (Timm et al.,  
706 2013). All of these signalling regulations provide mechanisms of integration between growth,  
707 metabolism and stress responses in cross-talk with hormonal regulations (Valluru and Van  
708 den Ende, 2011; Xiong and Sheen, 2014). Their major effects consist in regulation of genes  
709 involved in catabolism (proteolysis, amino acid catabolism, sugar degradation, lipid  
710 mobilisation), especially of genes involved in Asn and BCAA metabolisms (Valluru and Van  
711 den Ende, 2011). As shown in the present work (Figs 4, 6, 7), changes of Asn and BCAA  
712 metabolisms were indeed important features of chemical stress responses in *Lolium perenne*.  
713 Further work is therefore needed to analyse relationships between regulation of amino acid

714 metabolism genes and SnRK1 functioning under conditions of subtoxic chemical stress.

715

## 716 **Conclusions**

717 *Lolium perenne* was therefore shown to undergo major metabolic changes under conditions of  
718 adjustment to chemical stresses and in the absence of major physiological and developmental  
719 alterations. These changes resulted in major reorientations of central carbon (sucrose, glucose,  
720 fructose) and nitrogen (asparagine, branched-chain amino acids, photorespiration)  
721 metabolisms. Some of these changes showed correlations with slight effects on physiological  
722 traits such as root length and leaf length. The extent of these metabolic responses did not  
723 however translate into long-term loss of fitness, thus reflecting adaptive flexibility of  
724 metabolism rather than deregulation of cellular and metabolic homeostasis. Moreover, this  
725 metabolic flexibility was shown to occur in response to diverse xenobiotic and heavy-metal  
726 stresses, indicating common underlying response mechanisms. These mechanisms were  
727 associated with complex correlation networks between amino acids and soluble sugars and  
728 with contrasted dynamic ranges of responses among closely-related metabolites.  
729 Carbohydrate metabolites such as sucrose showed important variations, in line with other  
730 situations of abiotic stress, whereas other key metabolites such as Glc-6-P followed a pattern  
731 of limited variations, which may thus be important links between metabolic flexibility and  
732 long-term tolerability to subtoxic chemical stresses. It was also noteworthy that parallel  
733 metabolomic analysis of diverse chemical stresses revealed common response patterns in a  
734 background of differing modes of action and stressor-specific mechanisms, thus suggesting  
735 that general lines of defence were induced under low-intensity chemical stress. However,  
736 given the wide range of ecosystemic functions associated with grasslands and pastures,  
737 higher-level impacts of metabolic changes in chemically-stressed *Lolium perenne* cannot be  
738 excluded.

739 **SUPPLEMENTARY DATA**

740

741 The following supplementary data are available in the online version of this article.

742 **Figure S1.** Principal component analysis (PCA) and hierarchical classification of global  
743 metabolic responses to chemical stressors.

744 **Figure S2.** Relative variations of levels of metabolites in *Lolium perenne* under chemical  
745 stress in comparison to control conditions (Log<sub>2</sub> ratio).

746 **Method S1.** Metabolite profiling method.

747 **Table S1.** Plant metabolites analysed by GC/MS method.

748 **Table S2.** Organ-specific physiological trait-metabolite and metabolite-metabolite correlation  
749 matrix.

750 **Table S3.** Global physiological trait-physiological trait, physiological trait-metabolite and  
751 metabolite-metabolite correlation matrix.

752 **Table S4.** Global and modality-specific Glc-6-P-metabolite and Fru-6-P-metabolite  
753 correlation matrix.

754

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755

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**Table 1. Major metabolic effects of subtoxic levels of chemical stressors on *Lolium perenne*.**

Chemical stressors and stress conditions are described in Figure 1. Physiological effects are shown in Figure 1. Major metabolic changes were identified from response patterns shown in Fig. 5 and Supplementary Figure S2.

| <b>Stressor</b>                  | <b>Physiological effects</b>                         | <b>Major metabolic modifications</b>   | <b>Organ</b> | <b>Potential impact</b>  |
|----------------------------------|--|--|--------------|--|
| <b>AMPA</b>                      | Root growth inhibition                               | Quinate depletion<br>Asn accumulation  | Leaf         | N metabolism disturbance<br>Stress-induced proteolysis   |
|                                  |  | Arabinose depletion  | Root         | Cell wall metabolism disturbance   |
| <b>Glyphosate</b>                | Root growth inhibition                               | Quinate accumulation   | Root         | Quinate toxicity   |
| <b>Glyphosate + Tebuconazole</b> | Leaf growth inhibition<br><br>Root growth inhibition | Quinate accumulation<br>Asn accumulation   | Root         | Quinate toxicity<br>N metabolism disturbance<br>Stress-induced proteolysis                                     |
| <b>Tebuconazole</b>              | Leaf growth inhibition                               | Asn accumulation   | Leaf         | N metabolism disturbance<br>Stress-induced proteolysis   |
|                                  | Root growth inhibition                               | Citrate depletion<br>Asn accumulation  | Root         | TCA limitation<br>N metabolism disturbance<br>Stress-induced proteolysis                                       |
| <b>Fluoranthene</b>              | Leaf growth inhibition                               | Fru accumulation<br>Glc accumulation<br>Asn accumulation<br>Ser accumulation<br>Pro accumulation | Leaf         | C metabolism modification<br>N metabolism disturbance<br>Stress-induced proteolysis<br>Osmotic stress response |
|                                  | Root growth enhancement                              | Fru accumulation<br>Glc accumulation<br>Ser accumulation<br>Arabinose accumulation               | Root         | C metabolism modification<br>N metabolism disturbance<br>Cell wall metabolism modification                     |
| <b>Copper</b>                    | Root growth inhibition                               | Citrate depletion<br>Succinate depletion   | Root         | TCA limitation<br>Exogenous metal complexation   |

## FIGURE LEGENDS

**Figure 1. Effects of subtoxic levels of chemical stressors on *Lolium perenne*.** AMPA (A), glyphosate (G), tebuconazole (T), glyphosate plus tebuconazole (GT), fluoranthene (F) and copper (Cu) were tested. Values (mean  $\pm$  SEM) of root length (A, E), leaf length (B, F), chlorophyll and carotenoid levels (C, G) and PSII efficiency ( $F_v/F_m$ ) (D, H) are shown. In transfer experiments, seedlings were grown during 7 d under control conditions and then transferred to fresh medium in the presence of chemical stressors for 4 d (A, B, C, D). In direct exposure experiments, seedlings germinated and grew in the presence of chemical stressors for 11 d (E, F, G, H). Statistical analysis between means were carried out using the Mann-Whitney-Wilcoxon test. Statistical significance of differences ( $P \leq 0.05$ ) between treatments is indicated by different letters above bars.

**Figure 2. Long-term effects of subtoxic levels of chemical stressors on *Lolium perenne*.** Chemical stressors are described in Fig. 1. Treatments consisted of direct exposures where seedlings germinated and grew in the presence of chemical stressors for 30 d. Values (mean  $\pm$  SEM) of root length after 30 d of growth are shown. Statistical analyses were performed as described in Fig. 1.

**Figure 3. Principal component analysis (PCA) and hierarchical classifications of metabolic responses of *Lolium perenne* to chemical stressors according to condition of exposure and to plant organ.** Chemical stress treatments are described in Fig. 1. PCA was carried out on the correlation matrix of averages of metabolite levels measured for two conditions of stress exposure and in two different organs: root (A, TR: root after transfer exposure) and leaf (B, TL: leaves after transfer exposure) metabolite levels for transfer experiments; root (C, DR: root after direct exposure) and leaf (D, DL: leaves after direct exposure) metabolite levels for direct exposure experiments. Position of chemical treatments, distribution of metabolic parameters on the first plane (Dim1 and Dim2) and corresponding hierarchical classifications are shown. Treatment groups obtained by hierarchical classifications are circled.

**Figure 4. Heatmap and average linkage hierarchical clustering of chemical stress-responsive metabolites.** Heatmap and hierarchical tree result from relative levels of the 42 metabolites under study [ $\log_2(\text{metabolite level under stress}/\text{metabolite level in control})$ ]

according to roots (R) and leaves (L), to six chemical stress treatments (A: AMPA, G: glyphosate, GT: glyphosate plus tebuconazole, T: tebuconazole, F: fluoranthene, Cu: copper) and to two stress exposures [transfer experiment (T) and direct exposure (D)]. The first two letters correspond, respectively, to the type of exposure and the type of tissue; the letter after the underscore corresponds to stress treatment. Metabolites are separated in different clusters (named A to H) according to hierarchical classification.

**Figure 5. Effects of subtoxic levels of chemical stressors on root and leaf metabolite concentrations of *Lolium perenne*.** Effects of AMPA (A), glyphosate (G), tebuconazole (T), glyphosate plus tebuconazole (GT), fluoranthene (F) and copper (Cu) on *Lolium perenne* are shown for direct growth, which induced greater impacts on all traits (Fig. 1). Values (mean  $\pm$  SEM) of root metabolite levels and leaf metabolite levels in nmol mg<sup>-1</sup> of DW are shown respectively in grey bars and light bars. For each metabolite, the corresponding cluster (as defined in Fig. 4) is indicated into brackets.

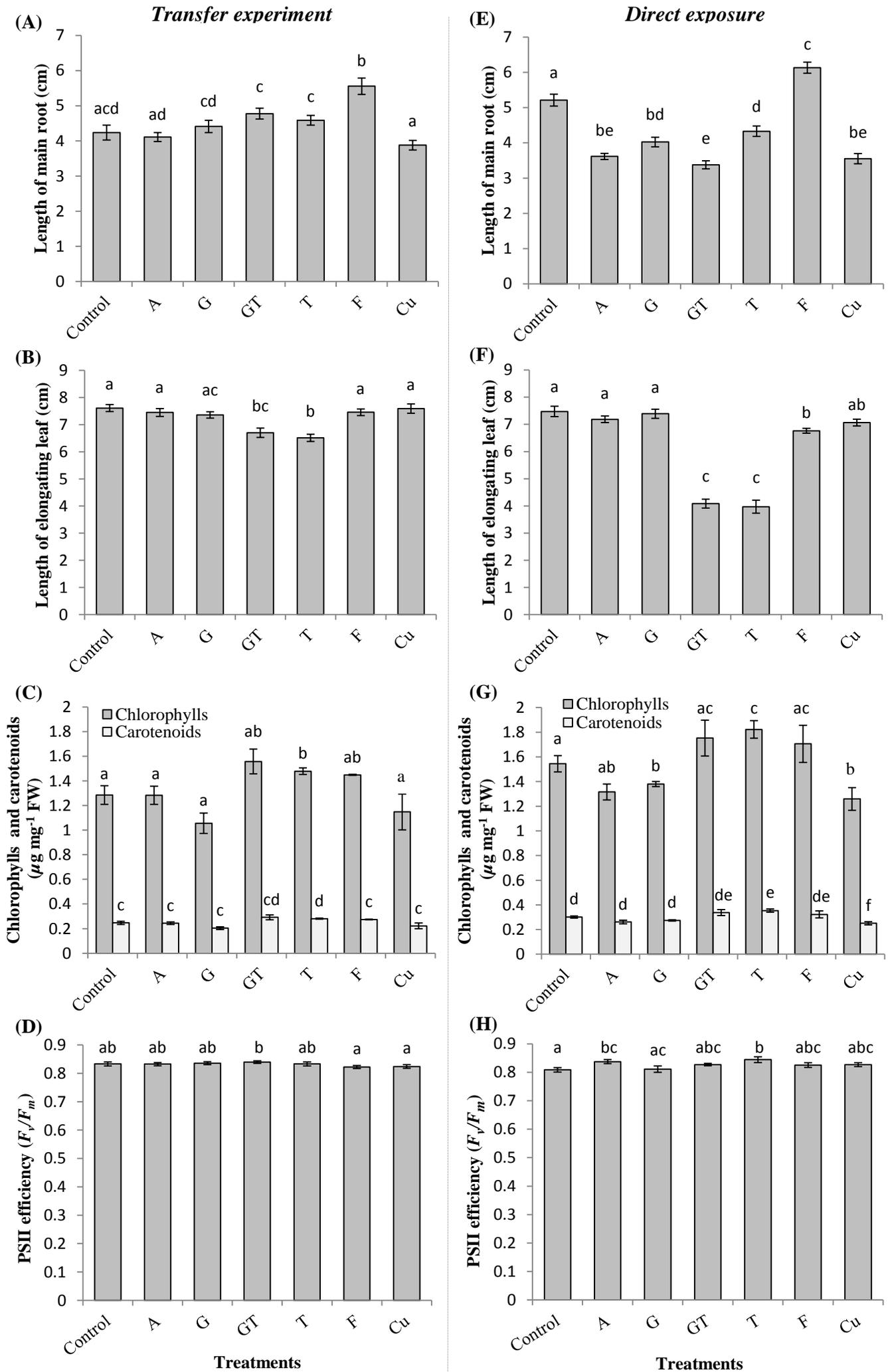
**Figure 6. Involvement of metabolic correlations in the effects of chemical stressors.** Organ-specific physiological trait-metabolite correlations are shown. For each metabolite, the corresponding cluster (as defined in Fig. 4) is indicated into brackets. For metabolites of cluster B, graphs correspond to underlined metabolites. Inserted graphs represent relative variations of physiological traits or metabolites against control conditions [ $\log_2(\text{ratio})$ ]. Each inserted graph shows, from left to right, changes in trait responses and in metabolite levels under direct growth and transfer exposure for each treatment (A: AMPA, G: glyphosate, GT: glyphosate plus tebuconazole, T: tebuconazole, F: fluoranthene, Cu: copper). The Pearson correlation coefficient (r) for each pair of parameters is shown. Asterisks indicate significant correlations: \*P $\leq$ 0.05, \*\*P $\leq$ 0.001, \*\*\*P $\leq$ 0.0001.

**Figure 7. Correlations of chemical-stress-related metabolic networks with carbohydrate, tricarboxylate and photorespiratory metabolites.** Red and green filled arrows represent, respectively, positive and negative Pearson correlations between directly-connected metabolites; red and green hashed arrows represent, respectively, positive and negative Pearson correlations between indirectly-connected metabolites. For each cluster, a typical metabolite is shown. For each metabolite, the corresponding cluster (as defined in Fig. 4) is indicated into brackets. Shaded metabolites are correlated to physiological parameters (Fig. 6). BCAA: Branched-chain amino acids; PP: photorespiratory pathway; TCA: tricarboxylic

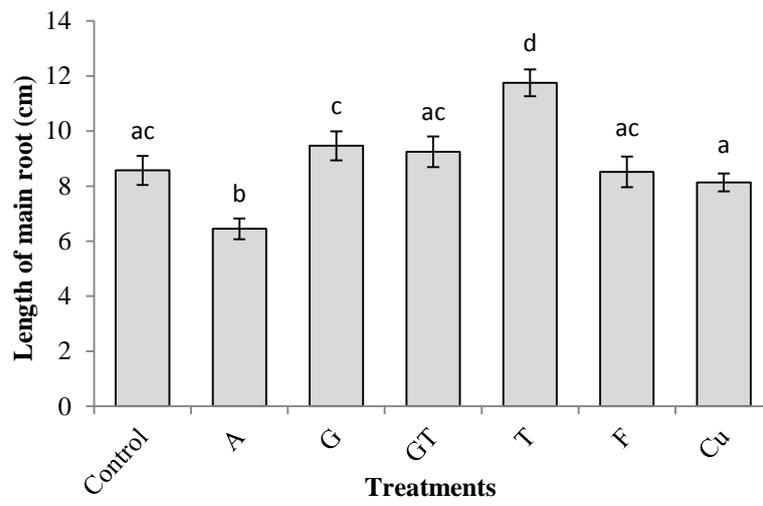
acid. The Pearson correlation coefficient ( $r$ ) for each pair of metabolites is shown. Asterisks indicate significant correlations: \* $P \leq 0.05$ , \*\* $P \leq 0.001$ , \*\*\* $P \leq 0.0001$ .

**Figure 8. Integration of soluble sugar dynamics and Glc-6-P regulation.** Red and green filled arrows represent, respectively, positive and negative Pearson correlations between directly-connected metabolites in a pathway; red and green hashed arrows represent, respectively, positive and negative Pearson correlations between indirectly-connected metabolites. Colorless arrows represent metabolic pathways without significant Pearson correlation. For each metabolite, the corresponding cluster (as defined in Fig. 4) is indicated into brackets. Shaded metabolites are correlated to physiological parameters (Fig. 6). The Pearson correlation coefficient ( $r$ ) for each pair of metabolites is shown. Asterisks indicate significant correlations: \* $P \leq 0.05$ , \*\* $P \leq 0.001$ , \*\*\* $P \leq 0.0001$ .

**Figure 1.**

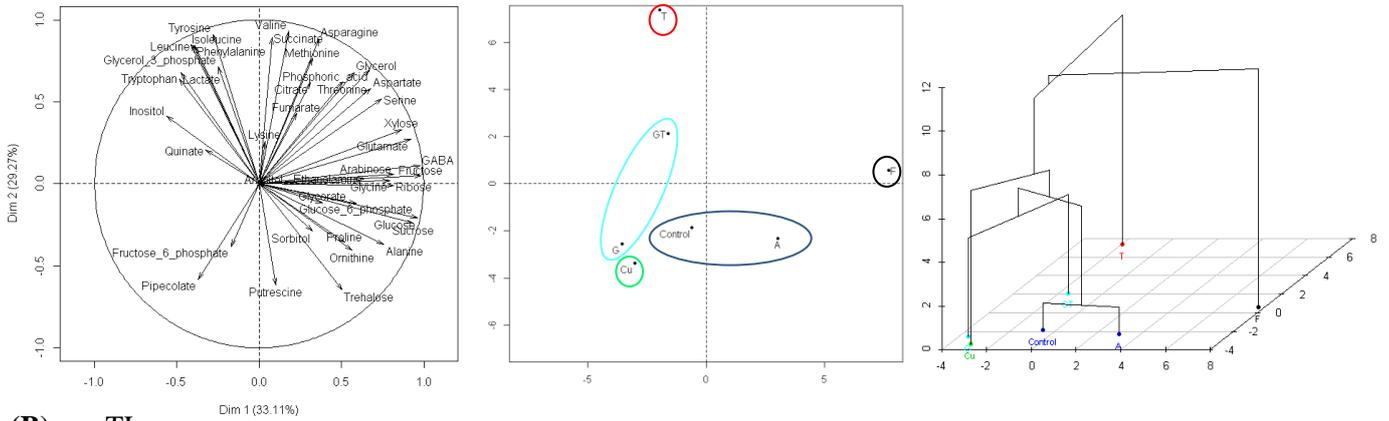


**Figure 2.**

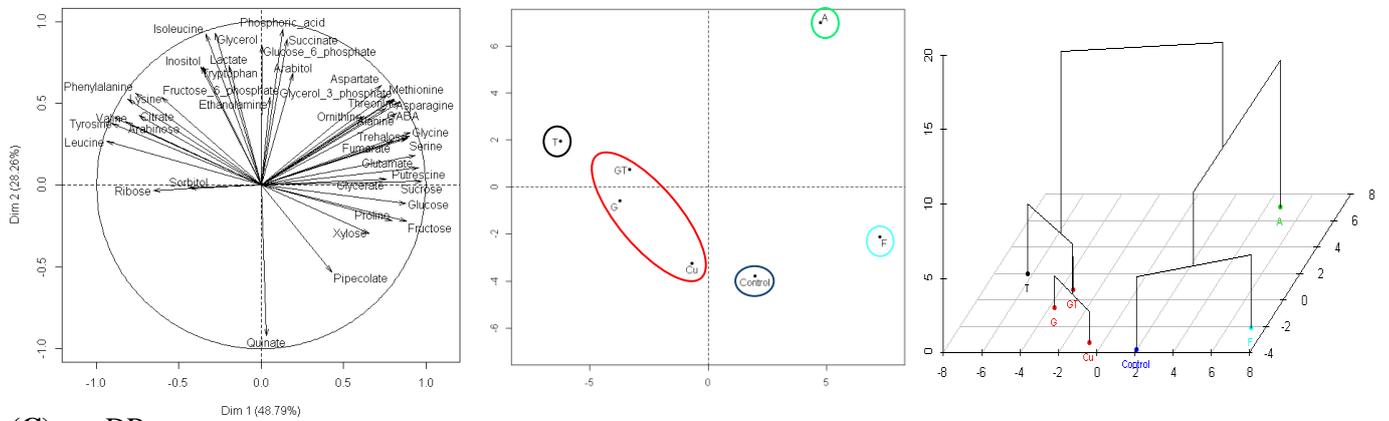


**Figure 3.**

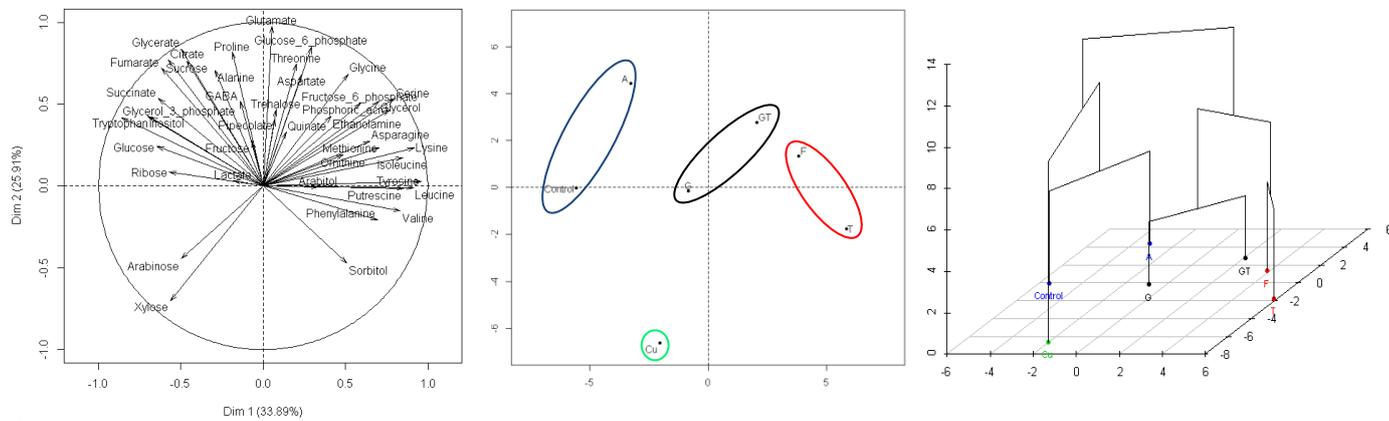
**(A) TR**



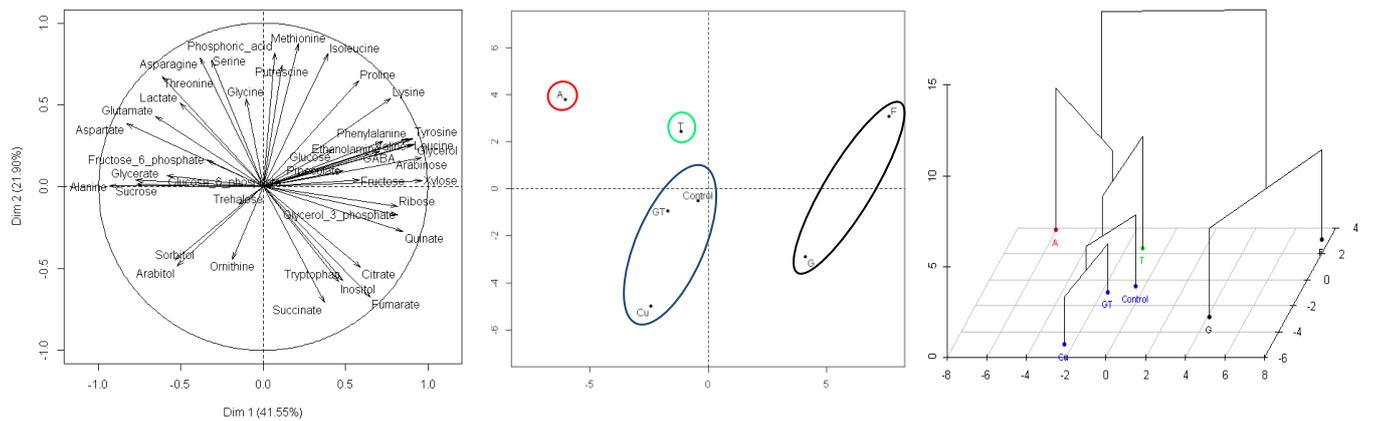
**(B) TL**



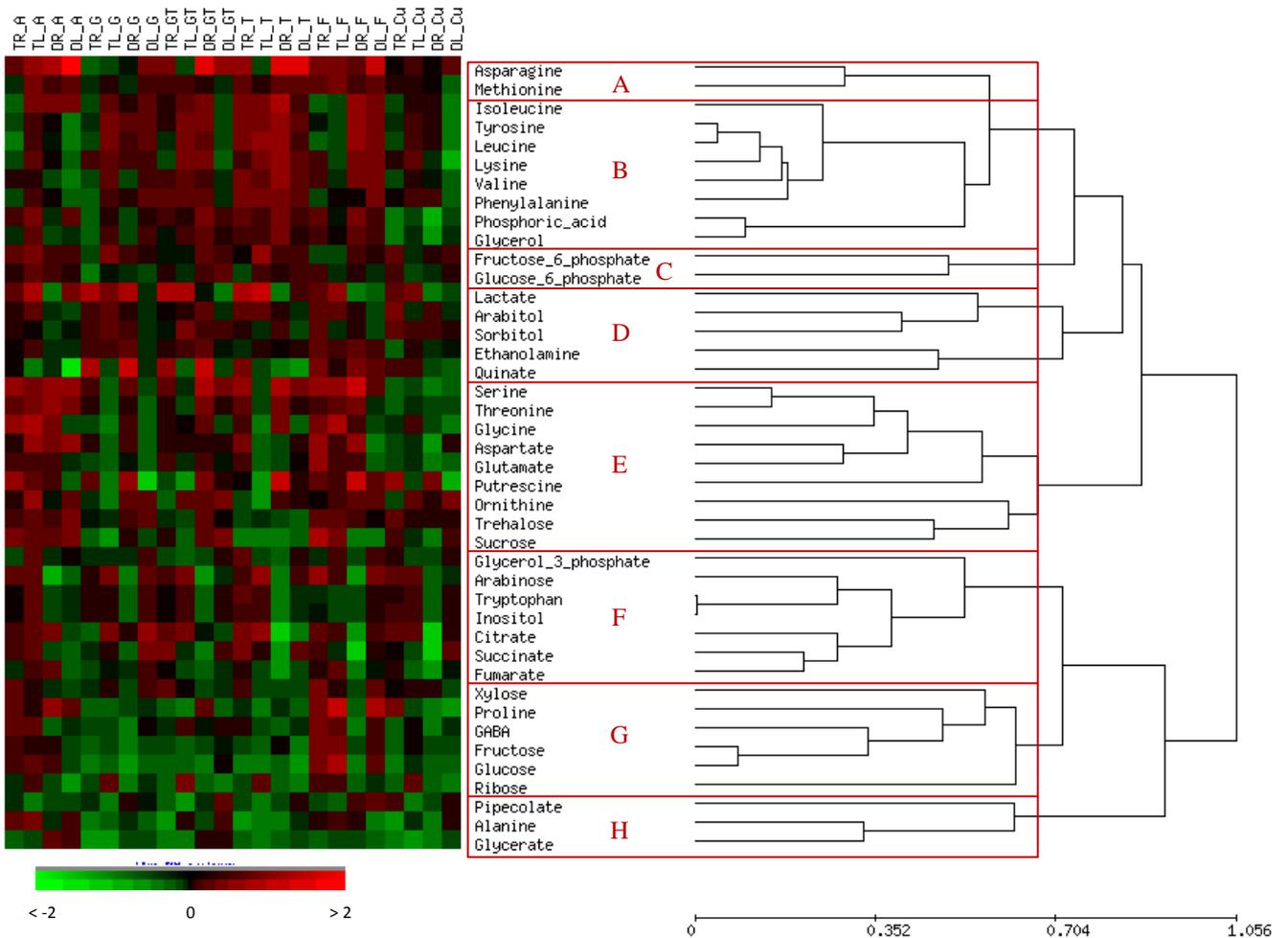
**(C) DR**



**(D) DL**



**Figure 4.**



**Figure 5.**

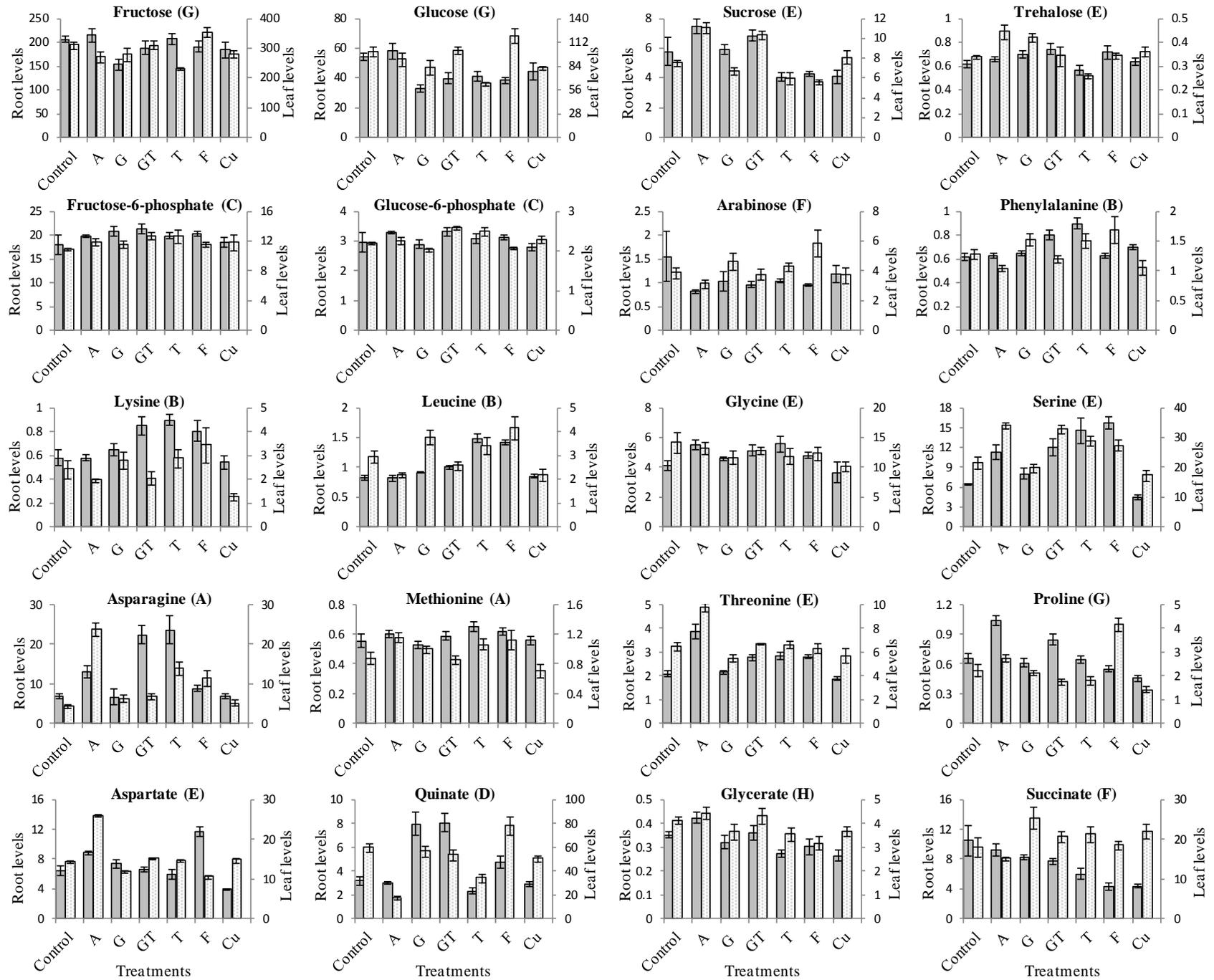


Figure 6.

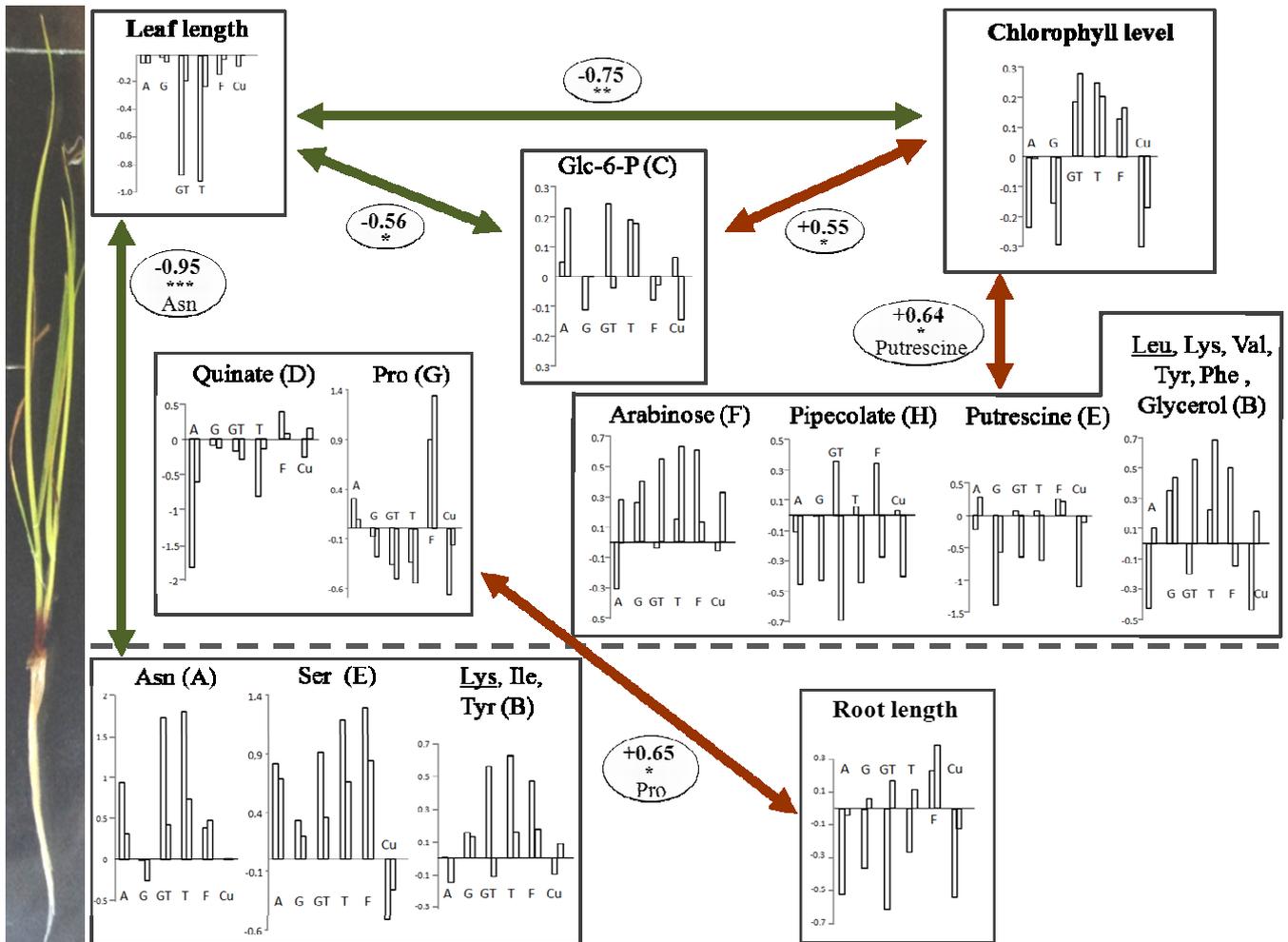


Figure 7.

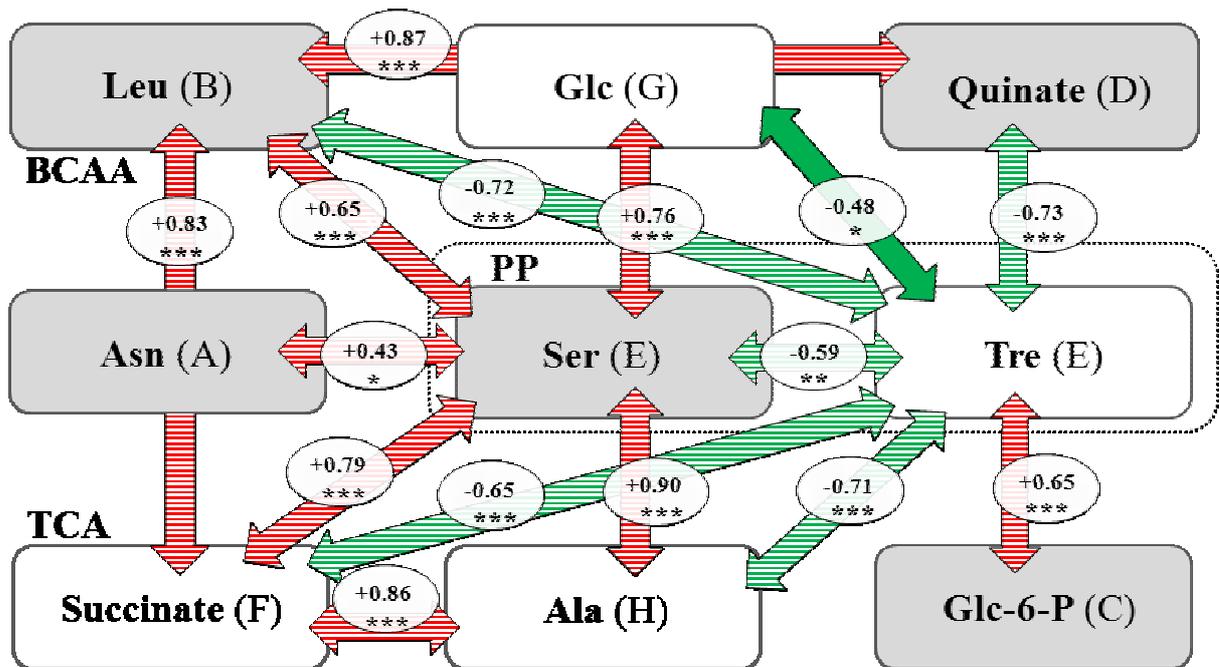
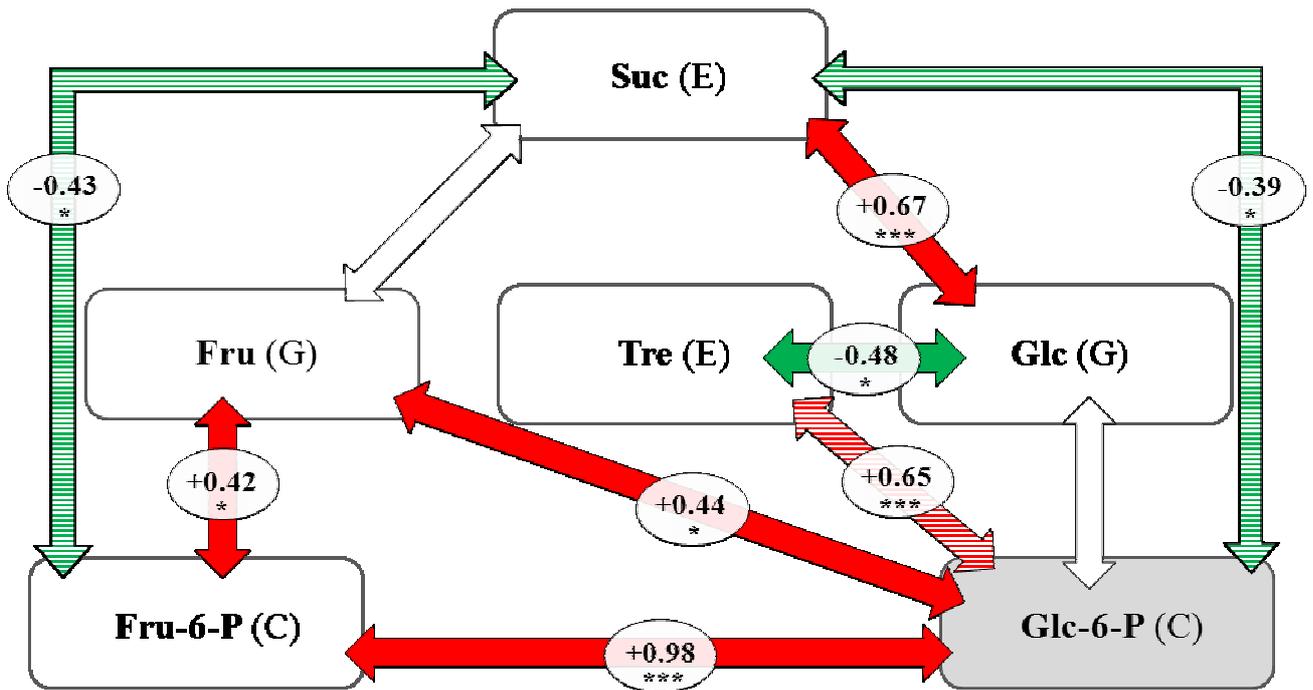


Figure 8.



**Manuscript Title:** Metabolic profiling of *Lolium perenne* shows functional integration of metabolic responses to diverse subtoxic conditions of chemical stress

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## **Supplementary Method S1: Metabolite profiling method**

### Metabolite derivatization

The dried aliquots were re-suspended in 30  $\mu$ L of 20 mg L<sup>-1</sup> methoxyamine-pyridine solution, and placed under automatic orbital shaking at 40°C for 1 h. Thirty  $\mu$ L of N-methyl-N-trimethylsilyl trifluoroacetamide were added and derivatization was conducted at 40°C for 1 h under agitation. All the derivatization process was automatized using a CTC CombiPal autosampler (GERSTEL GmbH and Co.KG, Mülheim an der Ruhr, Germany), thus ensuring identical derivatization time and process for all samples.

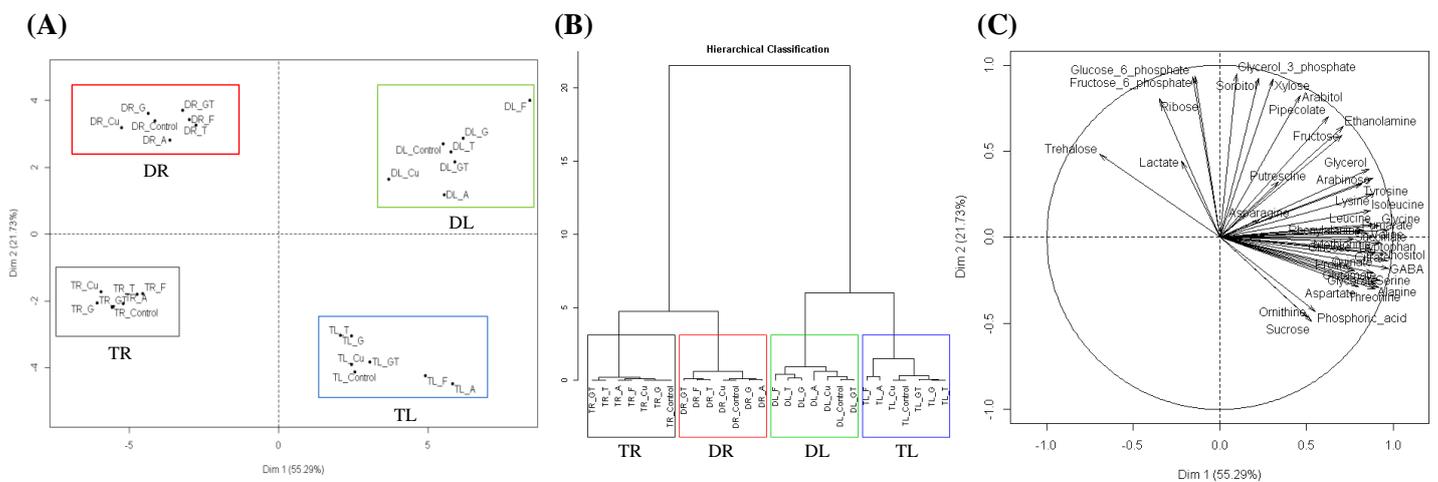
### Parameters of injection and of chromatography

The injector temperature was held at 250°C. The oven temperature ranged from 70 to 170°C at 5°C min<sup>-1</sup>, from 170 to 280°C at 7°C min<sup>-1</sup>, from 280 to 320°C at 15°C min<sup>-1</sup>. The oven then remained at 320°C for 4 min. A 30 m fused silica column (95% dimethyl siloxane, 5% phenyl polysilphenylene-siloxane, v/v) was used with helium as the carrier gas at a constant rate of 1 mL min<sup>-1</sup>. One microliter of each sample was injected using the split mode (25:1).

**Supplementary Table S1: Plant metabolites analysed by GC/MS method**

| <i>Amino acids</i>   | <i>Amines</i>        |
|----------------------|----------------------|
| Alanine              | Cadaverine           |
| Asparagine           | Dopamine             |
| Aspartate            | Ethanolamine         |
| Citrulline           | Putrescine           |
| GABA                 | Spermidine           |
| Glumataate           | Spermine             |
| Glycine              | Triethanolamine      |
| Isoleucine           | Tyramine             |
| Leucine              | <i>Nucleobase</i>    |
| Lysine               | Cytosine             |
| Methionine           | <i>Organic acids</i> |
| Ornithine            | Ascorbate            |
| Phenylalanine        | Citrate              |
| Proline              | Fumarate             |
| Serine               | Galactonolactone     |
| Threonine            | Glycerate            |
| Tryptophane          | Lactate              |
| Tyrosine             | Malate               |
| Valine               | Phosphoric acid      |
| <i>Oses</i>          | Pipecolate           |
| Arabinose            | Quinate              |
| Fructose             | Succinate            |
| Fructose-6-phosphate | <i>Polyols</i>       |
| Galactose            | Arabitol             |
| Galacturonic acid    | Erythritol           |
| Glucose              | Galactitol           |
| Glucose-6-phosphate  | Glycerol             |
| Maltose              | Glycerol-phosphate   |
| Mannose              | Inositol             |
| Ribose               | Mannitol             |
| Sucrose              | Sorbitol             |
| Trehalose            | Xylitol              |
| Xylose               |                      |

**Supplementary Figure S1: Principal component analysis (PCA) and hierarchical classification of global metabolic responses to chemical stressors.** Analysis was carried out on root (R) and leaf (L) metabolic responses of *Lolium perenne* to two types of chemical stress exposure [transfer experiment (T) and direct exposure (D)] to various chemical stressors as described in Fig. 1. PCA was carried out on the correlation matrix of averages of metabolite levels. The first two letters correspond, respectively, to the condition of stress exposure and the type of organ. The letter after the underscore corresponds to chemical treatment, as described in Fig. 1. The position of treatments (A), the corresponding hierarchical classification (B), and distribution of metabolic parameters on the first plane (Dim1 and Dim2; C) are shown. The four groups correspond to combinations of the two exposure modalities with the two organs: TR and TL for, respectively, root and leaves after transfer exposure, and DR and DL for, respectively, root and leaves after direct exposure.



**Supplementary Figure S2: Relative variations of the levels of metabolites in *Lolium perenne* under chemical stress in comparison to the control condition (Log2 ratio).**

