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# Nitrogen supply and cyanide concentration influence the enrichment of nitrogen from cyanide in wheat (*Triticum aestivum* L.) and sorghum (*Sorghum bicolor* L.)

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Stephen Ebbs, Dylan K Kosma, Elizabeth H Nielson, Marylou Machingura, Alan J M Baker, and Ian E Woodrow

1 **TITLE: Nitrogen supply and cyanide concentration influence the enrichment of**  
2 **nitrogen from cyanide in wheat (*Triticum aestivum* L.) and sorghum (*Sorghum bicolor***  
3 **L.)**

4

5 **Running head: Nitrogen supply influences enrichment of cyanogenic nitrogen**

6

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18 **Abstract**

19 Cyanide assimilation by the  $\beta$ -cyanoalanine pathway produces asparagine, aspartate, and  
20 ammonium, allowing cyanide to serve as alternate or supplemental source of nitrogen.  
21 Experiments with wheat and sorghum examined the enrichment of  $^{15}\text{N}$  from cyanide as a  
22 function of external cyanide concentration in the presence or absence of nitrate and/or  
23 ammonium. Cyanogenic nitrogen became enriched in plant tissues following exposure to  
24  $^{15}\text{N}$ -cyanide concentrations from 5 to 200  $\mu\text{M}$ , but when exposure occurred in the absence of  
25 nitrate and ammonium,  $^{15}\text{N}$  enrichment increased significantly in sorghum shoots at solution  
26 cyanide concentrations of  $\geq 50 \mu\text{M}$  and in wheat roots at 200  $\mu\text{M}$  cyanide. In an experiment  
27 with sorghum using  $^{13}\text{C}^{15}\text{N}$ , there was also a significant difference in the tissue  $^{13}\text{C}:^{15}\text{N}$  ratio,  
28 suggestive of differential metabolism and transport of carbon and nitrogen under nitrogen-  
29 free conditions. A reciprocal  $^{15}\text{N}$  labeling study using  $\text{KC}^{15}\text{N}$  and  $^{15}\text{NH}_4^+$  and wheat  
30 demonstrated an interaction between cyanide and ammonium in roots in which increasing  
31 solution ammonium concentrations decreased the enrichment from 100  $\mu\text{M}$  cyanide. In  
32 contrast, with increasing solution cyanide concentrations there was an increase in the  
33 enrichment from ammonium. The results suggest increased transport and assimilation of  
34 cyanide in response to decreased nitrogen supply and perhaps to ammonium supply.

35

36 Key words: cyanide, ammonium, nitrogen, wheat, sorghum, stable isotope

37 **INTRODUCTION**

38 Cyanide is easily recognizable as an inhibitor and metabolic uncoupler in biological systems.  
39 Most notably, the binding of cyanide to the mitochondrial cytochrome *c* oxidase blocks  
40 electron flow, impairing ATP production. Cyanide is also an inhibitor of electron carriers  
41 such as plastocyanin and a number of metalloenzymes, including superoxide dismutase,  
42 peroxidase, catalase, and Rubisco (Solomonson, 1981). Despite its inhibitory action at  
43 higher concentrations, cyanide at lower concentrations acts as an endogenous regulatory and  
44 signalling molecule in plants, influencing several processes associated with growth and  
45 development including plant resistance to viral attack (Grossmann, 1996), ethylene synthesis  
46 (Smith & Arteca, 2000), seed dormancy, and seed germination (Esashi *et al.*, 1996,  
47 Hasegawa *et al.*, 1995b, Maruyama *et al.*, 1996, Oracz *et al.*, 2008). Cyanide has also been  
48 postulated to influence germination indirectly via the mitochondrial alternative oxidase,  
49 reactive oxygen species, modulation of glycolysis and the pentose phosphate cycle, or the  
50 modification and/or turnover of certain proteins, carbohydrates, or other metabolites (Oracz  
51 *et al.*, 2008). The stimulation of germination in the presence of cyanide is accompanied by a  
52 significant increase in the concentration of amino acid in seeds (Esashi *et al.*, 1996,  
53 Hasegawa *et al.*, 1995b, Maruyama *et al.*, 1997). Cyanide is also known to regulate nitrogen  
54 metabolism as a competitive inhibitor of nitrogenase (Li, Burgess & Corbin, 1982) and as an  
55 inactivator of nitrate reductase (Barr *et al.*, 1995, Echevarria, Maurino & Maldonado, 1984,  
56 Solomonson & Barber, 1990, Somers *et al.*, 1983).

57

58 Plants are exposed to cyanide from both endogenous and exogenous sources. Cyanide is  
59 produced normally by several biochemical processes in plants. Cyanide is a co-product of

60 ethylene synthesis (Peiser *et al.*, 1984), produced in a 1:1 stoichiometric ratio with that  
61 gaseous plant growth regulating molecule. Cyanide is released during the hydrolysis of  
62 cyanogenic glycosides and glycolipids in those species that produce these compounds  
63 (Selmar, Grochowski & Seigler, 1990, Vetter, 2000) and from the glyoxylate oxime arising  
64 from photorespiration (Solomonson & Spehar, 1981). Exogenous cyanide can arise from  
65 both anthropogenic and natural sources. The primary sources of anthropogenic cyanide are  
66 mining discharges, organic chemical synthesis, plastics synthesis, electroplating, metal and  
67 aluminum works, and the manufactured gas industry (ATSDR 1997). Natural sources of  
68 cyanide in the soil and water environment include the decomposition of plant tissues  
69 containing cyanogenic glycosides (Widmer & Abawi, 2002) and bacterial cyanogenesis.  
70 Cyanogenesis has been reported in several different families of bacteria including  
71 *Chromobacterium*, *Anacystis*, *Nostoc*, *Plectonema* and certain free-living forms of *Rhizobium*  
72 (Antoun *et al.*, 1998, Castric, 1981, Gallagher & Manoil, 2001) and rhizospheric  
73 *Pseudomonas* spp. (Gallagher & Manoil, 2001, Kremer *et al.*, 1990, Laville *et al.*, 1998).  
74 Cyanide concentrations in excess of 100 mg kg DW<sup>-1</sup> soil have been reported in the  
75 rhizosphere of some plants colonized by cyanogenic bacteria (Kesler-Arnold & O'Hearn,  
76 1990, Owen & Zdor, 2001). Concentrations of cyanide in soils in proximity to industries that  
77 release cyanide can exceed 1,000 mg kg DW<sup>-1</sup> while concentrations in wastewaters may be  
78 an order of magnitude higher (Grosse, 1990, Henny, Hallock & Hill, 1994). Cyanide sorbs  
79 weakly to mineral phases in soils but more strongly to organic phases (Dzombak, Ghosh &  
80 Young, 2005), so the solubility of cyanide and the potential for plant uptake is greater in  
81 mineral soils and would approximate the total concentration.  
82

83 Plants detoxify cyanide by assimilating this molecule directly into primary metabolism via  
84 the  $\beta$ -cyanoalanine pathway. The first step of this pathway (Figure 1), mediated by  $\beta$ -  
85 cyanoalanine synthase (EC 4.4.1.9) and cysteine synthase (EC 4.2.99.8), replaces the  
86 sulfhydryl group on a cysteine molecule with cyanide, forming the nitrile cyanoalanine with  
87 concomitant release of hydrogen sulfide (Warrilow & Hawkesford, 1998, Watanabe *et al.*,  
88 2008). The subsequent step is mediated by an enzyme (E.C. 4.2.1.65) with both nitrilase and  
89 nitrile hydratase activity. The nitrilase activity results in the formation of asparagine while  
90 the nitrile hydratase forms aspartate and ammonium (Piotrowski, Schonfelder & Weiler,  
91 2001, Piotrowski & Volmer, 2006). Asparagine can be hydrolyzed by the two subtypes of  
92 asparaginase (E.C. 3.5.1.1) to aspartate and ammonium (Bruneau, Chapman & Marsolais,  
93 2006, Lea, Sodek & Parry, 2007). An additional benefit of this assimilatory pathway is that  
94 the nitrogen from the cyanide molecule can be incorporated directly into primary  
95 metabolism. For example, it is the  $\beta$ -cyanoalanine pathway that contributes to the  
96 aforementioned increase in amino acids pools through the assimilation of cyanide (Hasegawa  
97 *et al.*, 1995a, Hasegawa *et al.*, 1995b, Maruyama *et al.*, 1997).

98

99 While the production of asparagine and aspartate are directly associated with cyanide  
100 assimilation, the activity of the  $\beta$ -cyanoalanine pathway would provide plants with an  
101 additional source of ammonium. If true, this raises the question as to whether cyanide from  
102 the rhizosphere might be perceived by plants as an opportunistic source of nitrogen to  
103 augment, or as a substitute for, soil nitrate or ammonium. The research here examined the  
104 uptake and assimilation of cyanide by wheat (*Triticum aestivum*) and sorghum (*Sorghum*  
105 *bicolor*). The goal was to investigate the enrichment of cyanogenic nitrogen as a function of

106 cyanide concentration and as influenced by the presence or absence of other nitrogen sources.  
107 These species were used because the  $\beta$ -cyanoalanine pathway is active in both of these  
108 important crops (Goudey, Tittle & Spencer, 1989, Kosma, 2005, Wurtele, Nikolau & Conn,  
109 1984), yet they differ in the synthesis and turnover of cyanogenic glycosides. Wheat is only  
110 weakly cyanogenic and produces far lower concentrations of the cyanogenic glycoside  
111 dhurrin as compared to sorghum (Erb, Zinsmeister & Nahrstedt, 1981). The inclusion of  
112 sorghum was primarily to observe whether the response of sorghum as a highly cyanogenic  
113 plant species differed from wheat. Both species also harbor cyanogenic bacteria in their  
114 rhizosphere (Funnell-Harris, Pedersen & Marx, 2008, Mavrodi *et al.*, 2006). The majority of  
115 the literature on cyanide assimilation by plants emphasizes the importance of this pathway to  
116 cyanide detoxification, yet paradoxically, the cyanide concentrations plants are exposed to in  
117 the natural environment are far lower than those that are detrimental to plant metabolism.  
118 The more recent literature cited above has drawn attention to the regulatory and signalling  
119 role that endogenous cyanide plays *in planta* as well as the role in amino acid metabolism.  
120 The potential links between naturally-occurring exogenous cyanide and plant nitrogen  
121 metabolism have however been largely overlooked. The goal of this research then was to  
122 provide a study which demonstrated that non-toxic, environmentally realistic concentrations  
123 of cyanide interact with plant nitrogen metabolism. The results are expected to prompt  
124 additional questions about the role of cyanide in natural or agronomic systems where  
125 cyanogenic microorganisms are present or where anthropogenic cyanide has been introduced.

126

## 127 **MATERIALS AND METHODS**

### 128 **Nitrogen supply- and concentration-dependent accumulation of cyanide**



129 Wheat (*Triticum aestivum* cv. Wheaton) and sorghum (*Sorghum bicolor* cv. Pacer Elite) seed  
130 were planted in sterile potting mix (sorghum) or perlite:vermiculite (1:1 ratio, wheat) and  
131 grown for 14-18 d in a greenhouse under ambient light and temperature conditions to  
132 establish biomass. Seedlings were then transferred to 2 L pots containing a hydroponic  
133 solution with the following composition: 1.2 mM KNO<sub>3</sub>, 0.8 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.1 mM  
134 NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.2 mM MgSO<sub>4</sub>, 50 μM KCl, 12.5 μM H<sub>3</sub>BO<sub>3</sub>, 1 μM MnSO<sub>4</sub>, 1 μM ZnSO<sub>4</sub>, 0.5  
135 μM CuSO<sub>4</sub>, 0.1 μM NiSO<sub>4</sub>, and 0.1 μM H<sub>2</sub>MoO<sub>4</sub> (Ebbs *et al.*, 2003). The solution was  
136 aerated and buffered with 1 mM *n*-morpholinoethanesulfonic acid (MES), titrated to pH 6.0  
137 with KOH. Iron was provided as 10 μM Fe-EDTA. The plants were grown for 7 d at which  
138 point there was evidence of new root growth. The plants were then transferred to 2 L pots  
139 containing either this complete nutrient solution (referred to hereafter as nitrogen-replete  
140 nutrient solution) or the same nutrient solution minus nitrate and ammonium (referred to  
141 hereafter as nitrogen-free nutrient solution). No iron was added to the nutrient solutions and  
142 the solutions were not aerated at this point to prevent losses of added cyanide to precipitation  
143 or volatilization (Ebbs *et al.*, 2003, Ebbs *et al.*, 2008). For the experiment with sorghum,  
144 four plants were present in each pot, with each pot representing one replicate for a particular  
145 treatment. Because of biomass limitations realized later (see below), 8-10 wheat plants were  
146 used per pot (i.e., per replicate). For the nitrogen-free solution, the counterions present with  
147 the nitrate (i.e., K<sup>+</sup>, Ca<sup>2+</sup>) and ammonium (PO<sub>4</sub><sup>3-</sup>) were provided in an equivalent  
148 concentration as the chloride and potassium salts, respectively. The hydroponic solutions  
149 were amended with KC<sup>15</sup>N at 100% by mass to final concentrations of 5, 50, 100, 150, or  
150 200 μM, with each treatment replicated four times. Plants were grown in the presence of the  
151 cyanide treatments for 7 d. Measurements of chlorophyll fluorescence (F<sub>V</sub>/F<sub>M</sub>) were taken on

152 a randomly selected fully expanded leaf in the morning and early afternoon on the last day of  
153 the treatment period as a general measure of plant physiological status and a means of  
154 determining if the cyanide concentrations used caused any phytotoxicity to the plants. For  
155 sorghum, measurements were made using a Plant Efficiency Analyser (Hansatech  
156 Instruments, Norfolk, UK) while an OS1 field fluorometer (Opti-Sciences Inc., Hudson, NH  
157 USA) was used for wheat. At harvest, roots were thoroughly rinsed with deionized water,  
158 blotted dry with paper towels, and the plants separated into roots and shoots. The tissues  
159 were snap-frozen in liquid nitrogen, freeze-dried, and ground. It was discovered that the  
160 snap-freezing and freeze drying steps caused some sorghum shoot and root samples to be  
161 lost. Since fully replicated ( $n=3-4$ ) treatments could be provide only for sorghum shoots,  
162 those were the only tissues for which stable isotope data is reported. The number of plants  
163 used per replicate was increased for the wheat experiment as indicated above so that both  
164 roots and shoots of wheat could be analyzed for  $^{15}\text{N}$ .

165

166 Sorghum seed was germinated and grown as described above, except that plants were grown  
167 for 14 d in sterile potting mix and 14 d in nutrient solution. Plants were then transferred to  
168 200 mL of either the complete nutrient solution or the nitrogen-free nutrient solution above.  
169 The solutions were amended with double-labeled  $\text{K}^{13}\text{C}^{15}\text{N}$  (Cambridge Isotopes, Andover,  
170 MA, USA) at a final concentration of 100  $\mu\text{M}$ . The labeled compound was added at a rate of  
171 100% by mass and there were four replicates of each treatment. The plants were grown  
172 under ambient glasshouse conditions for 4 d and then harvested as described above. The root  
173 and shoot tissue were freeze-dried, ground, and prepared for stable isotope analysis. Using  
174 the  $^{13}\text{C}$  and  $^{15}\text{N}$  atom% obtained and the corresponding total carbon and nitrogen content,

175  $^{13}\text{C}:^{15}\text{N}$  ratios were calculated for each tissue in each treatment. Direct measurements of  
176 tissue cyanide could not be conducted on sorghum because the cyanogenic nature of that  
177 plant would have complicated the interpretation of those results.

178

### 179 **Interaction between cyanide and ammonium**

180 A reciprocal  $^{15}\text{N}$  labeling experiment was carried out using wheat to examine potential  
181 interactions between cyanide and ammonium. Seeds were germinated in sterile  
182 perlite:vermiculite for 7 d and then single seedlings were transferred to a 125 mL Erlenmeyer  
183 flask containing 100 mL of aerated nutrient solution with the same composition as above.  
184 Plants were grown for 7 d in a growth chamber (Percival Scientific, Boone, IA, USA) under  
185 a 16 h photoperiod, at a light intensity of  $350 \mu\text{Mol m}^{-2} \text{ s}^{-1}$  with 60-70% relative humidity.  
186 The wheat seedlings were then used to establish the reciprocal experiments, each of which  
187 used a randomized block design with one plant per replicate. The first experiment included  
188 three concentrations of ammonium (0, 10, 100  $\mu\text{M}$ , as  $\text{NH}_4\text{H}_2\text{PO}_4$ ), two concentrations (10  
189 and 100  $\mu\text{M}$ ) of isotopically-labeled  $\text{KC}^{15}\text{N}$  (Cambridge Isotopes, Andover, MA, USA), and  
190 four replicates. The reciprocal experiment was also established, with three concentrations of  
191  $\text{KCN}$  (0, 10, 100  $\mu\text{M}$ ), two concentrations (10 and 100  $\mu\text{M}$ ) of isotopically-labeled  $^{15}\text{NH}_4\text{Cl}$   
192 (Cambridge Isotopes, Andover, MA, USA), and four replicates. The stable isotopes were  
193 added at a rate of 100% by mass such that all added  $\text{KC}^{15}\text{N}$  or  $^{15}\text{NH}_4$  in the respective  
194 experiments was labeled with the stable isotope. For both experiments, the cyanide-  
195 ammonium regimes were established without the addition of iron or aeration. The plants  
196 were cultured in the growth chamber for a 48 h period of exposure and then harvested. At  
197 harvest, roots were thoroughly rinsed with deionized water, blotted dry with paper towels,

198 and the plants separated into roots and shoots. The tissues were dried at 65° C to constant  
199 mass before being ground and prepared for stable isotope analysis.

200

### 201 **Stable isotope analysis**

202 For isotopic analysis, ~5 mg of the ground tissue was weighed into 8x5 mm tin capsules

203 (Elemental Microanalysis Ltd, Manchester, MA, USA). Samples were submitted to the

204 Stable Isotope Facility at the University of California-Davis for <sup>15</sup>N and <sup>13</sup>C analysis.

205 Laboratory stable isotope standards used during the analysis had been previously calibrated

206 against select NIST Standard Reference Materials. The data returned included the atom%

207 <sup>15</sup>N, total N and, where applicable, total carbon and δ <sup>13</sup>C (‰ PDB). Conversion of the <sup>15</sup>N

208 data to enrichment units (δ <sup>15</sup>N ‰) used standard methods (Shearer & Kohl, 1993).

209

### 210 **Statistical analyses**

211 Statistical analysis of the results from experiments with three or more mean values used a

212 one-way or two-way ANOVA as dictated by the number of main effects. The post-hoc test

213 used with each ANOVA analysis was Tukey's HSD. For the two-way ANOVA, where

214 significant interactions were observed between the two main effects, the data were

215 reanalyzed using a one-way ANOVA treating each combination of main effects as a single

216 treatment. In cases where there was no interaction between the main effects, the results of

217 post hoc analysis are not shown. The Student's *t*-test was used in experiments where two

218 means obtained from an experiment were compared. All statistical analyses were conducted

219 using the SPSS software package for Windows (ver 13.0).

220

221 **RESULTS**

222 **Nitrogen supply- and concentration-dependent accumulation of cyanide**

223 Photosystem II photochemistry ( $F_v/F_M$ ) and biomass measurements were taken to  
224 demonstrate that the cyanide concentrations used had no adverse effect on the sorghum or  
225 wheat seedlings. Within each species, there were no significant differences for either of the  
226 main effects, KCN concentration or nitrogen supply. The ratios for  $F_v/F_M$ , which had means  
227 ranging from 0.75 to 0.79 in the morning and 0.74 to 0.76 in the afternoon for the various  
228 treatments, were not significantly different within a sampling time from control plants grown  
229 in the full hydroponic solution in the absence of cyanide (data not shown). These data, and  
230 the absence of any significant difference in biomass (data not shown) or overt visual signs of  
231 stress, were taken as an indication that the KCN concentrations had no adverse effects on the  
232 plants used here, an observation for wheat consistent with an earlier study (Kosma, 2005).  
233 Treatment with all five KCN concentrations produced substantial increases in  $^{15}\text{N}$  enrichment  
234 ( $\delta^{15}\text{N} \text{ ‰}$ ) in the shoot tissues. For sorghum plants grown in nitrogen-replete solution, the  
235  $^{15}\text{N}$  enrichment values for shoots ranged from ~1,000 for the 5  $\mu\text{M}$  treatment to values of  
236 ~2,000 for the remaining treatments, but were not significantly different between treatments  
237 (Figure 2). However, when grown in nitrogen-free nutrient solution, significantly greater  $^{15}\text{N}$   
238 enrichments in shoots were observed in sorghum shoots from 50, 100, and 200  $\mu\text{M}$   $\text{KC}_{15}\text{N}$   
239 treatments, with enrichment values >5,000 obtained. These enrichments were significantly  
240 greater than the 5  $\mu\text{M}$  and 150  $\mu\text{M}$  treatment and all of the treatments in the nitrogen-replete  
241 nutrient solution ( $p \leq 0.05$ ). It is not immediately clear why the enrichment in the 150  $\mu\text{M}$   
242 treatment in nitrogen-free solution was significantly lower than the corresponding 100 and  
243 200  $\mu\text{M}$  treatment. Three of the four replicates showed only modestly elevated atom%

244 values over background while the fourth replicate had an atom% value more consistent with  
245 those observed for the 100 and 200  $\mu\text{M}$  treatment. The concentration of KCN in the uptake  
246 solution was not measured before treatment was initiated, so the three indicated replicates in  
247 this particular treatment may simply not have been labeled with the full quantity of  $\text{KC}^{15}\text{N}$   
248 intended. There was no significant difference in total root or shoot nitrogen between  
249 treatments. Shoots in the nitrogen-replete and nitrogen-free solutions had nitrogen  
250 concentrations ranging from 1.7 – 3.7% (DW basis) and 1.1 – 3.6%, respectively.  
251  
252 For wheat in the nitrogen-replete nutrient solution, enrichment from cyanide was not  
253 significantly different across cyanide treatments in the roots or the shoots (Figure 3).  
254 However, when cyanide was the only nitrogen source in the solution (i.e., nitrogen-free  
255 nutrient solution),  $^{15}\text{N}$  enrichment was significantly higher only in roots for the 200  $\mu\text{M}$   
256 treatment. A visual comparison of the combined magnitude of the enrichment in roots and  
257 shoots in the 100, 150, and especially the 200  $\mu\text{M}$  cyanide treatments (Figure 3A, 3B)  
258 illustrated that the amount of cyanogenic  $^{15}\text{N}$  was greater in the whole plant when other  
259 sources of nitrogen were absent. Total nitrogen in roots and shoots between treatments did  
260 not differ significantly. Roots in the nitrogen-replete and nitrogen-free solutions had  
261 nitrogen concentrations ranging from 1.7 – 2.5% and 1.5 – 2.0%, respectively, while shoot  
262 concentrations ranged from 4.6 – 6.0% and 4.6 – 5.0%, respectively. Compared to sorghum  
263 there was a greater overall enrichment in wheat tissues for both nitrogen regimes (Figures 2-  
264 3). However, when looking within a species across the range of cyanide concentrations used,  
265 and for the nitrogen-free treatment, significant enrichments in sorghum shoots were observed  
266 in the nitrogen-free nutrient solution when solution cyanide concentrations were as low as 50

267  $\mu\text{M}$ , yet no significant differences were evident in wheat until 200  $\mu\text{M}$  and then only in the  
268 root.

269

270 In the experiment to examine the simultaneous uptake of  $^{13}\text{C}$  and  $^{15}\text{N}$  from cyanide, the  
271 sorghum exposed to  $\text{K}^{13}\text{C}^{15}\text{N}$  for 4 d had similar patterns of enrichment for the two stable  
272 isotopes. The  $^{15}\text{N}$  enrichment values were  $>3,200$  for shoots, but the  $^{15}\text{N}$  enrichment value  
273 was not significantly different between treatments (Figure 4A). The  $^{15}\text{N}$  enrichment for roots  
274 in both the normal and nitrogen-free treatments was  $>10,000$  (Figure 4B), but the value for  
275 the nitrogen-free treatment was significantly greater ( $p \leq 0.05$ ). For the  $^{13}\text{C}$  the trend was the  
276 same (Figure 4C, 4D). In the roots, the  $^{13}\text{C}$  enrichment was significantly greater in the  
277 nitrogen-free treatment, although there was no significant difference in the shoots. When the  
278  $^{13}\text{C}$  and  $^{15}\text{N}$  atom% and the tissue carbon and nitrogen content were used to calculate  $^{13}\text{C}:^{15}\text{N}$   
279 ratios for the plant tissues, values of 1.1 were obtained for roots of plants from both the  
280 nitrogen-replete and nitrogen-free treatments, suggestive of uptake and translocation of the  
281 intact cyanide molecule. For shoots, the ratios were significantly different ( $p \leq 0.04$ ), with  
282 values of 1.1 and 1.3 for shoots of plants from the nitrogen-replete and nitrogen-free  
283 treatments, respectively. Within a tissue (root or shoot) there was no significant difference in  
284 biomass at harvest (data not shown) or total N. Root and shoot nitrogen concentrations  
285 between the two treatments were 2.9% and 3.4%, respectively.

286

### 287 **Interaction between cyanide and ammonium**

288 The exposure of 14-day-old wheat plants to  $\text{K}^{15}\text{N}$  in the presence of 10 mM nitrate and one  
289 of three ammonium concentrations resulted in a clear enrichment of  $^{15}\text{N}$  in tissues, although

290 more so in the higher  $\text{KC}^{15}\text{N}$  treatment and in roots more than shoots (Figure 5). There was  
291 no relationship between  $^{15}\text{N}$ -cyanide enrichment and ammonium concentration for the  $10\ \mu\text{M}$   
292 cyanide treatment (Figure 5A, B). When the  $\text{KC}^{15}\text{N}$  treatment concentration was  $100\ \mu\text{M}$ , an  
293 inverse relationship was observed between  $^{15}\text{N}$  enrichment in roots and solution ammonium  
294 concentration (Figure 5B), with enrichment decreasing significantly ( $p \leq 0.04$ ) as solution  
295 ammonium increased. Total nitrogen content of roots and shoots of the wheat plants was not  
296 significantly different across the  $\text{KC}^{15}\text{N}$  and ammonium treatments. Root nitrogen ranged  
297 from 2.8 to 3.3% while shoot nitrogen ranged from 5.6 to 6.2%. For the reciprocal  $^{15}\text{N}$ -  
298 ammonium experiment, there was no clear pattern of  $^{15}\text{N}$  enrichment evident in the data for  
299 shoots at either concentration of ammonium (Figure 5C). In roots, however, there was a  
300 positive albeit not statistically significant increase in  $^{15}\text{N}$  enrichment from the labeled  
301 ammonium as the cyanide concentration increased (Figure 5D). There was again no  
302 significant difference in total nitrogen content of roots or shoots across the  $^{15}\text{N}$ -ammonium  
303 and cyanide treatments. The range of nitrogen concentrations was nearly the same as that  
304 reported above (roots 2.7 to 3.1%; shoots 5.7 to 6.1%).

305

## 306 **DISCUSSION**

307 Nitrogen is the mineral nutrient required by plants in the largest amount. Although plants  
308 primarily utilize nitrate and ammonium from the soil (Glass *et al.*, 2002, Harrison, Bol &  
309 Bardgett, 2007, Harrison, Bol & Bardgett, 2008), because of the energetic demand for ATP  
310 and reducing equivalents required for nitrate assimilation it is not surprising that plants are  
311 also opportunistic in their acquisition of reduced organic forms of nitrogen from the soil  
312 (Nasholm, Kielland & Ganeteg, 2009). Plants species utilize a wide range of organic



313 nitrogen compounds found in the soil, demonstrating for amino acids for example, a  
314 preference for simpler amino acids over more complex ones such as phenylalanine (Harrison,  
315 Bol & Bardgett, 2007). Cyanide is a simple nitrogenous compound in soils that arises from  
316 both anthropogenic and natural sources and can be present at concentrations comparable to  
317 (Kesler-Arnold & O'Hearn, 1990, Owen & Zdor, 2001) or greatly exceeding (for  
318 anthropogenic cyanide) concentrations of inorganic nitrogen in fertilized or unfertilized soil.  
319 Several studies have shown that plants are capable of acquiring cyanide from the external  
320 media (Ebbs *et al.*, 2003, Larsen, Trapp & Pirandello, 2004, Larsen, Ucisik & Trapp, 2005,  
321 Yu *et al.*, 2004). The extensive literature on the  $\beta$ -cyanoalanine pathway clearly  
322 demonstrates that cyanide can be assimilated via this pathway (for reviews, see Ebbs, 2004,  
323 Siegień & Bogatek, 2006). There has been no concerted effort however to create linkages  
324 that relate cyanide uptake and assimilation to the larger context of plant nitrogen metabolism.  
325 The effort here examined two aspects relevant to this larger goal, namely the enrichment of  
326 the cyanogenic nitrogen as a function of cyanide concentration and as influenced by the  
327 presence or absence of other nitrogen sources  
328  
329 Under the nitrogen-replete conditions here and over the range of cyanide concentrations used  
330 there was no significant difference within a species and within a tissue in  $^{15}\text{N}$  enrichment  
331 (Figure 2-3). This is contrary to the expectation of a dose-dependent  $^{15}\text{N}$  enrichment. Given  
332 the pH of the nutrient solutions used, HCN would be the predominant chemical form  
333 (Dzombak, Ghosh & Young, 2005, Ghosh, Dzombak & Wong-Chong, 2005). With an  
334 octanol-water partition coefficient ( $\log K_{ow}$ ) of -0.25 (Larsen, Ucisik & Trapp, 2005),  
335 cyanide displays a lipid solubility that allows this molecule to penetrate biological

336 membranes by simple diffusion (Borowitz, Isom & Nakles, 2005). Theoretically HCN could  
337 also be transported via mass flow through aquaporins since those channels reportedly  
338 mediate the transport of neutral solutes (Eckert *et al.*, 1999). Some authors have inferred  
339 from transport and kinetic data that there may be a protein-mediated aspect to cyanide  
340 transport (Bushey, Ebbs & Dzombak, 2006, Bushey *et al.*, 2006), which may represent  
341 aquaporin-mediated transport of HCN or protein-mediated transport of the CN<sup>-</sup> anion, but  
342 neither have been demonstrated. The enrichment data here under nitrogen-replete conditions  
343 nonetheless imply some regulation of uptake. Regardless of the chemical species of cyanide  
344 and the associated mechanism of transport, cyanide uptake was seemingly constant and  
345 independent of external cyanide concentration.

346

347 In contrast, under nitrogen-free conditions, there was a significantly greater enrichment of  
348 <sup>15</sup>N from cyanide in plants at a solution cyanide concentrations  $\geq 50$   $\mu$ M for sorghum shoots  
349 and at 200  $\mu$ M for wheat roots (Figures 2-3). The lack of a significant difference in tissue  
350 nitrogen suggests that cyanogenic nitrogen was used to supplement plant nitrogen status at  
351 least over the limited time frame of the experiments here. The increase in enrichment from  
352 cyanide under nitrogen-free conditions is probably not a simple response to differences in  
353 solution conditions as the pH and ionic strength (due to the substitution of ions for the  
354 omitted nitrate and ammonium) were not changed relative to the nitrogen-replete solution.  
355 One possible explanation for the increased enrichment under nitrogen-free conditions is that  
356 an increase in the activity of enzymes associated with cyanide assimilation may have drawn  
357 down the internal concentration of cyanide rapidly. Preliminary data have shown a  
358 significant increase in both  $\beta$ -cyanoalanine synthase and asparaginase activity in wheat

359 shoots when exposed to cyanide under similar nitrogen-free conditions (Ebbs and  
360 Machingura, unpublished results). This could have maintained if not increased the diffusion  
361 gradient for cyanide across the plasma membrane, facilitating passive entry and increasing  
362 the enrichment. The significant difference in the  $^{13}\text{C}:^{15}\text{N}$  ratios observed between plants  
363 from nitrogen-replete and nitrogen-free solutions (Figure 4) are further suggestive of a  
364 biological response that caused differential metabolism and transport of carbon and nitrogen  
365 to shoots, or redistribution of those elements, under the nitrogen-free treatment conditions  
366 which may be related to differences in cyanide metabolism or partitioning of derived  
367 metabolites.

368

369 Additional data from this study imply that ammonium supply plays a role in the extent to  
370 which cyanide is accumulated and used as a nitrogen source. The results from Figure 5B  
371 suggest an inverse relationship between solution cyanide and ammonium concentrations at  
372 the root level, with enrichment from cyanide decreasing with increasing solution ammonium.  
373 This interaction was clearly not competitively reciprocal because increasing solution cyanide  
374 did not have a negative effect on enrichment from  $^{15}\text{NH}_4^+$ , rather cyanide had a somewhat  
375 positive effect on enrichment (Figure 5D). Given the pH of the nutrient solution, ammonium  
376 would be the only species of ammonia/ammonium present. Transport of ammonium is  
377 mediated by high- and low-affinity membrane carriers and can be thermodynamically active  
378 or passive, respectively, depending upon factors such as the external ammonium  
379 concentration while uptake is being examined, the supply of ammonium during the growth  
380 period preceding the uptake experiment, and the cytosolic ammonium concentration (Glass *et*

381 *al.*, 1997). The mechanisms associated with the membrane transport of ammonium differ  
382 fundamentally from those associated with HCN or CN<sup>-</sup>, precluding a direct interaction.

383

384 The seemingly positive relationship between increasing concentrations of solution cyanide  
385 and ammonium in Figure 4D could reflect an indirect effect arising from the inactivation of  
386 nitrate reductase by cyanide (Barr *et al.*, 1995, Echevarria, Maurino & Maldonado, 1984,  
387 Solomonson & Barber, 1990, Somers *et al.*, 1983). Cyanide inactivates nitrate reductase by  
388 directly binding to the over-reduced valence of the molybdenum cofactor (Echevarria,  
389 Maurino & Maldonado, 1984). As the solution cyanide concentration increased, the degree  
390 of nitrate reductase inhibition in roots may have increased. Nitrate supply regulates the  
391 posttranslational expression of AMT uniports associated with ammonium transport (Yuan *et*  
392 *al.*, 2007), so it may also be possible that inhibition of nitrate reductase activity may also  
393 exert a regulatory effect over some aspects of ammonium transport, or expression of the  
394 underlying transporter genes.

395

396 The comparison of the data from wheat to the limited data from sorghum (Figures 2-3) also  
397 provided evidence that the responses of cyanogenic plants to cyanide exposure may differ  
398 from acyanogenic species. Studies which have compared the rate of cyanide transport,  
399 assimilation, and/or  $\beta$ -cyanoalanine synthase activity in different plant species have found  
400 that the rates are higher for cyanogenic species as compared to acyanogenic species, but the  
401 magnitude of the difference may be limited. The authors of such studies have suggested that  
402 cyanogenic species do not appear by default to have a greatly enhanced capacity to assimilate  
403 cyanide (Larsen, Trapp & Pirandello, 2004, Wurtele, Nikolau & Conn, 1984, Yu *et al.*,

404 2004). Most studies that have examined differences in cyanide transport or assimilation  
405 between cyanogenic and acyanogenic plants do so at the shoot level, so differences at the  
406 root level, or in whole plant distribution, may not be accounted for yet may be relevant to the  
407 difference in transport and assimilation between acyanogenic and cyanogenic plant species.

408

409 In conclusion, the results here suggest that the extent to which these species transport, and  
410 potentially assimilate, cyanide varies in response to the presence or absence of other nitrogen  
411 sources and in response to the external supply of ammonium. While there is available  
412 information on the assimilation of endogenous cyanide into nitrogen metabolism, the degree  
413 to which exogenous sources contributes to plant nitrogen metabolism remains an open  
414 question. The natural presence of cyanide in some communities has already been described  
415 in the context of cyanide “microcycles” (Allen & Strobel, 1966, Thatcher & Weaver, 1976).  
416 These microcycles consist of cyanogenic organisms and organisms that assimilate cyanide as  
417 a source of carbon and nitrogen for growth. An understanding of the degree to which such  
418 cycles scale up to higher levels of organization will be critical if future efforts are to  
419 demonstrate a broader physiological and/or ecological relevance of cyanide to communities  
420 and ecosystems. Bacterial and fungal cyanogenesis introduce cyanide into the rhizosphere  
421 but the implications for plants are not clear beyond the general promotion of growth that has  
422 been observed (Antoun *et al.*, 1998, Deka Boruah *et al.*, 2003). Since these soil  
423 microorganisms secrete suites of compounds into the rhizosphere, the specific impact of  
424 cyanide has not been established. Soil bacteria and fungi also have multiple pathways for the  
425 degradation and/or assimilation of cyanide (Ebbs, 2004) and there is little doubt that these  
426 organisms would opportunistically use cyanide as a nitrogen source, perhaps further limiting

427 the extent to which rhizospheric cyanide is perceived or utilized by plants. In fact, the  
428 capacity of microorganisms to degrade cyanide has been seized upon as an opportunity to  
429 remove cyanide contamination from industrial waste streams (Adjei & Ohta, 2000, Kao *et*  
430 *al.*, 2003, Oliveira, Reis & Nozaki, 2001, Oudjehani, Zagury & Deschênes, 2002, Patil &  
431 Paknikar, 2000). The release of anthropogenic cyanide from various industries may also  
432 argue in support of the questions explored here. Natural sources may not provide a  
433 significant supply of cyanide to plants on larger scales, but human activities provide a  
434 sustained input of cyanide into some terrestrial and aquatic ecosystems, so additional  
435 consideration of the potential role of cyanide as a nitrogen source to plants should be  
436 considered at least in that context. In which case, innate differences between plant species  
437 (including cyanogenic and acyanogenic) in the capacity to assimilate cyanide may become  
438 increasingly important, particularly when external cyanide concentrations increase beyond  
439 values typically encountered in soils or when cyanogenic compounds (e.g., metal cyanide  
440 complexes) other than simple cyanide are present.

441

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445

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628



629 **List of figures**

630

631 **Figure 1.** Assimilation of cyanide by the  $\beta$ -cyanoalanine pathway into the amino acids  
632 asparagine and aspartate with concomitant and/or subsequent production of ammonium.  
633 Formation of cyanoalanine from cyanide and cysteine is mediated by either cyanoalanine  
634 synthase or cysteine synthase. A bi-functional enzyme with both nitrilase and nitrile  
635 hydratase activity converts cyanoalanine into either asparagine or aspartate and ammonium,  
636 respectively. Asparaginase mediates the release of ammonium from asparagine to form  
637 aspartate.

638

639 **Figure 2.** Enrichment of  $^{15}\text{N}$  in shoot tissues of sorghum following a 7 d exposure to  $\text{KC}^{15}\text{N}$   
640 solution concentrations ranging from 5 to 200  $\mu\text{M}$  in the presence of nutrient solutions that  
641 were either nitrogen-replete (+N, +CN) or nitrogen-free (-N, +CN) nutrient solutions. Bars  
642 denote the mean and standard error ( $n=3-4$ ), with different letters used to indicate when  
643 values were significantly different from one another.

644

645 **Figure 3.** Enrichment of  $^{15}\text{N}$  in shoot (**A**) and root (**B**) tissues of wheat following a 7 d  
646 exposure to  $\text{KC}^{15}\text{N}$  solution concentrations ranging from 5 to 200  $\mu\text{M}$  in the presence of  
647 nutrient solutions that were either nitrogen-replete (+N, +CN) or nitrogen-free (-N, +CN)  
648 nutrient solutions. Bars denote the mean and standard error ( $n=4$ ). Within a tissue, bars with  
649 different letters are used to indicate when values were significantly different from one  
650 another.

651

652 **Figure 4.** Enrichment of  $^{15}\text{N}$  (**A, B**) and  $^{13}\text{C}$  (**C, D**) in sorghum shoots (**A, C**) roots (**B, D**)  
653 following a 4 d exposure to 100  $\mu\text{M}$   $\text{K}^{13}\text{C}^{15}\text{N}$  in the presence of nutrient solutions that were  
654 either nitrogen-replete (+N, +CN) or nitrogen-free (-N, +CN). Bars denote the mean and  
655 standard error ( $n=4$ ). Within a tissue, bars with different letters are used to indicate when  
656 values were significantly different from one another.

657

658 **Figure 5.** Enrichment of  $^{15}\text{N}$  in shoots (**A, C**) and roots (**B, D**) of wheat following a 48 hr  
659 hydroponic exposure to two concentrations of  $\text{KC}^{15}\text{N}$  (**A, B**) or ammonium (as  $^{15}\text{NH}_4\text{H}_2\text{PO}_4$ )

660 (C, D) in response to varying concentrations of unlabeled  $\text{NH}_4^+$  or KCN, respectively. Bars  
661 denote the mean and standard error ( $n=4$ ). While significant differences between main  
662 effects were observed, there was no interaction between the effects so no post hoc results are  
663 shown.  
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