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

Running head: Nitrogen supply influences enrichment of cyanogenic nitrogen

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Abstract

Cyanide assimilation by the β-cyanoalanine pathway produces asparagine, aspartate, and ammonium, allowing cyanide to serve as alternate or supplemental source of nitrogen. Experiments with wheat and sorghum examined the enrichment of ^{15}N from cyanide as a function of external cyanide concentration in the presence or absence of nitrate and/or ammonium. Cyanogenic nitrogen became enriched in plant tissues following exposure to ^{15}N-cyanide concentrations from 5 to 200 μM, but when exposure occurred in the absence of nitrate and ammonium, ^{15}N enrichment increased significantly in sorghum shoots at solution cyanide concentrations of $\geq$50 μM and in wheat roots at 200 μM cyanide. In an experiment with sorghum using $^{13}C^{15}N$, there was also a significant difference in the tissue ^{13}C:^{15}N ratio, suggestive of differential metabolism and transport of carbon and nitrogen under nitrogen-free conditions. A reciprocal ^{15}N labeling study using $KC^{15}N$ and $^{15}NH_4^+$ and wheat demonstrated an interaction between cyanide and ammonium in roots in which increasing solution ammonium concentrations decreased the enrichment from 100 μM cyanide. In contrast, with increasing solution cyanide concentrations there was an increase in the enrichment from ammonium. The results suggest increased transport and assimilation of cyanide in response to decreased nitrogen supply and perhaps to ammonium supply.

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

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_3, 0.8 mM $Ca(NO_3)_2$, 0.1 mM
134 $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5
135 μM $CuSO_4$, 0.1 μM $NiSO_4$, and 0.1 μM H_2MoO_4 (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^+, Ca^{2+}) and ammonium (PO_4^{3-}) were provided in an equivalent
148 concentration as the chloride and potassium salts, respectively. The hydroponic solutions
149 were amended with $KC^{15}N$ 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 ($F_{V/}F_M$) were taken on

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

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

175 ^{13}C:^{15}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}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 μMol m^{-2} 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 μM, as $NH_4H_2PO_4$), two concentrations (10
189 and 100 μM) of isotopically-labeled $KC^{15}N$ (Cambridge Isotopes, Andover, MA, USA), and
190 four replicates. The reciprocal experiment was also established, with three concentrations of
191 KCN (0, 10, 100 μM), two concentrations (10 and 100 μM) of isotopically-labeled $^{15}NH_4Cl$
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 $KC^{15}N$ or $^{15}NH_4$ 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 65º 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 ^{15}N and ^{13}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 ^{15}N, total N and, where applicable, total carbon and $\delta\ ^{13}C$ ($^{0}/_{00}$ PDB). Conversion of the ^{15}N
208 data to enrichment units ($\delta\ ^{15}N\ ^{0}/_{00}$) 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

RESULTS

Nitrogen supply- and concentration-dependent accumulation of cyanide

Photosystem II photochemistry (F_v/F_M) and biomass measurements were taken to demonstrate that the cyanide concentrations used had no adverse effect on the sorghum or wheat seedlings. Within each species, there were no significant differences for either of the main effects, KCN concentration or nitrogen supply. The ratios for F_V/F_M, which had means ranging from 0.75 to 0.79 in the morning and 0.74 to 0.76 in the afternoon for the various treatments, were not significantly different within a sampling time from control plants grown in the full hydroponic solution in the absence of cyanide (data not shown). These data, and the absence of any significant difference in biomass (data not shown) or overt visual signs of stress, were taken as an indication that the KCN concentrations had no adverse effects on the plants used here, an observation for wheat consistent with an earlier study (Kosma, 2005). Treatment with all five KCN concentrations produced substantial increases in ^{15}N enrichment ($\delta\ ^{15}N\ ^{0}/_{00}$) in the shoot tissues. For sorghum plants grown in nitrogen-replete solution, the ^{15}N enrichment values for shoots ranged from ~1,000 for the 5 μM treatment to values of ~2,000 for the remaining treatments, but were not significantly different between treatments (Figure 2). However, when grown in nitrogen-free nutrient solution, significantly greater ^{15}N enrichments in shoots were observed in sorghum shoots from 50, 100, and 200 μM $KC_{15}N$ treatments, with enrichment values >5,000 obtained. These enrichments were significantly greater than the 5 μM and 150 μM treatment and all of the treatments in the nitrogen-replete nutrient solution ($p \leq 0.05$). It is not immediately clear why the enrichment in the 150 μM treatment in nitrogen-free solution was significantly lower than the corresponding 100 and 200 μM treatment. Three of the four replicates showed only modestly elevated atom%

values over background while the fourth replicate had an atom% value more consistent with those observed for the 100 and 200 μM treatment. The concentration of KCN in the uptake solution was not measured before treatment was initiated, so the three indicated replicates in this particular treatment may simply not have been labeled with the full quantity of $KC^{15}N$ intended. There was no significant difference in total root or shoot nitrogen between treatments. Shoots in the nitrogen-replete and nitrogen-free solutions had nitrogen concentrations ranging from 1.7 – 3.7% (DW basis) and 1.1 – 3.6%, respectively.

For wheat in the nitrogen-replete nutrient solution, enrichment from cyanide was not significantly different across cyanide treatments in the roots or the shoots (Figure 3). However, when cyanide was the only nitrogen source in the solution (i.e., nitrogen-free nutrient solution), ^{15}N enrichment was significantly higher only in roots for the 200 μM treatment. A visual comparison of the combined magnitude of the enrichment in roots and shoots in the 100, 150, and especially the 200 μM cyanide treatments (Figure 3A, 3B) illustrated that the amount of cyanogenic ^{15}N was greater in the whole plant when other sources of nitrogen were absent. Total nitrogen in roots and shoots between treatments did not differ significantly. Roots in the nitrogen-replete and nitrogen-free solutions had nitrogen concentrations ranging from 1.7 – 2.5% and 1.5 – 2.0%, respectively, while shoot concentrations ranged from 4.6 – 6.0% and 4.6 – 5.0%, respectively. Compared to sorghum there was a greater overall enrichment in wheat tissues for both nitrogen regimes (Figures 2-3). However, when looking within a species across the range of cyanide concentrations used, and for the nitrogen-free treatment, significant enrichments in sorghum shoots were observed in the nitrogen-free nutrient solution when solution cyanide concentrations were as low as 50

μM, yet no significant differences were evident in wheat until 200 μM and then only in the root.

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

Interaction between cyanide and ammonium

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

290 more so in the higher $KC^{15}N$ treatment and in roots more than shoots (Figure 5). There was
291 no relationship between ^{15}N-cyanide enrichment and ammonium concentration for the 10 μM
292 cyanide treatment (Figure 5A, B). When the $KC^{15}N$ treatment concentration was 100 μM, an
293 inverse relationship was observed between ^{15}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 $KC^{15}N$ 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}N-
298 ammonium experiment, there was no clear pattern of ^{15}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}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}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

nitrogen compounds found in the soil, demonstrating for amino acids for example, a preference for simpler amino acids over more complex ones such as phenylalanine (Harrison, Bol & Bardgett, 2007). Cyanide is a simple nitrogenous compound in soils that arises from both anthropogenic and natural sources and can be present at concentrations comparable to (Kesler-Arnold & O'Hearn, 1990, Owen & Zdor, 2001) or greatly exceeding (for anthropogenic cyanide) concentrations of inorganic nitrogen in fertilized or unfertilized soil. Several studies have shown that plants are capable of acquiring cyanide from the external media (Ebbs *et al.*, 2003, Larsen, Trapp & Pirandello, 2004, Larsen, Ucisik & Trapp, 2005, Yu *et al.*, 2004). The extensive literature on the β-cyanoalanine pathway clearly demonstrates that cyanide can be assimilated via this pathway (for reviews, see Ebbs, 2004, Siegień & Bogatek, 2006). There has been no concerted effort however to create linkages that relate cyanide uptake and assimilation to the larger context of plant nitrogen metabolism. The effort here examined two aspects relevant to this larger goal, namely the enrichment of the cyanogenic nitrogen as a function of cyanide concentration and as influenced by the presence or absence of other nitrogen sources

Under the nitrogen-replete conditions here and over the range of cyanide concentrations used there was no significant difference within a species and within a tissue in ^{15}N enrichment (Figure 2-3). This is contrary to the expectation of a dose-dependent ^{15}N enrichment. Given the pH of the nutrient solutions used, HCN would be the predominant chemical form (Dzombak, Ghosh & Young, 2005, Ghosh, Dzombak & Wong-Chong, 2005). With an octanol-water partition coefficient (log K_{OW}) of -0.25 (Larsen, Ucisik & Trapp, 2005), cyanide displays a lipid solubility that allows this molecule to penetrate biological

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

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

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

Additional data from this study imply that ammonium supply plays a role in the extent to which cyanide is accumulated and used as a nitrogen source. The results from Figure 5B suggest an inverse relationship between solution cyanide and ammonium concentrations at the root level, with enrichment from cyanide decreasing with increasing solution ammonium. This interaction was clearly not competitively reciprocal because increasing solution cyanide did not have a negative effect on enrichment from $^{15}NH_4^+$, rather cyanide had a somewhat positive effect on enrichment (Figure 5D). Given the pH of the nutrient solution, ammonium would be the only species of ammonia/ammonium present. Transport of ammonium is mediated by high- and low-affinity membrane carriers and can be thermodynamically active or passive, respectively, depending upon factors such as the external ammonium concentration while uptake is being examined, the supply of ammonium during the growth 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^-, 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 β-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.*,

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

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

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

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List of figures

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

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

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

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

Figure 5. Enrichment of ^{15}N in shoots (**A, C**) and roots (**B, D**) of wheat following a 48 hr hydroponic exposure to two concentrations of $KC^{15}N$ (**A, B**) or ammonium (as $^{15}NH_4H_2PO_4$)

(**C, D**) in response to varying concentrations of unlabeled NH_4^+ or KCN, respectively. Bars denote the mean and standard error (*n*=4). While significant differences between main effects were observed, there was no interaction between the effects so no post hoc results are shown.