Cyanide Production by Rhizobacteria and Potential for Suppression of Weed Seedling Growth

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Abstract. Rhizobacteria strains were characterized for ability to synthesize hydrogen cyanide and for effects on seedling root growth of various plants. Approximately 32% of bacteria from a collection of over 2000 isolates were cyanogenic, evolving HCN from trace concentrations to >30 nmoles/mg cellular protein. Cyanogenesis was predominantly associated with pseudomonads and was enhanced when glycine was provided in the culture medium. Concentrations of HCN produced by rhizobacteria were similar to exogenous concentrations inhibiting seedling growth in bioassays, suggesting that cyanogenesis by rhizobacteria in the rhizosphere can adversely affect plant growth. Growth inhibition of lettuce and barnyardgrass by volatile metabolites of the cyanogenic rhizobacteria confirmed that HCN was the major inhibitory compound produced. Our results suggest that HCN produced in the rhizospheres of seedlings by selected rhizobacteria is a potential and environmentally compatible mechanism for biological control of weeds.

The rhizosphere is inhabited by a diversity of organisms, including a component known as the rhizobacteria, which are characterized by aggressive colonization and subsequent establishment on plant roots. Currently many of the traits that contribute to the competitiveness and colonization of bacteria in the rhizosphere are not well defined [25]. Growth-inhibitory bacteria, or deleterious rhizobacteria (DRB), are generally regarded as non-parasitic, primarily causing deleterious effects through production of harmful metabolites that are absorbed by the root [21, 24]. Metabolites that have been implicated in deleterious activity include phytohormones in high concentrations, various unidentified phytotoxins, and cyanide [17].

Cyanide is a potential inhibitor of enzymes involved in major plant metabolic processes including respiration, CO_2 and nitrate assimilation, and carbohydrate metabolism, and may also bind with the protein plastocyanin to block photosynthetic electron transport [11]. Cyanogenesis by bacteria is widely documented [9]; however, little is known about the role of cyanide-producing bacteria

associated with plants. About 50% of rhizobacteria on potato (*Solanum tuberosum* L.) roots produced cyanide, which was implicated in measurable inhibition of potato growth [5]. A growth-suppressing strain of *Pseudomonas fluorescens* inoculated in soil produced cyanide in sufficient quantities to inhibit growth of beans [1].

Descriptions are available for only a few cyanogenic rhizobacteria, which indicates that they are typically host-specific and associate only with roots of the host plant. Several DRB that reduce seed germination, seedling vigor, and subsequent plant growth have been isolated from roots of various weed seedlings [14]. Although the potential of DRB as biological control agents of weeds is being actively investigated, little is known of the mechanisms of phytotoxicity other than that due to crude phytotoxins found in culture filtrates of specific isolates. Cyanide production by DRB associated with weed seedlings as a possible phytotoxic mechanism has not been investigated. Such DRB could selectively colonize weed seedling roots, thereby localizing cyanide production and minimizing potential deleterious effects on growth of desirable plants. DRB specifically associate with germinating weed seeds and seedlings in soil [6].

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Successful establishment of cyanogenic DRB in weed rhizospheres would be more economical than chemical synthesis and/or field application of growth-suppressive compounds [1]. The overall objective of this investigation was to develop a clearer understanding of the mechanisms responsible for the phytotoxicity of DRB on weed seedlings. Specific objectives were to assess several rhizobacterial isolates for their ability to synthesize hydrogen cyanide; and to determine the effect of selected rhizobacteria on root growth of various plant seedlings relative to known quantities of hydrogen cyanide.

Materials and Methods

Rhizobacterial isolates, originating from roots of *Euphorbia* spp. collected from various sites in Europe and North America, were compared for cyanide production. Isolates were identified with the API Rapid NFT diagnostic kit (Bio Merieux Vitek Inc., Hazelwood, MO, USA). Classification of bacterial isolates was verified with gas chromatography-fatty acid methyl ester analysis [20]. All isolates were maintained on quarter-strength King's B agar [19] and stored at 4°C.

Isolates, subcultured on quarter-strength King's B agar for 48 h, were initially screened qualitatively for production of cyanide by using picrate/Na_2CO_3 saturated filter paper fixed to the underside of Petri dish lids [16], which were sealed with parafilm before incubation at 28°C. Color change of the filter paper from yellow to light brown, brown, or reddish brown was recorded at 4, 24, and 48 h as an indication of weak, moderate, or strong cyanogenic potential, respectively. Reactions from inoculated plates were visually compared with corresponding control plates containing no culture.

For quantitative cyanide determinations, selected rhizobacteria were cultured on quarter-strength King's B agar with or without a glycine (5.8 mM) amendment in Petri plates with lids fitted with a Whatman no. 1 filter paper saturated with 1.0 M NaOH and sealed with parafilm. After 48 h of incubation at 28°C, filter papers were removed, extracted with 5.0 ml 1.0 M NaOH, and titrated with 4.25 ml acetic acid. Cyanide extracted into NaOH was quantified after reacting with barbituric acid-pyridine reagent; and the absorbance spectrophotometrically was read at 575 nm [15]. Bacterial cultures on each plate were suspended in sterile water, extracted with trichloroacetic acid, resuspended in 0.1 M NaOH in 2% Na_2CO_3, and analyzed for protein by using a modified Lowry procedure [18], with bovine serum albumin as a standard. All measurements were made on three replicate plates for each treatment, and experiments were repeated at least once.

Plants used to assess the effects of bacteria or standard concentrations of cyanide included lettuce as a standard indicator species, barnyardgrass and green foxtail to represent major monocotyledon weeds, and field bindweed to represent a major dicotyledon weed. Seeds of each species were surface-sterilized and allowed to germinate overnight on 1.0% agar at 28°C [23]. Pregerminated seeds with uniform radicles (≤2 mm long) were evenly distributed on 1.0% agar (16 seeds/plate). Two-day-old cultures of each bacterial isolate on King's B plates were suspended in 5 ml of 0.1 M $MgSO_4$, and a 30-μl suspension containing approximately 10^6 cfu was applied to each seed. Control seeds received 0.1 M $MgSO_4$ without bacteria. The effects of known concentrations of cyanide were determined with cyanide as KCN added to agar prior to placing pregerminated seeds on the agar. For both experiments, plates containing treated seeds were sealed with parafilm prior to incubation; root lengths were measured 48 h after inoculation and were compared with control roots. Each experiment was repeated at least once.

Effects of rhizobacterial volatile metabolites on root growth were assessed by using a seedling bioassay based on Alström and Burns [1]. Pregerminated surface-sterilized lettuce or barnyardgrass seeds (ten per plate) were placed equidistantly apart on the surface of 1.0% agar. Rhizobacterial isolates were streaked on quarter-strength King's B agar and incubated for 24 h. After incubation, each inoculated plate was paired with a plate containing the pregerminated seeds. Both plates (without lids) of the paired-plate assembly, the lower containing seeds and the upper containing the isolate, were sealed with parafilm and incubated in the dark at 27°C. Each paired-plate assembly was replicated four times, and seedling root lengths were measured after 48 h. Assemblies containing noninoculated King's B agar served as controls. This experiment was conducted four times.

Data from quantitative cyanide determinations and all seedling bioassays were subjected to analyses of variance. Where F-values were significant at $p < 0.05$, means were compared by using Fisher's protected least significant differences (LSD) test.

Results and Discussion

Rhizobacterial isolates tested for cyanogenic activity by using the qualitative assay represented about 32% of a culture collection numbering over 2000 isolates from *Euphorbia* spp. sampled at 25 sites in Europe and North America. A subset of the culture collection for in-depth investigation of cyanogenesis and effects on seedling growth was selected for the study reported here. DRB isolates showing positive reactions for HCN production varied qualitatively in the amounts detected based on color intensity developed on the picrate/Na_2CO_3 impregnated papers (Table 1). Several *Pseudomonas* isolates produced high quantities of HCN with some evolving detectable HCN in ≤4 h after adding the developing reagents, indicating very strong cyanogenic potential [13]. Overall, cyanogenesis was predominantly associated with *Pseudomonas* spp. although not restricted to fluorescent pseudomonads as previously reported [1, 8]. Cyanogenesis by whole-cell cultures of DRB was related to seedling growth inhibition of lettuce and barnyardgrass test species; however, some non-cyanogenic DRB (i.e., *Pseudomonas* sp. 437) also inhibited seedling growth. When in direct contact with developing seedlings, all isolates significantly inhibited growth of lettuce, and 8 of 10 inhibited barnyardgrass. Inhibitory effects of the non-cyanogenic isolates were likely due to phytotoxins other than HCN. This is analogous to the reported production of two or more secondary metabolites by some rhizobacteria in causing plant growth inhibition [4, 22]. The relatively weak correlation between plant growth effects and cyanogenesis has been reported previously for DRB on lettuce seedlings [1].

In determining potential effects of HCN derived from rhizobacteria on seedling growth, the different plant seedlings exhibited a wide range in response (measured as root length) to exogenous cyanide (Fig. 1). Concentrations of 12.5 to 100 μmoles CN significantly reduced

Table 1. Qualitative detection of HCN production and effects of cell cultures of various rhizobacteria on lettuce and barnyardgrass seedling root length in agar bioassays

Isolate	HCN production[a]	Lettuce root length (mm)	Barnyardgrass root length (mm)
Pseudomonas sp.42	+++[b]	9.6 (40)[c]	14.8 (30)
Pseudomonas sp.74	+++[b]	9.0 (44)	12.4 (46)
Pseudomonas sp.473	+++	10.3 (35)	10.0 (56)
P. fluorescens 297	+++[b]	8.9 (44)	9.9 (56)
P. fluorescens 126	+++[b]	9.0 (44)	9.8 (57)
P. aeruginosa 136	++[b]	9.1 (44)	10.8 (52)
P. fluorescens 672	+	8.8 (45)	19.4 (15)
P. syringae 81	+	2.5 (84)	15.8 (40)
Pseudomonas sp. 437	−	10.4 (35)	12.7 (44)
P. aureofaciens 52	−	7.0 (56)	18.9 (17)
Control	−	16.0	22.8
LSD (0.05)		4.0	5.8

[a] Intensity of HCN reaction with picrate indicator: none, −; weak, +; moderate, ++; strong, +++.
[b] Reaction detectable at 4 h after initiation of HCN assay.
[c] Values in parentheses are % reduction in root length relative to the control.
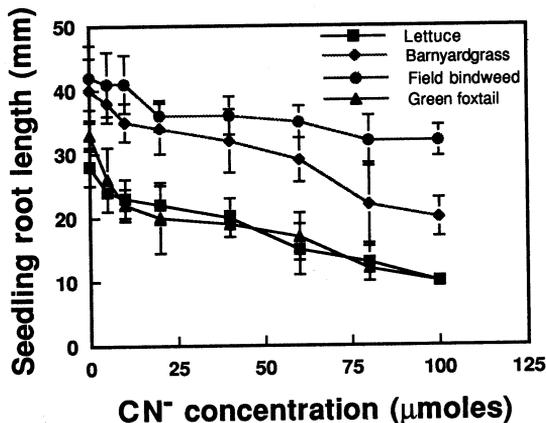

Fig. 1. Effect of exogenously applied cyanide as KCN on seedling root growth of selected plant species. Vertical bars indicate standard error of the mean.

Table 2. Quantitative HCN production by cell cultures of various rhizobacteria

	HCN (nmoles/mg cellular protein)	
Isolate	Culture broth	Culture broth + glycine
Pseudomonas sp.473	10.0	10.2
Pseudomonas sp.74	10.5	2.5
P. fluorescens 297	17.5	48.9
P. fluorescens 126	0.5	16.7
P. aeruginosa 136	34.0	52.0
P. fluorescens 672	8.8	11.2
P. syringae 81	0.5	5.8
Pseudomonas sp. 437	0.02	0.05
P. aureofaciens 52	0.04	0.10
Control	0.0	0.0
LSD (0.05)	7.5	10.0

root growth of lettuce, barnyardgrass, and green foxtail. Field bindweed growth was inhibited only at concentrations of 60 μmoles and above. Inhibition of seedling growth by low concentrations of cyanide has been previously reported for lettuce [1] and barnyardgrass [12].

Results of indicator assays for cyanogenesis by DRB were confirmed by using quantitative assays which revealed that four isolates liberated 10–34 nmoles HCN/mg protein (Table 2). All cyanogenic isolates except *Pseudomonas* sp. 74 evolved increased concentrations of HCN when glycine was added to the culture medium, indicating that the majority of DRB assayed was able to produce HCN through catabolism of glycine [3]. The levels of HCN produced by strongly cyanogenic DRB are similar to KCN concentrations that inhibited

seedling root growth (Fig. 1), suggesting that cyanogenesis by DRB inhabiting the rhizosphere environment could adversely affect root growth. Also, glycine, a common root exudate of many plants [10], would likely be available in the rhizosphere as a precursor for HCN synthesis by DRB.

Root growth of lettuce and barnyardgrass seedlings was inhibited by volatile metabolites during growth of cyanogenic DRB in the paired-plate assemblies (Table 3). Although the non-cyanogenic DRB *P. syringae* 81, *P. aureofaciens* 52, and *Pseudomonas* 437 developed abundant growth on the culture medium, these isolates did not inhibit growth of either indicator plant. This strongly suggests that HCN was the major component of volatile metabolites of cyanogenic DRB responsible for inhibiting root growth. Bacterial volatile metabolites

Table 3. Effect of volatile HCN produced by various rhizobacteria on seedling root length of lettuce and barnyardgrass

Strain	Lettuce		Barnyardgrass	
	Root length (mm)	% Reduction	Root length (mm)	% Reduction
Pseudomonas sp.42	2.7	85	3.8	83
Pseudomonas sp.74	5.6	68	10.5	54
Pseudomonas sp.473	3.9	78	3.1	86
P. fluorescens 297	3.9	78	2.8	88
P. fluorescens 126	4.1	77	7.5	67
P. aeruginosa 136	5.1	72	6.5	71
P. fluorescens 672	3.8	78	4.6	80
P. syringae 81	15.2	16	18.8	18
P. fluorescens 1942	4.6	74	10.6	54
Pseudomonas sp. 1035	5.2	71	3.8	84
Flavimonas oryzihabitans 7511	4.7	74	5.0	78
Pseudomonas sp. 437	19.0	5	21.3	6
P. aureofaciens 52	16.8	6	21.5	6
Control	18.0	—	22.8	—
LSD 0.05	0.7	—	1.6	—

were considerably more inhibitory toward root growth (Table 3) relative to inhibition by the same DRB isolates assayed in direct contact with seedling roots (Table 1). Similar observations reported previously suggested that HCN produced by bacteria in contact with root surfaces may be rapidly dispersed, degraded, or deactivated, more so than if roots were exposed to high levels of HCN [7]. Growth inhibition by non- or slightly cyanogenic DRB (Table 1) was apparently due to mechanisms other than HCN production.

On the basis of results of our assays, we infer that production of HCN at rates greater than 5 nmoles/mg cellular protein (Table 2) may contribute to growth inhibition of seedlings by cyanogenic DRB. Recognition of HCN as a major factor in growth inhibition of plants is supported in several previous studies [1, 7, 11]. To our knowledge, this is the first report demonstrating the inhibition of weeds by cyanogenic DRB as a potential biological control mechanism. Cyanogenic DRB selected for specific weeds could be applied inundatively to fields to inhibit weed seedling emergence and growth, leading to reduced competition with crop plants and limiting the use of synthetic herbicides that may impact the environment. Alternatively, native soil bacteria might be manipulated to express cyanogenesis through application of precursors (i.e., glycine) or cultivation of cyanide-resistant crops that select cyanogenic rhizobacteria in their rhizospheres through root exudation of precursors, thus serving as sources of DRB for weed suppression. Because soil conditions are more complex than those in vitro, further studies of edaphic factors affecting cyanogenesis by DRB should guide the selection of biotic agents and methods for expressing biological control in order to develop this approach as a feasible and environmentally sound biological weed management method.

ACKNOWLEDGMENTS

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