Enzymatic Activity in Root Exudates of Dahurian Wild Rye (*Elymus dauricus*) That Degrades 2-Chlorobenzoic Acid

Keywords: *Enzyme; phytoremediation; 2-chlorobenzoic acid; Elymus dauricus; pseudomonads*

INTRODUCTION

Bacterial seed inoculants can enhance contaminant degradation in soil as plants grow (Siciliano and Germida, 1997a; Crowley et al., 1996). Previously we found that only certain plant–bacteria associations reduced levels of 2-chlorobenzoic acid (2CBA) in soil. One association, Dahurian wild rye (*Elymus dauricus*) inoculated with a 1:1 mixture of *Pseudomonas aeruginosa* strain R75 and *Pseudomonas savastanoi* strain CB35, reduced levels of 2CBA in soil by 46%. We postulated that these inoculants either increased plant growth or augmented the ability of the rhizosphere microbial community to degrade compounds. Subsequently, we found that inoculating Dahurian wild rye had little effect on plant growth but increased the ability of the rhizosphere microbial community to degrade monochlorinated benzoic acids (Siciliano and Germida, 1997b). This supports our augmentation hypothesis. However, how inoculants augmented the degradative ability of the rhizosphere is still unclear.

Noninoculated Dahurian wild rye decreases the levels of 2CBA in solution during hydroponic growth, suggesting that either the roots or some compound in the root exudate mediates the decrease in 2CBA levels. Supporting this idea, plant tissue cultures are known to metabolize trichloroethylene (Newman et al., 1997), root surface peroxidases polymerize phenols in solution (Adler et al., 1994), and a root-associated compound stimulates atrazine degradation in water (Burken and Schnoor, 1996). In this paper, we report the presence of an enzyme in the root exudate of Dahurian wild rye that degrades 2CBA.

MATERIALS AND METHODS

The root exudates of Dahurian wild rye, meadow brome (*Bromus biebersteinii*), and streambank wheatgrass (*Agropyron riparum*), all previously shown to reduce 2CBA levels in soil (Siciliano and Germida, 1997a), were collected during hydroponic growth and tested for their ability to reduce levels of 2CBA in solution. The plants were either noninoculated or inoculated with a mixture of *P. aeruginosa* strain R75 and *P. savastanoi* strain CB35 as previously described (Siciliano and Germida, 1997a) and grown in a hydroponic system described by van Overbeek and van Elsas (1995) with the exception that the M9 growth solution contained 300 mg of 2CBA L^{-1}. The sterility of noninoculated treatments was assessed by plating out 0.1 mL of hydroponic solution onto 1/10th strength trypticase soy broth solidified with agar (TSA) and incubating for 48 h at 38 °C. The survival of the inoculant mixture was assessed on TSA supplemented with antibiotics (Siciliano and Germida, 1997b). After 28 days of growth, a 4 mL aliquot of hydroponic solution was filter sterilized (0.2 μm, cellulose acetate membrane) and 2CBA levels in this aliquot were determined every day for 3 days by HPLC (Siciliano and Germida, 1997b). To determine if plants contained a catalytic compound that degraded 2CBA, we triturated Dahurian wild rye roots with a mortar and pestle and resuspended them in low-salt buffer (Adler et al., 1994). The extract was filter sterilized and tested for 2CBA-degrading activity by measuring 2CBA levels in solution. Protein levels in the hydroponic solution and root extracts were determined according to the Lowry and Bradford assays with bovine serum albumin as a standard (Daniels et al., 1994). These experiments were each repeated three to five times with three replicates per treatment.

We characterized the 2CBA-degrading activity in Dahurian wild rye root exudate at pH values ranging from 5.9 to 8.1. Aliquots (0.5 mL) of root exudate were analyzed for 2CBA, mixed with 1.5 mL of 300 mg of 2CBA L^{-1} amended M9 medium, adjusted with either HCl or NaOH to the desired pH, and maintained at 23 °C; the 2CBA level was determined every 12 h (over a 3 day period) by HPLC analysis. We also characterized the 2CBA-degrading activity in root exudate at temperatures ranging from 10 to 50 °C at a pH of 6.6. The dependence of the reaction rate on the initial substrate level was characterized by varying the amount of 2CBA in the M9 solution from 0.32 to 10 μmol. Analysis of velocity versus substrate plots was performed as described by Cornish-Bowden and Wharton (1988). We determined if the catalytic activity in Dahurian wild rye root exudates was protein in nature by assaying the sensitivity of 2CBA degradation to protease. An assay similar to that described above was run for a period of 10 days, followed by the addition of 200 μL of a filter-sterilized (0.2 μm) solution containing 10 mg of protease mL^{-1} (*Streptomyces caespitosus* type IV, Sigma P-0384), and 2CBA levels were followed for a further 5 days. We determined if other chlorinated benzoic acids were degraded by the catalytic compound in root exudates. The assay was similar to that described above, but the M9 medium was amended with 100 mg L^{-1} 3-chlorobenzoic acid (3CBA), 2,3-dichlorobenzoic acid (23diCBA), or 2,5-dichlorobenzoic acid (25diCBA) instead of 2CBA.

The presence of this catalytic activity during the phytoremediation of 2CBA in soil was investigated by extracting proteins from the rhizosphere of inoculated or noninoculated Dahurian wild rye. Dahurian wild rye was inoculated as described above but planted in uncontaminated soil (Typic Haploborolls) or soil contaminated with 2CBA (51 mg kg^{-1}). This soil was initially contaminated with solid 2CBA at a concentration of 467 mg kg^{-1} and used in a study designed to screen the degradative ability of forage grasses (Siciliano and Germida, 1997a). At the end of that study, all contaminated soil was bulked together and stored in metal cans for 3 years. For the present study, the stored soil was thoroughly mixed and the residual extractable 2CBA level determined on six subsamples by HPLC analysis. Since inoculating soil with strains R75 and CB35 reduces 2CBA levels (Siciliano and Germida, 1997a), we determined if the inoculants or other soil microflora in the absence of a plant produced a compound that catalyzes the reduction in 2CBA levels. Nonplanted, inoculated treatments were amended every 2 days with 5 mg of glucose and 1 mg of yeast extract to simulate the stimulation of bacteria by root lysate. Twenty-one days after planting, the root system was extracted from pots and shaken vigorously. The rhizosphere sample was extracted by adding $\approx$10 mL of low-salt buffer (Adler et al., 1994) to 1 g of roots and soil in a 50 mL centrifuge tube. This was shaken on its side at 120 rpm for 90 min and centrifuged at 1000 rpm for 20 min, and the supernatant was filter sterilized (0.2 μm). The assay for 2CBA-degrading activity was similar to that used for the hydroponic exudate. This experiment was repeated twice with five replicates per treatment.

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Table 1. Degradation of 2CBA by Filter-Sterilized Root Exudates of Dahurian Wild Rye

plant root exudate[a]	inoculated[b]	protein (mg)	2CBA degraded[c] (nmol/day)
Dahurian wild rye	no	0.26	28
	yes	0.31	24
streambank wheatgrass	no	0.31	3
	yes	0.61	2
meadow brome	no	0.14	1
	yes	0.10	3
LSD (0.05)		0.42	25

[a] The rate of 2CBA degradation in the absence of plants was zero. [b] The bacterial strains R75 and CB35 were grown for 48 h and inoculated onto plant seed (10^6 cfu seed^{-1}), and the plants were grown in a hydroponic system for 21 days. [c] Degradation of 2CBA calculated in exudate containing ca. 7.6 μmol of 2CBA.

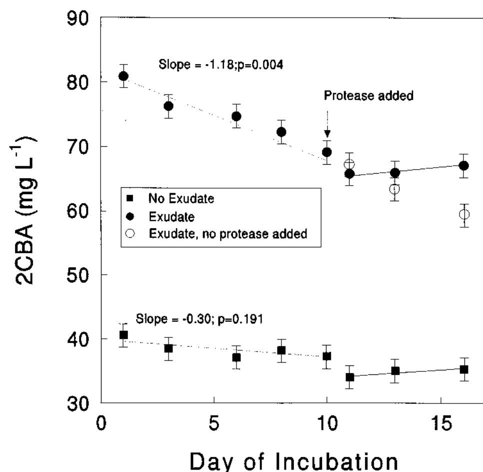

Figure 1. Elimination of 2CBA-degrading activity by the addition of protease. At day 10, 200 μL of a solution containing 10 mg of protease mL^{-1} (*S. caespitosus* type IV) was added to the reaction vials and the 2CBA concentration determined by HPLC.

RESULTS AND DISCUSSION

Only the hydroponic exudate of inoculated and noninoculated Dahurian wild rye degraded 2CBA in solution (Table 1). Although the inoculant survived in the hydroponic solution (data not shown), it had no effect on 2CBA degradation by filter-sterilized root exudates. Thus, of the plants that degrade 2CBA in soil, only Dahurian wild rye degraded 2CBA in hydroponic solution. Furthermore, the root exudate contained low levels of protein, suggesting that 2CBA degradation was related to an enzyme. Although substantial amounts of protein (1.28 mg) were present in the root extract, little 2CBA-degrading activity (9 nmol day^{-1}; standard error = 8) was detected, suggesting that a specific protein was involved in 2CBA degradation. Thus, it appears that the root-associated 2CBA-degrading activity was present only in root exudates.

The highest 2CBA-degrading activity was obtained at pH 6.3–6.6 with little activity observed at pH 5.9 or 7.3. The reaction rate increased with temperature, doubling from 18 to 38 nmol day^{-1} as temperature increased from 23 to 40 °C with no activity observed at 10 and 50 °C. The rate of 2CBA degradation followed Michaelis–Menten kinetics with apparent V_{max} and K_m of 13.4 μmol day^{-1} and 657 nmol of 2CBA, respectively. Degradation of 2CBA was linear for a 10 day period, and the addition of protease stopped the reaction (Figure 1). No degradation of 3CBA, 23diCBA, or 25diCBA occurred in solution. Collectively, these results indicate the presence of an enzyme in the root exudates of Dahurian wild rye that specifically catalyzes the reduction of 2CBA levels in solution.

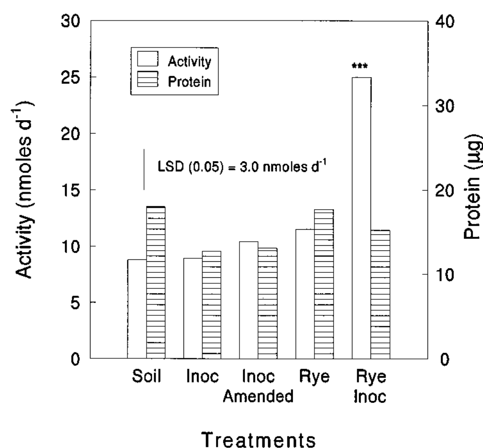

Figure 2. Protein levels and 2CBA-degrading activity in rhizosphere extracts of Dahurian wild rye. For treatments with only bacterial inoculants (Inoc), autoclaved Dahurian wild rye seeds were inoculated (10^6 cfu seed^{-1}) with strains R75 and CB35 and planted into soil. Amended treatments (Inoc Amended) had 5 mg of glucose and 1 mg of yeast extract added every 2 days to soil. Dahurian wild rye seed ($n = 10$) was either noninoculated (Rye) or inoculated (Rye Inoc) with strains R75 and CB35 and grown in soil for 21 days. Bars are the average of two experiments with five replicates per treatment. Treatments significantly different from controls ($p < 0.001$) are marked with ***.

Inoculating Dahurian wild rye increased ($p = 0.001$) the 2CBA-degrading activity in rhizosphere extracts but had little effect on protein levels (Figure 2). In contrast to hydroponics, there was little observed activity in the rhizosphere of noninoculated plants. It is well-known that plant physiology differs between hydroponics and soil (Curl and Truelove, 1986), and this may be one reason for the difference in 2CBA-degrading activity of Dahurian wild rye in hydroponics and soil. Despite this difference, the 2CBA-degrading activity in rhizosphere extracts was eliminated by protease treatment in a manner similar to that seen for hydroponics (data not shown). Adding glucose and yeast extract to soil had no effect on protein levels or 2CBA-degrading activity in the rhizosphere extract, suggesting that the activity seen in rhizosphere extracts was derived from the plant. In addition, contaminated soil had little effect on the 2CBA-degrading activity of rhizosphere extracts, which suggests that this activity is involved in other plant metabolic processes and reduces 2CBA serendipitously. While there have been previous reports in a non-peer-reviewed format of extracellular plant enzymes that degrade contaminants (Schnoor et al., 1995), to the best of our knowledge, no extracellular plant-produced 2CBA-degrading activity has been described previously. However, our results are limited to describing the reduction in 2CBA levels. It is possible that the enzyme is not degrading 2CBA but instead may be transforming 2CBA into a compound which is not amenable to our HPLC analysis. In addition, it is unlikely that 2CBA is being sorbed by some compound in root exudate because degradative activity was (i) seen in two different experimental systems, (ii) induced by bacterial inoculation, and (iii) specific for 2CBA with no degradation of 3CBA, 23diCBA, or 25diCBA observed. Future plans include the purification and further characterization of this enzyme, as well as a determination of its role during the phytoremediation of 2CBA by Dahurian wild rye.

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S. D. Siciliano,[†] H. Goldie,[‡] and J. J. Germida*[,†]
Department of Soil Science and Toxicology Group and Department of Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5A8

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[†] Department of Soil Science and Toxicology Group.
[‡] Department of Microbiology.