ASSOCIATIONS OF *PSEUDOMONAS* SPECIES AND FORAGE GRASSES ENHANCE DEGRADATION OF CHLORINATED BENZOIC ACIDS IN SOIL

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ABSTRACT

Microorganisms and plants can be used as bioremediation agents to clean up contaminated soil sites in a cost effective and environmentally friendly manner. Furthermore, associations of plants and bacteria can act synergistically, and thereby eliminate difficulties encountered when using a single agent. The purpose of my thesis research was to develop and characterize associations of plants and bacteria that degraded chlorinated benzoic acids (CBA) in soil. The contaminants were used as model compounds because they are present in soils contaminated with polychlorinated biphenyls (PCB) or chlorinated pesticides.

Sixteen forage grasses in combination with 12 bacterial inoculants were screened for their ability to promote the degradation of CBA in soil. The CBAs were added to soil as single isomers, or in tertiary mixtures. The effect of inoculants on the root associated microbial community was assessed by fatty acid methyl ester (FAME) profiles as well as carbon substrate utilization as determined by the Biolog system. In addition, inoculant stimulation of the CBA degradative activity of roots or rhizosphere soil was determined by *in vitro* and hydroponic systems. Degradative enzymes were isolated from root exudates using ultrafiltration and chromatography.

Five associations of plants and *Pseudomonas* species degraded CBA to a greater extent than plants without bacterial inoculants. Plant-bacterial associations that increased 2-chlorobenzoic acid (2CBA) degradation had little effect on di-chlorinated benzoic acid degradation. Furthermore, the effective inoculants altered the root-associated microbial community of *Bromus biebersteinii* and simultaneously increased the CBA degradative activity of roots. Although these *Pseudomonas* species had little effect on the microbial

community composition of *Elymus dauricus*, they stimulated a plant enzyme capable of degrading 2CBA in the rhizosphere.

Bacterial inoculants stimulated CBA degradation by some plant species by altering the microbial community present on the root surface, and thereby increasing the ability of this community to degrade CBA. Alternatively, with other plant species inoculants stimulated the production of a plant enzyme(s) that degraded 2CBA. My research has demonstrated that specific interactions between plants and bacteria promote contaminant degradation in soil, and suggests that new remediation strategies can be developed based on such interactions.

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1. INTRODUCTION

Contaminated soil poses a significant threat to human and ecosystem health in Canada. There are estimated to be in excess of 10,000 contaminated soil sites in Canada and the cost associated with the remediation of these sites is in the billions of dollars (Hrudey and Pollard 1993). The use of biological organisms to clean up hazardous waste sites *i.e.* bioremediation, has the potential to reduce the cost associated with remedial action, and plants are one such possible organism (Cunningham et al. 1995). Plants have an extensive root system which allows them to explore a large volume of soil, and they enhance microbial activity in soil which can stimulate contaminant degradation (Anderson et al. 1993; Shimp et al.1993). These attributes have led numerous investigators to explore the potential of plants to remediate contaminants in soil (Aprill and Sims 1990; Walton and Anderson 1990; Ferro et al. 1994; Perkovich et al. 1996; Reilley et al. 1996; Xu and Johnson 1995). However, there are some obstacles facing the use of plants as bioremediation agents: 1) their capacity to degrade pollutants is unknown and 2) the phytotoxicity of many contaminants limits their growth. For example, only recently have plants been shown to degrade common pollutants such as polychlorinated biphenyls (PCB), trichloroethylene (TCE) or nitroglycerin (Goel et al. 1997; Newman et al. 1997; Wilken et al. 1995). Furthermore, contaminants such as PCB, TCE, pentachlorophenol (PCP), hydrocarbons and chlorinated benzoic acids (CBA) reduce the growth of some plants (Xu and Johnson 1995; Siciliano et al. 1997; Newman et al. 1997; Pfender 1996; Wilken et al. 1995). Consequently, strategies need to be found to overcome these and other limitations of phytoremediation systems.

Bacteria are another possible organism to use in bioremediation systems and have been extensively studied as bioremediation agents (Providenti et al. 1993; Neilson 1996). The catabolic versatility of bacteria is widely known, but many bacterial bioremediation

inoculants do not survive, move or remain metabolically active once inoculated into soil (Devare and Alexander 1995; Haluska et al. 1995). This can be a significant problem during bioremediation and ways need to be found to increase microbial survival, transport and activity in contaminated soil (National Research Council 1993).

One possible solution to the problems associated with both plants and bacteria bioremediation agents is to combine the two organisms into one remedial technology. Table 1.1. compares the advantages and disadvantages of plant- and bacteria-based bioremediation systems. It is apparent that the advantages of plant systems offset the disadvantages of bacteria remedial systems and vice versa. For example, bacteria are known to promote plant growth (Glick 1995) and may reduce contaminant phytotoxicity (Pfender 1996; Shann 1995; Krueger et al. 1991). Hence, this would allow plant growth in contaminated sites whose phytotoxicity would preclude traditional phytoremediation approaches. In addition, bacteria which degrade contaminants can be inoculated into the rhizosphere and thereby augment the ability of the plant to degrade contaminants. For example, PCB degradation genes can be inserted into bacteria which then express these genes in the rhizosphere (Brazil et al. 1995). Such an approach would offset the unknown, and perhaps limited, capabilities of plants to degrade contaminants. Thus, bacterial inoculants can overcome the limitations found in plant based bioremediation systems.

Similarly, plants can be used to circumvent many of the problems associated with bacteria based bioremediation systems. For example, bacterial seed inoculants are known to be protected from predators, have increased plasmid stability, remain metabolically active, and survive on plant roots for over a year in field conditions (Smit et al. 1996; Crowley et al. 1996; Rattray et al. 1995; Hirsch and Spokes 1994). Thus plants can provide a protective niche for micro-organisms which may mitigate the poor survival traditionally seen with bacteria inoculated into soil. In addition, bacteria move with roots as roots explore soil (Hekman et al. 1995) and bacterial metabolism is influenced by root exudates (van Overbeek and van Elsas 1995). Thus plants will

increase bacterial inoculant transport through soil thereby increasing the effectiveness of inoculants and may, via their root exudates, induce the bacteria to continue degrading pollutants in the face of other, more amenable carbon sources. Hence, inoculating bacteria that degrade pollutants onto plant seeds might mitigate many of the problems encountered with traditional bacterial bioremediation inoculants.

TABLE 1.1 Advantages and disadvantages of plant and bacteria remediation systems.

PLANT SYSTEMS		BACTERIA SYSTEMS	
Advantages	Reference	Disadvantages	Reference
Protective niche for micro-organisms	Rattray et al. , 1995	Poor survival in soil	Havel and Reineke, 1992
Extensive root systems	Aprill and Sims, 1990	Limited transport through soil	Devare and Alexander, 1995
Root exudate release	Anderson et al. , 1993	Regulation of desired catabolic activity	Barriault and Sylvestre, 1993
Disadvantages	Reference	Advantages	Reference
Phytotoxicity of contaminated sites	Pfender, 1996	Plant growth promotion/ Detoxification	Glick, 1995; Krueger et al. , 1991
Unknown catabolic versatility	Siciliano and Germida, 1997	Catabolic versatility	Brazil et al. , 1995

The purpose of my thesis research was to investigate plant-bacteria associations and determine if they would promote contaminant degradation in soil. I utilised CBA as model contaminants. These compounds are present in many contaminated sites, and arise from the degradation of PCB and chlorinated herbicides (Fava et al. 1996). Furthermore, these compounds are known to adversely affect the PCB degradation

pathway (Sylvestre 1995). Chlorinated benzoic acids have a wide range of solubility and recalcitrance, ranging from the labile and soluble 3-chlorinated benzoic acid (3CBA) to the recalcitrant and poorly soluble 2,5-dichlorobenzoic acid (25diCBA). The metabolism of these components includes a variety of pathways with one common aerobic degradation route illustrated in Figure 1.1. Because of these attributes many other investigators have utilized CBA as model compounds (Crowley et al. 1996; Haby and Crowley 1996; Fava et al. 1996; Barriault and Sylvestre 1993).

My research project had two goals:

1) Develop specific plant-bacteria associations that degrade halogenated aromatics in soil.
2) Determine how plant-bacteria interactions stimulate remedial activity.

This was accomplished through a series of studies designed to :

1) Identify plants tolerant of contaminants in soil.
2) Identify bacterial seed inoculants which increased or initiated contaminant degradation by plants.
3) Determine the mechanism by which bacterial inoculants increase contaminant degradation by plants.
4) Determine how differences between plant species affect the ability of bacterial inoculants to stimulate contaminant degradation.
5) Isolate biochemical compounds produced by the plant which play a role in contaminant degradation.

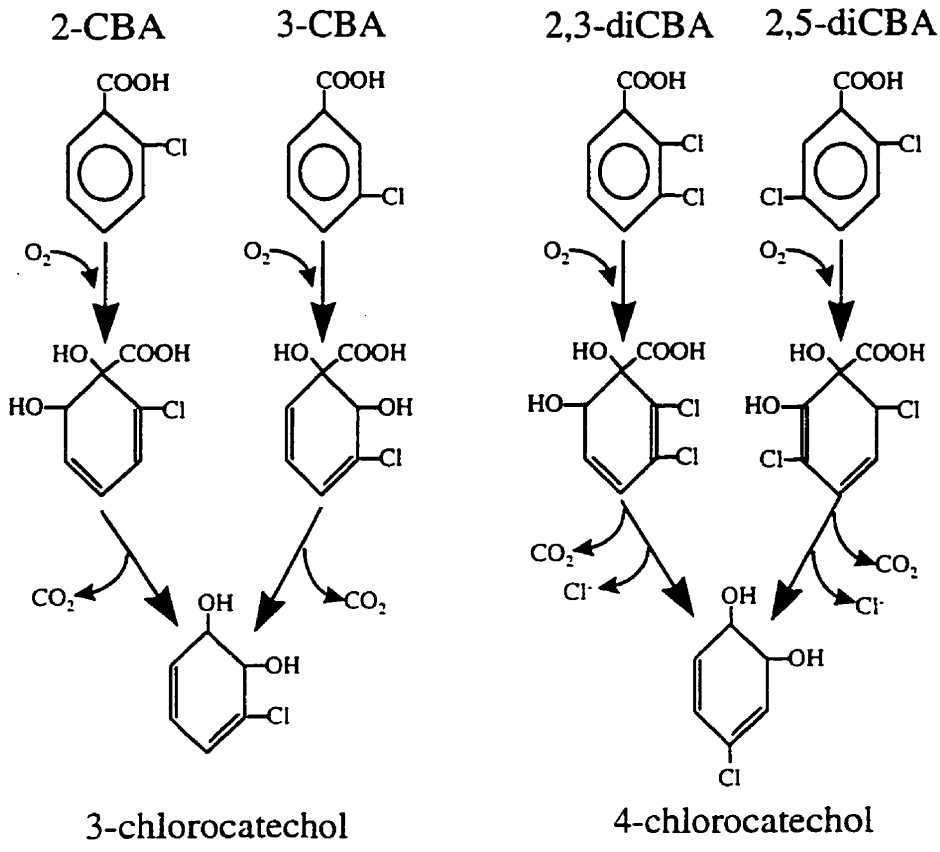

Fig. 1.1. A common aerobic degradation route of di- and mono-chlorinated benzoic acids. 2-CBA is 2-chlorobenzoic acid; 3-CBA is 3-chlorobenzoic acid; 2,3-diCBA is 2,3-dichlorinated benzoic acid; 2,5-diCBA is 2,5-dichlorinated benzoic acid. Adapted from Hickey and Focht (1990)

In Chapter 2 I developed a theoretical framework to describe possible roles of plants and bacteria in phytoremediation systems. When this thesis research was begun, bacteria-phytoremediation research was still largely a matter of speculation and was driven by the hypothesis of Anderson et al. (1993) regarding mutualistic associations between plants and rhizosphere organisms. Since that time, there have been several studies into the ecological and biochemical mechanisms of phytoremediation activity and it has become clear that plants and bacteria interact in specific and non-specific manners during the remediation of contaminants in soil. The empirical portion of this thesis explores the possible associations between plants and bacteria in contaminated soils.

In order to develop specific plant-bacteria associations, it was first necessary to identify those plants tolerant of CBA in soil. In Chapter 3, I studied the response of 17 different forage grasses to 2-chlorobenzoic acid (2CBA) contamination. I found that while some plants tolerated a range of 2CBA levels, many were very sensitive to 2CBA and PCB levels in soil. Thus, I investigated the potential of these sensitive grasses to act as bioindicators of soil contamination.

In Chapter 4 I identified plant-bacteria relationships that reduced 2CBA levels in soil. The efficacy of these associations was tested in three different Saskatchewan soils and at three different initial contamination levels. In Chapter 5, I evaluated the screening procedures used in Chapter 4 for their effectiveness in selecting plant-bacteria associations that reduced other CBAs such as 3-chlorobenzoic acid (3CBA), 2,3-dichlorobenzoic acid (23diCBA) or 2,5-dichlorobenzoic acid (25diCBA), in soil. In this chapter I also determined if plant bacteria associations can reduce levels of CBA present in mixtures because contaminated sites often contain mixtures of such compounds. Collectively the results presented in these two chapters indicated that plant-bacteria associations were successful in reducing CBA levels in soil, and suggested that the formation of such associations was due to specific plant-bacteria interactions. I postulated that there were two possible plant-bacteria interactions which gave rise to increased contaminant degradation in soil:

1) inoculants stimulated plant or rhizosphere activity and thereby increased the degradative effect of the indigenous microflora in soil, or
2) inoculants enhanced the ability of the rhizosphere to degrade contaminants by either stimulating degradative processes, or degrading the contaminants themselves.

I investigated the validity of these hypotheses in Chapter 6 by exploring the mechanism by which a mixture of two bacterial inoculants, *Pseudomonas aeruginosa* strain R75 and *P. savastanoi* strain CB35, increased the 2CBA degradation by Dahurian wild rye (*Elymus dauricus*). I found that inoculants increased the catabolic activity of the rhizosphere while not affecting plant parameters. Furthermore, this increase in catabolic activity was limited to mono-chlorinated benzoic acids with no effect seen on the potential of rhizosphere soil to degrade di-chlorinated benzoic acids.

Thus, Chapter 7 investigated how bacterial inoculants stimulated catabolic activity without increasing plant growth. In this chapter I studied the effect of inoculating a mixture of the strains R75 and CB35 on the rhizoplane community and catabolic activity of Dahurian wild rye and meadow brome (*Bromus biebersteinii*). Results in Chapter 7 demonstrated that bacterial inoculants stimulated phytoremediation in different manners between plant species. The microbial community associated with the rhizoplane of meadow brome was altered by inoculants, whereas there was no effect on the community associated with Dahurian wild rye's rhizoplane. Furthermore, there was a substantial increase in the potential of the meadow brome rhizoplane to degrade 2CBA but no such effect for Dahurian wild rye. Chapter 8 investigated how inoculants stimulated 2CBA degradation by Dahurian wild rye without altering the rhizoplane community. Here I found a protein released from Dahurian wild rye roots that reduced levels of 2CBA in solution. Furthermore, I found that inoculating Dahurian wild rye increased expression of this protein in soil.

2. MECHANISMS OF PHYTOREMEDIATION: BIOCHEMICAL AND ECOLOGICAL INTERACTIONS BETWEEN PLANTS AND BACTERIA.

2.1. ABSTRACT

The use of plants to reduce contaminant levels in soil is a cost effective method of reducing the risk to human and ecosystem health posed by contaminated soil sites. This review concentrates on plant-bacteria interactions which increase the degradation of hazardous organic compounds in soil. Plants and bacteria can form specific associations in which the plant provides the bacteria with a specific carbon source that induces the bacteria to reduce the phytotoxicity of the contaminated soil. Alternatively, plants and bacteria can form non-specific associations in which normal plant processes stimulate the microbial community which in the course of normal metabolic activity degrades contaminants in soil. Plants can provide carbon substrates and nutrients as well as increasing contaminant solubility. These biochemical mechanisms increase the degradative activity of bacteria associated with plant roots. In return, bacteria can augment the degradative capacity of plants or reduce the phytotoxicity of the contaminated soil. However, the specificity of the plant-bacteria interaction is dependent upon soil conditions which can alter contaminant bioavailability, root exudate composition and nutrient levels. In addition, the metabolic requirements for contaminant degradation may also dictate the form of the plant-bacteria interaction *i.e.* specific or non-specific. No systematic framework has emerged which can predict plant-bacteria interactions in a contaminated soil, but it appears that the development of plant-bacteria associations that degrade contaminants in soil may be related to the presence of allelopathic chemicals in the rhizosphere. Therefore, investigations into plants which are resistant to, or produce allelopathic chemicals, is suggested as one possible method

of identifying plant-bacteria associations which can degrade contaminants in soil.

2.2. INTRODUCTION

Contaminated soil poses a significant threat to human and ecosystem health in Canada. There are estimated to be in excess of 10,000 contaminated soil sites in Canada and the cost associated with the remediation of these sites is in the billions of dollars (Hrudey and Pollard, 1993). The use of organisms to clean up hazardous waste sites *i.e.* bioremediation, can potentially reduce the cost of remedial action, and plants are one such possible organism (Cunningham et al., 1995). Plants have an extensive root system that explores a large volume of soil and they enhance microbial activity, which can stimulate contaminant degradation (Anderson et al., 1993; Shimp et al., 1993). These attributes have led numerous investigators to explore the potential of plants to remediate contaminated soil (Aprill and Sims, 1990; Xu and Johnson, 1995; Reilley et al., 1996; Perkovich et al., 1996; Ferro et al., 1994; Walton and Anderson, 1990). However, the mechanism by which plants stimulate the disappearance of hazardous organics from soil is not fully understood.

2.2.1. Modes of Phytoremediation

Plants are known to sequester, degrade and stimulate the degradation of organic contaminants in soil (Anderson et al., 1993; Shimp et al., 1993). Disentangling these three processes from each other is a significant challenge in phytoremediation research with important consequences because it will, 1) suggest methods of enhancing phytoremediation activity, and 2) highlight areas of toxicological concern. For example, the sequestration of heavy metals by plants is an effective method of reducing heavy metal contamination in soil (Cunningham et al., 1995), but biomagnification and entry of toxicants into the human food chain are possible consequences of this approach. Hence, phytosequesteration technologies must take special precautions to prevent the ingestion of contaminated plants by livestock or wildlife.

Sequestration of toxicants by plants is an important area of phytoremediation research. Plants are known to accumulate a variety of toxicants from soil (Paterson et al., 1990) but the importance of the soil-plant route of bioaccumulation for lipophilic compounds may be minimal (Simonich and Hites, 1995). Most lipophilic pollutants do not pass beyond the root surface due to the high proportion of lipids on the root's surface and, hence, are not translocated within the plant (Trapp et al., 1990; Paterson and Mackay, 1994; Wang and Jones, 1994; Simonich and Hites, 1995). Thus it is unlikely that plants will accumulate significant amounts of lipophilic contaminants. However, there are exceptions, e.g. zucchini can accumulate polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F) (Hulster et al., 1994). This suggests that not all plants use a passive uptake process for lipophilic compounds. More research is required to identify and characterize plants with active uptake processes for contaminants.

In contrast to lipophilic compounds, hydrophilic toxicants (i.e. log K_{ow} = 1 to 3 [K_{ow}, octanol-water partition coefficient]), have the potential to be taken up and transformed in the plant (Simonich and Hites, 1995). For example, Ferro et al. (1994) found that hycrest crested wheatgrass (developed by hybridizing *Agropyron cristatum* (L.) Gaertn. crossed with *A. destorum*) reduced levels of C^{14} labelled pentachlorophenol (PCP) in soil, however 30-40% of the initial ^{14}C label was recovered in plant tissue, suggesting that hycrest crested wheatgrass accumulated PCP or degradation products in it's biomass. Other authors have also noted that plants can bioaccumulate a wide range of organic compounds that are hydrophilic (Hope, 1995; Paterson et al., 1990; McFarlane et al., 1987). The potential of plants to transform such compounds makes the interpretation of bioaccumulation data difficult. For example, in the case of Ferro et al. (1994), the chemical form of the labelled compound present in the plant was not determined. Hence it is not known if hycrest crested wheatgrass accumulated PCP, a PCP metabolite, or a carbon exudate of bacteria degrading PCP in soil.

Plant tissue cell cultures degrade contaminants such as trichloroethylene (TCE), polychlorinated biphenyls (PCB) or nitroglycerin (Schalk et al., 1994; Newman et al, 1997; Wilken et al, 1995; Goel et al., 1997). However, the behaviour of plant tissue cell cultures does not always mimic that seen *in vivo*. Why and how plants metabolize such xenobiotics is not fully understood. There are some suggestions that contaminant metabolism in plants is not due to a specific detoxification reaction, but instead may simply be due to the non-specificity of hydrolytic enzymes. For example, the plant cytochrome P450 enzyme CYP73A1, which is a cinnamate 4-hydroxylase expressed in *Helianthus tuberosus*, was found to react with low efficiencies with small, planar xenobiotics including some herbicides (Schalk et al., 1994). Further, these investigators found that a wide range of herbicides were competitive inhibitors of this enzyme (Schalk et al., 1994). The degradation of TCE and PCBs is also thought to be mediated through P450 systems (Newman, 1997; Wilken et al., 1995). Wilken et al. (1995) speculated that plant P450 systems work in a similar fashion to that seen in mammalian systems, i.e. hydroxylation followed by conjugation. However, such speculation awaits more experimental verification.

Plant transformations of contaminants may cause problems due to the production of toxic metabolites. For example, Wilken et al. (1995) found a number of hydroxylated intermediates of PCB during degradation by various cell cultures. Hydroxylated intermediates may play a role in the increased toxicity, mutatoxicity and genotoxicity often seen during PCB degradation (Barriault and Sylvestre, 1993; Meier et al., 1997). However, because the transformation enzyme systems of plants are different from those of bacteria, toxic metabolites which normally occur during bacterial biodegradation may not occur with phytoremediation. For example, Newman et al. (1997) found that the production of the toxic intermediate, chloral hydrate, during TCE degradation was minimal and postulated that this was due to chloral hydrate's short half life in plant systems. The differences between the degradation pathways of plants and bacteria require further research so that these

differences may be exploited to develop remediation systems which avoid toxic metabolite production.

Besides sequestering or metabolizing contaminants, plant roots may increase contaminant degradation *in situ* via their root systems. Plant roots and their exudates increase microbial numbers in the soil surrounding their roots by one to two orders of magnitude, thus increasing microbial activity. Many authors postulate that this stimulation will lead to enhanced degradation of contaminants in soil (Gunther et al., 1996; Anderson and Walton, 1995; Haby and Crowley, 1996). For example, the rhizosphere soil of *Kochia* sp. supported increased microbial numbers and pesticide degradation (Perkovich et al., 1996). The authors postulated that an increase in microbial numbers in the rhizosphere due to root exudates was the reason for increased pesticide degradation. However the interactions between plants and bacteria that increase contaminant degradation are not fully understood.

For the purpose of this review, interactions between plants, bacteria and soil will be grouped into two categories, biochemical and ecological. Biochemical interactions are the physical-chemical interactions which alter soil chemistry, or lead to contaminant degradation. Ecological interactions involve the interactions between species which modulate biochemical activity. For example, the ability of an organism to degrade a contaminant is a biochemical process that does not by itself guarantee that this organism will reduce contaminant levels once inoculated into soil. Other factors such as susceptibility to predation, movement through soil, genetical exchange with other organisms can substantially affect the ability of the inoculant to degrade contaminants in soil (Goldstein et al., 1985; Ramadan et al., 1990; Devare and Alexander, 1995; Steffensen and Alexander, 1995). Understanding biochemical and ecological interactions is important for developing a strategy to improve the efficiency of phytoremediation. An ecological understanding of plant-bacteria interactions during contaminant degradation will suggest strategies for enhancing degradation. Understanding the biochemical basis of these interactions will outline

the methods needed to accomplish the strategy. The purpose of this review is to critically examine the role of bacteria in the ecological and biochemical interactions that occur during phytoremediation.

2.3. THE ECOLOGICAL QUESTION OF PLANT-BACTERIA INTERACTIONS

What is the specificity of the interaction between plants and bacteria that leads to contaminant degradation? The answer to this question will determine the strategy used to develop and evaluate phytoremediation methods. For example, Donnelly et al. (1994) suggest that plants specifically increase degradation of certain contaminants in soil by providing the soil microflora with polyphenolic compounds. These compounds in turn will induce bacterial enzymes that can degrade a variety of pollutants such as TCE or PCB. Consequently, they screened a wide range of plants for production of polyphenolics that support PCB degrading bacteria, and identified Mulberry (*Morus rubra* L.) as a possible plant species well suited to remediating PCB contaminated soil sites (Fletcher and Hegde, 1995; Hegde and Fletcher, 1996). However, it is not clear if these plants would increase exudation in the presence of contaminants. In contrast, other groups have suggested that stimulation of bacteria may occur indirectly due to nutrients released from roots i.e., a non-specific relationship. These nutrients, often low molecular weight organic acids, increase microbial biomass and activity but do not normally induce specific enzymatic processes that degrade xenobiotics. Consequently, plant species with deep, fibrous roots that can grow in stressed environments are used in phytoremediation studies (Gunther et al., 1996). Thus, answering the question of how different plants stimulate soil microorganisms will have important implications for phytoremediation technology development.

2.3.1. Role of the plant in phytoremediation: Specific Interactions

Walton et al. (1994b) propose that plants produce specific signals in response to a contaminant. As a result bacteria detoxify contaminants in soil and the plant

provides root exudates which either supply an energy source or in some other way increase microbial detoxification activity in the rhizosphere (Fig. 2.1). The key point to this association is that the plant alters its behaviour in contaminated soil to stimulate microbial communities which degrade contaminants. Walton et al. (1994b) use evolutionary theory to support their hypothesis. Plants which encounter toxicants in soil will not survive unless they can find a way to detoxify the contaminant. Over the millennia, plants have developed means of using rhizobacteria as a method to detoxify toxins in soil.

However, because contamination occurs rarely and normally at low levels, plant mechanisms of stimulating degrading bacteria are not likely to be as specific as that seen in legume-*rhizobium* relationships where very specific plant-bacteria interactions have developed. For example, *Bradyrhizobium* not native to the soil from which *Amphicarpaea bracteata* originated from, caused a 39% decrease in seed biomass (Parker, 1995). Similarly, it is unlikely that bacteria have developed the means to induce the production and excretion of specific compounds from plant roots like that seen in crown gall disease. In crown gall disease, *Agrobacterium spp.* induce the formation of a tumour on the root which excretes specific compounds called opines which the *Agrobacterium* then uses (Moore et al., 1997). In both of these examples, genetic alterations have occurred within the plant and bacterium as a consequence of continous evolutionary pressure. Contaminants are present in soil rarely or at low concentrations and thus, there is no continous evolutionary pressure. Consequently, specific plant-bacteria interactions still occur in phytoremediation but may not be based on the strict genetic alteration seen between legumes and rhizobia. For example, Siciliano and Germida (1997) found that a combination of pseudomonads enhanced the phytoremediation activity of three different forage grasses while having no effect on other grasses. One of these strains was isolated as a plant-growth promoting rhizobacteria of wheat whereas the other was isolated from 2-chlorobenzoic acid contaminated soil. Thus it is unlikely that genetic alterations in the plant or bacteria are the basis for the enhanced phytoremediation activity seen when these two organisms are combined.

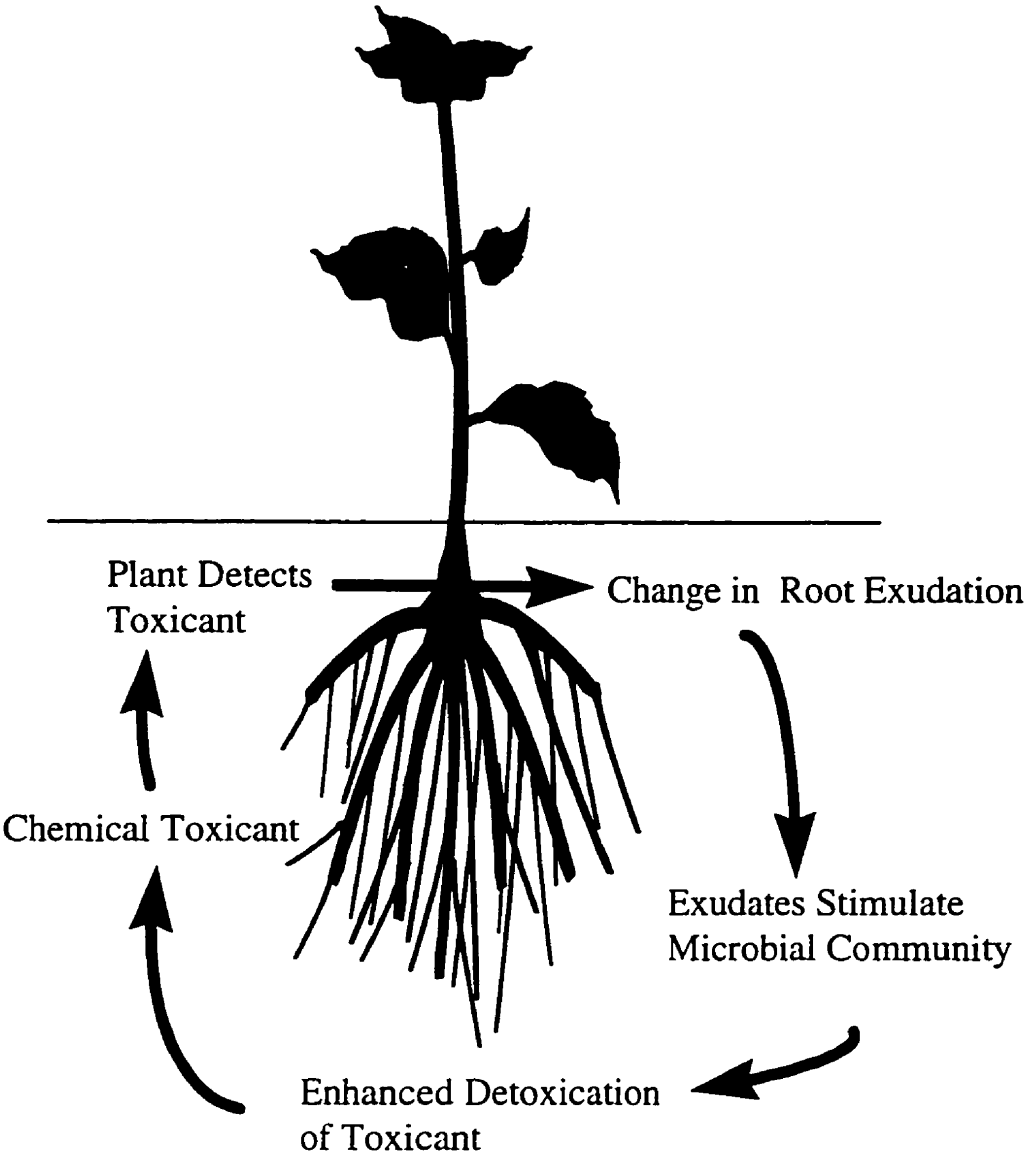

Fig. 2.1. Hypothetical mechanism by which the rhizosphere microbial community may be influenced by the host plant to promote detoxication of an organic substance in soil. By this scenario, a chemical toxicant in the soil would be detected by the plant and the plant would respond with a change in either the quality or quantity of root exudates. This change in exudation would evoke an increase in numbers of rhizosphere microorganism or an increase in the relative abundance of those strains best able to metabolize the toxicant. This proposed pathway would operate as a positive feedback loop until the concentration of the toxicant in soil was sufficiently reduced that the plant returned to a normal pattern of root exudation. Adapted from Walton et al., 1994b.

A specific association does not imply that contaminant levels in soil will be decreased. Instead, it is possible that plants have developed other mechanisms such as intercellular-metabolism, receptor desensitization or sequestration which allow the plant to grow in contaminated soil. For example, Siciliano and Germida (1997) found that 5 out of 16 tested plants were tolerant of 2-chlorobenzoic acid (2CBA) contamination in soil but that only 3 out of those 5 reduced 2CBA levels in soil. Furthermore, Siciliano et al. (1998) found that the expression of a plant enzyme involved in 2CBA metabolism was increased in the presence of only certain bacterial inoculants. Thus, specific associations between the plant and bacteria might allow the plant to survive in contaminated soil but not reduce contaminant levels.

If specific interactions are the norm, then in the absence of the contaminant there should be little stimulation of contaminant degrading microorganisms. This follows from the evolutionary argument, in that plants devoting a portion of their root exudate to maintaining a specific population of bacteria which do not in turn promote plant growth, would be at a competitive disadvantage to their counterparts. Investigators have attempted to determine whether plants select for bacteria that degrade contaminants only in the presence of contamination as predicted by mutualism. Nichols et al. (1997) found that levels of bacteria capable of degrading a mixture of hazardous organic chemicals were increased in the rhizosphere of alfalfa and bluegrass when these plants were grown in contaminated soil. However, these results are complicated by the observation that the number of bacteria degradaing organic chemicals was also increased to a similar extent in bulk soil when soil was contaminated. Thus it was not possible for this study to demonstrate selective pressure. Jordahl et al. (1997) found that the populations of benzene, toluene and xylene degrading microorganisms in the rhizosphere of poplar trees were 5 times that seen in contaminated bulk soil. In contrast, the population of total heterotrophs in the rhizosphere was only 3.4 times that seen in bulk soil. This suggests that the rhizosphere may have selected for degrading bacteria, but further experimental verification is needed. Both of these studies attempted to demonstrate that the

rhizosphere of plants specifically enhances contaminant degrading bacteria. However both studies were confounded by other ecological interactions occurring simultaneously. For example, it is well known that contaminating soil increases the number of bacteria capable of degrading the contaminant, and that the presence of roots increases the microbial population. Thus higher populations of degrading bacteria in contaminated compared to non-contaminated rhizospheres can not demonstrate selective enhancement of degrading populations. Similarly, increased levels of degrading bacteria in the rhizosphere compared to the bulk soil can not be taken as proof of selective enhancement. Separating the "rhizosphere effect" and the effect of contaminating soil from the effects of a phytoremediation plant on soil bacterial populations is a challenge for future research in this area.

Another difficulty involved in testing for specific interactions is finding a plant-soil situation in which the soil is not "polluted" with a toxicant or toxin. It is well known that plants secrete allelopathic compounds and that microorganisms can produce phytotoxins. It is likely that these compounds were the original reason for the development of detoxifying communities in the rhizosphere of plants. For example, Rutherford and Powrie (1993) found that *Euphorbia burmannii* was sensitive to *Acacia cyclops* exudates in *in vitro* toxicity tests but not in a field setting. This suggests that *E. burmannii* may have developed microbial defense against *A. cyclops*' allelopathic agents. Given that many allelopathic agents are similar to xenobiotics (Fig. 2.2.), it is likely that plants may routinely have a rhizosphere population capable of at least minimal contaminant degradation. The role of these pre-existing degradative communities as well as the signals plants use to combat alleopathic chemicals, in the degradation of contaminants warrants further attention.

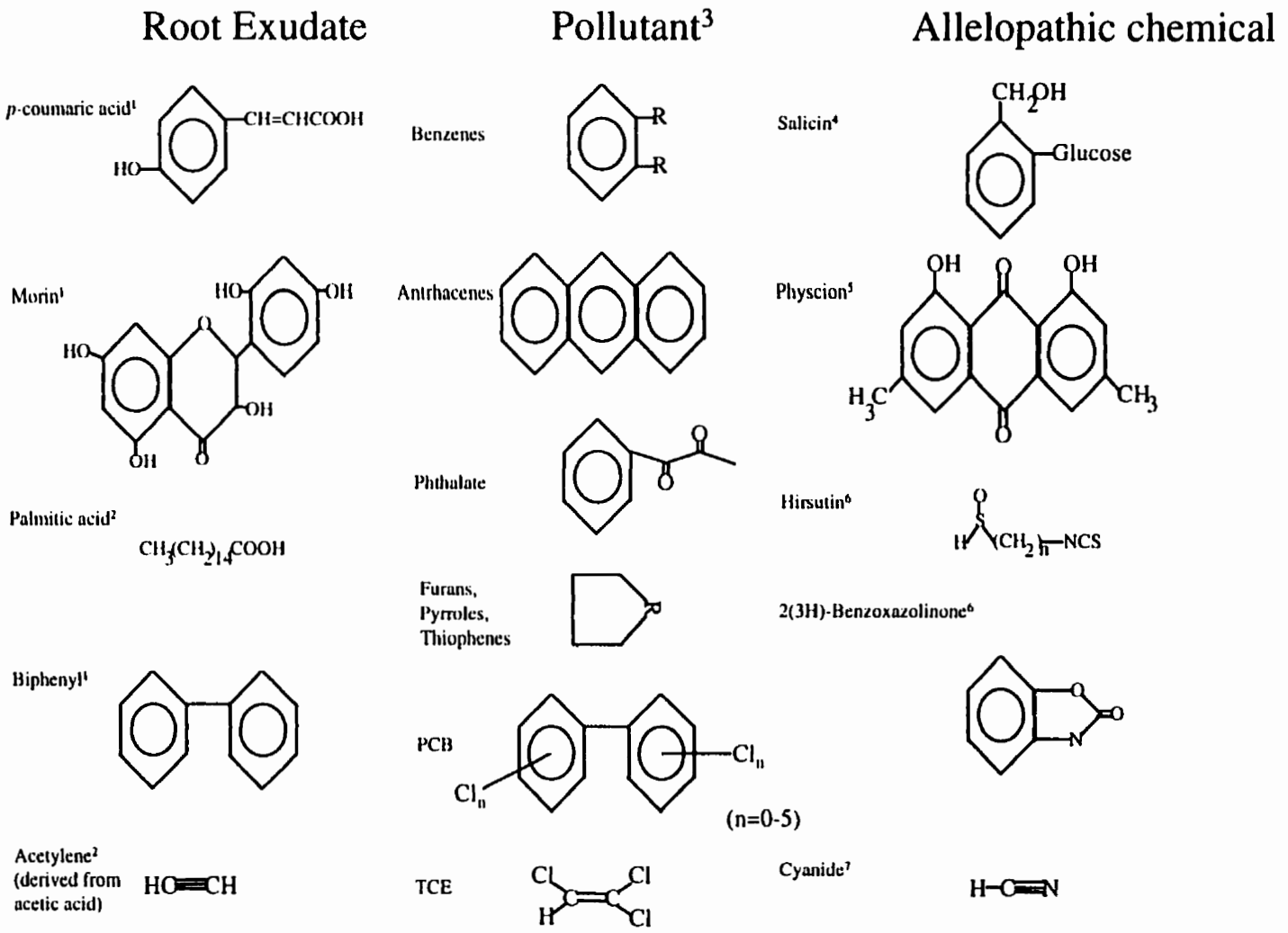

Fig. 2.2 Similarities between root exudates, pollutants and known allelopathic chemicals. Superscript numbers refer to references identifying the compound as belonging to the root exudate, pollutant or allelopathic class. [1]Donnelly et al. 1994; [2]Curl and Truelove, 1986; [3]Hrudey and Pollard, 1993; [4]Weston et al., 1987; [5]Nishimura and Mizutani, 1995; [6]Fujimoto et al., 1995.

Plants may need to specifically alter the rhizosphere community to enhance degradation of certain compounds. A general increase in microbial biomass often does not lead to enhanced degradation. For example, Schmidt et al. (1987) found that glucose stimulated *p*-nitrophenol degradation only when *p*-nitrophenol was above 10 $\mu g\ L^{-1}$. Below this threshold level glucose had no effect on biodegradation, despite an increase in bacterial biomass. The authors postulated that there were "critical" levels below which contaminant concentration was too low for enzyme induction and contaminant transport into the cell. Similarly, adding labile carbon sources known to enhance microbial activity did not enhance TCE degradation in soil (Fan and Scow, 1993). These authors speculated that the necessary catabolic enzymes were not induced, and hence increasing microbial biomass had little effect on TCE degradation. Thus it appears that in cases where specific enzymatic induction is required, simply increasing biomass does not lead to contaminant degradation. In contrast, the degradation of other contaminants which can be readily co-metabolized e.g. 3-chlorobenzoic acid (3CBA), may be enhanced by simply increasing biomass (Haby and Crowley, 1996). Understanding the relationship between specific and non-specific stimulation, and chemical characteristics is a major challenge for phytoremediation research.

If plants can alter their rhizosphere community to specifically detoxify a certain contaminant, the question remains, how do they accomplish this? One possible mechanism by which plants can quickly alter their rhizosphere community in response to environmental stresses is via root exudates. Root exudates directly affect the rhizosphere microflora, and variation in the microbial community associated with roots is likely due to different exudation patterns. For example, Zak et al. (1994) found that rhizosphere communities from different plants have different substrate utilization abilities, and postulated that this might be due to differences in root exudate patterns. Similarly, Garland (1996) found that the composition of the rhizosphere community shifted with the state of plant, once again suggesting that rhizosphere community composition is intimately linked with root exudate

composition.

If root exudates are the means by which plants control rhizosphere communities, then what effects root exudate patterns? Root exudate patterns are known to be dependent upon plant species and the stage of plant development. Furthermore, environmental factors such as soil type, soil nutrient levels, pH of soil, water availability, temperature, oxygen status of soil, light intensity, atmospheric carbon dioxide concentration and the presence of microorganisms are all known to significantly affect the type and quantity of root exudates (Gedroc et al., 1996; Schlapfer and Ryser, 1996; Grayston et al., 1996). Thus root exudates, and associated microbial communities, are likely to be a site-time-plant specific phenomenon. In addition, the type of contamination will also affect the site-time-plant interaction because contaminants damage secretory root cells differently between plant species (Fayez and Kristen, 1996) which in turn will alter root exudate patterns. A challenge to future research is to understand the influence of environmental factors on phytoremediation activity.

2.3.2. Role of the plant in phytoremediation: Non-Specific Interactions

Plants provide soil bacteria with substrates in the form of cell lysates which increase microbial activity and in turn, this may stimulate degradation of contaminants. The difference between specific and non-specific interactions is that a specific interaction results from the plant secreting specific compounds in response to environmental stimuli. In contrast, non-specific interactions arise from normal plant processes that increase microbial activity *e.g.* cell lysates. For example, root exudation of phenolic compounds which induce toluene monooxygenase, has been postulated to be the mechanism by which plants stimulate TCE degradation in soil (Anderson and Walton, 1995). Such exudation might not occur or be substantially less in the absence of TCE if the interaction was specific. A non-specific interaction would result in plants exudating phenol would occur regardless of TCE contamination. This concept is illustrated in a study by Haby and Crowley (1996)

who found that the growth of rye-grass stimulated the number and activity of 3CBA degrading bacteria in the presence as well as the absence of 3CBA in soil. The authors speculated that the general increase in microbial numbers in the rhizosphere was responsible for increased degradation.

Non-specific interactions may occur because many root exudate compounds are chemically similar to certain pollutants (Fig. 2). Compounds in the lysate or leachate may act as toxicant analogs or sources of cometabolite. For example, amending soil with biphenyl increases PCB degradation (Barriault and Sylvestre, 1993). Similarly, adding phenol to soil induces toluene monooxygenase which is involved in TCE degradation (Fan and Scow, 1993). Roots may provide these substances. Thus Hegde and Fletcher (1996) suggest that "*roots can be thought of as a closely interwoven, biologically mediated injection system of cometabolite*". Non-specific interactions would occur if the plant has little control over the nature of cometabolite excreted via this injection system.

If non-specific interactions are the norm, then augmentation of specific bacterial populations in soil should not be necessary for biodegradation to occur because of the catabolic activities inherent in the soil population. These inherent catabolic activities can be increased by stimulating microbial activity. For example, Fulthorpe et al. (1996) found that pristine soils from six different ecosystems yielded a large number of bacterial isolates capable of mineralizing 2,4-dichlorophenoxy acetic acid (2,4-D) and 3CBA. Hence, a non-specific stimulation of the rhizosphere community should lead to a decrease in 2,4-D or 3CBA levels in soil. Other authors have speculated that contaminating soil may accelerate the selection of a microbial community with diverse metabolic pathways. Atlas et al. (1991) found that microbial communities disturbed by pollutants have enhanced substrate utilization capabilities, and suggested that generalized physiological versatility may be an adaptive trait of communities in contaminated soils. In fact, some species of bacteria appear to be especially suited to degrading a wide variety of rarely occurring

substrates present at low concentrations. For example as shown in Fig. 2.3, the central catabolic pathway of pseudomonads allows these bacteria to degrade a variety of aromatic substances such as toluene, *m*-xylene or naphthalene without synthesizing a large number of different enzymes (Houghton and Shanley, 1994). It may be that the bacterial communities of many contaminated soils have the unrealized potential to degrade pollutants and thus, a non-specific stimulation of microbial activity results in increased contaminant degradation. However, Carmichael and Pfaender, (1997) found that the degradation of weathered (> 60 years) polyaromatic hydrocarbons (PAH) was not related to microbial biomass or catabolic potential, and the authors speculated that contaminant bioavailability was limiting PAH degradation, despite a large and active population of bacteria that could degrade PAH. Other authors have found that surfactants can increase degradation of weathered contaminants in soil (Madsen and Kristensen, 1997), which supports the idea that chemical factors and not the absence of bacterial strains capable of degrading the contaminant, limits biodegradation. Thus proponents of non-specific interactions argue that specific stimulation of selected bacterial groups in soil may not be necessary for the plant to enhance contaminant degradation.

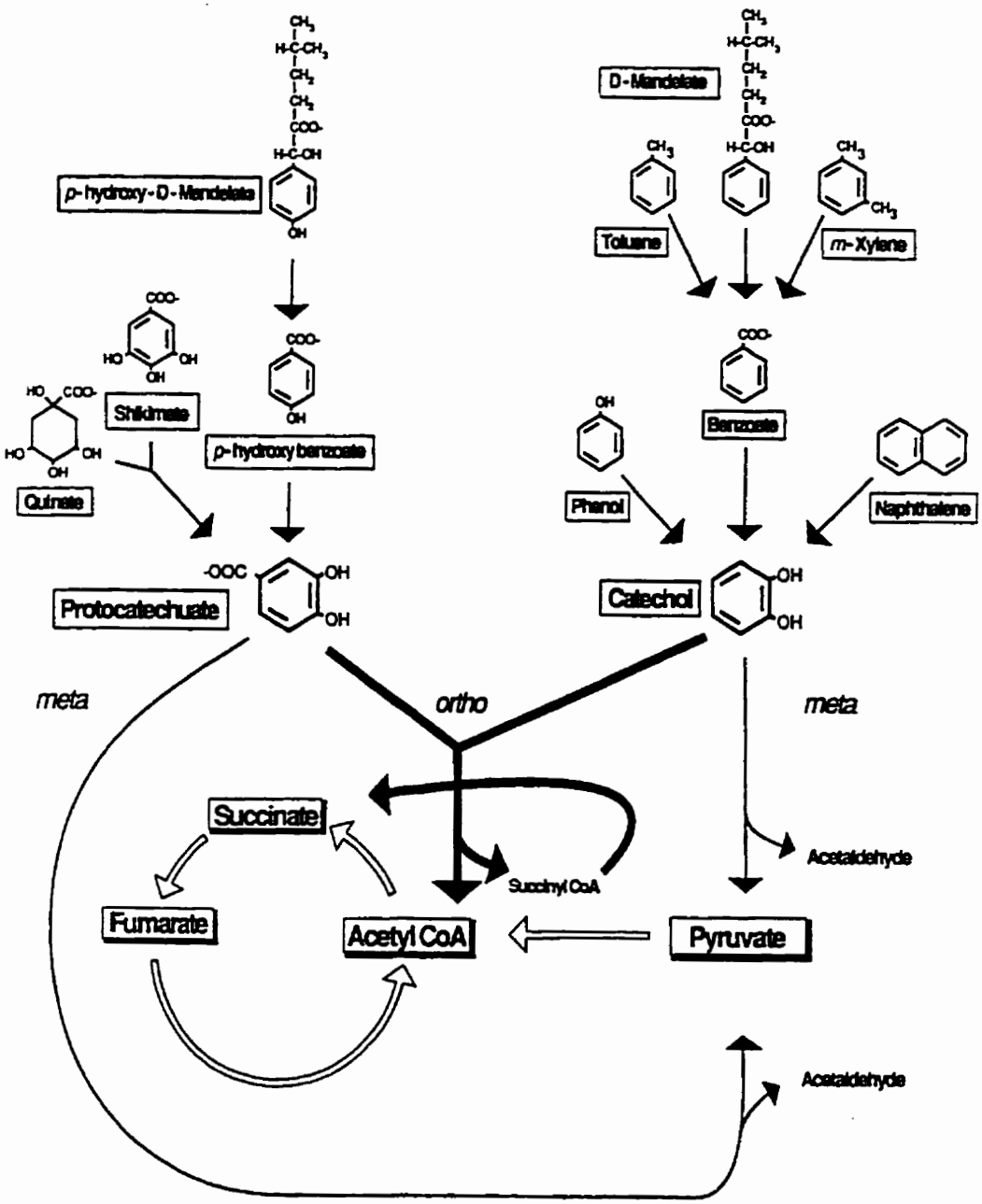

Fig. 2.3. Convergent aromatic catabolism. Dissimilation of a variety of aromatic compounds into the Krebs cycle (unfilled arrows) channelled through a few "key" biochemical intermediates, two of which are protocatechuate and catechol. Further catabolism of both these compounds can be achieved by either *ortho*- (heavy arrows) or *meta*-pathway enzymes (light arrows). Reprinted with permission (Houghton and Shanley., 1994). Copyright 1994 Timber Press.

2.3.3. Bacterial roles in phytoremediation: Detoxification

Bacteria have an important role to play in phytoremediation systems. Plants promote bacterial growth and explore large volumes of soil. However, the phytotoxicity of many contaminants can render phytoremediation ineffective by preventing plant growth. For example, Xu and Johnson (1995) investigated the effect of field pea and barley on the degradation of hydrocarbons. They found that the hydrocarbons significantly reduced plant growth. Furthermore, Siciliano et al. (1997) found that chlorinated benzoic acids and PCBs can be inhibitory to a wide range of prairie grasses. Such grasses are often suggested as potentially good phytoremediation plants (Aprill and Sims, 1990; Siciliano and Germida, 1997). To solve this problem, some investigators have used bacterial seed inoculants to reduce the phytotoxicity of contaminants in soil. For example, Siciliano and Germida (1998) found that inoculating Daurian wild rye with two pseudomonads allowed plant growth in a soil contaminated with a mixture of 2,3-dichlorobenzoic acid and 3CBA, but did not result in reduced levels of these contaminants in soil. Thus, bacteria have reduced the phytotoxicity of these chlorinated benzoic acids but did not degrade these compounds. Alternatively, bacteria can reduce phytotoxicity by degrading compounds. Pfender (1996) found that inoculating millet (*Panicum milaceum* L.) with *Pseudomonas* strain SR3, capable of degrading PCP, reduced PCP phytotoxicity and allowed plant growth. Other authors have found that inoculating pea (Ferry Morse, Alaska Pea) sensitive to dicamba with dicamba degrading bacteria allowed plant growth in dicamba amended soil (Krueger et al., 1991). Thus one possible role of bacteria in a phytoremediation system is to reduce the phytotoxicity of the contaminant to the point that the plant can grow in soil and thereby stimulate degradation of other non-phytotoxic contaminants.

2.3.4. Bacterial roles in phytoremediation: Augmentation of catabolic activity

Another problem associated with phytoremediation is the unknown capacity of plants to degrade pollutants. As discussed earlier, there is little information on plant transformations of priority pollutants, but even less information is available on root

associated metabolism of such pollutants. In fact, to the best of our knowledge, only Adler et al. (1994) have reported a root surface enzyme of a terrestrial plant and Siciliano et al. (1998) a root exudate of a terrestrial plant, capable of detoxifying contaminants. Other groups have found that plant peroxidases are effective in detoxifying a wide range of phenols, but the peroxidase preparation techniques (i.e. chopping the plant up and adding the pieces to contaminated waste water) are not amenable to those used in phytoremediation (Dec and Bollag, 1994; Roper et al., 1996). Root studies by Burken and Schnoor (1995) report preliminary findings on a root associated enzyme that reduces levels of atrazine in water but details were not presented. In contrast to plants, degradation of pollutants by bacteria is well characterized, and inoculating bacteria onto plants can increase degradation. For example, meadow brome (*Bromus biebersteinii*) did not stimulate degradation of 2CBA in soil when 2CBA was initially present at 200 mg kg^{-1}. However, when inoculated with a mixture of two pseudomonads, the amount of extractable 2CBA in soil was significantly reduced (Siciliano and Germida, 1997). Therefore, another role of bacteria in phytoremediation may be to augment the ability of the rhizosphere to degrade contaminants.

Rhizobacteria can degrade a variety of substances (Zablotowica et al. 1991). Hence there are potentially many different inoculants capable of augmenting degradative activity in the rhizosphere. Diez et al. (1995) found an *Azotobacter* sp. that fixed dinitrogen under aerobic conditions and used monochloroacetate as a sole source of carbon and energy. Similarly, *Rhizobium* sp. degrade organophosphate pesticides to obtain phosphorus (Liu et al., 1991).

Instead of selecting bacteria that can degrade substances and then determining whether they are effective in the rhizosphere, other investigators have attempted to modify rhizosphere microorganisms to degrade a contaminant. For example, Brazil et al. (1995) inserted a transposon containing the genes (*bph*), which encodes the biphenyl degradative pathway into a rhizosphere pseudomonad. They found that the

bacterium colonized the rhizosphere of sugar beet and the *bph* gene products were expressed *in situ*. Controlling the activity of such engineered organisms in the rhizosphere might be possible by combining a catabolic genetic sequence like one used by Brazil et al. (1995), with others sequences known to respond to root exudates. For example, Tepfer (1988) found that a plasmid in a *Rhizobium* sp. encodes for the catabolism of specific root exudates. Therefore, it appears that genetic engineering of microbial inoculants has the potential to provide tailored bacterial inoculants whose catabolic activity and survival are controlled by the inoculated plant.

2.3.5. Bacterial roles in phytoremediation: Plant growth promotion

Increasing the amount of root, or root activity in a given area of soil, should in turn increase any degradation mediated by the root (Siciliano and Germida, 1997). Hence, the use of bacterial seed inoculants that increase plant growth may in turn increase the degradation of contaminants in soil by plants. For example, Siciliano and Germida (1997) found that inoculating Dahurian wild rye with *P. aeruginosa* strain R75, a known plant-growth promoting rhizobacteria (PGPR), increased the degradation of 2CBA in soil. However, increases in plant biomass were not related to increased degradation and thus, the inoculant may have altered root activity. Inoculants are known to increase exudation (Prikryl and Vancura, 1980), alter exudate composition (Lawson et al., 1996) and even produce biosurfactants (Vermani et al., 1995) any of which may result in increased degradation by stimulating the rhizosphere community, altering the community structure or increasing the bioavailability of the contaminant. Therefore, it is possible that inoculants alter the rhizosphere to promote degradation of contaminants even if the inoculants themselves do not degrade the contaminants.

2.3.6. Susceptibility of chemicals to phytoremediation

A complicating factor in determining the role plants and bacteria play during phytoremediation is the that certain chemicals appear to be more susceptible to phytoremediation than others. For example, Gunther et al. (1996) found that the growth of rye-grass decreased aliphatic hydrocarbons in soil to a greater extent than polyaromatic hydrocarbons. Similarly, Knaebel and Vestal (1992) found that only certain surfactants were degraded in corn rhizospheres. Siciliano and Germida (1997a) found that levels of 2CBA and 3CBA, but not 2,3-dichlorobenzoic acid (23diCBA) or 2,5-dichlorobenzoic acid (25diCBA) were reduced in the rhizosphere of Dahurian wild rye. Other authors have also found that root exudates themselves stimulate the degradation of only certain chemicals. For example, amending soil with sterile root exudate from bush bean (*Phaseolus vulgaris* cv. Tender Green) specifically stimulated *O,O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate (parathion) but not *O,O*-diethyl-*O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate (diazinon) degradation (Hsu and Bartha, 1979). Similarly, Boyle and Shann (1995) reported greater stimulation of 2,4-D, compared to 2,4,5-T, degradation by root exudates. In contrast, Anderson et al. (1994) found that atrazine, a nitrogen heterocycle, and metolachlor, an aromatic amine, were degraded to a similar extent in rhizosphere soil of *Kochia* sp. Thus some chemicals can be readily phytoremediated, whereas others are not.

The susceptibility of soil contaminated by specific chemicals to phytoremediation may be linked to two predictors, the capability of the rhizosphere community to degrade a contaminant and contaminant bioavailability. If the degradation of a compound requires highly specialized microorganisms, then it is likely that phytoremediation in the absence of a specific inoculant will not work. For example, Siciliano and Germida (1998) found that Altai wild rye (*Elymus angitus*) did not reduce levels of 25diCBA unless this plant was inoculated with a mixture of *P. aeruginosa* strain R75 and *P. savastanoi* strain CB35. Similarly, Crowley et al.

(1996) found that a pseudomonad inoculant of bean (*Phaseolus vulgaris*) increased 25diCBA degradation in soil. Thus it appears that only specific inoculants with appropriate genes can induce 25diCBA degradation.

In addition to the genetic ability of the bacterial population to degrade a compound, the bioavailability of a compound affects the ability of plants to stimulate its degradation. For example, Nichols et al. (1997) found that the least soluble compound in a mixture of organic chemicals (i.e. pyrene), was the most persistent. Similarly, other investigators have found that bioavailability plays an important role in remedial efforts (Providenti et al., 1993; Alexander, 1995). Thus, an important factor determining the nature of plant-bacterial interactions is likely to be contaminant dependent with microbial communities requiring specific stimulation to degrade certain contaminants *e.g.* 25diCBA and non-specific stimulation for other contaminants *e.g.* 3CBA. Table 1 lists all the chemicals whose extractable levels in soils are known to be reduced by growing plants in soil. It is evident from this list that substantially more work needs to be completed before we understand factors influencing a chemical's susceptibility to phytoremediation.

Table 2.1. Chemicals amenable to phytoremediation[a]

Chemical	Plant	Effect of Plant	Reference
Atrazine	Corn	Inoculating plant with a bacterial consortia reduced levels to 16% of control soil	Alvey and Crowley, 1996
Atrazine, Metolachlor, Trifluralin	*Kochia* sp.	Only 30 to 60% of the parent compound remained in rhizosphere soil dosed with herbicides	Anderson et al., 1994
Trichloroethylene	*Lespedeza cuneata* (Dumont), *Pinus taeda* (L.), *Solidago* sp., *Glycine max*	Rhizosphere soil had >26% mineralization compared to <9% for control and 15% for non-vegetated	Anderson and Walton, 1995
	Clones from the crosses, *Populus trichocarpa* X *P. deltoides*, *P. trichocarpa* X *P. maximowiczii* (282-190)	Plants were found to metabolize and transpire TCE.	Newman et al., 1997

2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid	Red clover (*Trifolium pratense* L.), Daisy fleabane (*Erigeron annuus* (L.) Pers.), Barnyardgrass (*Echinochloa crusgalli* (L.)), Fall panicum (*Panicum dichotomiflorum* Michx.), Early goldenrod (*Solidago juncea* Aiton.), Chicory (*Chicorium intybus* L.), Timothy grass (*Phleum pratense* L.), Green foxtail (*Setaria virius* (L.) Beauv.)	Mineralization by monocot rhizosphere soil was two to three times that of control soil. Mineralization by dicot rhizosphere soil was 30 to 50% greater than control soil.	Boyle and Shann (1995).
Atrazine	Imperial Carolina hybrid popular (*Populus deltoides nigra* DN34)	After 80 d only 29% labelled remained in planted soil compared to 79% in unplanted soil.	Burken and Schnoor, 1996

2,5-dichlorobenzoic acid	Bean (*Phaseolus vulgaris*)	Plant decreased contaminant by 30% compared to control and inoculation with a pseudomonad resulted in complete contaminant degradation	Crowley et al., 1996
2-chlorobenzoic acid, 3-chlorobenzoic acid, 2,3-dichlorobenzoic acid, 2,5-dichlorobenzoic acid and mixtures of these chemicals	Dahurian wild rye (*Elymus dauricus*), Altai wild rye (*E. angitus*), Meadow brome (*Bromus biebersteinii*) Streambank wheatgrass (*Agropyron riparum*)	Inoculating plant with pseudomonads increased degradation by 56 to 74%	Siciliano and Germida, 1997; In Press
Pentachlorophenol	Hycrest crested wheatgrass, (hybrid of *Agropyron cristatum* (L.) Gaertn. and *A. desertorum*).	Planted soil evolved 15% more radiolabel than unplanted soil.	Ferro et al., 1994
	Proso millet (*Panicum miliaceium* L.)	Inoculation of millet with PCP degrading bacteria allowed plant growth	Pfender, 1996

Hydrocarbon mixture including n-alkanes, isoalkane, n-alkene, and polycylic aromatic hydrocarons	Ryegrass (*Lolium perenne* L.)	After 22 weeks, planted soil contained only 14% of the contaminant present in unplanted soil	Gunther et al., 1996
Pyrene, anthracene	Alfala, fescue, switchgrass, sudan grass	Levels of anthracene were 31-47% lower in rhizosphere soil 24 wk after planting, levels of pyrene were 36% lower in fescue, sudangrass and switchgrass rhizosphere 24 wk after planting	Reilley et al., 1996

3-chlorobenzoic acid	ryegrass (*L. multiflorum* X *perenne*)	3CBA degradation in rhizosphere soil from an uncontaminated site was faster compared to non-rhizosphere soil, this effect disappeared if soil was previously contaminated	Haby and Crowley, 1996
Diazinon, parathion	Bush bean (*Phaseolus vulgaris* cv. Tender Green)	Rhizosphere flasks evolved 11 to 13% more radiolabelled carbon than non-rhizosphere flasks	Hsu and Bartha, 1979[b]
Dicamba	Pea (Ferry Morse, Alaska Pea)	Inoculation with dicamba degrading bacteria allowed pea to tolerate dicamba	Krueger et al., 1991

[a] Readers are referred to Anderson et al. (1993) for phytoremediation references published prior to 1990.
[b] The authors have included this older study because they feel it is an exceptional paper.

2.4. BIOCHEMICAL MODES OF PLANT-BACTERIA INTERACTIONS IN PHYTOREMEDIATION: AN EVEN TOUGHER QUESTION.

It appears that plant-bacteria interactions in phytoremediation systems can either be specific or non-specific, and that bacteria can either act to reduce phytotoxicity to the plant or degrade the pollutant. However, the question immediately arises- what are the biochemical means by which plants and bacteria interact in contaminated soils to increase pollutant degradation? If plants and bacteria form specific associations, how do the two organisms communicate with one another such that contaminant degradation is optimized? Similarly, if plants and bacteria form non-specific associations, what are the root exudates that provide the stimulation and how do bacteria use these root exudates to aid in contaminant degradation?

2.4.1. Plant mediated increases in nutrient bioavailability

Plants increase nutrient availability by secreting cationic chelators, organic acids or specific enzymes such as phosphatase into the soil systems (Grayston et al., 1996; Gobran and Clegg, 1996; Jones and Darrah, 1995; Vinton and Burke, 1995). Readers are referred to a recent review for details on the influence of abiotic and biotic factors on root exudates (Grayston et al., 1996). The influence of nutrients on biodegradative activity is well known (Providenti et al., 1993; Steffensen and Alexander, 1995). For example, Cutright (1995) found that increasing nitrogen and phosphorus increased polycyclic aromatic hydrocarbon degradation by the soil fungus *Cunninghamella echinulata var. elegans*. Other authors have found that minor elements such as calcium and magnesium can also affect contaminant degradation rates (Widrig and Manning, 1995). Furthermore, competition for these nutrients by degrading and non-degrading species will influence the amount of contaminant degraded (Steffensen and Alexander, 1995). Hence, increases in nutrient availability brought about by plant growth may be one mechanism by which plants stimulate biodegradation. Supporting this, Cheng and Coleman (1990) found that living roots and fertilizer had equivalent stimulatory effects on straw decomposition.

Furthermore, atrazine degradation by an inoculated consortium was similar in treatments receiving fertilizer and those in which corn plants were grown (Alvey and Crowley, 1996).

However, the effect of plant mediated increases in available nutrients in a field setting is not known because plants use substantial amounts of inorganic nutrients themselves, and thus may be in competition with biodegrading microorganisms. The competition between bacteria and plants for elements is still not fully understood. For example Kaye and Hart (1997) argue that while plants and bacteria may not compete for inorganic nitrogen, competition for organic nitrogen might be crucial for the success of each organism. In addition, other authors have noted that the competitive relationship between rhizosphere bacteria and plants is often a fine line between mutualism/commensalism and antagonism (Nehl et al., 1997). Thus, stimulation of biodegradation by increases in nutrient levels may occur, but more work is required to understand how such processes actually work and to demonstrate this mechanism in a direct manner.

2.4.2. Root exudate solubilisation of contaminants

Besides increasing the availability of nutrients, plants may also increase the bioavailability of the contaminant. Contaminant bioavailability often limits biodegradation, and increasing it can stimulate degradation (Providenti et al., 1993). Root exudates can increase contaminant bioavailability by competing with the contaminant for binding sites on the soil matrix. For example, Reilley et al. (1996) found that adding a solution of low molecular weight organic acids (15 μM succinic acid and 10 μM formic acid) to rhizosphere soil increased the initial degradation rate of pyrene. This suggests that root exudates increase degradation of pyrene by increasing contaminant availability and/or stimulating microbial activity. The authors speculated that organic acids in the root exudate competed with pyrene for adsorption sites, and hence increased the amount of pyrene available to microorganisms. However, confirmation of this hypothesis awaits further

experimental verification. This competition need not be limited to specific binding sites, but may simply be related to alterations in soil surfaces. Root exudates and proteinaceous material have been shown to bind to a wide range of clay materials in soil (Boyd and Mortland, 1990). Furthermore, non-ionic, hydrophobic organic chemicals e.g. PCBs, interact weakly and non-specifically with mineral surfaces (Mader et al., 1997; Kowalska et al., 1994). Thus root exudates may alter the interaction between mineral surfaces and contaminants.

It may be possible for root exudates to increase the bioavailability of contaminants to degrading bacteria without simultaneously increasing the bioavailability of contaminants to toxic receptors. Otherwise, increases in contaminant bioavailability in the rhizosphere would lead to increased phytotoxicity. For example, Laor et al. (1996) found that pyrene sorbed to humic acids was bioavailable only to a mixture of organisms enriched from coal tar contaminated soil. Similarly, biphenyl sorbed to porous acrylic beads was available to degradation by *Bacillus licheniformis* without desorption of biphenyl into solution (Calvillo and Alexander, 1996). Further, White and Alexander (1996) found a consortium which could utilize polycyclic aromatic hydrocarbons (PAHs) sorbed to soil without desorbing the PAHs first. Thus it appears that bacteria may not require that contaminants be in an aqueous phase before degradation occurs. The ability of bacteria to use compounds sorbed into an organic phase is not fully understood, but it has important implications for phytotoxicity and requires more research.

2.4.3. Surfactant production

In addition to organic acids and aromatic compounds, plants also secrete surfactants (i.e. lipids and sterols), which lubricate the root as it passes through soil (Curl and Truelove, 1986). Surfactants reduce surface tension and solubilize contaminants, thereby increasing contaminant bioavailability. For example, Barriault and Sylvestre (1993) found that degradation of Aroclor 1242 by *Pseudomonas testerosteroni* B356 was enhanced by co-inoculating microcosms with a strain of

Acinetobacter faecalis B556 that produces a surfactant. However, this effect was limited to certain congeners and there was not a general increase in PCB degradation. Surfactant concentrations in the rhizosphere are likely to be below the critical micelle concentration and therefore may form hemicelles. Hemicelles can attach to a surface and promote solubilisation of compounds onto that surface (Haigh, 1996). The root surface, with it's high bacterial population, may be an ideal environment for this to occur. Biodegradation of contaminants inside these hemicelles, however, may be limited by the type of surfactant and the microbial strains present on the root. For example, Guha and Jaffe (1996) found that the bioavailability of phenanthrene was strongly influenced by the type of surfactant. Furthermore, surfactants can have toxic effects on bacterial populations that degrade contaminants (Sylvestre, 1995). Hence, specific bacteria-surfactant pairings may need to be present to take advantage of the conditions present in the rhizosphere.

The actual solubilisation of contaminants by surfactants may not need to occur. For example, Yeom et al. (1996) showed that surfactants can swell soil colloids and thereby expose non-soluble substances to microbial attack. Furthermore, microorganisms can attach themselves directly to particles containing sorbed contaminants and degrade the contaminant without producing surfactant (Calvillo and Alexander, 1996). In addition to increasing their bioavailability, surfactants also alter biochemical reactions of contaminants. Huang and Rusling (1995) found that the formal reduction potential of PCB was considerably more positive in a mixture of water and surfactant compared to water alone. Unfortunately, surfactants and their influence on extracelluar enzymatic reactions have not been studied. Nevertheless, the rhizosphere is known to contain both plant and bacterial extracelluar enzymes, and some of these have been implicated in biodegradation (Siciliano et al., 1998; Mawdsley and Burns, 1994; Adler et al., 1994). Thus rhizosphere surfactants with their effects on contaminant bioavailability and chemical reactivity warrant further study.

2.4.4. Plants and Contaminant Movement Through Soil

Water uptake by plants has a significant effect on water flow and thereby transport of contaminants through the soil matrix. Most contaminants are sparingly soluble in water and are sorbed onto soil colloids (Hrudey and Pollard, 1993). Increasing the amount of water flow through soil can increase the amount of contaminant desorbed from the soil matrix (Hamby, 1996). While many remediation strategies use the technique of "soil washing" to remove contaminants from soil, plants naturally draw water from the surrounding bulk soil towards their root systems (Stirzaker and Passioura, 1996). For example, TCE uptake and degradation was dependent upon plant water use, with TCE sorption to plant biomass correlated with water use (Anderson and Walton, 1995). Furthermore, Aprill and Sims (1990) found that plant growth reduced hydrocarbon leachate collected from hydrocarbon contaminated soil cores. They speculated that plants reduced water flow through bulk soil and re-directed it towards the root system, thereby limiting the movement of hydrocarbon down the soil column.

The effect of transpiration driven contaminant uptake may differ between contaminants. For example, transpiration driven uptake of bromacil, nitrobenzene or phenol by soybeans was contaminant specific, and could not be predicted by the octanol/water partitioning coefficient of the contaminants (McFarlane et al., 1987). Predicting the effect of water uptake on contaminant degradation may be difficult due to uncertainty regarding the physicochemical characteristics that govern plant uptake of non-dissociating organic chemicals (Trapp et al., 1994; Paterson and Mackay, 1990). Thus water uptake may be an important factor for certain contaminants, but further work is required to determine the relationship between plant water use and contaminant degradation in the rhizosphere.

2.4.5. The role of soil

It is important not to forget that all these reactions are occurring in a dynamic gas, solid and liquid system i.e. the soil. Thus differences between soils that alter contaminant-soil reactions will in turn affect the influence of plants on microorganisms. For example, Bachman and Kinzel (1992) found that the level of amino acids, sugars, carbon dioxide respiration and certain enzyme activities in the rhizosphere was largely dependent upon soil type and that only certain plants, e.g. *Medicago sativa* (M.), had similar levels of amino acids, sugars and enzymes across different soil types. In addition, Hoflich et al. (1995) found that the efficacy of plant growth-promoting rhizobacteria varied across soil types. Hence the application of phytoremediation systems may be limited to only specific soils. Alternatively, only certain plants may be able to stimulate degradation across a variety of soils. For example, Knaebel et al. (1992) found a significant difference between soil types in the degradation of surfactants by corn. However, Siciliano and Germida (1997) investigated the performance of phytoremediation systems across three different Saskatchewan soils and found that the systems were effective in reducing 2CBA levels across all soil types. Hence, more investigation into the influence soil properties have upon phytoremediation performance is needed.

2.5. Conclusion

This review has attempted to piece together various parts of the phytoremediation puzzle. There are possible roles for each participant in phytoremediation. The plant may act specifically or non-specifically to promote degradation. Bacteria may act to either reduce phytotoxicity, increase catabolic potential or increase plant growth. Phytoremediation is an emerging and potentially useful technique to reduce the risk to human and ecosystem health from contaminated soil but it is evident that due to the degree of complexity, phytoremediation systems are still a long way from being understood.

3. EVALUATION OF PRAIRIE GRASS SPECIES AS BIOINDICATORS OF HALOGENATED AROMATICS IN SOIL.

3.1. ABSTRACT

The purpose of this study was to assess the potential of prairie grasses as bioindicators of toxicants in soil and to investigate the effect different soil types and organisms have upon the germination of plant bioindicators. As a model compound, I used 2CBA, a hydrophillic, polar compound present in the degradation pathways of halogenated aromatics. The germination response of prairie grasses to Aroclor™ 1260 (a commercial mixture of polychlorinated biphenyls-PCBs) contaminated soil with concentrations ranging from 13 to 133 $\mu g\ kg^{-1}$ ΣPCBs also was investigated. The grasses responded to a wide range of contamination levels with a 11 and 9 fold difference in the sensitivity of grass species to 2CBA and Aroclor™ concentrations respectively. Canada blue grass (*Poa compressa*) and slender wheatgrass (*Agropyron trachycaulum*) were selected for further study of the effects of soil type and biological treatments on bioindicator response to 2CBA. Canada blue grass response in three out of four soils was characterized by $Y = 110 - 26 \times \ln(X)$ while in the fourth soil it was $Y = 94 - 1.6 \times X$, where Y equals percent germination and X the 2CBA concentration. Slender wheatgrass response was $Y = 140 - 23 \times \ln(X)$ with no significant difference between soil types. Previous biological treatments of soil significantly affected the response of slender wheatgrass as a bioindicator. Growing plants or plants inoculated with bacteria (*i.e.*, bioremediation treatments) in non-contaminated soil previous to planting the bioindicator Slender wheatgrass inhibited emergence, changing the logarithmic relationship between germination and 2CBA concentrations to $Y = 100 - 0.28 \times X$. Prairie grasses are potentially useful bioindicators of chlorinated aromatics in soil but biological interactions may alter the bioindicator response.

3.2. INTRODUCTION

Chemical analysis of contaminated soil can be expensive and uninformative regarding environmental hazards associated with polluted soil (Hund and Traunspurger 1994; Ongley et al. 1988; Belkin et al. 1994). The use of bioassays to evaluate hazardous chemical waste sites provides a direct, inexpensive and integrated estimate of contaminant toxicity (Belkin et al. 1994; Wang and Freemark 1995; Mueller et al. 1991). Effective bioassays require a rapid and reliable methodology that characterizes the extent of contamination, minimizes worker exposure and reduces artifacts induced by sampling the soil (Ronnpagel et al. 1995; Burmeier 1995).

One promising approach is the use of prairie grasses as *in situ* bioindicators of soil pollution. These grasses have many advantages over other types of bioindicators. For example, grass seed is often readily available from local seed suppliers and plant bioindicator methodology is uncomplicated (Wang and Freemark 1995; Shirley 1994). In addition, prairie grasses form an integral part of the prairie food chain (Braband 1986; Fletcher and Johnson 1990). Many different animals utilize grass stalks as shelter or grass seed as a food source (Shirley 1994). Hence, determining the impact of contaminants on these grasses is relevant to the prairie ecosystem, an important factor in selecting bioindicators (Chapman 1995; Cairns 1993). All these factors suggest that grass bioindicators would be useful in estimating the toxicological hazard of a contaminated soil site.

Bioindicators must give interpretable response curves across a range of environmental parameters (Hund and Traunspurger 1994; Adema and Henzen 1989; van Leeuwen 1990). Otherwise, environmental effects upon bioindicator response may confound extrapolations meant to depict the bioavailability and toxicity of contaminants in soil (Hund and Traunspurger 1994; van Leeuwen 1990). In addition, soil parameters fluctuate at the landscape level and different bioindicator calibration curves for each landscape position would be impractical for routine bioindicator use. An estimate of bioindicator response to different soil parameters independent of the effect of these

parameters on contaminant bioavailability will allow one to assess the utility of using bioindicators in a variety of soils and landscape positions.

Bioindicators integrate measurement of contaminant bioavailability and toxicity (Bacci 1994). Differences in either one of these components will alter the toxicological hazard associated with a contaminated site. Implicit in this measurement of bioavailability and toxicity is the independence of bioindicator response to other organisms in the ecosystem (van Leeuwen 1990). However, it is well documented that many organisms produce toxins designed to minimize competition (Curl and Truelove 1986c). Therefore, this assumed bioindicator independence may not exist, especially in those contaminated sites receiving remedial treatments such as the plant-bacterial systems suggested by Siciliano and Germida (1995).

The purpose of this investigation was to assess the potential of prairie grasses as bioindicators of chlorinated aromatics in soil and to investigate whether different soil parameters and other organisms influence the bioindicator response. I selected seed germination as a bioindicator response endpoint because of its simple methodology, moderate sensitivity to toxicants (Linder et al. 1990) and potential for *in situ* use.

3.3. MATERIALS AND METHODS

3.3.1. Soil collection and preparation

Four soils (0-15 cm) were collected from different soil climatic zones in southern Saskatchewan, Canada. Soil was air dried and sieved to pass a 4.75 mm sieve (USA Series Equivalent No.4). The surface "soil" of a parking lot contaminated with Aroclor™ 1260 transformer oil was collected. An analysis (Alberta Research Council, Method No. G106.0) of the PCB concentration in soil by Saskatchewan Research Council [Saskatoon, SK, Canada] indicated that the mean concentration of Aroclor™ 1260 was 130 μg kg^{-1} ΣPCBs soil. Soils were analyzed by EnviroTest labs formerly Plains Innovative Laboratory Services [Saskatoon, SK, Canada.]. Selected chemical and physical characteristics of these soils are presented in Table 3.1.

3.3.2. Forage grasses

Seventeen forage grass species (Table 3.2.) were obtained from a local seed supplier [Early's Farm and Garden Centre, Saskatoon, SK, Canada]. Grasses were selected because of their prevalence in the undisturbed prairie ecosystem or use in stabilizing marginal land. Seeds were stored in plastic bags at 4°C. Seed viability was assessed using a standard germination test in wet paper towels (22°C, 7 days) before use in growth chamber experiments. Only seeds with greater than 90% germination were used.

3.3.3. Bioindicator selection

Initially, the range of 2CBA concentrations grasses responded to was determined. The four agricultural soils were amended with 100 mg kg^{-1} 2CBA, placed in seedling trays (300 wells, 30 × 60 × 2.5 cm tray) and maintained at -33 KPa for 10 days. Grass seeds (n=10) were planted in wells (n = 5) containing either soil amended with 2CBA or non-amended soil, and covered with a opaque plastic bag. Seven days after planting (DAP), seed emergence was determined.

A second experiment assessed the response of representative grasses to a range of 2CBA concentrations. Canada blue grass had a low (*c.a.* 4%) percent emergence at 100 mg kg^{-1} so it was seeded (n = 6) in soil with 2CBA concentrations ranging from 0 to 50 mg kg^{-1} soil in five intervals of 10 mg kg^{-1}. Slender wheatgrass had a higher (*c.a.* 30%) percent emergence and consequently, was seeded (n = 6) in soil with 2CBA concentrations ranging from 0 to 250 mg kg^{-1} soil in five intervals of 50 mg kg^{-1}. All other grasses (*e.g.* Crested wheatgrass), germinated extremely well, *c.a.* 80% and were seeded (n = 6) in 2CBA contaminated soil containing 250 mg kg^{-1} soil. This was repeated twice.

TABLE 3.1. Selected characteristics of soils used in this study.

Site	Soil Type[a]	Texture	pH[b]	Conductivity[b] (mS/cm)	Organic Matter (%)	Extractable NO_3-N (mg kg^{-1})[c]	Extractable P[c] (mg kg^{-1})
Outlook	Brown Chernozem (Typic Haploborolls)	Sandy Loam	7.9	1.1	2.4	45	17
Allan	Dark Brown Chernozem (Typic Haploborolls)	Clay Loam	8.7	0.2	2.4	12	15
Paddockwood	Grey Chernozem (Typic Cryoboroll)	Loam	8.5	0.3	4.6	41	9.2
Lanigan	Black Chernozem (Aquic Cryoboroll)	Loam	7.4	0.4	13.5	55	31
McEown	----------[d]	Sandy Loam	8.6	0.2	0.5	9	6
Outlook-2[e]	Brown Chernozem (Typic Haploborolls)	Sandy Loam	7.9	0.9	2.5	61	19

[a] American designation in parenthesis.
[b] 1:2 soil:water dilution.
[c] NO_3-N determined colorimetrically on 0.001 M $CaCl_2$ extracts and P on 0.5 M $NaHCO_3$ extracts (Olsen et al. 1954).
[d] Parking lot gravel; soil classification not applicable.
[e] Outlook soil (200 g) was seeded (n=10) with meadow brome inoculated with *Pseudomonas aeruginosa* strain R75 (10^6 cfu $seed^{-1}$). Plants were grown for 42 days, roots and shoots removed, and soil analyzed.

TABLE 3.2. Grasses used in this study and their tolerance to 2CBA or Aroclor™ 1260.

Common Name	Scientific Name	Contaminant Tolerance[a]	
		2CBA (mg kg^{-1})	Aroclor™ 1260 (μg kg^{-1})
Canada blue	*Poa compressa*	50	>130
Slender wheatgrass	*Agropyron trachycaulum*	250	>130
Dahurian wild rye	*Elymus dauricus*	>250	>130
Common brome	*Bromus inermis*	>250	130
Intermediate wheatgrass	*Agropyron intermedium*	>250	>130
Streambank wheatgrass	*Agropyron riparum*	>250	110
Meadow brome	*Bromus biebersteinii*	>250	>130
Tall wheatgrass	*Agropyron elongatum*	>250	>130
Northern wheatgrass	*Agropyron dasystachyum*	>250	>130
Reed canary	*Phalaris arundinacea*	>250	>130
Perennial rye grass riviera	*Lolium perenne*	>250	>130
Russian wild rye	*Elymus junceus*	>250	>130
Crested wheatgrass	*Agropyron cristatum*	>250	130
Altai wild rye	*Elymus angustus*	>250	>130
Timothy	*Phleum pratense*	>250	>130
Orchard grass	*Dactylis glomerata*	>250	33
Sheep fescue	*Festuca ovina*	>250	>130

[a] Contaminant concentration at which percent emergence 7 days after planting was less than 10%. Grasses with more than 80% emergence at the highest contaminant level tested are marked by a greater than symbol. 2CBA results combined for four agricultural soils and the PCB results are from a parking lot contaminated with Aroclor™ 1260.

A further experiment assessed the response of prairie grasses to Aroclor™ 1260 contamination. The protocol was similar to that above with the exception that the maximum Aroclor™ concentration was 130 μg kg^{-1} soil. Contaminated soil was diluted with non-contaminated soil from the same location to obtain different contamination levels ranging from 13 to 130 μg kg^{-1} ΣPCBs soil in intervals of 33 μg kg^{-1}.

3.3.4. Effect of soil type on bioindicator response

Canada blue grass and slender wheatgrass were assessed for their response to 2CBA contamination in 4 different soils. Canada blue grass and slender wheatgrass were planted at 2CBA concentrations ranging from 0 to 50 mg kg^{-1} or 0 to 250 mg kg^{-1} soil respectively, watered to -0.33 KPa and emergence determined 7 DAP. Duplicate experiments with 7 replicates each were performed for each soil.

3.3.5. Effect of biological treatments on bioindicator response

To determine if bioindicator response was affected by bioremediation treatments, we investigated the effect of the biological treatments described by Siciliano and Germida (1995) on slender wheatgrass emergence in the Outlook and Paddockwood soils. Each experiment had five replications and the Paddockwood experiment was replicated twice. The biological treatments consisted of growing either streambank wheatgrass, Dahurian wild rye or meadow brome with or without a bacterial inoculant (*c.a.*. 10^6 cfu seed^{-1}) in 200 g (195 ml styrofoam cups) of either contaminated (*c.a.*. 200 mg 2CBA kg^{-1} soil) or non-contaminated soil. The bacterial inoculant was a 48 hour trypicase soy broth culture of *Pseudomonas aeruginosa* strain R75 and/or a 9 day 0.3 g 2CBA L^{-1} minimal salts broth culture (Farrell et al. 1993) of *P. savastanoi pv. fraxinus* strain CB35. Previously, I found that these bacteria stimulated the degradation of 2CBA in soil (see Chapter 3). Seeds were inoculated as described in Section 3.3.4. Plants were grown in a growth chamber (350 μmol s^{-1} m^{-2}, 16-8 h day-night cycle, 24°-18°C day-night temperature) for 42 days. The shoots and roots were harvested, soil mixed, 2CBA concentration in soil determined on a subsample and then the bioindicator seeded. Ten seeds were planted in each pot and emergence measured 7 DAP.

3.3.6. Determination of 2CBA levels in soil

Five grams of soil (dried at 110°C for approximately 24 hr basis) and 5 mL of aqueous sodium benzoate (600 mg L^{-1}) were added to a 50 mL Erlenmeyer flask and then 2 mL of 18.8 M sulphuric acid added. Dichloromethane (DCM) (15 mL) was added to the flask which was then sealed with neoprene stoppers and agitated over night on a rotary shaker (120 rpm) at room temp.

A small portion of DCM (*c.a.* 0.5 ml) was transferred to a clean scintillation vial and silyated with 10 μL of N,O-*bis*-(Trimethylsilyl)-Acetamide (Chromatographic Specialities Inc.). The silyated solution was passed through a pasteur pipette plugged with silane treated glass wool (Supelco), containing (*c.a.*. 2 g) Sephadex G-75 gel (Pharmacia) and (*c.a.*. 2g) anhydrous sodium sulphate. The clean up column was flushed with (*c.a.*. 0.5 mL) DCM twice and the elutent collected in a 1.8 ml GC injection vial.

One μL of the extract was injected into a split/splitless Varian 3500 gas chromatograph equipped with a flame ionization detector. The GC utilized 2 columns in series, the first a 15 m Supelco SPB-5 (0.53 I.D.) followed by a second 30 m Supelco SPB-1 (0.25 I.D.). The injector was held at 220°C, detector at 250°C and the column ramped in the following manner: 60° for 1 min, ramp at 20°min^{-1} to 122°, hold for 5 min, ramp at 20°min^{-1} to 250° hold for 5 min. The elution times of silyated benzoic acid and 2CBA were compared with authentic standards and found to be 8.07 min +/- 0.05 for silyated benzoic acid and 13.3 min +/- 0.08 for silyated 2CBA. The 2CBA concentration was determined by calculating the response ratio between 2CBA and benzoic acid. Trials consisting of soil contaminated with 2CBA ranging from 10 to 900 mg kg^{-1} 2CBA soil were performed and the relationship between the response ratio and 2CBA concentration in soil was linear (2CBA:Benzoate = 0.00124 × [2CBA]; r^2 = 0.983, $P \leq 0.01$).

3.3.7. Data analysis

An experimental unit consisted of 10 seeds placed in one well. Each unit was replicated 5-10 times depending upon the experiment. A Skewness and Kurtosis analysis of the data by methods outlined by Sokal and Rohlf (1981) indicated that the data were normally distributed so the data was analyzed using ANOVA procedures. The regression analysis was performed using the statistical software, CoStat (Cohort Software, CA, USA).

3.4. RESULTS

3.4.1. Bioindicator selection

The germination response of the 17 grass species differed over a wide range of 2CBA and Aroclor™ concentrations (Table 3.2.). Canada blue grass was especially sensitive to 2CBA contamination with 4 percent emergence observed at 50 mg kg^{-1} soil. This species had reduced germination to 2CBA concentrations between 10 and 50 mg kg^{-1} soil. Slender wheatgrass was moderately sensitive to 2CBA contamination with a response range between 20 and 250 mg kg^{-1} soil. Fifteen grass species were tolerant to at least 250 mg kg^{-1} soil.

Orchard grass had the highest sensitivity to Aroclor™ with 6% emergence observed at 33 μg kg^{-1} soil. This species had a response range between 13 and 33 μg kg^{-1} soil. Streambank wheatgrass was moderately sensitive to Aroclor™ contamination with a response range between 32 and 110 μg kg^{-1} soil. Common brome and Crested wheatgrass were almost as sensitive to Aroclor™ as streambank wheatgrass with a response range between 52 and 130 μg kg^{-1} soil. The remaining 13 grass species were tolerant (*i.e.* $\geq$ 80% emergence), of 130 μg kg^{-1} soil.

3.4.2. Effect of soil type on bioindicator response

The germination response of Canada blue grass to 2CBA was different ($P \leq 0.08$) in the Paddockwood soil compared to the Allan, Lanigan and Outlook soils (Fig. 3.1.). The response curve, where Y is percent emergence and X the concentration of 2CBA,

was linear ($Y = 94 - 1.6 \times X$; $r^2 = 0.986$, $P \leq 0.01$) in Paddockwood soil, whereas it was logarithmic ($Y = 110 - 26 \times \ln(X)$; $r^2 = 0.915$, $P \leq 0.01$) in the other three soils.

In contrast to Canada blue grass, the germination response of slender wheatgrass to 2CBA contamination was not significantly dependent upon soil type (Fig. 3.2). A logarithmic equation ($Y = 140 - 24 \times \ln(X)$; $r^2 = 0.949$, $P \leq 0.01$) characterized the germination across all soil types. Thus, slender wheatgrass was selected for further studies assessing the impact of biological treatments on bioindicator response.

3.4.3. Effect of biological treatments on bioindicator response

In non-contaminated soil, growing either prairie grasses by themselves, (*e.g.* Dahurian wild rye), or grasses inoculated with bacteria, (*e.g.* meadow brome inoculated with R75), for 42 days, and then planting Slender wheatgrass (*i.e.*, the bioindicator), lowered ($P \leq 0.05$) the percent emergence of slender wheatgrass (Fig. 3.3.). Similar effects were also seen with meadow brome inoculated with a combination of R75 and 2CB35, and with streambank wheatgrass inoculated with R75. When meadow brome inoculated with R75 was grown in contaminated soil, the emergence of slender wheatgrass was no longer related to the amount of 2CBA in the soil by the original logarithmic curve ($Y = 140 - 24 \times \ln(X)$; $r^2 = 0.949$, $P \leq 0.01$), but was described by a linear relationship ($Y = 100 - 0.28 \times X$; $r^2 = 0.817$, $P \leq 0.01$) (Fig. 3.4.). Similar results were found for other biological treatments (*e.g.* Dahurian wild rye, meadow brome inoculated with a combination of R75 and CB35 or streambank wheatgrass inoculated with R75) that significantly affected slender wheatgrass emergence in non-contaminated soil. In contrast, treatments which did not significantly affect bioindicator response in non-contaminated soil (*e.g.* streambank wheatgrass), did not significantly alter the response curve of slender wheatgrass to 2CBA contamination. However, germination was marginally increased in the presence of biological treatments. For example, in the presence of streambank wheatgrass, the bioindicator response was characterized by a logarithmic relationship ($Y = 220 - 38 \times \ln(X)$; $r^2 = 0.820$, $P \leq 0.01$) similar to the logarithmic curve obtained in the absence of remedial treatments (Fig. 3.5.).

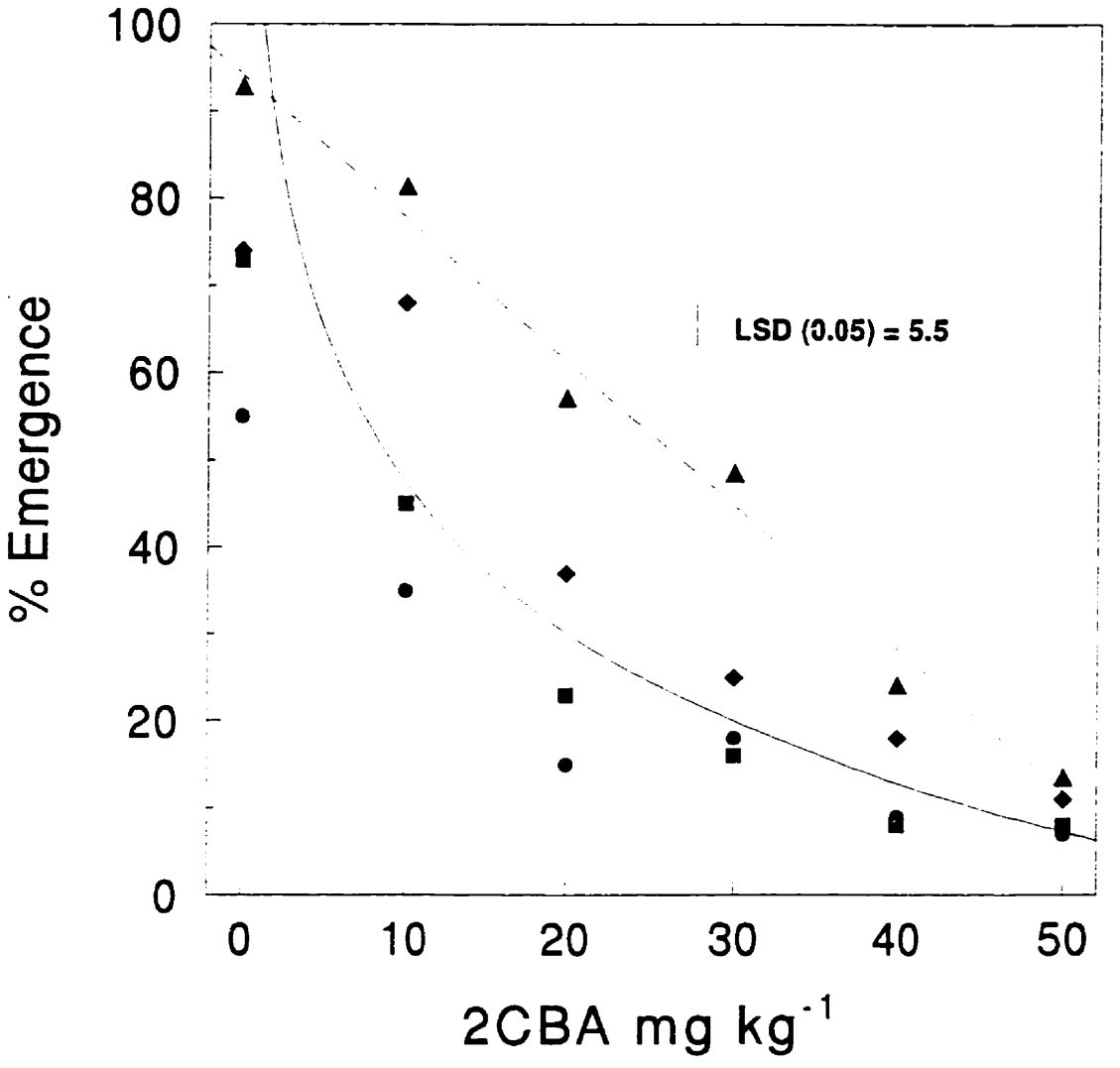

Fig. 3.1. The germination response of Canada blue grass in 2CBA contaminated soils 7 days after planting. Each symbol is the mean of two independent experiments (n=6 replicates). ▲, Paddockwood; ●, Lanigan; ■, Outlook; ◆, Allan. Dashed line represents the linear relationship for Paddockwood soil and solid line represents the logarithmic relationship for the combined results of the other three soils.

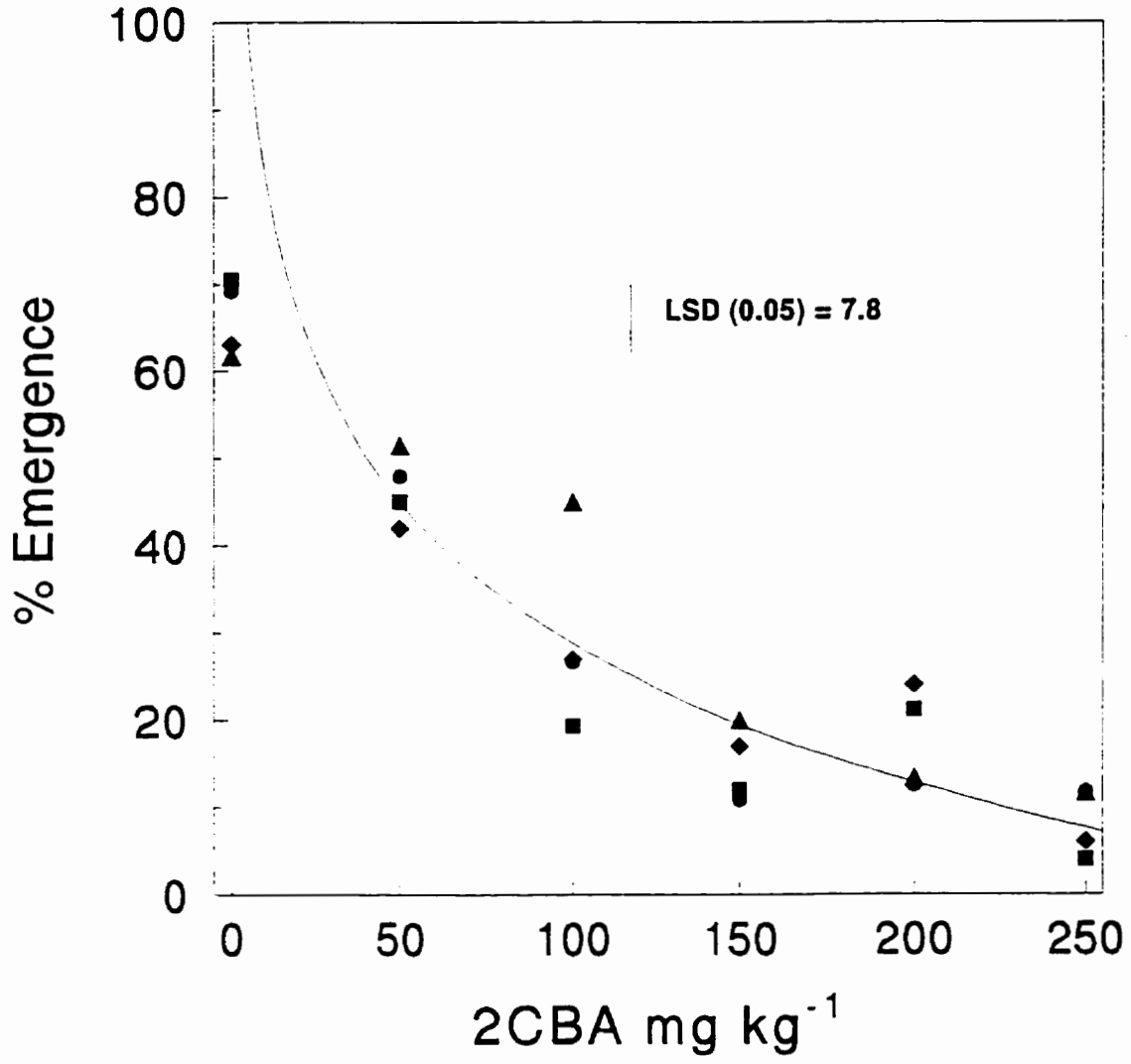

Fig. 3.2. The germination response of slender wheatgrass in 2CBA contaminated soil 7 days after planting. Each symbol is the mean of two independent experiments (n=6 replicates). ▲, Paddockwood; ●, Lanigan; ■, Outlook; ●, Allan. Solid line represents the logarithmic relationship for the combined results of the four soils.

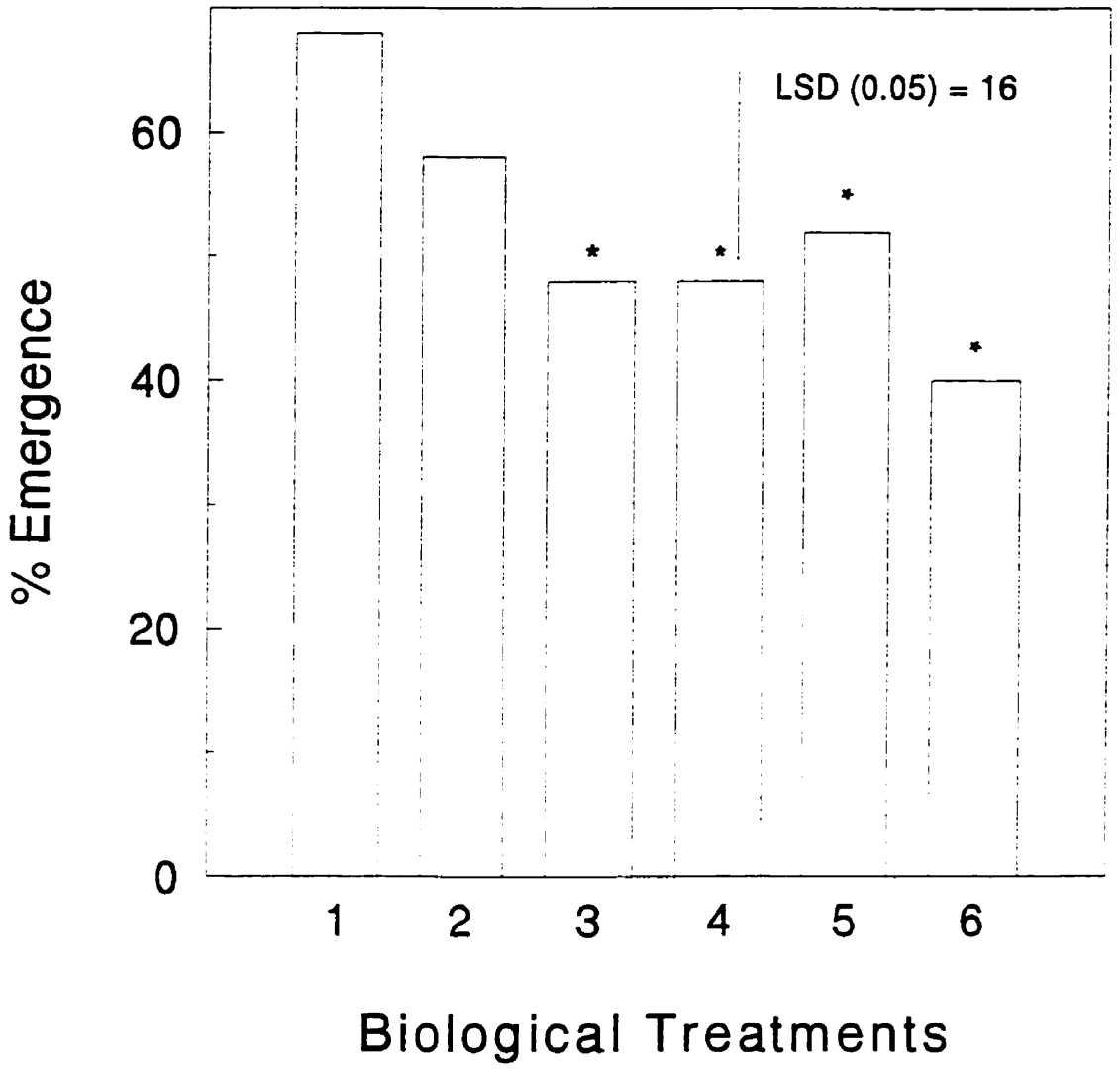

Fig. 3.3. The inhibitory effect of biological treatments on the germination response of slender wheatgrass in non-contaminated soil 7 days after planting. (1) non-planted control; (2) streambank wheatgrass; (3) streambank wheatgrass + R75; (4) Dahurian wild rye; (5) meadow brome + R75 & CB35; (6) meadow brome + R75. Each bar is the average of three independent experiments; two in the Paddockwood soil (n=5 replicates) and one in the Outlook soil (n=5 replicates). Bars marked with * are significantly ($P \leq 0.05$) different from non-planted control.

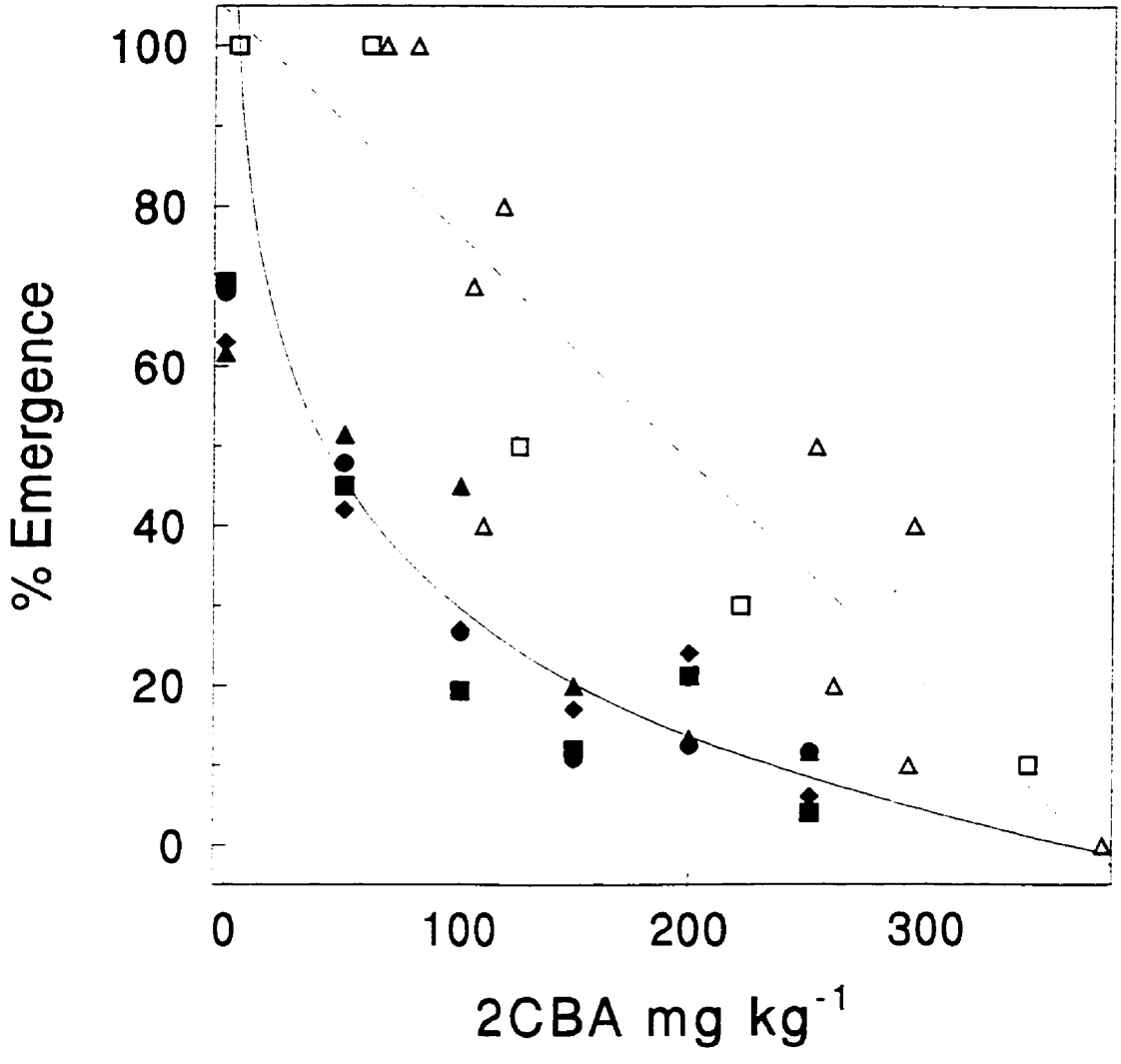

Fig. 3.4. The effect of the biological treatment, meadow brome inoculated with R75, on the relationship between slender wheatgrass emergence and 2CBA contamination. Dashed line represents the linear relationship in the presence of meadow brome + R75. Open symbols are data from two experiments in Paddockwood soil (n=5 replicates) and one experiment in Outlook soil (n=5 replicates) in the presence of meadow brome + R75. △, Paddockwood; □ Outlook. Solid line represents the logarithmic relationship in the absence of meadow brome + R75. Solid symbols are the mean of two independent experiments (n=6 replicates) in the absence of meadow brome + R75. ▲, Paddockwood; ●, Lanigan; ■, Outlook; ●, Allan.

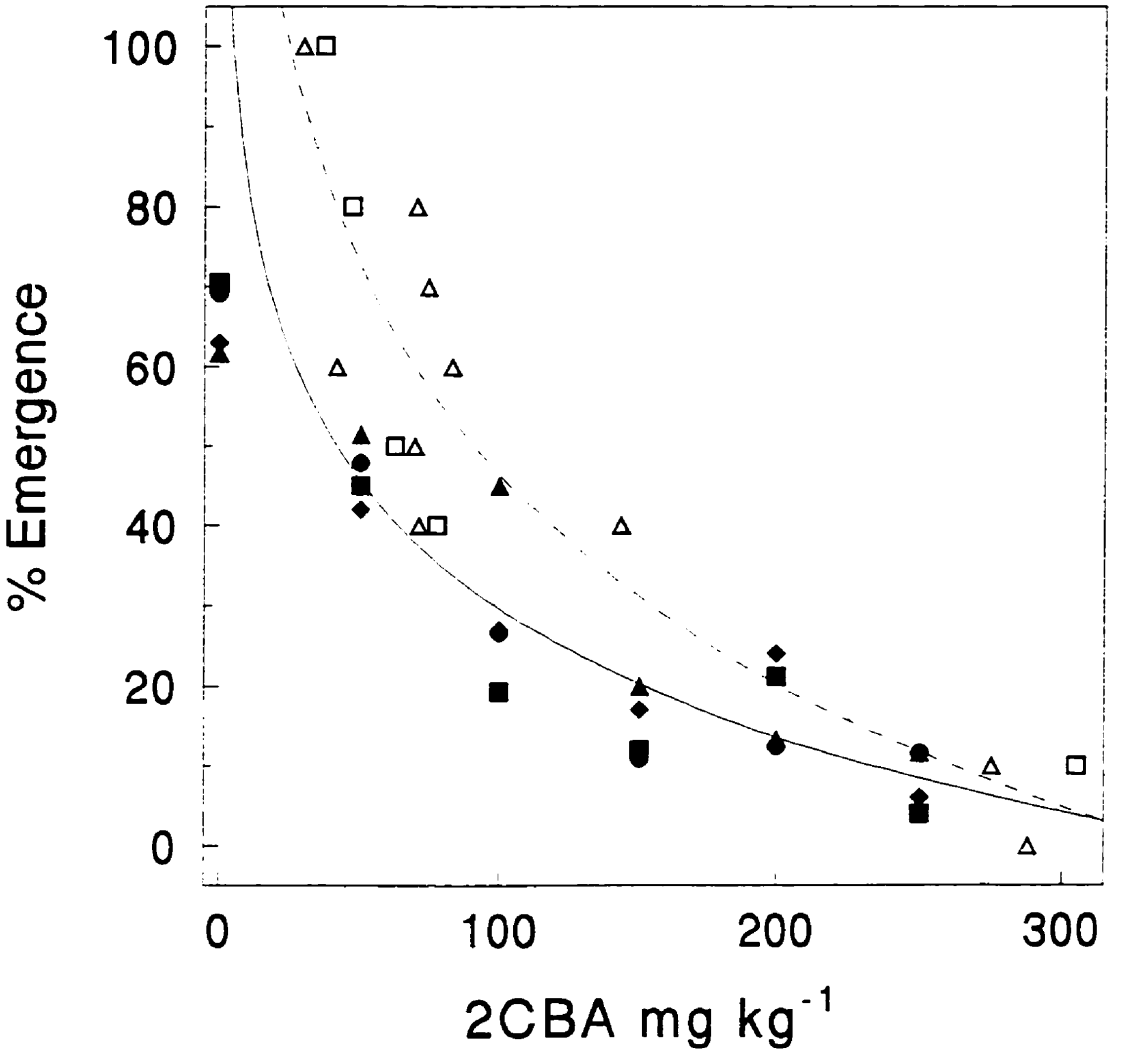

Fig. 3.5. The negligible impact of the biological treatment, streambank wheatgrass, on the relationship between slender wheatgrass emergence and 2CBA contamination. Dashed line represents the logarithmic relationship in the presence of streambank wheatgrass. Open symbols are data from two experiments in Paddockwood soil (n=5 replicates) and one experiment in Outlook soil (n=5 replicates) in the presence of streambank wheatgrass. △, Paddockwood; □ Outlook. Solid line represents the logarithmic relationship in the absence of streambank wheatgrass. Solid symbols are the mean of two independent experiments (n=6 replicates) in the absence of streambank wheatgrass. ▲, Paddockwood; ●, Lanigan; ■, Outlook; ●, Allan.

3.5. DISCUSSION

Plants are good bioindicators of toxicants in soil and respond to a wide range of chemicals at various concentrations (Wang and Freemark 1995; Fletcher and Johnson 1990). The concept of using a prairie grass to act as a bioindicator in prairie ecosystems is supported by Chapman (1995) and Cairns (1993) who both suggest that the use of indigenous species will increase the relevance and reliability of bioindicator testing. The collection of 17 grass species I screened as bioindicators had a tolerance of 2CBA at concentrations ranging from 20 to 250 mg kg^{-1} soil. This is a 12 fold difference in 2CBA sensitivity among grass species. Similar results were obtained with Aroclor™ 1260, with tolerances of Aroclor™ ranging from 13 to 130 μg kg^{-1} soil or a 9 fold difference in grass species sensitivity. This is comparable to the sensitivity range found by Fletcher and Johnson (1990) who, in an analysis of the Phytotox database which contains data from 3,500 published papers on plant sensitivity to chemicals, calculated that there was an average 11 fold difference in plant species sensitivity to chemicals. The logarithmic response curves obtained with slender wheatgrass and Canada blue grass are similar to curves obtained by Wang (1985) in his tests of millet (*Panicum miliacecum*), radish (*Raphanus sativus*) and velvetleaf (*Abutilon theophiasti* Medic) as bioindicators of phenol pollution. Logarithmic response curves of this sort have a high sensitivity to chemical concentration changes in the middle part of the response curve (Klaassen and Eaton 1991). Hence, selecting a bioindicator with the appropriate response range can result in a precise measurement of environmental pollution. Such precision was observed by Miller et al. (1985) in an investigation of herbicide toxicity, where it was found that the coefficient of variation in plant bioindicators was only 10%, compared to 35% for algae and 33% for daphnia bioindicators. Therefore, the ecological relevance, wide response range and precision of prairie grass bioindicators support my contention that prairie grasses have significant potential as bioindicators of toxicants in soil.

Canada blue grass response to 2CBA differed between soil types, but slender wheatgrass did not. It is known that plant sensitivity to toxicants can vary substantially with environmental conditions such as organic matter, pH, ligands and toxicant interactions and this sensitivity can vary on a species by species basis (Wang and Freemark 1995). This is one possible explanation. On the other hand the two grasses might have differed because one species, Canada blue grass, was tested outside of it's normal habitat range whereas slender wheatgrass' habitat encompasses the regions from which the four soil types were obtained (Looman 1982). Additional investigations utilizing soils from regions other than Saskatchewan are required to confirm either of these hypotheses.

Alteration in bioindicator response by remedial treatments has been observed by other investigators (Hund and Traunspurger 1994; Belkin et al. 1994). In these cases, the authors assumed that the alteration in bioindicator response was caused by a toxic metabolite of the remediation process. In my study, the bioindicator was inhibited by treatments in the absence of a contaminant. Hence, the formation of toxic metabolites derived from the contaminants by these bioremediation treatments could not have occurred. This suggests that indigenous or introduced organisms may have produced a chemical that affected the bioindicator.

Our hypothesis that a chemical affected the bioindicator can be assessed according to the six weight of evidence criteria, *i.e.* chronological order, strength of association between cause and effect, specificity of the interaction, consistency of the interaction, biological plausibility of the hypothesis and intensity of the effect, suggested by Chapman (1995) for analyzing the validity of ecotoxicological relationships. Chronologically, I found that the inhibitory effect was only seen after specific plants or bacteria had been added and allowed to grow in the test soil. This occurred consistently in three different experiments that used two soil types, thereby reducing the possibility of this effect being a soil or experimental artifact. Furthermore, the inhibitory effect was observed under specific circumstances. Only remedial biological treatments which

affected bioindicator response in non-contaminated soil affected the bioindicator's relationship to 2CBA levels. In contrast, no change was seen in bioindicator response to 2CBA by remedial treatments which had little effect on bioindicator response in non-contaminated soil. The biological plausibility of the hypothesis is supported by the abundance of evidence documenting that many plants and bacteria produce compounds that inhibit the growth of other organisms (Curl and Truelove 1986c). Two alternative biological hypotheses also present themselves, the production of toxic metabolites or nutrient depletion by the biological treatments. However, since the biological treatments in the absence of the contaminant caused an inhibition in bioindicator emergence, the toxic metabolite hypothesis is eliminated. A nutrient analysis of soil in which meadow brome and R75 had grown for 42 days indicated that there was no depletion of nutrients by this biological treatment (Table 3.1.). Alterations in soil nutrient status would have to be extreme in order to impact upon a germinating seed within 7 DAP because the seed draws off internal nutrient sources for its initial growth spurt. In addition, the inhibition of bioindicator emergence was seen both in the Paddockwood and Outlook soils which had different soil characteristics (*e.g.* see Table 3.1), furthering the case against the nutrient depletion hypothesis. The inhibition of bioindicators by a chemical produced by biological treatments fulfils five out of Chapman's (Chapman 1995) six criteria. The sixth criterion, the intensity of the effect, was not investigated.

This investigation has demonstrated the potential of prairie grasses to act as bioindicators of contaminants in soil. It has also given support to the hypothesis that local organisms are the most reliable bioindicators. However, these results indicate that remedial treatments may be interacting with bioindicators in a manner not related to xenobiotic degradation.

If remedial treatments are producing allelopathic or toxic substances that impact on the bioindicator, then the nature of the allelopathic-toxicant effect upon the bioindicator should be determined because this relationship will alter the indication of toxicity by the bioindicator. Until the antagonistic, additive or potentiating nature of the allelopathic-toxicant relationship is determined, correcting the bioindicator calibration curve for the impact of remedial biological treatments may not be possible. How to integrate such considerations into hazardous waste site risk assessment needs investigation.

4. BACTERIAL INOCULANTS OF FORAGE GRASSES THAT ENHANCE DEGRADATION OF 2-CHLOROBENZOIC ACID IN SOIL

4.1. ABSTRACT

Biological remediation of contaminated soil is an effective method of reducing risk to human and ecosystem health. Bacteria and plants might be used to enhance remediation of soil pollutants in situ. This study assessed the potential of bacteria (12 isolates), plant (17 forage grasses) and plant-bacteria associations (selected pairings) to remediate 2CBA-contaminated soil. Initially grass viability was assessed in 2CBA-contaminated soil. Soil was contaminated with 2CBA, forage grasses grown under growth chamber conditions for 42 or 60 days and the 2CBA concentration in soil determined by gas chromatography. Only 5 out of 17 forage grasses grew in 2CBA (816 mg kg^{-1}) treated soil. Growth of common brome had no effect on 2CBA concentration, whereas intermediate wheatgrass, meadow brome, streambank wheatgrass and Dahurian wild rye decreased 2CBA relative to non-planted control soil by 32-42%. The 12 bacteria isolates were screened for their ability to promote the germination of the five grasses in 2CBA-contaminated soil. Inoculation of streambank wheatgrass with strain R75, a proven plant growth promoting rhizobacterium, increased seed germination by 80% and disappearance of 2CBA by 20% relative to non-inoculated plants. Inoculation of Dahurian wild rye with a mixture of strain CB35, a 2CBA degrading bacterium, and strain R75 increased disappearance of 2CBA by 112% relative to non-inoculated plants. There was no clear relationship between enhanced 2CBA disappearance and increased plant biomass. These results suggest that specific plant-microbial systems can be developed to enhance remediation of pollutants in soil.

4.2. INTRODUCTION

Grasses are potentially inexpensive and environmentally friendly remediation agents. For example, prairie grasses increase the disappearance of polycyclic aromatic hydrocarbons (PAH), reduce PAH leachate from soil columns (Aprill and Sims 1990; Reilley et al. 1996) and have a PAH biological concentration factor of only 0.04 (Hoylman 1994). Aprill and Sims (1990) proposed that grasses would be good remediation agents because of their dense rooting pattern and the wide diversity of species present throughout the biosphere. Similarly, Ferro et al. (1994) found that Hycrest crested wheatgrass (*Agropyron desertorum* (Fisher ex Link) Shultes) increased PCP degradation in soil and had a PCP biological concentration factor of only 0.3. In addition to PAH and PCP contaminants, rhizosphere soil has the catabolic diversity to degrade TCE, atrazine, metolachlor, trifluralin and 3CBA (Anderson et al. 1994; Anderson and Walton 1995; Walton and Anderson 1990; Haby and Crowley 1996). However, while plants have the potential to remediate a variety of contaminated sites (Anderson et al. 1993; Shimp et al. 1993; Aprill and Sims 1990), the impact of toxicants upon plants can be substantial (Xu and Johnson 1995) and methods need to be found to ameliorate this impact. One possible solution is the use of bacterial seed inoculants that enhance the degradation of toxicants in soil.

Bacteria are well recognized as remediation agents (Morgan and Watkinson 1989). Their catabolic diversity is established (Chaudhry and Chapalamadugu 1991; Haggblom 1992), but their remedial activity in soil is variable (Providenti et al. 1993). Several factors limit the effectiveness of bacterial inoculants of field soils for bioremediation: in situ expression of catabolic activity (Guilbeault et al. 1994); inoculant survival (Havel and Reineke 1992; Ramadan et al. 1990); inoculant transport to the microsite of contamination (Devare and Alexander 1995); and nutrient requirements for growth of inoculants (Ramadan et al. 1990). Inoculants also need to successfully compete with indigenous soil populations for nutrients and habitats (van Elsas et al. 1991).

I propose that bacterial seed inoculants intended to remediate contaminated soil may be more effective than bacterial inoculants of bulk soil. Unlike inoculants of bulk soil, bacterial inoculants of seed may persist in soil under field conditions (de Freitas et al. 1994), exhibit activity in the rhizosphere (Rattray et al. 1995) and remain associated with the rooting system of an inoculated plant as it progresses through the soil profile (Hekman et al. 1995; Kluepfel et al. 1991). In addition, the rhizosphere provides nutrients and a physical environment conducive to rhizosphere bacteria (Curl and Truelove 1986b). In return, rhizosphere bacteria may attenuate the toxic impact of contaminated soils upon plant growth (Walton et al. 1994b).

Bacterial seed inoculants that enhance remediation might either (1) augment degradative activity in the rhizosphere by enhancing microbial or plant activity in the rhizosphere (*i.e.* plant growth promoting rhizobacteria-PGPR), or (2) increase the catabolic capacity of the indigenous rhizosphere community, thereby increasing the rate of contaminant degradation (*i.e.* degradative bacteria). This study assessed the potential of rhizosphere inoculants to enhance the degradation of 2CBA in soil.

4.3. MATERIALS AND METHODS

4.3.1. Soil collection and preparation

Three surface (0-15 cm) soils were collected from different uncontaminated field sites in Saskatchewan and prepared as described in Section 3.3.1. I contaminated soil by mixing a small portion of the soil with solid 2CBA and then mixing this soil into the remainder of the soil to be amended. Soil was watered to -0.33 KPa and allowed to equilibrate for 10 days. Preliminary experiments (data not shown) indicated that little or no degradation of 2CBA occurred within this 10 day equilibration period.

4.3.2. Bacterial strains and growth conditions

A 2CBA metabolizing bacterium was isolated from the Lanigan soil by enrichment culture on a minimal salts medium (Farrell et al. 1993) containing 0.1 g L^{-1} 2CBA as the sole carbon source (2CBM). After 96 h on a rotary shaker (160 rpm) at

22°C, a 0.2 ml aliquot of the enrichment broth was transferred to a new flask containing 50 ml 2CBM and incubated on a rotary shaker for a further 96 h. After 96 h, a 0.2 ml aliquot was transferred to a new flask; this was repeated twice. A 0.1 ml aliquot of the final enrichment flask was spread plated onto 2CBM solidified with 1.5% Difco agar and incubated at 28°C for 48 h. Colonies were transferred to 2CBM agar plates containing either 0.1, 0.2 or 0.3 g L^{-1} 2CBA and one isolate demonstrated enhanced growth on 0.3 and 0.2 compared to 0.1 g L^{-1} 2CBM agar plates. This isolate was designated strain CB35, and tentatively identified based on fatty acid methyl ester analysis using MIDI Corporation's (Newark, DE, USA) software and extraction procedure as a strain of *Pseudomonas savastanoi pv. fraxinus* (similarity index of 0.734). Six known PGPR strains [30] including *P. cepacia* R55, *P. cepacia* R85, *P. aeruginosa* R61, *P. aeruginosa* R75, *P. fluorescens* R111 and *P. putida* R104 and 5 other PGPR strains (J.J. Germida, unpublished observations) also were used in this study. All bacteria were stored at -40°C in 50% (w/w) glycerol/tryptic soy broth.

In order to inoculate seed with bacteria, 0.3 ml of a frozen bacterial culture was transferred to 100 ml of 1/10th strength tryptic soy broth (TSB) in a 500 ml Erlenmeyer flask and incubated on a rotary shaker (160 rpm) at 22°C for 48 hours. For PGPR isolates, these cells (*c.a.*. 10^9 cfu/ml on 1/10th strength TSA) were washed twice in sterile reverse osmosis water by centrifugation (15 min at 1700 × *g*, 5°C) and inoculated onto seeds (see below). Strain CB35 cells were washed twice in sterile reverse osmosis water by centrifugation (15 min at 1700 × *g*, 5°C) and re-suspended in 10 ml sterile water. A 5 ml sub-sample was transferred to 100 ml 2CBM containing 2CBA (0.3 g L^{-1}) in a 500 ml Erlenmeyer flask and incubated on a rotary shaker (160 rpm) at 22°C until mid-log phase (*c.a.*. 10^8 cfu/ml on 1/10th strength TSA). These cells were then inoculated onto seeds (see below).

4.3.3. Screening forage grasses for tolerance to 2CBA

Seventeen forage grass species (Table 3.2.) were obtained and stored as described in Section 3.3.2. Plants were assessed for their ability to grow in 2CBA contaminated soil as described in Section 3.3.3. with the exception that only Lanigan soil amended with 816 mg kg^{-1} 2CBA was used and that plant growth was assessed at 39 DAP. A second experiment assessed the ability of those plants that grew in contaminated soil to enhance 2CBA disappearance. Five seeds were planted in 200 g of 2CBA (816 mg kg^{-1}) contaminated soil in 6 replicate styrofoam cups (170 ml capacity). Cups were placed in a growth chamber and maintained at conditions similar to the trays. The 2CBA levels in soil were determined (see below) at 60 DAP.

4.3.4. Selection of bacterial seed inoculants

Bacterial seed inoculants were selected on the basis of their ability to promote germination of selected forage grasses in contaminated soil. The PGPR strains and CB35 isolate were tested for their ability to promote seed germination of selected forage grasses: 1) meadow brome, 2) streambank wheatgrass, 3) Dahurian wild rye and 4) common brome. Seed (5 g) was soaked in 8 ml of centrifuged (15 min at 1700 × *g*, 5°C) washed bacterial cells for 30 min, coated with 15 grams $CaCO_3$ and air dried for 30 minutes. Control seed was coated with autoclaved bacterial cells in a similar fashion. Fifteen seeds (*c.a.*. 10^6 cfu $seed^{-1}$) were placed in duplicate glass Petri plates containing either 20 g of 2CBA contaminated (816 mg kg^{-1}) or non-contaminated Lanigan soil, watered (-0.33 kPa), sealed with parafilm and incubated in the dark at 10°C. Seed germination was assessed 14 DAP.

4.3.5. Effect of bacterial seed inoculants on remediation by forage grass

The Paddockwood and Outlook soils were amended to 2CBA concentrations of 200, 400 and 800 mg kg^{-1}. Contaminated soil (160 g) was placed in styrofoam cups and watered to -0.33 kPa. Forage grass seeds (n=5) inoculated with strain R75 (*c.a.*. 10^6 cfu $seed^{-1}$), strain CB35 (*c.a.*. 10^6 cfu $seed^{-1}$) or a mixture of R75 & CB35 (*c.a.*. 10^7 combined cfu $seed^{-1}$) were planted in replicate cups (n=6). The ratio of R75:CB35 in

the mixed inoculant was estimated to be *c.a.*. 8:1. Pots were placed in a growth chamber under similar conditions to those described above. At 42 DAP, the shoots were harvested and the rooting system extracted from soil, shaken and washed in cold water. The plant material was dried (48 h, 60°C) and weighed. The 2CBA concentrations in soil and plant material were determined (see below).

4.3.6. Determination of 2CBA in soil and plant tissue

The method outlined in Section 3.3.5. was used to determine the concentration of 2CBA in soil. Plant tissue (0.2 g) was analyzed for the presence of 2CBA in a manner similar to that used for contaminated soil. The detection limit for 2CBA in roots and shoots was determined to be 3 mg/kg.

4.3.7. Statistical analysis

The experiment was designed as a complete factorial experiment with replicate experiments designated as random factors. Each treatment was replicated five times and each soil experiment was performed twice. The results were analyzed by ANOVA, and means separated by Student's T test.

4.4. RESULTS

4.4.1. Selection of remedial forage grasses and bacterial inoculants

Nine of the seventeen forage grasses tested germinated in 2CBA-contaminated Lanigan soil (816 mg kg^{-1}), but only five exhibited growth (Table 4.1.). In non-contaminated soil all seventeen forage grasses germinated well and by 39 DAP had overgrown their pots.

TABLE 4.1. Growth of forage grasses in soil contaminated with 816 mg 2CBA kg^{-1}

Common Name	Scientific Name	Growth[a]
Common brome	*B. inermis*	+++
Dahurian wild rye	*E. dauricus*	+++
Intermediate wheatgrass	*A. intermedium*	++
Meadow brome	*B. biebersteinii*	++
Streambank wheatgrass	*A. riparum*	++
Tall wheatgrass	*A. elongatum*	+
Northern wheatgrass	*A. dasystachyum*	+
Reed canary	*P. arundinacea*	+
Altai wild rye	*E. angitus*	+
Canada blue	*P. compressa*	-
Crested wheatgrass	*A. cristatum*	-
Perennial rye grass riviera	*L. perenne*	-
Russian wild rye	*E. junceus*	-
Sheep fescue	*F. ovina*	-
Slender Wheatgrass	*A. trachycaulum*	-
Orchard grass	*D. glomerata*	-
Timothy	*P. pratense*	-

[a] Gradient of visual responses determined at 39 days after planting: -, no seed germination; +, seed germination; ++, seed germination with leaf at coleoptile tip; +++, seed germination with first leaf through coleoptile.

Growing streambank wheatgrass, Dahurian wild rye, meadow brome or Intermediate wheatgrass in 2CBA-contaminated Lanigan soil decreased ($P \leq 0.05$) 2CBA levels relative to unplanted soil (Table 4.2.). Because common brome had no effect on 2CBA levels and intermediate wheatgrass grew poorly in 2CBA-contaminated soil, these grasses were not used in subsequent experiments. The levels of 2CBA in unplanted soil also were reduced just by incubating soil for 42 or 60 days (Table 4.2.). However, this disappearance was only significantly different from the initial nominal 2CBA concentration in the Lanigan soil and may be related to the high soil organic matter content (Table 3.1.). No 2CBA was found in plant tissue at any contamination level.

The ability of forage grasses to remediate 2CBA was not limited to a specific soil type, but was influenced by the initial level of contamination. For example, at the 800 mg kg^{-1} level, streambank wheatgrass decreased ($P \leq 0.05$) 2CBA levels in the Paddockwood soil by 42% and in the Outlook soil by 41% (Table 4.2.). Similarly, Dahurian wild rye decreased 2CBA levels by 37% and 19% ($P \leq 0.05$) in these soils, respectively. However, with the exception of Dahurian wild rye in Outlook soil (Table 4.2.), none of the forage grasses significantly reduced 2CBA levels when the initial contamination level was 400 or 200 mg kg^{-1}.

There was no significant difference in 2CBA disappearance between the soil types, but the grasses were consistently more effective in the Paddockwood compared to the Outlook soil at the 800 and 400 mg kg^{-1} contamination levels. For example, at the 400 mg kg^{-1} level, meadow brome decreased 2CBA by 28% in the Paddockwood soil, but inhibited disappearance by 35% in the Outlook soil. The above trend was reversed at the 200 mg kg^{-1} contamination level, with grasses being more effective in the Outlook compared to the Paddockwood soil. For example, Dahurian wild rye decreased 2CBA by 2% in the Paddockwood soil but decreased ($P \leq 0.05$) 2CBA by 64% in the Outlook soil.

TABLE 4.2. Levels of 2CBA in different soils after growing forage grass

Soil	Treatment	2CBA level (mg kg^{-1})		
		Initial 2CBA level		
		200	400	800
Lanigan[a]	Unplanted soil	-[b]	-	480
	Streambank wheatgrass	-	-	278
	Dahurian wild rye	-	-	302
	Meadow brome	-	-	292
	Intermediate wheatgrass	-	-	326
	Common brome	-	-	576
	LSD (0.05)[c]			135
Paddockwood[d]	Unplanted soil	191	329	708
	Streambank wheatgrass	223	273	404
	Dahurian wild rye	188	369	444
	Meadow brome	207	236	540
Outlook[d]	Unplanted soil	198	340	720
	Streambank wheatgrass	146	444	426
	Dahurian wild rye	72	449	582
	Meadow brome	122	459	647
	LSD (0.05)	108	143	174

[a] Plants grown for 60 days; [b] not tested; [c]least significant difference at $P \leq 0.05$ calculated from replicate experiments with 5 replicates each; [d] plants grown for 42 days.

Strain R75 increased ($P \leq 0.05$) the germination of streambank wheatgrass in 2CBA contaminated soil by 80% and increased (*c.a.*. 30%) the germination of other grasses in contaminated soil. Similarly, CB35 increased ($P \leq 0.05$) the germination of Dahurian wild rye in contaminated soil by 133%, and marginally increased (*c.a.*. 11%) the germination of meadow brome and streambank wheatgrass in contaminated soil. Some bacteria-plant combinations reduced germination in contaminated soil, *e.g.* inoculation of common brome with CB35 decreased germination by 47%. I also assessed the ability of strains R75 and CB35 to degrade 2CBA in soil in the absence of plants. Neither strain significantly enhanced disappearance of 2CBA compared to unplanted soil (Fig. 4.1), although a mixture of R75 and CB35 increased 2CBA disappearance by 12% and 15% at the 800 and 400 mg kg^{-1} levels, respectively.

4.4.2. Effect of bacterial inoculants on 2CBA disappearance by forage grasses

Inoculation of meadow brome with either strain R75, CB35 or a mixture of these strains increased ($P \leq 0.05$) 2CBA disappearance (Fig. 4.2.). Inoculation treatments enhanced 2CBA disappearance similarly at both 400 and 800 contamination levels by 18-28% with little difference between treatments and no difference between soil types. Although bacterial inoculants increased the biomass of meadow brome, there was no consistent trend and no apparent relationship to 2CBA disappearance (data not shown).

Inoculation of streambank wheatgrass with either strain R75, CB35 or a mixture of these strains increased ($P \leq 0.05$) 2CBA disappearance compared to the unplanted control to a similar extent in all three tested soils (Fig. 4.3.). However in contrast to meadow brome, inoculation of streambank wheatgrass affected 2CBA disappearance differently at 400 and 800 mg kg^{-1} contamination levels. For example, the mixed inoculant was the best treatment with this forage grass at the 400 mg kg^{-1} level, but had little effect at the 800 mg kg^{-1} level when compared to the uninoculated plant. Again, there was no apparent relationship between disappearance and plant biomass (data not shown). The response of Dahurian wild rye to inoculants and their effect on 2CBA disappearance was similar to that observed for streambank wheatgrass, and the mixed inoculant was the most effective inoculant of Dahurian wild rye at both contamination levels (Fig. 4.4.).

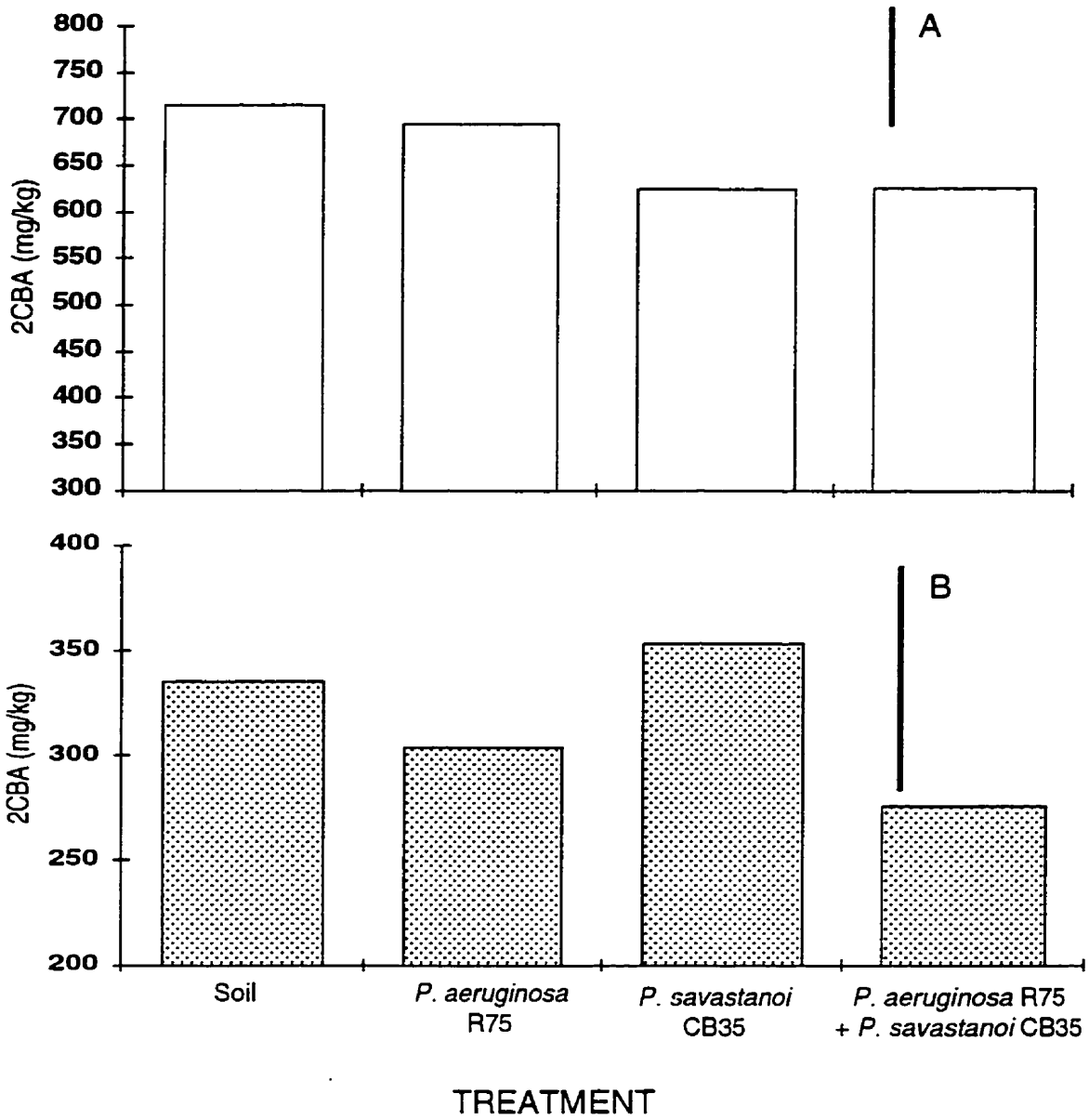

Fig. 4.1. The effect of inoculating dead seed with bacteria on 2CBA levels in soil initially amended with (A) 800 mg kg^{-1} and (B) 400 mg kg^{-1}. Combined results for the Paddockwood and Outlook soils. Streambank wheatgrass seed was autoclaved, inoculated with *c.a.*. 10^6 cfu seed, 10 seeds added to 160 g of contaminated soil and the soil incubated for 42 days in a growth chamber. Least significant ($P \leq 0.05$) difference between treatments is 122 mg kg^{-1} for (A) and 100 mg kg^{-1} for (B). Each bar is the combined average of replicate experiments in Paddockwood and Outlook soils.

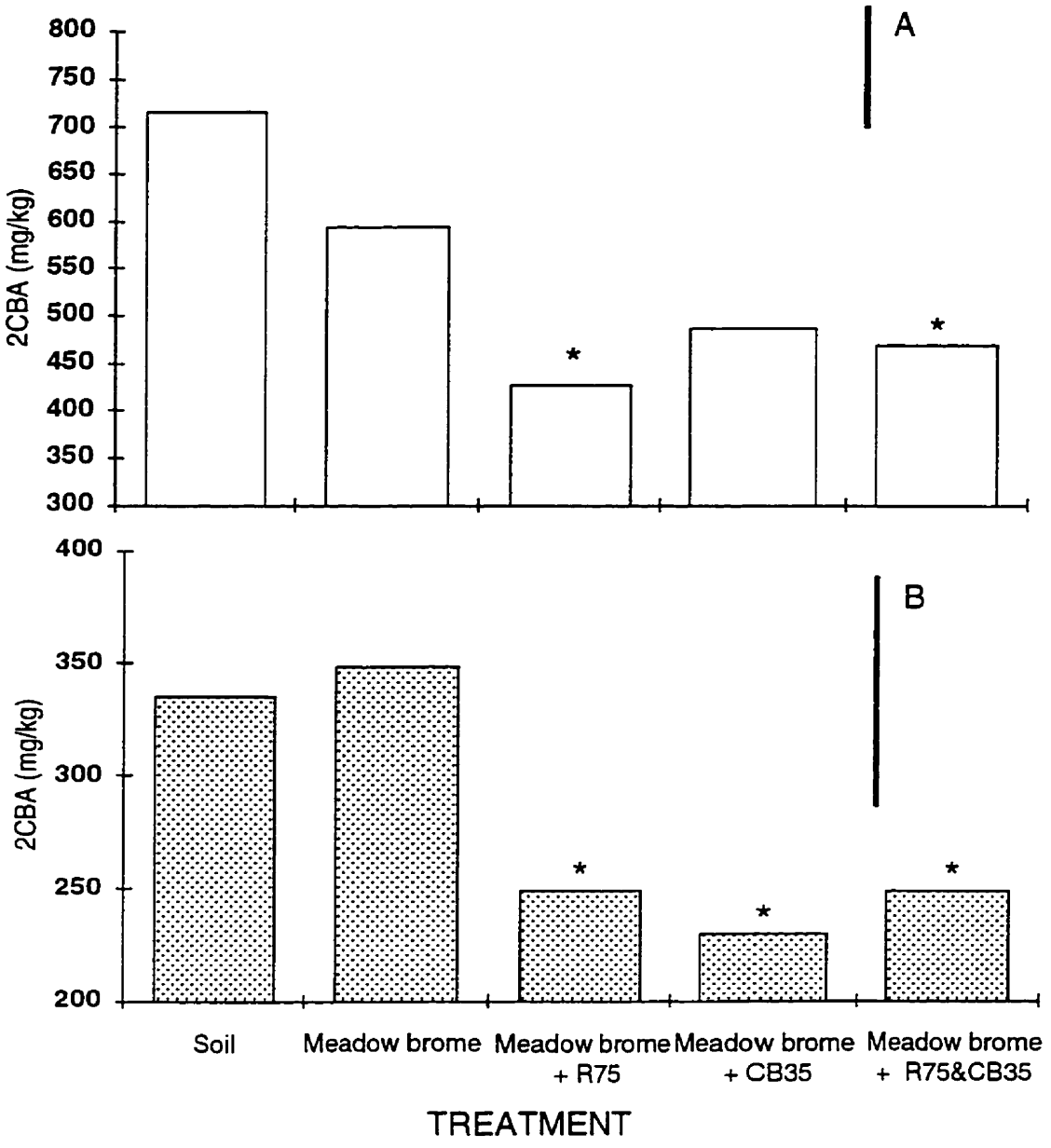

Fig. 4.2. The effect of meadow brome and bacterial seed inoculants on 2CBA levels in soil initially amended with (A) 800 mg kg^{-1} and (B) 400 mg kg^{-1}. meadow brome was inoculated with strain R75 (*c.a.*. 10^7 cfu $seed^{-1}$), strain CB35 (*c.a.*. 10^6 cfu $seed^{-1}$) or equal volumes of R75 & CB35 (*c.a.*. 10^7 combined cfu $seed^{-1}$) and grown for 42 days in a growth chamber. Least significant ($P \leq 0.05$) difference between treatments is 122 mg kg^{-1} for (A) and 100 mg kg^{-1} for (B). Bars significantly ($P \leq 0.05$) different from the uninoculated plant are marked with *. Each bar is the combined average of replicate experiments in Paddockwood and Outlook soils.

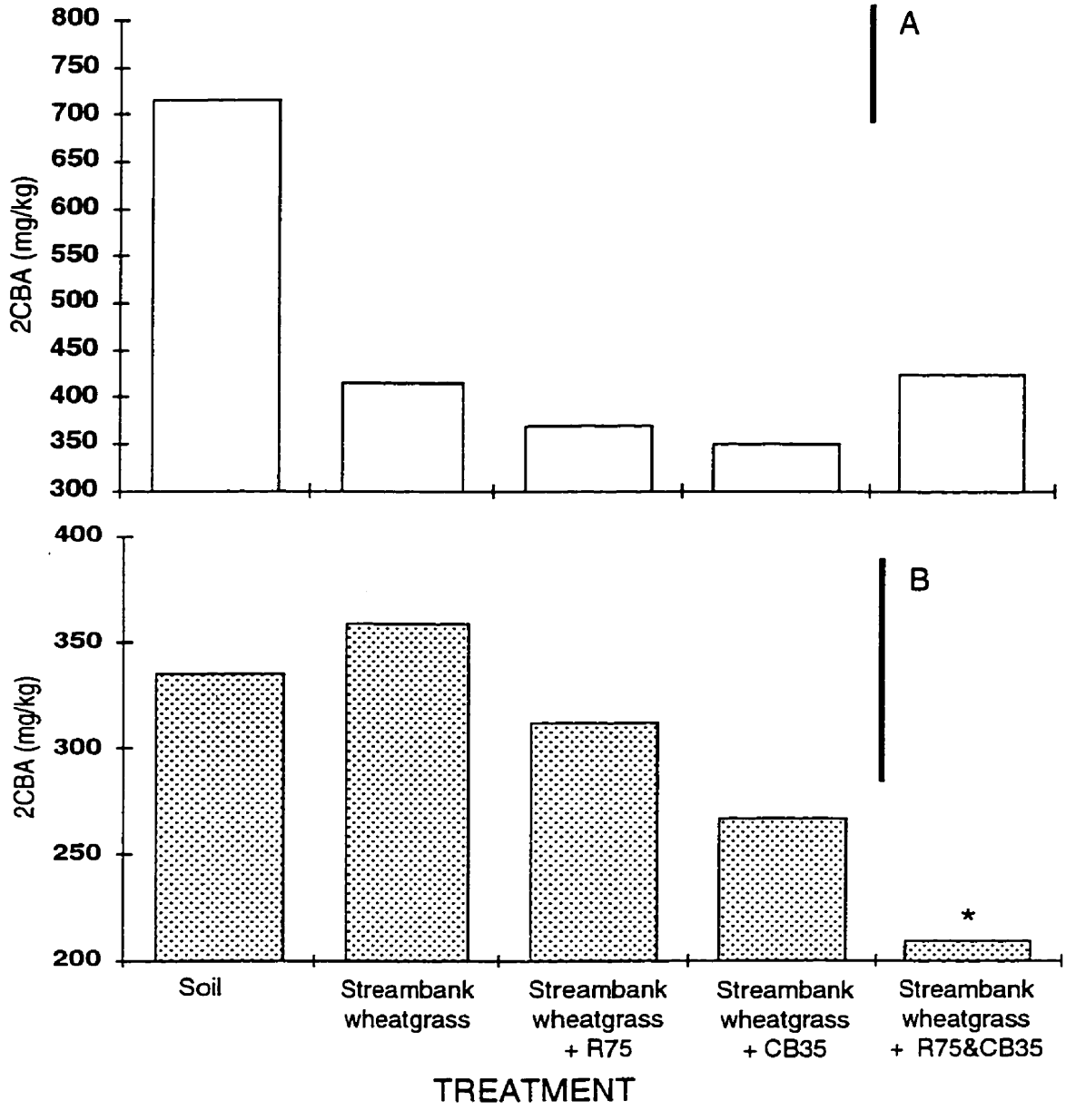

Fig. 4.3. The effect of streambank wheatgrass and bacterial seed inoculants on 2CBA levels in soil initially amended with (A) 800 mg kg^{-1} and (B) 400 mg kg^{-1}. Streambank wheatgrass was inoculated with strain R75 (*c.a.*. 10^7 cfu $seed^{-1}$), strain CB35 (*c.a.*. 10^6 cfu $seed^{-1}$) or equal volumes of R75 & CB35 (*c.a.*. 10^7 combined cfu $seed^{-1}$) and grown for 42 days in a growth chamber. Least significant ($P \leq 0.05$) difference between treatments is 122 mg kg^{-1} for (A) and 100 mg kg^{-1} for (B). Bars significantly ($P \leq 0.05$) different from the uninoculated plant are marked with *. Each bar is the combined average of replicate experiments in Paddockwood and Outlook soils.

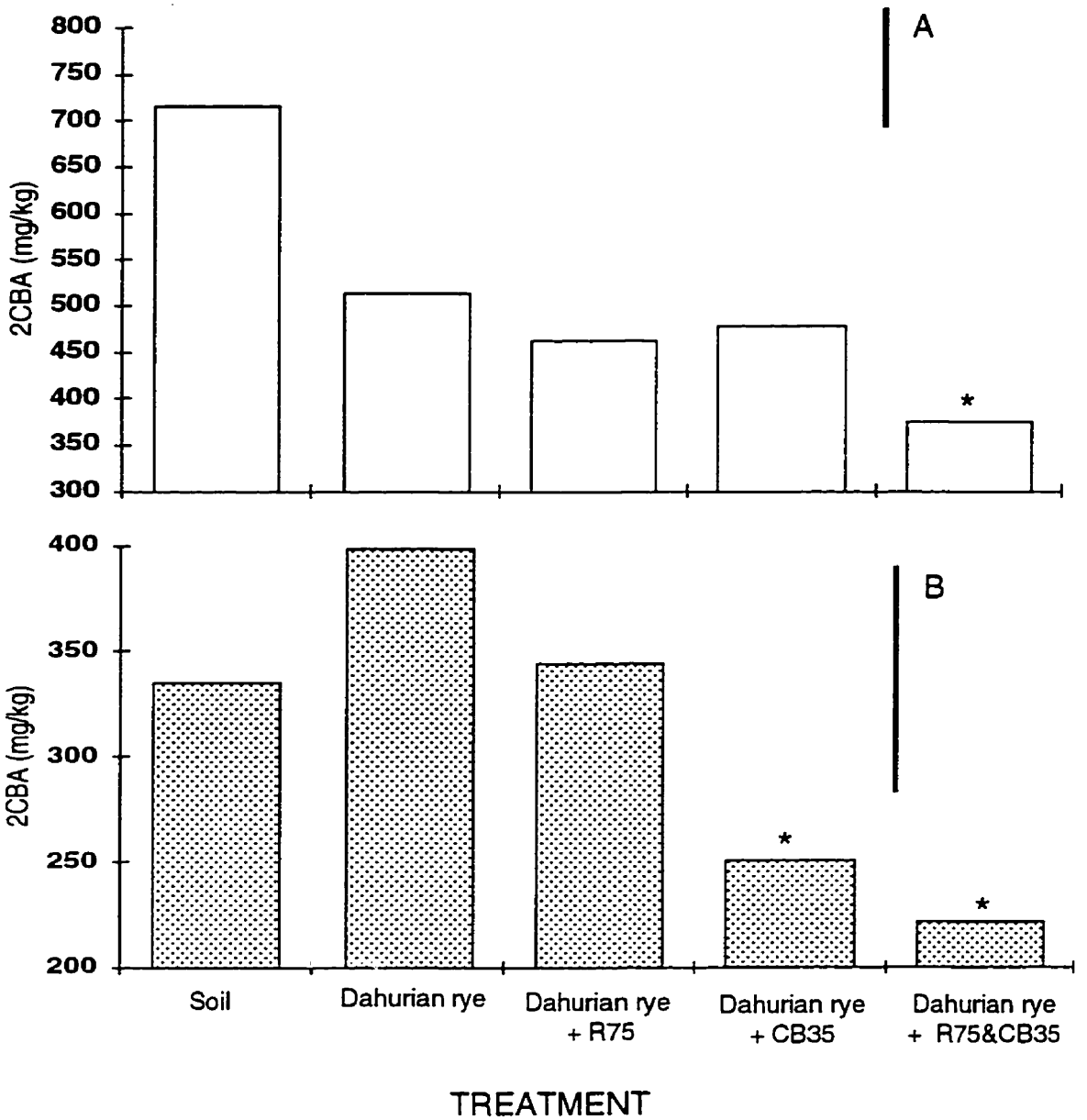

Fig. 4.4. The effect of Dahurian wild rye and bacterial seed inoculants on 2CBA levels in soil initially amended with (A) 800 mg kg^{-1} and (B) 400 mg kg^{-1}. Dahurian wild rye was inoculated with strain R75 (*c.a.*. 10^7 cfu $seed^{-1}$), strain CB35 (*c.a.*. 10^6 cfu $seed^{-1}$) or equal volumes of R75 & CB35 (*c.a.*. 10^7 combined cfu $seed^{-1}$) and grown for 42 days in a growth chamber. Least significant ($P \leq 0.05$) difference between treatments is 122 mg kg^{-1} for (A) and 100 mg kg^{-1} for (B). Bars significantly ($P \leq 0.05$) different from the uninoculated plant are marked with *. Each bar is the combined average of replicate experiments in Paddockwood and Outlook soils.

4.5. DISCUSSION

I found that the prairie grasses meadow brome, Dahurian wild rye and streambank wheatgrass not only grew in 2CBA contaminated soil, but also enhanced the disappearance of this chlorinated aromatic. These plants enhanced any disappearance that occurred in non-planted soil by 11 to 63%. The growth of plants in contaminated soil is known to reduce contaminant levels (Anderson et al. 1993; Anderson et al. 1994; Anderson and Walton 1995; Aprill and Sims 1990; Cunningham et al. 1995; Walton and Anderson 1990; Ferro et al. 1994; Reilley et al. 1996; Shimp et al. 1993). However, seldom have investigators described the process by which they selected their plant species or the number of species screened to obtain a plant which degrades contaminants. This study suggests that many forage grasses (*i.e.* 5/17), have the potential to degrade chlorinated aromatics in soil, and thus a modest sampling of grass species common to a geographical region may be a reasonable first step in identifying potentially useful plants for the disappearance of contaminants in soil. My results support the assertion of Aprill and Sims (1990) that the genetic diversity and dense, fibrous rooting system of prairie grasses make them good candidates for remediation agents, and confirms the fact that a wide variety of plants can enhance the disappearance of contaminants in soil. Thus, it appears that phytoremediation may be an appropriate technology for the *in situ* clean up of shallowly contaminated hazardous waste sites or *ex situ* use in land-farming operations.

There are three suggested mechanisms for the stimulation of soil contaminant disappearance by plants: (1) non-specific enhancement of microbial communities by the plant (Haby and Crowley 1996), (2) enhancement of microbial activity in the rhizosphere that protects the plant from contaminants and results in disappearance (Walton et al. 1994b), or (3) the development of specific degradative microbial communities in the rhizosphere (Ferro et al. 1994). My results for uninoculated plants only partially support the first mechanism. For example, four of the five plants that grew in 2CBA contaminated soil enhanced disappearance of 2CBA by 48 to 63%. Furthermore, common brome grew in 816 mg kg^{-1} 2CBA contaminated soil but had little

effect on 2CBA levels. Similarly, the growth of streambank wheatgrass and Dahurian wild rye had little effect on 2CBA disappearance at the 400 mg kg^{-1} level. Therefore, some plants grew in contaminated soil, but failed to support the degradative activity required for contaminant disappearance. It is well known that the growth of plants results in an increase in the number of microorganisms present in the rhizosphere (*i.e.*, the rhizosphere effect). Since I did not assess the population of 2CBA degrading microorganisms in the rhizosphere of grasses, I can only assume at this time that the enhancement of rhizosphere microorganisms was an important mechanism for the disappearance of 2CBA.

It is unclear if the growth of tolerant plants reduced the toxicity of 2CBA in soil to plants as suggested by Walton et al. (1994b). I found no relationship between the amount of 2CBA degraded and plant biomass, *e.g.* inoculation of streambank wheatgrass with CB35 increased 2CBA disappearance by 20% but had no effect on plant biomass. Because plant biomass would normally increase as the toxicity of contaminants in soil decreased, it does not appear that the rhizosphere detoxified 2CBA in soil. Plant biomass was inversely ($P \leq 0.04$) related to the initial level of contamination in soil (data not shown), indicating that plant biomass was sensitive to 2CBA levels in soil. Other investigators also have found that plants are good bioindicators of contaminant levels in soil (Cunningham et al. 1995; Fletcher et al. 1988; Shimp et al. 1993; Wang and Freemark 1995). The detoxification of 2CBA in soil by the rhizosphere of streambank wheatgrass or Dahurian wild rye may have occurred, but the benefits the plant received from this process was not reflected by increased growth. Furthermore, it is possible that a stressed plant may be necessary in order to create the conditions (via root exudates etc.) under which degradative bacterial communities flourish.

In my study, the development of specific bacteria-plant associations stimulated contaminant disappearance. This supports the idea that specific degradative communities are responsible for contaminant disappearance (Ferro et al. 1994). I found that specific rhizosphere inoculants affected bacteria-plant-soil activity such that enhanced

disappearance of 2CBA occurred. For example, inoculation of meadow brome, streambank wheatgrass and Dahurian wild rye with bacterial isolates such as strain R75 and strain CB35 resulted in a substantial (13%-45%) increase in the amount of 2CBA degraded compared to uninoculated plants. Although the seed inoculants R75 and CB35 increased germination of streambank wheatgrass and Dahurian wild rye, this increase in germination did not lead to increases in plant biomass (data not shown). Hence, it is doubtful that this increased germination is the basis of the increased 2CBA disappearance seen in inoculated plants. Therefore, the development of specific bacteria-plant associations was an important mechanism for the disappearance of 2CBA.

Generally, inoculants containing the 2CBA-degrading bacterium, CB35, performed the best at all 2CBA contamination levels. This supports my hypothesis that a seed inoculant could be used to increase the catabolic capacity of the indigenous rhizosphere community, and thereby increase the rate of contaminant disappearance. The isolate CB35 may have extended the catabolic capacity in the rhizosphere, thereby increasing the rate of contaminant disappearance. This extension of degradative capacity, while stimulated by CB35 inoculation, may also be the consequence of plant mediated interactions. Recent work by Haby and Crowley (1996) demonstrates that the growth of plants in soil increases the number of organisms capable of degrading 3CBA as a sole or cometabolitic carbon source. They hypothesized that this enhancement was due to the provision of labile nutrients by the plant. Similar work has shown that the addition of synthetic root exudates to soil increases the disappearance of parathion or pyrene (Anderson et al. 1993; Reilley et al. 1996). In a like manner, meadow brome, streambank wheatgrass or Dahurian wild rye may have released root exudates or other labile nutrients which increased the degradative activity of CB35.

I found that the enhancement of degradative capacity by bacterial seed inoculants was influenced by other microorganisms. Inoculation of R75, a known PGPR and incapable of 2CBA disappearance in pure culture, enhanced 2CBA disappearance without proportionally increasing plant biomass. Furthermore, inoculation of Dahurian wild rye with a combination of R75 and the 2CBA degradative strain CB35, resulted in a 12 to 22% increase in 2CBA disappearance over inoculation with CB35 only. Apparently, R75 altered rhizosphere processes or root growth in such a way as to increase the effectiveness of CB35. This supports my alternative hypothesis, that an inoculant might augment degradative rhizosphere processes by enhancing microbial or plant activity in the rhizosphere. It is possible that R75 altered the rhizosphere community by influencing root exudation of the host plant and hence the activity of CB35. Bacteria-bacteria interactions can increase disappearance by degrading toxic metabolites of other organisms (Davison et al. 1994), producing surfactant (Steffensen and Alexander 1995) or alternatively, negatively affect biodegradation by consuming scarce nutrients (Steffensen and Alexander 1995) or producing toxic substances which result in inoculant die off (Havel and Reineke 1992; Barriault and Sylvestre 1993). Alternatively, strain R75 may have expressed co-metabolic abilities in the rhizosphere due to the presence of root exudates or other organisms.

The initial 2CBA level of contamination also influenced the success of inoculants. Community composition and abundance of specific members are influenced by the type and level of a contaminant (Hicks et al. 1990; Komulainen and Mikola 1995) and this alteration in the indigenous microbial community will affect the performance of inoculated strains (van Elsas et al. 1991). Hence, the level of contamination may have altered the indigenous microbial community and the effectiveness of the bacterial inoculants. Alternatively, the distribution of the contaminant at the lower contamination levels may have resulted in a patchy contaminant distribution in the soil matrix and hence, degradative bacteria were unable to come into contact with the contaminant.

My results demonstrate that different forage grasses can be used to enhance the disappearance of 2CBA in soil, and that inoculation of these grasses with specific bacteria can increase contaminant disappearance in soil. This increase in disappearance was not related to increased plant biomass, and was dependent upon the initial level of contamination in soil. The best inoculant consisted of a mixture of strain CB35, a 2CBA degrading bacterium, and strain R75, a PGPR.

5. DEGRADATION OF CHLORINATED BENZOIC ACID MIXTURES BY PLANT-BACTERIA ASSOCIATIONS.

5.1. ABSTRACT

Phytoremediation technologies must degrade mixtures of contaminants, as most contaminated sites contain mixtures of compounds. This study assessed the ability of plant-bacteria associations to degrade mixtures of mono- and di-chlorinated benzoic acids. Seventeen forage grasses, and combinations of these grasses with several bacterial inoculants, were screened for growth in soil contaminated with various concentrations of mono or di-chlorinated benzoic acids. Dahurian wild rye inoculated with a combination of strain R75 and strain CB35 reduced 3CBA levels in soil by 74% (i.e., 583 to 149 mg kg^{-1}). Meadow brome inoculated with *Alcaligenes* sp. strain BR60 reduced 2,3-dichlorobenzoic acid (23diCBA) levels in soil by 56% (i.e., 125 to 55 mg kg^{-1}). Altai wild rye (*E. angitus*) inoculated with strains R75 and CB35 reduced 25diCBA levels in soil by 46% (i.e., 211 to 113 mg kg^{-1}). Two plant-bacteria associations and uninoculated Dahurian wild rye also degraded mixtures of 3CBA, 23diCBA or 25diCBA. When 25diCBA was mixed with 23diCBA, uninoculated Dahurian wild rye reduced levels of 25diCBA in soil by 31%, and reduced the levels of both 25diCBA and 3CBA by up to 64% when these two contaminants were present in a mixture. Similarly, meadow brome inoculated with BR60 reduced 23diCBA and 3CBA levels by up to 50%. Levels of all three chlorinated benzoic acids were reduced by 53-63% by Altai wild rye inoculated with strains R75 and CB35. These results indicate that plant-bacteria associations can tolerate and degrade mixtures of contaminants in soil but that predictions about phytoremediation of mixed contaminants may not be straightforward.

5.2. INTRODUCTION

Many different plant species enhance contaminant degradation, and a wide range of contaminants are amenable to phytoremediation. For example, rye-grass (*Lolium perenne L.*) promotes n-alkane degradation (Gunther et al. 1996), rhizosphere soil of *Kochia scoparia* (L.). Roth. enhances atrazine degradation (Perkovich et al. 1996), Dahurian wild rye and streambank wheatgrass promote 2CBA degradation (Siciliano and Germida 1997c) and crested wheatgrass (*Agropyron desertorum* (Fischer ex Link) Schultes) promotes PCP degradation (Ferro et al. 1994). However, there are few details on protocols used to select these grasses. Recently, a basis for the selection of plant species with phytoremediation potential has begun to emerge. For example, Donnelly et al. (1994) found that PCB degrading bacteria, *Alcaligenes eutrophus* H850, *Pseudomonas putida* LB400 and *Corynebacterium* sp. MB1 grew on phenolics found in root exudates. Following up on this, Fletcher and Hedge (1995) screened 17 plants for production of these phenolics and calculated that the rhizosphere of *Morus rubra* L. (mulberry) had phenolic levels (11 μg/ml of water thought to be in the rhizosphere zone) sufficient to support PCB degradation. Similarly, Siciliano and Germida (1997c) postulated that plants and bacteria might form beneficial associations that would degrade toxicants in soil, and developed a screening methodology to identify these associations. This latter screening procedure consisted of 1) assessing the germination of test plants in contaminated soil, and 2) inoculating grasses that grew in contaminated soil with either known plant growth-promoting rhizobacteria or bacteria capable of contaminant degradation.

Chlorinated benzoic acids are common contaminants and arise as byproducts of PCB or chlorinated herbicide degradation (Barriault and Sylvestre 1993; Stratford et al. 1996). For example, Fava et al. (Fava et al. 1996) observed increases in 2CBA and 25diCBA concentrations during 150 hours of PCB degradation by *Pseudomonas* spp. strain CPE1, in a bioreactor. Furthermore, PCB-contaminated

sites will contain mixtures of chlorinated benzoic acids as the PCB mixture present in commercial PCB formulations (e.g. Aroclor™ 1260), degrades (Stratford et al. 1996). The occurrence of contaminant mixtures may pose a problem in site remediation because one toxic component of the mixture may limit degradation of other contaminants. For example, Stratford et al. (1996) found that 2,3-dichlorobenzoic acid (23diCBA) inhibited 2CBA degradation by the bacterium *Burkholderia cepacia* strain JHR22, otherwise capable of 2CBA degradation.

This study was designed to address two questions. Is the selection procedure I developed for plant-bacteria associations capable of degrading 2CBA effective for other chlorinated benzoic acids i.e. 3CBA, 23diCBA or 25diCBA? Are phytoremediation systems developed to degrade a single contaminant, effective when that contaminant is present in a mixture?

5.3. MATERIALS AND METHODS

5.3.1. Soil and Forage Grasses

Surface soil was collected near Outlook, SK, Canada and prepared as described in section 3.3.1. I contaminated the soil as described in section 4.3.1 with the exception that solid 3CBA, 23diCBA or 25diCBA were used instead of 2CBA. Seventeen forage grass species (Table 3.2.) were obtained and stored as described in Section 3.3.2.

5.3.3. Bacterial Seed Inoculants

The following bacterial inoculants were screened for their ability to promote phytoremediation: a 8:1 combination of *Pseudomonas aeruginosa* R75 and CB35 known to promote phytoremediation of 2CBA (Siciliano and Germida 1997c); *Alcaligenes* sp. BR60 known to degrade 3CBA and 3,4-dichlorobenzoate (Nakatsu and Wyndham 1993); *P. aureofaciens* 3732 RN-L11 (*lacZY* Rif^r Nal^r) which was originally isolated from wheat and whose presence in non-sterile soil can be determined through the use of selective media supplemented with 5-bromo-4-chloro-

3-indolyl-β-D-galactopyranoside (X-Gal) (Angle et al. 1995). All bacteria were stored at -40°C in 50% (w/w) glycerol/tryptic soy broth.

To inoculate seed, bacteria were grown for 48 h in 1/10th strength TSB, centrifuged (15 min at 1700 × *g*, 5°C), re-suspended in 10 ml sterile tap water and enumerated on TSA (*c.a.* 10^9 cfu ml^{-1}). Seeds were inoculated in a manner similar to that described by Nijhuis et al. (1993). Cells (0.5 ml) were added to 3 ml of 40 % (w/w) gum arabic and added to seeds (5 g). Inoculated seed (*c.a.* 10^6 cfu $seed^{-1}$) was mixed with five grams $CaCO_3$ and used immediately. For treatments involving only bacteria and no plant, bacteria were inoculated onto autoclaved meadow brome seed (*c.a.* 10^6 cfu $seed^{-1}$) and five seeds added per pot.

5.3.4. Study #1: Selection of Degradative Plant-Bacteria Associations

To select plants tolerant of CBA, 20 seeds of one of the 16 species being screened were added per pot and growth assessed 14 DAP. Seeds of species that grew in contaminated soil were inoculated with either strain BR60, strain 3732 RN-L11 or a combination of strains R75 and CB35, and planted (n=5) in replicate cups (n=5). Cups were placed in a growth chamber with a 16 h day (23°C) and 8 h night (18°C). Illumination was 350 μmol s^{-1} m^{-2}. At 28 DAP, pots were harvested and the levels of CBA in soil determined by HPLC.

5.3.5. Study #2: Effect of Contaminant Mixtures on Phytoremediation

Plant-bacteria associations that successfully remediated 3CBA, 23diCBA or 25diCBA as individual compounds were tested for their effect on combinations of these contaminants. Soil was contaminated with various combinations of 3CBA (400 mg kg^{-1}), 23diCBA (100 mg kg^{-1}) and 25diCBA (100 mg kg^{-1}). Contaminated soil was seeded as described above with the exception that only four replicates were used per treatment and only four plant-bacteria associations were investigated: 1) Dahurian wild rye, 2) Dahurian wild rye + R75&CB35, 3) Altai wild rye + R75&CB35 and 4) meadow brome + BR60. Plants were grown for 28 days and CBA levels

determined by HPLC.

5.3.6. High Pressure Liquid Chromatography

Five grams of soil were placed in a 25 ml erlenmeyer flask amended with 10 mls of pH 9 reverse osmosis water and shaken (140 rpm) on a rotary shaker overnight (22°C). An aliquot (2 ml) was centrifuged (10 min, 10,000 rpm) and the supernatant injected (50 μL) into a HPLC system equipped with a model 510 pump, an autoinjector (Waters 700 Satellite WISP) and a UV detector (Waters 486 Tunable Absorbance Detector) set at 229 nm. The chlorobenzoic acids were separated on a Waters' Nova-Pak C-18 reverse-phase column (15 cm × 3.9 mm i.d.) maintained at 22°C. The mobile phase was a 1:1 mixture of methanol and 1% (v/v) acetic acid and the flow rate was 1 ml min^{-1}. Chromatograms were processed by a Baseline 810 Chromatography Work Station. Under these conditions, 23diCBA eluted at 5.2 minutes, 25diCBA at 6.7 minutes, and 3CBA at 8.2 minutes. Extraction efficiencies for single contaminants ranged from 82% for 25diCBA to 95% for 3CBA. Extraction of chlorinated benzoic acid mixtures had a similar extraction efficiency to single contaminant extraction.

5.3.7. Statistics

Study #1 was repeated three times with five treatment replicates per experiment. Study #2 was repeated twice with four treatment replicates. Data were analyzed by ANOVA and means separated using a protected Student's t-test.

5.4. RESULTS

5.4.1. Study #1: Selection of Degradative Plant-Bacteria Associations

Fourteen of the 17 grasses tested germinated in soil contaminated with 800 mg kg^{-1} 3CBA, but only meadow brome, Dahurian wild rye and Altai wild rye exhibited substantial growth 14 DAP (Table 5.1.). No plants grew in soil contaminated with 800 or 400 mg kg^{-1} 23diCBA or 25diCBA, and only meadow brome and Altai wild rye grew substantially at 200 mg kg^{-1}. Hence, the 200 mg kg^{-1} level was used for the remainder of the study. Eight out of 17 grasses germinated in 25diCBA (200 mg kg^{-1}) contaminated soil but only meadow brome and Altai wild rye

TABLE 5.1. Growth of forage grasses 14 DAP in soil contaminated with CBA.

Common Name	Scientific Name	Plant Growth[a]		
		Contaminant Levels (mg kg^{-1})		
		3CBA (800)	23diCBA (200)	25diCBA (200)
Common brome	*B. inermis*	+	+	+
Dahurian wild rye	*E. dauricus*	+++	+	+
Intermediate wheat grass	*A. intermedium*	-	-	-
Meadow brome	*B. biebersteinii*	+++	+++	+++
Streambank wheat grass	*A. riparium*	+	-	-
Tall wheat grass	*A. elongatum*	+	-	-
Northern wheat grass	*A. dasystachyum*	+	-	-
Reed canary	*P. arundinacea*	+	-	+
Altai wild rye	*E. angitus*	+++	+++	+++
Crested wheat grass	*A. cristatum*	+	-	-
Perennial rye grass	*L. perenne*	++	+	++
Russian wild rye	*E. junceus*	+	+	+
Sheep fescue	*F. ovina*	+	-	-
Slender wheat grass	*A. trachycaulum*	-	-	-
Orchard grass	*D. glomerata*	+	-	+
Canada blue grass	*P. compressa*	-	-	-
Timothy	*P. pratense*	+	-	-

[a] Gradient of visual responses: -, no seed germination; +, seed germination; ++, seed germination with leaf at coleoptile tip; +++, seed germination with first leaf through coleoptile.

grew substantially by 28 DAP. After selection of grasses tolerant of CBA, I determined if the grasses by themselves or in combination with selected bacterial inoculants could decrease chlorinated benzoic acid levels in soil.

Levels of 3CBA in unplanted, non-inoculated soil decreased from 800 mg kg^{-1} to 583 mg kg^{-1} over the 28 day incubation period (Table 5.2.). Surprisingly, non-inoculated Altai wild rye survived in contaminated soil but died when inoculated with strain BR60 or a combination of strains R75 and CB35. Inoculation of Altai wild rye with strain 3732 RN-L11 had no effect on 3CBA levels. In contrast, inoculation of Dahurian wild rye with a combination of strains R75 and CB35 decreased ($P \leq 0.05$) 3CBA levels to 33% that of uninoculated plants or 26% that of control soil. The other inoculants, strain BR60 or strain 3732 RN-L11, had no effect on 3CBA degradation by Dahurian wild rye. Meadow brome, which initially germinated in contaminated soil, died by 28 DAP. Interestingly, strain BR60 which reduced 3CBA levels to 74% that of control in the absence of a plant, had no effect on 2CBA degradation by Dahurian wild rye and caused the death of Altai wild rye and meadow brome. The combination of strains R75 and CB35 had no effect on 3CBA levels in the absence of a plant.

Levels of 23diCBA in unplanted, non-inoculated soil decreased by 38% during the course of the study from 200 mg kg^{-1} to 125 mg kg^{-1} (Table 5.2.). Furthermore, inoculation of Altai wild rye with a combination of strains R75 and CB35 decreased ($P \leq 0.05$) 23diCBA levels to 67% that of uninoculated plants, whereas inoculation with strain 3732 RN-L11 had no effect on 23diCBA levels and strain BR60 caused plant death. Inoculation of meadow brome with strain BR60 decreased ($P \leq 0.05$) 23diCBA levels to 44% that of the uninoculated plant. In contrast, inoculation of meadow brome with the combination of strains R75 and CB35 or strain 3732 RN-L11 caused plant death. Inoculants had no effect on 23diCBA levels in the absence of a plant.

TABLE 5.2. Effect on plant-bacteria associations on 3CBA, 23diCBA or 25diCBA levels 28 DAP.

Treatment		Contaminant (mg kg^{-1})		
Plant	Inoculant[a]	3CBA (800)	23diCBA (200)	25diCBA (200)
Control	None	583	125	211
Altai wild rye	None	547	140	171
Altai wild rye	R75 and CB35	ND[b]	94	113
Altai wild rye	3732 RN-L11	630	132	225
Altai wild rye	BR60	ND	ND	ND
Dahurian wild rye	None	450	ND	ND
Dahurian wild rye	R75 and CB35	149	ND	ND
Dahurian wild rye	3732 RN-L11	590	ND	ND
Dahurian wild rye	BR60	572	ND	ND
Meadow brome	None	ND	125	237
Meadow brome	R75 and CB35	ND	ND	192
Meadow brome	3732 RN-L11	ND	ND	ND
Meadow brome	BR60	ND	55	ND
None	R75 and CB35	568	151	160
None	3732 RN-L11	447	163	231
None	BR60	430	117	201
LSD (0.05)		175	42	49

[a] Forty eight hour cultures (*c.a.* 10^9 cfu $seed^{-1}$) of strain R75 and CB35, *P. aureofaciens* strain 3732 RN-L11 or *Alcaligenes* sp. BR60 were inoculated onto seeds (*c.a.* 10^6 cfu $seed^{-1}$). For inoculants without plants, seed was first autoclaved, inoculated and planted in soil (10^4 cfu g^{-1} of soil).

[b] ND, contaminant level not determined because plants died over course of experiment.

Unlike 3CBA or 23diCBA, incubating soil for 28 days had no effect on 25diCBA levels. Altai wild rye inoculated with a combination of strains R75 and CB35 decreased ($P \leq 0.05$) 25diCBA levels to 66% that of the uninoculated plant. Inoculation with strain BR60 caused plant death and strain 3732 RN-L11 had no effect on 25diCBA levels. A combination of strains R75 and CB35 inoculated onto meadow brome had no effect on 25diCBA levels, and inoculation with strains 3732 RN-L11 or BR60 resulted in plant death. A combination of strains R75 and CB35 in the absence of plants decreased ($P \leq 0.05$) 25diCBA levels to 76% that of control soil.

5.4.2. Study #2: Effect of Mixtures on Phytoremediation

A mixture of 23diCBA (100 mg kg^{-1}) and 25diCBA (100 mg kg^{-1}) did not degrade in unplanted control soil by 28 DAP (Fig. 5.1.A.). Dahurian wild rye decreased ($P \leq 0.05$) 25diCBA levels to 69% that of control soil but had no effect on 23diCBA levels. Inoculation of Dahurian wild rye with a combination of strains R75 and CB35 caused plant death. Altai wild rye inoculated with strains R75 and CB35 had no effect on a mixture of these contaminants. Meadow brome inoculated with strain BR60 survived in soil contaminated with a mixture of 23diCBA and 25diCBA (100 mg kg^{-1} each) but had no effect on CBA levels.

The combination of 25diCBA and 3CBA was lethal to most plant bacteria associations and no 25diCBA or 3CBA degraded in soil over a 28 day period (Fig. 5.1.B.). Only uninoculated Dahurian wild rye survived in this soil, and it decreased ($P \leq 0.05$) 25diCBA levels to 51% and 3CBA to 36% that of control soil.

In a mixture of 23diCBA and 3CBA, levels of 3CBA in control soil were reduced from an initial 400 mg kg^{-1} to 190 mg kg^{-1} by 28 DAP but no 23diCBA was degraded (Fig. 5.1.C.). This mixture was only lethal to uninoculated Dahurian wild rye. Inoculation of Dahurian wild rye with strains R75 and CB35 allowed Dahurian wild rye to survive in contaminated soil, but had no effect on CBA levels. Altai wild rye inoculated with strains R75 and CB35 decreased 3CBA levels to 48% but had no

effect on 23diCBA levels. The most successful treatment was Meadow brome inoculated with strain BR60 which decreased levels of 23diCBA to 61% and 3CBA to 50% that of control soil.

In a mixture of 23diCBA, 25diCBA and 3CBA no degradation of CBA was observed in control soil (Fig. 5.1.D.). This mixture was lethal to inoculated and non-inoculated Dahurian wild rye. Altai wild rye inoculated with strains R75 and CB35 decreased levels of 23diCBA to 47%, 25diCBA to 42% and 3CBA to 37% that of control soil. Inoculation of meadow brome with strain BR60 had no effect on CBA levels.

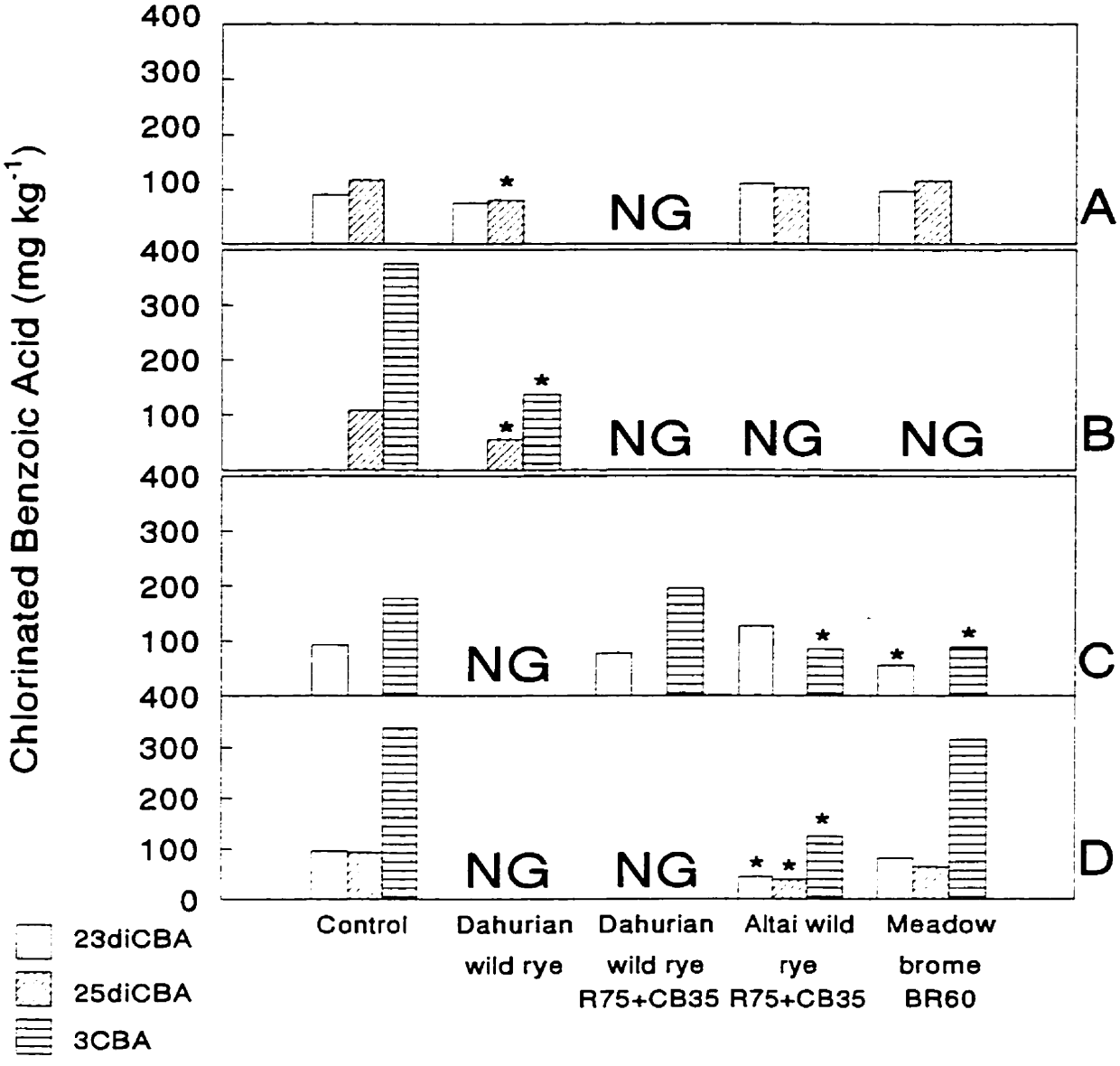

Fig. 5.1. Effect of contaminant mixtures on the survival and degradative activity of plant-bacteria associations 28 days after planting. (A); 23diCBA plus 25diCBA, (B); 25diCBA plus 3CBA, (C); 23diCBA plus 3CBA, (D); 23diCBA plus 25diCBA plus 3CBA. The symbol NG indicates that plants were dead by 28 days after planting and contaminant levels were not determined. Di-chlorinated benzoic acids were initially added at levels of 100 mg kg^{-1} and 3CBA at 400 mg kg^{-1}. Each bar is the average of two experiments with four replicates each. Bars significantly different than control are marked with; * $P \leq 0.10$, ** $P \leq 0.05$ or *** $P \leq 0.01$.

5.5. DISCUSSION

Wackett and Allan (1995) noted the difficulty in developing plant systems able to degrade contaminants in soil due to the wide range of possible plant-microbe-soil interactions. They suggested that a directed research agenda should be developed aimed at identifying specific plant-microbe associations that degrade contaminants in soil. In this study I evaluated a screening procedure for identifying plant bacteria associations and successfully isolated three associations that degraded three different contaminants: Dahurian wild rye inoculated with strains R75 and CB35 degraded 3CBA, meadow brome inoculated with strain BR60 degraded 23diCBA and Altai wild rye inoculated with strains R75 and CB35 degraded 25diCBA. Furthermore, in a previous study I isolated two plant bacteria associations that degraded 2CBA in soil (Siciliano and Germida 1997c). Hence, it appears that the selection procedure developed by Siciliano and Germida (1997c) is effective in identifying plant-bacteria associations that degrade CBA in soil. Several investigators have suggested that specific plant-bacteria associations are required to reduce levels of contaminants in soil (Crowley et al. 1996; Pfender 1996; Siciliano and Germida, 1995), and my present study supports this view. Some plants grew in contaminated soil but did not degrade the contaminant. For example, Altai wild rye grew in 25diCBA contaminated soil but had no effect on 25diCBA levels, and meadow brome grew in 23diCBA contaminated soil with no effect on 23diCBA levels. Similarly, Alvey and Crowley (1996) found that corn grew in atrazine-contaminated soil but had no effect on atrazine levels, and Pfender (1996) found that millet grew in PCP contaminated soil but had no effect on PCP levels. In my present study only inoculated plants reduced CBA levels in soil. However, unlike studies by Alvey and Crowley (1996) or Pfender (1996), my inoculants did not significantly enhance degradation in the absence of a plant. This suggests that specific plant-bacteria associations that reduce CBA levels in soil were formed during phytoremediation.

It is important to note that my results are limited to the readily extractable portion of CBA and can not differentiate between sequestration and degradation.

However, since some plants grew in contaminated soil but had no effect on the readily extractable levels of contaminants, any sequestration that occurred would be plant specific. In addition, it is well known that the toxicity of contaminants is related to their bioavailability (Alexander 1995; Hrudey and Pollard 1993) and thus, technologies that reduce levels of readily extractable contaminants may have a role to play during remedial actions.

How and why degradative plant-bacteria associations form is still in question. Pfender (1996) suggested that bacterial inoculants might protect plants from toxicants in soil, whereas Crowley et al. (1996) suggested that plants provide a niche for bacteria to maintain their degradative plasmids. In my study inoculants did not reduce CBA phytotoxicity and many inoculants caused plant death in contaminated soil. This may be related to a toxic intermediate being produced in the degradative pathway of chlorinated compounds as noted by Barriault and Sylvestre (1993), or production of a bacterial toxin in response to contamination. Siciliano and Germida (1995) found that growth of certain plant-bacteria associations in soil resulted in soil that was phytotoxic to other plants. Other investigators have also suggested that bacterial toxin production may be the cause of inoculant death in soil microcosms (Havel and Reineke 1992). On the other hand, plants may provide a specific niche for bacterial inoculants and conversely, inoculants may require specific plants to reduce contaminant levels in soil. For example, Dahurian wild rye inoculated with strains R75 and CB35 had no effect on the soil's potential to degrade 25diCBA (Siciliano and Germida, unpublished results) and in this study, inoculated Dahurian wild rye did not grow in 25diCBA contaminated soil. Furthermore, this inoculant had no effect on 25diCBA in the absence of a plant. However when inoculated onto Altai wild rye, the R75 and CB35 inoculant reduced 25diCBA levels. Hence, it appears that Altai wild rye provides an environment suitable for the degradation of 25diCBA by strains R75 and CB35. Similarly, strain BR60 in association with meadow brome significantly reduced 23diCBA levels but was not effective in the absence of a plant, and caused the death of other plant species.

Some plants appear to have intrinsic bioremediation activity, whereas others require bacterial inoculants to reduce contaminant levels in soil. In this study, Dahurian wild rye decreased 3CBA to 77% of control soil and inoculation with strains R75 and CB35 decreased 3CBA levels to 26% that of control soil. Similarly, I previously noted that Dahurian wild rye reduced 2CBA levels in soil and inoculation increased this degradation (Siciliano and Germida 1997c). Other investigators have found that bean (*Phaseolus vulgaris*), rye-grass and crested wheatgrass degraded contaminants in soil without being inoculated (Gunther et al. 1996; Crowley et al. 1996; Pfender 1996). The intrinsic bioremediation activity may be due to enzyme production as suggested by Schnoor et al. (1995), root peroxidases as described by Adler (1994) or plant uptake of toxicants. In contrast, meadow brome had no effect on 23diCBA levels, but upon inoculation with BR60 the 23diCBA levels were reduced to 44% that of control soil. Similarly, Pfender (1996) found that millet had no effect on PCP levels in soil until inoculated with a PCP degrading bacterium. This suggests some sort of niche creation by the plant for the inoculum as postulated by Crowley et al. (1996). Supporting this, Hedge and Fletcher (1996) found that phenols released by plants can support the growth of PCB degrading bacteria and suggested that phenol provision via root exudates may allow the development and maintenance of PCB degrading communities in rhizosphere soil.

In this study, plant-bacteria associations developed for degrading a specific compound such as 3CBA, rarely degraded this compound when it was present in a mixture of other CBA. Other contaminants present in the mixture may have been phytotoxic or inhibited catabolic activity. For example, Dahurian wild rye inoculated with strains R75 and CB35 significantly reduced 3CBA levels when it was present as a single contaminant but did not survive most contaminant mixtures, and when it did so had no effect on 3CBA levels in soil. I found that 2CBA was toxic to a wide range of grasses (Siciliano et al. 1997) and that 23diCBA, 25diCBA and 3CBA were also toxic (Siciliano and Germida, unpublished results). Despite this phytotoxicity, some plant-bacteria associations grew in contaminated soil but had no effect on contaminant levels.

For example, inoculated Altai wild rye and meadow brome survived a mixture of 23diCBA and 25diCBA but had no effect on CBA levels in soil. This may be because the degradation of CBA was lethal to the bacterial inoculant as noted by Havel and Reineke (1992) or interfered with the catabolic activity of bacteria (Barriault and Sylvestre 1993; Stratford et al. 1996). Understanding the effect of mixtures on inoculant strain surival will require further empirical work.

The interaction between mixtures of contaminants and plant-bacteria associations needs further investigation. For example, meadow brome inoculated with BR60 reduced levels of 23diCBA only when 25diCBA was not present in the contaminant mixture. In the absence of 23diCBA, this plant-bacteria association did not survive 3CBA contaminated soil. Hence, it appears that 23diCBA protected meadow brome plus BR60 from 3CBA toxicity, and 25diCBA prevented this association from degrading other CBA. Similarly, Altai wild rye inoculated with R75 and CB35 survived in the presence of 23diCBA and CBA levels were reduced only in the presence of 3CBA. The ability to isolate plant-bacteria associations that degrade contaminants in soil is an important first step in developing a phytoremediation technology. However, the inability to predict phytoremediation activity in contaminant mixtures may hamper efforts to implement phytoremediation technologies in the field.

6. ENHANCED PHYTOREMEDIATION OF CHLOROBENZOATES IN RHIZOSPHERE SOIL.

6.1. ABSTRACT

The use of plants to detoxify contaminated soil sites has the potential to be a cost effective alternative to traditional remediation technologies. However, plant-bacteria interactions in contaminated soils are not well understood. In this study I investigated the effect of bacterial seed inoculants on the rhizosphere community during the reduction of 2-chlorobenzoic acid (2CBA) levels by Dahurian wild rye (*Elymus dauricus*). Soil was amended with 450 mg kg^{-1} 2CBA and stored for 2 years, at which time the detectable 2CBA level was 61 mg kg^{-1}. Dahurian wild rye inoculated with either *Pseudomonas aeruginosa* strain R75, *P. savastanoi* strain CB35 or a 1:1 mixture of these bacteria was grown in contaminated soil for 56 days in a growth chamber. The potential of rhizosphere soil to degrade 3-chlorobenzoic acid (3CBA), a contaminant with a similar bacterial degradation pathway to 2CBA, versus 2,3-dichlorobenzoic acid (23diCBA) or 2,5-dichlorobenzoic acid (25diCBA), contaminants with bacterial degradation pathways dissimilar to 2CBA, was also assessed. Inoculating Dahurian wild rye with the mixed inoculum decreased the extractable 2CBA from 61 mg kg^{-1} to 29 mg kg^{-1}, 56 days after planting but had no effect on plant growth. Inoculating Dahurian wild rye with a mixture of strains R75 and CB35 increased the potential of rhizosphere microorganisms to reduce 3CBA levels by 17% but had no effect on levels of 23diCBA or 25diCBA. In a sterile hydroponic plant growth system, inoculation of Dahurian wild rye had no effect on 2CBA levels; although, the inoculum became established and grew in the hydroponic solution indicating that inoculants required an unknown soil factor to degrade 2CBA. Bacterial seed inoculants selectively enhanced the potential of the rhizosphere community to degrade certain compounds without affecting heterotrophic

bacterial populations.

6.2. INTRODUCTION

Phytoremediation uses plants and their associated microbial communities to degrade, sequester or immobilize toxicants in soil. It is well known that microbes in rhizosphere soil are capable of degrading a variety of contaminants (Alvey and Crowley 1996; Günther et al., 1996; Perkovich et al., 1996). Furthermore, inoculation of plants with selected bacteria can increase phytoremediation activity. Siciliano and Germida (1997) found that certain plants decreased 2-chlorobenzoic acid (2CBA) levels in soil, and that inoculation of these plants with specific bacterial seed inoculants increased 2CBA degradation. For example, growth of Dahurian wild rye (*Elymus dauricus*) increased 2CBA degradation by 200% compared to bulk soil, and 2CBA levels were further reduced by inoculating seeds with *Pseudomonas aeruginosa* strain R75, *P. savastanoi* strain CB35 or a mixture of these two strains. Similarly, Crowley et al. (1996) found that inoculation of bean (*Phaseolus vulgaris*) with *P. fluorescens* 2-79 RLD increased 2,5-dichlorobenzoic acid (25diCBA) degradation. Furthermore, inoculation of corn with a consortium of *Clavibacter michiganese, Pseudomonas* sp., and *Cytophaga* sp. increased atrazine mineralization (Alvey and Crowley 1996).

Ecological interactions between plants, indigenous bacteria and inoculated bacteria that increase degradation in contaminated soil are not completely understood. Inoculants may increase phytoremediation activity by enhancing the "rhizosphere effect" (Siciliano and Germida, 1997). This effect, *i.e.* a non-specific increase in microbial numbers and activity due to nutrient release by plants, has been postulated by a number of researchers to be a possible mechanism in phytoremediation systems (Haby and Crowley, 1996; Günther et al., 1996). Bacterial seed inoculants are known to increase root biomass, length or exudation (Höflich et al., 1995; Schippers et al., 1995), any of which might increase the "rhizosphere effect". Alternatively, bacterial inoculants might selectively increase the capacity of the rhizosphere community to degrade contaminants

(Siciliano and Germida, 1997). For example, Pfender (1996) found that inoculating millet (*Panicum miliaceium* L.), sensitive to pentachlorophenol (PCP), with a PCP degrading pseudomonad reduced the phytotoxicity of PCP to millet and thereby allowed millet to grow in PCP contaminated soil.

The purpose of this study was to determine if bacterial inoculants increased phytoremediation of 2CBA by either enhancing the "rhizosphere effect", the catabolic capacity of the rhizosphere community or both simultaneously. To differentiate between these two processes, degradation of 3-chlorobenzoic acid (3CBA), 2,3-dichlorobenzoic (23diCBA) or 2,5-dichlorobenzoic acid (25diCBA) after the soil had been subjected to phytoremediation was assessed. Microbial degradation of 2CBA via the 1,6-dioxygenase pathway produces 3-chlorocatechol which is also an intermediate in the 3CBA degradation pathway. Hence, 2CBA and 3CBA often can be degraded by similar organisms. In contrast, degradation of 23diCBA and 25diCBA produces 4-chlorocatechol which is not involved in the 2CBA degradation pathway because 1,2-dioxygenation of 2CBA results in the spontaneous dechlorination and decarboxylation of 2CBA resulting in catechol (Hickey and Focht, 1990). Thus, if strain CB35 enhanced the catabolic capacity of the rhizosphere in a selective manner, I would expect to see increased degradation of 3CBA but not 23diCBA or 25diCBA due to the different catabolic pathways involved. Alternatively, if strain CB35 enhanced the rhizosphere effect in a non-selective manner, I would expect to see enhanced degradation of all three contaminants. Further, I used hydroponic systems to determine if the inoculants required the presence of other soil microorganisms or abiotic factors to increase the reduction in 2CBA levels by plants. The use of sterile hydroponic systems allowed us to differentiate between plant mediated 2CBA degradation and those processes requiring introduced or indigenous microorganisms.

6.3. METHODS

6.3.1. Soil

Outlook soil initially contaminated during the study described in Section 4.3.1., was used in this study. At the end of the study described in Section 3, all contaminated soil was bulked together, allowed to stand in pots for one week and stored moist *c.a.* 30% moisture holding capacity, in metal cans for two years. For the present study, the stored soil was thoroughly mixed and the residual extractable 2CBA level determined on six sub-samples (see below) was 63 mg kg^{-1} with a standard deviation of 5.1.

6.3.2. Forage grasses and bacterial inoculants

The plant-bacteria combinations of Dahurian wild rye-*Pseudomonas aeruginosa* strain R75, Dahurian wild rye-*P. savastanoi* strain CB35 and Dahurian wild rye-strains R75+CB35 were used in this study. Bacteria were stored and inoculated onto seeds as described in Section 4.3.2. For treatments involving only inoculants and no plants, bacteria were inoculated onto autoclaved Dahurian wild rye seed.

6.3.3. Effect of plant bacteria associations on 2CBA levels in soil

Initially, I investigated the degradation of "aged" 2CBA in soil by Dahurian wild rye-bacterial inoculant associations. Seeds (n=5) inoculated with strain R75 (*ca.* 10^6 cfu $seed^{-1}$), strain CB35 (*ca.* 10^6 cfu $seed^{-1}$) or a mixture of strains R75 and CB35 (*ca.* 10^7 combined cfu $seed^{-1}$) were planted in replicate cups (n=5) containing 160 g of contaminated or uncontaminated soil maintained at -0.33 kPa moisture capacity. Cups were placed in a growth chamber with a 16 h day (23°C) and 8 h night (18°C). Illumination was 350 μmol s^{-1} m^{-2}. Replicate samples (n=5) for each treatment were harvested at 14, 21, 28, 35, 49 and 56 days after planting (DAP). At harvest, the 2CBA level in soil was determined by high pressure liquid chromatography (HPLC).

The effect of bacterial seed inoculants on plant growth parameters and the soil's heterotrophic bacterial population was determined. Shoot and root dry weight were

determined by standard procedures. A sub-sample (0.5 g) of the root system was rehydrated for 2 hours, stained with 1% (w/w) crystal violet for 24 hours and root length determined by computer image analyses (Farrell et al., 1993). Sub-samples of soil collected from pots were frozen at -20°C and the heterotrophic soil bacteria population assessed on 1/10 TSB solidified with 1.5% agar (TSA) plates.

The effect of bioremediation treatments on the rhizosphere soil's potential to degrade mono-chlorinated benzoic acids *i.e.* 3-chlorobenzoic acid (3CBA) and di-chlorinated benzoic acids *i.e.* 2,3-dichlorobenzoic acid (23diCBA) and 2,5-dichlorobenzoic acid (25diCBA) was determined. Soil (5 g) from cups harvested in the growth chamber study was placed into a 20 ml scintillation vial (n=3) and amended with 1 ml of a 400 mg L^{-1} solution of 3CBA, 23diCBA or 25diCBA for a final soil concentration of 80 mg kg^{-1}. Mercuric chloride was added (0.5ml of 3% $HgCl_2$) to a replicate soil sample as an abiotic control. The vials were placed in a plastic bag and incubated for 7 days at 22°C. The level of 3CBA, 23diCBA or 25diCBA in soil was determined by HPLC.

6.3.4. Hydroponic experiments

The ability of Dahurian wild rye and the bacterial inoculants to reduce 2CBA levels in hydroponic solution was assessed. The system was similar to that described by van Overbeek and van Elsas (1995), and consisted of a 250 × 20 mm glass tube containing 20 mls of M9 growth medium and 2.5 grams of dried, washed perlite. Dahurian wild rye was used as a positive phytoremediation plant and Meadow brome (*Bromus biebersteinii*) was used as a negative control unable to degrade low levels *i.e.*, 200 mg kg^{-1}, of 2CBA in soil (see Chapter 3). Seed was surface sterilized by a 15 minute wash in 2.5% (w/v) sodium hypochlorite containing a drop of Tween 20 and rinsed twice with sterile water. Bacteria were grown for 48 hours in TSB, centrifuged (15 min at 1700 × *g*, 5°C), re-suspended in 10 mls sterile, tap water and enumerated on TSA (*ca.* 10^9 cfu ml^{-1}). The inoculation procedure was similar to that used by Nijhuis et al. (1993). Cells (0.5 ml) were added to 3 ml of 40% (w/w) gum arabic and

added to surface sterilized seeds (5 g). Inoculated seeds (*ca.* 10^6 cfu seed^{-1} enumerated on TSA) were mixed with $CaCO_3$ (5 g) and used immediately. One seed was planted per tube, covered with sterile perlite (0.5 g) and allowed to grow for 21 days with a 16 h day (23°C), 8 h night (18°C) and 350 μmol s^{-1} m^{-2} illumination. The 2CBA level in hydroponic solution was determined as described in Section 5.3.6.

Bacteria were enumerated on two different media. The heterotrophic bacteria in the hydroponic solution were enumerated on TSA. Strains R75 and CB35 were enumerated on a selective medium (TSA+AB) composed of TSA supplemented with the antibiotics (Sigma), 75 mg Chloramphenicol L^{-1}, 75 mg Novobiocin L^{-1}, 5 mg Vancomycin L^{-1} and 100 mg Cycloheximide L^{-1}. The efficiency and selectivity of this medium were determined by spread plating known concentrations of pure cultures of R75 and CB35 onto both TSA and the selective medium. In addition, soil slurries from three different soils, Outlook (Sandy Loam), Paddockwood (Loam) and Allan (Clay Loam) were plated out onto TSA and TSA+AB.

6.3.5. Statistical Analysis

The growth chamber experiment was designed as a 2(plant) × 4(inoculant) × 6(sampling time) factorial experiment with sampling times designated a random factor. Tukey's honestly significant difference method was used for unplanned comparisons. The rate of 2CBA degradation of inoculated and non-inoculated Dahurian wild rye was determined by linear regression (Cohort Solutions, CA) and significance tests performed using the parallel line approach detailed by Mead et al. (1993). The Pearson correlation between percent 2CBA degraded and the potential of the soil to degrade other compounds was determined using CoStat's correlation program (Cohort Solutions, CA). The hydroponic experiment was designed as a 3(plant) × 2(inoculant) factorial repeated twice.

6.4. RESULTS

6.4.1. Reduction of "aged" 2CBA levels in soil by plant-bacteria associations

Growth of Dahurian wild rye decreased ($p \leq 0.05$) 2CBA levels by 36% compared to non-planted soil (Fig. 6.1.). Furthermore, inoculating Dahurian wild rye with the mixed inoculant increased ($p \leq 0.05$) degradation by 26% compared to the non-inoculated plant. Inoculating Dahurian wild rye with the individual inoculants R75 or CB35 decreased 2CBA levels only slightly *ca.* 10% compared to the uninoculated plant and *ca.* 44% compared to control soil. Inoculating soil with bacterial inoculants in the absence of Dahurian wild rye reduced ($p \leq 0.05$) 2CBA levels by *ca.* 18% compared to control soil.

The reduction of 2CBA levels by plant-inoculant treatments was strongly dependent ($p \leq 0.003$) on time. For example, 2CBA levels remained unchanged in unplanted, uninoculated soil, whereas the growth of Dahurian wild rye decreased 2CBA levels by 0.68 mg 2CBA day^{-1} ($r^2=0.85$; $p \leq 0.01$). Inoculation of Dahurian wild rye with strain R75 increased ($p \leq 0.05$) the rate of 2CBA degradation to 1.13 mg 2CBA day^{-1} ($r^2=0.98$; $p \leq 0.01$). Inoculation with strain CB35 or a mixture of strains R75 and CB35 reduced 2CBA levels by 0.75 ($r^2=0.70$; $p \leq 0.02$) and 0.78 mg 2CBA day^{-1} ($r^2=0.86$; $p \leq 0.01$), respectively.

The bacterial seed inoculants had no effect on plant parameters during the course of the experiment (Fig. 6.2.). The R75 inoculated plants tended to have greater shoot mass and root length compared to the uninoculated control. By 35 days after planting, root mass and length appeared to plateau and shoot mass only increased for plants inoculated with R75. Similarly, inoculants had little effect on plant growth in non-contaminated soil. Plant growth in non-contaminated soil was similar to that seen in contaminated soil, with shoot mass approximately 12% greater and root length 30% longer in non-contaminated compared to contaminated soil.

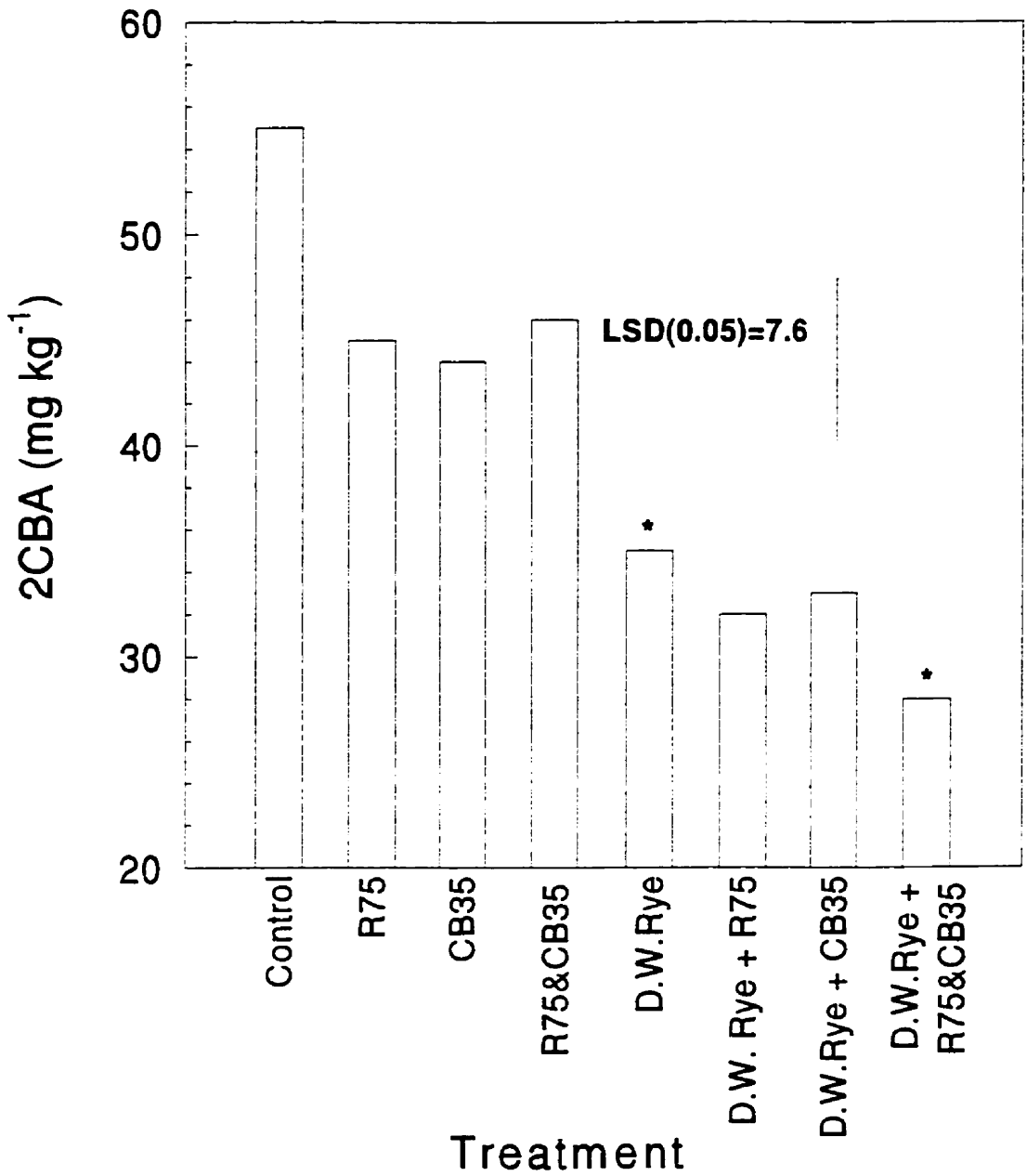

Fig. 6.1. Reduction of 2CBA levels in soil by Dahurian wild rye, bacterial inoculants (10^4 cfu g^{-1}) and Dahurian wild rye inoculated with bacteria (10^6 cfu $seed^{-1}$) at 56 days. Dahurian wild rye was inoculated with *P. aeruginosa* strain R75, *P. savastanoi* strain CB35 or an equal mixture of these two bacteria. Bars are the average of 30 samples taken over a 56 day period. Bars significantly ($p \leq 0.05$) different than the control treatment are marked with an *.

The effect of inoculants on the potential of rhizosphere microorganisms to degrade contaminants other than 3CBA was determined. In non-planted, uninoculated soil (*i.e.*, not subjected to a bioremediation treatment), only 53% of added 3CBA remained after seven days of incubation (Table 6.1.). 3-chlorobenzoic acid degradation was higher ($p \leq 0.05$) in soils in which Dahurian wild rye had grown (43% remaining) compared to non-planted treatments (53% remaining). Further, inoculating Dahurian wild rye with R75 and CB35 increased ($p \leq 0.05$) 3CBA degradation (26% remaining) in soil subjected to bioremediation treatments. Non-planted, uninoculated soil reduced 23diCBA to 38% and 25diCBA levels to 50% of the initial amount added. However, no phytoremediation treatments significantly increased 23diCBA or 25diCBA degradation beyond this level. In fact some treatments inhibited degradation with

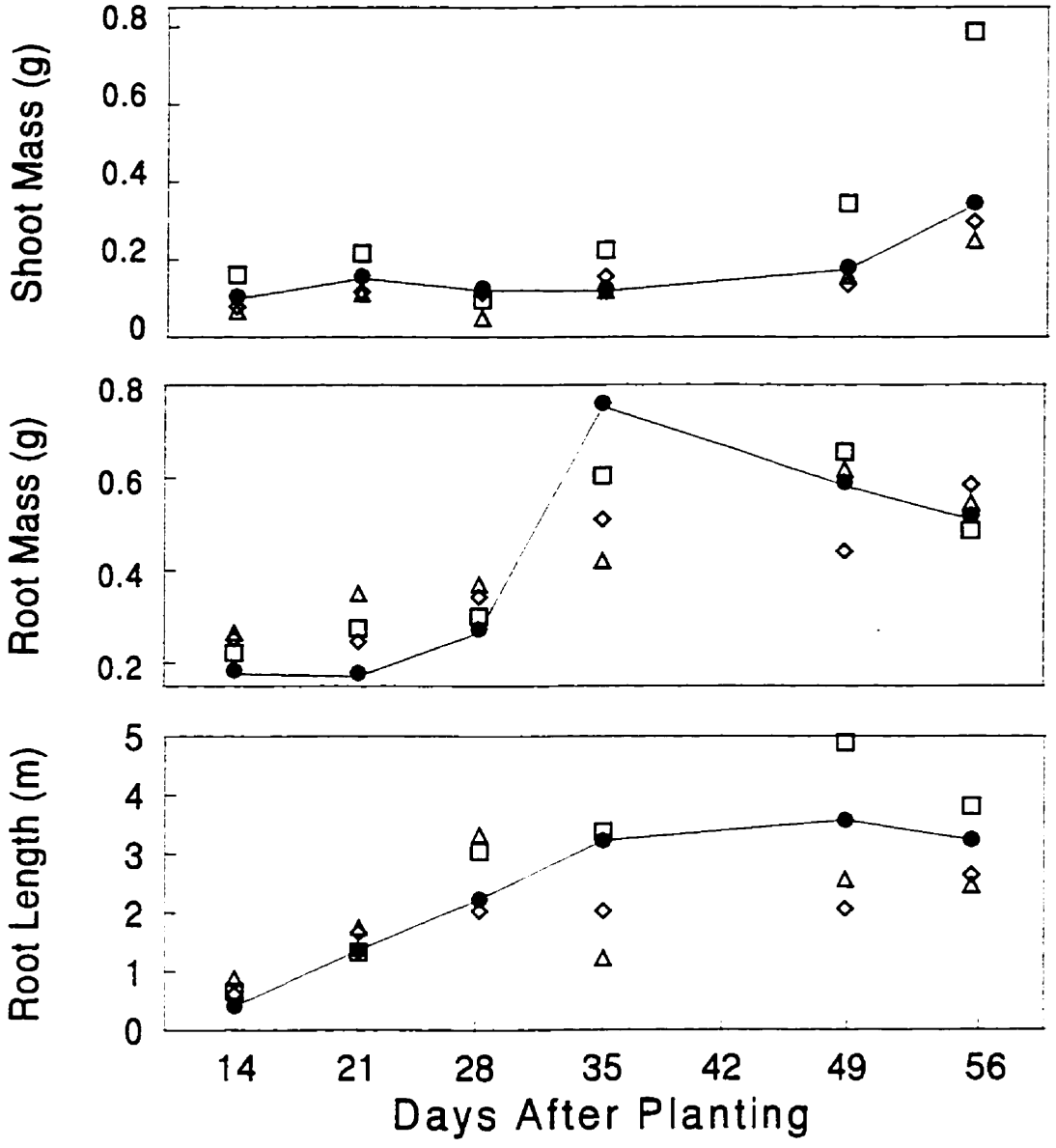

Fig. 6.2. Shoot and root biomass, and root length of inoculated Dahurian wild rye. Dahurian wild rye was inoculated (10^6 cfu seed^{-1}) with *P. aeruginosa* strain R75, *P. savastanoi* strain CB35 or an equal mixture of these two bacteria over 56 days. ● uninoculated Dahurian wild rye, □ Dahurian wild rye + strain R75, ◇ Dahurian wild rye + strain CB35, ▵ Dahurian wild rye + strains R75 and CB36. Points are the average of five pots and standard error for shoot mass was 0.053 g, root mass 0.15 g and root length 0.94 m.

inoculation of Dahurian wild rye with strain CB35 decreasing ($p \leq 0.05$) 25diCBA degradation (76% remaining) compared to control soil (50% remaining). Additionally, there was no effect of any inoculants on colony forming units (cfu) of total heterotrophs in soil which ranged from a high 1×10^7 cfu g^{-1} soil for the uninoculated plant to a low of 6×10^6 cfu g^{-1} soil for the strain R75 inoculated plant.

6.4.2. Hydroponic Studies

Dahurian wild rye and its associated microorganisms significantly ($p \leq 0.05$) reduced 2CBA levels in a hydroponic system whereas Meadow brome did not (Fig. 6.3.). Bacterial inoculation of plants resulted in the establishment of the inoculum in the growth solution, but only in the case of Meadow brome did this decrease 2CBA levels. Survival of the inoculum was increased ($p \leq 0.05$) by 13-fold in planted treatments compared to the unplanted treatment. Although the hydroponic solutions were not sterile, the inoculant dominated the microbial populations, comprising between 92 to 98% of the total bacterial population. The results of soil slurries indicated that the TSA+AB medium was selective for strains R75 and CB35 while inhibiting growth of bacteria from all three soil slurries.

TABLE 6.1. Degradation of other chlorinated benzoic acids in soils previous exposed to phytoremediation treatment.

Previous Treatment[a]	% Remaining after 7 days		
	3CBA[b]	23diCBA	25diCBA
Mercuric chloride (0.15%)	82	95	89
No plant, no inoculant	53	38	50
Strain R75	38*	49	63
Strain CB35	41*	41	48
Strains R75 + CB35	30*	38	61
D.W.Rye	43	50	53
D.W.Rye + strain R75	35*	37	48
D.W.Rye + strain CB35	36*	46	76
D.W.Rye + strains R75 + CB35	26*	44	55
LSD (0.05)	6	14	19

[a] Soil subjected to various bioremediation treatments was sampled (n=3) at 14, 21, 28, 35, 49 and 56 days after planting and incubated with 80 mg kg^{-1} contaminant for 7 days.
[b] 3CBA, 3-chlorobenzoic acid; 23diCBA, 2,3-dichlorobenzoic acid; 25diCBA, 2,5-dichlorobenzoic acid.
* indicates treatment significantly ($p \leq 0.05$) less than the control treatment.

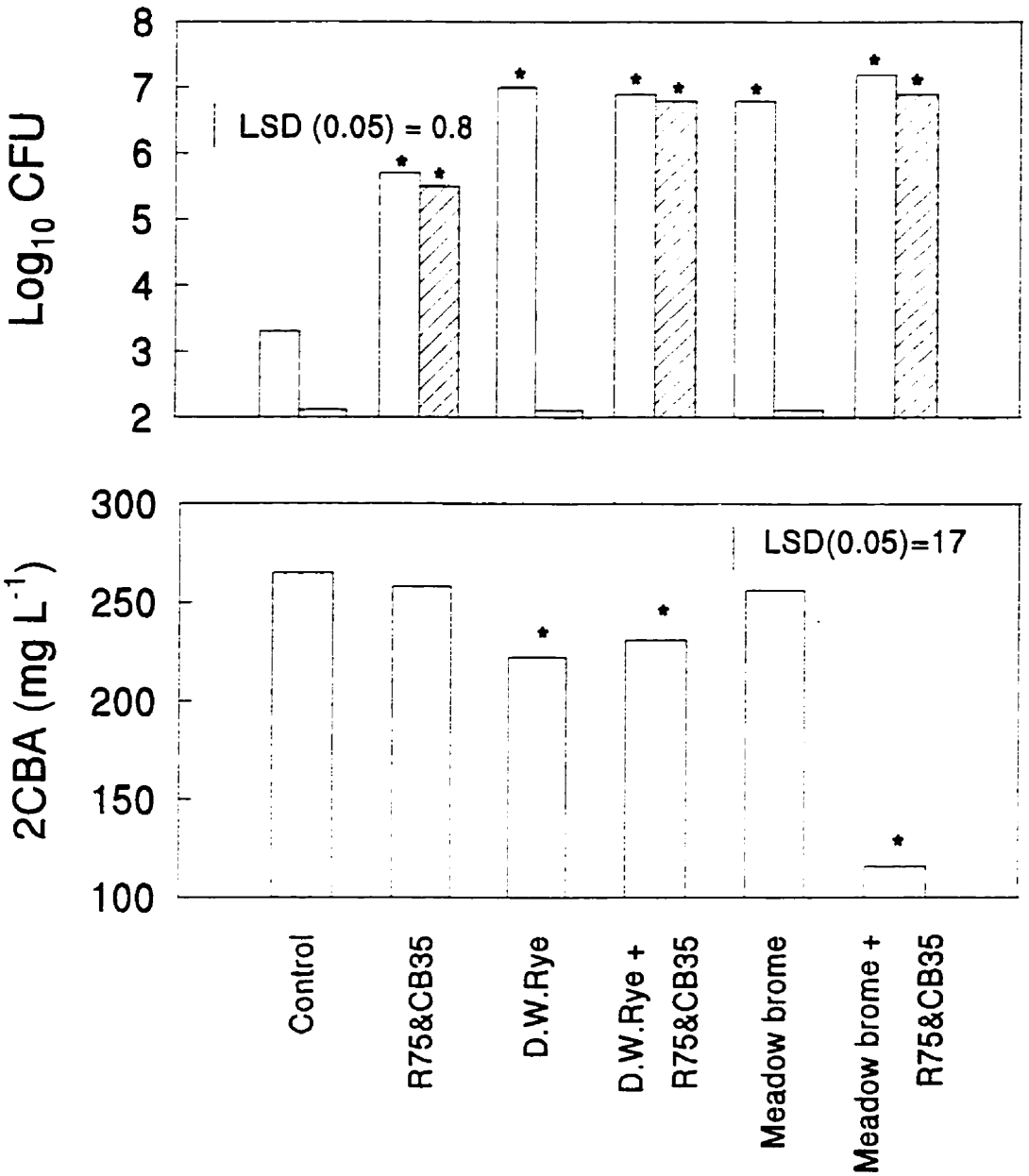

Fig. 6.3. Bacterial population numbers and 2CBA levels in a hydroponic phytoremediation system 21 days after planting. Dahurian wild rye and Meadow brome were inoculated with an equal mixture of *P. aeruginosa* strain R75 and *P. savastanoi* strain CB35. Open bars are colony forming units (cfu)/ml on non-selective media and hatched bars are cfu/ml on media selective for inoculant strains. Bars significantly ($p \leq 0.05$) less than the control treatment are marked with an *.

6.5. DISCUSSION

Previous studies indicated that 2CBA levels were reduced in uninoculated, non-planted soil with freshly added 2CBA at initial soil contamination levels ranging from 200 to 800 mg kg^{-1} (Siciliano and Germida, 1997a). Furthermore, growth of Dahurian wild rye enhanced this 2CBA degradation, and inoculating Dahurian wild rye further increased this effect. In the present study, growing Dahurian wild rye in soil decreased already low levels (*c.a.* 63 mg kg^{-1}) of 2CBA that had been present in soil for over two years. In contrast to my previous results, 2CBA levels remained unchanged in uninoculated, non-planted soil. This suggests that the contaminant in this study was in the asymptotic region of degradation, as noted for other compounds present in the soil for extended periods of time (Beck et al., 1996). As contaminants reside in soil, they move of the chemical into soil organic matter or nanopores (Alexander, 1995). The degradation and toxicity of these contaminants are known to be different compared to contaminants freshly added to soil and are often more recalcitrant (Alexander, 1995). To the best of my knowledge, this is the first report of the phytoremediation of contaminants present in soil for over a year.

It is important to note that my results are limited to the readily extractable portion of 2CBA and can not differentiate between plant uptake, soil sequestration and degradation. Previous studies have indicated that not all bacterial inoculants (2 out of 12) stimulate degradation by Dahurian wild rye (Siciliano and Germida, 1997). Hence, if plant uptake is a major route of 2CBA degradation, bacterial inoculants appear to play a role in this process. In addition, different inoculants reduced 2CBA levels to a differing extent with the same plant species, suggesting that any soil sequestration reactions are likely a specific effect of the plant rhizosphere inoculated with certain bacteria. Furthermore, it is well known that the toxicity of contaminants is related to their bioavailability (Alexander, 1995; Hrudey and Pollard, 1993), and thus remediation technologies that reduce levels of readily extractable contaminants may have a role to play during the remediation of contaminated soil, regardless of the exact mechanism by which this occurs.

In this study, bacterial seed inoculants capable of degrading 2CBA, increased the ability of rhizosphere microorganisms to reduce levels of 3CBA but had no effect on compounds with a different degradative pathway, *i.e.*, 23diCBA and 25diCBA. Levels of 23diCBA and 25diCBA were reduced in control soil, indicating that the potential to reduce these compounds was present in soil. The inoculants did not increase this potential. Furthermore, the soil's ability to degrade 2CBA and 3CBA, two compounds with similar degradation pathways, was only correlated ($p \leq 0.01$, $r=0.95$) in soil planted to plant-bacterial associations. Therefore it appears that inoculants increased the ability of rhizosphere microorganisms to degrade certain related compounds but not others. In addition, the inoculants had no effect on total heterotrophic bacterial populations in the rhizosphere.

The mechanism by which inoculants increased catabolic activity is not clear. Inoculants had no effect on any plant growth parameter suggesting that a simple increase in microbial activity in the rhizosphere was not responsible for the increased 3CBA and 2CBA degradation. The inoculants may have established themselves in the rhizosphere and degraded 2CBA and 3CBA directly. In the absence of plants, the inoculants reduced soil 2CBA levels by 17% and increased the potential of soil to degrade 3CBA by 23%. Yet in hydroponics, the mixed inoculum had no effect on 2CBA levels either alone or in the presence of Dahurian wild rye. Therefore, it appears that there may be some compound present in soil which allows the inoculants to degrade 2CBA. Alternatively, it is possible that inoculants are affecting the rhizosphere community and selectively enriching a specific population *i.e.* 2CBA and 3CBA degraders, in soil. This may occur by the inoculant's altering root exudates which are known to influence rhizosphere community composition (Lynch, 1993). Differentiating between inoculant establishment and rhizosphere community alteration is the subject of current research.

Inoculation of Meadow brome with the mixed inoculant enhanced 2CBA degradation in hydroponic solution. In contrast, inoculation of Dahurian wild rye did not affect 2CBA levels in hydroponic solution, although in both cases the inoculum was present in substantial numbers. This suggests that meadow brome provides some essential co-factor necessary for the inoculants to reduce 2CBA levels. However in soil, the mixed inoculum increased 2CBA degradation by both plant species (Siciliano and Germida, 1997). I propose that, in the case of Dahurian wild rye, the inoculants enhanced degradation that was already occurring. In contrast, in Meadow brome's case, inoculants induced degradation that was previously not occurring. Therefore, the mechanisms by which bacterial seed inoculants work may differ between plant species and the mechanisms by which plant species enhance degradation of toxicants may also differ.

In summary, my results show that plant-bacterial associations reduced 2CBA levels in soil. Furthermore, inoculating plants with certain bacteria increased the capacity of rhizosphere microorganisms to degrade similar contaminants but had no effect on total heterotrophic populations in the rhizosphere or upon plant growth parameters. Thus, it appears that inoculants do not increase phytoremediation by enhancing the rhizosphere effect but instead increase the capacity of rhizosphere microorganisms to degrade certain contaminants. It is not clear from my results if inoculants enhance phytoremediation by altering plant behaviour or indigenous bacteria. Alternatively, the inoculants may be the bacterial species primarily responsible for contaminant degradation. Understanding this process will allow rationale design and development of phytoremediation systems.

7. BIOLOG ANALYSIS AND FATTY ACID METHYL ESTER PROFILES INDICATE THAT PSEUDOMONAD INOCULANTS THAT PROMOTE PHYTOREMEDIATION ALTER THE ROOT-ASSOCIATED MICROBIAL COMMUNITY OF MEADOW BROME.

7.1 ABSTRACT

Inoculating Dahurian wild rye or meadow brome with a combination of *Pseudomonas aeruginosa* strain R75 and *P. savastanoi* strain CB35 increases degradation of 2CBA in soil. In this study the impact of these inoculants on the composition and activity of the root surface microbial community of these plants was investigated. The diversity of substrates utilized by the root-associated microbial community was assessed using Biolog™ GN and GP plates. The communities were also characterized by extracting fatty acid methyl esters (FAME) from roots of the plant-bacteria associations grown in soil. The capacity of the root surface and rhizosphere soil to degrade 2CBA also was assessed. Inoculating Dahurian wild rye increased the potential of rhizosphere soil to degrade 2CBA by 30%, but had no effect on Biolog™ substrate utilization patterns or on root FAME profiles. In contrast, inoculating meadow brome increased the potential of the root surface and associated microorganisms to degrade 2CBA by 250%, and also increased the utilization of amine, amide and polymer substrates. A cluster analysis of FAME profiles indicated that inoculation had a greater effect on root-associated microbial communities of meadow brome compared to Dahurian wild rye. The combination of strains R75 and CB35 increased the potential of Dahurian wild rye and meadow brome in different manners. Inoculating Dahurian wild rye had little effect on the root surface microbial community and increased rhizosphere soil's potential to degrade 2CBA. Conversely, inoculating meadow brome altered the root surface

microbial community but increased the potential of the root surface and associated microorganisms to degrade 2CBA. This suggests that the mechanism by which bacterial inoculants promote phytoremediation differs between plants.

7.2. INTRODUCTION

Some plants enhance degradation of contaminants in soil, and inoculation of plants with certain bacteria increases this degradation (Crowley et al. 1996; Siciliano and Germida 1997c). How inoculants increase phytoremediation is not understood. One possibility is that inoculants stimulate catabolic enzymes. Inoculants are known to increase plant enzyme activity (Mawdsley and Burns 1994) and plant enzymes can degrade contaminants. For example, Adler et al. (1994) found that peroxidases on the root surface of waterhyacinth (*Eichhornia crassipes* (C. Mart) Solms-Laub) and tomato (*Lycopersicon esculentum* L.) polymerized phenolic compounds. Similarly, plants can metabolize PCB or nitroglycerin (Wilken et al. 1995; Goel et al. 1997). A second possibility is that inoculants may become an established component of the microbial community on the root surface and thereby increase contaminant degradation. For example, Crowley et al. (1996) found that 25diCBA levels were reduced by inoculating bean (*Phaseolus vulgaris*) with a bioluminescent strain of *Pseudomonas fluorescens* which could degrade 25diCBA. The numbers of luminescent bacteria on bean roots were increased, suggesting that this bacterium had established itself on the root surface and thereby increased 25diCBA degradation. In contrast, other investigators have found that rhizosphere soil degrades contaminants faster than bulk soil and postulated that this is due to root exudation (Anderson et al. 1995; Haby and Crowley 1996). Similarly, inoculants may increase degradation of contaminants in the rhizosphere by indirectly stimulating rhizosphere activity. For example, Siciliano and Germida (1997a) found that inoculation of Dahurian wild rye with strain R75, a plant growth-promoting rhizobacterium, increased degradation of 2CBA in soil.

The purpose of this study was to investigate how the bacterial inoculants, *Pseudomonas aeruginosa* strain R75 and *P. savastanoi* strain CB35, increased phytoremediation by Dahurian wild rye and meadow brome. These plant species differ in their response to inoculants. Uninoculated Dahurian wild rye degrades 2CBA levels in soil and inoculating Dahurian wild rye with a combination of R75 and CB35 increases the degradation. In contrast, meadow brome has no effect on 2CBA levels in soil unless inoculated with a combination of strain R75 and CB35. This study was designed to answer two question: i) do inoculants affect the potential of the root surface and associated microorganisms or the rhizosphere soil to degrade 2CBA, and ii) do inoculants alter the microbial community present on the root surface?

7.3. MATERIALS AND METHODS

7.3.1. Soil and forage grasses

The collection of Paddockwood soil and the 2CBA amendment procedure are described in section 4.3.1. Dahurian wild rye and meadow brome seed were obtained and stored as described in section 3.3.2.

7.3.2. Bacterial species

The collection, storage and growth of strains R75 and CB35 were similar to those procedures described in section 4.3.2.

7.3.3. Inoculation of forage grasses

The inoculation procedure was similar to that used by Nijhuis et al. (1993). Briefly, strain R75 or CB35 was grown for 48 hours at 22°C in a 300 ml Erylenmeyer flask containing 50 ml of 1/10th TSB on a rotary shaker (250 rpm). Cells were washed twice by centrifugation (10 min × 7000 *g*, 4°C) and strains R75 and CB35 were re-suspended in 10 ml sterile tap water (10^9 cfu ml^{-1}). An aliquot (0.5 ml) of the inoculum was added to 3 mL of 4% (w/v) arabic gum and the inoculum-gum combination was mixed with 5 g of seed. Seed was coated with 5 g

talc, air dried for 15 min and 10 seeds (10^5 cfu $seed^{-1}$, R75:CB35 ratio of 5:1) were planted in styrofoam cups containing 160 g of contaminated or uncontaminated soil. For treatments involving only inoculants and no plants, bacteria were inoculated onto autoclaved seeds. Plants were grown in a growth chamber with a 16 h day (23°C) and 8 h night (18°C) with 350 μmol s^{-1} m^{-2} illumination. At 28 DAP, cups were harvested and 2CBA levels in soil determined by HPLC (section 5.3.6.).

7.3.4. Potential of rhizosphere soil or roots and their associated microorganisms to degrade 2CBA

Degradation of 2CBA by rhizosphere soil was assessed by HPLC as described in section 6.3.3. The ability of roots and associated microorganisms to degrade 2CBA was also assessed. At 28 DAP, plants were removed from soil, the shoot removed and the roots were washed with cold water until no soil was adhering to roots. The roots were placed in 20 ml scintillation vials, an aliquot (10 ml) of water containing 750 mg 2CBA L^{-1} was added and the vials were covered with dark plastic. Abiotic controls were prepared by adding 1 ml of a 3% mercuric chloride solution to the vials. The vials were placed in the growth chamber described above and incubated for five days. Levels of 2CBA in solution and the amount of 2CBA sorbed onto roots was determined by HPLC.

7.3.5. High pressure liquid chromatography

The HPLC system was similar to that described in section 5.3.6. To extract 2CBA from plant tissue, a method similar to that used by Crowley et al. (1996) for analysis of 25diCBA in plant tissue was adopted. Briefly, 100 mg (fresh weight) of root material was placed in a scintillation vial, 10 ml of 95% ethanol added and this vial placed on its side on a rotary shaker (250 rpm) at 22°C for 2 h. The ethanol was filtered (0.45 μm), transferred to HPLC vials and 50 μL injected into the HPLC system as described above.

7.3.6. Fatty acid methyl ester (FAME) analysis of roots and associated microorganisms

Roots (100 mg fresh weight) were removed from soil, washed free of soil and extracted according to procedures outlined by Graham et al. (1995). The gas chromatograph (Hewlett-Packard 5890 Series 2) was fitted with a flame ionization detector and a capillary column (Hewlett-Packard Ultra 2; 25 m by 0.20 mm; cross-linked 5% methyl siloxane with a film thickness of 0.33 μm) with helium as the carrier gas. The FAME peaks were measured by a Hewlett-Packard 3392 integrator and the Microbial Identification System used to identify FAMEs of 9 to 20 carbon atoms in length (TSBA Library version 3.80; Microbial ID. Inc.).

7.3.7. Biolog™ utilization profile of rhizoplane communities

Microbial communities associated with the surface of roots were assessed for their ability to utilize 128 different carbon substrates using Biolog™ GN and GP plates. The microbial communities were removed from the root surface by adding 1 ml of low salt buffer containing 100 mM $MgSO_4$, 10 mM Na-acetate and 1 mM $CaCl_2$ at pH 6.0 (Adler et al. 1994) for every 10 mg of root (up to 100 mg total root weight) to a sterile 20 ml scintillation vial. The vial was placed on its side on a rotary shaker (200 rpm) at 22°C for 1 h and this extract diluted 10 fold. Each well of the GN and GP Biolog™ plates was inoculated with 100 μL of extract, and the plates were incubated at 27°C. As suggested by Haack et al. (1995) I accounted for any differences in microbial numbers between treatment inocula by reading the Biolog™ plates at multiple incubation times *i.e.* 24, 48 and 96 hours, and each well scored + or - for growth. This approach ensures that observed treatment differences are due to differences in the potential of the community to use Biolog™ substrates and not differences in the growth rate of microbial communities. In addition, bacteria in the extract were enumerated by spread plating onto TSA.

7.3.8. Statistical Analysis

This experiment was designed as a 3 (experiment) × 2 (contamination/non-contaminated) × 3 (plant type) × 2 (inoculation) × 4 (replicate) factorial experiment with experiments and replicates designated as random factors. The 2CBA analysis of soil, root and shoot was performed in all experiments. The FAME and Biolog™ analysis were performed for the last two experiments and each replicated three times.

The 2CBA levels were analyzed by ANOVA and means separated by student's T-test. The FAME data was analyzed by principal components and dendogram analyses (Ward's linkage, squared Euclidean distance) while the individual fatty acids were analyzed by ANOVA. The Biolog™ data were grouped into substrate guilds as described by Zak et al. (1994), except that the polymer and the amine/amide guilds were combined to increase the number of trials in this guild to 23 *i.e.* the expected value of each cell was greater than five. I analyzed the GN and GP plates separately by two-way ANOVA, principle components and cluster analysis.

7.4. RESULTS

7.4.1. Phytoremediation of 2CBA in Soil

Levels of 2CBA in uninoculated, unplanted control soil were reduced from 200 mg 2CBA kg^{-1} to 110 mg kg^{-1} after 28 days. Dahurian wild rye significantly (LSD(0.05)=33) reduced 2CBA levels in soil from 110 to 69 mg kg^{-1}, and inoculating Dahurian wild rye further reduced 2CBA levels to 32 mg kg^{-1}. Uninoculated meadow brome only reduced 2CBA levels from 110 to 83 mg kg^{-1}, but inoculating meadow brome reduced 2CBA levels to 47 mg kg^{-1}. Inoculating soil with a combination of strain R75 and CB35 had no effect on 2CBA levels (113 mg kg^{-1}).

7.4.2. Potential of rhizosphere soil or roots and their associated microorganisms to degrade 2CBA

We assessed the effect of bacterial inoculants on the potential of rhizosphere soil and roots and their associated microorganisms of plant-bacteria associations to degrade 2CBA. Inoculation increased the potential of rhizosphere soil taken from both plant-bacteria associations to degrade 2CBA (Table 7.1.).

TABLE 7.1. Potential of rhizosphere soil and roots with their associated microorganisms of plant-bacteria associations to degrade 2CBA over a five day period.

Treatment[a]	2CBA Degradation (μg/g soil)	
	Soil[b]	Roots[c]
Control	26	ND
Inoculated	75	ND
D.W. Rye	140	220
Inoculated D.W. Rye	200	260
Meadow brome	90	160
Inoculated Meadow brome	160	400
LSD (0.05)	50	130

[a] Plants were inoculated with a combination of strain R75 and strain CB35 (10^5 cfu seed^{-1}), grown in 2CBA contaminated soil for 28 days and harvested. For inoculated treatment with no plant, bacteria were inoculated onto autoclaved Dahurian wild rye seed.

[b] Soil collected from the various treatments at 28 days was amended with 750 μg of 2CBA and incubated at 21°C for five days. Degradation was calculated by subtracting the 2CBA level remaining in amended soil from the level remaining in a replicated sample treated with mercuric chloride (3 g kg^{-1}).

[c] Roots collected from plant-bacteria associations at 28 days were immersed in 10 ml of a 750 mg L^{-1} 2CBA solution for five days at 21°C. Degradation was calculated by subtracting 2CBA levels remaining from the initial amount of 2CBA added. Degradation was corrected for the amount of 2CBA sorbed to root tissue.

Rhizosphere soil from inoculated Dahurian wild rye degraded 25% more 2CBA than inoculated meadow brome rhizosphere soil. However, the inoculants increased the potential of the meadow brome rhizosphere soil to degrade 2CBA by 78% compared to only a 43% increase for the Dahurian wild rye soil. Soil taken from pots inoculated with only strains R75 and CB35 also had the potential to degrade 2CBA but this potential degradation was significantly less than all plant-bacteria associations except uninoculated meadow brome. This confirms my earlier study in which uninoculated meadow brome did not degrade 2CBA (Siciliano and Germida 1997c). Soil from plant-bacteria associations grown in non-contaminated soil exhibited little potential to degrade 2CBA, with 93% of the 2CBA remaining after five days incubation (data not shown).

In contrast to rhizosphere soil, inoculation increased the potential of roots and their associated microorganisms taken from meadow brome to degrade 2CBA by 250%, but had no effect on the potential of Dahurian wild roots to degrade 2CBA (Table 7.1.). Reducing microbial activity with mercuric chloride reduced 2CBA degradation by 75 to 100%. There was no difference in the potential of roots taken from plant-bacteria associations grown in contaminated or non-contaminated soil to degrade 2CBA. Sorption of 2CBA by roots was unaffected by inoculation (data not shown). Roots taken directly from soil (*i.e.*, before immersion in the 2CBA solution) contained undetectable 2CBA levels.

7.4.3. FAME analysis of rhizoplane communities

Cluster analysis indicated that inoculation of meadow brome had a greater effect on FAME patterns than inoculation of Dahurian wild rye (Fig. 7.1.). Inoculated and non-inoculated meadow brome FAME patterns were 168 and 40 squared Euclidean Distance (SED) apart for contaminated and non-contaminated communities, respectively. In comparison, inoculated and non-inoculated Dahurian wild rye FAME patterns were only 8 and 18 SED apart for contaminated and non-contaminated soils respectively. This suggests that inoculating meadow brome had a greater effect on root-associated microbial communities than inoculating Dahurian wild rye.

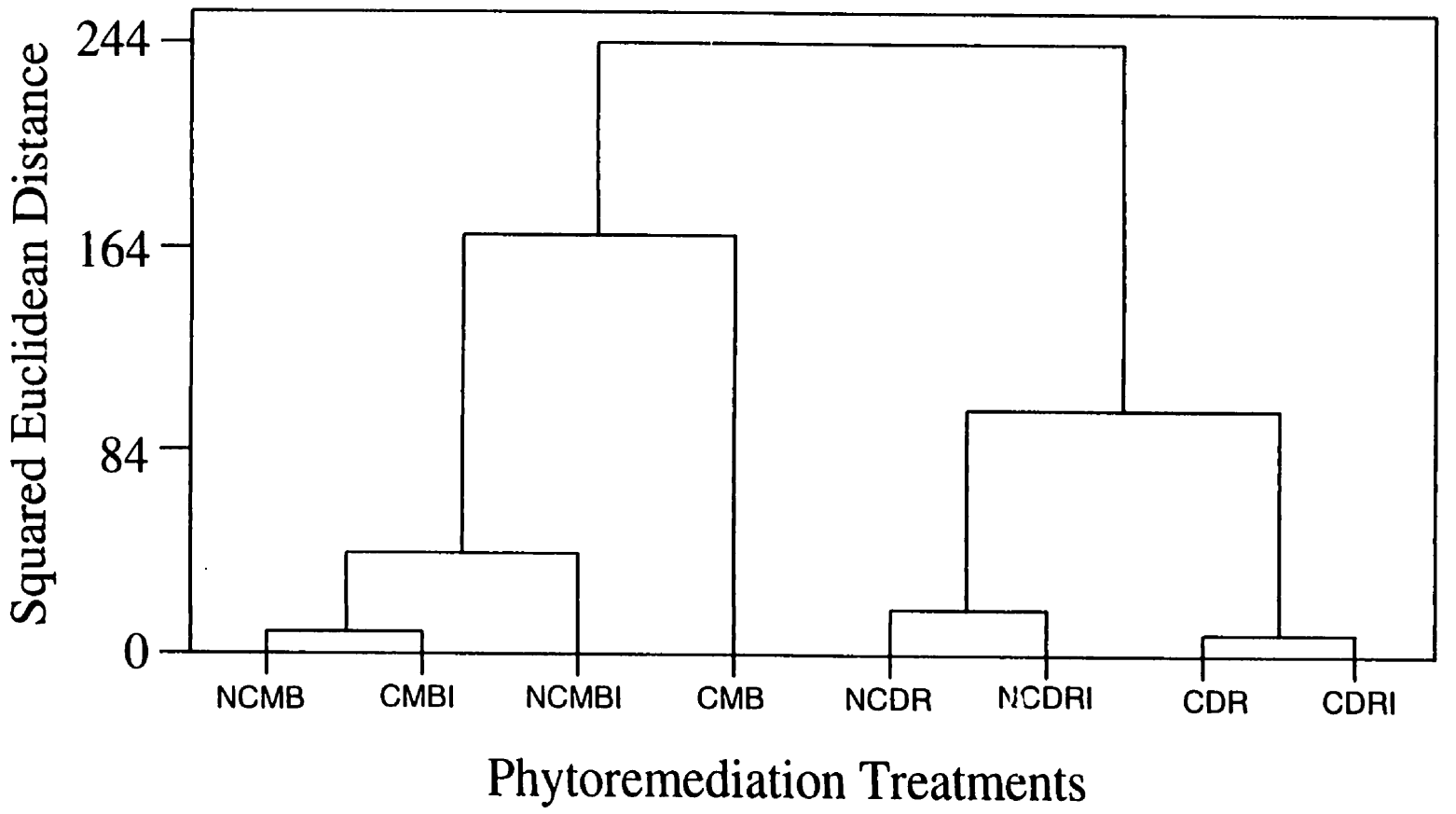

Fig. 7.1. Inoculating meadow brome alters fatty acid methyl ester root profiles to a greater extent than inoculating Dahurian wild rye based on differences in squared Euclidean distance calculations. Plants were inoculated or not, with a combination of strain R75 and strain CB35 (10^5 cfu seed^{-1}), grown in 2CBA contaminated soil (200 mg kg^{-1}) or not, for 28 days and fatty acids extracted from the roots with their associated microorganisms. Data were clustered using Ward's linkage. Each treatment is the average of 3 replicates taken from two independent experiments (n=6).

7.4.4. Biolog™ Utilization Analysis of Rhizoplane Communities

Inoculation increased Biolog™ substrate utilization by the rhizoplane community of meadow brome but had little effect on Dahurian wild rye's community. Inoculating meadow brome increased the capability of rhizoplane communities to use the amide/polymer guild in GN plates from 52 to 62% and from 44 to 63% in GP plates (Table 7.2.).

In addition, cluster analysis indicated that the communities were widely separated from non-inoculated meadow brome communities with a SED of 1140 (Fig. 7.2.). In contrast, there were no significant guild use differences between inoculated and uninoculated Dahurian wild rye communities, as the communities were only 444 SED distant. There were no significant differences in inoculum densities between the treatments with approximately 10^6 cfu ml bacteria in each extract. There was a significant ($P \leq 0.05$) time effect in all plates but no significant time-treatment interactions.

TABLE 7.2. Functional guild analysis of root-associated microbial communities of plant-bacteria associations.

Treatment	% Positives on GN Plates[a]				% Positives on GP Plates[a]			
	Carbohydrate	Carboxylic acid	Amine Polymer	Misc.	Carbohydrate	Carboxylic acid	Amine Polymer	Misc.
D.W. Rye	58	57	57	26	39	36	48	17
D.W. Rye plus R75 and CB35	54	55	59	26	39	35	47	13
M. brome	53	52	52	22	43	36	44	14
M. brome plus R75 and CB35	57	57	62	25	49	42	63	14
LSD (0.10)	5.7	10	4.2	2.4	11.2	9.6	11	8.6

[a] Values are the average of three observation times, 24, 48 and 96 hours. Guilds are a number of substrates in Biolog™ plates grouped on the basis of their chemical similarity. GN plates were originally developed by Biolog™ to identify gram negative bacteria by growing gram negative bacteria on 95 different substrates. GP plates were developed for gram positive bacteria.

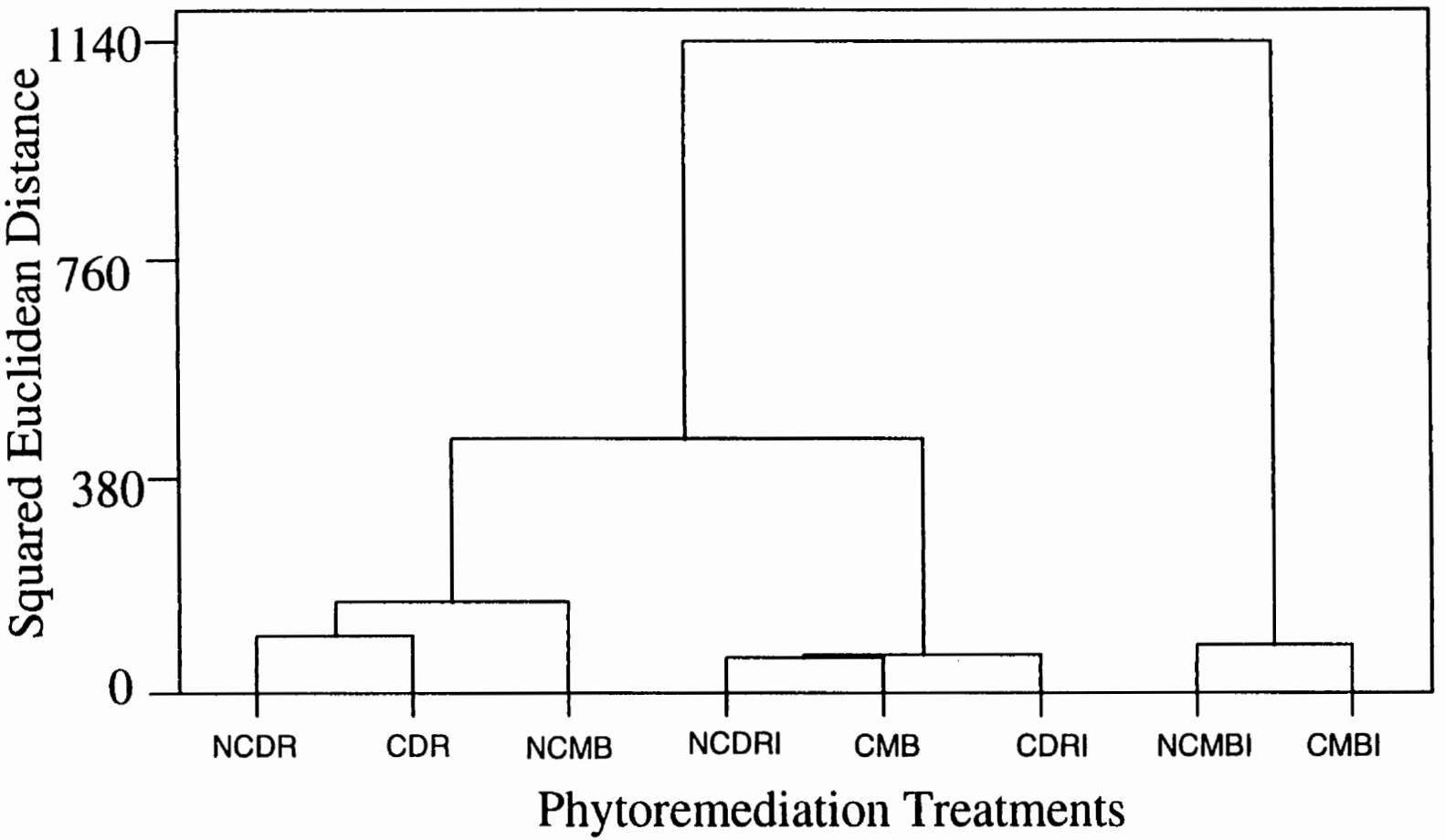

Fig. 7.2. Inoculating meadow brome alters the ability of root-associated microorganisms to use Biolog™ substrates to a greater extent than inoculating Dahurian wild rye based on differences in squared Euclidean distance calculations. Plants were inoculated or not, with a combination of strain R75 and strain CB35 (10^5 cfu seed^{-1}), grown in 2CBA contaminated soil (200 mg kg^{-1}) or not, for 28 days and the utilization of 128 Biolog™ substrates by microbial communities washed from the roots of plant-bacteria associations determined. Data were clustered using Ward's linkage. Each treatment is the average of 3 replicates taken from two independent experiments (n=6).

7.5. DISCUSSION

An inoculant mixture of strains R75 and CB35 does not degrade 2CBA in soil (Siciliano and Germida 1997c; this study). However, these bacteria stimulate 2CBA degradation when associated with roots of Dahurian wild rye grown in soil and also increase the potential of rhizosphere soil to degrade 2CBA and 3CBA. In this study, the inoculant mixture did not alter the microbial community present on the root surface as determined by FAME and Biolog™ analysis. Thus these inoculants must be altering the soil microbial community not directly associated with roots. One possible way for the inoculants to accomplish this would be by altering the root exudates of Dahurian wild rye. Root exudates are implicated in other plant-bacteria systems that degrade contaminants. For example, Shann (1995) found that root exudates of monocot species selectively stimulated degradation of 2,4-dichlorophenoxy acetic acid and 2,4,5-trichlorophenoxy acetic acid but had no effect on phenol or 2,4-dichlorophenol. Similarly, Hsu and Bartha (1979) found that root exudates of bush bean (*Phaseolus vulgaris* cv. Tender Green) specifically stimulated *O,O*-diethyle-*O*-*p*-nitrophenyl phosphorothioate (parathion) but not *O,O*-diethyl-*O*-(2-isopropyl-6-methyl-4-pyrimidinyl phosphorothioate (diazinon) degradation. However, in neither of these studies was the composition of the microbial community evaluated. My study shows that inoculants can enhance degradation of xenobiotics in the rhizosphere with relatively little impact on the root surface community.

The mechanisms by which strains R75 and CB35 increase 2CBA degradation by meadow brome appears to be quite different from that observed for Dahurian wild rye. In Dahurian wild rye, the inoculant mixture stimulates 2CBA degradation that was already occurring in the uninoculated plant. In contrast, inoculating meadow brome induces degradation that was previously absent. My evidence suggests that the inoculants accomplished this by altering the microbial community present on the root surface of meadow brome. Both Biolog™ and FAME analyses indicated that the microbial community was substantially altered by inoculation. It is not clear if the inoculants directly colonized the rhizosphere directly as was seen by Crowley et

al. (1996) who found that a pseudomonad inoculant established itself in the rhizosphere of bush bean and thereby increased degradation of 25diCBA. Alternatively, the inoculants may have altered the rhizosphere community without establishing themselves in the rhizosphere. For example, Haluska et al. (1995) found that degradation of polychlorinated biphenyls was not correlated with strain survival and postulated that other factors may influence polychlorinated biphenyl degradation in inoculated soils.

Understanding the nature of the plant-bacteria associations that occur in contaminated soils is important when developing phytoremediation technologies. Based on this study, the underlying mechanisms by which phytoremediation was enhanced by inoculants differed between plant species. In one case, inoculants alter rhizosphere processes and thereby stimulate 2CBA degradation (*e.g.*, Dahurian wild rye), whereas in the other, inoculants directly alter the root surface community (*e.g.*, meadow brome) which results in reducing 2CBA levels in soil. It is important to note that my results are limited to describing a reduction in extractable 2CBA from soil. It is possible that plant-bacterial associations may be immobilizing 2CBA, or in some other way reducing the amount of extractable 2CBA. However, it is well known that the toxicity of contaminants is related to their bioavailability, and thus technologies that reduce levels of readily extractable contaminants may have a role to play during remedial actions. I am presently studying how root exudates influence 2CBA degradation in the rhizosphere and how inoculants alter the root surface community of meadow brome.

8. ENZYMATIC ACTIVITY IN ROOT EXUDATES OF DAHURIAN WILD RYE THAT DEGRADES 2CBA.

8.1. ABSTRACT

Dahurian wild rye degrades 2CBA in soil and in hydroponics. This study assessed mechanisms responsible for this activity. Filter sterilized root exudates of hydroponically grown Dahurian wild rye degraded 2CBA in solution. This activity in root exudate had an apparent pH optimum of 6.3-6.6, a temperature optimum of 40°C, a V_{max} of 1.34 mmole 2CBA day^{-1} and a K_m of 657 pmoles of 2CBA. Furthermore, the 2CBA-degrading activity of the root exudate was eliminated by protease treatment. Filter sterilized rhizosphere extracts of Dahurian wild rye grown in soil also degraded 2CBA in solution and this activity was eliminated by protease treatment. My results demonstrate the presence of a protease sensitive degradative reaction in the root exudates of Dahurian wild rye which degrades 2CBA in solution.

8.2. INTRODUCTION

Bacterial seed inoculants can enhance contaminant degradation in soil as plants grow (Siciliano and Germida 1997c; Crowley et al. 1996). Previously I found that only certain plant-bacteria associations reduced levels of 2CBA in soil. One association, Dahurian wild rye inoculated with a 1:1 mixture of strains R75 and CB35, reduced levels of 2CBA in soil by 46%. I postulated that these inoculants either increased plant growth or augmented the ability of the rhizosphere microbial community to degrade compounds. Subsequently, I found that inoculating Dahurian wild rye had little effect on plant growth, but increased the ability of the rhizosphere microbial community to degrade mono-chlorinated benzoic acids (Siciliano and Germida 1997a). This supports my augmentation hypothesis. However, how

inoculants augmented the degradative ability of the rhizosphere is still unclear.

Non-inoculated 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 section, I report the presence of a protease senstivie reaction in the root exudate of Dahurian wild rye that degrades 2CBA.

8.3. MATERIALS AND METHODS

8.3.1. Analysis of Enzymatic Activity in Root Exudates

The root exudates of Dahurian wild rye, meadow brome and streambank wheatgrass, all previously shown to reduce 2CBA levels in soil (Siciliano and Germida 1997c), were collected during hydroponic growth and tested for their ability to reduce levels of 2CBA in solution. The plants were either non-inoculated or inoculated with a mixture of strains R75 and CB35 as previously described (Siciliano and Germida 1997c), and grown as described in section 6.3.4. The sterility of non-inoculated treatments was assessed by plating out 0.1 ml of hydroponic solution onto TSA and incubating at 38°C for 48 h. The survival of the inoculant mixture was assessed on TSA supplemented with antibiotics (Section 6.3.4). After 28 days of growth, a 4ml aliquot of hydroponic solution was filter sterilized (0.2 μm, cellulose acetate membrane) and 2CBA levels in this aliquot determined every day for three days by HPLC (Section 5.3.6). To determine if plants contained a catalytic compound that degraded 2CBA, I triturated Dahurian wild rye roots with a mortar and pestle, and re-suspended 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 albinum

as a standard (Daniels et al. 1994). These experiments were each repeated three to five times with three replicates per treatment.

8.3.2. Characterization of Degradative Activity in Root Exudates

I characterized the 2CBA degrading activity in Dahurian wild rye root exudate at pHs 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 2CBA L^{-1} amended M9 medium, adjusted with either HCl or NaOH to the desired pH, maintained at 23°C and the 2CBA level determined every 12 hours (over a 3 day period) by HPLC analysis. I 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 μmole. Analysis of velocity versus substrate plots was performed as described by Cornish-Bowden and Wharton (1988). I 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 protease mL^{-1} (*Streptomyces caespitosus* Type IV, Sigma P-0384), and 2CBA levels followed for a further five days. I determined if other CBA 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} 3CBA, 23diCBA or 25diCBA instead of 2CBA.

8.3.3. Soil Studies

The presence of this catalytic activity during the phytoremediation of 2CBA in soil was investigated by extracting proteins from the rhizosphere of inoculated or non-inoculated 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 for a concentration of 467 mg kg^{-1} and used in a study designed to screen the degradative

ability of forage grasses (Section 6.3.1.). At the end of that study, all contaminated soil was bulked together and stored in metal cans for three years. For the present study, the stored soil was thoroughly mixed and the residual extractable 2CBA level determined on six sub-samples by HPLC analysis. Since inoculating soil with strains R75 and CB35 reduces 2CBA levels (Siciliano and Germida 1997c), I determined if the inoculants or other soil microflora in the absence of a plant produced a compound which catalyses the reduction in 2CBA levels. Non-planted, inoculated treatments were amended every two days with 5 mg glucose and 1 mg yeast extract to simulate the stimulation of bacteria by root lysate. Twenty-one DAP, the root system was extracted from pots and shaken vigorously. The rhizosphere sample was extracted by adding approximately 10mls 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, centrifuged at 1000 rpm for 20 minutes and the supernatant 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.

8.4. RESULTS AND DISCUSSION

Only the hydroponic exudate of inoculated and non-inoculated Dahurian wild rye degraded 2CBA in solution (Table 8.1.). 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 nmole 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 to 6.6 with little activity observed at pH 5.9 or pH 8.3. The reaction rate increased with temperature, doubling from 18 to 38 nmole day^{-1} as temperature increased from 23°C to 40°C

with no activity observed at 10°C and 50°C. The rate of 2CBA degradation followed Michaelis-Menten kinetics with an apparent V_{max} and K_m of 13.4 μmol day^{-1} and 657 nmole of 2CBA, respectively. Degradation of 2CBA was linear for a 10 day period, and the addition of protease stopped the reaction (Fig. 8.1.). No degradation of 3CBA, 23diCBA or 25diCBA occurred in solution. Collectively, these results indicate the presence of a protein catalyzed reaction in the root exudates of Dahurian wild rye which specifically catalyses the reduction of 2CBA levels in solution.

Inoculating Dahurian wild rye increased ($p=0.001$) the 2CBA-degrading activity in rhizosphere extracts, but had little effect on protein levels (Fig. 8.2.). In contrast to hydroponics, there was little observed activity in the rhizosphere of non-inoculated plants.

TABLE 8.1. Degradation of 2CBA by filter sterilized root exudates of Dahurian wild rye.

Plant root exudates[1]	Inoculated[2]	Protein (mg)	2CBA Degraded (nmole/day)[3]
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

[1] The rate of 2CBA degradation in the absence of plants was zero.
[2] *Pseudomonas aeruginosa* strain R75 and *P. savastanoi* CB35 were grown for 48h, inoculated onto plant seed (10^6 cfu seed^{-1}) and the plants grown in a hydroponic system for 21 days.
[3] Degradation of 2CBA calculated in exudate containing *c.a.*. 7.6 μMoles.

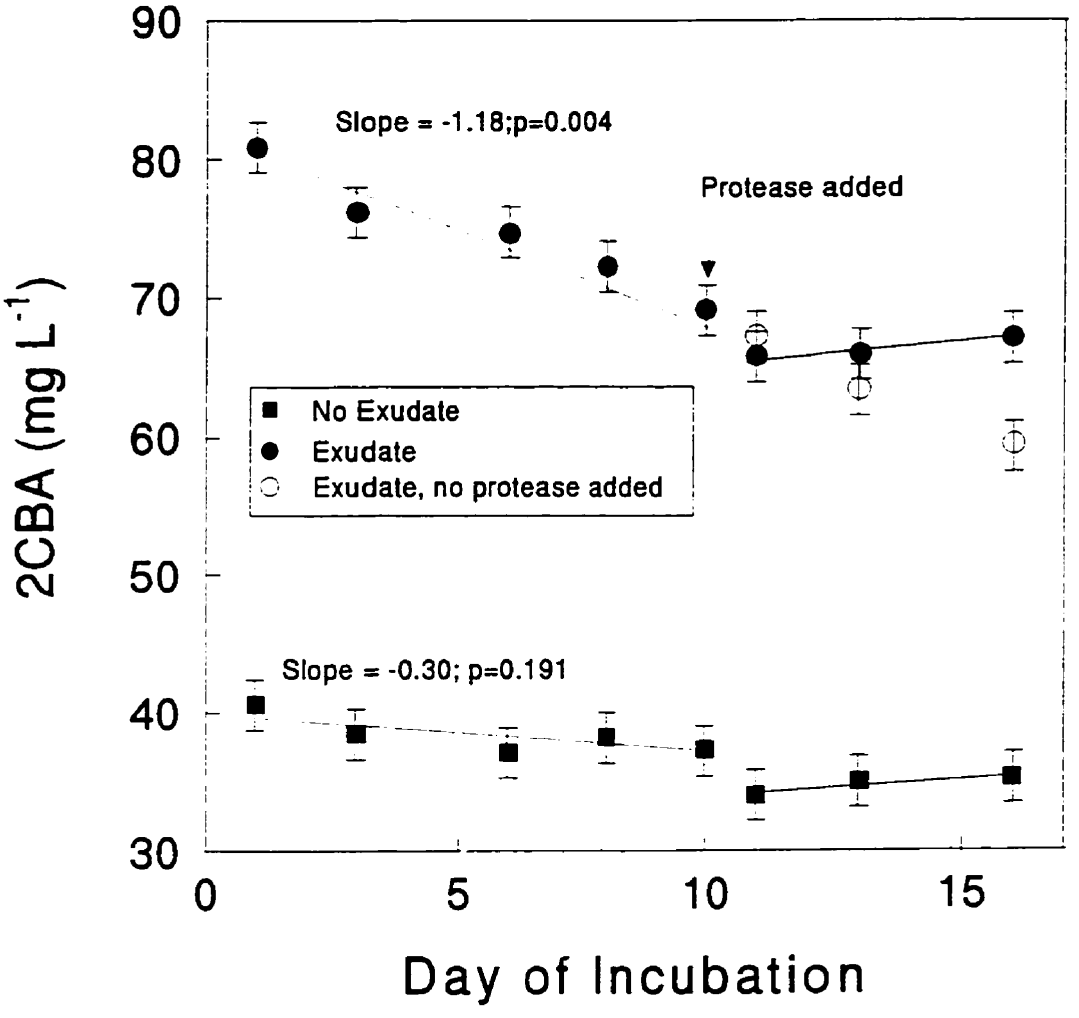

Fig. 8.1. Elimination of 2CBA degrading activity by the addition of protease. At day 10, 200 μL of a solution containing 10 mg of protease mL^{-1} (*Streptomyces caespitosus* Type IV) was added to the reaction vials and the 2CBA concentration determined by HPLC.

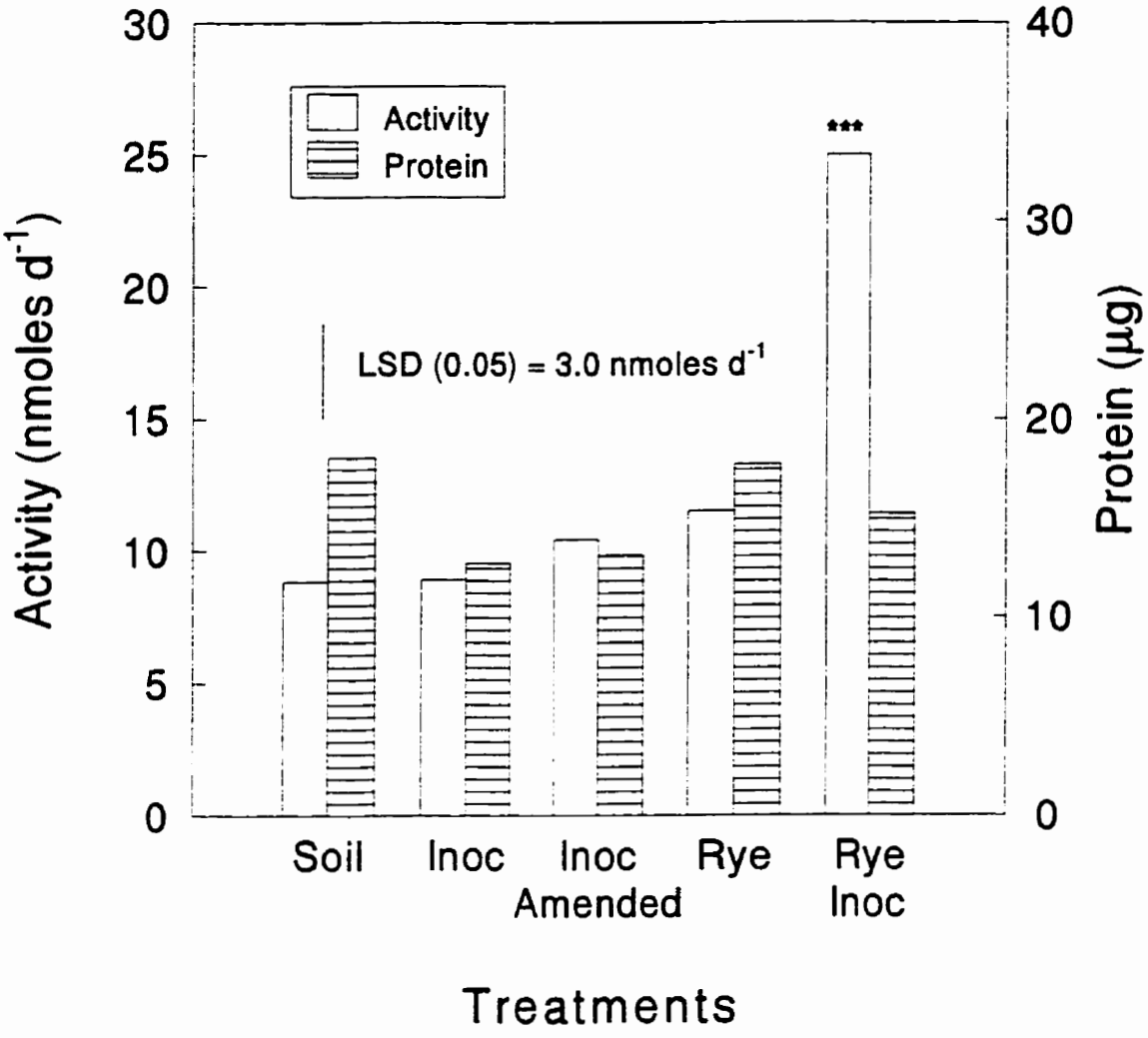

Fig. 8.2. Protein levels and 2CBA-degrading activity in rhizosphere extracts of Dahurian wild rye. For treatments with only bacterial inoculants (Inoc), autoclaved Dahurian wild rye seeds were inoculated (10^6 cfu seed^{-1}) with strains R75 and CB35 and planted into soil. Amended treatments (Inoc Amended) had 5 mg glucose and 1 mg yeast extract added every 2 days to soil. Dahurian wild rye seed (n=10) was either non-inoculated (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 ***.

It is well known that plant physiology differs between hydroponics and soil (Curl and Truelove 1986a) 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 extracelluar plant enzymes that degrade contaminants (Schnoor et al. 1995), to the best of my knowledge, no extracelluar plant produced 2CBA-degrading activity has been described previously. However, my results are limited to describing the reduction in 2CBA levels. It is possible that the protein is not degrading 2CBA but instead may be transforming 2CBA into a compound which is not amenable to my 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 reaction, as well as a determination of it's role during the phytoremediation of 2CBA by Dahurian wild rye.

9. CONCLUSIONS

The results from my thesis project indicate that plant-bacteria associations promote contaminant degradation in soil in pot experiments. Inoculating Dahurian wild rye with a mixture of pseudomonads decreases levels of 2CBA and 3CBA in soil whereas this same mixture of pseudomonads increased the degradation of 25diCBA by altai wild rye. Inoculating meadow brome with alcaligenes sp. BR60 increased the degradation of 23diCBA in soil. Furthermore, certain plant-bacteria associations were capable of degrading tertiary contaminant mixtures *i.e.*, altai wild rye inoculated with strains R75 and CB35 degraded a mixture of 3CBA, 23diCBA and 25diCBA.

The enhancement of contaminant degradation by plants and bacteria appears to only occur for certain plants and bacteria. During my initial screening, grasses displayed an 11 fold difference in sensitivity to 2CBA contamination. Thus, it appears that plants may have evolved a range of responses to toxicants in the environment from contaminant tolerance to degradation. Walton et al. (1994) postulate that evolutionary pressures would select for plants capable of tolerating toxins in soil. This ability to tolerate biologically produced chemicals might translate in certain instances to xenobiotics. Supporting this, I found that treatments which altered the soil biochemical environment modulated plant response. Therefore, plants interact in specific manners to certain chemicals in soil.

The discovery that select bacterial seed inoculants decreased levels of contaminants in soil, suggests that bacterial inoculants can modulate the behaviour of plants towards toxicants. The results from Chapters 3 and 4 indicate that this

modulation can be positive (*i.e.*, increasing degradation) or negative (*i.e.*, phytotoxic). The role of bacteria in phytoremediation is complex and appears to range from detoxifying, degradative, plant-growth promoting or even plant deleterious. The reasons for this complex interaction between plants and bacteria most likely lies in the ecological interactions occurring at the root-soil interface.

The results from this project indicate that bacteria inoculants dramatically affect the ecological interactions at the root-soil interface. In the case of meadow brome, pseudomonad inoculants alter the structural composition and functionality of the rhizoplane community. These inoculants thereby stimulate degradative activity in soil. In addition, inoculants can also interact directly with plant metabolic processes and thereby increase degradation. For example, inoculation of Dahurian wild rye increases the production of a protein that reduces 2CBA levels in soil. The molecular mechanisms by which inoculants alter the rhizoplane community or protein expression are not known.

Phytoremediation is an emerging technology to clean up contaminated soil and water sites. My project has demonstrated that bacteria can play an important role in phytoremedation. Furthermore, the use of bacteria in conjunction with plants has the potential to extend the number and type of contaminated soils sites in which phytoremediation systems may be applied.

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IMAGE EVALUATION
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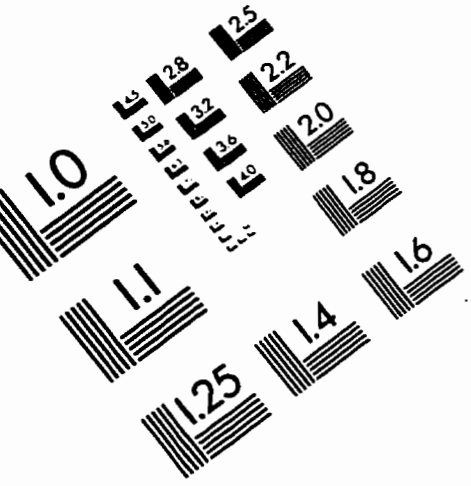

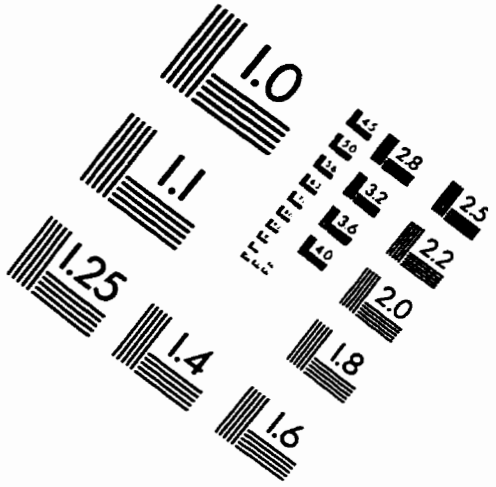

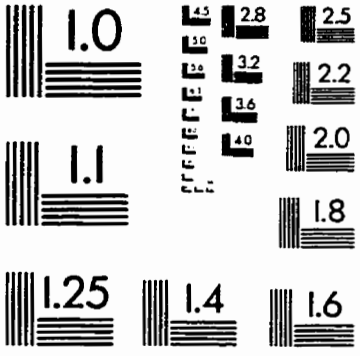

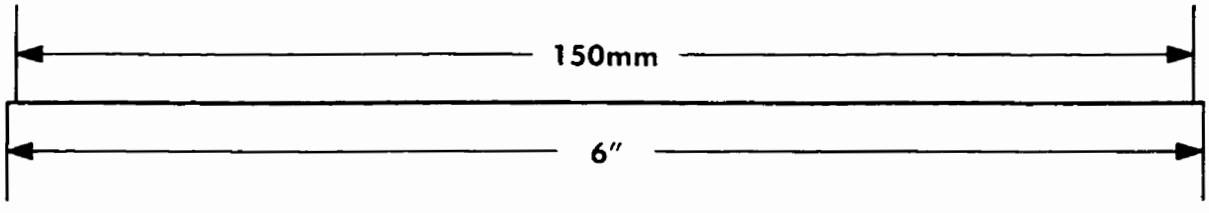

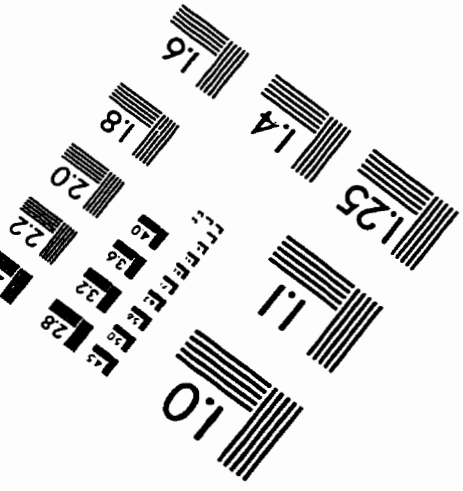

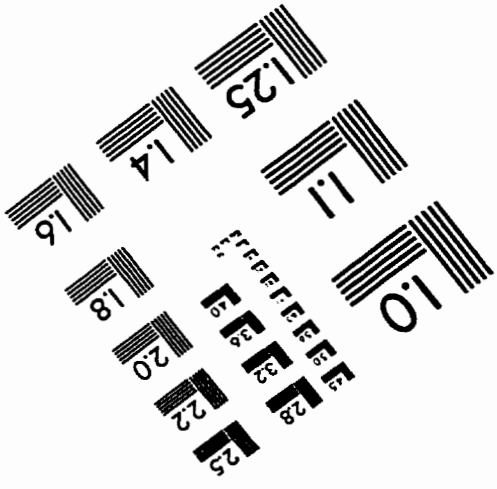

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