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Catechin, a novel root exudate of Centaurea stoebe, exhibits pleiotropic effects on the soil microbial community and individual soil isolates, and undergoes chemical changes with numerous abiotic factors from soil

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(±)-CATECHIN, A NOVEL ROOT EXUDATE OF *CENTAUREA STOEBE*, EXHIBITS PLEIOTROPIC EFFECTS ON THE SOIL MICROBIAL COMMUNITY AND INDIVIDUAL SOIL ISOLATES, AND UNDERGOES CHEMICAL CHANGES WITH NUMEROUS ABIOTIC FACTORS FROM SOIL

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(±)-Catechin, a novel root exudate of *Centaurea stoebe*, exhibits pleiotropic effects on the soil microbial community and individual soil isolates, and undergoes chemical changes with numerous abiotic factors from soil
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Allelopathy has been studied for (±)-catechin, a root exudate of spotted knapweed, as a plausible mechanism that facilitates knapweed invasion in North America. Phytotoxic effects are observed in invaded fields as well as greenhouse experiments when employing catechin or growing spotted knapweed, demonstrating similar effects on native plant species. However, many studies ignore the impact of catechin on microbial populations in soil. Understanding the impact of catechin on microbial processes will greatly aid our understanding of how spotted knapweed invasion can alter soil chemistry and biological processes. Chapter 2 will address the impact of soil and media parameters on catechin stability and extraction from soil. My results show catechin to be a highly reactive compound and that redox metals, the pH of the surrounding environment, and the aerobicity of the system can greatly alter catechin auto-oxidation rates. Chapter 3 expands on these findings by testing the impact of catechin-metal complexes on catechin phytotoxicity reported in the current literature. I demonstrate metals that lower the amount "pure" catechin in the system have differential effects on native plant species. I further reveal that conditions of increased environmental stress can further exacerbate this phytotoxic phenomenon. Chapter 4 addresses the impact of catechin on soil bacterial communities as well as individual bacterial populations. I confirm that catechin is not bacteriocidal, but instead should be categorized as bacteriostatic. In chapter 5, I extend the findings in chapter 4 to include a repressive effect to various bacterial genera but show that species capable of altering catechin stability can overcome this bacteriostatic effect. More importantly, I reveal that two simple organic acids were capable of restoring previously inhibited bacterial species. Chapter 6 focuses on the ability of catechin to prevent endospore formation, germination, and to alter cell morphology in many gram-positive endospore-forming bacteria commonly found in soil. The significance of this body of work is outlined in chapter 7 along with directions that future research might take to explore some of my more obscure findings.

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Chapter 1

INTRODUCTION

Overview

The term "allelopathy" was originally coined by Austrian Professor Hans Molisch in 1937 to describe the biochemical inhibition or stimulation by one plant of the growth of neighboring plants. Since then, Dr. Elroy R. Rice has revised the definition to include the release of biochemicals produced from one plant or microorganism into the environment to harm other plants or microorganisms (Rice, 1974). Since that time, many other studies have isolated and tested allelopathic chemicals produced by plant species capable of displacing other plant species (Einhellig & Souza, 1992, Kagan*, et al.*, 2003, Kato-Noguchi, 2004, Bertin*, et al.*, 2007), lending credence to the idea that allelopathy directly influences the success of invasive plants by inhibiting neighboring native plants. One such allelopathic compound is (±)-catechin, a racemic mixture exuded from the roots of spotted knapweed (Blair*, et al.*, 2006, Perry*, et al.*, 2007).

Several studies have used the pure chemical form of catechin to simulate the impact of spotted knapweed growth on both plants and microbes. Tests for its allelopathic potential have been performed using a variety of approaches, from greenhouse experiments using sand cultures or field soils (Inderjit*, et al.*, 2008, Inderjit*, et al.*, 2008, Duke*, et al.*, 2009, He*, et al.*, 2009; including chapter 3 of this dissertation; see Pollock*, et al.*, 2009), to petri dishes for seedlings or microorganisms (Callaway*, et al.*, 2005, Inderjit*, et al.*, 2008,

Duke*, et al.*, 2009, He*, et al.*, 2009), to field studies where catechin was directly added to soil near native plants (Inderjit*, et al.*, 2008, Inderjit*, et al.*, 2008).

While these studies have been important in understanding the phytotoxicity of catechin to other plants and have observed some antimicrobial effects, no study to date has directly assessed the impact of catechin on the total microbial community or its component populations. Moreover, many scientists have found biological effects of catechin to be specific to the enantiomer tested, either the positive (+) or the negative (-) form (Veluri*, et al.*, 2004, Duke*, et al.*, 2009). However, as spotted knapweed is reported to exude a racemic mixture of (±)-catechin into the rhizosphere (Blair*, et al.*, 2006, Perry*, et al.*, 2007), we included both compounds in our experiments to better test for the effects of exuded catechin on either plant or microbial species for consistency.

(±)-Catechin has been hypothesized as being a "novel weapon" for knapweed invasiveness in North America (Callaway & Ridenour, 2004). This hypothesis is based on the supposition that native plants and microorganisms are negatively affected by previously unseen chemical(s) produced by exotic plant species. Although some studies have shown catechin to have either low or no phytotoxic effects (Blair*, et al.*, 2005, Duke*, et al.*, 2009), the amounts added in those experiments may not have been at biologically active levels. To date, no studies have investigated mechanisms that might explain these seemingly contradictory findings.

With the research described herein, I propose to help to elucidate the effects of (±)-catechin on indigenous microbial and plant species. In order to investigate the impact of knapweed invasion via catechin production/exudation on native plant and microbial ecosystems, I have undertaken a series of studies to: i) determine if common soil chemistry parameters can alter the ability to effectively and precisely detect catechin concentrations in soils, ii) investigate the effect(s) of catechin on indigenous plant species under various scenarios of metal addition to determine what effect(s) catechin coupled with various metals has on phytotoxicity, iii) assess the impact of catechin on soil bacterial populations, and iv) determine the putative mechanism(s) responsible for these effects on soil bacterial isolates. Finally, my dissertation includes a concluding synthesis chapter discussing the impact of my research on the present level of understanding of the allelopathic effects of catechin and its contribution to the invasive success of spotted knapweed, as well as the seeming conditionality observed for these effects in different systems by different investigators. This summary will describe the implications of my findings on the effects of catechin on native plant phytotoxicity, as well as soil microbial communities via selective inhibition of soil bacterial populations.

Background and Origin of Research Questions

Allelopathy as a mechanism for invasive success

Allelopathy refers to the ability of a biochemical to alter growth of neighboring plants or microorganisms (Molisch, 1937, Rice, 1974). Currently, many scientists have focused much of their attention on plant:plant negative interactions, as the definition of allelopathy has been modified over the years (Putnam & Duke, 1978, Putnam, 1985, Boyette & Abbas, 1995, Pratley, 1996). Given the intimate associations of plants with fungi, bacteria, and soil invertebrates, it is naïve to ignore the impact of allelopathy on these other groups of organisms, especially given that negative impacts on any of these organisms can have profound effects on other trophic levels.

Spotted Knapweed Invasion

Centaurea stoebe has been an invasive species for over 100 years causing damage to crops estimated at $155.7 million/year to the agricultural industry as a result of displacement of crops as well as providing poor grazing materials for livestock (Bucher, 1984). *Centaurea stoebe* (spotted knapweed) negatively impacts indigenous flora, which can include plants used to produce hay for grazing animals in western North America (DiTomaso, 2000). Many invasive plant species are believed to negatively impact the growth of indigenous flora and outgrow them through a variety of potential mechanisms including predator/pathogen avoidance, and production of allelochemicals in roots, stems, or leaves. In the case of *C. stoebe*, the polyphenolic, enantiomeric compound (±)-catechin is exuded into the surrounding rhizosphere (Fig. 1). Early theories proposed that

this chemical is produced as a defense mechanism against insects, plants, and harmful microorganisms (Callaway*, et al.*, 1999).

(±)-Catechin contribution to invasive success

Additional experiments have attempted to better elucidate the role of catechin in invasion by spotted knapweed. (-)-Catechin (i.e. the minus enantiomer) has been shown to be phytotoxic to many North American plant species (Bais*, et al.*, 2003). By secreting this compound into its rhizosphere (Callaway*, et al.*, 1999, Callaway & Aschehoug, 2000, Tharayil & Triebwasser, 2010), *C. stoebe* appears to derive a selective advantage over native and agriculturally useful crops in western North America, contributing to its success as an aggressively invasive weed (DiTomaso, 2000). More recently, (+)-catechin (i.e. the plus enantiomer) has been reported to have antimicrobial effects on certain North American soil microbial isolates (Bais*, et al.*, 2002, Arunachalam*, et al.*, 2003). However, those experiments used variable (±)-catechin concentrations and pure cultures maintained under laboratory conditions and storage and might not reliably represent the impact that spotted knapweed/(±)-catechin might have on the native soil microbial community. This topic is of intrinsic interest to soil microbial ecologists, but may also play a significant role in knapweed invasiveness via perturbation of the composition and activities of the soil microbial community and the way that its processes and services in turn control the plant community at a given site.

It should be noted that experiments to date on the effects of catechin on plant and microbial populations do not always show (±)-catechin to have inhibitory effects on plant

species and the effects can vary substantially among species (Perry, *et al.*, 2005). Further complicating the situation, the reported natural soil concentrations of pure (±)-catechin vary greatly spatially and temporally and tend to be far lower than initially reported (Blair, *et al.*, 2005, Blair, *et al.*, 2006, Perry, *et al.*, 2007).

Catechin has previously been shown to bind with proteins and polysaccharides (Sarker, *et al.*, 1995, Hopper & Mahadevan, 1997), which might explain some of its antimicrobial activity. In addition, catechin has shown the ability to interact with various metals commonly found in soil (McDonald, *et al.*, 1996, Lim, *et al.*, 2005, Pollock, *et al.*, 2009), primarily as a result of the ortho-hydroxyl groups of (±)-catechin found on the B ring (Fig. 1). At least some of these metals are essential in organisms for several different metabolic processes; however, there is a paucity of knowledge in the current literature regarding the bioavailability and bioactivity of (±)-catechin-bound forms of these metals. This may have profound implications regarding the mechanism(s) that allow some plants and microbes to be resistant to spotted knapweed/(±)-catechin, while others are greatly inhibited. The concept of sequestration of metals from organisms by catechin is intriguing as other studies have shown that plants which produce a strong organic acid chelator to garner metals from the environment can up-regulate the amount of chelator exuded from their root systems when exposed to catechin (Weir, *et al.*, 2006). One such organic acid, oxalate, was shown to be helpful in protecting *Lupinus sericeus* and *Gaillardia grandiflora* from the harmful effects of catechin, and was also capable of protecting other native plants from catechin phytotoxicity (Weir, *et al.*, 2006). While the effects that metals have on (±)-catechin phytotoxicity have yet to be investigated, it is

possible that catechin-metal complexes (CMCs) are of great importance to the overall success of spotted knapweed.

Influence of soil chemistry on catechin phytotoxicity/stability

The ability of catechin to sorb to various soil components has interesting implications regarding current methods of catechin extraction and quantification from soil matrices (Blair*, et al.*, 2005, Thelen*, et al.*, 2005, Perry*, et al.*, 2007, Blair*, et al.*, 2009). Most researchers report low extraction efficiencies of catechin from soil, with the exception of Blair *et al.* (2005), who reported extraction and detection efficiency of catechin amended to soil of greater than 80% and that from the addition of as little as 5 ppm catechin. They also reported rapid reduction in extractable catechin in these systems as a function of time, which may help explain the seemingly contradictory results of other researchers. This is of particular importance to past, current, and future research on this topic as the concentrations of catechin reported for knapweed-infested field soils is highly variable, yet greatly influences the concentrations of catechin employed in a variety of laboratory and field experiments to assess its phytotoxicity and other effects. As chemically pure catechin is highly reactive as a function of pH, metal content, and other undetermined parameters, accurately monitoring catechin in the soil is extremely difficult. The rationale for this is that catechin exuded or otherwise introduced into soil may rapidly be converted to a derivative form (e.g. CMCs, oxidized forms, or polymeric complexes of catechin) that currently available analytical methods are not capable of detecting. As a result, it is never clear how much pure catechin is present in soil, or how many catechin-derived compounds exist in knapweed-invaded systems. Keeping in mind that derivative

forms of catechin may have more, less, or the same level of phytotoxicity, severely complicates attempts to understand the ecology and biochemistry of knapweed and catechin, and has certainly contributed to at least some of the current debate regarding its role and importance in knapweed invasiveness.

Evidence of this debate can be seen by reviewing studies that have shown a high degree of variability in regard to the ability of spotted knapweed to damage neighboring plant species, sometimes because of the broad range of catechin concentrations employed in these experiments (Blair*, et al.*, 2005, Perry*, et al.*, 2005). For example, some studies have found that knapweed and/or catechin had no effect in their soil system (Blair*, et al.*, 2005, Duke*, et al.*, 2009), while other studies report high levels of phytotoxicity (Bais*, et al.*, 2003). To be sure, researchers have yet to determine why spotted knapweed is a more aggressive invader in some environmental settings than in others.

Questions addressed within each chapter

The following questions have motivated the research of this dissertation in the examination of catechin interactions with soil chemistry, its impact on plant phytotoxicity, as well as its reported antimicrobial effects and the mechanisms that might underlie these.

1) Does soil chemistry affect catechin stability and influence the ability to effectively quantify catechin concentrations in the environment?

In chapter 2, I test the hypothesis that soil chemistry can greatly affect catechin stability, as well as our ability to quantify catechin in various soil systems. This study is the first of its kind to assess and compare the methods employed in other studies under a variety of media and soil parameters (e.g., metal and organic acid content, soil pH, moisture content). Because of its importance to this topic, I again note that current catechin quantification methods are based on the detection of chemically pure catechin in an HPLC-based assay, and that most or all chemically modified forms of catechin would not be detected by this approach regardless of residual toxicity. In some instances, catechin concentrations were immediately lowered and precipitates were formed in abiotic reactions with metals commonly found in soil. How this might affect metal availability to organisms is discussed in chapter 5, as well as in several extant and pending publications by this and other groups. One key study has shown that animals fed a diet high in tea catechins, similar in structure to catechin in our studies, exhibited a decrease in overall gut microflora biomass with the exception of lactic acid bacteria, which increased in number (Ishigami & Hara, 1993, Hara, 1997). Interestingly, this group of microbes is the only reported group of organisms that has no metabolic requirement for iron (Archibald, 1983, Boyaval, 1989, Bruyneel*, et al.*, 1989, Pandey*, et al.*, 1994), findings which support my conclusions regarding inhibitory effects of catechin on some soil microbial populations based on sequestration of essential metals.

My findings demonstrate that catechin is a highly reactive compound, subject to absorption to soil particles, pH effects, and interactions with metals, molecular oxygen, and organic acids. These reactions impede our ability to effectively quantify catechin in the laboratory and in the environment because altered forms of catechin differ in both

mobility and UV absorbance when analyzed by HPLC. My work helps demonstrate the instability of (±)-catechin and catechin-like compounds in the environment, and may help explain some of the discrepancies in the current literature regarding the amount of (±)-catechin in the environment and the concentrations of (±)-catechin that produce phytotoxic effects.

2) Do catechin-metal interactions impact the phytotoxicity of catechin to native plant species?

I hypothesize in chapter 3 that, by interacting with common soil metals (see chapter 2) and forming catechin-metal complexes, (±)-catechin can have conditional phytotoxic effects. This chapter includes work on catechin stability in a pH-buffered system (in contrast to the unbuffered system tested in Chapter 2). The change from an unbuffered system to this phosphate-buffered (pH 7) system was necessary, primarily because any addition of iron, lead, or copper to the solutions lowered the pH of the system dramatically and would thereby confound phytotoxicity assays. In these experiments, I determined the phytotoxic effects of catechin on two native Montana plant species and showed that the results are conditional based on the addition of different metals (e.g. iron or copper) to the system and that detectable catechin concentrations were highly variable dependent on the reacting metal. These findings address the variation of catechin phytotoxicity reported in other studies (Blair*, et al.*, 2005, Perry*, et al.*, 2005), and help to explain why spotted knapweed is a more aggressive invader in some environmental

settings than in others. This "conditionality" may also be important with regard to the impact of catechin on the microbial community or its component populations in soils.

3) Does exposure to knapweed, and by extension catechin, select for resistance or tolerance to the effects of catechin in soil microbial populations?

Chapter 4 is one of the first studies to test the overall impact of catechin exposure on native soil bacterial communities. First, I examined the hypothesis that catechin inhibition is dose-dependent and acts against only some bacterial populations while others are unaffected. I next tested the hypothesis that more catechin-resistant populations would be present in soils previously exposed to knapweed than in soil that had not been invaded by spotted knapweed (i.e. naïve soils). By testing soils from sites from Europe and the U.S. that have experienced spotted knapweed (as a native and an invasive plant, respectively) and sites in U.S. that have not been invaded by spotted knapweed, I have selected for a variety of soil bacterial communities and populations both naïve and experienced to (±)-catechin. This work has led to the isolation of several bacterial populations that appear to be unaffected by (±)-catechin. Interestingly, two groups exhibited normal growth in the presence of (±)-catechin; one with the ability to produce siderophores, presumably enhancing metal uptake capabilities, while the other group partially transformed (degraded) (±)-catechin, which presumably mitigated its inhibitory effects. Additionally, I demonstrated that when catechin is removed from the system, the bacterial population would rebound, effectively demonstrating a short-term bacteriostatic (rather than bactericidal) effect of catechin on bacterial species.

4) Does catechin influence transport of carbon sources into bacterial cells?

A previous soil microbial study has suggested an impact of catechin on bacterial metabolic processes related to nitrogen cycling in soil (Inderjit*, et al.*, 2009). Additionally, since shifts in predominant bacterial populations or in patterns of gene expression within the bacterial community can greatly impact nutrient cycling, understanding the impact of catechin on carbon acquisition/utilization by specific bacterial species is important to understanding its impact on microbially-controlled soil processes. In chapter 5, I address the influence of catechin on the transport of carbon sources commonly utilized by soil bacteria. This is the first study of its kind to examine the impact of catechin on specific metabolic processes of soil bacteria. I hypothesized that the chelating ability of catechin explored and discussed in chapters 2 and 3 might prevent bacteria from obtaining metals necessary for proper enzymatic function and thereby prevent normal cell growth and/or metabolism. These experiments ultimately demonstrated that bacterial cells increase in number yet do not divide normally, but clearly are not destroyed in the presence of catechin. These experiments also demonstrated that cells could still metabolize carbon normally and that that simple organic acids capable of chelating metals restored growth to catechin-inhibited bacterial populations.

5) Does catechin alter normal bacterial cellular functions (e.g., cell morphology, endospore formation, and endospore germination) via chelation of essential trace metals?

Many bacterial functions have been shown to be sensitive to metal concentrations, including cell division, endospore formation, and endospore germination (Nickerson &

Sherman, 1952, Rochford & Mandle, 1953, Shankar & Bard, 1955, Church & Halvorson, 1959). It is therefore plausible that catechin, through its ability to chelate metals (Gomah & Davies, 1974, McDonald*, et al.*, 1996, Lim*, et al.*, 2005, Pollock*, et al.*, 2009), could interfere with normal cellular functions by sequestering necessary metals from the surrounding environment, thereby making them unavailable to bacterial cells. Chapter 6 represents the first study to directly assess the effect of catechin on endospore-forming soil bacteria. I test the hypothesis that catechin will alter normal cell growth and morphology, and endospore formation and germination in endospore-forming Gram + bacteria commonly found in soil. These experiments also demonstrate that these effects mimic those produced by the omission of essential metals in catechin-free media, suggesting that the observed effects result from metal sequestration by catechin. Additionally I demonstrate that pyruvate and malate, two simple organic acids previously shown to restore growth to catechin-inhibited bacterial species (see Chapter 5), restore one or more of these functions to the cells/endospores. At least one plausible explanation for these observations is the ability of malate and pyruvate to chelate various metals (Gardner*, et al.*, 1983, Drechsel*, et al.*, 1993, Keerthisinghe*, et al.*, 1998, Chen & Liao, 2003). This may be occurring in competition with catechin in the system, thereby bringing these metals into cells via cellular transport mechanisms and thus restoring normal growth functions in the presence of catechin.

Broader significance of this work

Allelopathy is defined as the effect elicited by a chemical that is produced by an organism (either a plant or microorganism) that impacts another organism of a different species in either a beneficial or detrimental way (Molisch, 1937, Rice, 1974). However, in the current plant ecology literature, allelopathy is more commonly considered to be a chemical attack between plant species without regard to beneficial effects (Putnam, 1985, Boyette & Abbas, 1995) or microbial involvement (Putnam & Duke, 1978, Putnam, 1985, Pratley, 1996). Allelopathy is generally considered to be one of many mechanisms involved in invasive weed establishment and maintenance of spotted knapweed (Callaway & Aschehoug, 2000, Baldwin, 2003, Fitter, 2003, Callaway & Ridenour, 2004, Callaway*, et al.*, 2004). Most researchers studying allelopathy do not take into account other, more indirect, mechanisms by which one plant can affect the growth of another through chemical attack. My thesis contributes substantial new information on indirect mechanisms of catechin activity that involve biogeochemical interactions and modifications as well as effects on the bacterial component of the soil microbial community, both of which can greatly affect the growth of other plants in the community.

Root exudates provide several essential ecosystem services: carbon source additions to surrounding microbiota (Rovira, 1969), mineral and phosphate solublization (Rauser, 1999, Watanabe & Osaki, 2002, Thorpe*, et al.*, 2006), and toxic metal resistance (Kidd*, et al.*, 2001). Invasive plant establishment and subsequent root exudation directly alters native plant communities, in many instances forming near monocultures (Callaway*, et al.*, 1999, Rout & Chrzanowski, 2009). Knapweed invasion may also alter soil chemical

and biological properties altering soil bacterial assemblages through a variety of mechanisms related to the presence of catechin. Several groups have attempted to quantify the amount of catechin in soil (Bais*, et al.*, 2002, Blair*, et al.*, 2005, Perry*, et al.*, 2005, Blair*, et al.*, 2006, Perry*, et al.*, 2007). However, accurate determination of the amount of catechin in soil is clouded by limitations of extraction efficiency, the detection method employed, and differences in experimental design (e.g. pulsed versus single addition of catechin, selection of time-points for analysis). Additionally, it is well established that root exudate concentrations rapidly drop off with distance from the root (Kuzyakov*, et al.*, 2003, Sauer*, et al.*, 2006).

Thus, accurate detection of catechin concentrations in soil is essential to assessing the impact of catechin on native plant and microbial species. My work has compared currently used methods for extracting and measuring (±)-catechin concentrations in the environment and shows that the amount of pure catechin detected does not accurately represent the amount put into the soil, nor the biologically active concentrations that actually exist. This may explain the equivocal nature of data and discussions in this area of investigation.

Lastly, as catechin and other polyphenolic compounds are highly reactive with the chemistry of the system, be it media or soil, testing for catechin stability and transformation is instrumental in understanding how catechin functions in the soil environment. These and subsequent studies will lead to a better understanding of catechin effects on both plants and soil bacteria (and interactions between both) in the

complex ecosystem that soil represents. The work presented in this thesis provides insight into the ability of catechin to impact soil microbial populations, and assesses how soil chemistry influences catechin stability, toxicity, and quantification. This work also contributes information that extends on current knowledge in the fields of invasive plant ecology as well as the phenomenon of allelopathy.

My work has also elucidated some mechanistic aspects of the effects of (±)-catechin on soil bacterial populations and explores the potential ramifications of population-specific alterations of the bacterial community on plant : microbe interactions. I have also demonstrated that the ability of (±)-catechin to react to/with soil pH, metals, and organic acids alters its phytotoxic properties and potentially its interactions with the soil bacterial community. More importantly, these findings can help explain some of the seemingly equivocal and contentious reports on the effects of catechin related to knapweed invasiveness. I believe that, collectively, this knowledge might ultimately contribute to control of the spread of *Centaurea stoebe* as an invasive weed in North America and elsewhere.

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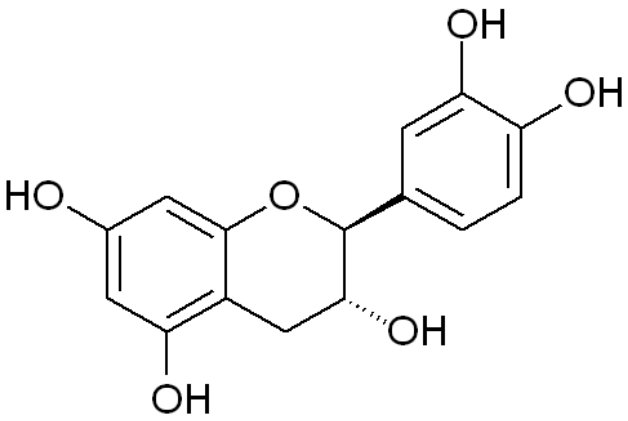
Figures

A)



B)

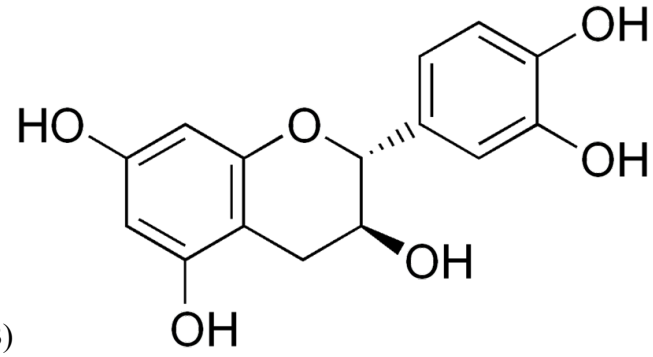


Figure 1.1

Chemical structure of catechin isomers. Panel A: (-)-Catechin; Panel B: (+)-Catechin.

Chapter 2

ABIOTIC FACTORS THAT IMPACT CATECHIN STABILITY AND EXTRACTION EFFICIENCY IN MEDIA, SAND, AND SOIL

Abstract

The topic of allelopathy is an area of intense and growing interest and affects the way we currently think about plant invasion and other ecological phenomena. However, determining the reality and efficacy of a putative allelopathic compound can be extremely difficult. The ability to attribute allelopathic effects to plant and microbial growth parameters in an invaded system is key to establishing treatments that can alleviate these effects, thereby allowing the native community and ecosystem to recover. *Centaurea stoebe* Lam. (spotted knapweed) exudes into its rhizosphere (±)-catechin, a polyphenolic substance whose activities in soil have yet to be fully studied and understood for both plant and microbial systems. Variable results regarding the phytotoxic impact (high, low, or none) of catechin on indigenous plants have led to conflicting views on the role and significance that catechin has as an allelopathic compound. Complicating things further, differences in experimental design details such as method of catechin application, quantification methods, and techniques for its extraction from soil vary greatly from lab to lab. As a consequence, the amount of catechin present in and extracted from soil is hotly debated between different research groups. However, knowing the true bioavailable concentration of catechin in a given system is key to the design of ecologically relevant experiments to assess its ecological role(s). Here, we report on how a variety of abiotic factors commonly associated with differences in soil composition

(e.g. pH, metals, aerobicity) affect catechin extraction and quantification efficiency. The relative efficiency of extraction of catechin from a variety of matrices (e.g. buffer, sand, soil) commonly used in experiments is also evaluated and discussed.

Introduction

Centaurea stoebe (spotted knapweed) has been shown to exude (±)-catechin, into the surrounding rhizosphere. This compound has been shown to have both phytotoxic effects on native plant species (Callaway*, et al.*, 1999, Pollock*, et al.*, 2009) in North America and bacteriostatic effects on some soil bacterial populations (Callaway*, et al.*, 2004, Inderjit*, et al.*, 2009). However, some researchers have reported only mild to no phytotoxic effects (Blair*, et al.*, 2005, Blair*, et al.*, 2006, Duke*, et al.*, 2009) and call into question the notion that catechin is serving a role in allelopathy by spotted knapweed. Although the purpose of each of these prior experiments was to test the potential for allelopathic effects from spotted knapweed, the levels of catechin used, their modes of application, and the soils involved varied from lab to lab. The catechin levels chosen by each research group were based on the amounts of catechin they extracted from field soils where knapweed was actively invading, and these varied widely from group to group, soil to soil, and even time to time. I suggest that the amount of catechin used in those prior experiments and the extraction and analytical techniques employed have contributed to the equivocal results obtained and have fueled the controversy over the effects and ecological role of catechin.

Given these issues, it is obvious that the ability to effectively detect catechin from field soils and in constructed environments is of great importance. The problems associated with catechin detection in environmental or laboratory samples are three-fold. First, the longevity of pure catechin in soil is highly variable (Blair*, et al.*, 2005), reportedly as a result of the unknown amount of catechin exudation into the soil (Weir*, et al.*, 2003,

Blair, *et al.*, 2005), the ability of catechin to be sorbed by soil (Blair, *et al.*, 2005, Furubayashi, *et al.*, 2007), and the ability of catechin to readily react with various chemicals commonly found in soil (McDonald, *et al.*, 1996, Es-Safi, *et al.*, 1999, Lim, *et al.*, 2005, Pollock, *et al.*, 2009). Second, the ability to effectively extract catechin, a polar chemical, from soil samples presents a significant challenge. Several methods have been developed to extract catechin from soil with varying degrees of success, presumably because of its ability to sorb to chemical components of soil that vary dramatically from site to site (Blair, *et al.*, 2006). Third, the extraction efficiency of catechin from soil has largely been tested through the ability to subsequently quantify chemically pure catechin. Currently, this is accomplished through high-performance liquid chromatography (HPLC), which is highly specific for single forms of a given compound based on its mobility (elution time) when passing through an HPLC column of a given composition and liquid elution phase. As such, even a slight modification of catechin (e.g. hydroxylation, loss of protons) can alter its mobility rendering it "invisible" to this assay. Beyond that, a review of the catechin literature shows that even this HPLC procedure is not standardized between laboratories, with scientists using a variety of solvents to produce catechin stock solutions, various HPLC columns and different mobile phases, and even different wavelengths utilized to detect the catechin peak as it is eluted (Bais, *et al.*, 2002, Weir, *et al.*, 2003, Blair, *et al.*, 2005, Perry, *et al.*, 2005, Pollock, *et al.*, 2009).

Herein, I examine abiotically the interactions of catechin with a number of soil, matrix, and media chemical parameters, both immediately upon introduction into the system, as well as over time. Principally important chemical parameters that directly impact

chemically pure catechin are pH and the metal concentrations in the system as well as its tendency to auto-oxidize. This new information may help to determine the environmental controls on naturally occurring concentrations of catechin in knapweed-infested soils and by extension the anticipated impact on native plant and microbial species in various soil types in North America.

Material and Methods

Sources of Chemicals

Suppliers for the chemicals used in these experiments (with the single-letter abbreviations used hereafter) were: Acros Organics (A; Acros Organics; Somerville, NJ), Sigma-Aldrich (S; Sigma-Aldrich, St. Louis, MO), Difco (D; Difco Laboratories Incorporated, Franklin Lakes, NJ), Fisher (F; Fisher Scientific, Pittsburgh, PA), JTBaker (J; JT Baker, Phillipsburg, NJ), and EM Science (E; EM Science, Gibbstown, NJ), unless otherwise stated, and all were of reagent grade quality or higher. Chemicals employed in these experiments were: 2-(N-morpholino)ethanesulfonic acid (MES) hydrate (A); sodium phosphate monobasic monohydrate (S); sodium phosphate dibasic heptahydrate (S); methanol (F); ferrous chloride tetrahydrate (S); ferric chloride (E); cupric chloride (F); calcium chloride dihydrate (S); magnesium chloride (S); lead chloride (F); manganous chloride (J); cobalt chloride (J); sodium chloride (F); aluminum chloride (EMD chemicals Inc., Gibbstown, NJ); sodium bicarbonate (S); phosphoric acid (J); ethyl acetate (J). A 50:50 racemic mixture of (±)-catechin·hydrate (>99% purity) was obtained from Shivambu International (New Delhi, India), hereafter referred to solely as catechin.

Sources of soils

Soils employed in these experiments were obtained from various locations. Bulk soil from the Clearwater Game Range, near Clearwater Junction, Montana (47.08°N/113.04°W) classified as a Winkler gravelly loam was collected, pooled and sieved to represent a naïve Montana soil that has never experienced *C. stoebe* invasion, nor been treated to prevent it (M. Thompson, *personal communication*). When employed in an experiment, this soil will be referred to as naïve MT soil.

Soil samples were collected from 5 sites in Romania, where *C. stoebe* is native, and 5 sites in Montana where *C. stoebe* is an aggressive exotic invader. The locations of the Romanian sites were at 47.13°N/26.29°E, 46.5°N/26.56°E, 47.09°N/27.35°E, 47.10°N/22.52°W, 45.51°N/27.26°E and the locations of the Montana sites were at 46.35°N/112.04°W, 46.51°N/113.59°W, 46.60°N/113.57°W, 46.10°N/113.46°W and 48.52°N/115.03°W. The soil samples were collected from grasslands in which *C. stoebe* was present, but were taken from the rhizosphere environment of the most abundant native grasses, rather than from the *C. stoebe* rhizosphere as in many of the other experiments.

In India, soil (sandy loam; pH, 7.7; organic matter, 0.95%) was collected from under native vegetation of Delhi, India (28.38°N/77.12°E). This soil has no history of exposure to any *Centaurea* species and is referred to as "naïve India soil" when employed. All soils, with the exception of the Clearwater Game Range soil, were collected courtesy of Giles Thelen and Inderjit.

HPLC analysis

All catechin concentrations in the experiments outlined below were determined by HPLC analysis as previously described (Paveto*, et al.*, 2004). In brief, catechin concentrations were determined by analysis of pre-filtered (0.22 μM) aqueous samples (15 μL injection volume) with UV detection at 280 nm on a Hewlett-Packard (HP) series 1100 HPLC (Hewlett-Packard, Palo Alto, CA) using a HP ODS Hypersil C18 column (5 μm, 125x4 mm) and a 100% methanol : 20 mM phosphoric acid (1:3 v/v) mobile phase at a flow rate of 1 $mL \cdot min^{-1}$. The sensitivity and upper limit of this protocol for the detection of catechin ranges from 5 $\mu g \cdot mL^{-1}$ to 2000 $\mu g \cdot mL^{-1}$ on a linear scale. A standard curve was generated by creating known concentrations of catechin dissolved in 100% methanol, ranging from 5 $\mu g \cdot mL^{-1}$ to 1000 $\mu g \cdot mL^{-1}$ (final concentration). These were used in every experiment described in this chapter to facilitate accurate quantification of unknown catechin samples.

pH stability of catechin

A common parameter that varies fairly widely between field soils is pH. Therefore, an experiment to test the stability of catechin across a wide range of pH values was performed. Two types of buffers: (100mM MES, effective buffer range pH 4-6; and 100mM sodium phosphate, effective buffer range pH 6-9) were used to determine whether catechin stability varied as a function of pH. A series of solutions buffered to specific pH values were autoclaved, allowed to cool, and amended with catechin. Catechin was added from a stock solution of 100 $mg \cdot mL^{-1}$ catechin in methanol to

provide the appropriate starting concentration of 1000 $\mu g \cdot mL^{-1}$. All mixtures were shaken to ensure thorough mixing and the pH of each solution was determined at time zero using a Corning 340 pH meter (Corning Inc., Corning, NY, USA). All solutions were incubated statically in the dark at room temperature. At each time point, the mixtures were vortexed briefly before sampling for analysis by HPLC.

Impact of various metals on catechin stability in an unbuffered system

Several studies have shown that catechin and similar compounds can chelate metals such as iron, zinc, copper, and aluminum (McDonald*, et al.*, 1996, Es-Safi*, et al.*, 1999, Kidd*, et al.*, 2001, Lim*, et al.*, 2005, Pollock*, et al.*, 2009). As such, it is important to determine if catechin reacts with metals that are common to soil environments or employed in laboratory media commonly used in related experiments. To assess this, catechin was dissolved at 1000 $\mu g \cdot mL^{-1}$ in sterile double distilled water by gentle heating. Aliquots of 15 ml of this solution were dispensed into 50 ml conical tubes in triplicate for each treatment. Metal chlorides were added to the catechin solution at equimolar concentrations (≈3.24mM) to test their reactivity with catechin. These were ferrous and ferric chloride, cupric chloride, calcium chloride, magnesium chloride, lead chloride, manganous chloride, cobalt chloride, sodium chloride, and aluminum chloride. The pH of each solution was determined at time zero and all treatments were incubated statically in the dark at room temperature. Before sampling at each time point, the mixtures were vortexed briefly and then filtered through 0.22 μm filters to remove any precipitates. Filtered samples were assayed for catechin concentrations by HPLC as described above.

A similar experiment in a pH-buffered system was performed with 5 types of metals as outlined in Fig. 1 of Chapter 3 (Pollock*, et al.*, 2009).

Concentration-based effects of iron on catechin stability

As ferric iron in the above experiment had an instantaneous reaction with catechin (immediately lowering the level of pure catechin), an iron concentration experiment was performed to determine whether this phenomenon is dose-dependent. As described in the previous experiment, 1000 $\mu g \cdot mL^{-1}$ of catechin was dissolved in sterile ddH_2O by gentle heating, and then 15 ml aliquots were dispensed into 50 ml conical tubes in triplicate for each treatment. Ferric chloride was added at half molar (~1.6 mM), equimolar (~3.2 mM), and 2X molar (~6.5 mM) concentrations relative to the catechin concentration. As before, all treatments were incubated statically in the dark at room temperature. Each mixture was vortexed briefly just prior to sampling and the samples were filtered using 0.22 μm filters prior to analysis by HPLC for pure catechin concentrations. The pH of each solution was determined before the start of the catechin readings as described above and recorded in Figure 3.

Catechin auto-oxidation under laboratory conditions

To determine if the initial concentrations of catechin used in petri dish-based plant germination experiments were stably maintained until seedling emergence, we tested the stability of catechin over time under the experimental conditions employed. Increasing concentrations of catechin (125, 250, 500 and 1000 $\mu g \cdot mL^{-1}$) were dissolved in sterile double distilled water by gentle heating (≈80° C). For each catechin concentration,

quadruplicate 5 mL aliquots were pipetted into sterile petri dishes containing a sterile Whatman #1 round filter paper. Immediately, 150 μl of liquid sample was removed from each plate, diluted 1:1 with 100% methanol, filter sterilized using a 0.2 μm filter, and analyzed by HPLC to precisely determine the starting catechin concentrations and assess whether catechin was reactive to the filter paper. Five seeds of *Festuca idahoensis* were distributed onto the filter paper in each petri dish and the dishes sealed with parafilm and incubated statically in the dark at room temperature. Upon seedling emergence (i.e. at 10 days), the catechin solutions were again sampled as just described and also upon completion of the seedling emergence experiment (i.e. at 14 days).

Extraction of catechin from sterile sand

Applied concentrations of catechin almost universally result in far lower measured concentrations in media, sand, and soil than predicted from the application rate (Inderjit, *et al.*, 2008). This may be due to interactions of catechin with components of the media, sand, or soil, or to catechin transformation or modification via auto-oxidation. This phenomenon makes the determination of phytotoxic or bioactive concentrations of catechin in natural plant/soil systems based on applied doses in controlled experiments extremely difficult. To better understand this phenomenon and in an attempt to calibrate the applied concentrations in the current experiments to measured soil concentrations from the literature, the fate of catechin added to sand was monitored over a seven-day period with quadruplicate replicates. A 300 μg ml^{-1} stock solution of pure catechin was prepared in ddH_2O by gentle heating as already described and added to 50 g of 20/30 grit sand to achieve a final concentration of 100 μg g^{-1} of sand. The samples were incubated

statically in the dark at room temperature and ¸1 g subsamples were taken for catechin extraction and analysis by HPLC at t = 1hr, 3 days and 7 days.

Impact of increased pH and lead content on catechin extraction from soil

The experiments described above examined the reactivity of catechin to both the pH of the system and metal species commonly found in soils. To test whether the extraction efficiency of catechin from soil is affected by either of these two parameters in a simple soil extraction experiment, we used a soil naïve to spotted knapweed, and by extension to catechin. Naïve MT soil was air-dried and sieved (2 mm), then brought up to 40% water holding capacity, and split soil into three containers (20 g each). One soil sample served as an unmodified control, while another was amended with sodium bicarbonate (20 mM final concentration) to raise the soil pH from 5.8 up to 7.5. The third soil sample was modified by increasing its lead (a representative soil metal) content to 5 $\mu g \cdot g^{-1}$ by the addition of lead chloride with thorough mixing. Catechin was dissolved in 100% methanol, and added to each of the soils to a final concentration of 1000 $\mu g \cdot g^{-1}$. All soils were thoroughly mixed before sampling for catechin analysis both initially (i.e. ~1hour after initial addition) and three days later. Eight 1g samples were taken from each treatment at each time-point for replication of analysis. Each individual sample was combined with 1mL of 100% methanol, vortexed for 30 seconds, centrifuged for 5 minutes at 13,000 rpm, and the supernatant filtered through a 0.22 μm filter prior to HPLC analysis for catechin.

Comparison of native (Romania) and invaded (Montana) soils for catechin retention

As soils can be highly variable in both chemical content and microbial community composition and activity, I also tested the stability and extractability of catechin in soils where *C. stoebe* grows. In this comparison, variation in the extracted amount of a single experimentally applied pulse of catechin among soils from 5 sites in Romania (native range) and 5 sites in Montana (invaded range) was assessed.

For each of the 10 sites (described in the *Sources of Soils* section above), 50 g of soil was placed into each of five 50 ml vials (standard borosilicate liquid scintillation vials were used). Each soil sample was treated with a single dose of 10 ml of 2,500 μg ml^{-1} of catechin dissolved into water by gradually warming the solution to ≈80° C, followed by thorough mixing into the soil samples to produce an initial soil concentration of 500 μg g^{-1}. The vials were incubated in the dark at room temperature because sunlight appears to increase the auto- or photo-oxidation rate of catechin (J. Pollock, personal observation). This 500 μg g^{-1}concentration is similar to the mean concentration of catechin pulses measured by Perry and co-workers (Perry*, et al.*, 2007) in soils surrounding *C. stoebe* plants. It is also worth noting that preliminary experiments by our group indicated that a pulse of this magnitude was necessary to detect even trace amounts of pure catechin in the soils after several days of incubation. Soil concentrations of catechin were measured immediately after application (t = 0) and after 1, 3 and 10 days of incubation using the extraction and HPLC methods described above. The effects of region of soil origin (fixed), and site (random and nested within a region) on catechin concentration were tested using a separate ANOVA for each time point (fixed). These tests were performed using the PROC GLM module within SAS using Type III sum of squares (version 9.1).

Effect of multiple pulse addition on soil catechin concentrations

To assess the ability of catechin to accumulate to high levels after multiple additions (pulses), we tested a naïve India soil in this format in conjunction with an experiment performed by our Indian collaborators. In both India and Montana, catechin was dissolved in 100% methanol and then further diluted 1:20 into ddH_2O to obtain final stock concentrations of 0, 500, 1,000, and 1,500 μg ml^{-1}. Soil microcosms were created by adding 50 g of soil to each of twenty-four 85-ml glass vials. Sets of six replicates for each concentration were repeatedly treated with catechin solutions at either 500, 1,000 or 1,500 μg ml^{-1} as follows: an initial application of 15 ml of catechin at each concentration was added, followed by three more applications with 5 ml of each treatment concentration at days 3, 7, and 10, followed by a final treatment with 4 ml of each treatment concentration of catechin on day 14. Thus, each set of replicate vials was treated with a total of 34 ml of either 0 μg, 500 μg, 1,000 μg or 1,500 μg ml^{-1} catechin in pulses that cumulatively produced additions of 0 μg (control), 340 μg, 680 μg and 1,020 μg catechin g^{-1} soil, respectively. To quantify catechin, a 1 g soil sample from each vial was extracted with 1 ml of 100% methanol, mixed by vigorous vortexing for 30 seconds, pelleted by centrifugation for 5 min at 13,000 rpm, and the supernatant filtered and analyzed by HPLC as described above. To determine the stability and maximum concentration of catechin accumulated in soil using this pulsed addition format (designed to represent what is thought to be the exudation behavior of plants), we sampled immediately after the initial application, after the applications on days 3 and 7, then both before and after application on days 10 and 14, and finally on day 17.

Testing the efficacy of various solvents for extraction of catechin from soil

Catechin extraction efficiencies have been reported to be highly variable (Blair*, et al.*, 2005). The ability to accurately detect pure catechin in field samples is of utmost importance to the topic of allelopathy, as measured values are used to guide experimental design to test for allelopathic effects. For this experiment, naïve MT soil was used to compare the efficacy of extraction of a known amount of added catechin, by a variety of extraction solvents. Catechin (20 mg) was dissolved in 1ml of 100% methanol, then added to 40 g of soil and thoroughly mixed to yield a final concentration of 500 $\mu g \cdot g^{-1}$ of soil. A suite of solvents that included 100% methanol, 20 mM phosphoric acid, water, ethyl acetate, and ethyl acetate:water (1:1 v/v) were individually tested (in triplicate) and compared for their efficacy in extracting this known amount of catechin immediately after its addition to soil. Extraction was performed by adding 1 g of soil to 1ml of each extraction solvent or mixture, followed by thorough mixing by vortexing for 30 seconds, then centrifugation for 5 min at 13,000 rpm. The supernatants were filtered through a 0.2 μm filter for analysis by HPLC as described above. Preliminary work by Branden Belknap (an undergraduate researcher in our group) had demonstrated that catechin was concentrated into the ethyl acetate phase during ethyl acetate:water extraction from soil (data not shown). Therefore, only the ethyl acetate phase was analyzed for this treatment.

Results

pH impacts catechin stability

Catechin was more stable under acidic conditions (Fig. 1). The lowest pH tested (4.0) showed only a 1.79 ± 0.25% [1SE] decrease from the starting concentration of 1000 $\mu g \cdot mL^{-1}$ after 21 days, while increasing pH values up to 9.0 resulted in increasingly greater reduction in the amount of pure catechin remaining to below detection levels (<5 $\mu g \cdot mL^{-1}$) after 21 days (Fig. 1). Interestingly, where the MES and PB buffers overlapped in pH, the PB solution showed less decrease in catechin concentration for a given pH value.

Metals common to soils have variable effects on catechin transformation

Various metals commonly found in soil have substantial impact on catechin stability, both instantaneously (≤1 hour) and over time (Fig. 2). The catechin only solution, and by extension each of the other solutions containing metals, started at 1000 ± 0.80 $\mu g \cdot mL^{-1}$ [1SE] catechin according to HPLC analysis, and loss due to simple auto-oxidation was modest with ≥90% of pure, soluble catechin remaining after 27 days of incubation. Ferric iron-catechin interactions resulted in immediate precipitate formation followed by a slower phase of additional precipitation, ultimately decreasing the concentration of catechin in solution to below 20%. Ferrous-catechin, and cupric-catechin interactions also formed precipitates by the first sampling point (~1 hr), but these were less than for ferric iron and subsequent precipitation proceeded more slowly, yet ultimately both reduced pure catechin concentrations in solution to a similar degree as for ferric iron. To be more specific, the initial decrease in catechin concentration for ferric iron reduced the pure catechin concentration in solution to 63.42 ± 0.15% [1SE] of the starting catechin concentration after 1 hr of exposure. However, ferric iron was not the most reactive

metal over the time course of the experiment since ferrous iron decreased the catechin concentration to 6.05 ± 0.26% after 27 days, whereas ferric iron decreased the catechin concentration to 15.22 ± 0.69% [1SE] within the same timeframe. Cupric copper-catechin interactions decreased catechin to 23.50 ± 1.68% of the starting concentration over the same time course.

Manganese and cobalt also exhibited an immediate though modest reduction of catechin concentration in solution, but over the 27-day course of the experiment only reduced the solution concentration of catechin to 80.0% and 88.9% of the starting concentration, respectively. Presumably, the precipitates that formed in each case were either catechin-metal complexes (CMCs) or polymerized catechin as will be further discussed in Chapter 3. Listed in terms of *initial reaction* (i.e. first sampling time point at ~1 hour), the order of reactivity to catechin was: $CAT \approx Ca^{2+} \approx Mg^{2+} \approx Na^{+} \approx Al^{3+} \approx Pb^{2+} < Fe^{2+} < Mn^{2+} < Co^{2+} \approx Cu^{2+} < Fe^{3+}$. After the 27-day incubation, the order of reactivity (least to greatest, based on pure catechin remaining in solution) was: $Al^{3+} \approx Mg^{2+} \approx CAT \approx Ca^{2+} \approx Na^{+} < Co^{2+} < Mn^{2+} < Pb^{2+} < Cu^{2+} < Fe^{3+} < Fe^{2+}$.

Ferric iron immediately lowers catechin levels in a dose-dependent manner

Upon finding that ferric iron had a high affinity for catechin, exhibiting immediate reactivity (refer Fig. 2 and previous section), the ability of ferric iron to react with a given amount of catechin at various metal concentrations was tested (i.e. a dose-response format was used for this experiment). Ferric iron again exhibited the ability to immediately (≤ 1 hour) lower the level of pure catechin remaining in solution. As

expected, the amount of decrease in pure catechin was proportional to the concentration of ferric iron added. For example, the initial loss was ≈21% for 1:2 Fe^{3+}:CAT ratio; ≈38% for 1:1 Fe^{3+}:CAT ratio; and ≈70% for 2:1 Fe^{3+}:CAT ratio (Fig. 3). However, the slower phase of reactivity of ferric iron with catechin over time did not appear to be dependent on the amount of ferric iron added, as the slopes for all three ferric iron additions appeared similar.

Catechin stability in seedling germination experiments

Consistent with my previous findings, catechin concentrations decreased over time in the petri dishes used for this experiment (Fig. 4). A browning of the roots that touched the catechin-soaked filter paper was also observed. However, the decrease in catechin by 10 and 14 days (averaging ~50% and ~90% loss, respectively) were significantly more than was observed in previous experiments (see Figs. 2 & 3). Whether this is a function of the greater surface area in petri dishes (i.e. the ability of O_2 to diffuse into the solution) or some poorly understood reaction with the filter paper, the plastic petri dish, or the seedlings is unknown. However, a similar study of catechin stability with a starting concentration of 500 $\mu g \cdot ml^{-1}$ employing sterile **glass** petri dishes (no seeds or filter paper were present) resulted in similar rates of catechin decrease (data not shown), suggesting that the broad surface area of the petri dish format for experiments was the driving mechanism behind the observed rapid decrease in catechin.

Catechin extraction from sterile sand

To test the ability to extract catechin from solid matrices, I first tested the stability and extraction efficiency of catechin added to sterile sand (Fig. 5), as there are far fewer complex and poorly understood variables in sand than in complex soil systems. Extremely poor extraction efficiency was obtained using 100% methanol, with less than 12% recovery after just one hour of incubation. However, extracted catechin levels were stable after this initial large decrease for at least 7 days (9±4 [1SE] $\mu g \cdot g^{-1}$ at day 3, and 10±4 [1SE] $\mu g \cdot g^{-1}$ at day 7), suggesting that sand may serve as a useful matrix for testing catechin phytotoxicity over time.

Two simple chemical properties can alter catechin readings from soil

Using a single soil, but modifying either soil pH or lead content resulted in poorer catechin extraction efficiencies compared to the unmodified control (Fig. 6). The effect of increased soil pH (from 5.8 to 7.5) significantly lowered the extractable catechin concentration immediately (25.3% versus 35.3% for the unmodified control). Also, lead (Pb^{2+}) at an enhanced soil concentration of 5 $\mu g \cdot g^{-1}$ lowered the extractable concentration of catechin over time. For example, in as little as three days detectable catechin levels were 15±2 [1SE] $\mu g \cdot g^{-1}$ in the lead amendment compared to 29±4 [1SE] $\mu g \cdot g^{-1}$ for the control (Fig. 6).

Comparison of catechin extraction efficiencies from Montana and Romanian soils

This experiment showed remarkable variability between soils, both in the ability to extract catechin efficiently (immediate extraction after 1 hour) and in the apparent stability of catechin over time (Fig 7). Soil concentrations measured immediately after

application of catechin in the 5 soils from Montana and the 5 soils from Romania ranged from almost zero for one Montana soil to concentrations that were ≈200 μg g^{-1} higher than the calculated application rate of 500 μg g^{-1} for the Br and PC soils. In the ANOVA, the effect of continent (fixed) was not significant ($F = 2.298$; df = 1,8; $P = 0.204$); the effect of site (nested, random) was not significant ($F = 1.224$; df = 8,4; $P = 0.424$), but the effect of time (fixed) after application was highly significant ($F = 10.535$; df = 4,160; $P<0.001$). The high concentrations of extracted catechin obtained early in the time series indicate that the extraction protocol was effective for these soils.

Multi-pulse additions of catechin do not enhance extracted concentrations of catechin

A multiple pulse experiment of increasing catechin concentrations was conducted on naïve India soil in collaboration with another laboratory to test the amount of pure catechin that can be detected. Interestingly, the total amount of catechin detected over the course of the experiment never reached levels approaching even 10% of the total added amounts (0 μg·g^{-1}, 340 μg·g^{-1}, 680 μg·g^{-1}, or 1020 μg·g^{-1}) (Fig 8). The mean extractable concentrations of catechin achieved over the course of the experiment were 0, 1.4 ± 1.4 μg [1SE], 14.5 ± 5.8 [1SE] μg, and 36.1 ± 10.2 [1SE] μg g^{-1}, respectively, and measurements made immediately after application never exceeded 77.3 ± 30.9 [1SE] μg g^{-1}, even for the highest application of 1020 μg·g^{-1}.

Different solvents have different catechin extraction efficiency

Of all solvents tested, ethyl acetate:water (1:1) exhibited the best extraction efficiency of 62.5±3.6 [1SE]% (Fig. 9). Listed in order of increasing extraction efficiency the efficacy

of the solvents was: water<phosphoric acid<methanol<ethyl acetate≈ethyl acetate:water (1:1; analyzing only the ethyl acetate fraction).

Discussion

Understanding the stability, biotic and abiotic transformations, biological activity of pure and derivative forms, and ecological role of an allelochemical in a plant/soil system presents myriad challenges to investigators. Given that soils are physically and chemically complex systems with ever-shifting biotic and abiotic parameters, it is imperative to be able to reliably and accurately detect the allelochemical and recognize its activities in a variety of widely different soil types. As most extant studies have used measured concentrations of allelochemicals as a starting point for laboratory experiments to study allelopathy (Bais*, et al.*, 2002, Blair*, et al.*, 2006, Inderjit*, et al.*, 2008), the described limitations in analytical capabilities and knowledge regarding ecologically relevant concentrations of such compounds in soil have likely led to misconceptions and controversy between researchers on this topic.

The findings presented in this thesis collectively demonstrate the highly reactive nature of catechin. The stability of chemically pure catechin is subject to many abiotic factors commonly associated with differences in field soils. Metal species and concentrations, and soil or media pH all appear to impact pure catechin stability, some immediately, some over a longer time period (days to weeks). Clearly, the immediate reactivity of catechin with ferric iron, ferrous iron, and cupric copper demonstrates its ability to undergo rapid alterations, impacting the ability to detect pure catechin even immediately

after addition to certain systems. Published findings from this dissertation have demonstrated the ability of catechin to complex with a variety of metals commonly found in soil (Pollock*, et al.*, 2009—see chapter 3 of this dissertation). Similar to the unbuffered metal reactivity experiment described in this chapter (refer to Fig. 2), the ability to detect pure catechin in the presence of metals in those published experiments was highly variable, dependent on the specific metal reacting with catechin. However, it is important to note that there were significant differences in the reactivity of catechin to the same metals in these unbuffered and buffered systems (compare Fig. 2 in this chapter and Fig. 1 in Chapter 3); specifically, calcium prevented catechin auto-oxidation over time in the buffered system, and copper rather than ferric iron was the most reactive metal both initially and over time in the buffered system.

The demonstration that low pH in solution stabilizes catechin over time (Fig. 1) was particularly interesting and congruent with prior findings in catechin amended sand (Blair*, et al.*, 2005), where the pH of the sand system was shown to greatly impact catechin extraction efficiency. This is noteworthy as differences in the amount of catechin detected in individual field samples could vary significantly based on the bulk soil pH rather than as a function of the concentration of catechin exuded into or present in the soil. Further, this observation opens the door for potential modification of experimental design and existing extraction protocols by modifying soil pH to enhance the stability of catechin or the efficiency of its extraction. Other abiotic interactions of catechin with pyruvate, malate, and citrate, discussed later in this dissertation, also

showed remarkable differences in pure catechin stability over time (see Chapter 5—Fig. 6) and that pyruvate is capable of lowering catechin concentrations in a buffered system.

Perhaps some of the most striking findings in this chapter were that catechin addition to soil as multiple pulses over the course of several days did not produce levels of extractable pure catechin above 10% of the expected amount (Fig. 8). This finding is particularly significant, since studies by other investigators typically employ levels of catechin based on previously detected catechin concentrations from the soil being used (Blair*, et al.*, 2005, Thelen*, et al.*, 2005, Blair*, et al.*, 2006, Inderjit*, et al.*, 2008) in the expectation that such levels are ecologically relevant. However, all indications based on the research in this and other chapters of this thesis suggest that levels of catechin in soil determined using existing protocols and analytical techniques grossly underestimate the amount of catechin (and derivative forms of catechin) that are exuded into and actually present in the soil, as well as the length of time catechin is stable in soil.

The best extraction of catechin from soil in the current work was 62.5% utilizing ethyl acetate:water (1:1) followed by 57.4% utilizing pure ethyl acetate as solvents to extract catechin from soil immediately after its addition. This is in contrast to the earlier findings of Blair *et al.* (2006) who reported extraction an efficiency from soil using ethyl acetate of only 12.7% but an efficiency of ~80% using their best method (75% acetone : 25% water : 0.1% phosphoric acid split), and in some instances nearing 100%. One possible explanation for this discrepancy is that in many of their studies the method of catechin addition to soil is not described (Blair*, et al.*, 2005, Blair*, et al.*, 2006), or that it was, to

my mind, inappropriately added as a dry powder to soil (Duke*, et al.*, 2009). As noted in Chapter 1, the method of catechin application to soil by different researchers is a highly variable procedure, with different studies dissolving catechin in any number of solvents or gently heating catechin in water to get it into solution before adding to soils or other media. In the absence of a comprehensive comparative study of all of these various methodologies, we can only guess at the impact of these approaches on catechin phytotoxicity in laboratory and greenhouse experiments. Worse yet, we know essentially nothing about the specific chemical and physical conditions/environment under which spotted knapweed exudes catechin into the surrounding rhizosphere through an as yet poorly understood process.

Some investigators have suggested that the amount of catechin produced by spotted knapweed could not be present in soil at levels sufficient to be toxic to native plants (Blair*, et al.*, 2006, Duke*, et al.*, 2009). However, those studies employed unique methods for catechin detection from sand and soil, arguing that original reports of catechin concentrations were unrealistically high in the field. Specifically, they suggested that poor extraction efficiencies of other methods would result in higher predicted amounts of catechin in soil than are currently reported, a seemingly paradoxical concept (Blair*, et al.*, 2006). However, work in this thesis clearly shows that many processes and parameters can alter the efficiency of chemical extraction of catechin and other closely related compounds from soil. Further, many of these processes and parameters result in an immediate decrease in detectable amounts of catechol-like compounds upon addition to soil (Furubayashi*, et al.*, 2007). That study emphasized the

ability of chemicals, including catechin, to adsorb to soil particles, interacting with the chemistry of the system and thereby lowering the ability to detect the compound as a "pure" sample. This also suggests that measured amounts of catechin from soil are more likely to be underestimates of the true concentration of catechin and its derivative compounds which might be more or less active than the parent compound.

Allelopathy is defined as the ability of an allelochemical produced by either a plant or microorganism to affect the growth of a neighboring plant or microorganism of another species (Molisch, 1937, Rice, 1974). Allelochemicals can be exuded into the surrounding environment during growth (e.g. sorgoleone, catechin) or as leachates from decaying plant material (e.g. cnicin). As such, demonstrating allelopathy requires determination of the production rate of the compound of interest, mechanisms and rates of transfer of the compound from the donor organism to the targeted species at concentrations sufficient to produce a positive or negative effect, and the ability to detect and measure the effect(s) on the target organism in both native and experimental systems.

In summary, the findings presented herein described a number of parameters that can alter catechin stability, extractability, and quantification in field, laboratory, and greenhouse experiments. Metal interactions, soil pH, the type of matrix used (e.g., sand, soil, media), and organic acid type and content can all impact catechin to varying degrees. These findings suggest that great care must be taken when designing and performing field and laboratory experiments on allelopathy towards plants, microbes, or fungi, as these factors can modify chemically pure catechin (introduced or produced by

spotted knapweed) to more or less "biologically active" forms and interfere with quantification of residual concentrations. It should be emphasized that the amount of catechin detected in soils or other matrices with current methods is not quantitative and cannot account for the multitude of derivative forms following modification reactions in the environment. It is entirely possible, even likely, that an unknown, undetected fraction of catechin and catechin-like compounds plays a crucial part in catechin phytotoxicity and bacteriostatic activity. Such effects likely contribute to the controversy surrounding the question of allelopathy caused by *Centaurea stoebe* as an important mechanism of its invasive success.

Acknowledgements

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Figures

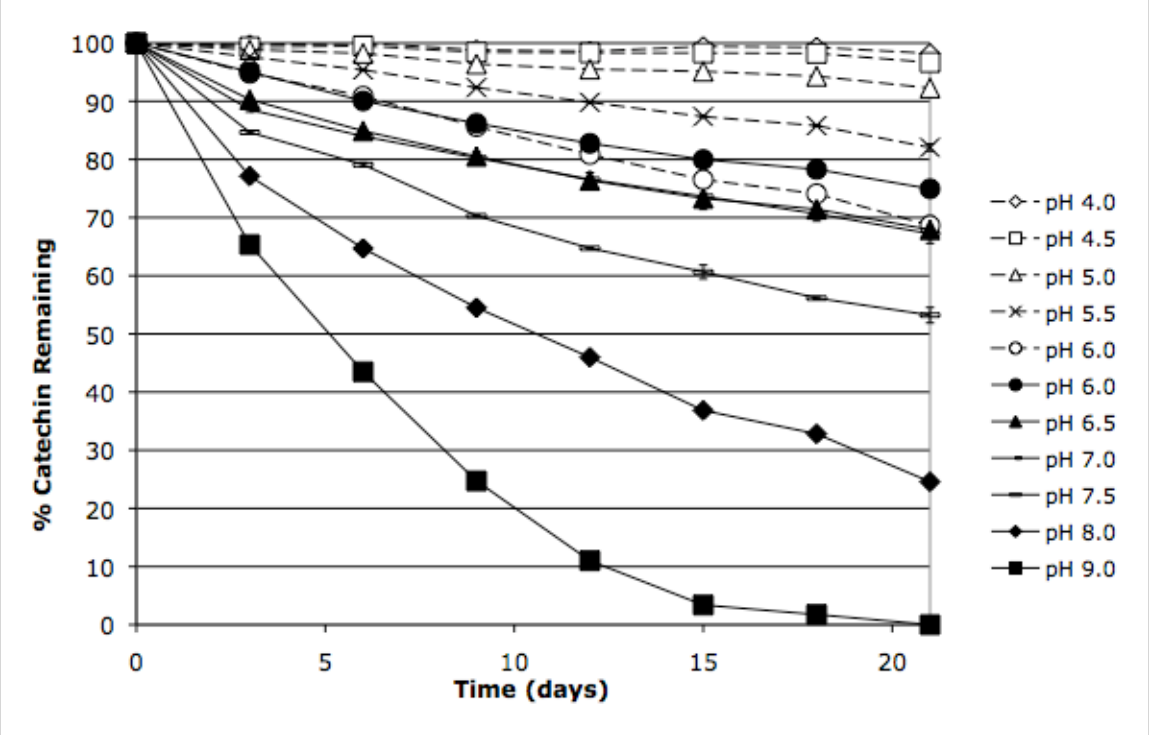

Figure 2.1

Impact of pH on catechin stability in solution. Two different buffers were used at 100 mM: MES buffer was used from pH 4.0 to 6.0 (open symbols); sodium phosphate buffer was used from pH 6.0 to 9.0 (closed symbols). Data points represent the average of triplicate determinations; error bars are ±1 SE and where not seen were hidden by the symbol. Starting catechin concentrations were 1000 $\mu g \cdot mL^{-1}$ for all pH solutions.

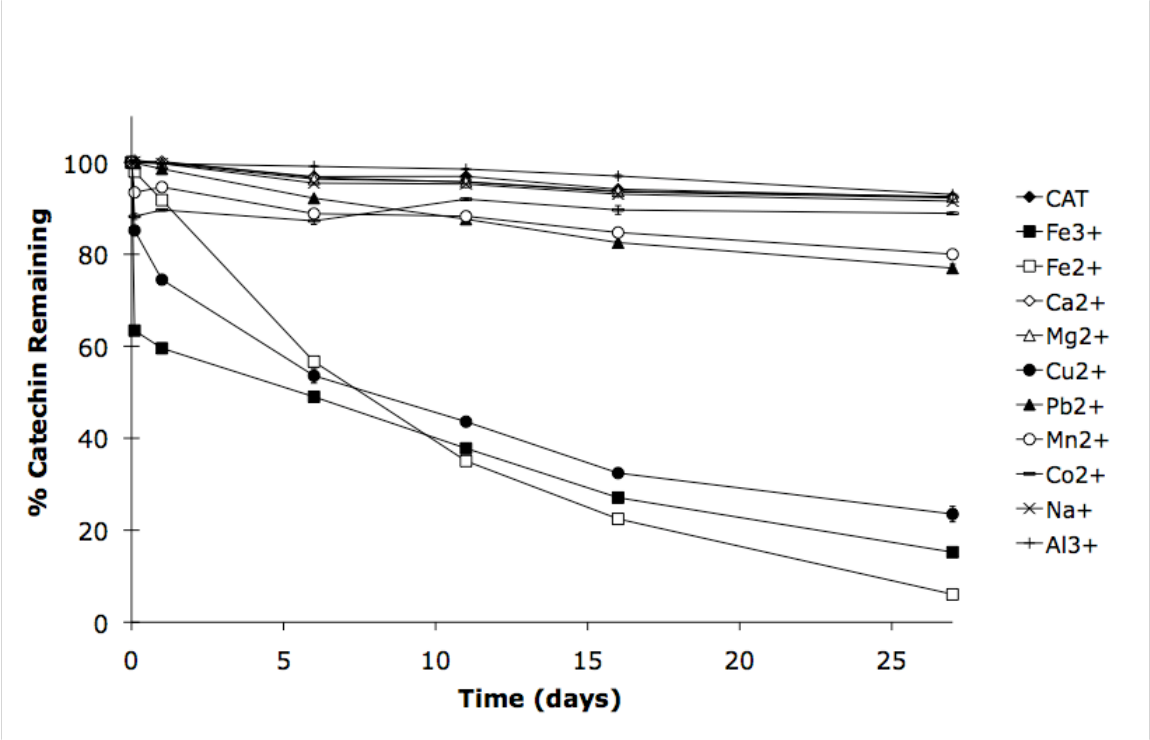

Figure 2.2

Impact of metal species on concentration of pure catechin. Starting catechin concentration for all combinations was 1000 $\mu g \cdot mL^{-1}$ (3.24mM) in double distilled water; equimolar concentrations of metal chlorides were added as indicated.

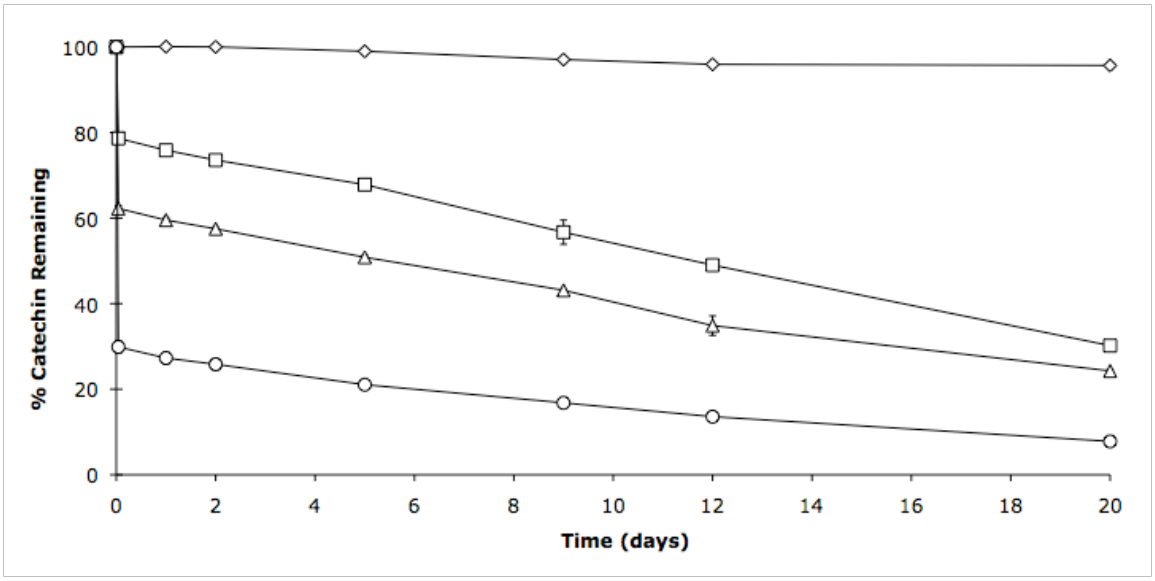

Figure 2.3

Concentration-based effects of ferric iron on catechin stability. Starting catechin concentration for all mixtures was 1000 $\mu g \cdot mL^{-1}$ (3.24mM). Moles of ferric chloride added were in proportion to the mM concentration of the catechin. pH change was noted next to the treatment. ◇=catechin (CAT) control (ph 4); □= half molar Fe:CAT (pH 2.5); △= equimolar Fe:CAT (pH2); and ○= 2X molar Fe:CAT (pH2). Symbols are the average of triplicate determinations; error bars are ±1SE.

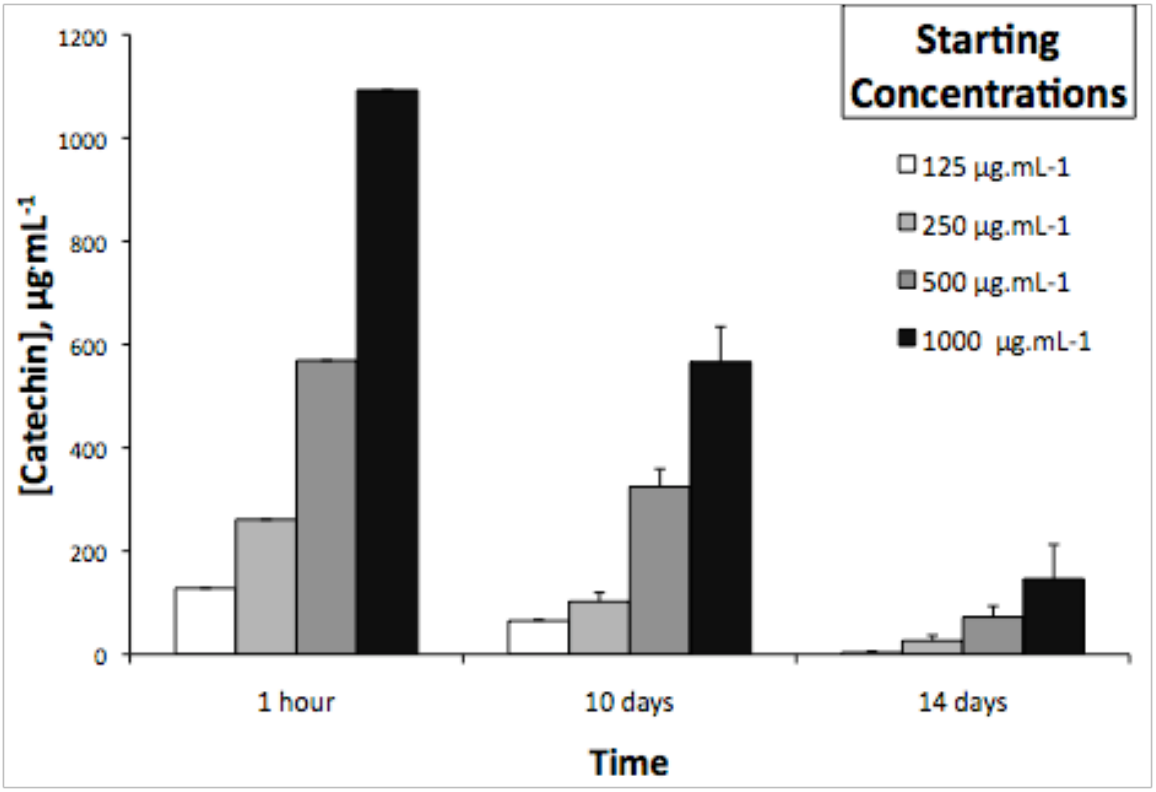

Figure 2.4

Catechin concentrations in seedling emergence experiment. Bars are the average of quadruplicate determinations; error bars are ±1SE. Expected starting concentrations are noted in the inset.

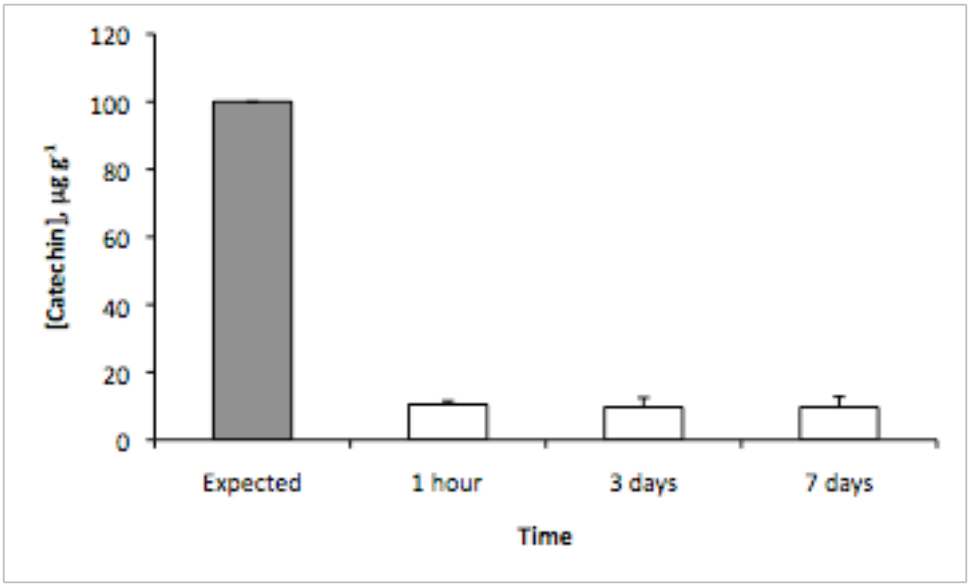

Figure 2.5

Catechin extraction efficiency from sterile 20-30 grit sand. Bars represent the average of quadruplicate measurements; error bars are ±1SE.

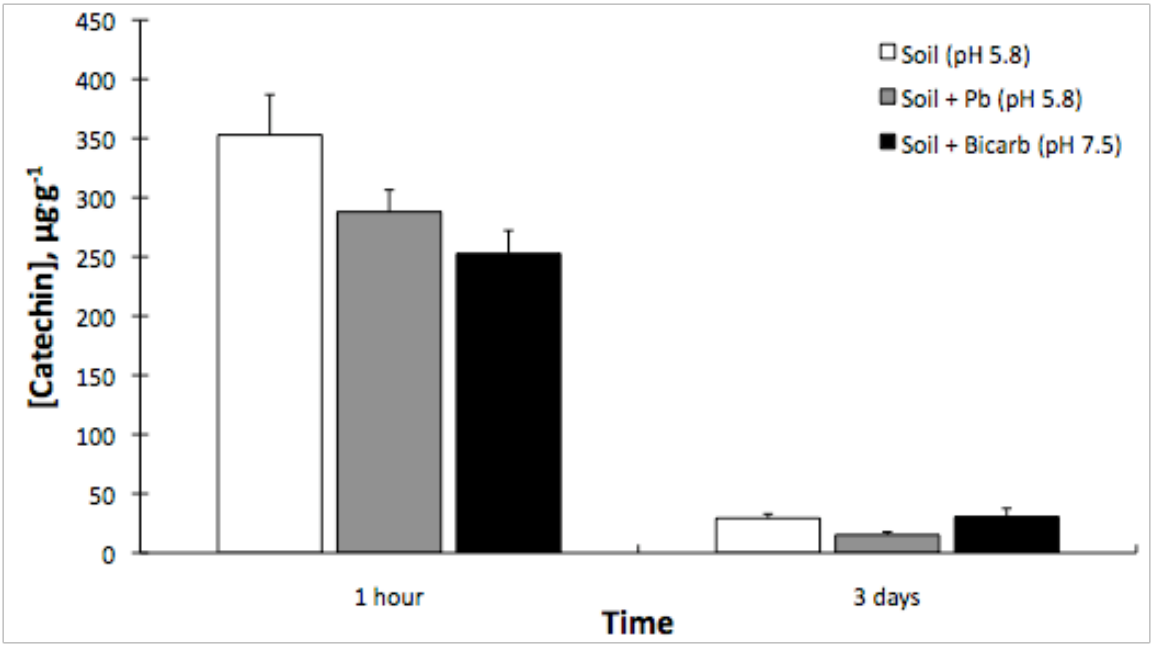

Figure 2.6

Impact of lead or pH shift on catechin extraction from a naïve MT soil. Catechin was added to a final concentration of 1000 $\mu g \cdot g^{-1}$ soil at the onset of the experiment. Lead was added as $PbCl_2$ at a lead concentration of 5 $\mu g \cdot g^{-1}$ soil; sodium bicarbonate was added to a final concentration of 20mM. Values given are the average of octuplicate determinations; error bars are ±1SE.

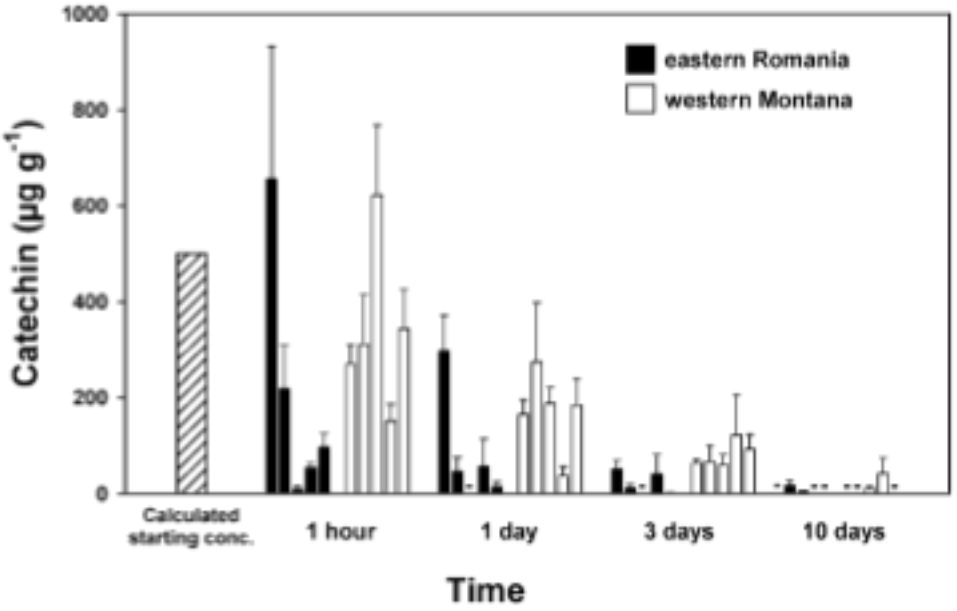

Figure 2.7

Measured soil concentrations of (±)catechin applied at 500 $\mu g \cdot g^{-1}$ to soils from eastern Romania (black bars) and western Montana (white bars) as a function of time after application. Bars represent the average of quintuplicate determinations; error bars show 1SE. ANOVA statistics are presented in the results.

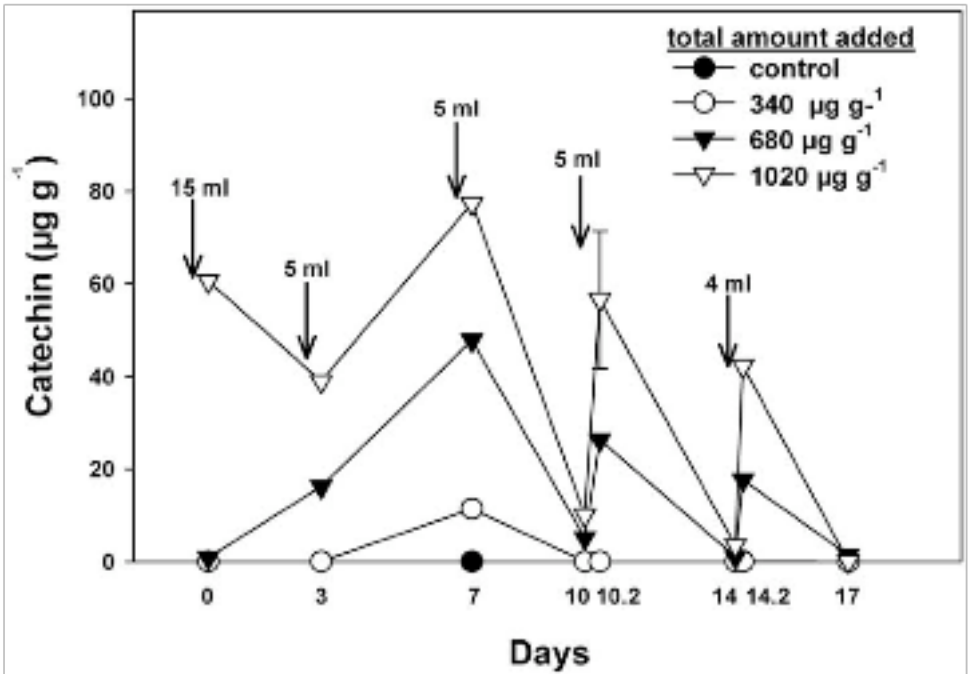

Figure 2.8

Measured concentrations of (±)-catechin in soil from India, derived from the application of pulsed deliveries of different concentrations shown in the inset. Values on x-axis denote days on which sampling was conducted. In detail, "10" and "14" denote sampling prior to the application of (±)-catechin pulses on those days, and "10.2" and "14.2" denote sampling two hours after application. The total (±)-catechin delivered to these soils over all pulses was 0, 340, 680, and 1020 $\mu g \cdot g^{-1}$, respectively. The single error bar shown indicates the largest 1SE.

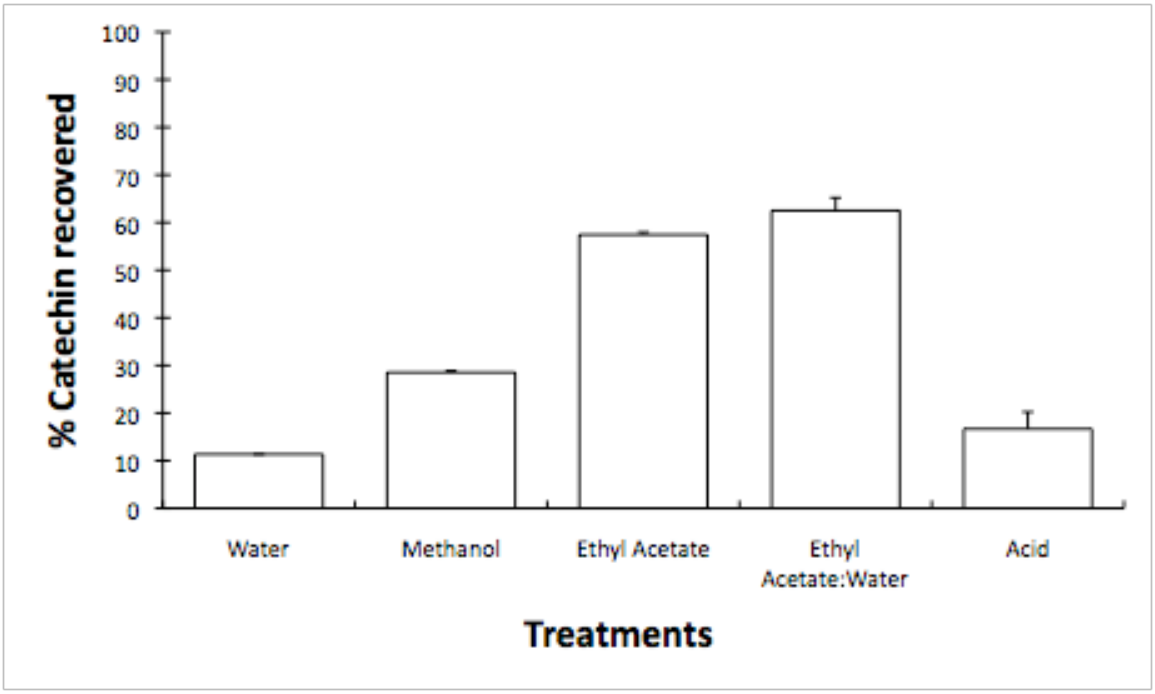

Figure 2.9

Catechin extraction efficiencies from sand for several solvents. Error bars represent the mean of triplicate determinations; error bars are ±1 SE.

Chapter 3

CATECHIN–METAL INTERACTIONS AS A MECHANISM FOR CONDITIONAL ALLELOPATHY BY THE INVASIVE PLANT *CENTAUREA STOEBE*

Summary

1. Evaluating variation, or "conditionality", in plant interactions is crucial to understanding their ecological importance and predicting where they might be at play. Much is known about conditionality for competition, facilitation, and herbivory, but not for allelopathy, which likely contributes to the equivocal nature of reports on this topic. *Centaurea stoebe* (spotted knapweed) is an invasive species in North America, whose success has been attributed, at least in part, to the allelochemical root exudate, (±)-catechin.
2. Understanding the ecological relevance of (±)-catechin necessitates determining how it interacts with various soil components. We found that some metals caused rapid declines in measurable (±)-catechin, while calcium impeded its auto-oxidation, maintaining concentrations higher than for (±)-catechin alone. Certain (±)-catechin—metal complexes were more phytotoxic than (±)-catechin alone, while others showed decreased toxicity.
3. The variable phytotoxicity of these complexes suggests that (±)-catechin effects are enhanced, mitigated, or otherwise affected by complexation with different metals and perhaps other soil components.

Synthesis. These findings serve to illustrate that the precise chemical forms, interactions, and effects of catechin in the environment are highly variable and that further

examination is warranted to increase our understanding of its role in invasion and allelopathy. The conditional effects observed for catechin detection and phytotoxicity likely extend to related allelopathic compounds, other root exudates, and potentially other systems involving chemically complex and spatially heterogeneous environments.

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Introduction

Considering variation, or conditionality, in the ways that plants compete for resources, facilitate, or indirectly interact with each other has been crucial for understanding the relative importance of these interactions in the organization of plant communities (Tilman, 1985, Wilson and Keddy, 1986, Kitzberger *et al.*, 2000, Levine, 2000, Brooker *et al.*, 2005, Baumeister and Callaway, 2006). Such conditionality may also be important for allelopathic interactions, but to our knowledge there have been no explicit, quantitative studies of conditional allelopathic effects or of the mechanisms that might cause them.

Allelopathic effects of the North American invasive plant, *Centaurea stoebe* Lam. (spotted knapweed), have been reported from leaves (Fletcher and Renney, 1963, Bohlmann *et al.*, 1973, Stevens, 1986, Kelsey and Locken, 1987) and roots (Ridenour and Callaway, 2001). Also, phytotoxic effects of (±)-catechin, a racemic phenolic compound exuded from the roots of *C. stoebe* (hereafter referred to simply as catechin), have been demonstrated *in vitro* (Buta and Lusby, 1986, Bais *et al.*, 2002, Iqbal *et al.*, 2003, Weir *et al.*, 2003, Weir *et al.*, 2006, Perry *et al.*, 2005b, D'Abrosca *et al.*, 2006, Furubayashi *et al.*, 2007, Rudrappa *et al.*, 2007, Simões *et al.*, 2008), in controlled experiments with field soils (Bais *et al.*, 2003, Inderjit *et al.*, 2008a, Inderjit *et al.*, 2008b); and in the field (Thelen *et al.*, 2005, Thorpe, 2006, Inderjit *et al.*, 2008a, Inderjit *et al.*, 2008b). However, experiments have not always shown catechin to have inhibitory effects (Blair *et al.*, 2005, Perry *et al.*, 2005a) and effects can vary substantially among species (Weir *et al.*, 2003, Perry *et al.*, 2005a, Thorpe, 2006). Furthermore, field

applications of catechin at the same site and to the same plant species show substantial variability between years (Thelen *et al.*, 2005, Thorpe, 2006). Even in the same growing season, application of catechin dramatically reduced the growth of *Geum triflorum* Pursh in open grassland (Thorpe, 2006), but the same concentration had no effect on *G. triflorum* in soils under *Pseudotsuga menzeisii* tree canopies at the same site (G.C. Thelen, R.M. Callaway, *unpublished results*). Complicating this variation even more, exudate production rates from seedlings and mature plants *in vitro* range from 0–2.4 $\mu g \cdot ml^{-1}$ (Blair *et al.* 2005), 5–35 $\mu g \cdot ml^{-1}$ (Weir *et al.* 2003, 2006), 0–113 $\mu g \cdot ml^{-1}$ (Ridenour *et al.*, 2008), 83–185 $\mu g \cdot ml^{-1}$ (Bais *et al.* 2003), and 0-33 $\mu g \cdot ml^{-1}$ (R.M. Callaway, J.L. Pollock, unpublished data). In a related system, Tharayil and co-workers found that 8-hydroxyquinoline exudation by the roots of *C. diffusa* varied on a diurnal basis (Tharayil *et al.*, 2009). Natural soil concentrations of chemically pure catechin also vary spatially and temporally, with recent measurements being far lower than those initially reported (Blair *et al.*, 2005, Blair *et al.*, 2006, Perry *et al.*, 2007). Collectively, these observations and reports suggest that substantial variability in the effects of both spotted knapweed and catechin may exist.

Variation in the effects of a putative allelopathic chemical and in its environmental concentrations could be due to many reasons, including: application of different chemical concentrations in experiments; structural differences in the chemical applied; age or size of target plants; seasonal timing of applications or soil collection; local temperature or moisture conditions; different analytical or methodological techniques; or local differences in the effects of soil chemistry or biota on the chemical. Polyphenolic root

exudates (catechin belongs in this class of compounds) have been shown to auto-oxidize as well as interact with various specific metals (Gomah and Davies, 1974, McDonald *et al.*, 1996, Lim *et al.*, 2005). Further, (Furubayashi *et al.*, 2007) suggested that several allelopathic root exudates containing catechol moieties, as does catechin, are particularly prone to as yet undefined chemical transformations and binding to soil.

Here, we focus on how metals that are commonly found in soils affect the concentration of pure catechin in laboratory and greenhouse conditions, and assess whether catechin—metal complexes (hereafter CMCs) affect target plants differently than catechin itself. It is important to note that current analytical methods for determining catechin concentrations (e.g. Blair *et al.*, 2005, Paveto *et al.*, 2004) depend on HPLC-based detection of chemically pure catechin as a discrete chromatographic peak at a given elution time whose area represents the concentration of the chemical. As a result, essentially any chemical modification through addition, oxidation, complexation with metals, or full or partial degradation could alter the absorption wavelength of the modified catechin or its retention time on the HPLC column. This would render altered catechin undetectable by current detection methods, even though it could still be present in the system in this modified form and may be more or less phytotoxic then pure catechin itself.

Here we hypothesize that catechin, through its ability to chelate metals, is highly variable in its ability to be detected as pure catechin. Further, interactions of catechin with metals commonly found in soils could potentially alter the phytotoxicity currently reported in

literature. These findings suggest that catechin, and perhaps other root exudates from other plants, may exist in multiple forms in soil after exudation, inducing variable and conditional effects on the surrounding plant community and perhaps other macro- and microbiota. This conditionality may explain the seemingly equivocal nature of various reports regarding allelopathic and phytotoxic activities of *C. stoebe* and catechin, as well as other potentially allelopathic chemicals.

Materials & Methods

Effect of metals on catechin in solution

To examine the effects of metals commonly found in soils on the concentrations of catechin in simple solutions, we created mixtures of catechin and the following minerals: ferric chloride ($FeCl_3 \cdot 6H_2O$); cupric sulfate ($CuSO_4 \cdot 5H_2O$); calcium chloride ($CaCl_2 \cdot 2H_2O$); magnesium chloride ($MgCl_2 \cdot 6H_2O$); and lead nitrate ($Pb(NO_3)_2$(anh)). (±)-Catechin hydrate ($C_{15}H_{14}O_6 \cdot H_2O$) (racemic mixture; >99% purity) was obtained from Shivambu International (New Delhi, India) and all other chemicals were obtained from Sigma (St. Louis, MO, USA). For all solutions, catechin was dissolved into sodium phosphate buffered solution (PB) [100 mM $NaPO_4$ (pH 7.0)] by stirring and gradually warming the solution to create a final concentration of 1000 $\mu g \cdot mL^{-1}$ (3.25 mM). This stock solution was used to make all other solutions of CMCs and also served as the "no metal" control. Although catechin is stable in ultra-pure water longer than in PB, using a buffered system allowed us to control for the effects of pH, which would vary substantially among these solutions if water were used rather than PB (J.L. Pollock & W.E. Holben, *unpublished results*).

After cooling, catechin solution was dispensed into 3 replicate sterilized tubes for each treatment, and individual metals were then added to establish the following final concentrations: 4 $mg \cdot mL^{-1}$ $FeCl_3 \cdot 6H_2O$; 3 $mg \cdot mL^{-1}$ $CuSO_4 \cdot 5H_2O$; 4 $mg \cdot mL^{-1}$ $Pb(NO_3)_2$(anh); 2.3 $mg \cdot mL^{-1}$ $MgCl_2 \cdot 6H_2O$; and, 1.7 $mg \cdot mL^{-1}$ $CaCl_2 \cdot 2H_2O$. This produced a suite of treatments of 3.25 mM catechin plus 12 - 15 mM for each individual metal, which was expected to saturate all potential binding sites of the added catechin. The metals are hereafter referred to simply as Fe, Cu, Pb, Mg, or Ca, respectively. All tubes were kept stationary in the dark at room temperature for the course of the experiment and sampled periodically to measure the amount of pure catechin remaining by HPLC. All tubes were vortexed briefly before use or sampling because precipitates formed in most of the treatments (Mg being the exception). For catechin analysis, 1 mL of each suspension was transferred into a sterile Eppendorf tube, centrifuged at 13,000 RPM for 10 min, and the resulting supernatants placed into HPLC vials to determine catechin concentrations as previously described (Paveto *et al.*, 2004). In brief, catechin concentrations were determined by analysis of aqueous samples (15 μL injection volume) with UV detection at 280 nm on a Hewlett-Packard (HP) series 1100 HPLC (Hewlett-Packard, Palo Alto, CA) using a HP ODS Hypersil C18 column (5 μm, 125 x 4 mm) and a 100% methanol:0.2% phosphoric acid (1:3 v/v) mobile phase at a flow rate of 1 $mL \cdot min^{-1}$.

Effect of metals on catechin extraction from sand

A number of studies have reported that catechin concentrations typically decrease rapidly following addition to soils (Blair *et al.*, 2005, Blair *et al.*, 2006, Furubayashi *et al.*, 2007, Inderjit *et al.*, 2008a), presumably because of auto-oxidation, sorption, and/or chelation. Therefore, it is inappropriate to consider the concentration of pure catechin added to soils or other substrates as the "effective" *in situ* concentration, which is likely much lower. For the growth and survival experiments described below, in which pre-sterilized silica sand was used as a simplified growth matrix for plants, we wanted to assess the extractable concentration of catechin and the various CMCs immediately after addition to the sand matrix. To accomplish this, unplanted pots were separately amended with PB, catechin, each metal alone, or suspended CMC, then immediately 1 g was sampled and extracted with 100% methanol for catechin analysis by HPLC as described above.

Effect of CMCs on plant growth and stress survival

To test the effects of catechin and CMCs on the growth and survival of native plant species, we applied each solution independently (except Mg—CMC, since Mg showed little effect on pure catechin concentrations in the initial abiotic experiment) to *Festuca idahoensis* (n = 10) and *Koeleria macrantha* (n = 9) plants grown in 300 g of pre-sterilized silica sand (20/30 grit, Lane Mountain Sand, Valley, WA) in 525 cm^3 'rocket' pots. For negative controls, 10 individuals of *Festuca* and 9 individuals of *Koeleria* were transplanted, watered, and fertilized as for the other treatments, but only PB was added. Both species were purchased as seed from Wind River Seed Co. (Manderson, WY). Initially, each plant species was grown from seeds for 33 days before administering the first of four doses of the corresponding treatment (one dose every week). However, early

and high mortality of these seedlings within 30 days of the onset of treatments (data not shown) indicated that multiple pulses of catechin or CMCs were highly toxic to seedlings. The experiment was therefore repeated, this time using more mature and established plants, which were initially grown in clean sand, then transplanted to the rocket pots containing the abovementioned CMC treatments (from which any surviving seedlings had been removed). Prior to transplanting, *Festuca* and *Koeleria* plants were grown for 64 days in clean potted sand in a greenhouse with natural and supplemental light (≈1600 $\mu mol^{-1}m^{-2}$ on a sunny day) at temperatures maintained at a mean daily low of 20 ^{o}C and mean daily high of 24 ^{o}C. Plants were watered with tap water every other day, and fertilized every 21 days with 50 mL of Peters Excel 15-2-20 Plus Fertilizer solution, mixed at 0.34 $mg \cdot mL^{-1}$. We maintained these same greenhouse conditions after transplanting, but ambient temperatures increased periodically to >30 ^{o}C as the greenhouse warmed during the summer.

The transplants were allowed to equilibrate for approximately two weeks following transplantation and were seemingly unaffected by the prior treatments with catechin or CMCs. Following this equilibration period, the pots were subjected to four more doses of the treatments (once a week for four weeks). For each time-point, all treatment doses (PB, pure catechin, each metal alone, each metal—CMC) were pre-aged for 21 days to allow auto-oxidation or CMC formation as in the initial abiotic experiment, and then used to treat the sand in the second experiment. As noted above, precipitates formed in all solutions except the PB and pure catechin treatments, so they were thoroughly suspended before 42 mL aliquots were added to the respective pots.

To directly assess catechin and CMC effects on plant growth, all green leaves on each plant were counted 7 days after transplanting (prior to treatment) to provide an initial leaf number and then again at day 43 following all catechin or CMC treatments, with the difference in green leaf number used as a measure of growth or inhibition. Leaf number data were analyzed using one-way ANOVA with each of the ten treatments used as an independent factor. For comparisons between all combinations of treatments, the ANOVA was followed by a Tukey HSD comparison of means and Bonferroni adjustment for the number of treatments.

To test the effect of catechin and CMCs on general plant mortality under controlled conditions, all of the plant treatments were exposed to harsh, but environmentally relevant, conditions after the 43-d time-point. Briefly, the pots were not fertilized or watered for 3 weeks under ambient greenhouse conditions ranging between 25°C and 35°C in daily cycles to simulate summer-like temperatures, drought, and nutrient-poor conditions. Plant mortality was recorded after 20 days under these conditions (on day 63). Plants were considered dead when no green leaves were left, and no plants that were recorded as dead later put on green leaves. The mortality of both species was summed for each treatment for analysis in order to provide adequate statistical power for logistic regression analysis. We recorded mortality and analyzed differences among treatments using logistic regression analysis (see Efron, 1988; SPSS 15.0, 2006).

Effect of CMCs on seedling establishment

Because the earlier experiment exhibited high levels of mortality in emergent seedlings with multiple catechin or CMC treatments, a second experiment was conducted using single doses at lower concentrations to assess the effect of catechin, CMCs, or uncomplexed metals on seedling establishment. In this experiment, Ca, Fe and catechin were tested alone or in CMC forms, since these showed enhanced, decreased, or control-level phytotoxicity, respectively, in the prior experiment. In this case, the solutions were made in sterile, ultra-pure water rather than PB to alleviate concerns that PB components or buffering may have somehow contributed to the observed toxicity in the seedling experiment.

To assess effects on seedling establishment, 'rocket' pots were set up with clean, sterile sand as before, except in this case five *F. idahoensis* or *K. macrantha* seeds were placed on the sand surface in each pot and the pots were treated just once with either 40 mL of ultra-pure water (negative control), 40 mL of catechin solution, 40 mL of a given metal chloride solution without catechin, or 40 mL of a given CMC; n = 10 pots, each containing 5 seeds, for each plant-treatment combination. Catechin and CMC preparations were made as before except that the catechin stock solution was 300 $\mu g \cdot mL^{-1}$, sterile ultra-pure water was used rather than PB, and they were used immediately following preparation. The pH of each resulting solution was measured using a Corning 340 pH Meter (Corning Inc., Corning, NY, USA). The concentration of pure catechin remaining in each solution at the time of application to the pots was measured by HPLC as described above. As before, precipitates formed in both CMC solutions and these were thoroughly suspended before application to the pots. Mortality was low during this

experiment, and we scored establishment as being only plants that germinated and survived to the end of the experiment. Differences in % emergence among treatments were analyzed using repeated measures ANOVA (SPSS 15.0, 2006), where establishment was measured over time (in days) and time was the repeat variable in the analysis.

Results

Effects of metals on catechin in solution

The metals tested varied substantially in their effects on pure catechin concentration in solution as determined by HPLC. All metals except Mg formed precipitates in the presence of catechin in PB. Color and textural differences clearly distinguishable from those observed in the "no-catechin" metal treatments used for the seedling growth and seed establishment experiments suggest that catechin reacted with metal phosphates to form catechin—metal complexes, catechin—metal—phosphate complexes, or both (for convenience and simplicity, we refer to these collectively as CMCs). Despite thoroughly suspending these precipitates prior to sampling for HPLC analysis, only the supernatant remaining after centrifugation of samples was analyzed by HPLC, meaning that insoluble forms of catechin were removed and only pure catechin remaining in solution was detected. Similarly, auto-oxidized catechin, though still in solution and not removed as precipitate, was not detected as pure catechin in these analyses due to its altered chemical form. It is important to note, however, that bound or oxidized forms of catechin may be more, less, or equally biologically active (i.e. phytotoxic) as pure catechin, and thus the entire suspension was added to pots in all experiments.

Pure catechin in solution alone or with individual metals decreased in concentration with time, presumably due to auto-oxidation when alone, to complexation with metals forming the corresponding CMCs, or possibly an additive effect of both types of reactions (Fig. 1). In PB, the concentration of pure catechin decreased from 978 ± 3 $\mu g \cdot mL^{-1}$ after ~1 h (the shortest possible time to analysis) to 606 ± 17 $\mu g \cdot mL^{-1}$ after 21 days of incubation, a 38% decrease. The presence of metals with catechin resulted in more dramatic and metal-specific effects on the stability of pure catechin in solution. The greatest impact was observed with Cu, which reduced the concentration of pure catechin from 738 ± 13 $\mu g \cdot mL^{-1}$ at ~1 h to undetectable levels (< 5 $\mu g \cdot mL^{-1}$; hereafter referred to as ND for "Not Detected") after 21 days. Fe decreased pure catechin in solution from 851 ± 10 $\mu g \cdot mL^{-1}$ at ~1 h to 78 ± 13 $\mu g \cdot mL^{-1}$ at day 21. Pb and Mg produced more moderate decreases in pure catechin after 21 days, from 953 ± 5 at ~1 h to 293 ± 7 $\mu g \cdot mL^{-1}$ at day 21 and 958 ± 4 to 514 ± 16 $\mu g \cdot mL^{-1}$, respectively. In contrast to the other metals, Ca caused the concentration of catechin in solution to remain significantly higher than for catechin alone, decreasing only from 966 ± 7 after ~1 h to 738 ± 19 $\mu g \cdot mL^{-1}$ at day 21; 22% higher than the catechin-only control. The effect of the nutrient solution (used to fertilize the plants) on pure catechin stability in PB was also tested and no significant effect was detected compared to the catechin-only control (data not shown).

Effect of metals on catechin extraction from sand

To assess the extractability of catechin from the sand matrix and the effects of metals on extraction, catechin solution or CMCs aged 21 days (as for the initial experiment) were applied to pots and then immediately sampled and analyzed for determination of

extractable pure catechin concentrations. While the initial input concentration of catechin in each treatment was identical, based on the concentrations of pure catechin remaining in the catechin-only solution and the various CMCs after the 21 day preincubation and the 42 mL that was added to each 300 g of sand, the calculated concentrations of pure catechin added to each pot at the onset of this experiment were: no-catechin control and metal-only controls = 0 $\mu g \cdot g^{-1}$ sand; catechin only = 85 $\mu g \cdot g^{-1}$ sand; Ca—CMC = 103 $\mu g \cdot g^{-1}$ sand; Pb—CMC = 41 $\mu g \cdot g^{-1}$ sand; Fe—CMC = 11 $\mu g \cdot g^{-1}$ sand; and Cu—CMC = ND.

The concentrations of pure catechin extracted from sand immediately after application of the above treatments were as follows: no-catechin control and metal-only controls = ND; catechin-alone = 8.0 ± 1.0 $\mu g.g^{-1}$ sand; Ca—CMC = 19 ± 2.0 $\mu g \cdot g^{-1}$ sand; Pb—CMC = 14 ± 0.45 $\mu g \cdot g^{-1}$ sand; Fe—CMC = 5.8 ± 0.72 $\mu g \cdot g^{-1}$ sand; and Cu—CMC = ND (Fig. 2). Interestingly, the Pb—CMC treatment yielded more pure catechin than the catechin-alone treatment. Thus, catechin-alone and all of the CMCs exhibited at least some loss of recoverable pure catechin to the sand matrix in this short time interval, with the catechin-only treatment showing the greatest magnitude of reduction.

Effect of CMCs on plant growth and stress survival

The effects of catechin and CMCs on plant survival and growth were measured in experiments where, following transplantation and treatment, increased leaf number compared to the PB-only control indicated increased growth, decreased leaf number indicated decreased growth, and visual indications of plant death were scored as

mortality. Plants of *Festuca idahoensis* treated with PB-only control solution added 7 ± 1 new green leaves over the course of the experiment (43 days). In contrast, *Festuca* exposed to the catechin-only treatment showed a decrease of 9 ± 2 leaves per plant (Fig. 3). When metals alone (in PB) were added to the pots, only *Festuca* exposed to Cu showed a significant decrease in growth. However, Ca—CMC, Pb—CMC, and Cu—CMC inhibited growth of *Festuca* significantly more than the PB-only control solution. Fe-alone and Fe—CMC showed no significant effect on leaf growth. Importantly, no CMC decreased growth significantly more than catechin alone (unlike the case for the mortality measurement), suggesting that catechin complexation into CMCs can create substantial conditionality in the phytotoxicity of catechin to *Festuca*.

For *Koeleria macrantha*, the effect of catechin-alone on leaf growth was significant when compared to the PB-only control, but not as strong as for *F. idahoensis* (Fig. 3). Applied without catechin, Ca and Pb did not affect leaf growth, but Cu, Fe and Fe—CMC significantly reduced growth, while Ca—CMC and Pb—CMC caused strong decreases in growth. Notably, Ca—CMC, Pb—CMC and Cu—CMC had stronger effects on leaf growth than metal alone, suggesting that complexation with metals *increases* catechin phytotoxicity to *Koeleria*, illustrating a species-specific element to catechin and CMC conditionality.

Metals alone had no significant effects on plant mortality relative to the PB-only control (Fig. 4). However, catechin-alone increased mortality relative to the PB-only control, 45% versus 15%, respectively. Ca—CMC and Pb—CMC induced even higher mortality

rates than catechin-alone, being 85%, and 75%, respectively. The effects of Cu—CMC and Fe—CMC on mortality did not differ significantly from either the catechin-only treatment or the control (Fig. 4). Visual inspection of roots for both plant species demonstrated a stunted morphology and visible darkening of the root system for all treatments treated with catechin or various CMCs when compared to the PB-only control.

Effect of CMCs on seedling establishment

The effects of catechin, Ca, and Fe (alone or in CMC form) on seedling establishment indicated Ca-alone, Fe-alone, and Fe—CMC had no effect relative to the water-only control on the establishment of seedlings for either plant species (Fig. 5). However, catechin-alone and Ca—CMC inhibited the establishment of both *Festuca* and *Koeleria*. Because these treatments were prepared in ultra-pure water and not buffered, the pH of each solution was measured just prior to use. The results were as follows: water-only control, pH 7.1; catechin-alone, pH 5.5; Ca-alone, pH 5.7; Fe-alone, pH 2.1; Ca—CMC, pH 5.3; Fe—CMC, pH 2.0.

Pure catechin concentrations in each treatment were determined by HPLC just prior to addition to the pots with the following results: water-only control, ND; catechin-alone, 291 $\mu g \cdot mL^{-1}$; Ca-only, ND; Fe-only, ND; Ca—CMC, 290 $\mu g \cdot mL^{-1}$; Fe—CMC, ND. Note that, unlike in the buffered treatments, Fe rapidly reduced pure catechin concentrations to undetectable levels in pure water. It is very important to the interpretation of these findings to note that these measures reflect the amount of pure catechin remaining in solution in the treatments at the time of application, and also that each treatment was

prepared with the same initial amount of catechin. As such, each treatment ultimately contains the same molar amount of catechin per se, but it exists in one or more different derivative forms in each catechin and CMC treatment applied.

Discussion

Numerous prior studies have reported catechin phytotoxicity (Bais *et al.*, 2002, Weir *et al.*, 2003, Perry *et al.*, 2005a, Thelen *et al.*, 2005, Thorpe, 2006, Rudrappa *et al.*, 2007, Simões *et al.*, 2008, Inderjit *et al.*, 2008a, Inderjit *et al.*, 2008b) and data from the current study corroborate those findings. Conversely, Blair et al. (2006) have suggested that *C. stoebe* does not have phytotoxic activity on failing to extract and measure significant amounts of catechin from *C. stoebe* inhabited soil in their own study. Based on the current study, we suggest that rapid transformation of catechin to oxidized or CMC forms, and perhaps pulsed releases, contribute to the observed variation in soil catechin concentrations where *C. stoebe* is present and may help explain conditionality in the effects of catechin and in the general allelopathic effects of *C. stoebe*.

We showed that common soil metals dramatically and differentially altered the concentration of pure catechin in solution, suggesting a specific mechanism for the loss of pure catechin after it is added to sand or soil, and for conditionality in the observed effects of catechin on plants. The results also demonstrate that very low or even undetectable concentrations of pure catechin in sand cultures can inhibit native grass species where it is complexed with certain metals (e.g. refer to the Cu—CMC data in Figures 2 and 3). These pure catechin concentrations are comparable to or lower than

recent extensive measurements in the field where *C. stoebe* is present (Blair *et al.*, 2006) and far lower than some periodic measurements previously observed (Perry *et al.*, 2007).

In addition, our results show that high amounts of pure catechin must be applied, even to clean, sterile sand in order to recover and measure pure catechin concentrations comparable to those observed in the field using published protocols (1-100 $\mu g \cdot g^{-1}$ soil) as recently suggested by Inderjit and co-workers for soils (2008a). However, even these concentrations can be ephemeral, as single large applications may not be detectable immediately after applying catechin. Indeed, our results show that even when 42 mL containing 738 $\mu g \cdot mL^{-1}$ of pure catechin is added to sterile sand and immediately extracted, the concentrations detected can be very low. This suggests that very high levels of catechin may need to be released from *C. stoebe* roots in order to reach field concentrations that have been recently reported (Blair *et al.*, 2006, Perry *et al.*, 2007). We note, however, that high soil concentrations of catechin have not been consistently observed, with the exception of what appear to be periodic pulses (Perry *et al.*, 2007).

It is striking that the most redox-active metals (Fe and Cu) caused the most rapid loss of pure catechin from solution under abiotic conditions (refer to Fig. 1). Less redox-active metals (Mg and Pb) also decreased the concentration of pure catechin in solution with time, but to a lesser extent. Catechin alone was subject to a modest rate of auto-oxidation, reducing the concentration of pure catechin during the course of the experiment, and that form was also least extractable from sand (Fig. 2). Interestingly, Ca seemed to protect catechin from auto-oxidation (Fig. 1).

Given the chelating properties of catechin and related compounds (Gomah and Davies, 1974, McDonald *et al.*, 1996, Lim *et al.*, 2005), it seems plausible that Pb, Cu or other metals in CMC form might have increased solubility and bioavailability relative to pure metals. This could result either in enhanced availability of micronutrients, as suggested for Fe by Tharayil et al. (2009), or in increased metal toxicity to plant species. The experiments and data presented in Figures 2 - 4 address this last possibility, but warrant careful interpretation because metals in CMC form also are complexed with catechin.

For example, using leaf number as a measure of growth (increased number) or senescence (decreased number) indicated that all CMC forms, with the possible exception of Ca—CMC, were less inhibitory than pure catechin for *F. idahoensis*, while pure catechin and all forms of CMCs showed similar inhibition of *K. macrantha*. This suggests species-specific variability in phytotoxicity in that the pure catechin form was most toxic to the former, while either pure or complexed catechin were equally toxic to the latter compared to metal-only controls (refer to Fig. 3). However, we also note that the metal-only controls in this experiment exhibited differential inhibition within and between plant species as well.

It is particularly interesting that Cu—CMC was more toxic than Cu-only, at least for *K. macrantha*, since the Cu—CMC solution added to the pots showed no detectable catechin (Fig. 2) and no pure catechin was recovered from the Cu—CMC treatment in the extraction experiment. This suggests that Cu—CMC exhibits a phytotoxic effect that is

not dependent on residual pure catechin concentrations. Extrapolation of this finding to soil suggests that *C. stoebe* may exert catechin-based phytotoxicity in the absence of measurable catechin, perhaps explaining the seemingly contradictory findings of Blair *et al.* (2006) who suggested that there was no evidence for phytotoxicity by *C. stoebe* in their study since there was no catechin detected.

The effects of iron on pure catechin retention in solution and its phytotoxicity were different than for Cu. Fe ultimately decreased the final concentration of pure catechin in solution by ~90% (Fig. 1). However, Fe—CMC did not cause greater stress-related mortality than the negative, metal-only, or catechin-only controls (Fig. 4). Fe—CMC also did not significantly decrease leaf growth of *Festuca* relative to these controls and apparently mitigated toxicity compared to the catechin-only control (Fig. 4). By contrast, Fe-alone and Fe—CMC both decreased leaf growth of *Koeleria* to the same extent as pure catechin. Collectively, these results suggest that, in contrast to the case with Cu, a substantial component of the effects of Fe—CMC may have been due to the metal itself, at least at the higher concentrations utilized in the first growth and mortality experiments. Interestingly, the lower concentrations of Fe-alone and Fe—CMC solution employed later had no effect on seedling establishment in sand (Fig. 5).

In contrast to Fe and Cu, Pb and Mg are far less redox-active, as reflected in their apparent reaction kinetics with pure catechin, showing slower rates of reduction and higher concentrations of pure catechin in solution after 21 days. Similarly, Pb- and Ca-only controls showed lower toxicity and mortality to plants, while their corresponding

CMC forms showed greater mortality than even the pure catechin control. Ca—CMC also exhibited enhanced inhibition of seedling establishment (Pb—CMC was not tested for this), and even showed a trend toward greater inhibition than the pure catechin control.

The mechanisms by which the various metals alter catechin concentration are not clear, but binding or chelation of catechin to a variety of di- and trivalent cations is the most likely explanation. Catechin is highly reactive, switching between its reduced form (the one commonly referred to as (±)-catechin), to a more oxidized form (possessing a (semi)-quinone moiety on the B-ring) and conversion to various catechin dimers when exposed to copper (Es-Safi *et al.*, 2003). It is probable that other metal species found in soils are capable of similar abiotic reactions with catechin and thereby decrease its phytotoxic effects by altering its available concentration. Thus, the most redox-active metals produced the greatest reduction in pure catechin concentrations, thereby limiting its toxicity and mortality, while less redox-active metals (e.g. Ca, Mg, and Pb) have less or even a positive effect on the persistence of pure catechin.

Chelation processes could affect plants in several different and context-dependent ways including, but not limited to, making toxic metals more bioavailable, mitigating toxic biochemical effects through competitive or transformational effects, increasing the stability and/or availability of phytotoxic biochemicals thereby creating synergistic effects (as suggested here for Pb and Ca), or otherwise altering the toxic effects of metals in the environment. If the stability of catechin is affected by different di- and trivalent

cations in the environment, soils varying in the concentration of different minerals may also vary in how they retain catechin and alter the phytotoxicity of the compound through purely abiotic reactions. Further, spatial heterogeneity in the distribution, speciation, chelation, etc. of metals and other soil components may have a profound effect on the local distribution and behavior, or even production, of particular root exudates.

Rapid oxidation (1-3 days) of catechin is commonly observed as a red coloration of media in Petri dishes (*our unpublished results*) and seedling establishment experiments (e.g. (Perry *et al.*, 2005a, Inderjit *et al.*, 2008a), or as a chemical darkening of roots (Bais *et al.* 2003; Weir *et al.* 2003); and therefore available pure catechin concentrations in such tests are likely to be much lower than the added amount. Where the chemical and physical properties of an environment are more complex (e.g. in soil), phenomena such as those reported here may enhance or inhibit the concentration and availability of pure catechin and thus either enhance or mitigate apparent phytotoxicity.

The high levels of plant mortality reported here for catechin and CMCs have not been reported before, either *in vitro* or in the field. The drought-like conditions imposed during the last three weeks of our experiment produced 15% mortality in the negative control plants, which is unusually high for such experiments (*our unpublished observations*), suggesting that there was substantial abiotic stress not associated with catechin treatments. We suggest that the plant mortality induced by catechin and CMCs in the current study was more prominent because of the abiotic stress imposed, and such stress may have exacerbated the effects of catechin and CMCs. For example, drying of

the sand substrate may have increased aqueous phase concentrations of catechin and CMCs at the root, leading to greater phytotoxic effects. Alternatively, the effects of catechin on mortality may have been to due to reduced root length and biomass as suggested by Perry *et al.* (2005a), and drought stress simply exacerbated a fundamental synergistic mechanism driving the deleterious effects of catechin on plants in the field. Such abiotic stresses might also contribute to the conditional effects of catechin.

To summarize, our experiments suggest strong but conditional effects of low and ecologically relevant concentrations of (±)-catechin on native North American plants. We observed different metal-specific effects on the amount of pure catechin in solution, its extractability from a sand-based matrix, and its inhibition of growth and exacerbation of mortality under stressful conditions. Such phenomena may help explain some of the observed spatial variation in soil catechin concentrations and toxicity, and the seemingly equivocal reports of the prevalence and importance of this root exudate to the invasive success of *Centaurea stoebe* and related systems.

These observations have significant impact on our thoughts regarding catechin phytotoxicity, bioavailability, and behavior in soil. Measurable pure catechin present in soil in the presence of *Centaurea* plants may have little to do with the observed degree of phytotoxicity at a given site. While this complicates the interpretation of data related to the role of catechin in *Centaurea* invasion, it serves to illustrate that the precise chemical forms, interactions, and effects of catechin in the environment are highly variable and unpredictable and that further examination is warranted to increase our understanding of

its effects and role in invasion. Finally, we suggest that the conditional effects observed for catechin likely extend to related compounds, other root exudates, and other systems involving chemically complex and spatially heterogeneous environments and that care should be taken in the design and interpretation of studies of such phenomena.

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Figures

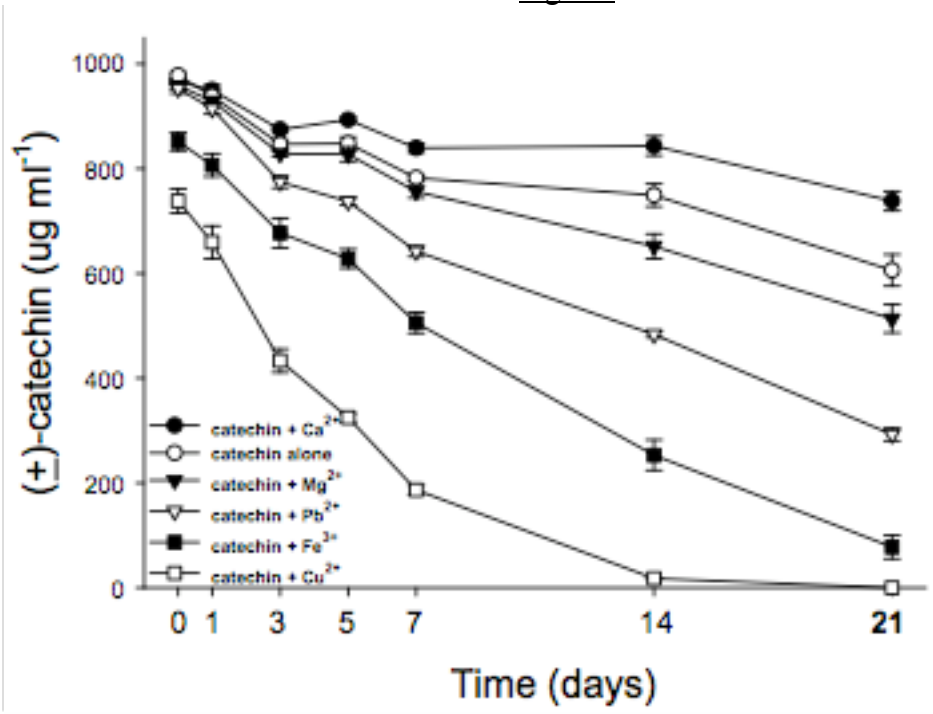

Figure 3.1

Mean concentrations of pure catechin in solution with time. Data shown are for catechin alone or in combination with various individual metals (CMCs). Error bars show ± 1 SE of the mean (n = 3).

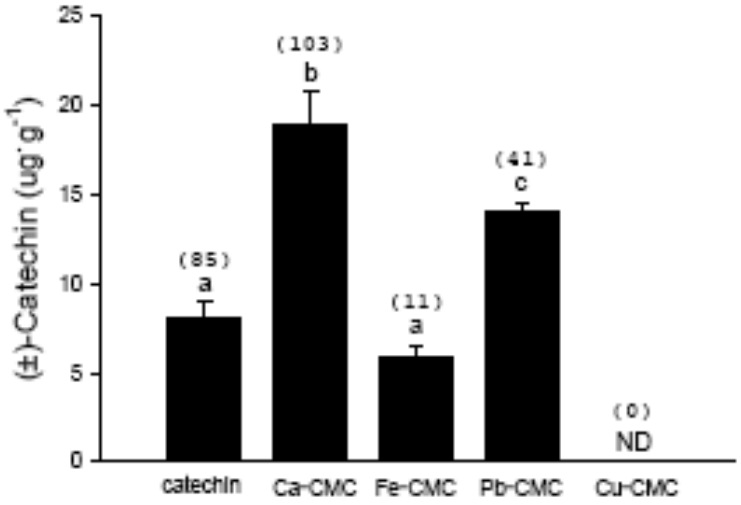

Figure 3.2

Pure catechin extracted from sand immediately after treatment with aged catechin or CMCs. Error bars show ± 1 SE of the mean (n = 6). Shared letters above indicate no significant difference in post-ANOVA HSD Tukey tests ($P < 0.05$). Numbers in parentheses above bars represent the calculated concentrations ($\mu g \cdot g^{-1}$) of pure catechin added to the sand for one dose.

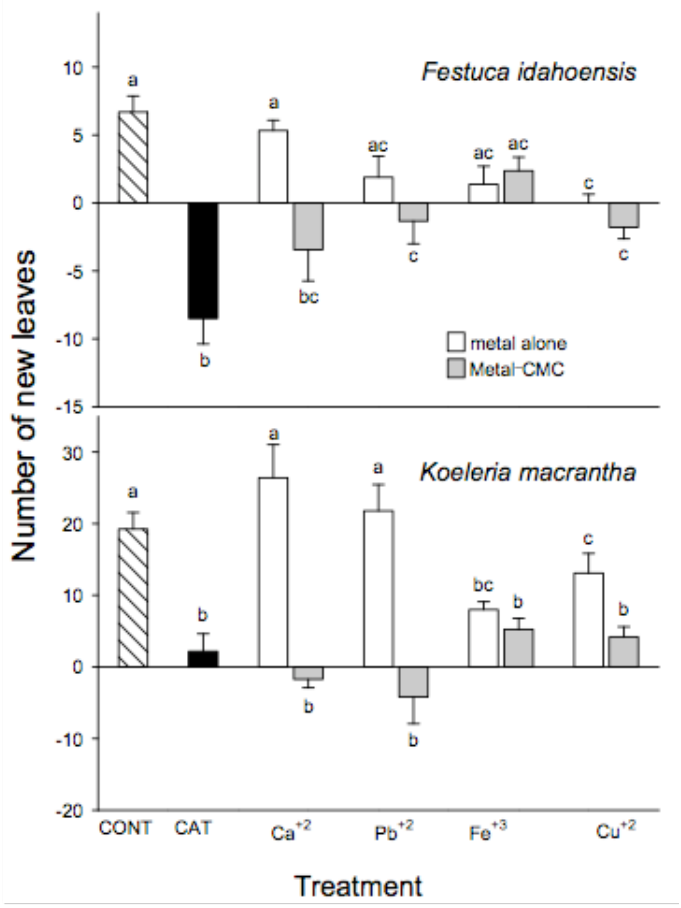

Figure 3.3

Number of new leaves produced by *Festuca idahoensis* and *Koeleria macrantha* in sand culture with the following solutions added: controls with no catechin added, CONT; catechin added, CAT; metals-alone, Metal-alone; and CMCs, Metal-CMC. Error bars show ± 1 SE of the mean. With each treatment considered independently in a single ANOVA, $F_{treatment} = 16.45$; df = 9,101; $P < 0.001$. Shared letters above indicate no significant difference in post-ANOVA HSD Tukey tests ($P < 0.05$).

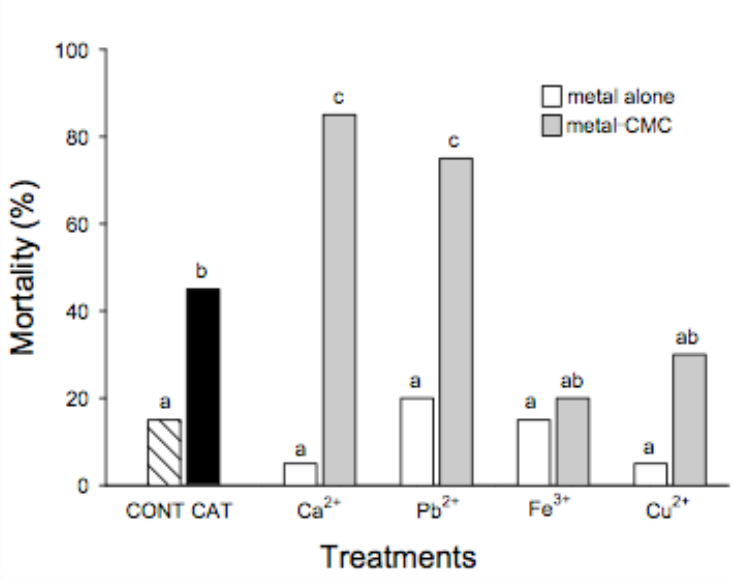

Figure 3.4

Percent mortality of *Festuca idahoensis* and *Koeleria macrantha* (combined) under drought-like conditions for controls with no catechin added, CONT; catechin added, CAT; metals-alone, Metal-alone; and CMCs, Metal-CMC. Shared letters above designate no significant differences among means ($P < 0.05$) as determined from paired logistic regression comparisons (SPSS 15.0, 2006).

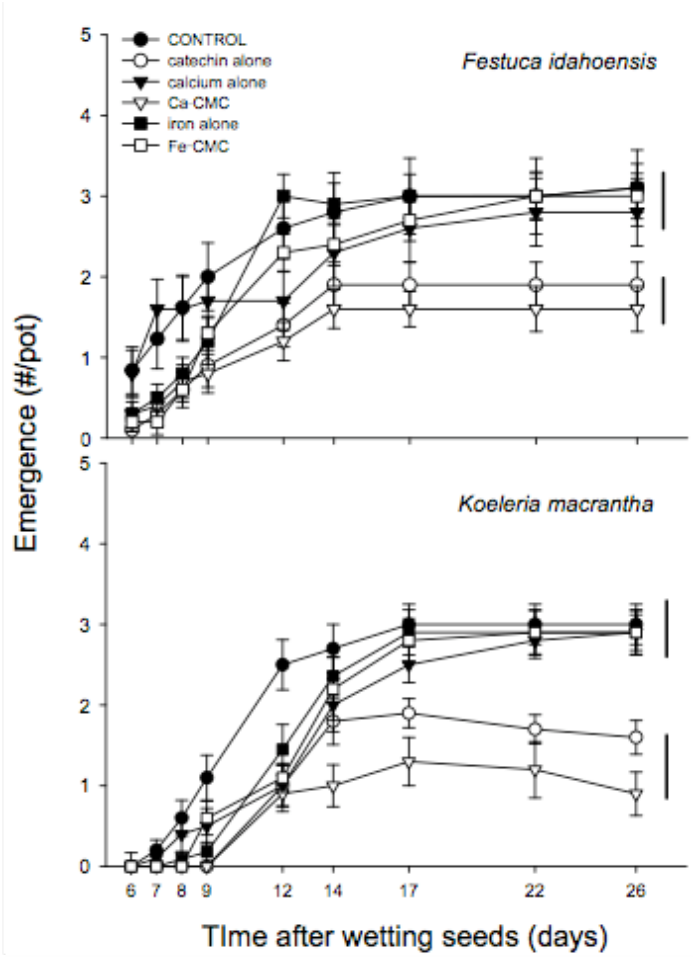

Figure 3.5

Impact of catechin, selected metals and CMCs on final seedling establishment of *Festuca idahoensis* and *Koeleria macrantha* in sand culture. Error bars show ± 1 SE of the mean. Final means sharing a vertical bar are not significantly different as determined by post-repeated measures ANOVA Tukey HSD tests. For *Festuca*, repeated measures ANOVA, $F_{treatment} = 6.464$; df = 5,55; $P < 0.001$). For *Koeleria*, repeated measures ANOVA, $F_{treatment} = 9.676$; df = 5,55; $P < 0.001$).

Chapter 4

(±)-CATECHIN, A ROOT EXUDATE OF *CENTAUREA STOEBE* LAM. (SPOTTED KNAPWEED), EXHIBITS BACTERIOSTATIC ACTIVITY AGAINST MULTIPLE SOIL BACTERIAL POPULATIONS

Abstract

Understanding the effects of allelopathic plant chemicals on soil microorganisms is critical to understanding their ecological roles and importance in exotic plant invasion. *Centaurea stoebe* Lam. (spotted knapweed), an aggressive invasive weed in North America, secretes a racemic mixture of (±)-catechin as a root exudate. This enantiomeric, polyphenolic compound has been reported to have allelopathic effects on surrounding flora and microflora. To better understand how catechin affects microbial communities in the rhizosphere of spotted knapweed, we assessed its impact on the total culturable bacterial component and numerous individual bacterial populations from Romanian (native range) and Montana (invaded range) soils. Catechin suppressed total culturable count numbers from the bacterial community and inhibited growth of some, but not all, soil bacterial populations tested. The native range bacterial community was generally more resistant to inhibitory effects of catechin than either the invaded or naïve soils. We further show that the inhibitory effect of catechin on nine different soil bacterial strains from seven genera was reversible, demonstrating that it acts via a bacteriostatic rather than bactericidal mechanism. These findings suggest that catechin is likely to have strong effects on bacterial community composition and activity in the rhizosphere.

Introduction

Centaurea stoebe Lam., spotted knapweed, is an invasive plant species of western North America that was introduced more than 100 years ago (Watson & Renney, 1974). Considered a noxious weed, spotted knapweed has been shown to flourish aggressively in North America relative to its behavior in its native range of Eastern Europe and Asia (Callaway*, et al.*, 1999). *C. stoebe* causes extensive damage estimated at $156 million per year resulting from displacement of crops and degradation of forage materials for grazing livestock (Bucher, 1984). Potential mechanisms behind the displacement of indigenous plant species and subsequent succession of *C. stoebe* are through evasion of natural predators and pathogens from its native habitat (Callaway*, et al.*, 1999, Callaway*, et al.*, 2004), allelochemical attack on native plant species in the invaded range, effects on soil fungal populations (Callaway*, et al.*, 2004, Mummey*, et al.*, 2005, Mummey & Rillig, 2006, Broz*, et al.*, 2007), and preliminary data suggesting effects on soil bacterial populations (Callaway*, et al.*, 2004, Inderjit*, et al.*, 2009).

C. stoebe exudes a racemic mixture of (±)-catechin (a flavan-3-ol) into its rhizosphere (Blair*, et al.*, 2006, Perry*, et al.*, 2007, Tharayil & Triebwasser, 2010). Recent research implicates (±)-catechin as both a plant and microbial toxin. The racemic (±) mixture has an inhibitory effect on other plant species including *Pseudoroegneria*, *Stipa*, *Koeleria*, and *Festuca* (Callaway*, et al.*, 2005, Pollock*, et al.*, 2009) and has also been reported to impact soil microorganisms *in vitro* (Callaway*, et al.*, 2005). Other evidence has demonstrated that *Centaurea stoebe* growth is facilitated by either promoting beneficial fungal interactions (Callaway*, et al.*, 2004) or by avoidance of harmful microbes

normally found in its native range of Europe (Callaway*, et al.*, 2004). Thus, important plant-microbe interactions could be disrupted by the presence of (±)-catechin in soil. However, research to date has not attempted to elucidate the effects that catechin has on specific soil microbial populations or the microbial community in general, nor the specific mechanisms by which it might exert such effects.

To understand how catechin affects soil and rhizosphere microbial communities, we investigated its overall impact on bacterial communities from Romanian (native range) and Montanan (both invaded and naïve) soils, as well as on individual soil bacterial isolates (populations) from these same soils. Additional experiments examined the effect of various catechin concentrations on bacterial growth kinetics, as well as the effect of removing catechin from the system in these inhibition studies. The relationship between catechin concentration and its impact on various bacterial soil isolates is discussed.

While there has recently been a tendency to rely heavily on molecular techniques to examine microbial community phenomena, with the exception of 16S rRNA gene classification of isolates, we were able to employ simple culture-based MPN assays and growth kinetics studies to establish that (±)-catechin has a general suppressive effect on the culturable aerobic, heterotrophic soil bacterial community in all soils. Further, we show that this suppression is manifested at the population-level as dose-dependent bacteriostatic inhibition that varied between the populations analyzed. These results suggest that catechin exuded by spotted knapweed will have differential effects at the bacterial population-level, which may interfere with, or enhance, important plant:microbe

(or microbe:microbe) interactions and thus play a role in the invasive success of this aggressive noxious weed.

Materials and methods

Source of Soil samples and soil microorganisms

Soil was obtained from a native grassland plant community including spotted knapweed in Timisesti, Romania (47.13°N/26.29°E) to represent the soil microbial community in the native range of *C. stoebe* and was collected on March 24, 2005. This sample was comprised of 5 individual soil samples (0-10 cm depth from the A horizon) from random locations within the grassland plot that were composited, mixed thoroughly, and sieved (2 mm) to provide a representative sample hereafter referred to as "native". The soils at this site are classified as Entic Hapludolls and Chernozems. Two different soils (both collected in mid-June of 2005) from western Montana were used to represent invaded and naïve soil microbial communities, respectively. Soil from the Bandy Ranch, near Ovando, Montana (47.06°N/113.26°W) classified as Perma gravelly loam was collected, pooled and sieved as described above to represent a typical spotted knapweed-invaded location, hereafter referred to as "invaded". Similarly, soil from the Clearwater Game Range, near Clearwater Junction, Montana (47.08°N/113.04°W) classified as a Winkler gravelly loam was collected, pooled and sieved to represent a naïve Montana soil that has never experienced spotted knapweed invasion, nor been treated to prevent it (M. Thompson, *personal communication*). This sample is hereafter referred to as "naïve". Multiple bacterial isolates representing phenotypically diverse (based on colony

morphology) populations were obtained from each soil type using R2 broth and R2A agar as described below for use in population-level catechin inhibition experiments.

Two other Montana soil samples: one from the rhizosphere of spotted knapweed plants within the Bob Marshall Wilderness (47.80°N/113.03°W) on April 6, 2005; and the other from the rhizosphere of spotted knapweed plants on Waterworks Hill near Missoula, Montana (46.88°N/113.98°W) on April 5, 2005 were the source of additional catechin-sensitive and -resistant rhizosphere bacterial strains. As soil had to be air-dried for shipment at 4 °C from Romania to the USA, all other soil samples were also air-dried and held at 4 °C prior to analysis for the sake of consistency. Soil samples were sent to Soil, Water, & Forage Testing Lab (College Station, TX USA) for chemical analysis (Table 1).

Pure culture isolates were recovered from the rhizosphere of knapweed plants at the Waterworks Hill and Bob Marshall Wilderness sites via initial growth on R2A + cycloheximide (300 $\mu g \cdot ml^{-1}$) and subsequently restreaked onto R2A to ensure purity. Sixteen pure isolates were shown to represent diverse genera and species based on 16S rRNA gene affiliation (see below) and used to test for catechin sensitivity and recovery from inhibition (further below). Of the sensitive strains, *Burkholderia* strain G-5 was selected for use in detailed growth kinetics experiments to further elucidate the bacteriostatic effects of catechin.

Identification of bacterial isolates based on 16S rRNA gene affiliation

To ensure the phylogenetic diversity of the isolates used for catechin sensitivity and recovery assays, their 16S rRNA gene was PCR amplified, sequenced, and compared to known 16S rRNA genes using standard techniques. Briefly, PCR was performed using the generally conserved 16S rRNA gene primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3'), 536f (5'-CAGCMGCCGCGGTAATWC-3'), 907r (5'-CCGTCAATTCMTTTRAGTTT-3'), and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') as described previously (Holben*, et al.*, 2004). PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and the manufacturer's recommended protocol and then sequenced using the Sanger dideoxy method at the University of Montana Murdock Molecular Biology Facility. The obtained sequences were compared with their closest relatives using the Sequence Match function at the RDP II website (Cole*, et al.*, 2005). Strain designations and closest phylogenetic matches to the Genus level are provided in Table 2.

Bacterial growth media and chemicals

R2 broth (Difco, Detroit, MI) was prepared according to the manufacturer's suggested protocol and, after autoclaving and cooling to 65 °C, amended to a final concentration of 300 $\mu g\,ml^{-1}$ cycloheximide (to inhibit fungal growth) by addition of 10 ml of a 30 $mg\,ml^{-1}$ stock solution (in 100% ethanol) to 1 L R2 broth. R2A plates were prepared in the same fashion as R2 broth, except that 15 g Bacto-agar (Becton Dickinson, Sparks, MD) was added prior to autoclaving. Where minimal medium plus glucose was employed, MMO minimal salts medium was prepared as described previously (Stanier*, et al.*, 1966), and amended with 500 $\mu g\,ml^{-1}$ glucose and 300 $\mu g\,ml^{-1}$ cycloheximide. Whenever (±)-

catechin was amended to liquid or agar media for use in community- and population-level analyses, these were prepared as described below.

(±)-Catechin hydrate (>99% purity) (hereafter referred to simply as catechin) was obtained from Shivambu International (New Delhi, India) and all other chemicals were obtained from Sigma (St. Louis, MO, USA). Due to its limited solubility in water, stock solutions of catechin (150 $mg \cdot ml^{-1}$) in 100% methanol were made fresh for use in community- and population-level inhibition experiments (see below). All liquid and agar media were stored in the dark prior to and during use in experiments. Liquid and agar media amended with various concentrations of catechin were made by adding (after autoclaving and cooling the media to 65 °C) the appropriate volume of catechin stock solution, along with the appropriate volume of 100% methanol, so that a constant volume of methanol (20 $ml \cdot L^{-1}$) was added to all media and only catechin varied in concentration between 0 and 3,000 $\mu g \cdot ml^{-1}$. This was done to normalize for any methanol-based effects on bacteria, although these are unlikely since the methanol was assumed to have evaporated from media prior to use in experiments.

Community-level inhibition of bacteria by catechin

To examine general inhibition of the total cultivable bacterial community by catechin, naïve soil was used to enumerate the fraction of the total bacterial community capable of aerobic, heterotrophic growth on general medium. The numbers of bacteria that grew in R2 broth (containing cycloheximide) with or without catechin were determined using the three-tube most-probable-number (MPN) approach (Banwart, 1981). To achieve this,

naïve soil was split into four equal proportions (11.23 g dry weight each) and placed into four sterile 50 ml screw-top flasks. All four subsamples were hydrated drop-wise with sterile double distilled water (ddH_2O) to 40% water holding capacity. These were subsequently amended with solutions of catechin in 10 ml of 10% methanol to yield final soil concentrations for catechin of 500, 1000, and 3000 $\mu g\ g^{-1}$. Control treatments with 0 $\mu g\ g^{-1}$ catechin were similarly amended with 10 ml of 10% methanol for the reason described above. All treatments were brought to 100% water holding capacity after catechin addition. After 8 days of incubation in the dark at 24 °C, 500 mg aliquots from each flask were added to 4.5 ml of R2 broth amended with either the same concentration of catechin that was present in the original incubation (0, 500, 1000, or 3000 $\mu g\ ml^{-1}$, respectively) to simulate continuous exposure to catechin, or with no catechin to assess the effect of its removal from the system. These suspensions also contained 1% sodium pyrophosphate and were mixed vigorously by vortexing to aid in detaching cells from soil particles. Aliquots of each of these suspensions (100 μL) were then serially diluted into 900 μL of the same type of medium (but without pyrophosphate) with vigorous mixing after each dilution. After 7 days of incubation at room temperature with orbital shaking at 150 rev min^{-1} in the dark, each set of tubes was visually scored for bacterial growth based on increased turbidity.

Population-level inhibition of bacteria by catechin

To compare the degree to which catechin inhibited individual aerobic, heterotrophic bacterial populations, the three soils (native, invaded, and naïve) were sampled using serial dilution plating to obtain individual isolates from each site. To achieve this, 10 g of

soil was placed into 90 ml sodium phosphate buffer (100 mM, pH 7.0), shaken vigorously by hand for 2 min, after which large particles were allowed to settle for 1 min. Decadal serial dilutions into phosphate buffer were performed to 10^{-8}, after which 100 μL of each dilution was spread onto R2A supplemented with cycloheximide. All plates were incubated at room temperature in the dark for ~14 days, after which 140 to 150 phenotypically distinct colonies (based on colony size, color, texture, and rate of growth) from appropriate dilutions (producing between 30-300 colonies per plate) for each soil type were sequentially replica-plated onto R2A agar containing: 500 μg ml^{-1} catechin; 1,000 μg ml^{-1} catechin; 3,000 μg ml^{-1} catechin; and, finally 0 μg ml^{-1} catechin to confirm growth of the transferred colony in the absence of catechin (each plate also contained 300 μg ml^{-1} cycloheximide). Colonies were transferred by touching the chosen colony with the blunt end of a sterile, plain wood applicator (Fisher Scientific Pittsburgh, PA USA Cat# 01-340) and then touching each of the above four plates in succession in an ordered array. All plates were incubated at room temperature in the dark and colony growth at each position was recorded at 1, 2, 4, and 6 days to qualitatively assess colony growth rate as a function of catechin concentration. Only colonies that grew successfully on the final control plate (i.e. no catechin added) were scored on the catechin-amended plates. Each bacterial population was categorized as either Not Affected (NA), Semi-Inhibited (SI), or Completely Inhibited (CI) based on its rate of growth (colony formation) on catechin-amended plates compared to the "no catechin" control plate.

To establish whether catechin inhibition was bacteriostatic and therefore reversible, or bacteriocidal, nine bacterial strains (representing seven bacterial genera) that were

inhibited by catechin on agar plates were tested for their ability to grow in liquid medium before and after removal of catechin from the medium (Table 2). All strains were initially grown in MMO medium with 500 μg ml^{-1} glucose to mid-log phase. These cultures were then transferred (1:100 inoculum) into MMO medium containing 500 μg ml^{-1} glucose and the following amendments: 0 μg ml^{-1} catechin; 2000 μg ml^{-1} catechin; and 2000 μg ml^{-1} catechin that was removed after the corresponding no catechin control culture had reached stationary phase. The catechin concentration of 2000 μg ml^{-1} was selected as being intermediate in the previous inhibition experiments. Catechin was removed by collecting the inhibited cells by centrifugation, carefully pouring off the supernatant, resuspending and washing the cells once with sterile PB (100 mM phosphate buffer; pH 7), collecting by centrifugation, then resuspending the cells in MMO medium with 500 μg ml^{-1} glucose without catechin. All treatments were performed in triplicate and incubated at room temperature in the dark with rotary shaking at 150 rev min^{-1}.

One of these catechin-sensitive soil bacterial isolates, *Burkholderia* strain G-5, representative of the inhibited populations, was used to examine in detail the kinetics of growth in the absence, presence, and following removal of catechin from the medium. Growth kinetics for strain G-5 were obtained by 1:100 dilution of triplicate fresh overnight cultures in MMO medium with 500 μg ml^{-1} glucose into fresh MMO + glucose medium amended with: 0 μg ml^{-1} catechin; 2000 μg ml^{-1} catechin; and 2000 μg ml^{-1} catechin that was subsequently removed. For this last treatment, catechin was removed by collecting the inhibited cells by centrifugation after 36 hours (when the parallel cultures of strain G-5 without catechin had reached stationary phase), carefully pouring

off the supernatant, resuspending and washing the cells once with sterile PB, collecting by centrifugation, then resuspending the cells in fresh MMO medium containing 500 μg ml^{-1} glucose but no catechin. All treatments were performed in triplicate and incubated at room temperature in the dark with rotary shaking at 150 rev min^{-1}. Cell growth was monitored based on optical density at 550 nm using a Spectronic 20D+ spectrophotometer (Milton Roy USA, Ivyland, PA). As catechin exhibits limited auto-oxidation with resultant absorbance at 550 nm, two sets of blanks were used for this experiment. The blank for cultures grown without catechin was MMO medium with 500 μg ml^{-1} glucose, while the blank for cultures grown in the presence of catechin was MMO medium with 500 μg ml^{-1} glucose and 2000 μg ml^{-1} catechin. Both blanks were incubated under conditions identical to the cultures. To avoid adding methanol to these cultures, catechin was completely dissolved by gentle heating in 5 ml of sterile ddH_2O and immediate addition to the MMO + glucose medium effectively diluting the catechin before it could precipitate.

Results

Inhibition of the soil bacterial community by catechin

MPN enumeration using R2 broth was used to assess the effect of catechin on the aerobic heterotrophic bacterial community of soils. This was accomplished by determining the fraction of the total bacterial community capable of growth in the presence of various concentrations of catechin. The number of culturable heterotrophs ranged from 4.27 ± 2.14 (standard error, SE) × 10^8 cells (g of soil)$^{-1}$ with no catechin to as low as 4.27 ± 2.14 (SE) × 10^6 cells (g of soil)$^{-1}$ when high levels of catechin were present (Fig. 1). Dose-

dependent inhibition of growth with increasing catechin concentration was observed when catechin concentrations were maintained. Interestingly, samples treated with catechin but then released from its presence showed a complete reversal of catechin inhibition and their MPN values were comparable to or exceeded those observed in the no-catechin control tubes.

Inhibition of growth of individual populations by catechin

Having shown that catechin had a general depressive effect on the growth of the aerobic, heterotrophic bacterial community, the effect of catechin at the level of individual bacterial populations was investigated through ordered replication of colonies on media with various concentrations of catechin or no catechin. Higher concentrations of catechin showed greater inhibition in terms of percent of populations capable of growth (Fig. 2). Further, when the growth of individual populations was classified as being not affected (NA), semi-inhibited (SI) (i.e. late colony formation and slow growth relative to the no-catechin controls), or completely inhibited (CI) relative to no-catechin controls, populations from the native range exhibited trends toward greater resistance to catechin than from the naïve and invaded ranges, with larger proportions of NA and lower proportions of SI and CI populations for all concentrations of catechin.

Catechin inhibition is bacteriostatic

To further investigate the seeming reversal of inhibition that was observed in the MPN analysis, a suite of soil isolates were tested for sensitivity or resistance to the inhibitory effects of catechin, and the sensitive isolates tested for their ability to resume growth after

removal of catechin from the media (Table 2). Of the sixteen isolates tested, seven were unaffected by catechin and, interestingly, six of these were identified as *Pseudomonas* sp. representing five different taxa based on their 16S rRNA gene sequence while the seventh was identified as *Rhodococcus* sp. (Table 2). Eight of the remaining nine strains tested were completely inhibited by catechin, while one, *Bacillus* strain C-5, was semi-inhibited. Growth of all nine of the inhibited strains (including C-5) was completely restored after removing catechin from the medium by washing the cells and placing them into fresh MMO plus glucose medium without catechin (Table 2). Seven different genera were represented in the group of nine sensitive but recoverable isolates, suggesting that catechin may be a fairly broad inhibitor of growth across bacterial populations, but clearly not all are affected.

Catechin effects on bacterial growth kinetics

To better examine the inhibitory effects of catechin on bacterial growth, *Burkholderia* strain G-5 was subjected to detailed growth kinetics assays in the absence, presence, and following removal of catechin in the medium (Fig. 3). When catechin was present, growth of strain G-5 was essentially completely inhibited relative to the no catechin control, even after prolonged (~200 h) incubation. When catechin was removed from the medium after 36 h of completely inhibited growth (by washing the cells and placing them into fresh MMO plus glucose medium), normal growth kinetics essentially indistinguishable from the no-catechin controls resumed following a substantial (≥80 h) lag period (Fig. 3).

Discussion

The impact of catechin on indigenous plant species of North America has been extensively studied (Buta & Lusby, 1986, Iqbal*, et al.*, 2003, Inderjit*, et al.*, 2008), while its effects on soil bacterial communities have not been thoroughly examined. The few reports to date on this topic have suggested that catechin is "antimicrobial" to certain laboratory strains relevant to soils (e.g. *Xanthomonas campestris*, *Agrobacterium radiobacter*, *Erwinia carotovora*, and *Erwinia amylovora*) (Veluri*, et al.*, 2004), but have not examined the nature of the inhibitory effect(s) or their generality against a broad array of soil microbial populations.

In the current study, we demonstrated a general dose-dependent suppressive effect of catechin on the aerobic, heterotrophic bacterial community of soils that reduced MPN enumeration values by up to two logs (99%) at the highest concentration tested (Figure 1). The inhibitory effect on total community numbers was fully relieved upon transfer to catechin-free medium, suggesting that its action is bacteriostatic rather than bactericidal. We note that the higher MPN numbers observed following release from catechin inhibition in this MPN assay (Figure 1) suggest the potential existence of some poorly understood rebound effect upon release from catechin suppression, since the control treatments that never experienced catechin did not exhibit this same increase.

It is also interesting to note that a small proportion of the bacterial community in these soils (~1% based on the MPN assay) was capable of forming a soluble red compound in the presence of catechin (data not shown). This compound may represent some catechol-

like intermediate of catechin breakdown by bacterial populations in the soil. While this interpretation is consistent with the observations, it is purely speculative in the absence of additional data and the basis for and significance of this phenomenon remain unknown at this time.

The data from the replica-plating assay with individual bacterial populations (refer to Fig. 2) are also indicative of dose dependent catechin inhibition in that, for each soil type, the number of NA populations decreased, while the number of SI or CI populations increased with catechin concentration. Figure 2 also demonstrates that the soil bacterial community from the native range is generally more resistant to the inhibitory effects of catechin than either the invaded or naïve sites in Montana as evidenced by its generally higher numbers of NA populations and generally lower numbers of SI or CI populations at each catechin concentration. This phenomenon may reflect long-term selection for catechin-tolerant bacterial populations in the native range of *Centaurea stoebe.*

These results raise intriguing possibilities regarding soil bacterial population dynamics in the face of invasion by spotted knapweed. Populations showing no sign of inhibition by catechin at any concentration (NA) have likely been selected for over time in the native range such that the community has overall greater resistance or tolerance to the bacteriostatic effects of catechin. By contrast, NA populations in the naïve and invaded ranges represent potential successor populations in the presence of catechin, which could produce a shift in soil microbial community composition to populations resistant (or tolerant) to the bacteriostatic effects of catechin with prolonged exposure. This scenario

could enhance the invasive success of spotted knapweed if those populations either facilitate its growth or suppress growth of native plant populations. CI populations, on the other hand, represent catechin-sensitive taxa that might be supplanted by resistant or tolerant ones during knapweed infestation, although moderate numbers of CI populations were also detected in the native range. Such population shifts could have dramatic effects on soil nutrient cycling or other important processes depending on the identities and functions of the inhibited populations.

The SI populations also merit consideration. These were observed from all soils and there may be several reasons for their partial state of inhibition. Among these is the observed instability of catechin over time (Blair*, et al.*, 2005, Blair*, et al.*, 2006, Perry*, et al.*, 2007, Broeckling & Vivanco, 2008, Inderjit*, et al.*, 2008). Since catechin effects are dose-dependent, biotic or abiotic transformation of catechin into a biologically inert form might decrease its effective concentration below the inhibitory level for these populations over time, thereby restoring growth. Another possible explanation might be the ability of some microbial populations to mitigate the inhibitory effects of catechin. Since catechin is a known metal chelator (Gomah & Davies, 1974, McDonald*, et al.*, 1996, Lim*, et al.*, 2005, Pollock*, et al.*, 2009), delayed growth may represent a lag period prior to induction or activation of pathways for sequestration of metals or other micronutrients required for growth. Indeed, experiments examining the effects of catechin and various related analogs (present as natural products in teas, wines, and certain other food products) on gastrointestinal microflora showed that various bacterial populations were differentially impacted by these compounds (Ishigami & Hara, 1993, Hara, 1997). Among those not

affected were the Lactic Acid Bacteria, which, interestingly, are reported to have no metabolic requirement for iron (Archibald, 1983, Boyaval, 1989, Bruyneel*, et al.*, 1989, Pandey*, et al.*, 1994, Imbert & Blondeau, 1998). Finally, since catechin and similar compounds have variously been reported to damage cell membranes (Hoshino*, et al.*, 1999), block cellular transport systems (Shimizu*, et al.*, 2000, Song*, et al.*, 2002), and damage DNA (Hayakawa*, et al.*, 1999), bacterial populations with less extensive damage or more robust repair systems may be immune to (e.g. NA), or capable of overcoming with time (e.g. SI), inhibitory effects of catechin. Given the genetic, metabolic and physiological diversity of soil bacteria, it is perhaps not surprising that a full spectrum of responses to catechin exposure was observed across these populations.

Sixteen soil bacterial populations isolated from the rhizosphere soil of spotted knapweed and representing nine different genera were tested for their ability to grow in the presence of catechin (Table 2). Nine microbes exhibited either complete or partial inhibition of growth by catechin, which was shown to be restored after its removal. To better illustrate that catechin was capable of fully inhibiting growth and that growth could be fully restored by removing catechin, the kinetics of growth of one of the populations, *Burkholderia* strain G-5, were examined in detail. The results clearly demonstrated complete inhibition of growth of G-5 by catechin, but that normal growth was fully restored following catechin removal.

The results presented herein collectively demonstrate that catechin is a bacteriostatic, not bactericidal, inhibitor of some, but not all, bacterial populations in soils. This is

ecologically important in this invasive plant system because, if catechin were somehow removed (e.g. through knapweed suppression followed by biodegradation or abiotic transformation), inhibited bacterial populations could potentially rebound and resume normal growth. This scenario may be of great relevance in attempts to mitigate knapweed invasion and restore the ecology of native systems.

In these experiments, we used a range of catechin concentrations ranging from 0 – 3,000 $\mu g \cdot ml^{-1}$. It is salient to note that reports in the literature have indicated catechin concentrations in soil harboring knapweed that range from 1.1 $\mu g\ g^{-1}$ to ~650 $\mu g\ g^{-1}$ (Blair*, et al.*, 2006, Perry*, et al.*, 2007). However, those prior studies measured catechin in bulk soil samples using a procedure involving extraction and detection of chemically pure catechin by HPLC. We suggest that the concentrations of catechin a bacterium would encounter at the point of exudation into the soil (i.e. in the rhizoplane/rhizosphere) would be much higher and that the range of concentrations used in the current study are ecologically relevant for microbes living at the root - soil interface. We also suggest that a steep gradient of catechin concentration exists as a function of distance from knapweed roots, as is commonly found for other root exudates (Kuzyakov*, et al.*, 2003, Sauer*, et al.*, 2006), and that the inhibitory effects of catechin on soil microbial populations thus decrease with distance from the knapweed rhizoplane. We also note that catechin has been reported to be chemically unstable in media, sand, and soil (Blair*, et al.*, 2005, Blair*, et al.*, 2006, Perry*, et al.*, 2007, Inderjit*, et al.*, 2008, Pollock*, et al.*, 2009), presumably being transformed through biotic and/or abiotic processes into derivative compounds (e.g. the aforementioned soluble red compound) and that such compounds would not be

detected as catechin by the currently available HPLC-based methods. Finally, as we have shown recently, derivative compounds of pure catechin may have greater or less toxicity than chemically pure catechin (Pollock*, et al.*, 2009). In other words, it is difficult to determine precisely how much biologically active catechin is present in various compartments of soil ecosystems.

Catechin exuded by *C. stoebe* potentially disrupts a number of important plant:microbe interactions, both beneficial and antagonistic. Such disruptions may play an important role in the invasive success of spotted knapweed as they impact both the plant and microbial components of the system (Callaway & Ridenour, 2004) by acting at the level of individual soil bacterial populations (this work). Thus, catechin may facilitate the invasion of spotted knapweed into naïve soils through a variety of potential mechanisms including suppression of pathogens of *C. stoebe*, facilitation of pathogens specific for native plants, suppression of microbes promoting growth of competitors, or facilitation of microbes promoting growth of *C. stoebe*.

Conclusions

Our results show that a diverse set of bacterial populations from soils native to spotted knapweed, invaded by spotted knapweed, and naïve to spotted knapweed were differentially affected by catechin. More specifically, the data show that: 1) the inhibitory effect is dose-dependent at both the community and the population level; 2) individual microbial populations are differentially sensitive to catechin; 3) catechin is bacteriostatic to those populations that are affected; and, 4) removal of the catechin from

the system allows normal bacterial growth kinetics to resume. We further suggest that catechin could perturb normal soil bacterial community structure and function as well as plant-microbe relations, and that removal of catechin in soil by degradation or transformation could allow an impacted soil microbial community to resume its normal structure and function. This last suggestion may have profound ramifications for attempts to restore knapweed-infested ecosystems.

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Tables

Table 4.1 List of soils and soil characteristics

Soil	pH	Conductivity (umho/cm)	NO_3^- ppm	P ppm	K ppm	Ca ppm	Mg ppm	S ppm	Na ppm	OM %
Bandy Ranch	6.0	344	135	36	342	2054	213	22	80	5.05
Romania	6.4	444	85	10	485	2962	420	18	70	7.11
Clearwater	5.5	313	112	31	229	1642	187	28	69	4.30
Soil C	6.5	340	119	36	495	2254	266	26	59	4.52
Soil G	5.0	356	110	57	301	1710	209	16	59	6.18

Table 4.2 List of microorganisms isolated from Petri plates, S_ab scores and their closest match to other known microorganisms based on 16S rRNA gene analysis, and their relationship to catechin when grown under aerobic glucose-oxidizing conditions. **Bold strains** are inhibited by catechin but are restored when catechin is removed from the media by washing. G denotes growth; PG denotes poor growth; NG denotes No growth.

Strain	S_ab score (based on # nucleotides)	Closest Phylogenetic Match (Genus level)	Gram reaction	Glucose	Glucose + Catechin	Post Wash
C-1	0.913 (1246)	***Aeromicrobium***	**positive**	**G**	**NG**	**G**
C-2	0.957 (1344)	***Chryseobacterium***	**negative**	**G**	**NG**	**G**
C-4	0.975 (1325)	***Arthrobacter***	**positive**	**G**	**NG**	**G**
C-5	0.996 (1397)	***Bacillus***	**positive**	**G**	**PG**	**G**
C-6	0.985 (1285)	*Pseudomonas*	negative	G	G	NT[a]
C-7	0.978 (1443)	*Pseudomonas*	negative	G	G	NT
C-8	0.984 (1423)	*Pseudomonas*	negative	G	G	NT
C-9	0.987 (1222)	***Flavobacterium***	**negative**	**G**	**NG**	**G**
C-10	0.977 (1230)	***Arthrobacter***	**positive**	**G**	**NG**	**G**
G-1	0.773 (1412)	***Pedobacter***	**negative**	**G**	**NG**	**G**
G-2	1.000 (720)	*Rhodococcus*	positive	G	G	NT
G-4	0.995 (1386)	*Pseudomonas*	negative	G	G	NT
G-5	0.974 (1382)	***Burkholderia***	**negative**	**G**	**NG**	**G**
G-7	0.995 (1398)	*Pseudomonas*	negative	G	G	NT
G-8	0.995 (1400)	*Pseudomonas*	negative	G	G	NT
G-9	0.968 (1379)	***Flavobacterium***	**negative**	**G**	**NG**	**G**

[a]NT stands for Not Tested

Figures

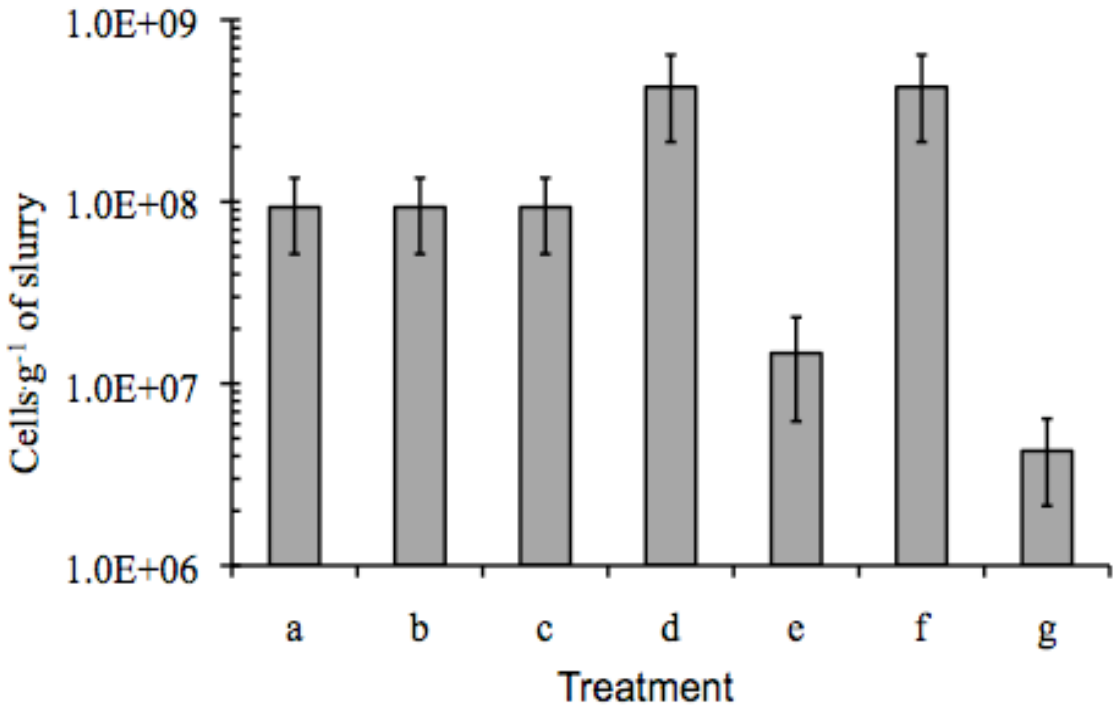
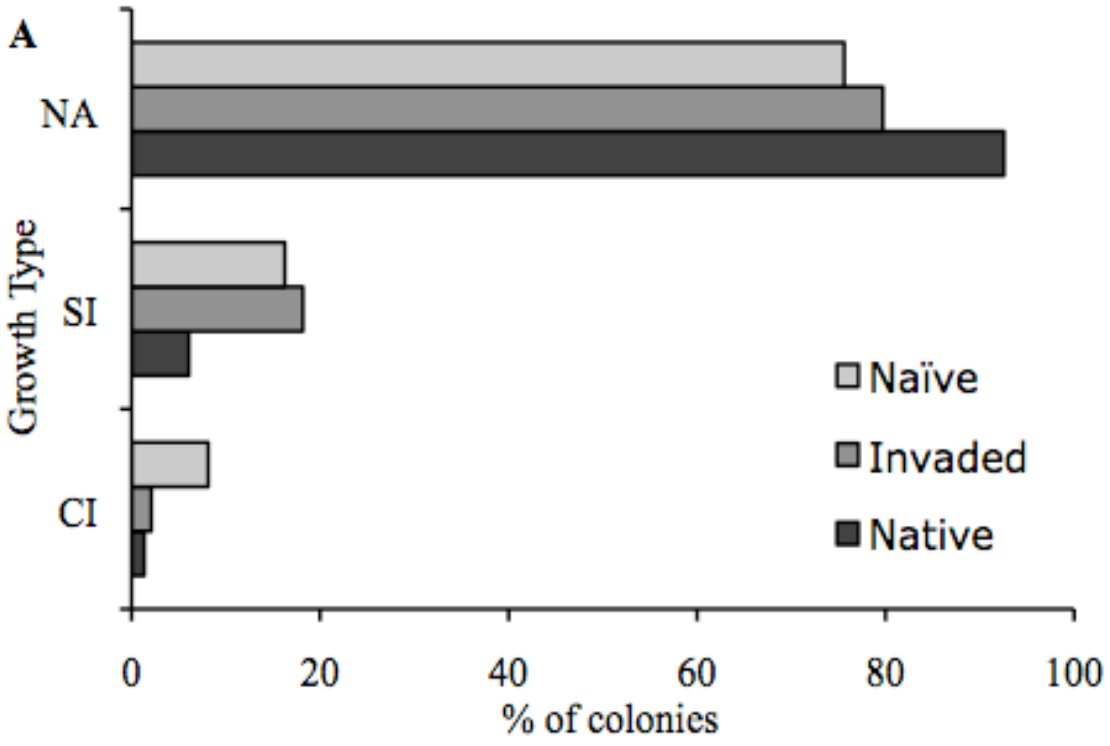
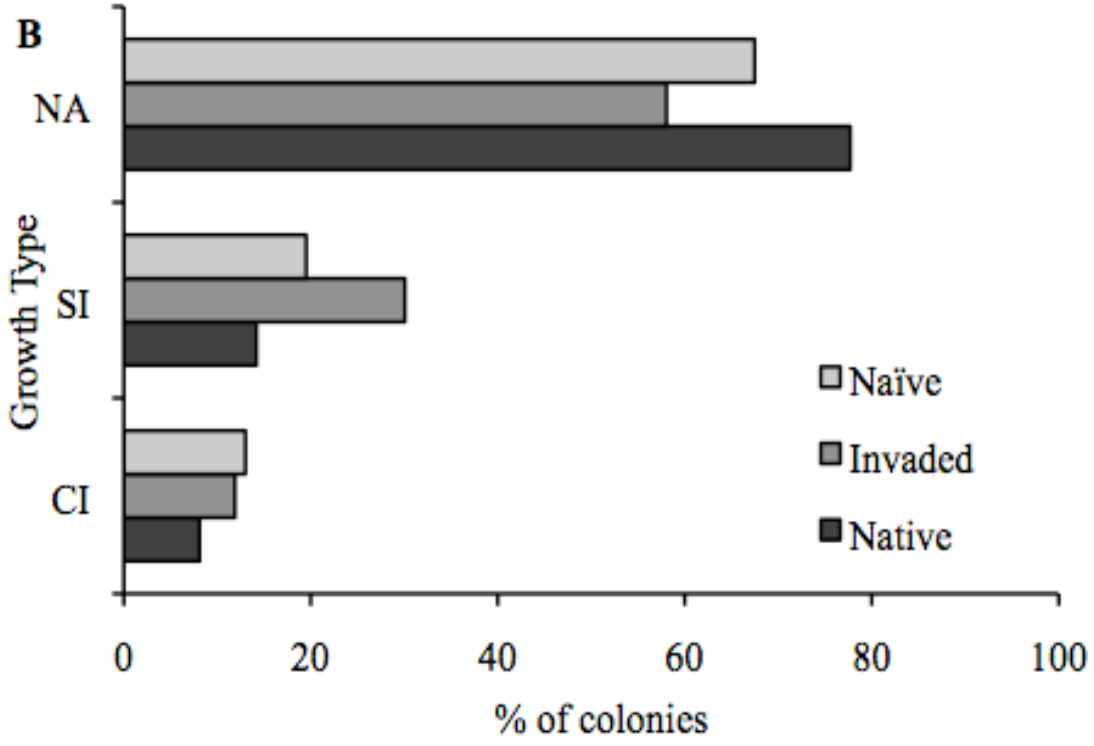


Figure 4.1

MPN analysis of the naïve soil bacterial community grown in R2 broth + cycloheximide medium with and without catechin. Bars represent the average of triplicate determinations ±1 SE. Treatments were as follows: **a)** control (0 μg g^{-1} and 0 μg ml^{-1} catechin in soil and media, respectively); **b)** 500 μg g^{-1} catechin in soil, 0 μg ml^{-1} catechin in media; **c)** 500 μg g^{-1} and 500 μg ml^{-1} catechin in soil and media, respectively; **d)** 1000 μg g^{-1} catechin in soil, 0 μg ml^{-1} catechin in media; **e)** 1000 μg g^{-1} and 1000 μg ml^{-1} catechin in soil and media, respectively; **f)** 3000 μg g^{-1} catechin in soil, 0 μg ml^{-1} catechin in media; **g)** 3000 μg g^{-1} and 3000 μg ml^{-1} catechin in soil and media, respectively.


A
NA
SI
CI
Growth Type
Naïve
Invaded
Native
0
20
40
60
80
100
% of colonies


B
NA
SI
CI
Growth Type
Naïve
Invaded
Native
0
20
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60
80
100
% of colonies

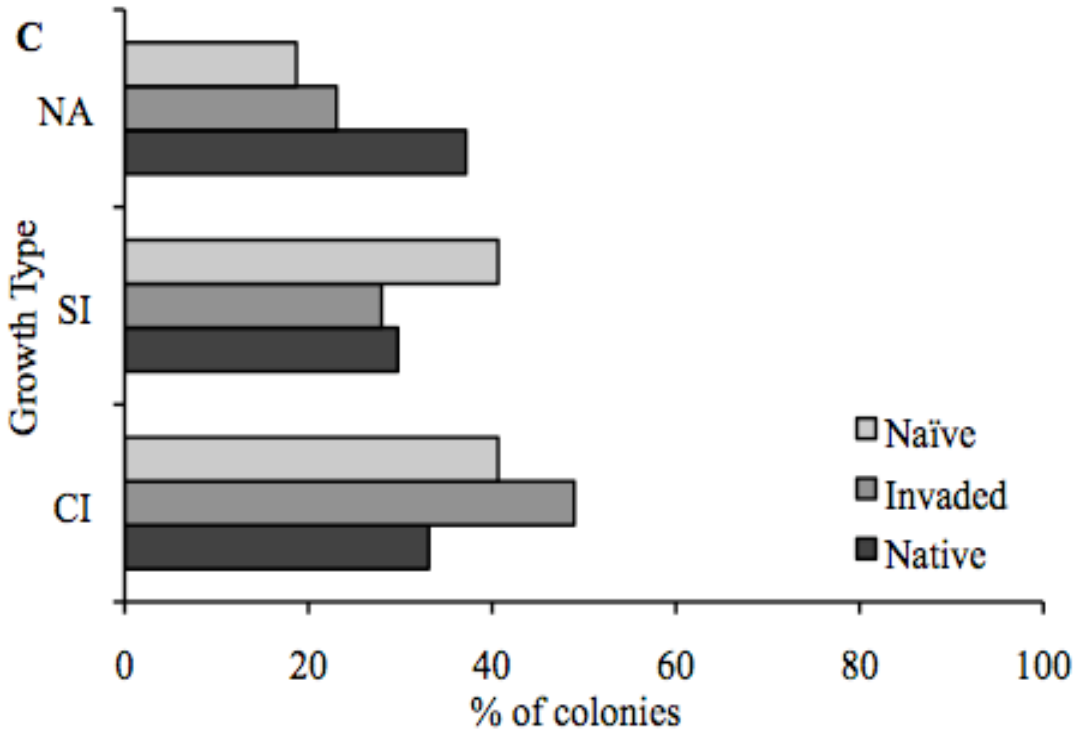

Figure 4.2

Isolates from naïve (123 colonies), invaded (143 colonies), and native (148 colonies) soils plated on R2A containing various concentrations of catechin. Panel A: colony growth under 500 μg ml^{-1} catechin conditions; Panel B: colony growth under 1000 μg ml^{-1} catechin conditions; Panel C: colony growth under 3000 μg ml^{-1} catechin conditions. Growth type as a result to exposure to catechin was characterized as: NA (Not Affected), SI (Semi-Inhibited), and CI (Completely Inhibited) and colony growth categories were compared to growth on control plates (0 μg ml^{-1} catechin) and normalized to the percentage of colonies tested.

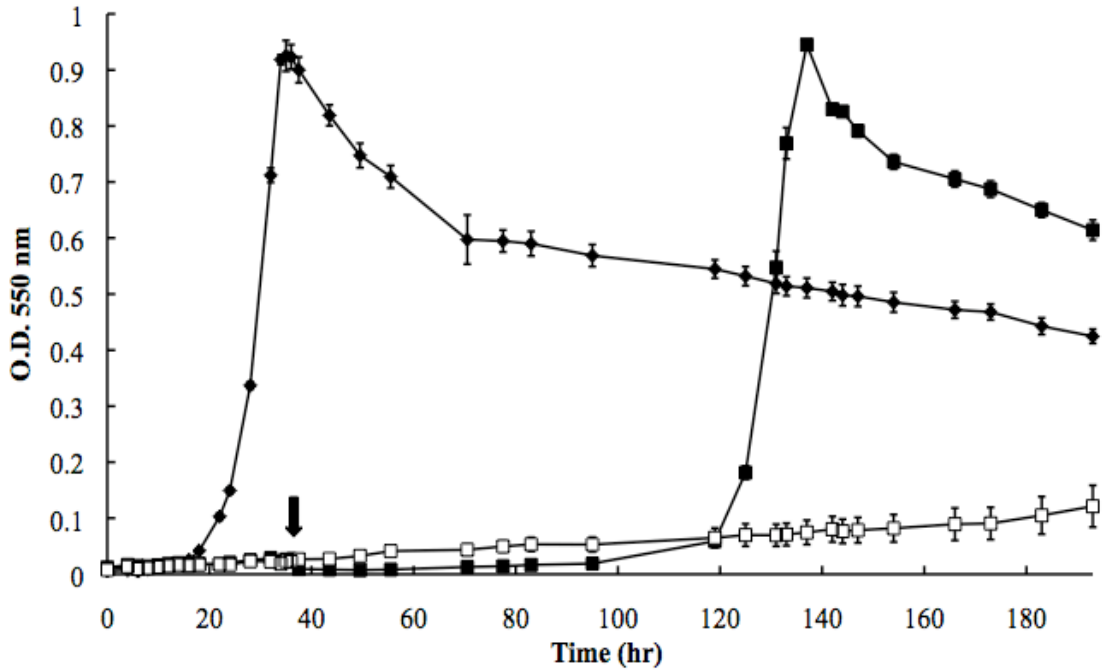

Figure 4.3

Growth of strain G-5 in the presence, absence or following removal of catechin. Analyses were performed in triplicate and error bars indicate ±1 SE. ◆, MMO + glucose; □, MMO + glucose + catechin; and ■, MMO + glucose + catechin. For ■, catechin was removed and cells washed at t = 36 hours (indicated by arrow) and placed into fresh MMO medium with glucose but no catechin. All glucose concentrations were 500 μg ml^{-1} and catechin concentrations were 2000 μg ml^{-1}.

Chapter 5

(±)-CATECHIN, A ROOT EXUDATE OF *CENTAUREA STOEBE* LAM., INHIBITS GROWTH BUT NOT GLUCOSE METABOLISM OF SOME SOIL BACTERIAL POPULATIONS

Abstract

Determining the impact of allelochemicals on plants, soil bacteria and other biota is critical to understanding their effects on soil ecosystems. (±)-Catechin, a root exudate of the North American invasive plant *Centaurea stoebe*, has been shown to have detrimental effects on both plants and soil microorganisms. Research demonstrating phytotoxic effects of catechin on various indigenous plant species is well documented, while work from this lab has revealed suppression of both total bacterial community growth parameters and growth characteristics of individual bacterial populations from several soils. During testing of bacteria sensitive to the bacteriostatic effect of catechin, we discovered that isolates from the genera *Flavobacterium*, *Burkholderia*, *Arthrobacter*, *Pedobacter*, and *Aeromicrobium* were incapable of normal cell growth in the presence of catechin when utilizing a variety of carbon sources. Further analysis of the impact of catechin on carbon utilization revealed that, unlike the other carbon sources tested, pyruvate and malate allowed growth of all isolates capable of using them as sole carbon sources, even when catechin was present. We thus present another aspect of the catechin bacteriostatic effect on bacterial isolates and discuss the implications this effect has for

bacterial communities in the rhizosphere. Collectively, current research reveals that (±)-catechin inhibits growth of soil bacterial isolates of various genera and that this effect can be ameliorated by the addition of pyruvate or malate.

Introduction

Centaurea stoebe Lam. (spotted knapweed) is an invasive weed of North America and as a result of its poor grazing quality and displacement of crops, spotted knapweed has caused an estimated damage of \$150 million·year^{-1} in the United States (Bucher, 1984). The invasive success of spotted knapweed is attributed to various mechanisms, including evasion of natural predators and pathogens (Callaway*, et al.*, 2004) and the ability of spotted knapweed to exude (±)-catechin, a phytotoxic and putatively allelopathic compound, from its root system (Blair*, et al.*, 2006). Catechin is a polyphenolic compound that has been shown to possess both phytotoxic (Callaway*, et al.*, 1999, Duke*, et al.*, Pollock*, et al.*, 2009) and bacteriostatic/antimicrobial effects (Callaway*, et al.*, 2004, Inderjit*, et al.*, 2009, Pollock *et al.*, in review). However, no research to date has attempted to evaluate the mechanism(s) underlying the bacteriostatic inhibitory effect that (±)-catechin has on some soil bacterial populations.

Our previous findings (Pollock *et al.*, in review) have shown that catechin inhibits growth of a large proportion of the culturable bacterial populations from a variety of soils. The current study extends on those findings and suggests mechanism(s) that underlie the bacteriostatic effect of catechin. Here, we report that catechin inhibits cell growth for many, but not all, bacterial isolates, yet doesn't interfere with their metabolism of glucose. Additionally, two simple organic acids, pyruvate and malate, restored growth to all tested soil isolates, provided they could metabolize that organic acid when catechin was not present.

Materials & Methods

Source of Chemicals and Soils

Suppliers for the chemicals used in these experiments were Sigma-Aldrich (S; Sigma-Aldrich, St. Louis, MO), Difco (D; Difco Laboratories Incorporated, Franklin Lakes, NJ), Fisher (F; Fisher Scientific, Pittsburgh, PA), JT Baker (J; JT Baker, Phillipsburg, NJ), and EM Science (E; EM Science, Gibbstown, NJ), Becton Dickinson (B; Becton Dickinson, Sparks, MD) unless otherwise stated, and all were of reagent grade quality or better and their single-letter abbreviations appear in parentheses below.

Chemicals employed in these experiments were: The components of R2A medium [Casamino Acids (D); Yeast Extract (F); Bacto Peptone (D); Soluble Starch (S); Glucose (F); Sodium Pyruvate (S); Dipotassium Phosphate (anh) (S); and, Magnesium Sulfate Heptahydrate (S)]. The components of MMO medium [Disodium Phosphate (anh) (J); Potassium Phosphate (anh) (S); Sodium hydroxide (F); Calcium Chloride Dihydrate (S); Disodium Nitrilotriacetic Acid (S); Magnesium Sulfate Heptahydrate (S); Ammonia Molybdate Tetrahydrate (S); Ferrous Sulfate Heptahydrate (S); Sulfuric Acid (E); Sodium EDTA (F); Zinc Sulfate Heptahydrate (J); Manganese Sulfate Hydrate (J); Copper Sulfate Pentahydrate (J); Cobalt Dinitrate Hexahydrate (J); Disodium Borate Decahydrate (J); and, Ammonia Sulfate (S)]; Galactose (D); D-Fructose (S); D-Xylose (F); L-Arabinose (S); D-Cellobiose (S); D-Lactose (S); Melibiose (S); Xylitol (S); Sodium Acetate (F); D,L-Sodium Lactate (S); Trisodium Citrate Dihydrate (S); Oxalic Acid Dihydrate (S); 2-Ketobutyrate (S); D,L-Malic Acid (F); Ethanol (AAPER alcohol

and chemical Co., Shelbyville, KY); Methanol (F); Acetone (J); Cycloheximide (S); and, D-Glucose UL ^{14}C (S).

Locations for the soil samples used in these experiments are outlined in Table 1. To obtain bacterial isolates from each soil, one gram of soil was added to 9 mL of sterile sodium phosphate buffer (PB) solution (100mM; pH 7), vortexed for 30 seconds, after which large particles were allowed to settle for one minute. Serial dilutions into PB were performed to 10^{-8}, and 100 μL of each dilution was spread-plated onto the various media as indicated below. All plates were incubated in the dark for 7 days at room temperature before counting colony forming units (CFUs) from an appropriate dilution plate (i.e. having colony numbers between 30 and 300 on a single plate).

Plating Conditions and Sources of Organisms

Several experiments were conducted to determine the effect of catechin on microbial populations under a variety of plating conditions. R2A plates (D) were prepared according to the manufacturer's suggested protocol and MMO minimal medium amended with 500 μg mL^{-1} glucose was made as previously described (Stanier*, et al.*, 1966), except that 15 g Bacto-agar (B) per liter of media was added prior to autoclaving. Various components from either R2A broth or MMO (at the concentrations used in the respective media; see Table 2) were tested individually in other agar media to determine if that component increased the antimicrobial activity of catechin, restored cell growth in the presence of catechin, or had no effect. After autoclaving and cooling to 55 - 65°C, all agar media were amended with cycloheximide (hereafter Cx) to inhibit fungal growth to

a final concentration of 300 $\mu g \cdot mL^{-1}$ by addition of 10 mL of a 30 $mg \cdot mL^{-1}$ stock solution (in 100% ethanol).

A 50:50 racemic mixture of (±)-catechin·hydrate (>99% purity) was purchased from Shivambu International (New Delhi, India), hereafter referred to solely as catechin. Where catechin was added to media, catechin was dissolved in 100% methanol to create a 200 mg mL^{-1} stock solution (unless otherwise noted) and 10 ml aseptically added to autoclaved and cooled media to a final concentration of 2000 $\mu g \cdot mL^{-1}$. Media not amended with catechin received the same volume of methanol to normalize for any potential effects of methanol on colony growth. However, it was assumed that, under the open storage and incubation conditions employed, that any added ethanol or methanol was fully evaporated from media before use in these experiments. Several soils were used to determine if resident bacteria in each were capable of growth in the presence of catechin (hereafter, + CAT) (refer to Table 1).

Identification of bacterial populations sensitive to catechin

Ten colonies each from two Montana knapweed rhizosphere soil samples from Waterworks Hill and Bob Marshall Wilderness (see Table 1) were picked from R2A + Cx agar and restreaked onto R2A agar to ensure purity. Ultimately, sixteen pure isolates were obtained and used for further characterization. To determine the phylogenetic identity of the isolates, the 16S rRNA gene of each was PCR amplified, purified, sequenced, and compared to known 16S rDNA genes as previously described (Pollock et al., *in review*).

Effect of catechin on bacterial carbon source utilization

Four isolates (C-1, C-4, G-1, and G-2) from the genera *Aeromicrobium*, *Arthrobacter*, *Pedobacter*, and *Rhodococcus*, respectively, were tested for their ability to utilize various carbon sources with and without catechin present. Upon successful initial growth in MMO + glucose, cultures were subsequently transferred by 1:100 dilution into MMO medium containing one of the suite of carbon sources at 500 $\mu g \cdot mL^{-1}$ (except for methanol, ethanol, and acetone, which were at 300 $\mu L \cdot L^{-1}$) and amended or not with catechin (Table 3). For this experiment, catechin was dissolved into sterile ultra-pure water by gradual warming, then quickly dispensed directly into the media to create a final concentration of 2000 $\mu g \cdot mL^{-1}$. With this approach, the catechin is rapidly diluted to prevent precipitation and the addition of methanol, a potentially confounding substrate for growth, is avoided. All cultures were incubated at room temperature with shaking at 150 rpm to ensure that aerobic conditions were maintained. Growth on individual carbon sources was confirmed by visual inspection of turbidity (Table 3). After discovering that malate or pyruvate, could support growth of these isolates in the presence of catechin (Table 3), all of the isolates were similarly tested for their ability to utilize malate, pyruvate, or glucose in the presence or absence of catechin (Table 4).

Measurement of growth kinetics of *Burkholderia* isolate G-5 on pyruvate plus catechin

To determine whether growth on pyruvate in the presence of catechin was truly normal, detailed growth kinetics assays in the absence or presence of catechin were performed using *Burkholderia* isolate G-5, which was inhibited by catechin under glucose-oxidizing

conditions (Pollock et al., *in review*). Triplicate cultures were grown on MMO + pyruvate (500 $\mu g \cdot mL^{-1}$) to mid-log phase at room temperature in the dark with shaking at 150 rpm, then inoculated by 1:100 dilution into fresh medium with and without catechin and incubated under the same conditions. Cell growth was measured based on optical density (OD) at a wavelength of 550 nm using a Spectronic 20D+ Spectrophotometer (Milton Roy USA, Ivyland, PA). As catechin naturally auto-oxidizes (especially in the presence of pyruvate—see figure 6) and thereby changes the absorbance of the medium, blanks were either MMO + 500 $\mu g \cdot mL^{-1}$ pyruvate or MMO + 500 $\mu g \cdot mL^{-1}$ pyruvate + 2000 $\mu g \cdot mL^{-1}$ catechin and treated to the same conditions as for the cultures. As in the prior experiment, catechin was completely dissolved by gentle heating in 5 mL of sterile double distilled water and the contents poured directly into 100 mL of MMO medium + pyruvate. HPLC analysis of the medium confirmed the presence of catechin at the appropriate starting concentration of 2000 $\mu g \cdot mL^{-1}$.

Abiotic impact of organic acids on catechin stability

As pyruvate and malate restored growth to all catechin-inhibited isolates that could use them as a sole carbon source (see Table 4), the abiotic interactions of these and citrate (another common, simple organic acid) on catechin stability over time were compared by HPLC analysis. These organic acids are hereafter referred to as Pyr, Mal, or Cit, respectively. For all solutions, catechin was dissolved into PB by stirring and gradually warming the solution to create a final concentration of 1000 $\mu g \cdot mL^{-1}$. The treatments were performed in triplicate as follows: CAT (no additional amendments); CAT + Pyr (500 $\mu g \cdot mL^{-1}$); CAT + Mal (500 $\mu g \cdot mL^{-1}$); and CAT + Cit (500 $\mu g \cdot mL^{-1}$). Although

catechin is stable in ultra-pure water longer than in PB (see Chapter 2 results), using this buffered system allowed us to better compare the results of this experiment to the phosphate-buffered, pH neutral media used in the microbiological experiments. All tubes were kept stationary in the dark at room temperature for the course of the experiment and sampled periodically to measure the amount of pure catechin remaining. To accomplish this, 1 mL of each solution was filtered and aliquoted into vials for HPLC analysis to determine catechin concentrations as previously described (Paveto*, et al.*, 2004). In brief, catechin concentrations were determined by analysis of aqueous samples (15 μL injection volume) with UV detection at 280 nm on a Hewlett-Packard (HP) series 1100 HPLC (Hewlett-Packard, Palo Alto, CA) using a HP ODS Hypersil C18 column (5 μm, 125x4 mm) and a 100% methanol : 20 mM phosphoric acid (1:3 v/v) mobile phase at a flow rate of 1 $mL \cdot min^{-1}$. The sensitivity and upper limit of this protocol for the detection of catechin range from 5 $\mu g \cdot mL^{-1}$ to 2000 $\mu g \cdot mL^{-1}$ on a linear scale.

Effect of catechin on glucose utilization

Because catechin and similar compounds have been shown to damage cell membranes (Ikigai*, et al.*, 1993, Hoshino*, et al.*, 1999) and block cell transport (Shimizu*, et al.*, 2000, Song*, et al.*, 2002), a ^{14}C-glucose experiment was performed to determine if catechin was preventing either uptake of radiolabeled glucose or its metabolism via oxidation, thereby resulting in the observed inhibition of growth. A culture of isolate G-5 was grown on MMO + 500 $\mu g \cdot mL^{-1}$ unlabeled glucose to mid-log phase and then 1 mL aliquots were added to each treatment. All flasks contained 20 mL of MMO medium + 10 μL (1,356,503 CPM) of ring-U-^{14}C glucose, and were incubated at room temperature in the

dark with rotary shaking at 150 rpm. The treatments were performed in triplicate as follows: CONT (no additional amendments); + Mal (23 $\mu g \cdot mL^{-1}$); + CAT (+ 2000 $\mu g \cdot mL^{-1}$); + Mal and CAT, and all were performed with uninoculated controls. Mineralization of glucose was measured as previously described (Mortensen & Jacobsen, 2004). In brief, sterile filter paper was soaked in sodium hydroxide (0.5 M) to convert $^{14}CO_2$ in the headspace into ^{14}C-sodium bicarbonate for analysis by liquid scintillation counting (LSC). In addition, 500 μL of culture was removed at each time point of which 100 μL was used to confirm that cell growth did not occur in the presence of catechin based on measuring optical density (OD_{550}). The remaining 400 μL was centrifuged to pellet the bacterial cells and the top 300 μL of supernatant was carefully decanted and analyzed by LSC to determine the CPM remaining in the medium. In addition, the pelleted cells were resuspended in the remaining 100 μL of medium and added to 16 ml of liquid scintillation fluid to monitor ^{14}C-uptake into cells attributable to radioactive glucose uptake and incorporation into cell biomass (after correction for the radioactive counts in the remaining medium).

Results

Media and media component effects on inhibition of bacterial growth by catechin

Several plating experiments were performed to ascertain whether bacteria from Romanian soil (native range of spotted knapweed) or Montana soils (representing both invaded and naïve range) were capable of growing in the presence of catechin under a variety of media conditions and various substrates for growth (refer to Table 2) since soil composition can be highly variable even within meters at the same site (Snaydon, 1962,

Frankland*, et al.*, 1963, Jackson & Caldwell, 1993). Addition of catechin to unsupplemented MMO agar or MMO + agar supplemented with casamino acids resulted in complete inhibition of growth of the bacterial community from all soils (Fig. 1). By contrast, on R2A agar, the addition of catechin resulted in an overall lowering of CFUs by one to two logs (i.e. 90% to 99%) for the bacterial community from all soils, but there was clearly still growth of part of the community on this medium (Fig. 1).

The previous experiment demonstrated that all soils exhibited complete inhibition of growth by catechin when grown on either MMO medium containing no supplemental carbon source or on MMO media amended with casamino acids. Previous work from this lab showed that catechin exhibits increased phytotoxic effects dependent on complexation with various metals commonly found in soils (Pollock*, et al.*, 2009). It was therefore plausible that one or more components of MMO were toxic to bacteria when coupled with the addition of catechin. To ascertain whether a component of MMO was toxic to cells in the presence of catechin, various components of MMO were incorporated into R2A agar + CAT. Four spotted knapweed rhizosphere soils (two from Montana, and two from Romania) were used to exemplify typical bacterial communities closely associated with spotted knapweed for this and all remaining plating experiments (Table 1). Cell numbers did not fluctuate significantly compared to the R2A + CAT controls when R2A amended with catechin was supplemented with MMO, or the individual components MMO A, MMO B, or MMO C (Fig. 2).

As neither MMO nor its components showed signs of bacterial inhibition in the previous experiment, it was hypothesized that one or more components of R2A allowed growth for at least part of the bacterial community in the presence of catechin. Two separate experiments were performed to determine the specific component of the complex R2A medium that was capable of overcoming the bacteriostatic effect exhibited by catechin for some bacteria. In the first experiment, R2A broth components were split into 3 categories (Rich Carbons, Defined Carbons, and Minerals; refer to Table 2) for preliminary tests on the ability of R2A broth to allow growth of some populations in the presence of catechin. MMO medium supplemented with either complete R2A broth (i.e. MMO + R2A) or the defined carbons (MMO + DefC) from R2A broth restored growth in the presence of catechin to some bacterial populations from all four soils. However, neither the addition of rich carbons (MMO + RichC) nor minerals (MMO + Min) allowed for growth of any bacterial populations in the presence of catechin (Fig. 3).

After determining that some component of the Defined Carbons mixture (glucose, pyruvate, or starch) was responsible for allowing growth of part of the bacterial community inhibited by catechin on MMO medium, a further experiment using MMO separately supplemented with each individual carbon source was performed. Neither MMO + glucose nor MMO + starch allowed growth of any of the bacterial populations in the presence of catechin (Fig. 4), while MMO + pyruvate supported growth of about 10% of the populations with catechin present that could grow when it was absent (Fig. 4). Pyruvate was thus determined to be the key component of R2A broth responsible for allowing growth of some bacterial populations in the presence of catechin.

Isolation of bacterial populations

To determine how this pyruvate phenomenon acted at the level of individual subpopulations, several soil isolates were obtained from two Montana soils previously exposed to spotted knapweed (Table 1). Of these 16 novel isolates, eight bacteria across six genera were incapable of growth in the presence of catechin when grown under glucose-oxidizing conditions (Table 4). Interestingly, all of these isolates were capable of growth utilizing pyruvate as the sole carbon source in the presence of catechin (Table 4).

Catechin alters carbon source utilization for several soil isolates

As pyruvate, a simple organic acid, allowed growth of all isolates inhibited by catechin when grown on glucose, additional alternative carbon sources were tested on three representative isolates for their ability to support growth in the presence of catechin. Three isolates normally inhibited by catechin (isolates *Aeromicrobium* C-1, *Arthrobacter* C-4, and *Pedobacter* G-1) were tested for growth in its presence on a broad selection of sugars (mono-, di-, and trisaccharides; 5 Carbon and 6 Carbon sugars), organic acids, ketones, and alcohols. Of the nineteen carbon sources tested, only malate and pyruvate allowed growth of the three isolates in the presence of catechin (Tables 3 and 4). After determining that malate also restored growth to these isolates, all other isolates were tested for their ability to grow using pyruvate or malate in the presence of catechin. Remarkably, all isolates inhibited by catechin under glucose-oxidizing conditions were

capable of growing in the presence of catechin using pyruvate or malate as carbon source provided they could use pyruvate or malate when catechin was not present (Table 4).

Of perhaps greater significance, eight isolates from three genera (one *Bacillus*, one *Rhodococcus*, and six *Pseudomonas* spp.) exhibited no inhibition by catechin when grown on any carbon source they could use when catechin was not present (Tables 3 & 4). Although all eight of these isolates grew in the presence of catechin, only the *Rhodococcus* isolate exhibited an ability to lower catechin concentrations while forming an undetermined metabolite (data not shown). Curiously, while these eight isolates exhibited growth in the presence of catechin in MMO liquid medium with glucose, they were incapable of growth on MMO + glucose + CAT agar (data not shown).

The bacteriostatic effect of catechin is mitigated but not eliminated by growth on pyruvate

As pyruvate was shown to allow growth of all isolates inhibited by catechin on other carbon sources (except malate), growth kinetics were measured for isolate *Burkholderia* G-5 in MMO + pyruvate, and MMO + pyruvate + CAT. Typical growth kinetics were observed for isolate G-5 under aerobic conditions on MMO + pyruvate (Fig. 5). However, with catechin present, its growth rate was lower (i.e. exhibited a longer doubling time) but ultimately reached nearly the same population density as the control culture (Fig. 5).

Pyruvate, but not malate, lowers catechin concentrations abiotically over time

Since both pyruvate and malate supported growth of some bacteria in the presence of catechin, an abiotic experiment was performed to determine if either organic acid had an impact on catechin stability. Another organic acid, citrate, did not support growth with or without catechin (except for the *Rhodococcus* strain which both grew on citrate and is uninhibited by catechin on any growth substrate) and was therefore used as a negative control for this experiment. Each treatment began with 1,000 $\mu g \cdot mL^{-1}$ of catechin in PB, which spontaneously degraded or transformed over time leaving 753 ± 9 $\mu g \cdot mL^{-1}$ of pure catechin after 21 days. Neither malate nor citrate exhibited a significant positive or negative effect on catechin stability, leaving 750 ± 9 $\mu g \cdot mL^{-1}$ and 785 ± 18 $\mu g \cdot mL^{-1}$ of pure catechin, respectively after 21 days (Fig. 6). In contrast, addition of pyruvate lowered the catechin concentration over the 21-day time course to 580 ± 12 $\mu g \cdot mL^{-1}$, a 23% increase in the degradation or transformation rate.

Catechin inhibits bacterial growth but not metabolism

One possible reason for the inability of glucose or starch to support growth in the presence of catechin might be that catechin negatively impacts substrate transport. Alternatively, catechin might be interfering with enzymatic processes essential for the metabolism of these compounds. In order to determine if catechin was preventing uptake or oxidation of glucose, a ^{14}C-glucose experiment was performed on *Burkholderia* isolate G-5, one of the isolates shown to be inhibited by 2000 $\mu g \cdot mL^{-1}$ catechin when grown under glucose-oxidizing conditions (this work and Pollock *et al.*, in review). Without catechin, production of $^{14}C\text{-}CO_2$ was concomitant with ^{14}C-glucose metabolism (decrease of ^{14}C-glucose in liquid fraction) (Fig. 7A & C). In addition, the cell pellets

progressively increased in radioactivity (^{14}C-glucose uptake into cells), indicating both ^{14}C-uptake and incorporation into cell biomass (Fig. 7B). Strikingly, in the presence of catechin, mineralization of ^{14}C-glucose by isolate G-5 was increased significantly (Fig. 7C) suggesting that glucose metabolism was still active in the growth-inhibited cell fraction. The addition of unlabeled malate to the treatments of ^{14}C-glucose + CAT + cells showed a marked trend of returning metabolism to normal levels (comparable to ^{14}C-glucose + cells without catechin) (Fig. 7A-C). Whether this is a result of malate somehow restoring cellular metabolism, or simply replenishing some unknown component (e.g. metals, a cofactor) to the inhibited cells when catechin is present, thereby restoring the ability to metabolize ^{14}C-glucose normally, is still unknown.

Discussion

Collectively, the findings described in this chapter demonstrate that catechin selectively, but also fairly generally, impacts the activities of bacteria from soil including the way carbon sources are utilized by a variety of bacterial populations. Catechin caused an overall inhibition of growth for the total bacterial community when grown on minimal media (Fig. 1). This suppressive effect was extensive (i.e. producing 90 – 100% inhibition) for bacterial populations from several different locations, including sites historically exposed to spotted knapweed, and by extension catechin. One striking finding was that having pyruvate in the medium was sufficient to allow growth of a portion (roughly 10%) of the bacterial community previously inhibited by catechin (refer to Fig. 4). Extending on this finding, I observed that all bacterial isolates that showed signs of inhibition by catechin under glucose-oxidizing conditions were capable of

growth using pyruvate or malate as an alternative carbon source when catechin was present provided that they could in its absence (Table 4). However, complete immunity to the inhibitory effects of catechin was not observed as exemplified by the retardation of the growth rate for isolate *Burkholderia* G-5 in the presence of catechin over the course of the detailed growth kinetics experiment (Fig. 5). The ability of pyruvate to transform or degrade pure catechin stability abiotically (Fig. 6) suggests a possible mechanism that may allow growth of some of the bacterial populations that are inhibited by catechin when grown on any other substrate tested (except malate). It is plausible that the amount of pure catechin in the medium was lowered by pyruvate to a level that was sub-inhibitory to many of the isolates. However, the growth of isolate G-5 on MMO amended with pyruvate in the presence of catechin exhibited an ~20-hour lag time before growth occurred. Alternatively, the interaction of pyruvate with hydrogen peroxide (see references Arakawa*, et al.*, 2002 and Hinoi*, et al.*, 2002 and also below) could explain how it supports and allows growth for all tested isolates. Note that catechin itself has not been proven directly responsible for plant and bacterial inhibition; thus, the formation of reactive oxygen species (ROS) as a result of catechin auto-oxidation may actually represent the indirect mechanism of catechin toxicity (Weir*, et al.*, 2004). In this scenario (below), pyruvate might act by both removing a toxic endproduct of natural catechin breakdown (H_2O_2) as well as serving as a substrate for growth as both pyruvate and acetate. Also in this scenario, rapid removal of H_2O_2 by pyruvate presumably enhances the rate of abiotic oxidation of catechin explaining the results observed in Fig. 6.

Proposed mechanism for increased catechin breakdown by pyruvate:

1) Catechin + O_2 → Oxidized Catechin + H_2O_2 (Arakawa*, et al.*, 2002)

2) Pyruvate + H_2O_2 → Acetate + CO_2 + H_2O) (Hinoi, *et al.*, 2002)

In contrast to the bacterial populations inhibited by catechin on most growth substrates but capable of growth in the presence of pyruvate or malate and catechin, a proportion of the bacterial community always showed signs of inhibition (Figs. 1, 3, & 4). One explanation for this partial restoration of bacterial community growth by pyruvate could be the inability of all bacterial species to utilize pyruvate (or malate) as an alternative carbon source. Indeed, some of the isolates tested in detail showed poor or no growth on pyruvate and malate, respectively as the sole carbon source (Table 4). However, unlike pyruvate, malate showed no discernable effect on pure catechin stability in PB (Fig. 6), which suggests that an alternative mechanism could be responsible for its support of bacterial growth in the presence of catechin. While not all carbon sources tested were used by every bacterial isolate even without catechin present, the clear effect of catechin inhibition of aerobic growth on numerous substrates for numerous bacterial populations likely would also alter the utilization of various carbon sources in soil and thereby prevent or slow the growth of those populations, while selecting for or at least facilitating the growth of other populations through suppression of catechin-sensitive ones.

My results further demonstrate that catechin prevents cell growth on glucose (in terms of CFUs and OD_{550}), but not its uptake or mineralization for isolate *Burkholderia* G-5 (Fig. 7A-C). Indeed, the presence of catechin actually increased total ^{14}C-CO_2 production (Fig. 7C). While this effect was not tested on all inhibited isolates and certainly not on all carbon sources that catechin prevented from being utilized to support growth, it is

conceivable that catechin forces growth-inhibited cells to metabolize these substrates primarily to CO_2 instead of using them to increase cell biomass. Note, however, that in the case of malate or pyruvate use as carbon source for growth of isolate G5, catechin only slowed, but did not prevent, cell growth as indicated by OD_{550} (Fig. 5).

An alternative hypothesis for the mechanism by which pyruvate and malate support growth in the presence of catechin involves the ability of catechin to bind to specific metals (McDonald*, et al.*, 1996, Es-Safi*, et al.*, 1999, Lim*, et al.*, 2005, Pollock*, et al.*, 2009), which might potentially sequester them from bacterial bioavailability, thereby interfering with growth. Malate and other organic acids are known to be exuded by many plant species in either mineral- or phosphorous-deficient systems, as they readily chelate metals and free-up phosphates bound to metals in soil (Gardner*, et al.*, 1983, Keerthisinghe*, et al.*, 1998). Malate and pyruvate might be competitively chelating metals in the medium that would be otherwise sequestered by catechin. Malate and pyruvate (and their bound essential metals) would subsequently be transported into bacterial cells, thereby supporting growth in the presence of catechin. Thus the ability of catechin to negatively impact bacterial and plant communities could be due to its metal sequestration properties, which can perhaps be overcome or at least mitigated by those bacterial populations capable of transporting and metabolizing pyruvate and/or malate.

A related study examined the inhibitory effects of 8-hydroxyquinoline (8HQ), an allelochemical produced by diffuse knapweed, a close relative of spotted knapweed, on microbial growth and found that the addition of excess iron restored growth to the

inhibited populations and also that this allelochemical selects for plant growth-promoting *Pseudomonas* species (Geels*, et al.*, 1985). More recent work on 8HQ showed a similar ability to liberate insoluble iron for diffuse knapweed uptake (Tharayil*, et al.*, 2009). Of special note, previous work from another laboratory demonstrated that oxalate, another common root exudate used for metal chelation, restored growth to several plant species inhibited by catechin (Weir*, et al.*, 2006). It is thus possible that metal chelators, produced by either indigenous plants, bacteria, other soil organisms, or even added as an amendment to soils, could serve to strip metals away from catechin, making them more bioavailable and restoring normal cell function to other plants and bacteria.

A somewhat confusing discovery from the work described herein was the isolation of bacteria that, while unable to grow on MMO + glucose + CAT agar, were capable of growth in MMO + glucose + CAT liquid media. Isolates from three genera (*Bacillus*, *Rhodococcus*, and *Pseudomonas*) showed no signs of inhibition by catechin when grown in MMO liquid medium on any carbon source tested (Tables 3 & 4). Unlike soil bacterial populations grown on plates, these cultures were shaken vigorously (150 rpm on a rotary shaker) to ensure thorough mixing and aeration of the medium. Catechin, being highly reactive with molecular oxygen, undergoes 'auto-oxidation'; a reaction that abiotically transforms catechin over time into a more oxidized form (Lim*, et al.*, 2005) and generates hydrogen peroxide as a byproduct as discussed above. This aeration might be expected to more rapidly oxidize catechin than would happen in agar plates, thereby reducing the pure catechin concentration and its toxicity. Another plausible explanation for this observation is that, catechin (and any harmful byproducts of its transformation) would

have much more limited diffusion in agar plates. Thus, microbes capable of somehow transforming catechin producing harmful metabolites could be building up localized gradients or zones of products harmful to the cells, inhibiting their own growth. This explanation is perhaps lent some credence by the observation that *Rhodococcus* isolate G-2 showed evidence of catechin transformation (as determined by HPLC analysis) when using glucose as an alternative carbon source and formed an as yet undetermined compound concomitant with catechin removal and bacterial growth (data not shown).

In contrast to the *Rhodococcus* isolate, the *Pseudomonas* isolates never showed any sign of altering catechin concentrations relative to the abiotic control when grown on MMO + glucose (data not shown). This suggests that whatever general effect catechin has on bacterial species (e.g., generation of ROS, metal sequestration), the six *Pseudomonas* isolates do not appear to be affected by it. While the mechanism for this resistance/immunity is still unknown, it is well documented that many members of the *Pseudomonas* genus have the ability to produce complex siderophores to provide adequate metal availability (Achouak*, et al.*, 2000, Delorme*, et al.*, 2002, Gardan*, et al.*, 2002, Munsch*, et al.*, 2002, Huston*, et al.*, 2004) and might thereby be competitively garnering metals for their own use. However, the presence of catechin, which is similar in structure to catecholate siderophores, confounded my ability to determine whether the six *Pseudomonas* isolates were actively producing siderophores in what presumably is a metal-limiting medium when catechin is present.

Summary

Centaurea stoebe (spotted knapweed) is an invasive weed for much of North America. Central to the invasive success of this noxious plant is thought to be the its ability to exude a racemic form of catechin into the surrounding rhizosphere (Bais*, et al.*, 2002). The experiments described herein used relatively high levels of catechin (1000 - 2000 $\mu g \cdot mL^{-1}$). The amount of catechin exuded into the soil (~0.1-650 $\mu g \cdot mg^{-1}$: Blair*, et al.*, 2005, Blair*, et al.*, 2006, Perry*, et al.*, 2007), and the length of time catechin exists in the environment (Furubayashi*, et al.*, 2007, Inderjit*, et al.*, 2008) make catechin, and through extension allelopathy by spotted knapweed, a somewhat controversial topic. However, I posit that the concentration of catechin on a microscale (i.e. at the rhizoplane where bacteria live and are exposed to catechin) could be far higher than the values in the literature, which are based on bulk soil analyses from soils with knapweed. Current literature suggests that root exudates, like catechin, are concentrated at the root and those concentrations rapidly decrease with distance from the root as a result of sorption to soil, microbial activity, and passive diffusion (Kuzyakov*, et al.*, 2003, Sauer*, et al.*, 2006). As such, experiments designed to mimic bulk soil catechin concentrations could easily underestimate the bioactive concentrations present in the knapweed rhizosphere.

The observed increase in mineralization of catechin-inhibited *Burkholderia* strain G-5 was an interesting finding (refer to Fig 7C). We originally believed that bacteria were essentially inert (completely static) when inhibited by catechin because there was no evidence of growth based on CFUs or OD_{550} (Pollock *et al*., in review). However, these findings suggest that the bacteriostatic effects of catechin are: i) prevention of cell growth for inhibited microbes; ii) increased mineralization of glucose and perhaps other growth

substrates; and, iii) blocked by the addition of malate, which restored cell growth and lowered glucose mineralization to control levels. This raises the interesting possibility that, in a spotted knapweed invaded system, microbial populations inhibited by catechin may still be actively metabolizing, perhaps at an enhanced rate of activity. What impact this might have on plant ecology, microbial ecology, or spotted knapweed invasion merits further attention but was beyond the scope of these studies.

Assuming that the effects of catechin outlined here for randomly selected knapweed rhizosphere bacterial isolates are common to numerous other soil bacterial populations, soil invasion by spotted knapweed and its subsequent exudation of catechin into its rhizosphere could alter the structure of bacterial communities. As a consequence, the dynamics of bacterial populations as well as their corresponding metabolic cycles could be disrupted. The impact that this type of perturbation would have on environmental food webs is unknown, but preliminary work on both catechin and spotted knapweed, for example, has demonstrated an impact on nitrogen cycling in soil (Inderjit*, et al.*, 2009).

In belowground processes, the ability of bacteria to utilize a broad spectrum of alternative carbon sources is an invaluable tool in remaining active in an ever-shifting chemical environment. Because catechin prevented various bacterial isolates in this study from effectively utilizing the full complement of their carbon source repertoire, it is plausible that invasion of spotted knapweed could alter the abundance of various bacterial populations, lowering those inhibited by catechin, while raising those not affected through lack of inhibition and competition. Importantly, the bacteriostatic effect that

catechin inflicts on numerous bacterial populations was shown to be alleviated or at least mitigated by two simple organic acids, pyruvate and malate. Since many plant species produce a variety of organic acids (like pyruvate and malate) under mineral and/or phosphate limiting conditions (Welch*, et al.*, 2002, Weir*, et al.*, 2006), it may well be that plants are to some degree "protecting" rhizosphere bacterial diversity and activity in soils invaded by spotted knapweed.

Our findings (this work and Pollock *et al.*, in review) have demonstrated the bacteriostatic effect of catechin on bacterial populations from a variety of soils. As this effect is alleviated by the removal of catechin from the system, any lowering of catechin concentrations would be of natural benefit to the plant and microbial community. Future work in this area might focus on potentially valuable tools that might promote the growth of indigenous plant and bacterial species in either naïve or knapweed-infested soils and thereby lessen the success of spotted knapweed invasion. These include: 1) bacteria that mineralize catechin; 2) compounds that competitively chelate metals away from catechin; or 3) chemicals that enhance the abiotic degradation or transformation of catechin.

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Tables

Table 5.1 List of soils used in Chapter 5 experiments.

Location of soil	Sample Designation	Description	GPS Location	Tested in Fig. No.
Romania 'Metro' site in Iasi	*Festuca* site 1	Loose *Festuca* soil	47.19234736° 27.4645539° 83 m elevation	1
Romania 'Metro' site in Iasi	*Festuca* site 2	Loose *Festuca* soil	47.19234736° 27.4645539° 83 m elevation	1
Romania 'Metro' site in Iasi	*Festuca* site 3	Loose *Festuca* soil	47.19234736° 27.4645539° 83 m elevation	1
Romania 'Metro' site in Iasi	*Festuca* site 4	Loose *Festuca* soil	47.19234736° 27.4645539° 83 m elevation	1
Romania Breasu	Breasu with Knapweed site 1	Loose *Festuca* soil	47.2214° 27.51980643° 180 m elevation	1
Romania Breasu	Breasu with Knapweed site 2	Loose Knapweed soil	47.2214° 27.51980643° 180 m elevation	1
Romania Breasu	Breasu with Knapweed site 3	Rhizosphere soil	47.2214° 27.51980643° 180 meters elevation	1,2,3,4
Romania Breasu	Breasu with Knapweed site 4	Rhizosphere soil	47.2214° 27.51980643° 180 m elevation	1,2,3,4
Montana	Grant Creek with *Festuca*	Rhizosphere soil	46.9232° -114.025° 1177 m elevation	1
Montana	Grant Creek with Knapweed	Rhizosphere soil from one plant	46.9232° -114.025° 1177 m elevation	1
Montana	Waterworks Hill with Knapweed	Rhizosphere soil	46.885836° -113.986972° 1091 m elevation	1,2,3,4
Montana	Bob Marshall Conifer site with Knapweed	Rhizosphere soil	46.8961° -113.92° 1230 m elevation	1,2,3,4

Table 5.2 List of media designations and composition for Ch. 5 experiments. All plates contained 15mg·mL^{-1} Bacto-agar and 300 µg·mL^{-1} of cycloheximide.

Medium or component designation[a]	**Components**	**Tested in Fig. No.**
R2A	500 µg·mL^{-1} yeast extract, 500 µg·mL^{-1} proteose peptone #3, and 500 µg·mL^{-1} casamino acids, 300 µg·mL^{-1} sodium pyruvate, 500 µg·mL^{-1} glucose, and 500 µg·mL^{-1} soluble starch, 300 µg·mL^{-1} dipotassium phosphate, and 50 µg·mL^{-1} magnesium sulfate (final concentration)	1,2,3,4
MMO	MMO minimal medium made as previously described (Stanier*, et al.*, 1966), basically 40 mL of MMO A, 20 mL of MMO B, and 10 mL of MMO C added per liter of media	1,2,3,4
+CAT	2000 µg·mL^{-1} catechin (final concentration)	1,2,3,4
+CAA	500 µg·mL^{-1} casamino acids (final concentration)	1
+MMO A	40 ml·l^{-1} of 536.59mM Na_2HPO_4/462.41mM KH_2PO_4 solution (pH 6.8)	2
+MMO B	20 ml·l^{-1} of Hutner's Vitamin Free Mineral Base (=H44 Salts) (see reference (Stanier*, et al.*, 1966))	2
+MMO C	10 ml·l^{-1} of 10% $(NH_4)_2SO_4$	2
+RichC	500 µg·mL^{-1} yeast extract, 500 µg·mL^{-1} proteose peptone #3, and 500 µg·mL^{-1} casamino acids (final concentrations)	3
+DefC	300 µg·mL^{-1} sodium pyruvate, 500 µg·mL^{-1} glucose, and 500 µg·mL^{-1} soluble starch (final concentrations)	3,4
+Min	300 µg·mL^{-1} dipotassium phosphate, and 50 µg·mL^{-1} magnesium sulfate (final concentrations)	3
+Pyr	300 µg·mL^{-1} sodium pyruvate (final concentration)	4
+Gluc	500 µg·mL^{-1} glucose (final concentration)	4
+Sta	500 µg·mL^{-1} casamino acids (final concentration)	4

[a] The + symbol denotes addition of component(s) to either MMO or R2A as the base media.

Table 5.3 Ability of four isolates to utilize various carbon sources in MMO medium aerobically with and without catechin. Symbols represent growth, G; poor growth, PG; or no growth, NG. Symbols before and in parentheses are for growth on carbon tested without and with catechin, respectively.

Carbon Source	Isolate C-1 (*Aeromicrobium*)	Isolate C-4 (*Arthrobacter*)	Isolate G-1 (*Pedobacter*)	Isolate G-2 (*Rhodococcus*)
Glucose	G (NG)	G (NG)	G (NG)	G (G)
Galactose	NG (NG)	G (NG)	G (NG)	G (G)
Fructose	G (NG)	G (NG)	NG (NG)	G (G)
Xylose	PG (NG)	NG (NG)	PG (NG)	PG (PG)
L-Arabinose	G (NG)	PG (NG)	NG (NG)	G (PG)
Cellobiose	PG (NG)	PG (NG)	G (NG)	PG (PG)
Lactose	NG (NG)	NG (NG)	G (NG)	G (G)
Melibiose	NG (NG)	G (NG)	PG (NG)	G (G)
Xylitol	PG (NG)	NG (NG)	NG (NG)	G (G)
Acetone	NG (NG)	NG (NG)	NG (NG)	G (G)
Pyruvate*	**G (PG)**	**G (PG)**	**G (PG)**	**G (G)**
Acetate	PG (NG)	NG (NG)	NG (NG)	G (G)
Lactate	PG (NG)	NG (NG)	NG (NG)	G (G)
Citrate	NG (NG)	NG (NG)	NG (NG)	G (G)
2-Ketobutyrate	PG (NG)	NG (NG)	NG (NG)	PG (PG)
DL-Malate*	**G (G)**	**G (G)**	**PG (PG)**	**G (G)**
Ethanol	PG (NG)	NG (NG)	NG (NG)	PG (PG)
Methanol	PG (NG)	NG (NG)	NG (NG)	PG (PG)
Phenol	NG (NG)	NG (NG)	NG (NG)	NG (NG)

Table 5.4 List of microorganisms isolated from Petri plates, S_ab scores and closest phylogenetic match to a known genus based on 16S rRNA gene analysis, and their reaction to catechin when grown under glucose-, pyruvate-, or malate-oxidizing conditions. **Bold isolates** are inhibited by catechin under some conditions. G denotes growth; PG denotes poor growth; NG denotes No growth.

Isolate	S_ab score (based on # nucleotides)	Closest Phylogenetic Match (Genus level)	Glucose (Glucose + Catechin)	Pyruvate (Pyruvate + Catechin)	Malate (Malate + Catechin)
C-1	0.913 (1246)	***Aeromicrobium***	G (NG)	G (PG)	G (G)
C-2	0.957 (1344)	***Chryseobacterium***	G (NG)	G (PG)	NG (NG)
C-4	0.975 (1325)	***Arthrobacter***	G (NG)	G (PG)	G (G)
C-5	0.996 (1397)	*Bacillus*	G (PG)	G (PG)	G (G)
C-6	0.985 (1285)	*Pseudomonas*	G (G)	G (G)	G (G)
C-7	0.978 (1443)	*Pseudomonas*	G (G)	G (G)	G (G)
C-8	0.984 (1423)	*Pseudomonas*	G (G)	G (G)	G (G)
C-9	0.987 (1222)	***Flavobacterium***	G (NG)	G (PG)	G (PG)
C-10	0.977 (1230)	***Arthrobacter***	G (NG)	G (PG)	G (PG)
G-1	0.773 (1412)	***Pedobacter***	G (NG)	G (PG)	G (PG)
G-2	1.000 (720)	*Rhodococcus*	G (G)	G (G)	G (G)
G-4	0.995 (1386)	*Pseudomonas*	G (G)	G (G)	G (G)
G-5	0.974 (1382)	***Burkholderia***	G (NG)	G (PG)	G (PG)
G-7	0.995 (1398)	*Pseudomonas*	G (G)	G (G)	G (G)
G-8	0.995 (1400)	*Pseudomonas*	G (G)	G (G)	G (G)
G-9	0.968 (1379)	***Flavobacterium***	G (NG)	G (PG)	NG (NG)

Figures

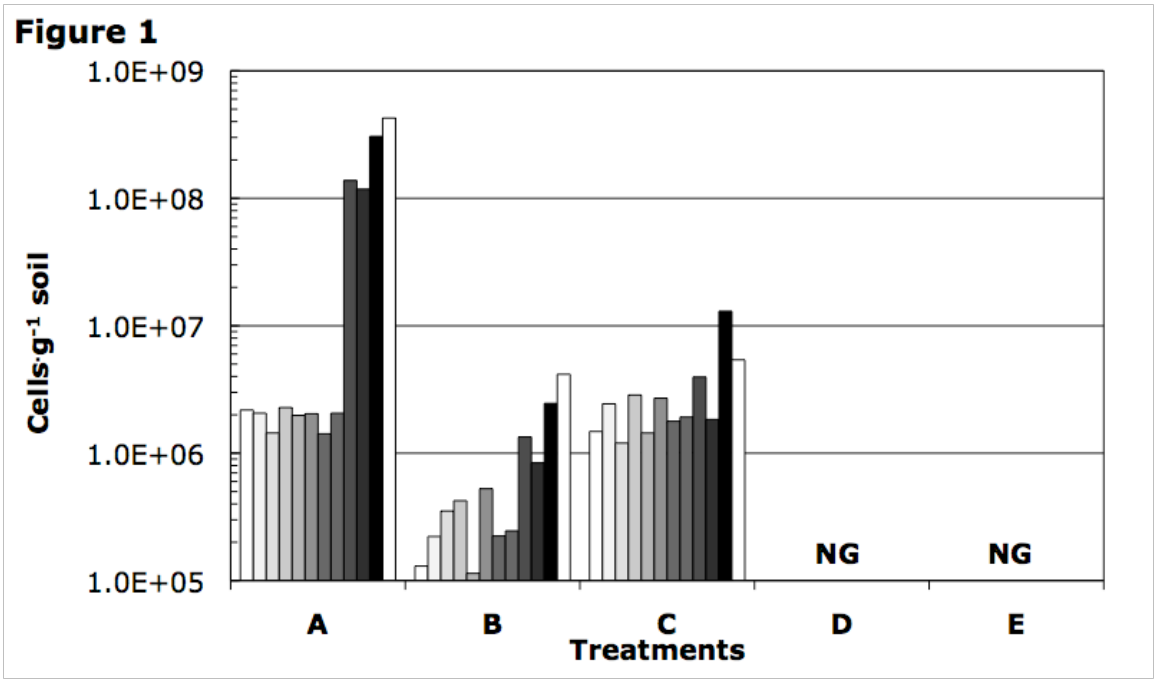

Figure 5.1

Soil microbial biomass based on CFUs from various soils with various media. Sources of soil samples for this experiment were (from left to right): a) Romania with Festuca site 1; b) Romania with Festuca site 2; c) Romania with Festuca site 3; d) Romania with Festuca site 4; e) Romania Breasu with Knapweed site 1; f) Romania Breasu with Knapweed site 2; g) Romania Breasu with Knapweed site 3 (rhizosphere soil); h) Romania Breasu with Knapweed site 4 (rhizosphere soil); i) Montana Grant Creek with Festuca (rhizosphere soil); j) Montana Grant Creek Knapweed (rhizosphere soil); k) Montana Waterworks Hill with Knapweed (rhizosphere soil); l) Montana Bob Marshall Wilderness Conifer area with Knapweed (rhizosphere soil). Media treatments were: **A)** R2A; **B)** R2A + CAT; **C)** MMO; **D)** MMO + CAT; **E)** MMO + Casamino Acids + CAT. **NG** denotes "No Growth" of any bacterial colonies over the course of the experiment.

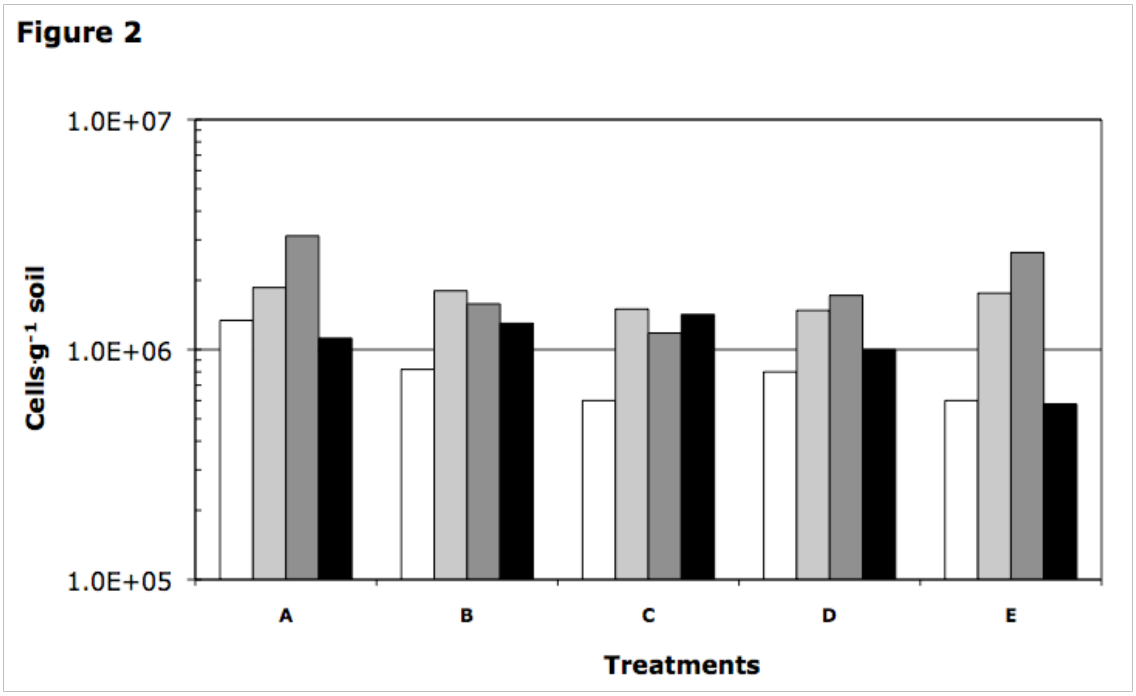

Figure 5.2

Soil microbial biomass based on CFUs from various soils with components of MMO minimal medium amended into R2A agar. Sources of soil samples for this experiment were (from left to right): (□)Romania Breasu with Knapweed site 3 (rhizosphere soil); (▒)Romania Breasu with Knapweed site 4 (rhizosphere soil); (▓)Montana Waterworks Hill with Knapweed (rhizosphere soil); (■)Montana Bob Marshall Wilderness Conifer area with Knapweed (rhizosphere soil). Media treatments were: **A)** R2A + CAT; **B)** R2A + MMO (complete) + CAT; **C)** R2A + MMO A + CAT; **D)** R2A + MMO B + CAT; **E)** R2A + MMO C + CAT.

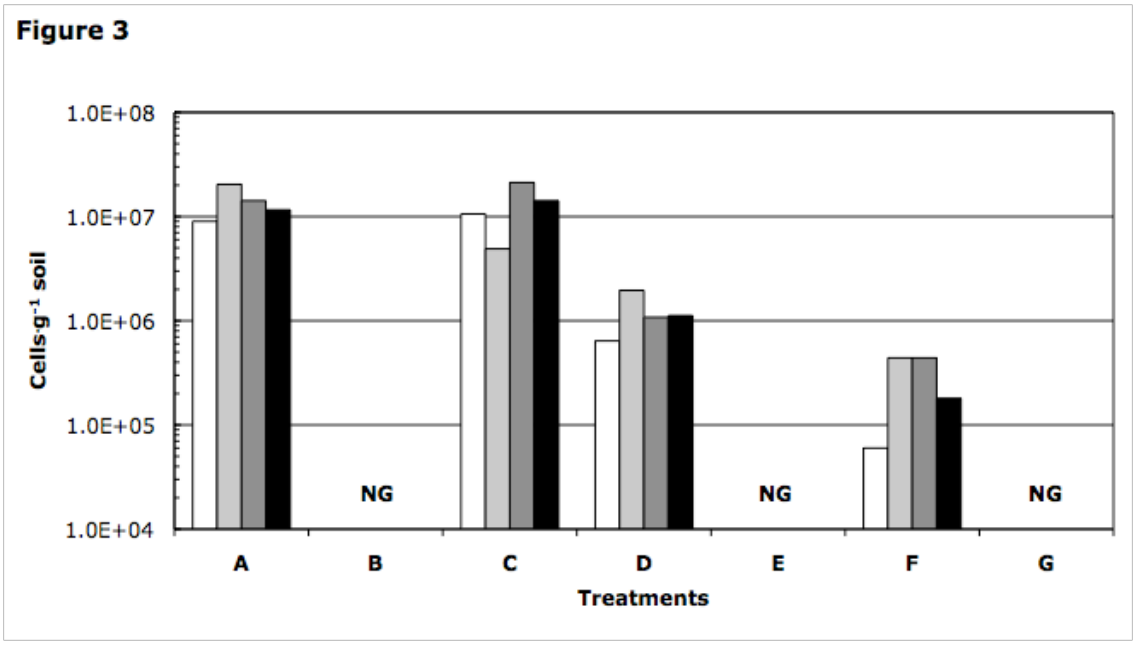

Figure 5.3

Soil microbial biomass based on CFUs from various soils with components of R2A medium amended into MMO minimal agar. Sources of soil samples for this experiment were (from left to right): (□)Romania Breasu with Knapweed site 3 (rhizosphere soil); (▒)Romania Breasu with Knapweed site 4 (rhizosphere soil); (▓)Montana Waterworks Hill with Knapweed (rhizosphere soil); (■)Montana Bob Marshall Wilderness Conifer area with Knapweed (rhizosphere soil). Media treatments were (refer to Table 2 for abbreviations): **A)** MMO (no additional amendments); **B)** MMO + CAT; **C)** MMO + R2A (complete); **D)** MMO + R2A (complete) + CAT; **E)** MMO + RichC + CAT; **F)** MMO + DefC + CAT; **G)** MMO + Min + CAT. **NG** denotes "No Growth" of any bacterial colonies over the course of the experiment.

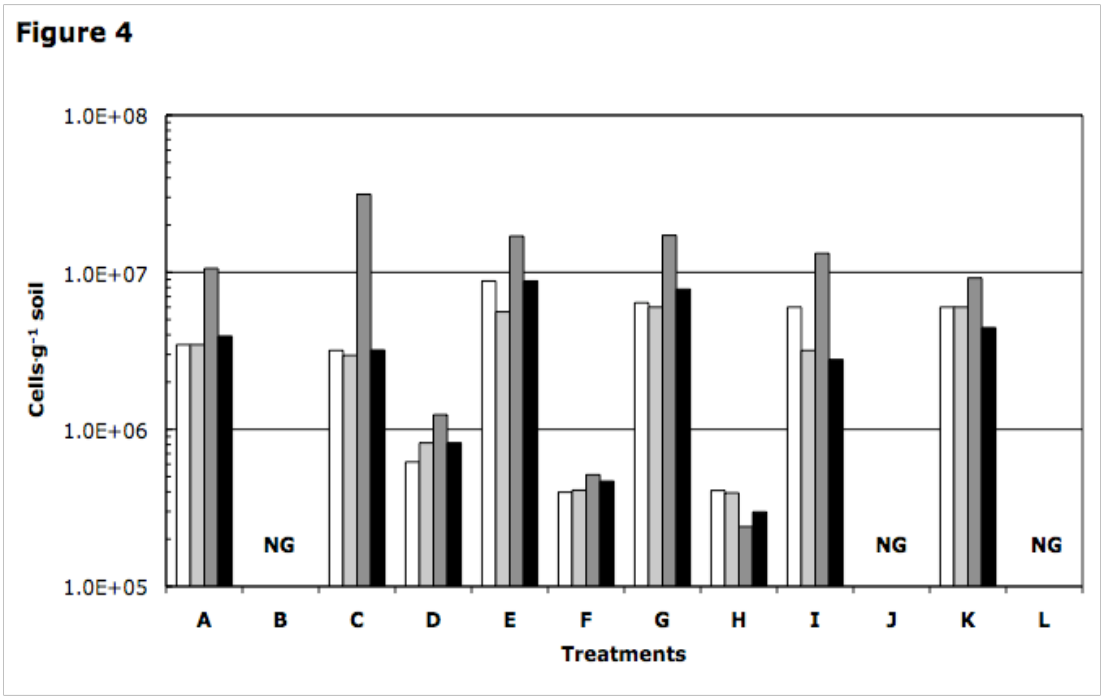

Figure 5.4

Soil microbial biomass based on CFUs from various soils with the individual defined carbon sources of R2A medium amended into MMO minimal agar. Sources of soil samples for this experiment were (from left to right): (□)Romania Breasu with Knapweed site 3 (rhizosphere soil); (▒)Romania Breasu with Knapweed site 4 (rhizosphere soil); (▓)Montana Waterworks Hill with Knapweed (rhizosphere soil); (■)Montana Bob Marshall Wilderness Conifer area with Knapweed (rhizosphere soil). Media treatments were (refer to Table 2 for abbreviations): **A)** MMO (no additional amendments); **B)** MMO + CAT; **C)** MMO + R2A (complete); **D)** MMO + R2A (complete) + CAT; **E)** MMO + DefC; **F)** MMO + DefC + CAT; **G)** MMO + Pyr; **H)** MMO + Pyr + CAT; **I)** MMO + Gluc; **J)** MMO + Gluc + CAT; **K)** MMO + Sta; **L)** MMO + Sta + CAT. **NG** denotes "No Growth" of any bacterial colonies over the course of the experiment.

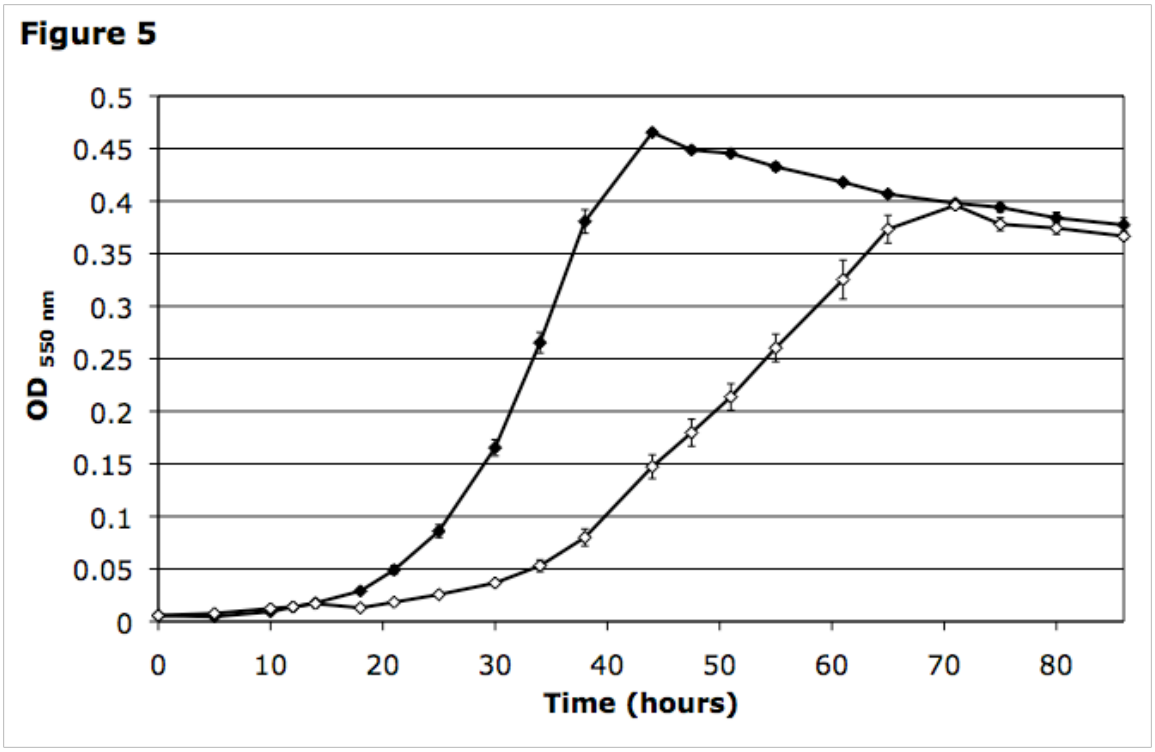

Figure 5.5

Burkholderia isolate G-5 growth on pyruvate in the presence and absence of (±)-catechin. All media contained MMO + pyruvate (500 $\mu g \cdot mL^{-1}$ final concentration. Medium with catechin contained 2000 $\mu g \cdot mL^{-1}$. Symbols are: (◆) CONT; (◇) + CAT. The experiment was performed with triplicate samples and average (mean) measurements are displayed ±1 SE.

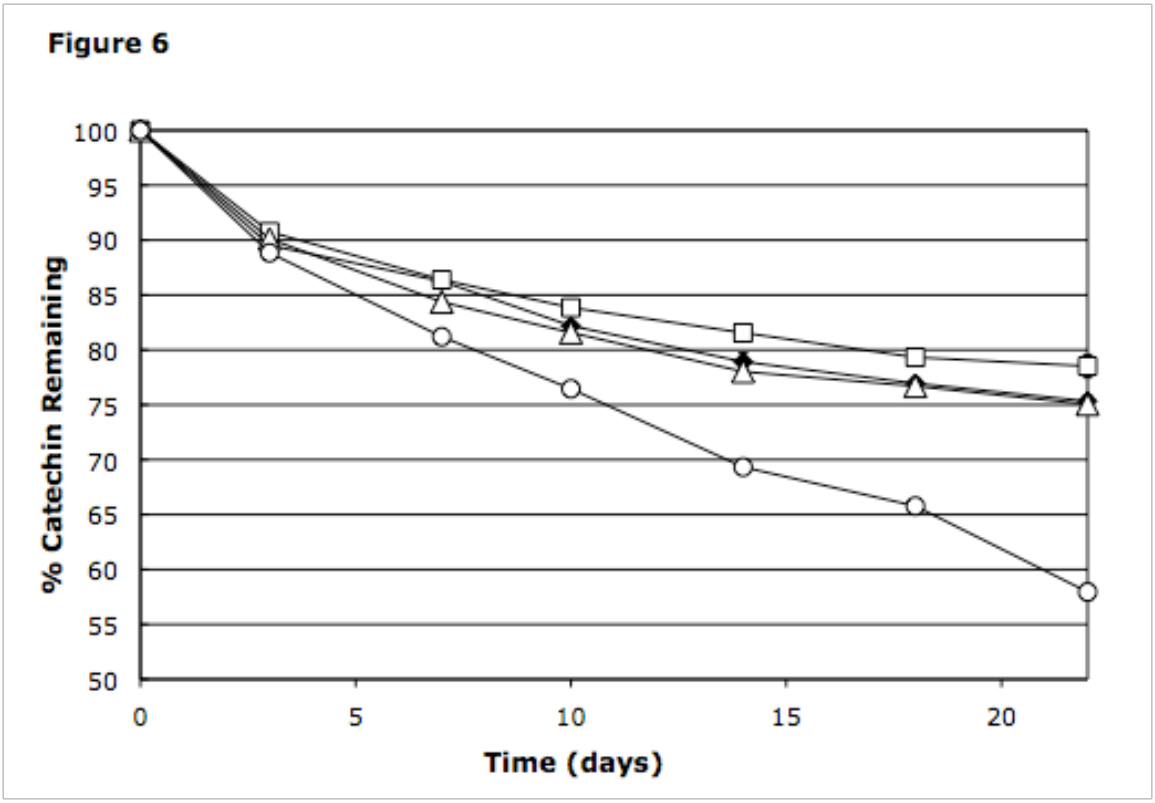

Figure 5.6

Impact of organic acids on catechin stability over time. All treatments contained PB + 1000 $\mu g \cdot mL^{-1}$ catechin (starting concentration). Organic acids were added at 500 $\mu g \cdot mL^{-1}$ each (final concentration). Treatments were: (◆) CAT (no additional amendments); (☐) CAT + Citrate; (△) CAT + Malate; (○) CAT + Pyruvate. Symbols represent the average (mean) of triplicate determinations; error bars are ± 1SE.

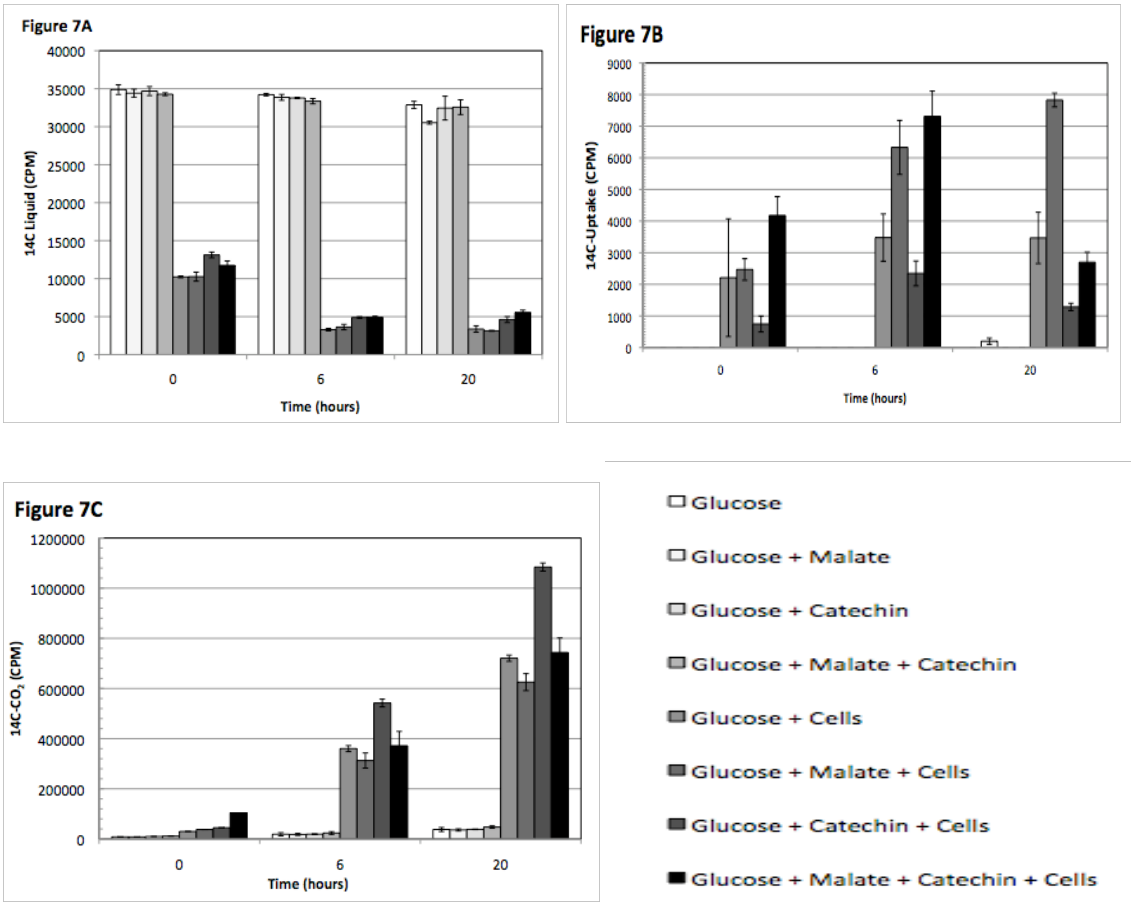

Figure 5.7

^{14}C-Glucose uptake and mineralization study of *Burkholderia* isolate G-5. Panel A; ^{14}C-glucose remaining in the liquid fraction; Panel B; ^{14}C-uptake into cells; Panel C; ^{14}C-CO_2 production over time indicating mineralized glucose; these values are cumulative over the course of the experiment. All flasks contained MMO + ^{14}C-glucose. The bars represent the average (mean) of triplicate determinations; error bars are ± 1 SE. Data from panels A and B were arithmetically normalized to account for the 100 µL carry-over of liquid medium to the bacterial cell pellets using the formula (CPM of 300 µL medium X 4/3) to obtain the true counts in the liquid medium; and the formula (CPM of cell fraction - CPM of 100 µL of liquid medium) to obtain the true counts associated with bacterial cells. Inset alongside Panel C represents the legend for Panels A-C.

Chapter 6

CATECHIN, A ROOT EXUDATE OF *CENTAUREA STOEBE* LAM., INTERFERES WITH CELL GROWTH, SPORULATION, AND GERMINATION IN SEVERAL GRAM-POSITIVE BACTERIA

Abstract

Understanding the effects of root exudates on plants and soil-borne bacteria can further our understanding of what constitutes allelopathy. *Centaurea stoebe*, spotted knapweed, has been an invasive species in the U.S. for more than a century and part of its invasive success is attributed to its ability to excrete the enantiomeric polyphenolic substance (±)-catechin from its roots into the surrounding rhizosphere. Recent research has demonstrated reversible (bacteriostatic) inhibition of growth by (±)-catechin on certain individual bacterial populations from a variety of locations. It has also been shown that pyruvate and malate restored growth to catechin-inhibited bacteria. The current study further explores specific mechanisms of catechin toxicity on a specific bacterial group, the Gram-positive endospore-forming bacteria. We demonstrate the ability of catechin to inhibit proper endospore germination and sporulation of two soil isolates of *Bacillus* and two *Paenibacillus* isolates. Catechin also altered normal cell growth patterns, resulting in long chains of cells rather than individual bacillus forms. This pattern was similar for cells of *B. megaterium* strain C-5 grown in metal-deficient media without catechin. The observed effects on cell growth, sporulation, and germination are attributed to the ability of catechin to sequester various metals necessary for these processes. Sporulation was allowed by the substitution of either pyruvate or malate as the sole source of carbon and

energy when catechin was present, although not to fully normal levels. However, cell morphology was still filamentous, suggesting interference with normal cell division. Interestingly, spore germination was also restored with pyruvate as growth substrate when catechin was present, but not by malate. These findings demonstrate differential effects of catechin on soil bacterial populations that may perturb microbial community composition, potentially facilitating the invasive potential of spotted knapweed.

Introduction

Soil bacterial communities are dynamic and competition between bacterial species under varying conditions likely influences shifts in community composition. The bases for such shifts include, but are not limited to, variations in plant diversity (Carney & Matson, 2005), plant carbon inputs into the rhizosphere (Broeckling*, et al.*, 2008), or traits specific to individual bacteria (e.g. Gram-positive vs. Gram-negative; Butler*, et al.*, 2003). Importantly, some microbial populations in low abundance can mediate ecologically important microbial processes such as nitrification (Holben*, et al.*, 2004). Therefore, even modest alterations in bacterial community composition or activities could have profound effects on soil processes and impact the persistence of individual plant species within a community (Bever, 2003; and references therein).

Gram-positive (hereafter G^+) endospore forming bacteria have a variety of protective features that other bacterial cell types do not possess. Resistance to UV damage, heat, desiccation, and chemical treatments, as well as the ability to survive during nutrient-limiting conditions are all capabilities contributing to the survival of endospore-forming G^+ bacteria under harsh environmental conditions (Russell, 1990, Setlow, 1995). Sporulation (i.e. spore formation) is a metabolically expensive process and is irreversible once initiated (Parker*, et al.*, 1996), resulting in the formation of an endospore. Soil-borne endospore-forming bacteria are involved in many important ecological processes. These include nitrogen fixation (*Bacillus* - Grau & Wilson, 1962), nitrate reduction (*Bacillus* & *Clostridium* - Caskey & Tiedje, 1980, Hoffmann*, et al.*, 1995), sulfate reduction (*Desulfotomaculum* - Newman*, et al.*, 1997), metal reduction (*Bacillus*-

Pollock, *et al.*, 2007), cellulose degradation (*Clostridium* - Petitdemange, *et al.*, 1984), plant and fungal growth promotion (*Bacillus* & *Paenibacillus*- Bai, *et al.*, 2002, Vessey, 2003, Hildebrandt, *et al.*, 2006) and plant pathogenicity (*Pasteuria* & *Bacillus* - Tian, *et al.*, 2007, Fang, *et al.*, 2009). If specific bacterial populations critical to such functions are inhibited in soil, many important soil processes could be perturbed to the point where ecosystem functions, including plant community dynamics, are affected.

Plant invasion can serve as a catalyst for shifts in both plant and soil bacterial communities (Wolfe & Klironomos, 2005, Batten, *et al.*, 2006, Mummey & Rillig, 2006). *Centaurea stoebe* (spotted knapweed) is an invasive plant species in the United States that originated in Eurasia (Callaway, *et al.*, 1999). Spotted knapweed is responsible for several millions of dollars of damage to the agricultural industry by displacing crops as well as being unsuitable grazing material for livestock (Bucher, 1984). The apparent success of spotted knapweed in the US is thought to be based on the ability of the plant to avoid natural predators and pathogens (Callaway, *et al.*, 1999, Callaway, *et al.*, 2004) and its allelochemical attack on other plant species (Bais, *et al.*, 2003), although the latter point is a matter of debate in the literature.

A racemic mixture of catechin is exuded through the roots of spotted knapweed into the surrounding rhizosphere (Perry, *et al.*, 2007, Tharayil & Triebwasser, 2010). This chemical has been shown to have both phytotoxic (Bais, *et al.*, 2002, Callaway, *et al.*, 2005, Pollock, *et al.*, 2009) and microbistatic effects (Chapter 4 and Bais, *et al.*, 2002, Callaway, *et al.*, 2004, Veluri, *et al.*, 2004, Callaway, *et al.*, 2005, Mummey, *et al.*, 2005,

Mummey & Rillig, 2006, Broz*, et al.*, 2007, Inderjit*, et al.*, 2009). Previous work has shown that catechin and close derivatives are capable of complexing with various metals (Gomah & Davies, 1974, McDonald*, et al.*, 1996, Lim*, et al.*, 2005, Pollock*, et al.*, 2009). Earlier work from our group (Chapters 4 & 5) and another laboratory (Weir*, et al.*, 2006) has suggested that catechin, through its ability to sequester metals away from various soil bacteria and native plant species, prevents them from growing. The production or addition of malate or oxalate, organic acids with known chelating ability, was suggested as a potential mechanism for replenishing pools of trace metals and thereby restoring growth to catechin-inhibited soil bacteria and native plants (Chapter 4 & 5 and discussions therein). Studies of the effects of catechin on soil bacterial populations have shown impact on growth (based on CFU enumeration or optical density) and metabolism of various carbon sources for many genera (Chapters 4 & 5). However, relatively little is known about the effect of catechin on other aspects of the metabolism and cellular processes of bacteria. Although a few studies have demonstrated inhibitory effects of catechin and related compounds on endospore-forming species (Sakanaka*, et al.*, 2000, Hara-Kudo*, et al.*, 2005, Friedman*, et al.*, 2006), most of these focused on catechins from tea and their effects on medically important bacteria. In contrast, the work presented herein examines the effects of catechin (in its role as a root exudate) on endospore-forming Gram-positive bacteria commonly isolated from soil.

Here, we demonstrate that catechin affects normal bacterial cell division, and prevents sporulation and endospore germination. The first two effects appear to be due to the ability of catechin to sequester various metals, some of which are required for these

functions, while germination is inhibited through some as yet unknown mechanism. Interestingly, pyruvate allowed endospore germination with catechin present, while both malate and pyruvate partially restored sporulation in the presence of catechin. However, only complete removal of catechin restored proper cell morphology (bacillus form versus filamentous form), along with sporulation and germination demonstrating the reversible nature of the bacteriostatic effects of catechin on cell functions related to spore formation and cell division.

Materials & Methods

Source of bacterial strains and chemicals

Strain C-5 (closest phylogenetic match = *Bacillus megaterium*) was isolated during the experiments described in Chapter 4. In brief, dilutions from a soil sample obtained from the Bob Marshall Wilderness (46.8961° -113.92° 1230 m elevation), a site covered with conifers and spotted knapweed were performed in sterile sodium phosphate buffer (100mM—pH 7) and spread-plated onto R2A plates amended with cycloheximide (300 $\mu g \cdot L^{-1}$ final concentration). All other endospore-forming bacteria were obtained from stock laboratory cultures at The University of Montana—Missoula.

Suppliers for the chemicals used in these experiments with single-letter abbreviations given were: Sigma-Aldrich (S; Sigma-Aldrich, St. Louis, MO), Difco (D; Difco Laboratories Incorporated, Franklin Lakes, NJ), Fisher (F; Fisher Scientific, Pittsburgh, PA), JT Baker (J; JT Baker, Phillipsburg, NJ), and EM Science (E; EM Science, Gibbstown, NJ), unless otherwise stated, and all were of reagent grade quality or higher.

Chemicals employed in these experiments were: Components of R2A [Casamino Acids (D); Yeast Extract (F); Bacto Peptone (D); Soluble Starch (S); Glucose (F); Sodium Pyruvate (S); Dipotassium Phosphate (anh) (S); and, Magnesium Sulfate Heptahydrate (S)]; Components of MMO [Disodium Phosphate (anh) (J); Potassium Phosphate (anh) (S); Sodium hydroxide (F); Calcium Chloride Dihydrate (S); Disodium Nitrilotriacetic Acid (S); Magnesium Sulfate Heptahydrate (S); Ammonia Molybdate Tetrahydrate (S); Ferrous Sulfate Heptahydrate (S); Sulfuric Acid (E); Sodium EDTA (F); Zinc Sulfate Heptahydrate (J); Manganese Sulfate Hydrate (J); Copper Sulfate Pentahydrate (J); Cobalt Dinitrate Hexahydrate (J); Disodium Borate Decahydrate (J); and, Ammonia Sulfate (S)]; D,L-Malic Acid (F); Ethanol (AAPER alcohol and chemical Co., Shelbyville, KY); Methanol (F); and Cycloheximide (S). A 50:50 racemic mixture of (±)-catechin hydrate (>99% purity) was obtained from Shivambu International (New Delhi, India). Stock solutions of glucose (20%), D,L-malic acid (20%), and sodium pyruvate (20%) were filter-sterilized (0.22μM) before addition to MMO broth and agar.

MMO minimal medium (liquid and plates) were made as previously described (Stanier*, et al.*, 1966) and amended with sterile glucose, malic acid, or pyruvic acid to (500 $\mu g \cdot ml^{-1}$ (0.05%) where indicated in sections below. R2A broth and agar were made as recommended by the manufacturer (Difco, Detroit, MI). R2A plates used for bacterial isolation directly from soil were amended with the fungal inhibitor cycloheximide at a final concentration of 300 $\mu g \cdot mL^{-1}$ (from a 30 $mg \cdot mL^{-1}$ stock solution in 100% ethanol). Catechin was gently heated in sterile ultrapure water to dissolve before adding to media that had been cooled to ~60° C after autoclaving.

Effects of catechin on *Bacillus megaterium* strain C-5

Previous work from this group demonstrated that the inhibitory effect of catechin is bacteriostatic, as removal of catechin from the system restored growth to all strains tested (Chapter 4). Growth of *Bacillus megaterium* strain C-5 on MMO agar + glucose with and without catechin was observed to ensure that catechin inhibition of this isolate. Catechin showed complete inhibition of strain C-5 on MMO + glucose plates (data not shown), but slight cell growth occured in liquid MMO + glucose relative to the no-catechin control, albeit after a significant lag (data not shown). In a subsequent detailed growth curve experiment, strain C-5 was grown to mid-log phase on MMO + glucose and then inoculated (by 1:100 dilution) into fresh medium with or without catechin. All treatments were incubated at room temperature with orbital shaking at 150 rpm in the dark (as catechin is somewhat photoreactive). Cell growth was measured based on OD_{550} using a Spectronic 20D+ Spectrophotometer (Milton Roy USA, Ivyland, PA). As catechin naturally auto-oxidizes (especially in the presence of pyruvate—see Chapter 5) and thereby changes the absorbance of the medium, blanks for this experiment were either MMO + glucose or MMO + glucose + 2000 $\mu g \cdot mL^{-1}$ catechin and treated to the same conditions. For this growth experiment the treatments consisted of MMO + glucose (control); MMO + glucose + catechin (2000 $\mu g \cdot ml^{-1}$); and MMO + glucose + catechin in which the catechin was removed from the media after the control cultures reached stationary phase (T= 19 hours) by centrifugation of the culture, decanting the supernatant, washing the cell pellet with PB, centrifuging again, decanting the supernatant, then resuspending the cells in 1ml of PB and then transferring into 100 mL of fresh MMO +

glucose medium without catechin. The culture incubation was continued as before and the OD measured using the MMO + glucose blank. All treatments were performed in triplicate.

Aliquots of all cultures in this experiment were taken at each time point to assess the impact of catechin exposure on cell morphology. Samples (5 mL) from each flask were removed, centrifuged at 13,000 RPM to pellet cells, the supernatant decanted, and the pellet washed once in PB and resuspended in a fixative solution of 5% (v/v) glutaraldehyde in sodium cacodylate buffer (20mM, pH 6) overnight at 4° C. Scanning- and transmission-electron microscopy (SEM and TEM, respectively) were performed on all samples using standard techniques by the EMTrix facility at The University of Montana—Missoula.

Direct microscopic determination of numbers of cells, filaments (chains of cells ≥4), and spores were made twice for each culture and treatment, at the onset of stationary phase (T = 12 h for the control cultures, T = 23 h for both treatments with catechin) and again after 48 additional h of additional incubation in stationary phase for each treatment. A Petroff-Hausser cell counting chamber (0.02-mm depth) and light microscope (100X objective with oil immersion) were used for these determinations. Endospores were counted as refractile bodies in the media (either within cells or released from cells). Subsequent TEM analyses of small cellular inclusions observed by light microscopy during the mid-log phase of growth confirmed the presence of poly-β-hydroxybutyrate (PHB) using the Sudan Black staining method (Burdon, 1946).

Comparing catechin effects to those of metal deficiency

As shown in the Results section of this chapter, catechin eliminates the biphasic increase in OD_{550} observed when catechin is absent. Since strain C-5 is a *Bacillus* species, this suggested the possibility of endospore formation being disrupted. Because normal sporulation requires several metals (Donnellan*, et al.*, 1964, Kolodziej & Slepecky, 1964, Splittstoesser & Farkas, 1966, Greene & Slepecky, 1972, Murray*, et al.*, 1998) and catechin has metal-chelating properties (Chapter 2 of this dissertation and Gomah & Davies, 1974, McDonald*, et al.*, 1996, Lim*, et al.*, 2005, Pollock*, et al.*, 2009), modified MMO medium lacking manganese (-Mn), calcium (-Ca), or both (-Ca/Mn) was prepared to observe and compare the effects of lack of these metals on endospore formation by strain C-5 to those of catechin.

Strain C-5 grown to mid-log phase on MMO + glucose used to inoculate triplicate flasks by 1:100 dilution for each treatment. These consisted of: MMO (complete) + glucose as a control; modified MMO (lacking Ca) + glucose; modified MMO (lacking Mn) + glucose; modified MMO (lacking Ca and Mn) + glucose; and MMO (complete) + glucose + catechin (2000 $\mu g \cdot ml^{-1}$). All cultures were incubated as described above and OD_{550} was measured using either MMO + glucose or MMO + glucose + catechin, as appropriate, as the blank. Direct microscopic enumeration of cells, filaments, and spores were performed as described above. SEM analyses were performed on cells at mid-log growth phase for each culture as outlined above.

Effect of magnesium deficiency on cell morphology

Previous work has demonstrated that magnesium is essential for normal cell morphology for many bacterial species including *Bacillus* (Rochford & Mandle, 1953, Shankar & Bard, 1955, Slepecky & Foster, 1959, Weiss, 2004). A simple growth experiment was performed to determine whether lack of magnesium (Mg) mimicked the filamentous cell morphology exhibited by strain C-5 when grown in the presence of catechin. An inoculum of strain C-5 grown to mid-log phase on MMO + glucose, then transferred 1:100 into MMO (complete) + glucose as a control or serially diluted into flasks containing 100 ml of modified MMO (lacking Mg) + glucose to test the effect of limited Mg on cell morphology. All cultures were incubated as described above subjected to visual inspection by light microscopy and also SEM and TEM as outlined above.

Effect of catechin on endospore germination

As catechin exhibited the ability to inhibit the formation of endospores when grown in glucose, the ability to prevent germination of normally-formed endospores was also tested. For this experiment, a culture of strain C-5 was grown on MMO + glucose for 4 d to ensure maximal sporulation, then, heated to 80° C for 20 minutes (i.e. pasteurized) to ensure that only endospores survived. One ml of this pasteurized suspension was transferred to each 100 ml of medium in triplicate flasks for each treatment. Treatments consisted of contained MMO + glucose (as control); .MMO + glucose + catechin (2000 $\mu g \cdot ml^{-1}$); and MMO + glucose + catechin, which was removed after the cells in the control treatment reached stationary phase (T = 41 hours) by pelleting cells, washing and resuspending in fresh MMO + glucose without catechin as described above. All

treatments were incubated as described above and OD_{550} was monitored using MMO + glucose or MMO + glucose + catechin blanks as appropriate.

Assessing the ability of organic acids to restore cell morphology and endospore formation

Previous work demonstrated the ability of pyruvate and malate to restore growth to catechin-inhibited bacterial populations (see Chapter 5). To determine whether pyruvate or malate were capable of restoring normal cell morphology and endospore formation in the presence of catechin, growth kinetics experiments using MMO amended with 500 $\mu g \cdot ml^{-1}$ pyruvate or malate in the presence and absence of catechin (2000 $\mu g \cdot ml^{-1}$) were performed on strain C-5. Cultures were grown on MMO + pyruvate or MMO + malate to mid-log phase before inoculating by 1:100 dilution into the respective treatments in triplicate. All treatments were incubated in the dark with shaking at 150 rpm at room temperature. The treatments included: MMO + pyruvate (no additional amendments); modified MMO (lacking Mn^{2+} and Ca^{2+}) + pyruvate; MMO + malate (no additional amendments); MMO + pyruvate + catechin; and, MMO + malate + catechin. Cell growth was monitored as outlined above using appropriate uninoculated blanks for each medium type and the effect these organic acids on cell morphology was assessed by SEM and TEM as outlined above.

Endospore numbers for this experiment were determined by pasteurization at 80° C for 20 min (72 hours after reaching stationary phase to ensure maximal endospore formation), followed by serial dilution to 10^{-8} in PB and spread-plating 100 µl of each dilution onto R2A. After 2 days of incubation at room temperature, all plates were

scored for CFUs at an appropriate dilution (~30-300 colonies on a plate). All CFUs were assumed to arise from heat-resistant endospores that successfully germinated.

Assessing the ability of organic acids to restore endospore germination

As pyruvate and malate allowed growth of bacteria in the presence of catechin in previous work (see Chapter 5), spore germination using either of these organic acids in the presence of catechin was assessed. Endospores were generated as described above and 1 ml of the pasteurized suspension was transferred to 100 mL of medium in triplicate flasks for each treatment. Treatments were as follows: MMO + pyruvate; MMO + pyruvate + catechin (2000 $\mu g \cdot ml^{-1}$); MMO + malate; and MMO + malate + catechin. Appropriate uninoculated blanks for each medium type were used to monitor OD_{550} as before.

Tests against other endospore-forming bacteria

Two other *Bacillus* species (*B. cereus* and *B. subtilis*) and two *Paenibacillus* species (*P. macqueriensis* and *P. borealis* strain HC, were also tested for alteration of cell morphology, and inhibition of endospore formation and germination by catechin. All strains were tested as described in detail above for strain C-5. R2A broth was used for these experiments as neither *B. cereus* or *P. borealis* would grow in MMO + glucose and all five species (including *Bacillus* strain C-5) were grown in R2A broth with and without catechin for consistency. The effect of catechin on cell morphology, sporulation, and germination was monitored by light microscopy as described above.

Comparison of origin of spotted knapweed on endospore formation in soil

As spotted knapweed does not invade in its native range but is highly invasive in North America, we tested for differences in the ability of different knapweed populations to impact the formation of endospores in a pot experiment using the naïve Clearwater Game Range soil described in chapter 4. *Centaurea stoebe* seeds from various locations in North America: Missoula, MT (MS), Butte, MT (BT), Bozeman, MT (BZ), Elko British Columbia (EK), Boise, Idaho (ID); and Eurasia: France ssp Rheaana (FR), Hungary Gyor (HU), Romania (#4) Falticeni (ROF), Romania (#2) Roman (ROR), Ukraine 1 (UK) were used in this experiment. Seeds were germinated and seedlings (starting with 6 and thinned to 2 per pot) were grown in the naïve soil in pots (n=5 per *C. stoebe* population) in the greenhouse at the University of Montana. Pots were watered twice weekly and greenhouse temperatures ranged between 20° to 30° C during the day and 15° to 20° C at night. After 60 days, each soil sample was tested for aerobic heterotrophic culturable cell counts and for endospore numbers (following soil pasteurization as described above) based on CFUs. To accomplish this, all soil samples were serially diluted to 10^{-8} in PB and 100 µl of each dilution was spread onto R2A agar amended with cycloheximide (300 $\mu g \cdot ml^{-1}$) to control fungal growth. All plates were incubated at room temperature in the dark for 2 days before enumeration of CFUs on plates of an appropriate dilution (colony number between 30 and 300). Colonies grown from pasteurized soils are assumed to have arisen from heat-resistant endospores, while colonies grown from unpasteurized soils are assumed to represent a combination of vegetative cells and endospores. Therefore, for the calculation of spore-to-cell ratios, the formula employed was: endospore to vegetative cell ratio = [heat-treated CFUs ÷ (untreated CFUs minus heat-

treated CFUs)]. The "no seed" control pots (pots of soil watered and incubated in the greenhouse just like the treatments) were also tested to obtain background spore-to-cell ratios in the absence of knapweed.

Results

Transitory effects of catechin on cell morphology and endospore formation

When grown in MMO media with glucose as the sole carbon source and electron donor, strain C-5 exhibited normal growth kinetics (Fig. 1) and cell morphology (Fig. 2A & 3A). However, when catechin was added to the medium, strain C-5 exhibited a long lag phase (>6 hours), slower growth kinetics, and lack of biphasic growth kinetics (Fig. 1). The second stage of this biphasic growth pattern is believed to be indicative of the formation of refractile endospores that would increase the OD_{550} even in the absence of additional cell division. Growth in the presence of catechin also resulted in at least a 1000-fold decrease in the number of endospores formed (Table 1), and filamentous (i.e. chained vs. normal bacilli) cell morphology (Fig. 2B & 3B). TEM micrographs (Fig. 3A, C, and E) also showed small inclusions during the mid-log phase of growth for all treatments. These inclusions were concluded to be PHB based on their appearance using the Sudan Black method.

Although cells in the + catechin treatment (Fig. 3C) had an altered, chained cell morphology, their cell walls within the filament appeared normal (data not shown). Also, these chained cells did not appear to have endospores in them during late stationary/death phase (Fig. 3B). For example, in the direct spore count (Table 1), only one lone spore

was detected in one of the three replicates. However, this may have been an endospore that never germinated once placed into catechin-amended media (refer to Fig. 8 results below) and the actual number of endospores formed would thus be zero (total inhibition) rather than the 1000-fold reduction due to this one spore that was detected.

Interestingly, these dramatic changes in catechin-treated cells are not permanent. When these abnormal cells are washed and placed into fresh MMO + glucose medium without catechin, normal cell growth kinetics occurred (Fig. 1), cell morphology returned to normal (Fig. 2D), endospores were formed (Fig. 3F), and spore numbers were comparable to the control (Table 1).

Effect of metal deficiency on the morphology of *Bacillus megaterium* strain C-5

As previously seen, when grown on MMO medium with glucose as sole carbon source and electron donor, strain C-5 exhibited normal cell growth kinetics (Fig. 4) and cell morphology (Fig. 5A). Omitting calcium, manganese, or both from the media did not inhibit cell growth appreciably, but did reduce the OD_{550} observed during the second phase of the biphasic growth curve (Fig. 4). The addition of 2000 μg ml^{-1} (±)-catechin to strain C-5 again resulted in the effects seen previously, a prolonged lag phase (~8 hours) and hampered growth kinetics (Fig. 4). As illustrated by Table 2 and Figs 5B-D, the omission of calcium, manganese, or both from the media resulted in filamentous cell morphology similar to that induced by catechin (Table 2; Fig. 5E). However, unlike for the catechin-amended media, the absence of either or both metals appeared to have no effect on spore number (Table 2).

Effect of Mg^{2+} concentration on cell morphology and endospore formation

When strain C-5 was grown on modified MMO + glucose with decreasing concentrations of Mg^{2+}, cell morphology became filamentous in a way that is similar to the effects seen with catechin, although to a somewhat lesser extent. Cultures grown at a Mg^{2+} concentration 10^{-4} (10,000-fold) lower than complete MMO still had many cells of normal morphology interspersed with a few sets of cells in filaments (Fig. 6B). At a Mg^{2+} concentration 10^{-6} (1,000,000-fold) lower than complete MMO exhibited cell morphology was evenly filamentous in nature (Fig. 6C), similar to that observed in the presence of catechin.

Catechin inhibits endospore germination

When grown in MMO medium with glucose, strain C-5 endospores were capable of normal endospore germination and vegetative growth (Fig. 8). Clearly, endospore germination was inhibited when endospores of strain C-5 were in the presence of catechin (Fig. 8). After 118 h of incubation, endospores of strain C-5 still did not germinate in the presence of catechin based on microscopic observation (data not shown). Monitoring for germination could not be pursued past 120 hours because the abiotic control with catechin oxidized to the point that it could no longer be used as a blank for absorbance measurement by spectrophotometry. However, when endospores inhibited by catechin were washed and placed into fresh MMO + glucose medium without catechin, endospore germination resumed and showed typical growth kinetics (Fig. 8). The addition of 1mM of l-alanine (a common germination trigger for many endospores;

McCann*, et al.*, 1996, Wuytack*, et al.*, 2000, Atluri*, et al.*, 2006) had no effect on catechin-inhibited endospores (Fig. 8).

Effect of organic acids on inhibition by catechin

When the above two experiments were repeated using either pyruvate or malate as carbon source in MMO media with and without catechin, the organic acids allowed partial endospore formation to take place, but did not exhibit normal cell morphology. As seen in Figure 9, the vegetative growth rate was almost normal in the presence of catechin when malate was the sole carbon source in the medium, although a lag phase of ~12 hours was observed. Interestingly, treatments with pyruvate and catechin showed essentially no lag phase, but the growth rate was still slower (Fig. 9). Neither organic acid produced normal cell morphology in the presence of catechin (Fig. 10A-E), and cells frown in MMO + pyruvate + catechin exhibited pinched cell and filamentous morphology (Fig. 10B). Also, as observed in Figure 11, when strain C-5 was grown with catechin and either malate or pyruvate, endospore numbers were not comparable to the corresponding controls with no catechin addition. However, the differences in endospore numbers for treatments between malate and malate plus catechin were 1000-fold, whereas, the difference between the pyruvate and pyruvate plus catechin treatments was 10,000-fold (Fig. 11).

Interestingly, pyruvate allowed endospore germination in the presence of catechin, but with a longer lag phase (~20 hours longer) than for the no-catechin control (Fig. 12).

Effect of spotted knapweed race on endospore formation in a naïve U.S. soil

A common garden pot experiment was used to test whether endospore formation in soil would be impacted by different races (populations from different locations) of spotted knapweed. Since others have suggested that knapweed from Europe behaves differently than the invasive form found in the U.S. (Müller, 1989, Ridenour*, et al.*, 2008, He*, et al.*, 2009), seeds from various sites in both locales were employed in this experiment. Four of five seed types from sources in North America germinated to produce plants and soil from all of these pots showed endospore to cell ratios that were not significantly different than the control level (Fig. 13). All five seed types from sources in Eurasia grew, and interestingly, three of these showed statistically higher endospore to cell ratios than for the unplanted control plots (Fig. 13).

Discussion

While the impact of catechin on indigenous plant species of North America has been extensively reported (Iqbal*, et al.*, 2003, Inderjit*, et al.*, 2008, Duke*, et al.*, 2009), its effects on soil bacterial communities are still poorly understood. One other research group has reported an "antimicrobial" aspect of catechin on bacterial isolates (Veluri*, et al.*, 2004). However, until now, there have been no data reported that have adequately addressed the specific mechanism(s) underlying these effects or their impact on specific bacterial processes.

In this thesis chapter, I report that catechin has many effects on common soil endospore-forming bacteria. Cell growth and morphology, and endospore formation and

germination are all altered or inhibited by catechin during growth on glucose as sole carbon source. Many of these altered cell functions can be attributed to metal deficiency of one or more types. Interestingly, the replacement of glucose with either pyruvate or malate allowed sporulation to occur in the presence of catechin. Further, growth on pyruvate supported germination of catechin-inhibited endospores.

Other studies have shown that catechin from tea, and related polyphenolic compounds, prevented endospore formation and germination in Gram+ bacteria relevant to the gastrointestinal tract and food industry. In one study, tea catechins exhibited the ability to prevent the germination of endospores responsible for food spoilage (Hara-Kudo*, et al.*, 2005). However, these studies focus on medicinal aspects of catechin application as they pertain to endospore-forming bacteria.

In the current study, I expand on these findings by studying the impact of catechin on cell morphology, endospore formation, and endospore germination of common soil isolates, with *Bacillus megaterium* strain C-5 typifying these effects. Normal cell growth, morphology, endospore formation and germination were all observed for strain C-5 when cells were grown in MMO media without catechin. However, the addition of catechin altered cell growth kinetics, changed cell morphology from single bacillus cells to filaments of cells, lowered endospore numbers to essentially undetectable levels (Tables 1 & 2 respectively), and prevented normal endospores from germinating successfully. These effects were all relieved upon the removal of catechin by washing and resuspending cells or spores in medium without catechin, suggesting that the effects are

not responsible for permanently damaging the cells or endospores, and strongly supporting the findings in Chapter 4 that the inhibitory effects of catechin are bacteriostatic rather than bactericidal.

It is also interesting that many of the effects of catechin on cell growth, morphology, and endospore formation, mimicked similar effects caused by deficiencies of essential metals in the media. We suggest that that the ability of catechin to chelate a variety of metals (McDonald*, et al.*, 1996, Es-Safi*, et al.*, 1999, Kidd*, et al.*, 2001, Lim*, et al.*, 2005, Pollock*, et al.*, 2009) is directly responsible for some of the effects observed in this study. It has long been known that decreased concentrations of magnesium ions in media can have deleterious effects on normal cell morphology for G^+ organisms like *Bacillus cereus* (Nickerson & Sherman, 1952), *Diplococcus pneumoniae* (Rochford & Mandle, 1953), and *Clostridium perfringens* (Shankar & Bard, 1955). Similarly, others have shown that supplementing media with calcium ions (Wright & Klaenhammer, 1981, Faguy*, et al.*, 1993) and manganese ions (Wright & Klaenhammer, 1983) ions results in the formation of non-filamentous individual cells, suggesting a role for these metals in normal cell growth, morphology and presumably division. It also seems likely that the decrease in endospore production is related to the cell's requirement for certain metals in order to form proper endospore components. For example, calcium dipicolinate is necessary for heat resistance in endospores (Church & Halvorson, 1959) and chelation of this compound in media containing catechin may have resulted in no or irregular spores, rendering all cells in these experiments susceptible to the pasteurization process used to

select for healthy endospores. Magnesium and calcium ions have been shown to be responsible for increased heat resistance of spores (Atrih & Foster, 2001).

We have shown that metal limitations result from catechin exposure, and that lack of calcium and manganese interferes with normal cell morphology and endospore formation. However, removal of calcium and/or manganese from catechin-free growth medium did not hamper endospore germination although the presence of catechin did (data not shown). Therefore, catechin is either: a) removing a different metal necessary for proper endospore germination from the medium; b) stripping the metal(s) necessary for endospore germination out of the endospores; or c) affecting spore germination through some as yet undefined mechanism. Alternatively, others have shown that superoxide radicals can stimulate germination (Baillie*, et al.*, 2005). Since catechin is reported to scavenge superoxide radicals (Mira*, et al.*, 2002, Proestos*, et al.*, 2005), it is possible that this ability is what is inhibiting endospore germination rather than metal limitation.

Since pyruvate and malate restored growth of catechin-inhibited soil bacteria in our previous work (see Chapter 5), we tested these organic acids for their ability to restore normal cell morphology, endospore formation and germination function to *Bacillus megaterium* strain C-5. Somewhat unexpectedly, cell filaments were still formed when strain C-5 is using pyruvate or malate as its sole carbon source in the presence of catechin (Fig. 10). However, both pyruvate and malate allowed endospore formation in the presence of catechin, although not nearly to the same levels obtained on either substrate

in the absence of catechin (Fig. 11). Several prior studies have clearly demonstrated the metal-binding ability of malate (Gardner*, et al.*, 1983, Keerthisinghe*, et al.*, 1998), and pyruvate is capable of chelating zinc (Chen & Liao, 2003) as well as complexing with iron and promoting growth, albeit poorly (Drechsel*, et al.*, 1993). It is therefore reasonable to assume that the organic acids employed in this study might chelate other metals necessary for proper cell function. Additionally, endospores did not germinate in medium containing malate, a known metal-chelating organic acid, regardless of whether catechin was present. By contrast, when pyruvate was the carbon source in the presence of catechin, endospores readily germinated (Fig. 12). Cumulatively, these data suggest that these two organic acids are probably not competitively sequestering (i.e. away from catechin) and making available to the cell divalent metal(s) necessary for normal endospore formation, cell growth, or cell morphology.

Alternatively, pyruvate or malate may competitively block some site of cell disruption by catechin. Two studies have demonstrated that catechin has the ability to competitively bind to enzymes involved in malate metabolism (Pairoba*, et al.*, 1996, Savitha, 2000). If cells are generating more malate metabolic enzymes as a result of having abundant malate as the sole carbon source, it is plausible that catechin has been effectively neutralized by the increase in abundance of these enzymes to which it binds. How this would translate into the ability of pyruvate to support normal cell functions in the presence of catechin is still unknown, although cell growth, endospore formation and germination are clearly different with malate compared to pyruvate, which leads to the

conclusion that the mechanisms by which these two organic acids allow growth in the presence of catechin could in fact be different.

To link these laboratory experiments to the potential impact of spotted knapweed on a naïve system, a study testing a common soil against spotted knapweed obtained from multiple sites in its native range (Europe) and its invaded range (North America) showed relatively minor differences in the ability of the plants to interfere with endospore formation in soil (Fig. 13). Most North American plants demonstrated endospore-to-cell ratios similar to the unplanted soil pots over the course of the experiment, while the European plants also exhibited very little impact on spore to cell ratios overall and three of the knapweed populations tested seemed to increase the ratio of endospores to total CFUs (Fig. 13). Overall, the results of this experiment were inconclusive and, as the first experiment of its type, do not allow us to draw any firm conclusions regarding whether catechin from knapweed interferes with endospore formation in natural soil systems.

Collectively, the results presented in this chapter increase our understanding of allelopathy by *C. stoebe* that might very well impact soil bacterial population dynamics in the face of invasion by spotted knapweed. For example, given that catechin causes altered cell morphology in the form of long filaments of cells rather than bacilli, movement of these altered cells through the soil could potentially be impacted. Also, the ability of endospore-forming bacteria to produce endospores when presented with harsh environmental conditions was compromised by the addition of catechin and perhaps by extension invasion of North American spotted knapweed.

What impact these catechin effects on bacteria have on plant-microbe relations, both antagonistic and beneficial, or the role they play in the invasive success of spotted knapweed is not known at this time. However, many endospore-forming soil species play vital roles in plant, insect, and nematode pathogenicity (Tian*, et al.*, 2007, Fang*, et al.*, 2009) and references therein). Thus, the inhibitory effect of catechin on endospore-formers might represent a mechanism by which knapweed protects itself from pathogens (Callaway*, et al.*, 2004). Catechin exudation into naïve soils may be just one mechanism facilitating the invasive success of North American populations of *Centaurea stoebe*. Spotted knapweed, through exudation of catechin into the surrounding rhizosphere, could alter normal soil bacterial community composition and activities by affecting the abundance of endospore-forming species through the mechanisms described herein and potentially alter the dynamics of carbon-utilization throughout the system (this work and Chapter 5). Further, removal of bioactive catechin in soil, whether by degradation, sorption, or transformation, could allow the impacted bacterial community to resume its normal structure and function. This last suggestion may have profound ramifications for attempts to restore knapweed-infested ecosystems.

The data from this study suggest the potential for substantive retardation of bacterial cell and endospore function for soil species exposed to catechin and similar compounds via root exudation or other methods of entry to the soil. These are the first studies that have directly demonstrated dramatic morphological changes in bacterial populations. They are also the first to demonstrate that certain growth substrates can allow or restore endospore

formation and germination in the presence of catechin. The work presented herein also suggests mechanisms by which these phenomena occur. These results clearly demonstrate that a variety of endospore-forming bacteria commonly found in soil can be negatively affected by catechin. More specifically, the data demonstrate that: 1) growth rates are reduced in the presence of catechin; 2) cell morphology was changed from bacilli to filamentous forms when growing in the presence of catechin; 3) catechin hampers endospore formation and is "sporostatic" but not "sporicidal"; 4) removal of the catechin restores bacterial growth kinetics, endospore formation and spore germination; and, 5) two different organic acids, pyruvate and malate, when used as the sole source of carbon allow endospore formation (but not to normal levels) in the presence of catechin, while pyruvate allows spore germination in the presence of catechin.

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Tables

Table 6.1 Growth of *Bacillus megaterium* strain C-5 on glucose without, with and after removing catechin from the medium. MMO + glucose (CONT); MMO + glucose + catechin (+ CAT); MMO + glucose + catechin, with catechin removed after CONT had reached stationary phase (+ WASH). The values given are numbers of cells, filaments, cells per filament, and endospores per ml of culture as determined by light microscopy. Numbers are the average for triplicate cultures for each treatment. Numbers in parentheses are ±1 standard deviation (SD). ND= Not detected.

Treatment	Individual cells (Number·ml^{-1})		Filaments (Number·ml^{-1})		Filamentous Cells (Number·ml^{-1})		Spores (Number·ml^{-1})	
	Stationary (±SD)	+ 48 hrs (±SD)	Stationary (±SD)	+ 48 hrs (±SD)	Stationary (±SD)	+ 48 hrs (±SD)	Stationary (±SD)	+ 48 hrs (±SD)
CONT	1.180 (±0.294) $^{x}10^{8}$	ND	ND	ND	ND	ND	ND	2.079 (±0.718) $^{x}10^{8}$
+ CAT	ND	ND	1.708 (±1.583) $^{x}10^{7}$	2.458 (±1.161) $^{x}10^{7}$	1.408 (±1.515) $^{x}10^{8}$	1.917 (±0.993) $^{x}10^{8}$	ND	***4.167 (±7.217) $^{x}10^{5}$**
+ WASH	1.117 (±0.914) $^{x}10^{8}$	1.667 (±1.909) $^{x}10^{6}$	ND	ND	ND	ND	ND	3.025 (±0.794) $^{x}10^{8}$

*****=The + CAT treatment had only one spore between all three samples.

Table 6.2 Comparison of growth of *Bacillus megaterium* strain C-5 on glucose in metal-deficient medium. complete MMO + glucose (CONT); modified MMO + glucose – calcium (- Ca); modified MMO + glucose – manganese (- Mn); modified MMO + glucose – calcium & - manganese (- Ca/Mn); complete MMO + glucose + catechin. The values given are numbers of cells, filaments, cells per filament, and endospores per ml of culture as determined by light microscopy. Numbers are the average for triplicate cultures for each treatment. Numbers in parentheses are ±1 standard deviation. ND= Not detected.

Treatment	Individual cells (Number·ml^{-1})		Filaments (Number·ml^{-1})		Filamentous Cells (Number·ml^{-1})		Spores (Number·ml^{-1})	
	Stationary (±SD)	+ 48 hrs (±SD)	Stationary (±SD)	+ 48 hrs (±SD)	Stationary (±SD)	+ 48 hrs (±SD)	Stationary (±SD)	+ 48 hrs (±SD)
CONT	8.875 (±3.733) $^{x}10^{7}$	ND	ND	ND	ND	ND	4.167 (±7.217) $^{x}10^{5}$	1.242 (±0.452) $^{x}10^{8}$
- Ca	4.792 (±4.646) $^{x}10^{7}$	ND	7.083 (±3.819) $^{x}10^{6}$	ND	6.083 (±4.254) $^{x}10^{7}$	ND	ND	8.583 (±2.538) $^{x}10^{7}$
- Mn	4.625 (±0.875) $^{x}10^{7}$	4.583 (±1.909) $^{x}10^{6}$	4.583 (±2.887) $^{x}10^{6}$	1.25 (±1.25) $^{x}10^{6}$	2.583 (±1.512) $^{x}10^{7}$	6.667 (±5.907) $^{x}10^{6}$	ND	2.242 (±0.5274) $^{x}10^{8}$
-Ca/Mn	7.917 (±2.887) $^{x}10^{6}$	8.333 (±14.434) $^{x}10^{5}$	1.167 (±0.260) $^{x}10^{7}$	ND	1.429 (±0.399) $^{x}10^{8}$	ND	ND	1.425 (±0.74) $^{x}10^{8}$
+ CAT	1.667 (±2.887) $^{x}10^{6}$	ND	1.583 (±0.641) $^{x}10^{7}$	1.625 (±0.250) $^{x}10^{7}$	2.275 (±0.410) $^{x}10^{8}$	1.204 (±0.277) $^{x}10^{8}$	ND	ND

Table 6.3 Impact of catechin on endospore-forming bacteria in R2A medium. *B.* = *Bacillus*, *P.* = *Paenibacillus*. Note: *B. subtilis* was unaffected by catechin in these experiments (highlighted in **bold**).

Strain name	Chained cell Morphology	≥2 log decrease in number (i.e. 1% or less of normal spore #)	Germination prevented?
B. megaterium C-5	Y	Y	Y
B. subtilis	**N**	**N**	**N**
B. cereus	Y	Y	Y
P. macqueriensis	Y	Y	Y
P. borealis	Y	Y	Y

Figures

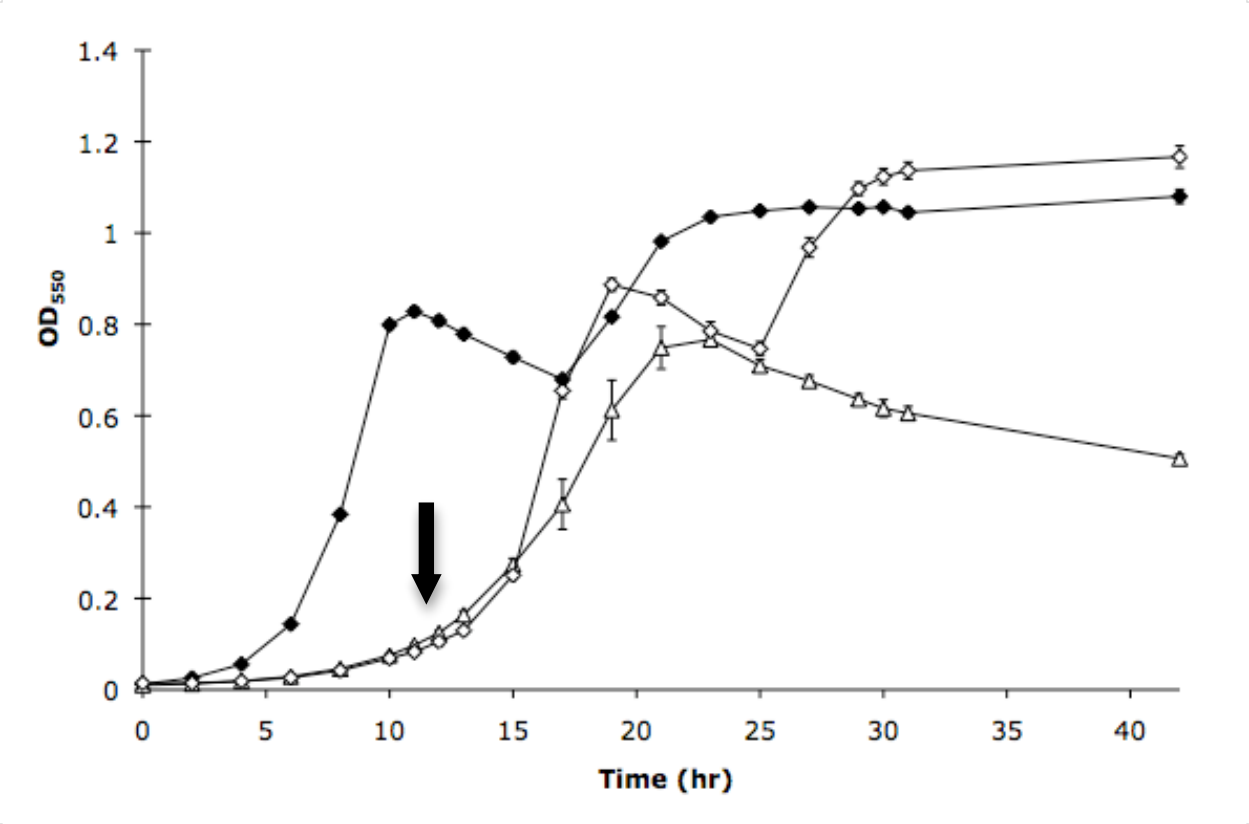
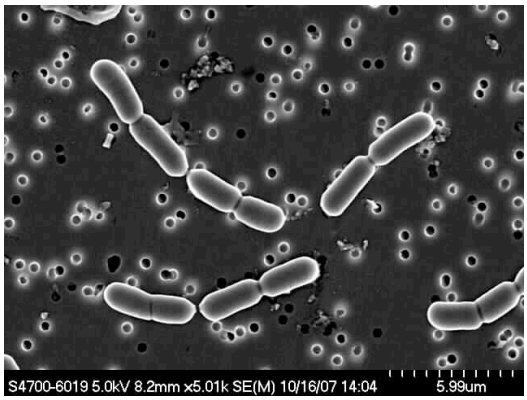
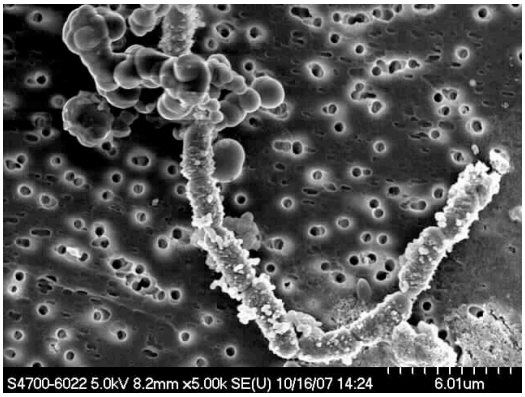
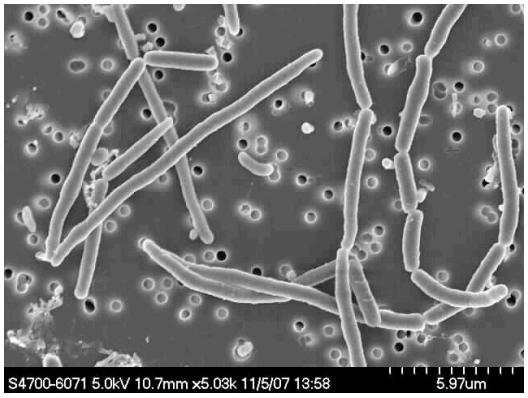


Figure 6.1 Inhibition of growth and endospore formation of *Bacillus megaterium* strain C-5 by catechin in MMO + glucose. Symbols are the average for triplicate cultures of each treatment; error bars = ±1 SE. ◆, MMO + glucose; △, MMO + glucose + catechin; ◇, MMO + glucose + catechin, with catechin removed after ◆ had reached stationary phase as indicated by the arrow.

A) 

B) 

C) 

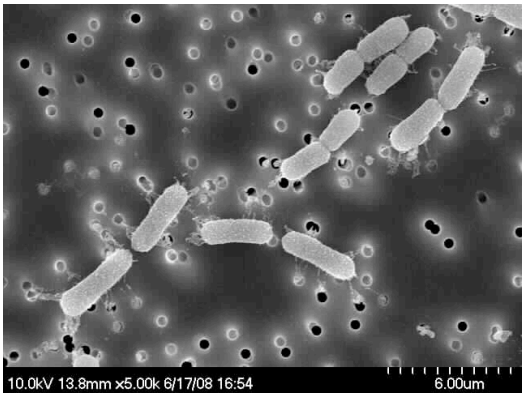
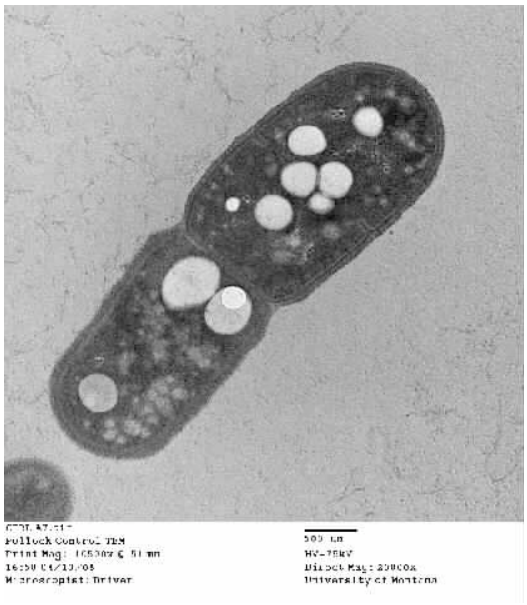
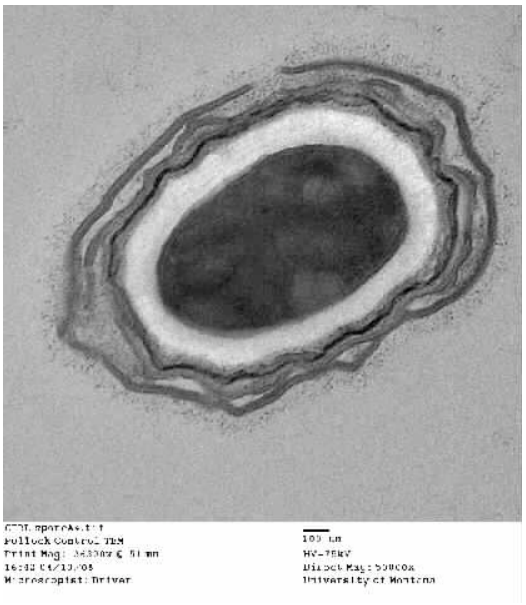
D) 

Figure 6.2 Scanning Electron Micrographs (SEMs) of *Bacillus megaterium* strain C-5. **Panel A**) Normal cells during mid-log phase of growth; **Panel B)** altered morphology in the presence of catechin during mid-log growth; **Panel C)** altered cells immediately after washing (note the lack of blebs compared to cells in Panel B); **Panel D)** washed cell morphology after resumption of growth in the absence of catechin.

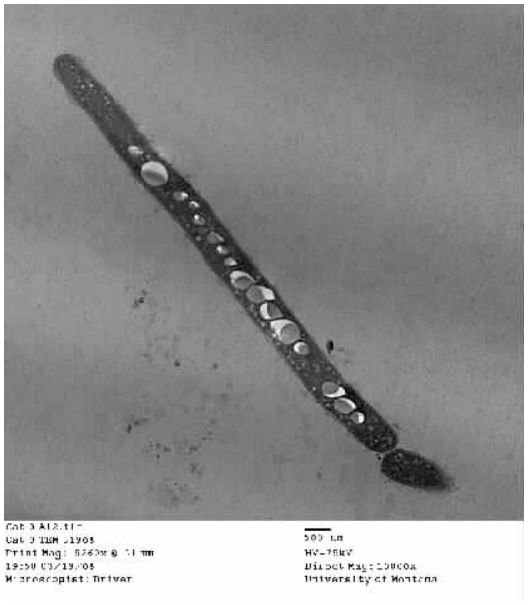
A)



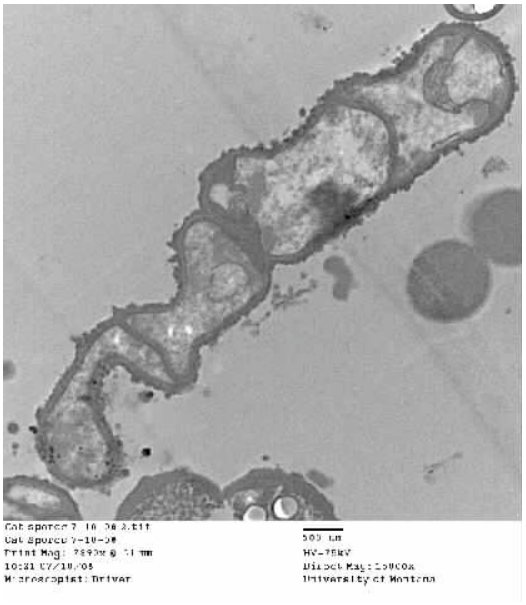
B)

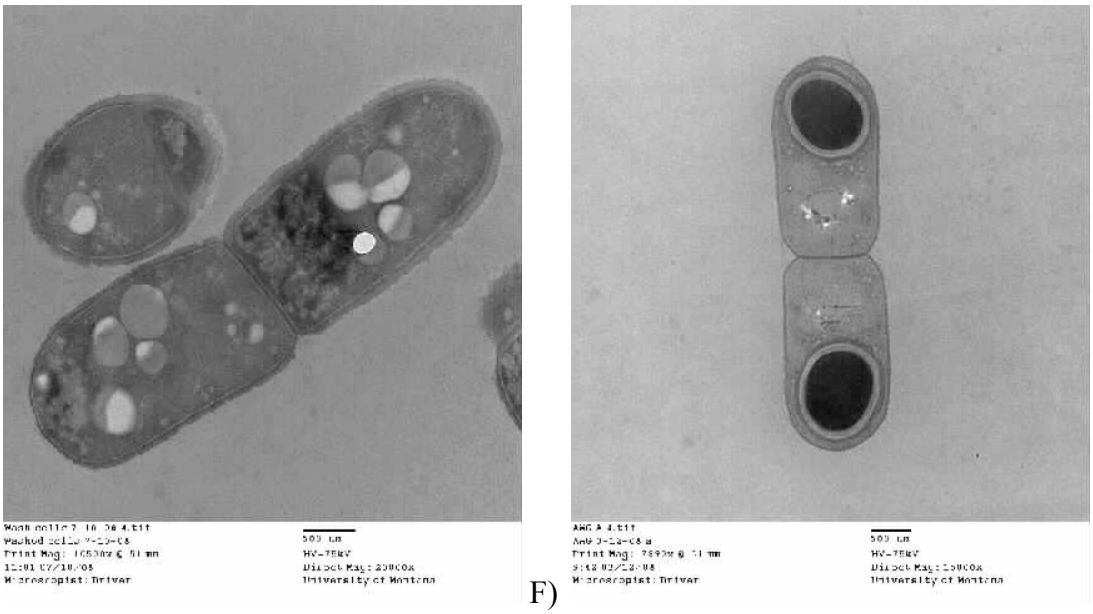


C)



D)





E) F)

Figure 6.3 TEMs of *Bacillus megaterium* Strain C-5 cells and spores. **Panel A)** Normal cells during mid-log phase of growth; **Panel B)** typical endospore formation; **Panel C)** altered cell morphology in the presence of catechin during mid-log growth; **Panel D)** absence of spores in the presence of catechin; **Panel E)** washed cell morphology after resumption of growth in medium without catechin; **Panel F)** normal spore formation after catechin removal from the medium. Note in panels A, C, and E, polyhydroxybutyrate (PHB) is formed internally (white inclusions confirmed with Sudan Black staining).

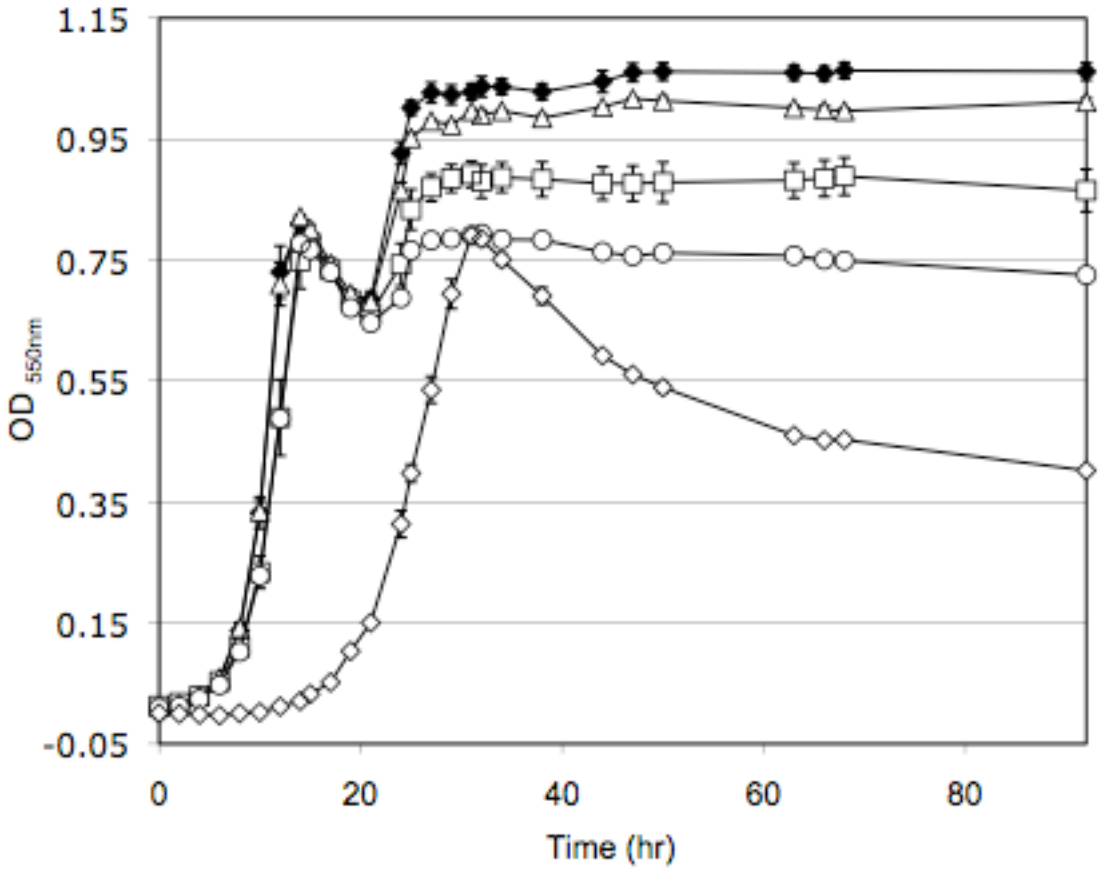
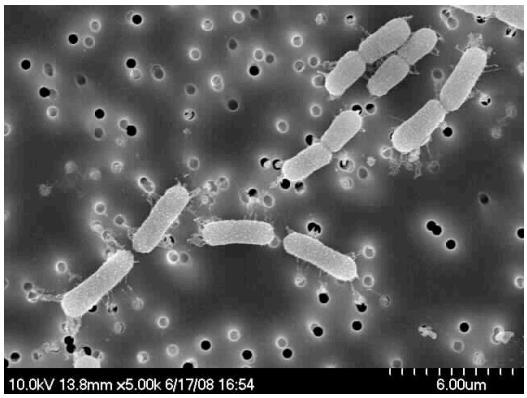
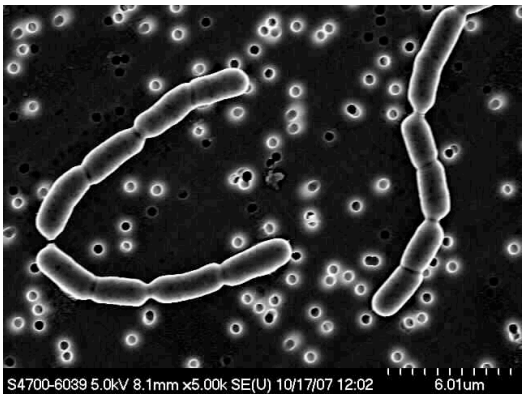
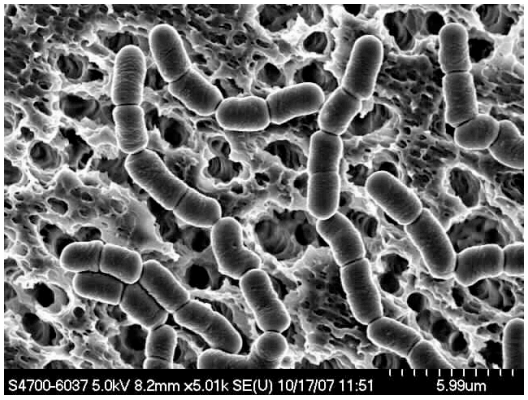
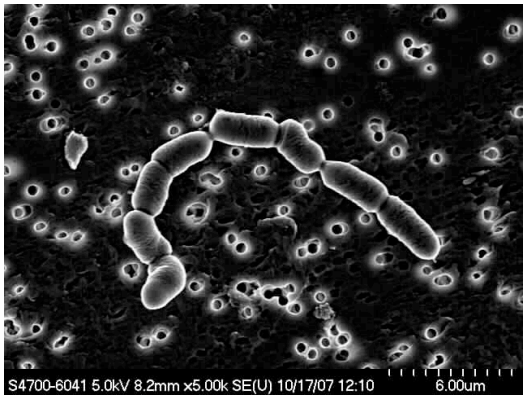


Figure 6.4 Growth curves of *Bacillus megaterium* strain C-5 grown on complete MMO + glucose, ◆; complete MMO + glucose + catechin, ◇; modified MMO omitting Mn^{2+} + glucose, △; modified MMO omitting Ca^{2+} + glucose, □; and modified MMO omitting Mn^{2+} & $Ca^{2+,}$ ○. Symbols are the average for triplicate cultures of each treatment. Error bars are ±1 standard error.

A) 

B) 

C) 

D) 

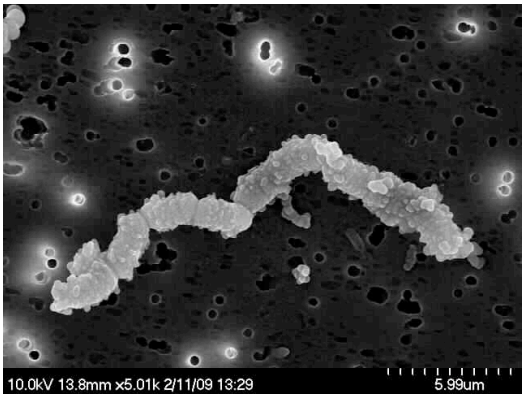
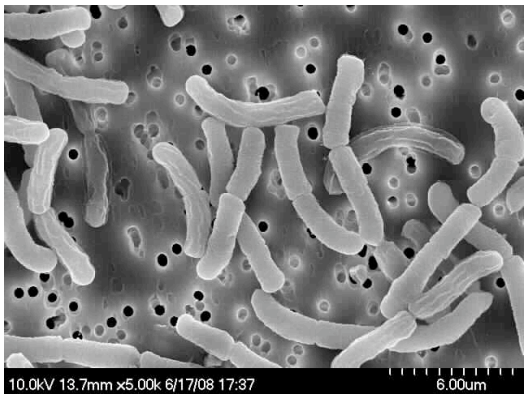
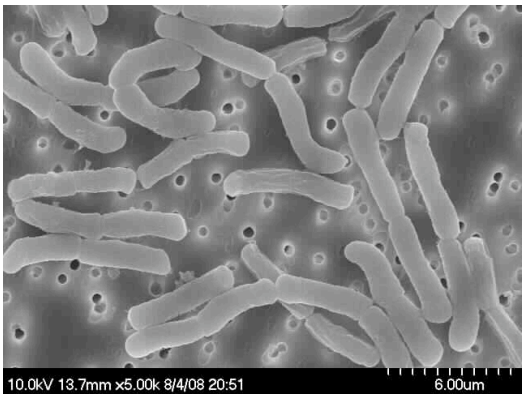
E) 

Figure 6.5 SEM micrographs of *Bacillus megaterium* strain C-5 treatments from Figure 4. **Panel A**, complete MMO + glucose (control); **Panel B**, modified MMO omitting Mn + glucose; **Panel C**, modified MMO omitting Ca + glucose; **Panel D**, modified MMO omitting Mn & Ca + glucose; and, **Panel E,** complete MMO + glucose + catechin. Note that in Panel A cells appear as individual rods, while in the absence of Ca and/or Mn (Figs 5b-d) cell morphology is filamentous similar to when catechin is present (Fig. 5e).

A) 

B) 

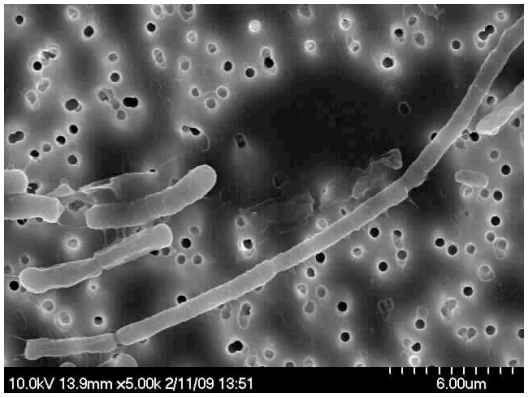
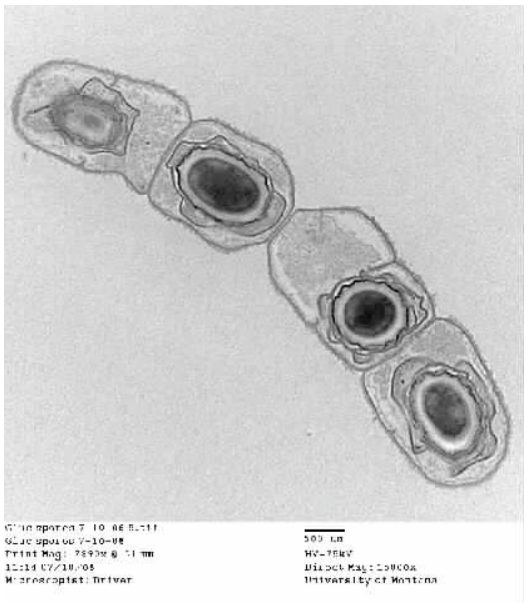
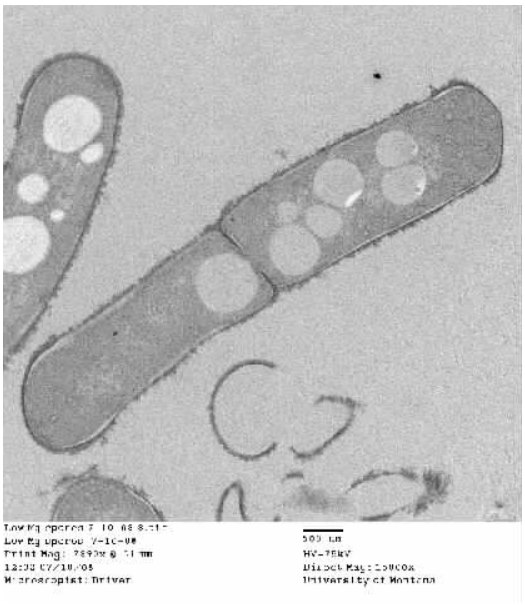
C) 

Figure 6.6 SEMs of *Bacillus megaterium* strain C-5 grown under decreasing magnesium concentrations. **Panel A)** Normal cells grown on MMO + glucose; **Panel B)** Cells grown at 1/10,000th concentration of normal Mg^{2+}; **Panel C)** Cells of strain C-5 at 1/1,000,000th concentration of normal Mg^{2+}. Note that in Panels B & C, that filamentous forms are apparent, but some cells are still bacilli.

A) 

B) 

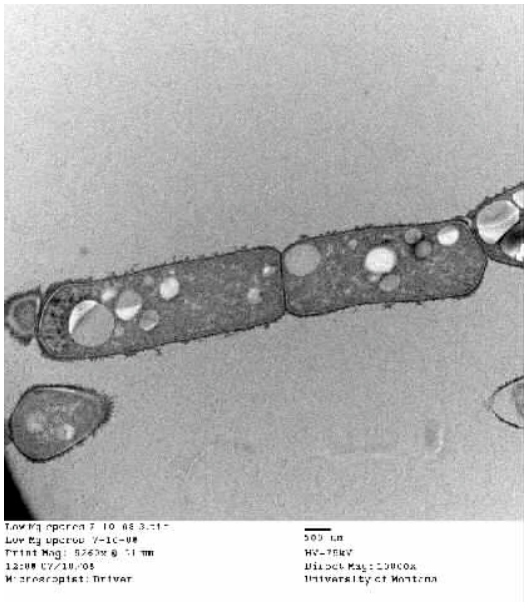
C) 

Figure 6.7 TEM analysis of *Bacillus megaterium* strain C-5 during spore stage exposed to decreasing amounts of Mg^{2+} corresponding to Figure 6. **Panel A)** Normal cells grown on MMO + glucose; **Panel B)** Cells grown at 1/10,000th concentration of normal Mg^{2+}; **Panel C)** Cells of strain C-5 at 1/1,000,000th concentration of normal Mg^{2+}. Note that the lack of endospores in Panels B & C.

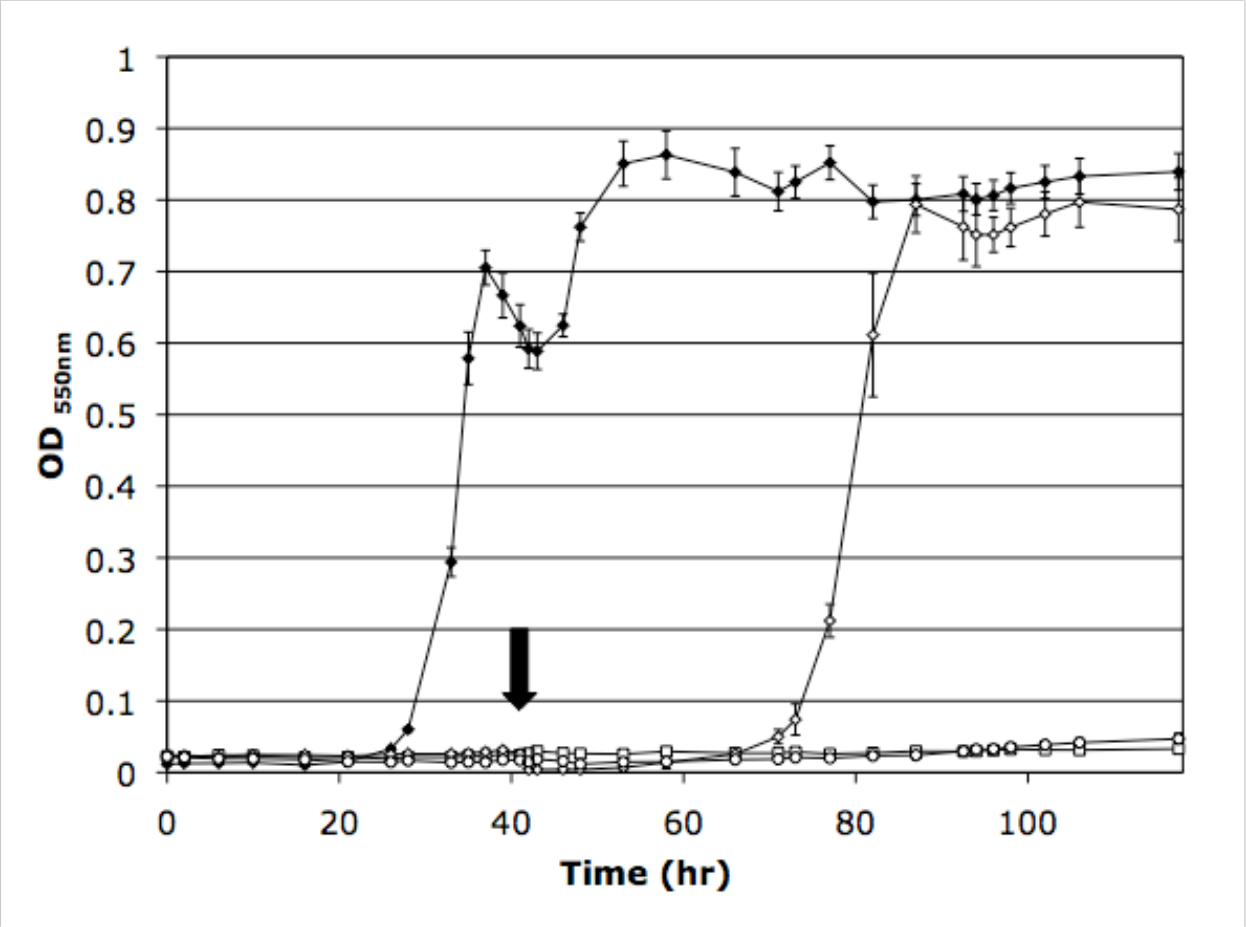


Figure 6.8 *Bacillus megaterium* strain C-5 endospore germination inhibition by catechin when germinated on MMO + glucose (◆); MMO + glucose + catechin, □; MMO + glucose + catechin with cells washed at t = 41 h, ◇; and MMO + glucose + catechin + alanine, ○.

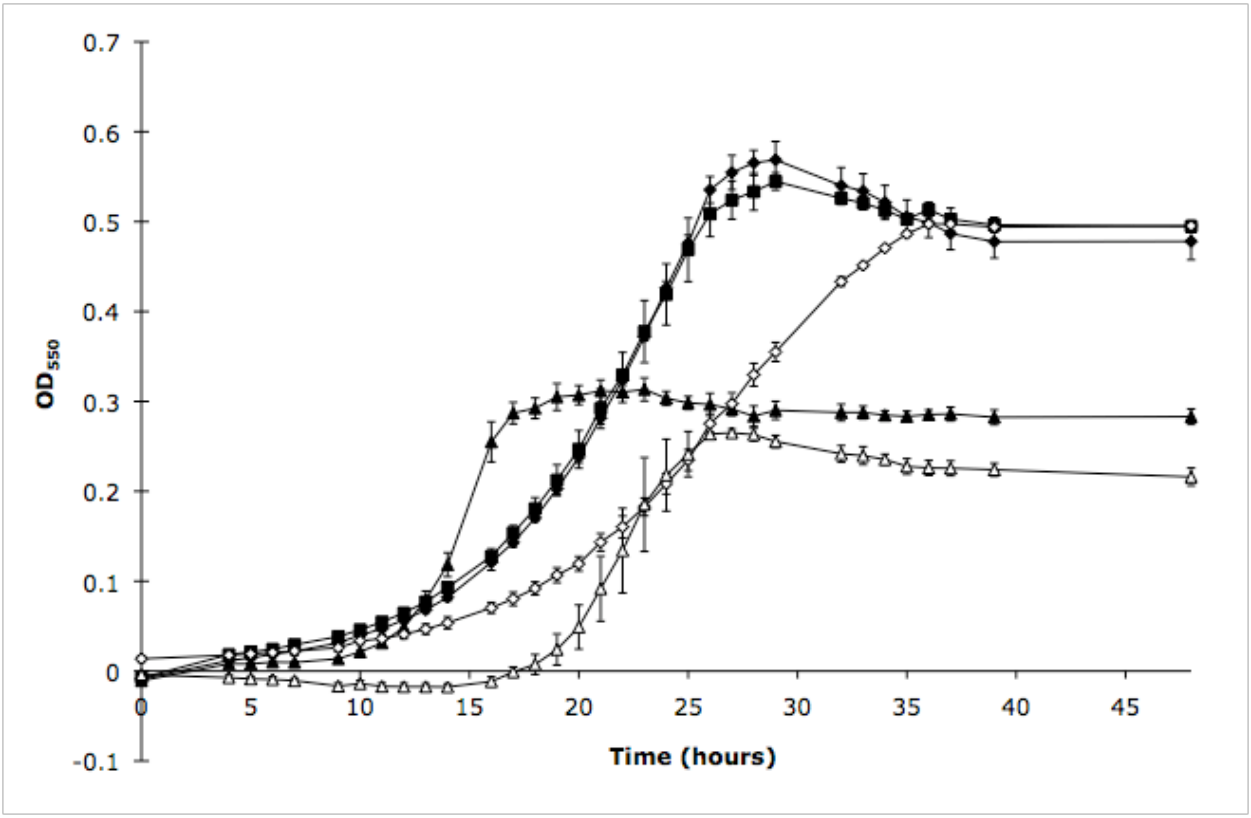
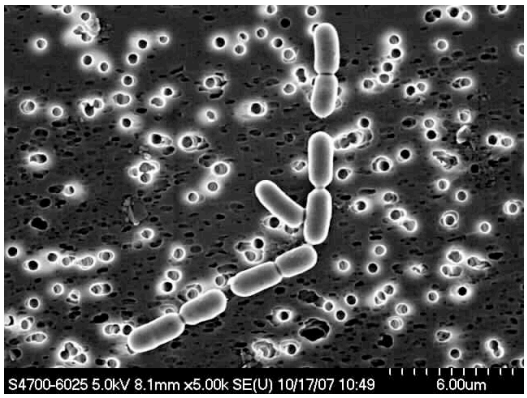
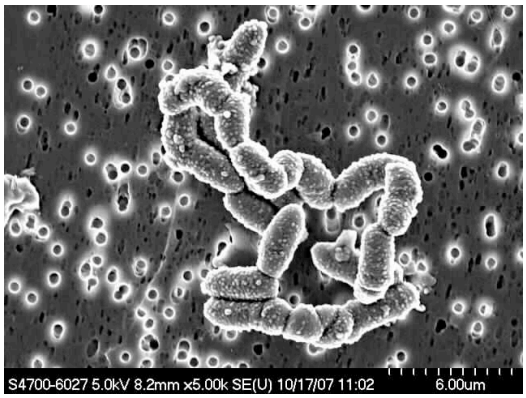
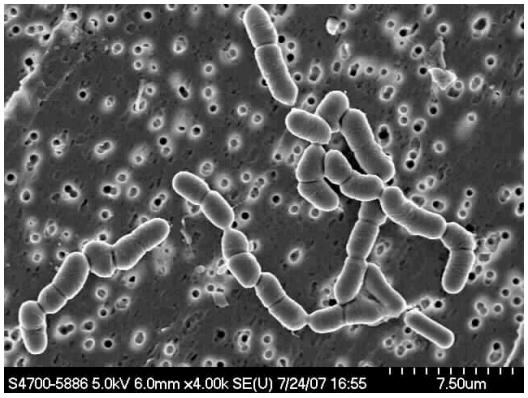
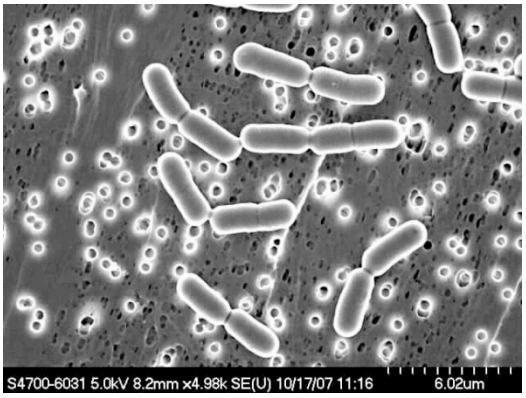


Figure 6.9 Effect of catechin on growth kinetics of *Bacillus megaterium* strain C-5 grown on pyruvate or malate. Symbols represent the average of triplicate cultures for each treatment; error bars are ±1SE. ◆=MMO + Pyruvate; ■=MMO - Mn/Ca + Pyruvate; ▲=MMO + Malate; ◇=MMO + Pyruvate + Catechin; △=MMO + Malate + Catechin.

A) 

B) 

C) 

D) 

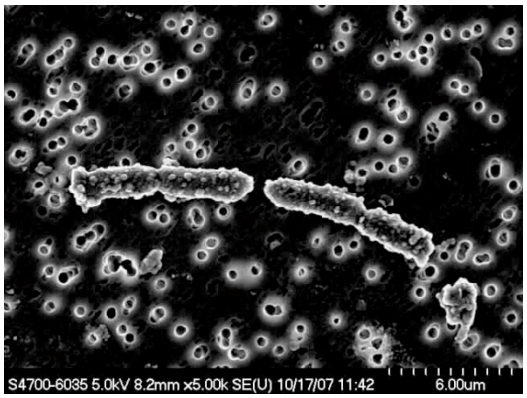
E) 

Figure 6.10 SEM analysis of *Bacillus megaterium* strain C-5 grown on pyruvate or malate. **Panel A,** mid-log phase on MMO + pyruvate; **Panel B**, mid-log phase on MMO + pyruvate + catechin; **Panel C**, mid-log phase on modified MMO lacking Mn & Ca + pyruvate; **Panel D**, mid-log phase on MMO + malate; and, **Panel E**, mid-log phase on MMO + malate + catechin.

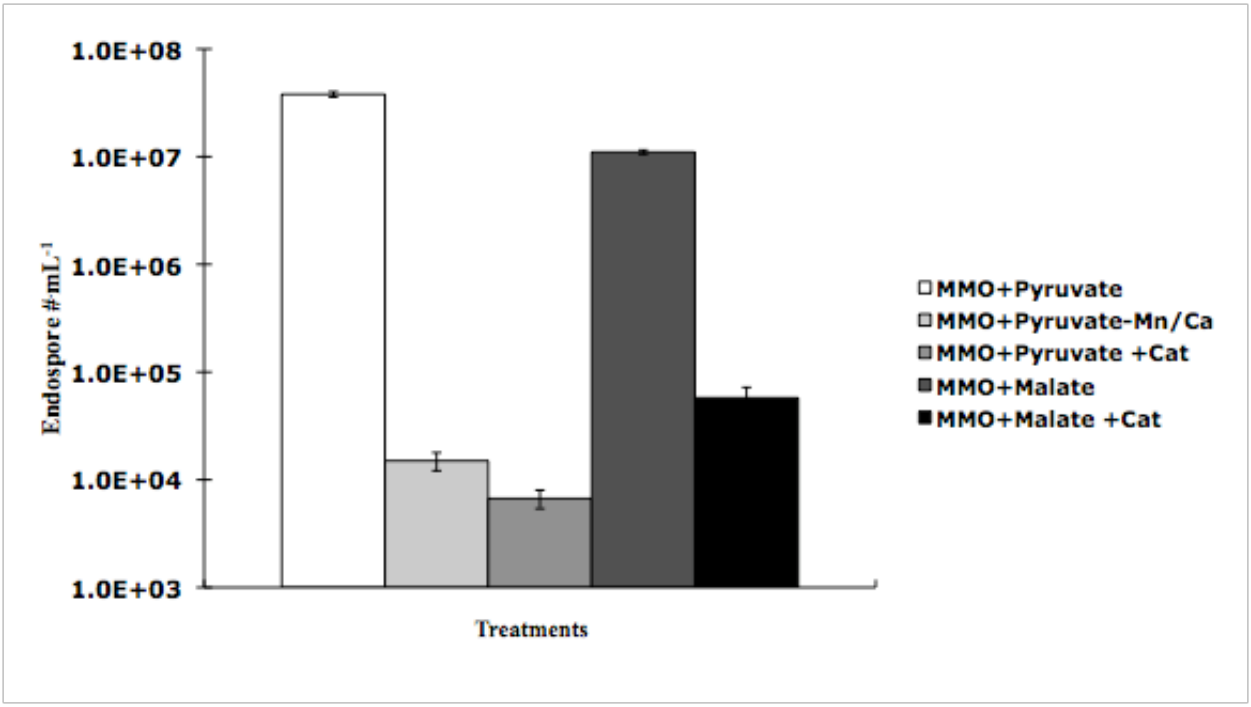


Figure 6.11 Inhibition of heat-resistant endospore-formation in *Bacillus megaterium* strain C-5 by catechin. Bars are the average of triplicate cultures for each treatment; error bars represent ±1SE. Endospore enumeration was performed 72 hours after stationary phase was achieved.

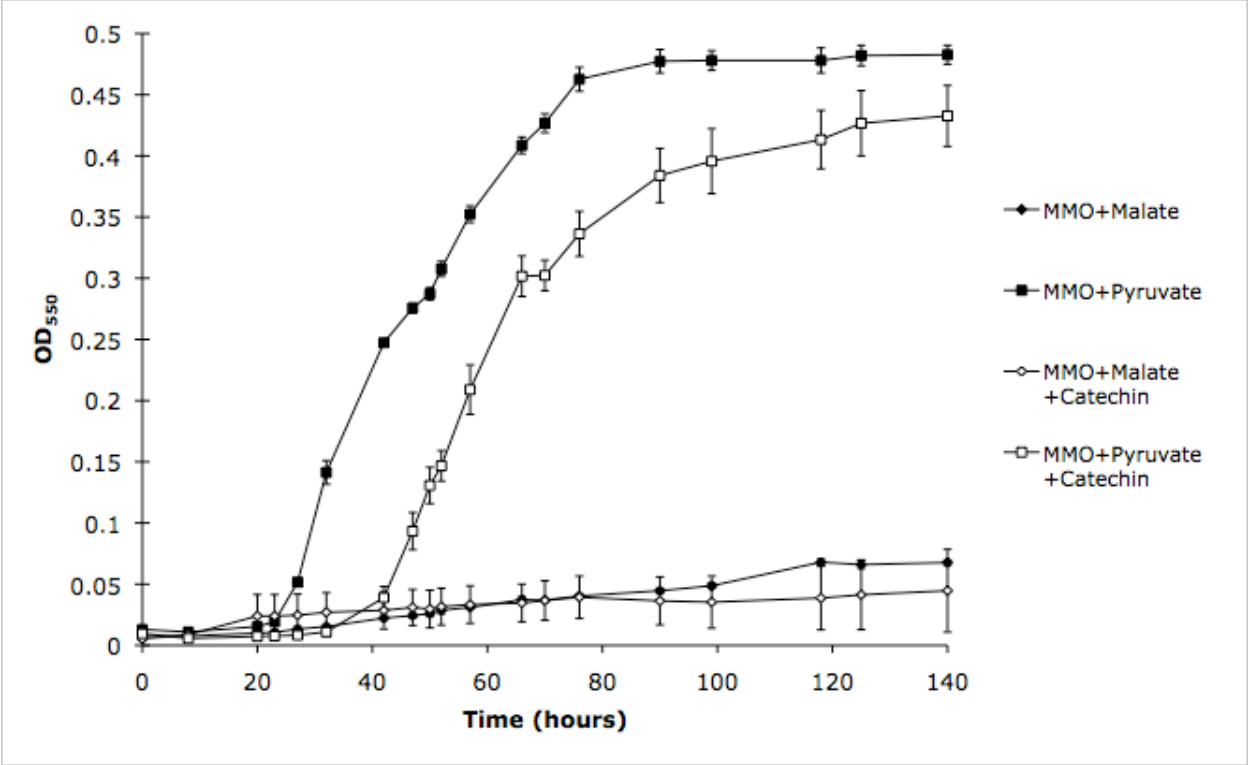


Figure 6.12 Pyruvate, but not malate, allowed proper endospore germination for *Bacillus megaterium* strain C-5 when grown in the presence of catechin. Symbols represent the average of triplicate cultures for each treatment; error bars are ±1SE.

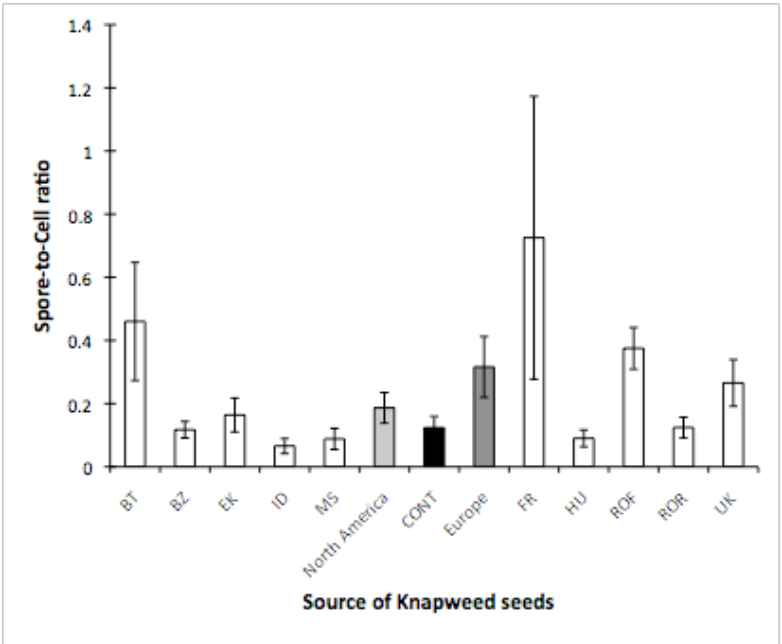


Figure 6.13 Impact of knapweed population origin on soil endospore-forming bacteria. Bars on the left are from North America, bars on the right are from Europe.

Chapter 7

SYNTHESIS: THE EFFECTS OF (±)-CATECHIN ON SOIL MICROBIAL ECOLOGY AND PLANT COMMUNITY STRUCTURE: A CONCEPTUAL MODEL

The body of work presented in this dissertation addresses how (±)-catechin, a root exudate of *Centaurea stoebe* (spotted knapweed), affects both or is affected by biotic and chemical components of invaded ecosystems. Specifically, the integration of observational studies and manipulative experiments described herein has established a causal link between the phytotoxic and antimicrobial effects of catechin and its ability to interact with some common soil chemical parameters. Conceptual models of these interactions as they pertain to effects on plant, microbial, or chemical processes in soil for catechin as a "pure" compound as well as in "derivative" forms, are presented in Figures 1 and 2. Also, as catechin exhibits differential (i.e. conditional) effects via these environmentally relevant forms, models of processes, both biotic and abiotic, that impact the stability or production of pure and derivative forms of catechin in the environment are discussed as well (Figures 3 & 4). Following is a discussion of these conceptual models and suggestions for future research to further substantiate or refute the role of catechin as an allelochemical. I will summarize and discuss my thesis research in the context of what was known, and then sum up the effects of and controls on catechin and its derivative forms in a series of schematic models at the end of the chapter.

When spotted knapweed invades a naïve field site, displacement of native plant species is a commonly observed effect, with knapweed in many cases establishing near

monocultures in the system (Callaway*, et al.*, 1999). Considered a noxious weed, spotted knapweed has been an exotic invader for over 100 years and is responsible for displacing crops in much of North America (Bucher, 1984). Principal to the success of spotted knapweed invasion is its ability to exude a 50:50 racemic mixture of (±)-catechin into its surrounding rhizosphere (Blair*, et al.*, 2006, Perry*, et al.*, 2007). This polyphenolic compound can have a multitude of negative effects on the local flora and microbiota.

Both derivative and pure catechin exhibit phytotoxic activity against many native plant species as exemplified by poor plant growth (biomass) above- and below-ground, resulting in a decrease in root surface area as well as a heightened sensitivity to drought (Chapter 3; Weir*, et al.*, 2006, Duke*, et al.*, 2009, Pollock*, et al.*, 2009). In the current work, enhanced phytotoxic effects were observed when catechin was bound to lead and to a lesser extent copper, presumably as it is placing the normally toxic, but usually insoluble, metals into a more biologically active state (Chapter 3).

A previously proposed mechanism for catechin phytotoxicity is the ability of catechin to produce reactive oxygen species (ROS), chemical compounds toxic to many organisms, as a result of natural auto-oxidation processes (Weir*, et al.*, 2004). In a previous study, *Lupinus sericeus* and *Gaillardia grandiflora*, two native plant species growing in fields alongside spotted knapweed, were shown to increase their amount of root exudation of oxalate upon exposure to catechin (Weir*, et al.*, 2006). The study further demonstrated that other neighboring native plant species could be protected from the negative effects of catechin by oxalate addition, purportedly representing a way to mitigate root damage by

ROS. Interestingly, plants commonly exude oxalate and other organic acids during both metal- and phosphate-limiting conditions (Gardner*, et al.*, 1983, Welch*, et al.*, 2002, Weir*, et al.*, 2006). I suggest that *C. stoebe*, through its ability to exude catechin which has been shown to chelate metals (Gomah & Davies, 1974, McDonald*, et al.*, 1996, Lim*, et al.*, 2005, Pollock*, et al.*, 2009), is locally creating a metal-limited system, and that subsequently catechin-resistant native plants (e.g. *Lupinus* and *Gaillardia*) respond to this limitation by increasing exudation of their own competing chelators. As such, another mechanism for catechin phytotoxicity could be its ability to sequester metals from sensitive species (e.g. with no chelators or siderophores), both plant and microbial, limiting their access to essential trace metals necessary for normal growth. This scenario would place the afflicted plant species at a competitive disadvantage to spotted knapweed and other native plant species that are resistant or tolerant to catechin (DiTomaso, 2000, Weir*, et al.*, 2006). In return, spotted knapweed flourishes, gaining in biomass and root surface area. The increased success of knapweed in invaded areas could thus be argued to result from lower competition for resources from damaged, neighboring plant species.

Alternatively or additionally, *C. stoebe* may realize a benefit of catechin mobilizing sparingly soluble phosphate as suggested by Thorpe and co-workers (Thorpe*, et al.*, 2006) and placing trace metals necessary for growth into more bioavailable forms accessible to spotted knapweed. As many plants increase the production of root exudates to increase both metal and phosphate solubility, it is plausible that catechin exudation from knapweed would increase in response to mineral or phosphate deficient conditions. Previously, Thorpe et al. (Thorpe*, et al.*, 2006) reported the ability of spotted knapweed

to increase phosphorous levels in the field, and catechin has been suggested to help put insoluble metal-phosphates into solution (DeLuca, T., *personal communication*). However, however neither of these investigators were testing for catechin production from spotted knapweed under these conditions, so enhanced phosphorous solubilization by catechin remains a suggestion that warrants further investigation.

Indeed, our own findings regarding the impact of catechin on bacterial processes have shown striking similarities to other metal-deficient systems (Chapter 6). The inhibition of bacterial sporulation, endospore germination, and altered cell morphology exhibited by endospore-forming bacteria in the presence of catechin bear a striking resemblance to the appearance of the same bacteria tested under metal-deficient conditions (refer to Chapter 6). Also, each of the six *Pseudomonas* isolates shown to be unaffected by catechin (see Chapter 5) were capable of siderophore production (data not shown), a common ability in this genus (Achouak*, et al.*, 2000, Delorme*, et al.*, 2002, Gardan*, et al.*, 2002, Munsch*, et al.*, 2002, Huston*, et al.*, 2004). This may explain their ability to grow in the presence of catechin despite its metal-chelating properties.

Similar studies regarding 8-hydroxyquinoline, an allelochemical exuded by diffuse knapweed (a close relative to spotted knapweed), have shown an ability of the this root exudate to chelate insoluble iron for uptake into the plant (Tharayil*, et al.*, 2009), and also to inhibit bacterial populations, an effect which was overcome by the addition of surplus iron to the medium (Geels*, et al.*, 1985). This last study also demonstrated the ability of this allelochemical to select for plant growth-promoting *Pseudomonas* species, a

discovery that is congruent with our findings for catechin and these apparently unaffected *Pseudomonas* populations (Chapter 5), although their ability to promote plant growth was not tested.

In a related vein, the mechanism that I propose to explain why malate and pyruvate allowed growth of catechin-inhibited bacterial populations centers on the ability of these two organic acids to chelate metals (Gardner*, et al.*, 1983, Drechsel*, et al.*, 1993, Keerthisinghe*, et al.*, 1998). Since growth rates, cell morphologies, and endospore processes were not fully restored by the addition of either of these organic acids in the presence of catechin (Chapters 5 & 6), I suggest that they provide sufficient levels of trace metals required for growth in the presence of catechin, but do not completely overcome its effects. Another possible explanation for this observation is that there is some unidentified metal essential for normal cell function that neither organic acid can sufficiently chelate away from catechin.

Bacterial resistance or tolerance to the effects of catechin might also be attributed to catechin modification, as exhibited by the *Rhodococcus* isolate G-2 in Chapter 5 of this work. Transformation-based resistance to the detrimental effects of catechin could confer a competitive advantage to bacterial populations over those vulnerable to catechin. In a separate study, researchers demonstrated that animals fed a diet containing tea catechins (which are structurally and reactively related to the *C. stoebe* catechin employed in my studies) increased the densities of one group of bacteria, the lactic acid bacteria, while

other populations decreased in abundance (Ishigami & Hara, 1993, Hara, 1997). This is particularly salient, as lactic acid bacteria have no metabolic requirement for iron (Archibald, 1983, Boyaval, 1989, Bruyneel*, et al.*, 1989, Pandey*, et al.*, 1994), which reinforces the notion that catechin resistant bacteria (whether through competing sequestration of metals, or indifference to iron availability) are capable of outcompeting sensitive populations in a given system in the presence of catechin.

The highly reactive nature of catechin to a variety of chemical parameters in soil (Chapters 2, 3, & 5; Pollock*, et al.*, 2009) in conjunction with variable extraction efficiencies, limitations of quantification has led to much controversy over the amount of bioavailable catechin in soil and its potential importance as an allelopathic chemical of *C. stoebe* (Blair*, et al.*, 2005, Blair*, et al.*, 2006, Perry*, et al.*, 2007). Catechin stability in soil can be both positively and negatively affected by abiotic and biotic interactions in the environment. I have demonstrated that catechin can be immediately modified by the addition of various redox active metals (e.g., Fe^{3+}, Fe^{2+}, Cu^{2+}) to a form undetectable by current HPLC methods (Chapters 2 & 3). Also, the ability of pyruvate to increase the rate of what is believed to be catechin auto-oxidation (Chapter 5) suggests that compounds or elements capable of "scrubbing" hydrogen peroxide from the system (e.g., ascorbic acid; Mehlhorn*, et al.*, 1996) may impact catechin stability, and also its toxicity, in media and soil. The stability of catechin at various pH values also plays a role in catechin concentrations and quantification as exemplified by my findings in Chapter 2, as well as by the work of Blair et al., who have shown catechin extraction from a simple sand matrix to be dependent on initial pH (Blair*, et al.*, 2005). Lastly, I have shown that,

immediately following addition to soil, catechin is extremely difficult to extract efficiently (Chapter 2), which is consistent with earlier suggestions that catechin is readily sorbed when added to soils (Furubayashi*, et al.*, 2007).

Of potentially greater significance is the discovery that calcium and low pH have a positive effect on catechin stability (Chapters 2 & 3; and Blair*, et al.*, 2005, Pollock*, et al.*, 2009). Consequently, soils with chemistries that mimic these findings might be expected to yield higher catechin extraction numbers. As such, environmental systems with low pH or unusually high calcium content might be invaded by spotted knapweed to a higher degree as its allelochemical would endure for longer periods of time. Others have found that dry soils result in better extraction efficiencies (Blair*, et al.*, 2005), however they fail to mention the method of catechin addition to soils, which makes reproducing their findings problematic. Still, in a natural system, dry soils could conceivably keep catechin concentrated at the root surface and may be another mechanism for explaining what appears to be increased root damage and plant mortality under drought conditions as observed in Chapter 3 (Pollock*, et al.*, 2009). These findings collectively suggest that catechin could be found at high soils levels, given low pH or water content, or high calcium concentrations of the system.

Resulting and Remaining Questions from this Research

Many of the findings reported herein suggest a connection between the ability of catechin to chelate metals and the effects of catechin on plant and bacterial growth and other cellular processes. A concerted effort do demonstrate and quantify CMCs and other

derivative forms of pure catechin in native range systems would greatly benefit research regarding this allelochemical by increasing our understanding of various catechin forms while providing better quantification of pure, transformed and sorbed catechin concentrations in soil ecosystems. Clearly, a better and more general method of extracting and quantifying not just pure catechin, but its many potential derivative forms, is warranted and necessary to understanding specific modes of activity as well as which "species" of catechin are actually biologically active.

In addition, studies with known metal-resistant plant species (e.g. aluminum resistant plants) grown in the presence of insoluble aluminum phosphates and either knapweed or catechin may shed some light on another potential mechanism of catechin phytotoxicity. For example, a previous study using varieties of maize demonstrated an increase in root exudation to alleviate aluminum toxicity (Kidd*, et al.*, 2001). Interestingly, one of the varietals, *Zea mays* vars Sikuani, demonstrated the ability to not only exude catechin from root tips, but substantially increased (>200 fold) the amount of exudation if pretreated with silicon. The high degree of phenolic versus organic acid exudation was posited to be a possible mechanism for aluminum resistance for this plant. Potentially, spotted knapweed might also increase catechin exudation under toxic metal stress and this phenomenon should be examined as another mechanism for explaining variability and conditionality in catechin exudation and toxicity.

Lastly, other research on both tea catechins (closely related to *C. stoebe* catechin) and condensed tannins (essentially polycatechins) has shown a wide variety of detrimental

effects on microbial species. Many of these effects closely mimic my own findings, from inhibition of endospore-forming organisms (Sakanaka*, et al.*, 2000, Hara-Kudo*, et al.*, 2005, Friedman*, et al.*, 2006) to altered cell morphology (Odenyo & Osuji, 1998). Also, many of these studies and reviews suggest toxicity via many of the same mechanisms I demonstrate or propose in my own work. As such, more research focused on the ubiquity of catechin production by plants, its role in controlling plant and microbial populations through direct and indirect effects, and its interactions to develop transformed or derived products that may have greater or less toxicity than catechin itself is warranted.

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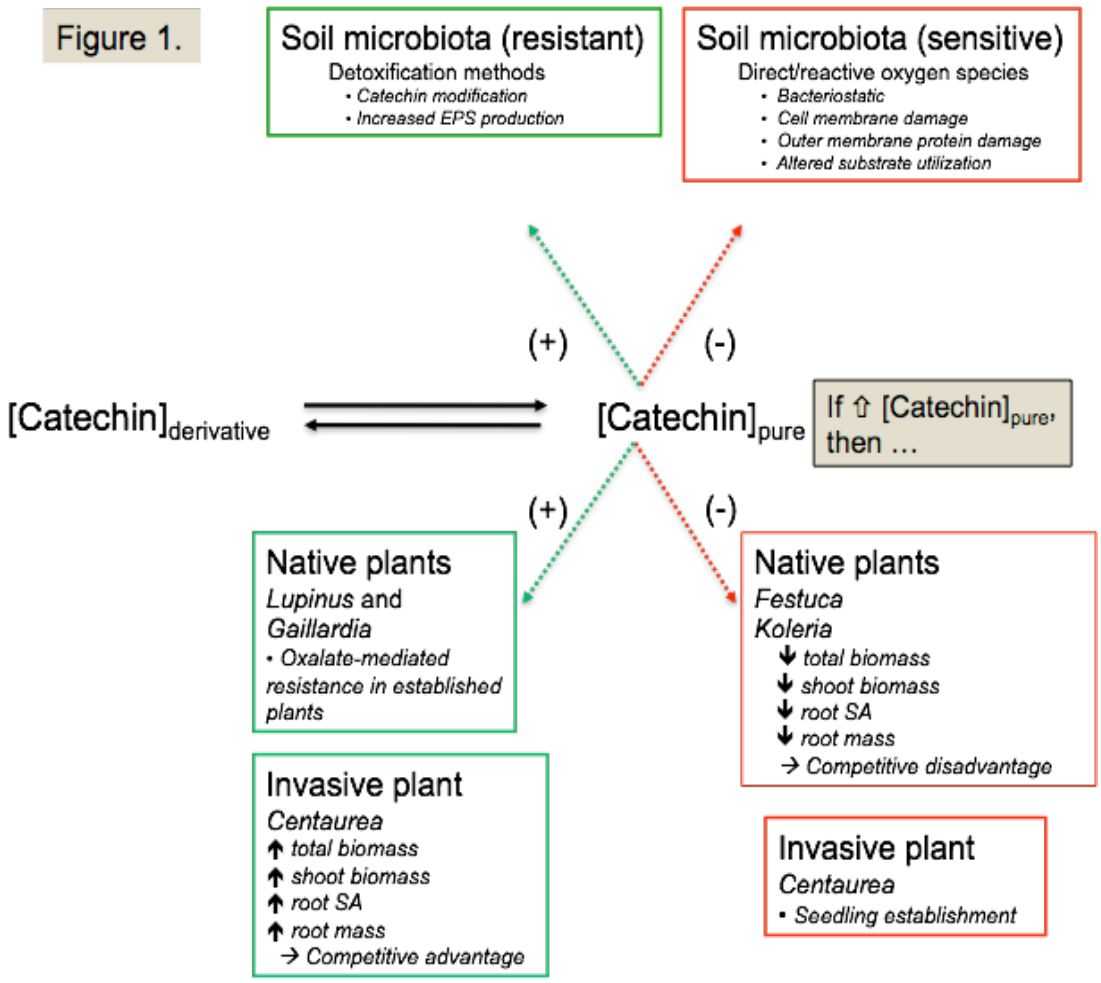

Figure 7.1

Summary of the ecological effects of pure catechin based on this work and what is known from the literature.

Figure 2.

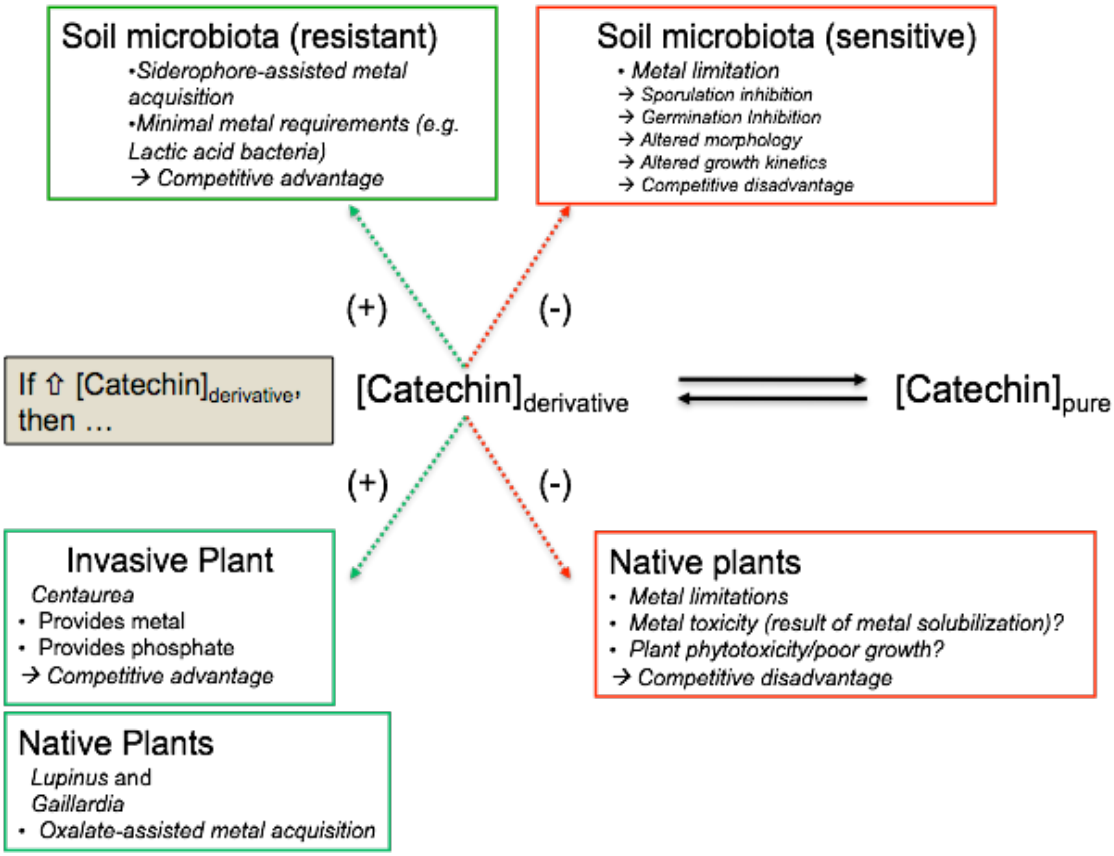

Figure 7.2

Summary of the ecological effects of spontaneous derivatives of catechin based on this work and what is known from the literature.

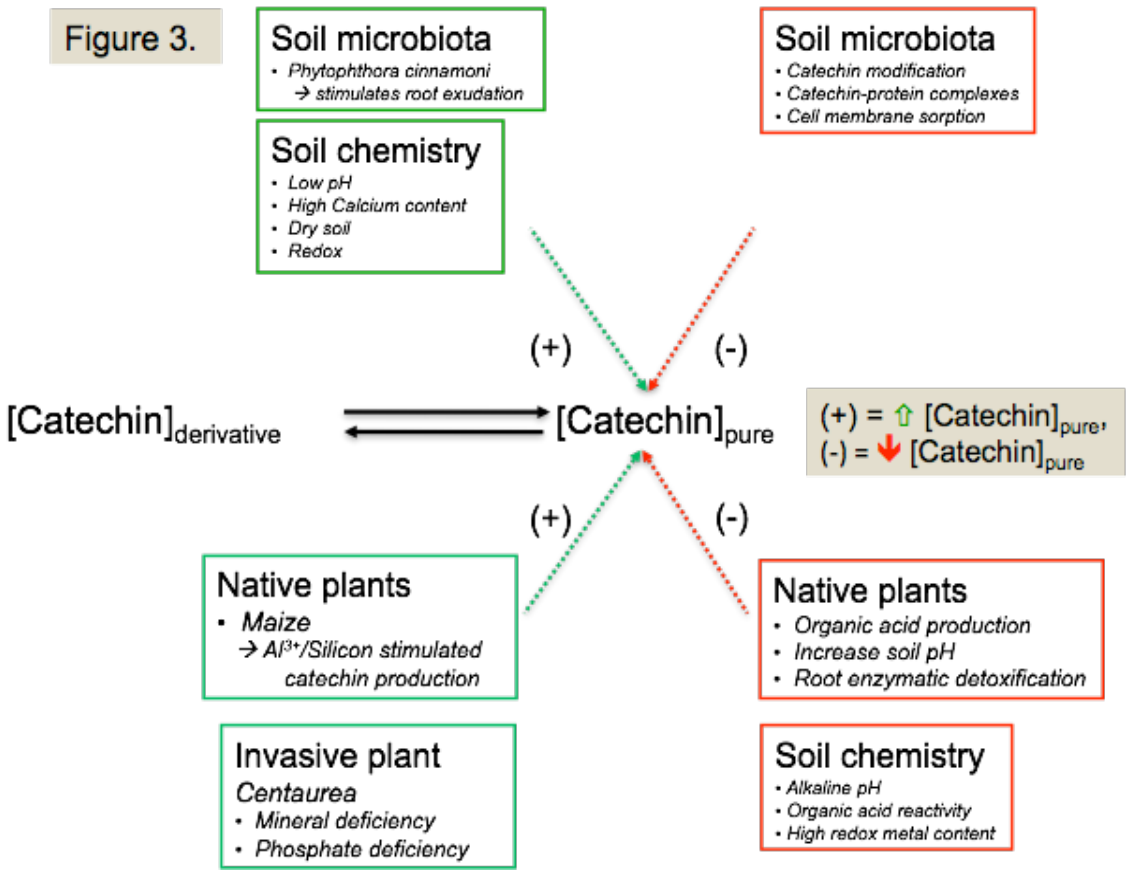

Figure 7.3

Summary of ecological reasons for pure catechin soil concentrations based on this work and what is known from the literature.

Figure 4.

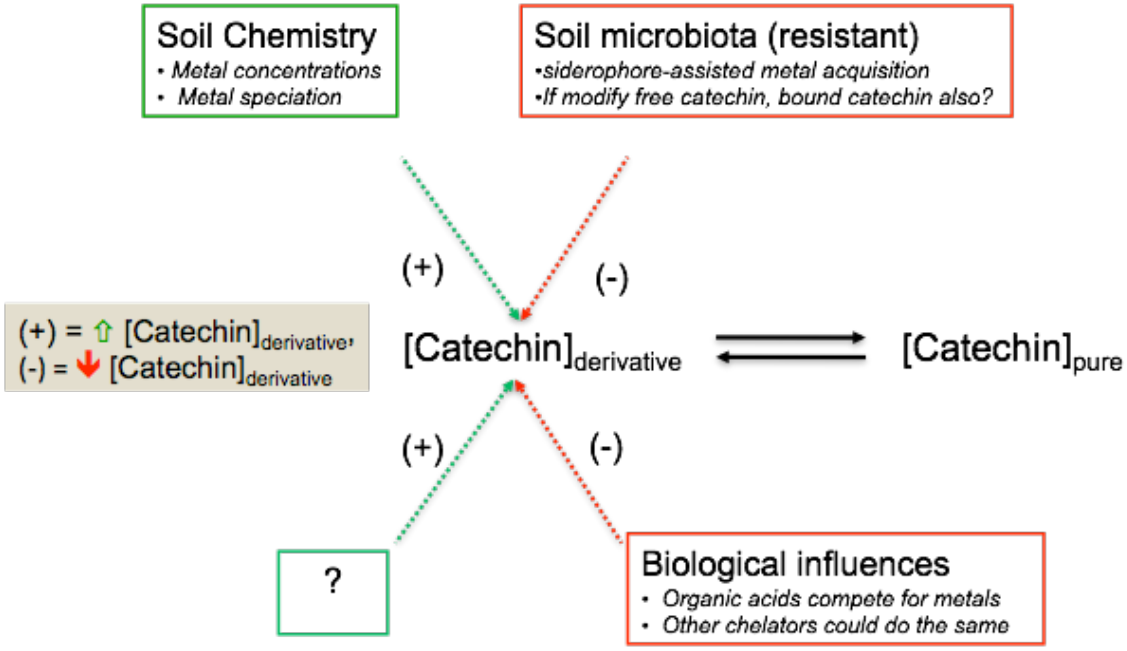

Figure 7.4

Summary of ecological reasons for catechin-derivative soil concentrations based on this work and what is known from the literature. "**?**" denotes that there have yet to be processes known to positively affect the formation of catechin derivatives by biological means.