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THE ROLE OF MICROBIAL ENDOSYMBIONTS IN *SORGHUM HALEPENSE*
INVASIONS: EVIDENCE OF A NEW INVASION STRATEGY, MICROBIALLY
ENHANCED COMPETITIVE ABILITY (MECA)

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Dissertation

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for the degree of

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The Role of Microbial Endosymbionts in *Sorghum halepense* Invasions: Evidence of a New Invasion Strategy, Microbially Enhanced Competitive Ability (MECA)

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Invasive plants can profoundly alter ecosystem processes, and tremendous economic costs are often associated with these disturbances. Attributes like higher growth rates, increased biomass, and enhanced chemical defenses have been documented in many invasive plants. When expanding into new ranges, these traits frequently allow invasive plants to outcompete native plant communities. Current theories suggest these invasive attributes are plant-regulated; however, my work shows that bacterial endosymbionts can regulate these traits in the invasive grass *Sorghum halepense*. Using culture and molecular approaches, I found the invasive grass harbors several bacterial organisms inside the roots and rhizomes. These bacterial endosymbionts were isolated from within plant tissues and identified using 16S-rRNA gene sequencing. Numerous physiological functions of these plant-associated bacterial isolates were confirmed using *in vitro* studies, including the capacity for N₂-fixation, iron siderophore production (iron chelation), phosphate solubilization, and production of the plant-growth hormone indole-3-acetic acid (IAA). In long-term field studies conducted within the Fort Worth Nature Center & Refuge spanning 46-months, alterations to several soil biogeochemical cycles across an *S. halepense* invasion gradient were documented. Heavily invaded soils had increased plant-available forms of essential macronutrients (nitrogen, phosphorus, potassium, and magnesium) and trace metals (copper, iron, manganese, and zinc) compared to moderately and non-invaded soils. Using a novel antibiotic approach, I restricted growth of the bacterial endosymbionts within the plant and found they significantly increased plant biomass, and altered resource allocation enhancing rhizomatous growth. Plants with endosymbionts significantly inhibited the growth of a native prairie grass, *Schizachyrium scoparium*, which is frequently displaced by the invader in tallgrass prairie ecosystems. Restricting bacterial growth completely removed these competitive effects. Plants with bacterial endosymbionts also had increased production of the herbivore-defense compound, dhurrin, contained in leaves. When leaves from plants with bacterial endosymbionts were fed to a generalist insect herbivore (*Tricoplusia ni*), the insect could not grow and experienced significant mortality. Restricting bacterial growth resulted in a 6-fold decrease in dhurrin, in conjunction with significant increases in insect growth and survival. These results suggest microbial endosymbionts significantly contribute to *S. halepense* invasions by enhancing the plant traits of biomass, growth rate, competitive effects, and herbivore-defense. This work shows that these invasive plant traits are microbially-mediated. This novel invasion strategy is referred to as Microbially Enhanced Competitive Ability (MECA), in which microbial associations significantly contribute to a range of plant traits that directly correspond to invasion success.

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CHAPTER 1

Soil biota and plant invasions: Biogeographical effects on plant-microbe interactions*

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Thou shalt suffer in alternate years the new reaped fields to rest ... thus by rotation like repose is gained nor earth meanwhile unearned and thankless left.

Virgil (29 BC)

INTRODUCTION

Ancient agriculturists realized that crops, if left in the same place for long enough, would begin to perform poorly (White 1970; Howard 1996; Altieri 2004). They also appeared to recognize that by removing crops from an area for a season or more, rotating different crops among years in the same place, or by planting other species with their crops, they could trade high short-term productivity for long-term sustainability of crop production at rates of lower productivity. There are several reasons why agricultural monocultures lose productivity over time, but among the most important causes are host-specific soil-borne diseases, which proliferate and have disproportionately strong effects when the densities of their hosts are unusually high. In the past century, modern agronomists have clearly shown that the accumulation of host-specific pathogenic soil organisms is a major cause of damage, productivity loss, and mortality in crop monocultures (Hoestra 1975; Magarey 1999). Charles Elton (1958) did not address soil biota as

important natural enemies in his landmark book but since then many studies have clearly shown that pathogenic soil organisms, or the lack of them, play a major role in the development of other kinds of monocultures: those caused by exotic plant invasions. Furthermore, the study of soil biota in the context of exotic plant invasions has led to an explosion in our understanding of the role of these organisms in community ecology in general (see Fig. 11.1). The primary aim of this chapter is to explore how our understanding of the role of soil biota in exotic plant invasions has increased since the time of Elton. First, we examine studies looking at the effects of soil pathogens in the context of exotic plant invasions which paved the way for the groundbreaking work on plant–soil feedbacks. We then discuss how biogeographical approaches have been used to explore shifts between invaded and native ranges in these feedbacks. Lastly, we turn to current research on various microbial mechanisms underlying successful invasions in light of the different effects invaders exhibit on soil biota and the biogeographical nature of these impacts.

BIOLOGICAL INVASIONS AND SOIL PATHOGENS

Since Elton, we have found that soil pathogens have powerful effects in natural plant communities, but with the exception of studies of soil biota in the context of the Janzen-Connell hypothesis for tropical tree diversity (Janzen 1970; Connell 1971), soil pathogens are just beginning to find their place in community theory. Inclusion of pathogens in community theory has been helped by efforts to understand the causes of exotic plant invasions as studies of pathogens and exotic invaders have provided new insights into the role of pathogens as important drivers of classic patterns and processes in plant communities (Mitchell & Power 2003). For example, Saara DeWalt et al. (2004) (Fig. 1) examined the role of fungi in the invasion of the Neotropical shrub *Clidemia hirta* on Hawaii. They planted *C. hirta* into understorey and open

habitats in its native range of Costa Rica and its non-native range in Hawaii and applied insecticide and fungicide to some treatments. They found that the survival of the invader in its native range increased with protection from these two groups of natural enemies. In contrast, suppression of pathogenic fungi and herbivores had no effect in the non-native range. It is important to note that this study inhibited fungi and insects via foliar leaf applications. Although soil fungal and insect populations were not directly manipulated, these novel experiments indicate that pathogens were important for invasive success. Additionally, this study indicated that pathogens had important effects on the local distribution and abundance of *C. hirta* in its native range. One of the most important early breakthroughs for understanding the importance of pathogens in natural communities, and specifically soil pathogens, was made by Wim van der Putten et al. (1988) (Fig. 11.1) who found that *Ammophila arenaria*, a European beachgrass, grew more poorly in ‘rhizosphere sand’ (sand in which the plant had been growing) than in fresh ‘sea sand’ (sand in which no *Ammophila* had grown). They sterilized the soil with gamma-irradiation and found that the biomass of *Ammophila* seedlings increased. They concluded that sand in which the grass had been growing accumulated biota harmful to *Ammophila*, which was reflected in the condition of the roots. In a later paper (Van der Putten et al. 1993) the role of soil biota on *Ammophila* was further investigated in the context of succession. *Ammophila* was suppressed by soil biota that accumulated in its rhizospheres, but plant species that followed *Ammophila* in succession were not. Van der Putten and others established that *Ammophila* was strongly inhibited by soil biota in its European native range, where it is naturally succeeded by other species.

In an extension of this work, others explored the role of soil biota on *Ammophila* in parts of the world where the grass has become highly invasive. In California, where *Ammophila* has

invaded coastal dunes, Beckstead and Parker (2003) measured the effects of belowground pathogens on *Ammophila*. They manipulated soil biota by sterilizing soils to eliminate all soil-dwelling organisms and found that seed germination, seedling survival, and seedling growth was reduced in nonsterilized soil compared with sterilized soil, thus failing to find a release from natural enemies. They also found several common fungal pathogens, but no pathogenic nematodes. The absence of nematodes could be an important factor contributing to successful invasions in California because they can have strong additive pathogenic effects on plant growth when combined with fungi from soils in the native range (De Rooij-van der Goes 1995; but see Van der Putten and Troelstra 1990). In a global survey of nematodes on *Ammophila*, Van der Putten et al. (2005) found that in the non-native ranges of *Ammophila* more specialist nematode taxa attacked the local native plant species than attacked the invasive *Ammophila*. Knevel et al. (2004) compared the effects of the soil biota from the native European range versus the non-native range in South Africa where *Ammophila* has also invaded. As shown before, biota in the soil from the native range had strong inhibitory effects on *Ammophila* grown in native soils, but unlike what was found in California, soil biota from the non-native range were far less damaging to *Ammophila* growing in non-native soils. Importantly, van der Putten's initial research on soil biota in the context of plant *Ammophila* invasions (*Ammophila*) stimulated a growing body of work on plant–soil feedbacks that has yielded insights into the nature of invasions and the forces that structure native communities.

PLANT–SOIL FEEDBACKS

Plant rhizospheres contain many organisms that inhibit plant growth (parasites) and organisms that promote plant growth (mutualists), and many in each group are bacteria and fungi. The

species-specific biochemistry of root exudates arguably plays a key role in determining the composition of the microbial community in the rhizosphere and suggests a ‘degree of co-evolution’ among plant–microbe interactions (Hoestra 1975). The relative accumulation of parasitic or mutualistic bacteria over time in rhizospheres can elicit ‘feedback’ responses in which a species declines or increases in growth or fitness as it affects its own rhizosphere community. Again, biological invasions have allowed comparisons of soil feedbacks in ways that have contributed tremendously to our overall understanding of the role of soil biota in ecology.

Jim Bever (1994) (Fig. 1) explored plant–soil feedbacks with four old-field plant species and biota that developed in the soil occupied by each plant species. He found that the survival of *Krigia* dandelion was lower when the plant was grown in soil previously occupied by conspecifics and the other three species had lower growth and root–shoot ratios when exposed to inoculum from soil previously occupied by conspecifics compared with the inoculum derived from other species. These effects were tentatively attributed to root pathogens. Mills and Bever (1998) then followed up the earlier study by testing the effects of the soil pathogen *Pythium* on several different old-field plant species. They found that some plant species were more susceptible to *Pythium* in general than other species and that some plant species accumulated *Pythium* at faster rates than other species. They made the case that this sort of species-specific accumulation of soil pathogens could explain negative plant–soil feedbacks.

In 2002, John Klironomos connected the effects of soil feedbacks to the relative abundance of both exotic and native plant species in communities (Fig. 11.1). The relative strength of feedback interactions between plants and soil biota was highly correlated with the relative abundance of species in old-field communities. In other words, species that developed strong negative feedbacks over time with soil biota were less common and species that

developed strong positive feedbacks with soil biota were more common in the community. In an explicit comparison of ‘rare plants’ to ‘invasive plants’ Klironomos found that all rare species tested experienced strong negative feedbacks, whereas four of five invasive species experienced significant positive feedbacks. He noted that these invasive species may have simply possessed inherent physiological traits that prevented large build ups of soil pathogens in their rhizospheres, but importantly that the invaders may have ‘escaped their harmful pathogens by invading foreign territory’. To understand whether invaders possess inherent resistance to soil pathogens or have escaped host-specific pathogens in their home ranges, several researchers have conducted explicit biogeographical comparisons of interactions between exotic species, pathogens and soil biota (Hierro et al. 2005).

BIOGEOGRAPHY OF INVASIONS

The most fascinating attribute of exotic invaders is that they become far more abundant or dominant in their new non-native ranges than they were in their native ranges; and this occurs regardless of the continent of origin or the continent of destination. Unless by some chance the abiotic conditions of non-native ranges happen to dramatically favour some non-native species over locally adapted native species, it would seem that powerful *biotic* forces are at work. Of a suite of potential biotic forces from which invaders may benefit, the overwhelming focus has been on escape from specialist insect herbivores, enemies that were fully recognized by Elton (Keane & Crawley 2002). Taking a novel tack on enemy escape, Mitchell and Power (2003) (Fig. 11.1) searched the literature and databases for evidence of infection by viruses, rust, smut and powdery mildew fungi on the above-ground parts of 473 plant species that had been introduced from Europe into the USA. They found that on average, 84% fewer fungi and 24%

fewer viruses infected plant species in the non-native North American range than infected the same species in the native range. They also found that species ranked as ‘invasive’ were more highly infected in their native ranges than species not ranked as invasive. These results suggest that many relatively host-specific leaf and flower pathogens occurred on plant species in their native ranges and that many of these were escaped when the species were introduced by humans into new ranges. Mark Van Kleunen and Markus Fischer (2009) tackled the same question but in the opposite biogeographical direction. They used global data for 140 North American plant species that had naturalized in Europe, for which fungus–plant host distributions were known. In the invaded European range, they found 58% fewer floral and foliar pathogen loads on these invasive North American plant species. However, when they also looked at the known distributions of the fungal pathogens, only 10.3% were not found in the European range, which suggests the enemy release hypothesis does not account for the successful distributions of these North American species. Most interestingly, the geographic spread of these non-native species in Europe was *negatively* correlated with decreased loads of fungal pathogens. They noted that North American plants may have escaped specific fungal species that control them in their native range, but based on total abundances of fungal species reported in the database, release from foliar and floral fungal pathogens does not explain the spread of North American plant species in Europe. It is important to note that van Kleunen and Fischer equated ‘geographic spread’ with ‘invasiveness’ owing to the nature of the data set, but species that have broad distributions are not necessarily invasive (Hierro et al. 2005). Very high densities and high negative impacts on other species are traits more connected to invasiveness. Nevertheless, these kinds of biogeographical comparisons are crucial for understanding invasions. These studies focused on pathogens that target above-ground plant components. However, soils are reservoirs for many

above-ground pathogens. For example, virtually all plant pathogenic fungi spend a relatively small fraction of their life cycle on their host plants, with most the life cycle spent in the soil or in plant detritus within soils (Pepper 2000). Thus survival and effects of even above-ground pathogenic fungi are primarily regulated by biotic and abiotic soil factors (Pepper 2000).

PLANT–SOIL FEEDBACKS AND THE BIOGEOGRAPHY OF INVASIONS

To our knowledge, Kurt Reinhart et al. (2003) (Fig. 1) were the first to undertake intercontinental biogeographical comparisons of soil biota feedback effects in the context of invasion. They studied *Prunus serotina*, a native tree in temperate North America, which is typically early successional and rarely abundant in its native range. In the native range, Packer and Clay (2000) found that the soil pathogen *Pythium* had stronger inhibitory effects on *P. serotina* seedlings near mature trees, where the fungus appeared to accumulate, than on seedlings occurring far from mature trees. When seedlings were grown in soil collected either 0–5 m or 25–30 m from *P. serotina* trees, sterilization of the soil collected near mature trees improved seedling survival, but sterilization of soil collected far from trees had no effect. In sharp contrast to the typical low densities of *P. serotina* in its native range, it has become very dense and highly invasive in parts of Europe. Reinhart et al. (2003) examined populations of *P. serotina* in the Netherlands and in Indiana and found that the densities of saplings and trees were roughly six to nine times higher in the non-native range than in the native range. This dramatic difference in tree density among ranges correlated with less negative soil feedbacks in the non-native range than in the native range where soil pathogens appeared to strongly limit seedling growth and tree density. Later, Reinhart et al. (2005) studied the generality of the negative feedbacks in the native range by sampling 22 populations over a substantial area of the eastern USA. In soils

collected under *P. serotina* trees, sterilization and fungicide treatments specific to oomycetes (including *Pythium*) improved seedling survival, suggesting that oomycetes were causing the death of seedlings. They also compared the effects of soil biota collected under other tree species and found that biota collected under *P. serotina* were more pathogenic to conspecifics than biota collected under other species. Considered together, these results indicate that relatively host-specific soil biota have important regulatory effects on *P. serotina* over large areas in its home range, but that this biotic constraint is escaped in Europe where the tree has successfully invaded.

In sterilization experiments similar to those conducted by Reinhart et al. (2003), Callaway et al. (2004a) found that soil microbes from several sites in the home range of the invasive exotic forb *Centaurea stoebe* (née *maculosa*) L. had stronger inhibitory effects on this species than soil microbes from several sites in the North American invasive range. They then tested feedback effects using soils from one site in each range. The microbial community in the soil from Europe was trained with either *C. stoebe* or *Festuca ovina*, a small perennial bunchgrass native to Europe. The microbial community in the soil from North America was pre-cultured by planting *C. stoebe* or *Festuca idahoensis*, a bunchgrass similar to *F. ovina* but native to western North America. The trained soils were used to inoculate substrate in which *C. stoebe* was planted alone or in competition with one of the two grass species. In the native European soil *C. stoebe* cultivated strong negative feedbacks with soil biota either alone or in competition. However, in soils from North America, *C. stoebe* cultivated strong positive feedbacks. The same treatments in sterilized soil eliminated feedback effects in soils from both ranges indicating the crucial role of soil biota, but they did not identify soil taxa that might have been involved. In related experiments, Callaway et al. (2004b) investigated the effects of soil fungi on interactions between *C. stoebe* and six species native to the grassland invaded by *C. stoebe*. Fungicide

reduced arbuscular mycorrhizal fungi (AMF) colonization of *C. stoebe* roots, but did not reduce non-AMF. In soils without fungicide *C. stoebe* grew larger when grown with *F. idahoensis* or *Koeleria cristata* than when grown alone. However, when fungicide was applied to the soil the positive effects of *Festuca* and *Koeleria* were not present. Fungicide reduced the competitive effects of the native bunchgrass *Pseudoroegneria* on *C. stoebe* and did not affect the way two native North American forbs, *Achillea millefolium* and *Linum lewisii*, competed with *C. stoebe*. These results suggested that *C. stoebe* invasion can be affected by complex and often beneficial effects of fungal communities in the non-native range. Interestingly the effects of soil fungi were not manifest as simple direct effects. Only when native plants, invasive plants, and soil microbial communities were interacting at the same time were the effects observed, suggesting indirect interactions among plants and soil microbes.

Positive effects of soil biota from the non-native range were also observed by Reinhart and Callaway (2004) for *Acer platanoides*, a widespread tree in Europe that has become invasive in North America, and *A. negundo*, a widespread tree in North America that has become invasive in Europe. They compared the relative importance of soil biota in the context of the ‘enemy release hypothesis’ and biogeographical differences in the effects of mutualisms. As found by Reinhart et al. (2003) for *P. serotina*, distances from focal trees to the nearest *Acer* conspecifics were 56–77% less in their non-native ranges than in their native ranges demonstrating higher densities in the non-native range. Unlike the experiments with *P. serotina* and *C. stoebe*, soil collected under *Acer* species in their non-native ranges decreased the growth of conspecific seedlings by 64–112%, but the soil associated with native tree species in the non-native ranges *increased* the growth of *Acer* seedlings. These results suggest that native soil biota initially boost invasion by the two *Acer* species, but over time soil biota communities become more pathogenic

to *Acers* as they establish over time. Like for *C. stoebe*, mutualistic interactions with soil biota may be relatively more beneficial to *Acer* species in their non-native ranges than in their native ranges. Similarly, Cui and He (2009) found that the native shrub *Saussurea deltoidea* altered soil biota in ways that increased the growth of the invader *Bidens pilosa* in China.

As noted above, some invaders benefit from soils trained by certain native species early in the invasion process, over time shifting the soil biota. These shifts in plant–soil feedbacks can not only enhance the initial invader, but can also be beneficial to other invasive species. For example, in the same intermountain grasslands invaded by *C. stoebe*, Jordan et al. (2008) measured the feedback effects of three other aggressive invaders: *Euphorbia esula*, *Bromus inermis* and *Agropyron cristatum*. They found that *B. inermis* and *A. cristatum* demonstrated strong positive plant–soil feedbacks. However, in a novel twist, *B. inermis* and *A. cristatum* also had facilitative effects on other invasive species through soil feedbacks. Jordan et al. (2008) argued that their results suggested that the alteration of native soil biota by invasives could promote self-invasion or ‘cross-facilitation’ of other invasive species. Studies of *Ageratina adenophora*, a species native to Mexico that has become highly invasive throughout the world in semi-tropical ecosystems, have provided a conceptually similar picture. In China, where *A. adenophora* has become an exceptionally aggressive invader, Niu et al. (2007) found that the soil biota collected from heavily invaded sites suppressed native plant species, but did not suppress *A. adenophora*. They also found that soil biota in the invaded site had greater positive effects on *A. adenophora* than soil biota in the non-invaded site, suggesting that the invader altered soil microbial communities in ways that favoured itself.

As noted above, soil biota include complex mixtures of deleterious and beneficial taxa. Van der Putten et al. (2007) explored the contribution of soil pathogens and AMF in the

feedback effects of an invasive and two native grasses in the Kalahari savanna in Botswana. The exotic grass *Cenchrus biflorus*, established neutral to positive soil feedbacks, whereas the two native grass species, *Eragrostis lehmanniana* and *Aristida meridionalis*, showed neutral to negative feedback effects. This study found two host-specific pathogens on the roots of *E. lehmanniana* that did not affect the invasive *C. biflorus*. Fungi were also isolated from the roots of the *C. biflorus*, but these fungi had no effects on any of the grasses. Native grasses showed higher diversities of AMF taxa in their roots than *C. biflorus*, but it was not clear what role these different species played in the general feedbacks generated by the different grasses.

Plant–soil feedbacks can impact native communities in ways that suggest community-level alterations are occurring, including those that directly inhibit native species as well as those that directly enhance invasives. One example of a native species experiencing direct inhibition by an invader was conducted by Batten et al. (2008) in a novel experiment on feedbacks in the Western USA. They tested the effects of the soil microbial biota found in the soil rhizosphere of the invasive grass *Aegilops triuncialis* and biota found in the rhizospheres of the natives *Lasthenia californica* and *Plantago erecta*, on the growth of the three species. *Lasthenia californica* experienced a delayed flowering date and reduced above-ground biomass when exposed to soil microbes that accumulated in the rhizospheres of *A. triuncialis*. They proposed that changes in soil microbes elicited by invaders might cause community-scale positive feedbacks (not necessarily positive feedbacks on the invader themselves) that ultimately lead to dominance by the invader. In other words, these observed changes in the native species contributed directly to their decreased abundance which could indirectly contribute to increased invader dominance. In a long-term field experiment on northern shrub steppe communities Andrew Kulmatiski et al. (2006) examined ‘soil history’, a proxy for feedbacks, and found that

exotics appeared to facilitate their own growth by maintaining small beneficial fungal populations. Whether or not soils were occupied by exotics or native was a crucial factor for determining exotic and native plant distributions. Following this with a major synthetic analysis, Kulmatiski et al. (2008) (Fig. 1) gathered the literature on these kinds of feedback studies with invasive species and used meta-analytical models to examine generality in the effects of plant–soil feedbacks on plant diversity and invasions. After including the literature on the subject to date, they found that plant–soil feedbacks were larger and more negative for native species than for invasive species.

The strong biogeographical patterns noted in several of the studies discussed above suggest that plants in a region affect the evolution of soil pathogens, and vice versa, in a way that leads to some degree of difference in host preference, host response, or host effect. Human-facilitated dispersal beyond natural geographic limits may then lead to escape from these pathogens. There is other evidence that plants can influence the evolutionary trajectories of their neighbours through the effects of microbial symbionts (Aarssen & Turkington 1985; Chanway et al. 1989). These studies showed that the legume *Trifolium repens* had increased biomass when grown with genotypes of the grass *Lolium perenne* with which they had coexisted (Aarssen & Turkington 1985). This increased biomass was due to increased effectiveness of nodule induction by nitrogen-fixing *Rhizobium* strains collected from co-existing *Trifolium–Lolium* communities compared with *Rhizobium* strains collected from *Trifolium*-only populations (Chanway et al. 1989). In sum, these studies showed that the plant–microbe symbiosis may not only be influenced by coevolution between the microbial partner and the respective host plant, but also by other neighbouring plant species. Evidence that invasive plants disrupt microbially

mediated ecological functions supports the notion that coevolution among plants and microbes may occur within communities.

EFFECTS OF INVASIVE PLANTS ON SOIL BIOTA

Invasive plants can alter soil biota in ways that do not explicitly lead to feedback relationships, and these effects can be positive, neutral or negative (Wolfe & Klironomos 2005). There are far more examples of this sort of invader–soil biota interaction in the literature than examples of feedbacks, thus we focus on only a few of these to illustrate the ranges of variation observed for these effects.

Some experimental studies have found no relationship between plant species diversity and microbial diversity (Gastine et al. 2003; Porazinska et al. 2003; Niklaus et al. 2007). Other experiments have found that plant diversity correlates positively with bacterial composition or diversity (Grüter et al. 2006; Wardle et al. 1999; see Wardle 2002). Yet others have found no correlation between plant diversity and bacterial diversity but strong correlations with fungal diversity (Millard & Singh 2009). Despite the lack of a consistent relationship between plant diversity per se and the diversity of soil microbes in general, the presence or absence of particular plant species or functional groups is often correlated with the composition or diversity of soil microbial communities (Grüter et al. 2006; Bremer et al. 2009; Zhang et al. 2010). The strong effects of different plant species on soil microbial composition and function may derive from differences in the carbon: nitrogen ratios of leaves (or other basic stoichiometric differences), changes in disturbance regimes or species-specific differences in secondary metabolites (Wolfe & Klironomos 2005). Regardless of the mechanism, the arrival of many new

plant species and the decline of native species has important implications for microbial communities, particularly their functions in ecosystem processes.

Biological invasions commonly drive marked decreases in the diversity of native plants (Rout & Callaway 2009), and whether caused by this decrease in diversity per se or the presence of a new plant species, soil biota often change in composition or decrease in diversity (Wolfe & Klironomos 2005 and references therein). In this context, the system that has been the most thoroughly studied is arid shrub-steppe invaded by the annual grass *Bromus tectorum*. During the past decade Jayne Belnap and colleagues have conducted a series of studies yielding a great deal of insight into the effects of this invader on soil microbes and their functions. Belnap et al. (2005) showed that sites historically invaded with *Bromus tectorum* had lower diversity measures of both plants and soil biota, while non-invaded sites had the greatest plant diversity and soil biota diversity. *Halogeton glomeratus* is an annual in the Chenopodiaceae from western Asia that has invaded grassland and shrubland throughout the western USA. Duda et al. (2003) found that the abundance of *H. glomeratus* correlated with increases in soil fertility (see also Liao et al. 2008, Rout & Callaway 2009) and increases in the diversity of soil bacteria. Whether invaders alter soil biota through their effects on the stoichiometry of nutrient cycling or through secondary metabolites is not known.

Taking a different approach to studying the effects of invaders on soil microbes, in 2006 Maarten Eppinga and colleagues in the Netherlands integrated experimental data with models to advance the theory that invasive species might accumulate local native generalist pathogens to a degree where native species are harmed more than the invasive species. They based their models on *Ammophila arenaria* arguing that in the non-native range ‘accumulation of local soil pathogens could enhance dominance and rate of spread of *A. arenaria* as a result of negative

specific soil community effects on native plant species'. Consistent with this idea, Mangla et al. (2007) found that soils from the rhizospheres of *Chromolaena odorata*, one of the world's most invasive tropical species, accumulated high concentrations of *Fusarium* (apparently *semitectum*), a generalist soil fungi, which was harmful to native plant species. Soils collected beneath *Chromolaena* in India inhibited growth of native species and contained over 25 times more spores of the pathogenic fungi than soils collected beneath neighbouring native species. Sterilization of the *Chromolaena* soils eliminated the inhibitory effect. In an experiment linking root exudates to the effect of the invader on soil biota *Chromolaena* root leachate increased *Fusarium* in spore density by over an order of magnitude. This exacerbation of the effects of native soil biota on native plants suggests a novel mechanism that might contribute to exotic plant invasion.

MICROBIAL MECHANISMS UNDERLYING PLANT INVASIONS

Plant invasions provide biogeographical contexts in which microbial ecological functions can be explored, and invasions demonstrate biogeographical patterns in ecological functions that might help to unify biogeographical concepts across all life forms (Green et al. 2004; Horner-Devine et al. 2004). Although recent studies suggest that regional or local evolutionary trajectories exist for microbes (McInerney et al. 2008), until recently, ecologists have focused on broad pathogenic or mutualistic functions of microbes and the broad functional role of microbes in nutrient cycling; processes suggesting that soil microbes rarely have restricted biogeographical distributions. However, invasive plants often interact differently with microbial functional groups in invaded ranges than they do with the same functional groups in native ranges, causing substantial shifts in the ecological nature of microbial mutualistic relationships (Parker et al. 2006), escape from

fungal pathogens (Reinhart et al. 2005) and functional changes in soil bacterial communities (Liao et al. 2008). These biogeographical effects of invasive plants soil microbes suggest a new paradigm for microbial biogeography – biogeographical patterns exist – which is the case for all other taxa on Earth.

Support for the idea that invasive plants affect soil biota differently in native and non-native ranges has been found in studies of bacteria and fungi. For example, biological invasions commonly disrupt ecosystem processes mediated by soil bacteria (Liao et al. 2008; Rout & Callaway 2009) (Fig. 11.1). In the context of biogeographical patterns for soil microbes, plant invasions reduce local plant species richness, but typically correlate with *increased* net primary productivity; a correlation that conflicts conceptually with the current diversity–ecosystem function paradigm; i.e. species richness increases net primary productivity (Tilman et al. 2001). Even more puzzling, invasions commonly increase net primary productivity but they generally do not deplete soil resources such as nitrogen; instead they generally correlate with increased soil nitrogen pools and total ecosystem nitrogen stocks (Liao et al. 2008; Rodgers et al. 2008; Rout & Chrzanowski 2009; Rout & Callaway 2009), all processes mediated by soil bacteria. Rout & Chrzanowski (2009) (Fig. 11.1) found that the ecosystem effects of *Sorghum halepense* may be caused through mutualistic endophytic bacteria, including nitrogen-fixers, housed in the rhizomes of the invader. The effects of invaders on these processes in their home ranges are unknown, but since many invaders are far less abundant in their home ranges similar effects are unlikely. The dramatic and consistent alterations of soil nutrient cycles associated with invaders in introduced ranges (Liao et al. 2008) suggests that they exert novel effects, relative to the effects of native plants, on soil bacteria. As mentioned, this differential effect exists for soil fungi

as well. Callaway et al. (2008) showed that one of North America's most aggressive invaders of undisturbed forest understories, *Alliaria petiolata*, had much stronger inhibitory effects on AMF in invaded North American soils than on AMF in European soils where *A. petiolata* is native. This biogeographical effect was also found for specific flavonoid fractions in extracts from *A. petiolata*. Together, these patterns suggest that ecological functions of soil microorganisms (as measured by various microbially mediated parameters like nutrient cycling, litter decomposition, etc.) have biogeographical patterns.

These patterns may provide a conceptual link with the differences in plant–soil feedbacks between native and invaded ranges (less negative in the invaded range) described above. If invaders affect microbially mediated soil processes in predictably different ways than natives (see Rout & Callaway 2009) two equally interesting hypotheses exist. Either plants with 'invasive' traits, as a group, also happen to affect soil microbes differently than plants without such invasive traits (but somehow do not display these traits in their native range), or microbes in various parts of the world are functionally similar but subtly different with respect to the plants with which they interact.

Future research on plant invasions requires attention how they alter the structure and function of soil biota. Invaders can have dramatic effects on above-ground community structure, most dramatically as decreases in species richness and functional diversity, and these decreases are likely to strongly soil biota. Disrupted ecosystem processes that are microbially mediated often appear to shift the balance of particular cycles in favour of the invaders. Recent research has explored how interactions between plants and their symbionts (both parasitic and mutualistic associations) respond to shifts in plant diversity (Nuismer & Doebeli 2004; Thrall et al. 2007).

There is growing interest in how invaders might affect mutualistic soil biota. This interest is associated with recent sea changes in plant ecology in which the effects of positive interactions (i.e. facilitation and mutualisms) are emphasized in shaping natural and invaded communities (Bruno et al. 2003; Richardson et al. 2000). Perhaps successful invasions are due in part to the lack of coevolved interactions with soil biota, thus invaders are interacting with microbial symbionts in ways that are more beneficial through positive feedbacks (Klironomos 2002; Packer & Clay 2000; Reinhart et al. 2003, 2005; Callaway et al. 2004a,b), increased soil nutrient pools (Liao et al 2008; Rodgers et al. 2008; Rout & Chrzanowski 2009), and perhaps the accumulation of more beneficial mutualisms.

CONCLUSIONS

As discussed, when humans disperse invasive species in new areas they can escape soil-borne natural enemies. Incorporating biogeographical approaches into research on plant invasions has contributed not only to our understanding of plant performance in invaded ecosystems, but also to our understanding of microbial processes underlying invasions. Clearly, interactions among plants and microbes are bidirectional: plant communities affect microbial communities and vice versa. Biogeographical differences in the pathogenic effects of soil biota can be due to different pathogen densities, the composition of pathogen communities, and differences in the virulence of individual species or genotypes in the soil biota. Interestingly, the phylogenetic relatedness of the invader relative to resident species might affect how susceptible exotic species are to resident pathogens (Gilbert & Webb 2007) further supporting the necessity for using biogeographical approaches to study. The effects of soil mutualists on invasions are understood less than the effects of pathogens, but mutualists also appear to play important roles. Invaders might be

limited by the absence of appropriate mutualists in their new ranges (Parker 2001) or benefit from mutualists they encounter in the soil of invaded ranges (Marler et al. 1999; Parker et al. 2007). Invaders can also suppress soil mutualists of other plant species in invaded ranges more aggressively than mutualists in their original range (Callaway et al. 2008) or have effects that are similar to the effects of nitrogen-fertilization on decreased mutualisms among coevolved organisms (see Denison & Kiers 2004; Thrall et al. 2007). Whether or not the benefits of new mutualistic relationships with soil biota in invaded regions are generally stronger, weaker or similar to mutualistic interactions in native regions remains an important unanswered question. Focusing on the role of microbial mutualisms in the invasion process might also advance our general understanding of how these interactions coevolve in general. Furthermore, whether invaders will in time acquire more effective parasites (see Parker & Gilbert 2004) remains a crucial yet unexplored area. Testing these and other processes in the context of invasion, and specifically in a biogeographical context, will provide insight into the function of natural communities and ecosystems.

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FIGURE LEGENDS

Figure 1. Timeline for some conceptual developments involving the role of soil biota in structuring plant communities and in exotic plant invasions.

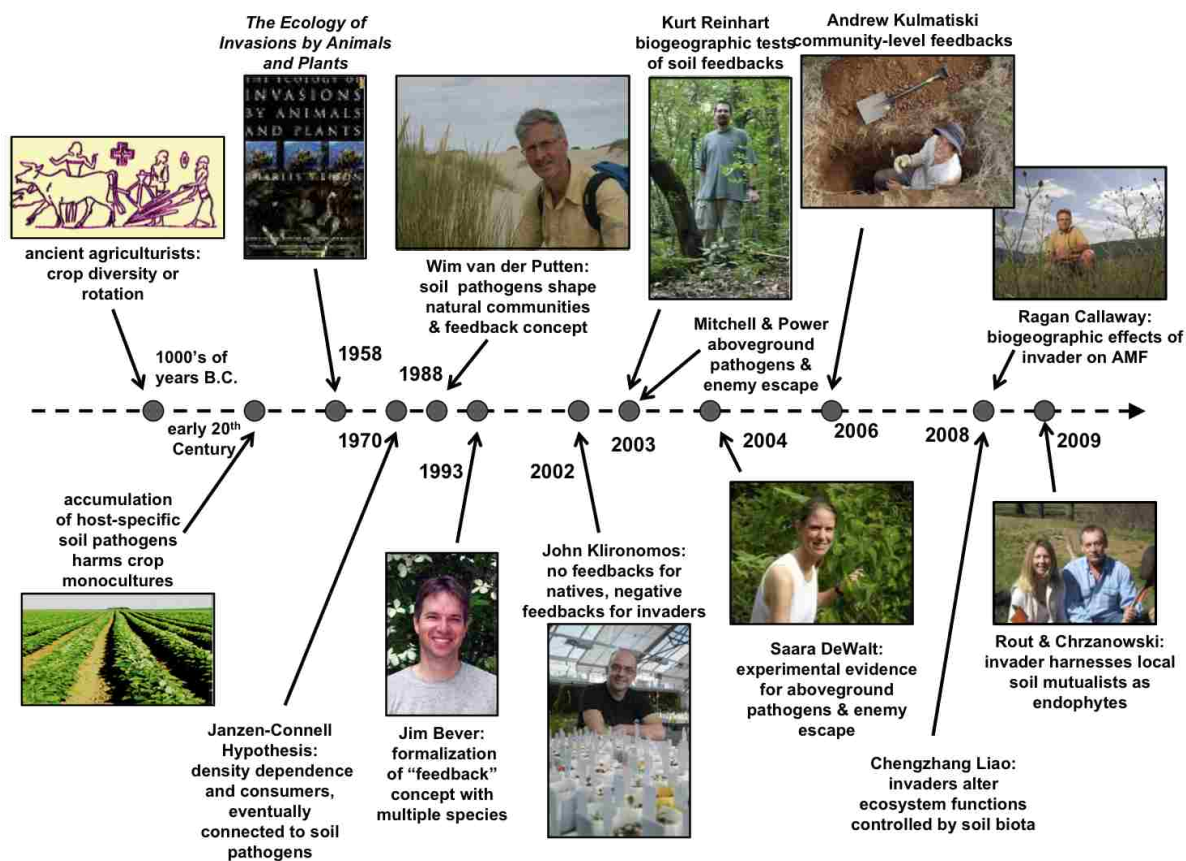


FIGURE 1

CHAPTER 2

Is everything everywhere? Plant invasions and microbial biogeography

Abstract

A major question in ecology and evolution is whether or not microbes demonstrate biogeographic distributions that occur for all higher taxa. The idea that microbes are free from dispersal limitations therefore the same basic taxa are everywhere on Earth, is a long held notion. However, recent research demonstrating genetic differences among microorganisms over geographic distances is challenging this century-old belief and suggesting biogeographic patterns for microorganisms. We expand the discussion by considering evidence that invasive plants disrupt microbially-mediated ecological functions. Effects of plant invasions on microbial ecology add to the argument that microbes are not exempt from biogeographic boundaries and that at some taxonomic, ecotypic, or functional level everything might *not* be everywhere.

What is a microbial “species”?

“Everything is everywhere, but the environment selects” as stated by Lourens Baas Becking [1], has provided the context for one of biology’s most intriguing issues. Microorganisms have been thought to be free from geographic boundaries and globally ubiquitous because of their great ancestral age, vast population sizes, environmental hardiness, and ability to disperse [2,3]. However, an initial inherent problem with determining whether or not biogeographic patterns exist for microbial taxa lies with the definition of a microbial “species”, both past and present definitions (Box 1).

The biogeographic aspect of microbiology emerged in the late 1800’s based on the theories of Baas Becking and studies of Beijerinck. At this time, Buffon’s law of biogeography - geographically separated areas that are environmentally similar have evolved regionally specific flora and fauna - was not applicable to “lower organized” taxa, which included not only bacteria, but also fungi, algae, and lichens [3-5]. Cosmopolitan distributions for lower-organized taxa were accepted because the same morphologies were consistently observed in geographically separated areas. It was thought that more simplified body organization allowed “lower” organisms to have wider ranges, and the absence of dispersal limitations allowed frequent migration across geographical borders that limit higher taxa [3,4]. Thus, “everything is everywhere” implied “lower-organized” taxa were not dispersal limited, while “the environment selects” implied ecological factors determined which organisms from the global species pool would proliferate upon arrival into new environments [3]. Regional evolutionary trajectories for microbes were presumed to be non-existent - until the emergence of molecular techniques.

Molecular methods moved beyond morphology, redefining microbial phylogenies and allowing us to rethink microbial biogeography. Initially, phylogenies were based on data obtained from the small subunit ribosomal RNA (SSU r-RNA). Identity is assigned by grouping the sequences into operational taxonomic units (OTUs), with consensus that sequences with 97% or greater OTU similarities are the same “species” [6]. The phylogenetic trees constructed from these OTUs were used to assess microbial biogeography [2,7-9], and challenged the tenet that everything is everywhere. This approach is not foolproof, as contradictory phylogenies can be constructed from functional genes, like RNA polymerase [10]. Thus, two microorganisms can have >97% OTU similarity based upon SSU r-RNA, but might possess genes that result in different ecological and biological functions [11,12].

As a solution, sequence-based approaches have been used to assess microbial community function. Methods include targeting functional genes relevant to the ecology of the organism, concatenation of sequences of multiple housekeeping genes (multilocus sequence analysis, MLSA), and metagenomic studies [11]. As entire genomes have been sequenced, it has become clear that microbial evolution is not only rapid, but also moves horizontally in bacteria, known as horizontal gene transfer (HGT), which can account for high proportions of bacterial genomes [see 13]. Rapid microbial generation times and the prevalence of HGT provide potential mechanisms for the development of regional genetic differences, possibly ecotypes, to arise.

Whether based on morphology or genes, phylogenetic scale determines the biogeographic interpretations - at some broad taxonomic or functional level everything does appear to be everywhere [14]. Put another way, nitrifiers and decomposers have been found in all biogeographic regions where sought. This does not negate the possibility of regional differences in evolutionary trajectories at all taxonomic or functional scales; much like the global

distribution of carnivores does not negate biogeographic patterns for carnivores. Are microbes exempt from fundamental biogeographic patterns characterizing all other life forms? This is a crucial question because geographical isolation is a primary mechanism for speciation – without biogeography in the evolutionary past how did we get different microbial species in the first place? Understanding the biogeography of all life forms at functional scales contributes to an understanding of their evolution.

Plant invasions and microbial biogeography

Recent genetic research has shown that microbial community composition changes with geographic distance - the relationship between sampled area and taxa accumulation is similar to that for macro-organisms [2, 7-9]. Redefining phylogenies with molecular characters has also led to the discovery of new local microbial “species”, suggesting the existence of biodiversity-based scaling rules. These advances have placed us on “the cusp of a complete reversal of opinion” about the biogeography of microbes [3], and have led to the suggestion to integrate ecological function with genetics- based phylogenies to unify microbial species definitions [11]. In this context, plant invasions provide ideal systems in which variation in microbial ecological functions can be explored in a biogeographic context. Invasions demonstrate biogeographic patterns in ecological functions that might help unify biogeographic concepts across all life forms, including soil fungi [8] and bacteria [9]. If plant invasions suggest regional or local evolutionary trajectories for microbes, and we now comprehend enough about microbial genomes to know that this is mechanistically possible [12], such functional biogeography would add to the argument that everything is not everywhere.

Until recently, ecologists have focused on the broad pathogenic or mutualistic functions of microbes and the broad functional role of microbes in nutrient cycling. From this perspective microbes function quite similarly, driving the same basic cycles everywhere on the planet. Yet invasive plants often interact differently with broad soil-microbial functional groups in invaded ranges than they do with the same functional groups in native ranges, causing substantial shifts in the ecological nature of microbial mutualistic relationships [15], escape from fungal pathogens [16], and functional changes in soil bacterial communities [17]. If plant-microbe interactions are substantially altered in the plant invasion process, either relative to interactions in the invader's home range, or relative to general plant-microbe interactions among native species, "everything is everywhere" is questioned while the "environment selects" is emphasized, but from an evolutionary perspective rather than through filtering the global species pool.

The first clue from plant invasions for microbial biogeographic patterns is that exotic invasions commonly disrupt processes mediated by soil bacteria. Plant invasions typically reduce local plant species richness; local richness has been repeatedly shown to increase ecosystem net primary productivity (NPP) [18]. Yet invasions commonly *increase* ecosystem NPP [17]. Even more puzzling, though invasions commonly increase NPP they generally do not deplete soil resources such as nitrogen; instead they generally correlate with *increased* soil nitrogen pools and total ecosystem nitrogen stocks [17,19, 20], all processes mediated by bacteria. This combination of changes is odd. Such changes might also occur when native species replace other natives, but a fundamental ecological paradigm is that short-term increases in NPP deplete soil resources, as in agricultural systems [21]. The effects of invaders on these processes in their home ranges are not known, but since many invaders are far less abundant in their home ranges similar effects are unlikely. The dramatic and consistent alterations of soil

nutrient cycles associated with invaders in introduced ranges [17] suggests that they exert novel effects, relative to the effects of native plants, on soil microbes. If invaders affect microbially-mediated soil processes in predictably different ways than natives, two equally interesting hypotheses exist. Either plants with “invasive” traits, as a group, also happen to affect soil microbes differently than plants without such invasive traits - but somehow do not display these traits in their native range - or microbes in various parts of the world are functionally similar (e.g. there are nitrifiers everywhere), but subtly different with respect to the plants with which they interact.

Mechanisms utilized by invaders to alter nutrient cycles are poorly understood, but they are not as consistent as the general pattern of increasing soil cycling rates, pools, and total stocks. Morphological and physiological traits of invaders are highly likely to alter nutrient cycles, but these changes cannot occur without concomitant responses by microbial, specifically bacterial, communities. For example, manipulation of invasive grasses in California showed that they doubled gross nitrification rates, relative to natives, by increasing the abundance and changing the composition of ammonia-oxidizing bacterial communities in comparison to native grass communities [22]. In other cases the unique biochemistry of invaders affects microbial decomposers [23], in turn altering nutrient mineralization rates. However, there have been no explicitly biogeographic studies comparing invader effects on ecosystem processes in home and away ranges; a major knowledge gap in ecology. However, the consistent trend in the effects of invaders on microbially-mediated soil ecosystem processes suggests the possibility of regional evolutionary trajectories [e.g.24] for soil microbes.

Bacteria

Broad ranges of microbial taxa were the initial focus of the hypothesis that everything is everywhere [4,5], but bacteria have received more attention recently. Bacteria, incapable of sexual reproduction, were thought to be genetically homogeneous around the globe. We now know that bacteria acquire novel genes through horizontal gene transfer (HGT). This is not trivial because it drives rapid evolution of antibiotic resistance [25], transfer of the *Rhizobium* nodulation plasmid [26], and accounts for 18-60% of the *Escherichia coli* genome [13,27].

Morphological similarities have also hindered examination of bacterial responses to plant invasions because organisms with virtually identical morphologies can vary subtly in their functional responses to different plant species. For sexually reproductive species, specifically macro-organisms and to large degree fungi, centuries of collection and identification across the world has allowed us to determine who is native where; yet, these historical collections do not exist for bacteria. Only recently have we been able to investigate taxonomic detail for bacteria beyond morphology and phenetics. The greatest insight into biogeographic patterns through studying invasions is gained from comparing basic ecological processes with combinations of species from home ranges and invaded ranges. But there have been no such comparisons of free-living nitrogen (N₂)-fixers, nitrifying bacteria, mineral chelating bacteria, or other such taxa between invaded and native ranges. While plant-associated microbial distributions have been studied, patterns of these plant-microbe interactions in the context of plant invasions have not.

Studies of rhizobia, N₂-fixing bacteria that form nodules and thus, a complex mutualism with plants, are an exception to this problem. These endophytic bacteria play a crucial role in agriculture and participate in a fascinating mutualism; many nodule-forming species are prominent invaders. Leguminous invaders have to either bring their symbionts with them, or possess the ability to form mutualisms with a wide range of bacterial taxa. Nodulating N₂-fixing

bacteria and their hosts can have species-specific relationships. Several studies have shown that exotic but non-invasive legumes were unable to nodulate when first introduced to a new geographical region, even though native legumes and their symbionts were present (reviewed by [15]). This suggests that biogeographic differences exist for these soil-dwelling bacteria. Functional variation in these mutualisms would also support the idea that everything is not everywhere.

Robinia pseudoacacia (black locust) is a leguminous tree native to North America, but globally invasive. Predictably, it interacts with different genotypes of N₂-fixers within a number of genera including *Rhizobium*, *Bradyrhizobium*, and *Mesorhizobium* [28]. Despite this wide range of N₂-fixing partners, there is some evidence for biogeographic differences in function. For example, a strain of *Rhizobium* isolated from invasive trees in Argentina was a superior symbiont to the “native soil born *Rhizobium*” [29]. *Cytisus scoparius* (scotch broom) is another root-nodulating legume that has become a major invader throughout the world. *Cytisus* seedlings transplanted into sites in the invasive range in North America, either with or without inoculum from other *Cytisus* plants, demonstrated significantly greater nodulation and biomass with inoculum [15]. In another study, the genetic diversity of *Bradyrhizobia* associated with the invasive leguminous tree, *Acacia longifolia*, was compared between Australian sites that varied in the time since invasion [30,31]. Long-invaded sites had higher genetic diversity of *A. longifolia*-associated rhizobia than sites currently undergoing invasion. Additionally, some phylogenetically related isolates showed overall low levels of genetic similarity.

We caution that the biogeographic origins of N₂-fixing organisms are not precisely known because large numbers of genotypes are introduced with legume seeds [30] or through agricultural inoculum [32], potentially to become invaders themselves. Rhizobia might be

everywhere, but symbiont limitation for some plant species, and biogeographical differences in symbiont functions, demonstrates a strong biogeographical aspect to their current distributions.

Fungi

Recently, several studies of invasive plants have shown that soil fungi can have geographic boundaries [16,33-35]. An uncommon North American tree, *Prunus serotina*, exploded in abundance when introduced in northern Europe. Fungal pathogens (specifically oomycetes) from North American soils inhibit the growth of *Prunus*, but pathogens from European soils do not [16]. Soil pathogens, including oomycetes, exist in European soils, but do not inhibit *Prunus*, suggesting strong biogeographical differences in the ecological function of soil biota. Striking biogeographic differences have also been demonstrated for the effects of European native *Alliaria petiolata*, a North American invader, on arbuscular mycorrhizal fungi (AMF) [33]. *Alliaria* is not mycorrhizal and biochemical exudates kill North American AMF in invaded soils. However, the effect of the plant and its chemical constituents are far weaker on AMF in soils collected near natural populations of European *Alliaria*. In soils where *Alliaria* had been experimentally grown, North American AMF-dependent species were smaller while European AMF-dependent species were unaffected. *Alliaria* had no effect on non-AMF dependant species.

Fungal endophytes are common in plants and can have striking effects on plant growth, defense and competitive ability. Genetic analysis of endophytic fungi found in *Centaurea maculosa* in both its native and invaded ranges showed that 85% occurred in only one of the two ranges [34]. In Europe, the native range, the most common endophyte was a haplotype of

Alternaria alternata. In the invaded range, no haplotype was dominant and many were novel. While some fungal endophytes were introduced with *C. maculosa*, the invader also acquired new endophytes after introduction [34]. Similarly, some fungal endophytes isolated from *C. maculosa* seeds caused significant declines in germination of *Festuca idahoensis*, a North American grass competitor [35]. These data suggest that endophytic effects have biogeographic differences and might occur indirectly through the invasive plant in this system.

Such biogeographical differences in plant pathogenic and mutualistic interactions indicate that meaningful evolutionary relationships develop for microbes within biogeographic boundaries that are similar to the boundaries observed for other evolved interactions [24]. Clearly, there is a need for more nuanced biogeographic theory for fungi, but such subtle differences in ecotypes are likely due to the effects of biotic interactions among species on natural selection, discernable through the disruptions of evolutionary trajectories caused by plant invasions.

Invasive soil microorganisms

Recent advances in molecular techniques (briefly described earlier) have enabled epidemiologists to reconstruct evolutionary trajectories among parasites and hosts, in some cases identifying origin of, and environmental factors responsible for disease spread [36]. Consequently, most research on invasive microorganisms has targeted disease ecology and infectious diseases, primarily among animals. There are very few studies, however, that convincingly demonstrate similar findings for invasive soil microbes [37]. However, invasive microbes that act as disease agents in plants clearly show biogeographic patterns of evolution with hosts. The majority of these are fungal pathogens capable of residing in the soil rhizosphere

[38], and many of these are invasive [37].

Cryphonectria parasitica causes chestnut blight. Introduced from Eurasia, this fungal pathogen almost drove the North American chestnut (*Castanea dentata*) to extinction [39]. The effects on *Castanea* hosts in the home range of the fungus are not as devastating. Hybridization among non-pathogenic soil fungi once biogeographically isolated, but brought together through human activity, might also result in new plant disease epidemics. For example, hybridization among previously isolated species within the genus *Phytophthora* is credited for the evolution of pathogenic isolates responsible for sudden oak death and potato blight [40]. The pathogenic *Phytophthora* isolates contain hybrid genotypes from non-pathogenic strains. Similarly, threats by invasive soil fungal pathogens were attributed to hybridization and the introduction of biological controls for invasive plants [41]. Hybridization among biogeographically isolated microbial taxa that results in more aggressive offspring suggests that everything is not everywhere.

Plant-soil feedbacks

The reciprocal effects of soil biota and plant species on each other in the same soil volume vary over time due to accumulation or attenuation of particular soil taxa that either harm or help the species. These changes are called “feedbacks” [42], and disproportional accumulation of pathogenic or parasitic soil taxa leads to negative feedbacks, while the disproportional accumulation of mutualistic taxa leads to positive feedbacks. These feedbacks show biogeographical patterns that are consistent with the notion that soil microbial communities are not the same in different parts of the world. For feedbacks involving invaders, the direction is typically neutral to positive, while feedbacks for native species are most often negative [43].

If everything were everywhere, plant-feedbacks should not show this general biogeographical difference.

A recent review found that invasive plants in invaded ranges experienced fewer negative soil feedbacks than either native species or exotic non-invasive species [44]. Similarly, comparison of plant-soil feedbacks between 10 taxonomic pairs of native and introduced old-field plants showed that exotics experienced far fewer negative soil feedbacks than natives [45]. Invasive exotics were not differentiated from non-invasive exotics or resident congeners; however, three of the four species in this study are well-recognized invaders and had the strongest positive feedbacks relative to congeners. Additionally, *Carpobrotus edulis* grown in sterilized soil had a 32% reduction in biomass when inoculated with rhizosphere soil from the native range (southern Africa), while no effect on biomass was observed when inoculated with rhizosphere soil from the invaded range (Mediterranean region) [46].

To date, only five invasive species have been subjected to biogeographical comparison, and all showed evidence for stronger negative feedbacks in home versus invaded soils [16,47-49]. However, it is important to point out that total soil biota used in some of these studies includes a wide range of taxa, and in some cases might incorporate effects of higher organisms (i.e., soil-dwelling invertebrates) which are taxa that have never been thought free from biogeographical constraints. Regardless, these consistent directional differences in plant-soil feedbacks between native and invaded ranges (less negative in the invaded range) strongly suggest that ecological functions of soil microorganisms (as measured by various microbially-mediated parameters like nutrient cycling, litter decomposition, etc.) have biogeographic patterns - everything is not everywhere. Plant-soil feedbacks suggest that microbial biogeography is both a function of limitations to dispersal and environmental selection on evolutionary trajectories.

These factors will remain intertwined until we can improve our technical abilities to sample at the micro-scale, or until we can identify two habitats that are identical at the micro-scale yet geographically isolated [50].

Conclusions

Separating the species specificity of plant-microbe interactions from the effect of invasions *per se* requires much more resolution, yet interactions between invasive plants and soil microorganisms, especially those compared between native and invaded ranges, support recent genetic evidence that soil microbes have evolved biogeographic patterns similar to those for macro-organisms. Perhaps dispersal is far more limited than previously thought, or rapid evolutionary relationships between soil microbes and plants might be occurring over a short enough time scale such that dispersal cannot disrupt the effects of geographic isolation. The “selection” part of Baas Becking’s famous quote has been interpreted as environmental filtering of a globally homogeneous species pool, but growing evidence suggests that evolutionary selection is occurring. Regionally specific patterns of plant-soil-microbial interactions, detectable in part through the “natural experiment” of exotic plant invasions, are revealing a geographical mosaic [24] of apparent evolution among plants and microbes in which everything is not everywhere.

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FIGURE LEGENDS

Box 1. Defining a microbial “species”: the evolution of the microbial species concept and the “everything is everywhere” debate

Augustin Pyramus de Candolle (1778-1841)

- Stated “lower organized taxa” were not dispersal limited and were ubiquitous
- Lower taxa included fungi, lichens, and algae.
- Foundation for the “everything is everywhere” debate.

Charles Darwin (1809-1882)

- Supported de Candolle’s assertion in *On the Origin of Species*, “...the lower any group of organisms is, the more widely it is apt to range” (1859).

Ferdinand Cohn (1828-1898)

Classified bacteria as plants; placed microscopic organisms (bacteria and fungi) into the “lower organized taxa” distinction of de Candolle.

Robert Koch (1843-1910)

- Developed Koch’s Postulate, which demonstrated consistent effects of microorganisms under identical physiological conditions supporting the uniformity assertion for microbial distributions.

Martinus Beijerinck (1851-1931)

- Empirically demonstrated the cosmopolitan nature of microbes, reinforcing the ubiquity of microbial taxa and the role of the environmental filter.

Lourens Baas Becking (1895-1963)

- Stated, “*Everything is everywhere; but the environment selects*” in 1934.
- This “law” was formulated based on the empirical laboratory work of Beijerinck and observational studies of salt lake microbes (algae and crustaceans).

Bergey’s Manual of Determinative Bacteriology (1st ed.1923- 8th ed.1994)

- This publication is the primary resource, from the first edition in 1923 to the eighth edition in 1994, for the identification of bacterial species.
- In the initial editions, attempts to classify prokaryotes according to phylogenetics were disregarded in lieu of phenetic taxonomy.
- As the definitive guide for bacterial species, the manual influenced the disregard of microbial biogeography until the late 1970’s with emergence of molecular techniques

CHAPTER 3

An Invasive Plant Paradox*

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INTRODUCTION

Why some plants can attain extremely high densities in communities where they are exotic yet remain at low densities in their native ranges is a mystery. This pattern has been called a “paradox” because it conflicts with long held ideas about the importance of local adaptation for the ecological performance of organisms (1). We find that this biogeographical shift may be connected to other apparent ecological paradoxes that occur with plant invasions; paradoxes involving processes mediated by soil microbes. Invasions decrease plant species diversity yet they also consistently increase productivity. Rather than depleting soil resources as productivity increases, invasions are often correlated with increased soil stocks, pools, and fluxes of nitrogen; all are transformations regulated by microbial communities.

Plant species richness and functional diversity increases local net primary productivity (NPP) (Fig. 1), predominantly through more complete utilization of resources, or “niche complementarity” (2). Exotic plant invasions locally reduce native plant diversity, often to the point of near monocultures (*e.g.* 3). However, contrary to what diversity-productivity experiments would predict, NPP typically increases with exotic invasions (4-6) (Fig. 1). In a recent meta-analysis of 94 studies, the average increase in annual NPP was over 80% in invaded ecosystems (6). This “invasion-diversity-productivity” paradox cannot be explained by “niche complementarity”, but differences in plant-soil-microbe interactions in the invaded and native ranges could provide part of the answer. Soil microbes can have strong density-dependent

effects on plants, often called plant-soil-microbe feedbacks (7). These feedbacks are virtually always neutral or negative for plants in soils from their native ranges, but are frequently positive for invasive plants in soils from invaded ranges (8, 9). This directional shift observed for invasive plants is likely due to the absence of evolved plant-pathogen relationships that are species specific (9). The absence of density-dependent effects of pathogenic microbes on plant populations likely enhances competitive dominance of plant species in new ranges and increases plant productivity.

Nitrogen is the primary factor limiting NPP in most ecosystems (10) and short-term increases in NPP (such as those produced by agricultural) typically deplete nitrogen and other soil resources. Contrary to this, plant invasions appear to increase soil nitrogen pools and total ecosystem nitrogen stocks (6, 11, 12). Ecosystem pools and stocks of nitrogen are regulated by the activity of soil-dwelling and mutualistic microbes. On average, invaders increase litter decomposition rates by twofold, and increase both soil nitrogen mineralization and nitrification by over 50% (6). For example, the invasive trees *Acer platanoides* and *Ailanthus altissima* increase net nitrogen mineralization, net nitrification, and soil nitrogen availability compared to native tree species, including the congener *Acer saccharum* (13).

How invasive plants might decrease species diversity, increase NPP, and increase soil nitrogen is difficult to explain in the context of our current understanding of these ecosystem functions. Invaders might possess general morphological or biochemical traits that consistently differ from those of native species in ways that increase nitrogen cycling. For example, if thinner chlorophyll-enriched leaves lower in structural carbon are important traits for invasive success, then higher deposition rates or increased litter quality (14) could explain increased nitrogen pools, stocks and fluxes. However, invaders vary widely in leaf traits and invasive plant

species do not appear to initiate the same chain of ecosystem changes in their home ranges. For example, *Spartina alterniflora* is native to eastern North America but is an aggressive invader in China where it has greater NPP and a greater leaf area index (5). Reciprocally, *Phragmites australis* is native to China but is a highly successful invader in North America where it has greater NPP (5). We know very little about these kinds of biogeographical differences of invasive plants on ecosystems. However, if invasive species enhance NPP and nitrogen cycling in invaded ranges but not in their native ranges, then the inherent traits of plants are unlikely to drive these processes.

Alternatively, invasive plants may undergo bottlenecks, or natural selection for these traits occurs only in invaded ranges. For example, *Ageratina adenophora*, an invader throughout the subtropics, appears to have evolved increased nitrogen allocation to photosynthesis and reduced allocation to cell walls in the absence of specialist herbivores (15). This would make leaves easier to decompose and suggests a potential mechanism by which invaders might possess leaves with traits that enhance nitrogen cycling in invaded ecosystems.

In the scenarios hypothesized above, soil microbes might simply be passengers in the process of increasing nitrogen pools and fluxes. However, invaders and soil microbes might interact in a biogeographically explicit way that allows the microbial community to drive changes in the nitrogen cycle that occur with plant invasions. These shifts in plant-soil-microbe feedbacks with invasions suggest that communities of soil microbes and plants have regional evolutionary trajectories in different parts of the world. If microbial communities responsible for the various ecosystem process discussed above (nitrogen fixation, nitrification, ammonification, and decomposition) interact with invasive plants in ways determined by evolution and biogeography, then this may help to explain the apparent paradox of increased nitrogen pools

and fluxes with plant invasions. To our knowledge, there are no biogeographical comparisons of soil microbial communities or of the processes by which they drive plant invasions, specifically in native and invaded ranges. However, one study demonstrated that invasion by exotic grasses corresponded with increased soil nitrification rates and higher abundance and diversity of ammonia-oxidizing bacteria in invaded ranges (16). Additionally, nitrification rates were positively correlated with changes in the bacterial community, suggesting a mechanism for increased nitrogen cycling in these invaded soils.

Through a series of apparent paradoxes in the way they affect native ecosystems and interact with native microbial communities, invasive plants offer unparalleled opportunities to reexamine ecological and evolutionary paradigms. We remain on the cusp of realizing these opportunities; as our understanding of microbial biogeography and associated functional differences expands, we may learn much about regional evolutionary relationships among plants and soil microbes and how this affects ecosystem functioning.

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FIGURE LEGENDS

Figure 1: Diversity and productivity. Plant productivity increases to an asymptote as plant diversity increases [solid line; derived from (2) with permission from the Ecological Society of America]. Higher productivity correlates with losses in native species richness, and invasives dominate [dashed line; estimated from (6); see (17)]. The asymptote remains higher due to invader presence in the system at lower relative densities. (Inset) The photo shows *A. adenophora*.

FIGURES

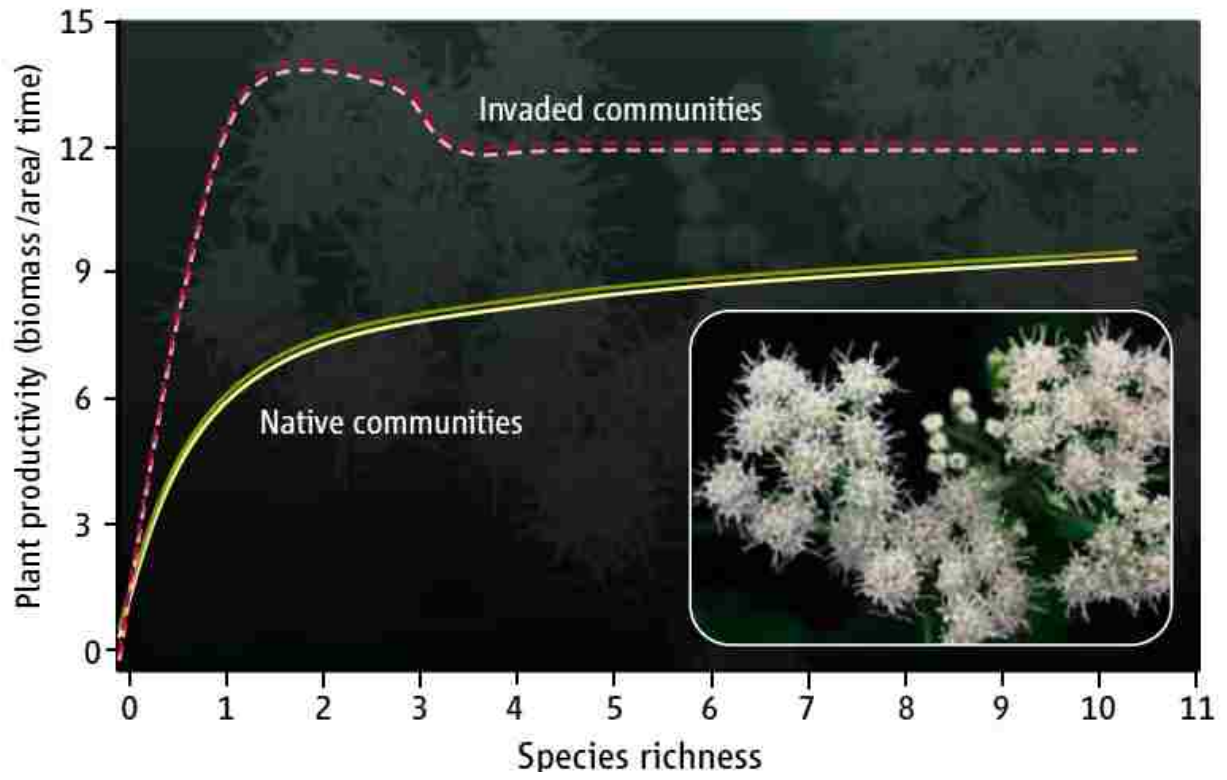


FIGURE 1

CHAPTER 4

The invasive *Sorghum halepense* harbors endophytic N₂-fixing bacteria and alters soil biogeochemistry*

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ABSTRACT

Exotic plants invading new habitats frequently initiate broad changes in ecosystem functioning. *Sorghum halepense* is an invasive grass capable of growing in nitrogen (N)-poor prairie soils that creates near monocultures in once phylogenetically diverse-communities. The biogeochemistry of soils invaded by *S. halepense* was compared to that of un-invaded native prairie soils. Invaded soils contained 2-4 times greater concentrations of alkaline metals, micronutrients, and essential plant nutrients than native prairie soils. The notable exception was Ca⁺², which was always significantly lower in invaded soils. The N-content of *S. halepense* above-ground biomass was 6.4 mg g⁻¹ (320 mg N plant⁻¹) and suggested a supplemental N source supporting plant growth. Altered soil biogeochemistry in invaded areas coupled with high above-ground biomass in N-poor soils suggested N₂-fixing activity associated with *S. halepense*. Nitrogenase activity of plant tissues indicated that N₂-fixation was occurring in, and largely restricted to, *S. halepense* rhizomes and roots. A culture approach was used to isolate these N₂-fixing bacteria from plant tissues, and 16s rRNA gene sequencing was used to identify these bacterial isolates. Nitrogenase activity of bacterial isolates indicated several were capable of N₂-fixation. In addition to N₂-fixation, other roles involved in promoting plant growth, namely mobilizing

phosphorus and iron chelation, are known for closest matching relatives of the bacterial isolates identified in this work. Our results indicate that these plant-growth promoting bacteria may enhance the ability of *S. halepense* to invade and persist by altering fundamental ecosystem properties via significant changes in soil biogeochemistry.

Keywords

N₂-fixing bacteria, endophytes, invasive plants, soil biogeochemistry, *Sorghum halepense*

Abbreviations

PBS Phosphate buffered saline

ARA Acetylene reduction assay

INTRODUCTION

The modification of resource availability (here broadly considered as nutrient cycling) by invasive plants is well documented (Vitousek and Walker 1989; Evans et al. 2001; Kourtev et al. 2002; Ehrenfeld 2003; Reinhart and Callaway 2006) and considerable attention has been focused on nitrogen (N) cycling, one of the most basic of ecosystem processes (Vitousek et al. 1987; Stock et al. 1995; Yelenik et al. 2004). In many cases, an invasive or newly introduced species may modify the habitat by simply adding a new functional trait to the ecosystem; for example, introduction of the N-fixing tree *Myrica faya* into N-limited volcanic soils where there were no or few native N-fixing plants introduced a completely novel ecosystem process, which subsequently modified the habitat (Vitousek 1986; Vitousek and Walker 1989). Alternatively, an invasive or newly introduced species may not introduce a new functional trait, but may instead alter existing nutrient cycles. For example, in ecosystems supporting a variety of N₂-fixing plants, invaders altering the N-cycle may bring about new interactions with soil microbial communities (Reinhart and Callaway 2006) or modify existing feedback loops (Klironomos 2002) that affect species diversity and overall ecosystem functioning. In either case, the changes in nutrient cycling brought about by invasive plants shed new light on the mechanisms leading to successful invasions.

Sorghum halepense is a globally-distributed allelopathic invasive-grass that resists displacement once established (Holm et al. 1977, Bais et al. 2006). This successful invader has many properties which suggest that it has a high N-demand and may impact the soil biogeochemistry of areas it invades. Most obvious among these properties is the presence of the N-rich constitutive-defense chemical, dhurrin, contained within leaves. In addition to containing dhurrin, *S. halepense* also has considerable above-ground biomass; it often grows as a densely-

packed monoculture, achieving an abundance of >90 ramets m^{-2} (Rout 2005) and exceeding 2 m in height (McWhorter 1981). In plants harvested from Fort Worth Prairie (south central U.S.), the N-content of *S. halepense* above-ground biomass was 6.4 mg g^{-1} , or $320 \text{ mg N plant}^{-1}$ (M. Rout, unpublished data). Across the southern portions of the U.S., *S. halepense* establishes and expands rapidly; surprisingly, even in soils that are exceptionally N-poor (as in Fort Worth Prairie soils where total N is $\sim 0.008 \text{ g kg}^{-1}$, Rout 2005). It seems paradoxical that this highly productive grass can persist and expand in N-poor soils. The N-content of the plant, when combined with large above-ground biomass and high abundance, suggests that *S. halepense* alters the N-availability, and consequently N-cycling, in areas in which it invades.

In this work we investigated the role of *S. halepense* invasion on soil biogeochemistry. We measured soil nutrient pools in areas invaded by *S. halepense* and compared them to soil nutrient pools in adjacent native-prairie. Dramatic differences in the pools of several elements, particularly N as nitrate (NO_3^-) between native and invaded prairie, prompted us to explore the hypothesis that this invasive grass harbors N_2 -fixing bacteria. We present the first evidence of the presence of N_2 -fixing and other growth promoting bacteria living endophytically within *S. halepense* rhizomes and those closely associated with roots. Based on the presence of these bacteria and the changes in nutrient pools of invaded soils, we present a speculative model describing a positive nutrient-cycling feedback loop based on N-cycling that might explain the modification of resource availability and bring about the observed changes in the biogeochemical environment.

MATERIALS & METHODS

Study site

The ~1,400 ha Fort Worth Nature Center and Refuge (32° 84' N, 97° 47' W, FWNCR) is located in Fort Worth, TX. The refuge stretches across an ecotone between Fort Worth (Grand) Prairie and Western Cross Timbers. Fort Worth Prairie is characterized by the dominant vegetation, *Schizachyrium scoparium* (little bluestem, Diggs et al. 1999), and has a shallow-gravel alkaline clay-based soil underlain by limestone (hereafter, native prairie). A section of the prairie (~12 ha) is undergoing extensive invasion by *S. halepense* (hereafter, invaded prairie). Historically (~25 years ago), this site was a contiguous native prairie remnant, devoid of the invasive *S. halepense* (S. Tuttle, FWNCR, personal communication). Now, there is a clear and unmistakable shift in the vegetation of the ecosystem: *S. halepense* is advancing as a distinct invasion wave and displacing the dominant native flora (Rout 2005). The invasion of *S. halepense* into native prairie creates a transition zone between invaded and native prairie that is characterized by *S. halepense* emergence from rhizomes in areas where the plant was not previously found. Along some sections of the invasion front, *S. halepense* has advanced into native prairie at a rate of approximately 3 m yr⁻¹ (Rout 2005). We functionally defined the transition zone as that area between native and invaded prairie where *S. scoparium* and *S. halepense* were equally abundant based on percent cover (data not shown).

Soil nutrient analyses

Soil analyses were conducted on soils collected quarterly spanning a one-year period; November 2006, through November (inclusive) 2007. Soil cores (3 to 5 discrete, ~3 cm diameter, 13 cm deep) were collected from replicate 1 m² plots (n = 4) established within native-prairie, invaded-prairie, and the transition zone (N = 12). Cores from each plot were pooled into a composite sample, air dried, homogenized, and plant debris removed. Soil analyses of pH, exchangeable

alkaline metals (Ca^{2+} , Mg^{2+} , K^+ , and Na^+), micronutrients (Zn^{2+} , Fe^{2+} , Cu^+ and Mn^{2+}), and essential plant nutrients (N as NO_3^{2-} and phosphorus as available PO_4^{3-}) were conducted commercially (Texas A&M University Soils Lab). Brief descriptions of the methods used by this facility are available at <http://soiltesting.tamu.edu/>.

Nitrogenase activity associated with *S. halepense* tissues

Rhizomes were harvested from random areas of invaded prairie in February 2006, planted in an initially-sterile sand-vermiculite mix, and grown under greenhouse conditions until plants attained a height of ~30 cm (~90 days). Leaves, roots, and rhizomes (0.25 – 0.75 g) were rinsed in sterile water (Milli-Q) to remove loose soil and surface debris. Tissues were surface sterilized (30 s sequentially in 1% Chloramine-T detergent solution, 95% ethanol, and 1.6% hypochlorite; tissues were rinsed 3x with sterile water between each step). The outer, coarse surface layer of rhizomes was removed after surface sterilization, leaving only tissue that did not come in contact with the soil. Plant tissue was aseptically transferred to glass tubes (16 x 150 mm) containing 200 μL sterile water and fitted with serum stoppers. Air (10% of headspace) was removed from each tube, replaced with an equal amount of acetylene gas, and incubated (26°C, 24 h). Acetylene conversion to ethylene (ARA) was determined using gas chromatography (SRI Instruments). Tubes without plant tissue, but injected with acetylene, served as controls. This procedure was repeated on rhizomes harvested from three randomly selected areas of invaded prairie in April 2008. Segments of individual rhizomes (0.6 – 2.0 g) were rinsed, surface sterilized, and outer tissue layers removed (as detailed above), and assayed for nitrogenase activity using ARA (Shimadzu GC-2014). An additional set of controls (in triplicate) were used in this assay to control for endogenous ethylene production, which consisted

of tubes containing rhizomes without acetylene injection.

Isolation and identification of bacteria associated with *S. halepense* tissues

Three ~30-cm diameter, 15-cm deep plots of *S. halepense* were harvested on two occasions during the summer (2006) from separate, randomly-selected areas of invaded prairie and transported to nearby laboratory facilities. Tissues (~0.25 g of leaves, roots, and rhizomes) were surface sterilized, and ground (sterile mortar and pestle) in 1 mL sterile phosphate buffered saline (PBS). Outer surfaces of rhizomatous tissues were removed (detailed above) after surface sterilization. Extracts from each tissue type were diluted (10^0 - 10^{-7} in PBS in 10^{-1} steps) and 0.1 mL of each dilution was inoculated into N-free semisolid medium (JNFb, Baldani et al. 1996) and incubated (30°C, 4 d). Turbidity or distinct below-surface veils or pellicles were taken to represent growth. Subsamples of cultures showing positive growth were plated (0.1 mL) onto JNFb yeast-extract agar (Baldani et al. 1996), colonies isolated (2 d growth), and repurified. Isolates were grouped by colony characteristics to yield 19 distinct morphotypes. Of these, the five that were most frequently isolated from plant tissues (i.e. the most frequently recovered) were identified by 16S rRNA gene sequencing.

Bacterial genomic DNA for comparative sequence analysis was extracted from pure cultures using the Ultra Clean Microbial DNA Isolation Kit (MoBio). Approximately 1400 nt of the 16S rRNA gene was amplified using the general bacterial primers, 27f (AGAGTTTGATYMTGGCTCAG) and 1492r (TACGGYTACCTTGTTACGACT). PCR products were purified (Ultra Clean PCR Clean-Up Kit, MoBio) to remove dNTPs and non-target DNA, and sequenced using the AB3130xl Genetic Analyzer (Applied Biosystems) at the University of Montana, Murdock DNA Sequencing Facility. Phylogenetic analysis was

performed using Laser Gene DNASTar software version 5.01 and sequences were aligned using the Ribosomal Database Project II (RDP, Cole et al. 2007) SeqMatch program incorporating closely related sequences from GenBank (<http://www.ncbi.nlm.nih.gov/entrez>).

Nitrogenase activity associated with bacterial isolates

Pure cultures of isolates were each inoculated into JNFb medium and incubated (30°C, 4 d) prior to ARA measurements (Shimadzu GC-2014) using methods described above, with the exception of incubation time (1 hr). Uninoculated JNFb medium injected with acetylene served as negative controls. *Hebaspirillum seropedicae* (ATCC 35892) was used as a positive control.

Statistical analyses

Soil biogeochemistries were analyzed separately with one-way Analyses of Variance (ANOVA; three levels of treatment application: native prairie, transition zone, invaded prairie). Normality and homogeneity of variances were examined and data were transformed when appropriate. Kruskal-Wallis ANOVA on ranks was applied when data failed normality or equal variance tests. Post hoc means comparisons were conducted using Tukey's HSD (ANOVA) or by Dunn's method (Kruskal-Wallis). All statistical analyses were conducted using SigmaStat 3.11 (Systat Software, Inc., San Jose, CA).

RESULTS

Soil nutrient analyses

Soils collected from within each prairie type were similar throughout the entire sampling period; in most cases the variability associated with alkaline metals, micronutrients, and essential plant-

nutrients was less than 10% of the mean (Fig. 1). Invaded and transition-zone soils had pH values slightly, but significantly, lower than those of native soils. The nutrients Mg^{2+} , K^+ , Zn^{2+} , Fe^{2+} , Mn^{7+} , NO_3-N and PO_4-P were significantly greater in invaded soils compared to native soils. In most cases, the concentration of a nutrient element was 2 to 4 fold greater in invaded soils than in native soils. The concentration of several nutrients increased progressively from native prairie through the transition zone and into fully invaded prairie (Fig. 1). Calcium was the only nutrient element significantly lower in invaded prairie than in native prairie. This pattern remained consistent throughout the study period and low standard errors of the means indicate that the observed patterns were not the result of a single pulse-event.

Nitrogenase activity associated with *S. halepense* tissues

The ARA indicated strong nitrogenase activity was associated with rhizomes collected from plants grown in the greenhouse (86% positive, max = $27 \mu\text{mol C}_2\text{H}_4 \text{g}^{-1} \text{d}^{-1}$, n = 7). Greenhouse grown plants also showed nitrogenase activity was associated with roots (57% positive, max = $3 \mu\text{mol C}_2\text{H}_4 \text{g}^{-1} \text{d}^{-1}$, n = 7) and leaves (23% positive, max = $0.9 \mu\text{mol C}_2\text{H}_4 \text{g}^{-1} \text{d}^{-1}$, n = 4), but the signal was variable and low compared to activity associated with rhizomes.

Similarly, the ARA indicated nitrogenase activity associated with field collected rhizomes (85% positive, n = 13). Nitrogenase activity was variable and rates of acetylene reduction were similar to those obtained from rhizomes collected from greenhouse grown plants. Nitrogenase activity ranged between 0 and $3.9 \mu\text{mol C}_2\text{H}_4 \text{g}^{-1} \text{d}^{-1}$ and the activity of nitrogenase positive rhizomes averaged $0.97 \mu\text{mol C}_2\text{H}_4 \text{g}^{-1} \text{d}^{-1}$ (n = 11).

Isolation and identification of bacteria associated with *S. halepense* tissues

Bacteria capable of growth in N-free medium were isolated from leaves, roots, and rhizomes. We cultured 54 isolates which were grouped by colony characteristics into 19 distinct morphotypes. Of all 54 original isolates, the majority were isolated from rhizomes (56%) and roots (41%). We selected five of the 19 morphotypes to be identified using 16s rRNA gene sequencing. These five morphotypes were chosen because they contained over 50% of the 54 original isolates. Their identities, the plant tissues they were isolated from, percent similarity match, and sequence match score (S_{ab}) are given in Table 2. A similarity score reports the percent sequence identity over all pairwise comparable positions when aligned with RDP sequences. The S_{ab} scores are the number of unique 7-base oligomers shared between the submitted sequence and a given RDP sequence, with the lowest number of unique oligos in either of the two sequences in the denominator. The rank order may differ between S_{ab} and pairwise identity scores; the top S_{ab} scores contain the nearest match sequence ~95% of the time (Cole et al. 2007). The percent similarity matches listed are all >97% to type strain isolates of near-full-length sequences (≥ 1200 bp). Given the sequences obtained in this work were near-full-length 16s rRNA sequences (1323 – 1445 bp), but were not perfect matches to the known type strains, it is possible that the bacterial isolates we obtained may be unique strains (Fox et al. 1992; Stackebrandt and Goebel 1994). We intend to fully characterize these isolates in future research.

Nitrogenase activity associated with bacterial isolates

The ARA indicated nitrogenase activity associated with three of the five bacterial isolates recovered from plant tissues (Table 3). Positive rates of nitrogenase activity ranged between 0.144 – 0.480 nmol C₂H₄ mL⁻¹ h⁻¹; these rates range between 25 % of, to greater than 100% of those recorded for the positive control (*H. seropedicae*). All bacterial isolates had turbidity

(distinct below-surface veils or pellicles) at the time of the ARA. Nitrogenase activity for negative controls (2) were averaged and subtracted from all ARA measurements reported for the bacterial isolates in Table 3. Since the closest match relatives of several isolates showing positive results for nitrogenase activity have not been previously reported as N₂-fixing organisms, it is likely the isolates obtained in this work may be unique strains.

DISCUSSION

There is a clear shift in the plant community structure of prairie ecosystems invaded by *S. halepense*. Growing as virtual monocultures, this grass rapidly displaces native plant communities in Fort Worth Prairie (Rout, 2005). Our work shows that prairie soils associated with *S. halepense* also undergo dramatic changes in resource availability resulting in increased concentrations of alkaline metals, micronutrients, and essential plant-nutrients (Fig. 1).

Interestingly, ANOVA revealed that the biogeochemical signatures of invaded prairie soils often differed significantly from that of native prairie soils despite their close proximity (<50 m) and shared geologic origins. The shift in biogeochemical signatures of these soils may be a consequence of organic acids released by *S. halepense* through root-rhizome exudates (see Bias et al. 2006); however, the N required to support the biomass of this plant suggests involvement of microbial processes associated with the N-cycle, specifically nitrification. We initially suspected that *S. halepense* invasion modified the habitat to promote the growth of free-living N₂-fixing bacteria which would subsequently supply the plant with the necessary N to support growth. However, a preliminary experiment using enrichment cultures suggested that free living N₂-fixing bacteria were lower (3x) in invaded soils compared to native soils (data not shown). An alternative source of N to support *S. halepense* growth could be from bacteria associated with

the formation of nodules (primarily seen in legumes). There are many non-legume species that can form nodules when in association with the actinomycete, *Frankia* (Lambers et al. 1998) as well as one non-legume taxon, *Parasponia*, that forms nodules when it associates with *Rhizobium* (LaFay et al. 2006). Since *S. halepense* is a non-leguminous plant that does not show the presence of nodules, we explored the possibility that it is associated with endophytic N₂-fixing bacteria, which have often been found within grasses (Reinhold-Hurek and Hurek 1998).

Other grasses are known to harbor N₂-fixing bacteria belonging to the genus *Herbaspirillum* (Kirchhof et al. 2001). *Sorghum bicolor*, one of the hybridization parents of *S. halepense*, harbors bacteria of this genus as endophytes (Baldani et al. 1996; James et al. 1997; Kirchhof et al. 2001). Thus it seemed likely that members of the genus *Herbaspirillum* would be associated with *S. halepense*. The culture techniques we utilized were specifically designed to recover members of the *Herbaspirillum* genus; however, *Herbaspirillum* was not recovered from *S. halepense*. We further probed for members of this genus via molecular techniques. DNA was extracted from rhizomes that were surface sterilized, as well as from those that were not surface sterilized, and probed for *Herbaspirillum* using the genus specific primer HERB68 (AGCAAGCTCCTATGCTGC) coupled with the general reverse primer 907r (Feris et al. 2003). Again, *Herbaspirillum* was not detected. It is possible that members of the *Herbaspirillum* genus were present, but at concentrations below detection.

The presence of endophytic and closely-associated root N₂-fixing bacteria was confirmed by nitrogenase activity associated with *S. halepense* rhizomes, roots, and pure culture bacterial isolates from these tissues. In the case of rhizomes, nitrogenase activity was confirmed in both greenhouse grown plants and plants collected directly from field sites. While the absolute rates of nitrogenase activity may be biased (see caution below), we were able confirm (both by culture

methods and ARA) that N₂-fixing bacteria were closely associated with plant roots and were living endophytically within rhizomes. Some caution must be applied when interpreting rates of nitrogenase activity associated with plant tissues since relatively long incubation times were used for these analyses (James 2006 and citations therein). We utilized shorter incubation times when assessing nitrogenase activity of bacterial isolates in pure culture (1 hr). Surprisingly, the known N₂-fixing isolate *Agrobacterium tumefaciens*, did not demonstrate nitrogenase activity when growing in JNFb medium. Nitrogenase activity was assessed on four day old cultures; thus, it is possible that the cultures capable of growth on the N-free medium but not testing positive for nitrogenase activity (*A. tumefaciens* and *Xanthomonas melonis*) were no longer actively fixing N₂ at the time of the ARA. Collectively, these findings suggested the presence of N₂-fixing organisms within the rhizomes of *S. halepense*; no such N₂-fixation has been reported for this invasive grass.

We did not seek to exhaustively examine the microflora associated with the plant. It appears that N₂-fixing bacteria are associated with *S. halepense* roots and rhizomes and these bacteria seem to be actively fixing N₂ (indicated by the ARA on pure cultures) and altering the N-cycle in invaded areas. It is likely that additional N₂-fixing bacteria were associated with the plant tissues and that these bacteria were not isolated by culturing, or were overlooked by our screening to simply detect and select the most common N₂-fixing bacteria. In addition to N₂-fixation, several interesting ecological roles are known for the closest matching organisms of the bacterial isolates recovered. These ecological roles include iron siderophore production, the ability to solubilize phosphate, and plant pathogenicity (listed in Table 2).

Through its association with bacteria, the plant appears to alter plant species diversity and resource availability, which subsequently modifies the habitat within the remnant prairie. Unlike

the situation where a newly introduced species adds a new functional trait to the ecosystem, the attribute of N₂-fixation in *S. halepense* does not introduce a new functional trait into this prairie ecosystem. Several native plant species harboring nodulating N₂-fixing bacteria co-exist in Fort Worth Prairie, including the tree *Prosopis glandulosa*, the forbs *Chamaecrista fasciculata*, *Indigofera miniata*, and several members of the *Lupinus* genus. Yet, *S. halepense* displaces this diverse native community along with the dominant native grass, *Schizachyrium scoparium* (Rout 2005). Our work shows alterations to resource availability coincide with heavily invaded areas. Clearly, the plant or the plant-microbe interaction brings about a change in the habitat.

The competitive ability of *S. halepense* (through the plant N-requirements) may be enhanced by microbial activities of closely-associated bacteria and the secondary microbial processes which condition soils and favor the persistence of this successful invader. A conceptual model of these processes is shown in Fig. 2. This conceptual model focuses on plant-soil feedback systems that seem to be driven by plant growth promoting bacteria and processes associated with soil microflora. A similar, but more advanced conceptual model has been proposed for the role of plant growth promoting bacteria in mangrove ecosystems (Bashan and Holguin 2002). Novotny et al. (2007) recently reported findings that lend support to our general model. They found that N-availability, changes in CO₂ levels, and changes in plant community diversity interact to affect both above-ground and below-ground processes. Additional research will be required to separate the effects of root exudates from those connected to plant growth promoting bacteria.

Many hypotheses have been put forth to explain successful plant invasions, and most focus on unusual traits of the invasive plant itself (empty niche, novel weapons, adaptation to humans) or escape from regulating consumers and pathogens (summarized by Mitchell et al.

2006). Microbial associations with plants, both bacterial and fungal, are certainly not unusual. In the case of plant invasions, associations with microbes are most often thought to have negative affects on plants rather than mutualistic relationships, accounting for the expansion of an invader when it escapes microbial pathogens. We have demonstrated that the highly invasive *S. halepense* establishes and persists in exceptionally N-poor soils and appears to do so, in part, as a consequence of a relationship with endophytic and closely associated root bacteria. This consortium of bacteria contains N₂-fixers and other plant growth promoting bacteria that alter biogeochemical cycles in soils harboring *S. halepense*. In this system, it appears that the microbial partners form a mutualistic relationship with the invasive *S. halepense*, which we refer to as microbial enhanced competitive affects (MECA). This idea of a plant-microbial mutualism enhancing the success of an invasive plant may shed new light on how some invasives, like *S. halepense*, acquire a competitive advantage over native plant communities not accounted for by exploitation of an empty niche or by enemy release. Thus, a key component to understanding invasive plant establishment, persistence, and the cascade of ecosystem changes that follow, may reside literally within the plants themselves, as appears to be the case for *S. halepense*.

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Table 1 Bacteria associated with tissue types of *S. halepense*

Tissue	Isolate	Similarity (%) / S _{ab} score	GenBank type strain	Notes on potential ecological role
Rhizomes	<i>Xanthomonas melonis</i> , strain LMG8670	99.6 / .971	Y10756	Plant pathogen ^a
Rhizomes	<i>Agrobacterium tumefaciens</i>	99.3 / .974	M11223	Plant pathogen ^b , organism fixes N ^b
Rhizomes- roots	<i>Sphingobium amiense</i> , strain YT	98.2 / .890	AB047364	N ₂ -fixation in genus ^c , nonylphenol degrader ^d
Rhizomes	<i>Pseudomonas jessenii</i> , strain CIP105274	99.6 / .951	AF068259	N ₂ -fixation in genus ^e , respires NO ₃ ^e , produces Fe-siderophores ^e , solubilizes PO ₄ ^e
Rhizomes	<i>Caulobacter vibroides</i> strain (ATCC) 15252T	99.1 / .971	AJ227756	Horizontal gene transfer reported with N ₂ -fixing organisms ^f

^a Vauterin et al. 1992, ^b Kanvinde and Sastry 1990, ^c Xie and Yokota 2006, ^d Ushiba et al. 2003, ^e Garrity et al. 2005, ^f Wong and Golding 2003. All submitted sequences were >1300 bp.

Table 2 ARA nitrogenase activity on bacterial isolates recovered from *S. halepense* tissues

Isolate	Nitrogenase Activity (nmol C ₂ H ₄ mL ⁻¹ hr ⁻¹)
<i>Herbaspirillum seropedicae</i> * strain (ATCC)35892	0.411
<i>Agrobacterium tumefaciens</i>	0.007
<i>Xanthomonas melonis</i> , strain LMG 8670	0.003
<i>Sphingobium amiens</i> strain YT	0.144
<i>Pseudomonas jessenii</i> , strain CIP105274	0.480
<i>Caulobacter vibroides</i> , strain (ATCC) 15252T	0.281

Values reflect calculations after accounting for negative controls. *This organism served as the positive control .

Figure Legends

Fig. 1. Mean concentrations of various mineral nutrients extracted from soils collected in native, transition-zone, and invaded prairie. Analyses of Variance indicated that, with the exception of Na and Cu, concentrations of various elements were significantly different among the soil types (F values between 16.2 and 64.6, p always < 0.001 ; H values between 11.9 and 38.8, p always < 0.003). Pairwise comparisons of the means are indicated by letters appearing at the base of columns in each figure. Statistically different means are indicated by different letters.

Fig. 2 A conceptual model of microbial processes occurring in the rhizosphere of *S. halepense*. Rhizosphere N_2 is fixed by endophytic N_2 -fixing bacteria closely-associated with rhizomes. NH_4^+ is shuttled into plant biomass and dhurrin synthesis. Some NH_4^+ is lost to the rhizosphere and converted rapidly into NO_3^- , the preferred form of external N for *S. halepense*. A portion of the NO_3^- remains in the soil, accounting for higher soil NO_3^- in *S. halepense* invaded areas compared to native soils. NH_4^+ and NO_2^- metabolism acidifies soils and promotes dissolution of hydroxyapatite ultimately resulting in an increase in available P, Ca^{2+} , and Fe^{2+} for plant uptake. These latter processes are also promoted by action of endophytic pseudomonads and contribute to overall higher soil P and Fe, and lower Ca in invaded soils.

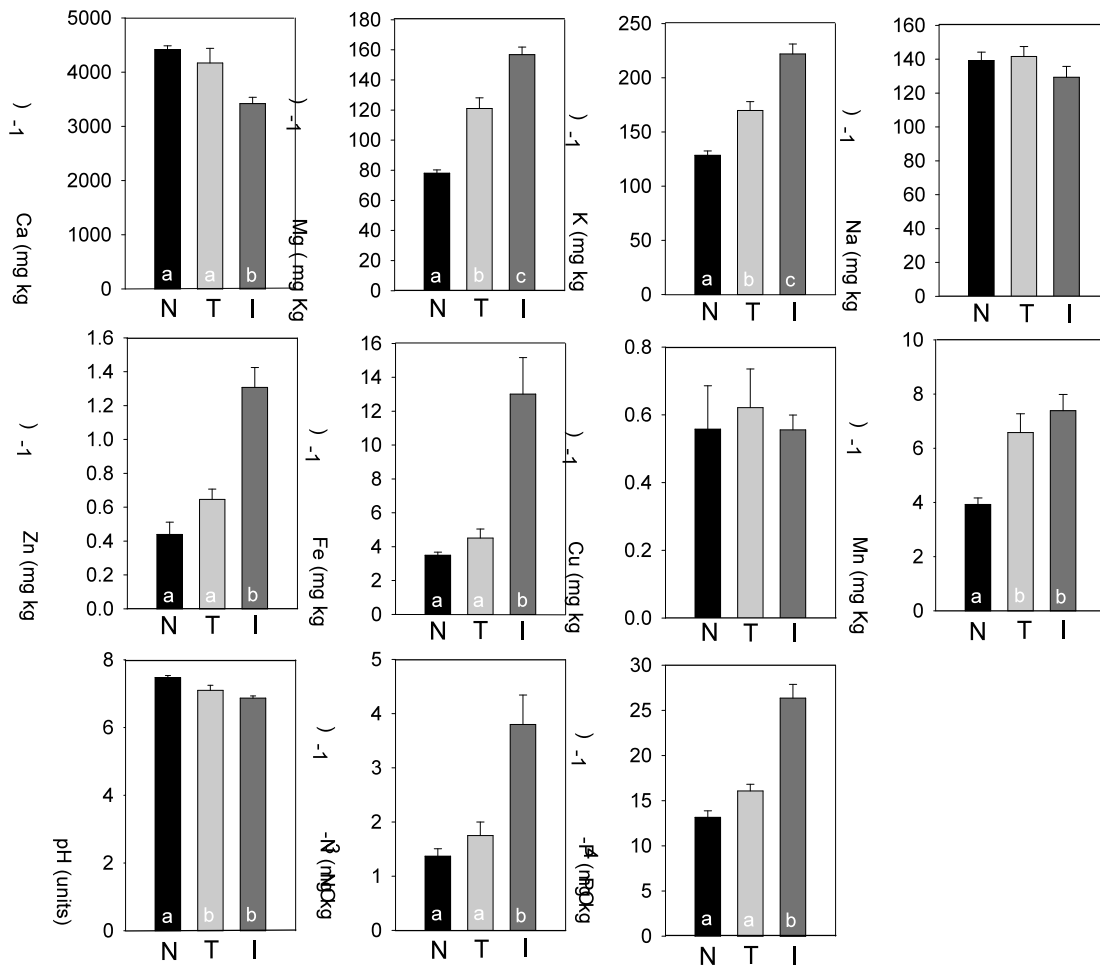


FIGURE 1

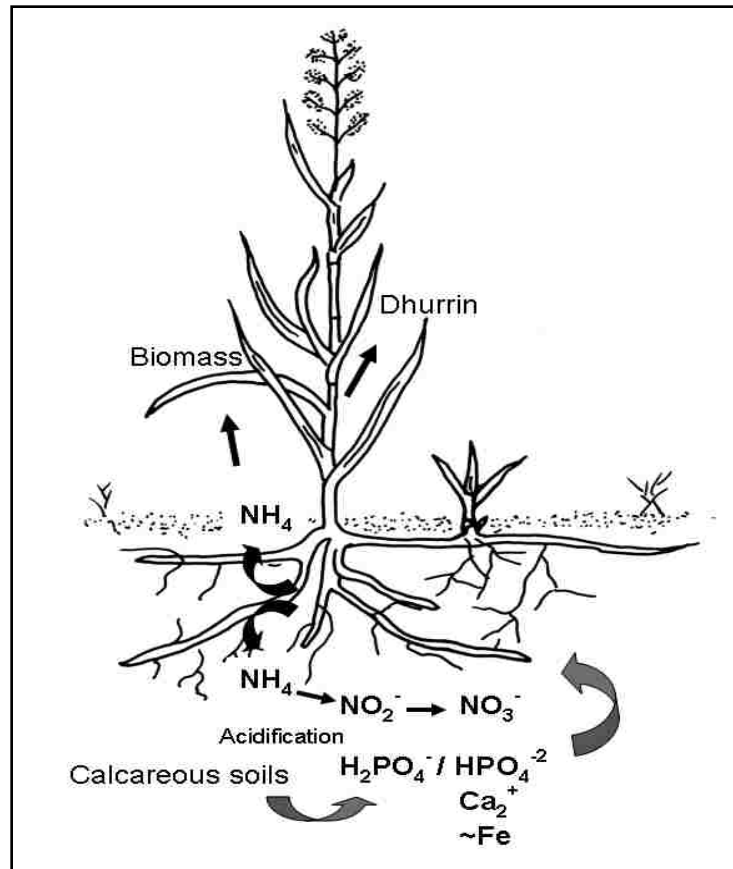


FIGURE 2

CHAPTER 5

Bacterial endophytes increase growth of *Sorghum halepense* and have physiologies that correspond with the invader's impacts on biogeochemical cycles

ABSTRACT

Invasive plants can alter soil microbial communities and profoundly alter ecosystem processes that are regulated by these microbes. The invasive grass *Sorghum halepense* has been shown to eliminate native species in North American grasslands and has strong effects on biogeochemical cycles. These effects may be due in part to the effects of N₂-fixing bacteria in the rhizomes of *S. halepense* on plant growth and the physiological effects of these bacteria on nutrient cycles. Here we found persistent, long-term alteration of eight biogeochemical cycles (including nitrogen, phosphorus, and iron) in soils recently invaded by *S. halepense*. Using five previously isolated N₂-fixing bacterial strains from *S. halepense*, we assessed *in vitro* their physiological potential to alter soil biogeochemical cycles and their ability to produce plant growth promoting substances. Two of the five bacterial isolates solubilized phosphate, all produced iron siderophores, and all produced the plant-growth hormone indole-3-acetic acid. Through a series of growth chamber studies we also showed that endophytes in *S. halepense* fixed large quantities of N and dramatically increased plant growth. We found that these bacteria were transmitted vertically within plants, and molecular analyses of bacterial community fingerprints from rhizomes indicated that endophytes can also be recruited from soils. Using the antibiotic tetracycline, we experimentally-inhibited bacterial infection in plants and found significant

declines growth rates, biomass, and in belowground resource allocation. These results are the first to demonstrate strong effects of bacterial endophytes on the growth of an invasive plant. Furthermore, this work shows that the physiological functions of these endophytes correspond closely with the effects of the invader on the biogeochemical cycles of nitrogen, phosphate and iron.

INTRODUCTION

Invasive plants can suppress or eliminate native species (Vilá and Weiner 2004, Maron & Marler 2008a, Inderjit et al., *in press*) and profoundly alter ecosystem functions (Vitousek et al. 1997; Gordon 1998; Ehrenfeld 2003; Liao et al. 2008; Rout & Callaway 2009). In a meta-analysis, Liao et al. (2008) reported that invaded ecosystems were characterized by increased concentrations of carbon (C) and nitrogen (N) in plant tissues and increased concentrations of various forms of N in soils. Others have reported that invaded ecosystems also have increased pools of various forms of soil phosphorus (P) (Ehrenfeld 2003; Allison & Vitousek 2004; Thorpe et al. 2006). Alterations of nutrient cycles, especially those associated with the N-cycle, are particularly interesting since bioavailable N is frequently limiting in terrestrial ecosystems and changes to ecosystem N pools may thus impact community structure across multiple trophic levels. Collectively, the combined increase of soil N and P pools in invaded ecosystems may partially explain one of the paradoxes associated with plant invasions: the significant increase in net primary productivity (NPP) despite the decrease in plant diversity (Liao et al. 2008; Rout & Callaway 2009).

Whether native or not to an ecosystem, plant species that form mutualisms with N₂-fixing bacteria ultimately increase soil N, (Stevenson & Cole 1999). However, N transformations within the soil N cycle are driven by microbiota and thus changes in soil N pools with invaded ecosystems are likely to be caused by major changes in plant-microbe or plant-soil-microbe interactions (Rout & Callaway 2009). Rout & Chrzanowski (2009) found that the invasive grass *Sorghum halepense* harbors N₂-fixing bacteria in its rhizomes and rhizosphere, and that soils invaded by this grass contained significantly higher concentrations of N, P, alkaline metals, and several micronutrients compared to native soils. Specific characteristics of the leaf tissues of *S.*

halepense may drive increases in rates of litter decomposition and nutrient cycling, but the endophytic bacterial symbionts may also affect biogeochemical cycling through their physiological functions, other than N₂-fixation. For example, some bacteria categorized as plant growth-promoting bacteria (PGPB) also solubilize P (Rodriguez & Fraga 1999) or can liberate iron (Fe) into bioavailable forms (Stevenson & Cole 1999), while other bacteria produce plant-growth hormones or pseudohormones (Patten & Glick 1996; Glick 1999; Hardoim et al. 2009).

Here we report on four years of altered soil biogeochemical nutrient pools occurring with *S. halepense* invasion, and measurements of several aspects of *S. halepense*-bacterial symbioses related to plant invasion and ecosystem impacts. Specifically, the effects of N₂-fixing bacteria on the growth and biomass of the invasive grass were quantified. In addition, *in vitro* physiology and metabolism measurements of the five bacterial endophytes previously isolated from *S. halepense* tissues (Rout & Chrzanowski 2009) that are relevant to the effects of the invader on soil biogeochemistry are reported.

MATERIALS AND METHODS

Site description and soil nutrient analyses

The ~ 12 ha study site was located in North Central Texas at the convergence of Fort Worth Prairie and Western Crosstimbers inside the Fort Worth Nature Center and Refuge (FWNCR) (32° 84' N, 97° 47' W, ~200 m elevation). The site is a remnant tallgrass prairie that has been undergoing extensive invasion by *S. halepense* within the last 25 years (Rout et al., accepted). *Sorghum halepense* has invaded on a broad linear front, indicating this expansion has been primarily via rhizomatous growth into native prairie, such that three general plant communities can be identified near the invasion – native prairie, transition prairie, and invaded prairie (Rout et

al., accepted).

Sorghum halepense invasion gradient and soil nutrients

Nutrient analyses were conducted on soils using methods of Rout & Chrzanowski (2009) from the same 1 m² plots used in this earlier study. Briefly, soil cores (three to five discrete, 60 cc volume) were collected quarterly throughout each year from the previously established replicate 1 m² plots inside each prairie community (native, transition, invaded; n = 4, N = 12). Cores from each plot were pooled into a composite sample, air-dried, homogenized, and plant debris removed. Soil samples were collected spanning nearly a 4-year period, November 2006 through July 2010, using methods and commercial analyses documented in Rout & Chrzanowski (2009).

Bacterial strains

The bacterial isolates harbored in the rhizomes of *S. halepense* and identified by Rout & Chrzanowski (2009) were examined for production of plant-growth-promoting substances (indole-3-acetic acid) and physiological/metabolic functions related to P solubilization and Fe chelation (hereafter iron siderophore production). These bacteria were classified as, or most closely related to the following taxa based on near full-length 16S rRNA gene homology and RDPII classification: *Agrobacterium tumefaciens*, *Caulobacter vibroides* ATCC strain 16262T, *Pseudomonas jessenii* strain CIP 105274, *Sphingobium amiense* strain YT, and *Xanthomonas melonis* strain LMG8670 (see Rout & Chrzanowski 2009 for detailed sequence analyses). Hereafter, these strains are referred to as: FWNCR-*Agrobacterium*, FWNCR-*Caulobacter*, FWNCR-*Pseudomonas*, FWNCR-*Sphingobium*, and FWNCR-*Xanthomonas*.

Plant-associated analyses

Overview: To determine whether endophytic N₂-fixing bacteria promoted plant growth via enhanced N availability or other mechanisms, it was necessary to obtain bacteria-free plants. First, we developed a seed surface-sterilization method and germinated and grew seedlings in either sterile sand or in antibiotic-amended sterile agar media. We also experimentally determined whether *S. halepense* plants were capable of recruiting bacterial partners from the surrounding physical matrix using surface-sterilized seedlings grown in sterile-sand supplemented with a variety of slurries prepared from invaded-prairie soil. The soil-slurries were differentiated as follows: 1) filtered to remove indigenous fungi, but retaining indigenous bacteria; 2) filtered to remove indigenous fungi and bacteria, then augmented with the five bacterial isolates (described above); or 3) filtered to remove indigenous fungi and bacteria. Detailed methodologies for each plant-associated microbial analysis are described in the sections below.

Seed sterilization: Seeds from 20 populations of *S. halepense* were collected from the south-central U.S. from the states of AR, LA, MS, OK, and TX in June 2007 and stored at 4°C prior to use. A random selection of seeds from these 20 populations were pooled for all experiments and surface-sterilized by soaking in sterile Milli-Q water for 5 min, followed by sequential submersion in 1% Chloramine-T detergent solution (5 min), 95% ethanol (1 min), then 1.6% hypochlorite (5 min), with rinsing 3x with sterile Milli-Q water between each step. Surface sterilization efficacy was assessed by randomly selecting 15 seeds and extensively streaking them (without tearing open the seed coat) across the surface of several standard microbiological media agar plates. Seed-streak plates were prepared in triplicate and incubated at 37°C for ~2 weeks to reveal the presence of bacterial or fungal contaminants. Six different

media were used; JNF agar (Baldani et al. 1996), R2 agar (Reasoner et al. 1979), Minimal Agar, Nutrient Agar, Mycobiotic Agar, and Potato Dextrose Agar (as described by Atlas 2004).

Experiments in sterile agar: Once surface-sterility of *S. halepense* seeds was confirmed (data not shown), the presence of bacteria inside the seeds was assessed in order to determine whether or not vertical transmission of bacteria could occur. Surface-sterilized *S. halepense* seeds were germinated on sterile Whatman #1 filter paper in sterile glass Petri dishes (90 mm diameter) and moistened with 2 mL of sterile Milli-Q water. Each dish contained 20 seeds and was sealed with parafilm and incubated in the dark at room temperature. Upon germination (7 d), seedlings were aseptically transferred into tissue-culture grade Magenta boxes (250 mL volume; Fisher Scientific) filled with 200 mL of Rout's Agar Hydroponic (RAH) medium, a semi-solid inert agar containing 5 g L⁻¹ of Difco Noble gar (Becton-Dickenson, Sparks, MD) and 12 mL of filter-sterilized liquid fertilizer (per 1 L from 1M stock solutions: 2 mL micronutrients; 2 mL Fe-EDTA; 2 mL MgSO₄; 1 mL KH₂PO₄) and were either supplemented with high nitrogen (1000 ppm NO₃-N L⁻¹) in the form of KNO₃ and Ca(NO₃)₂, or devoid of nitrogen (0 ppm N L⁻¹) in the form of KCl and CaCl₂. Thus, Magenta boxes were filled with RAH medium with two different N-amendments (+N, -N; n = 20, N = 40). The lids of the Magenta boxes were modified with a 1 cm diameter hole to accommodate seedlings and covered in aluminum foil to exclude light. Individual seedlings planted into RAH medium treatments were established for 17 d, when half in each fertilizer treatment (n = 10) were subjected to antibiotic application with 1 mL of tetracycline (12.5 mg mL⁻¹) injected into the RAH medium on two occasions (17 and 32 d post-planting), while the other half were injected with an equal volume of sterile water. Each seedling was grown for a total duration of 60 d post-planting. Tetracycline is a broad-spectrum bacteriostatic antibiotic that was previously demonstrated to be effective against the bacterial

isolates used in this study at the minimal inhibitory concentration listed above (data not shown). Thus, the experimental treatments described above consisted of two fertilizers (+N; -N) and plants that either harbored any putative endophytes (E) in an active state (+E), or in a tetracycline-inhibited state (-E), resulting in 4 treatments with 10 replicates each (+E +N; +E -N; -E +N; and -E-N). This suite of treatments allowed us to determine whether there was any effect of inhibiting putative endophytes on plant growth in N-rich and N-lacking substrates.

Experiments with microbial soil-slurries in sterile sand: Another set of surface-sterilized *S. halepense* seeds were used to determine whether plants were able to acquire bacterial endophytes from their surrounding environment (i.e. horizontal acquisition). To accomplish this, seeds were planted into thrice-autoclaved (on 3 separate days) silica sand contained in sterile receptacles (autoclave-sterilized plastic cups, ~946 mL, ~9 cm diameter, Kroger brand). Replicate receptacles planted with one seed each received one of three microbial slurry amendments that were based on soil slurries (10:1 sterile Milli-Q water: sieved soil, thoroughly mixed) generated from *S. halepense* invaded prairie soil obtained from the field site (FWNCR, described above). The microbial slurry amendments were as follows: 1.0 μM -filtered soil slurry (to remove the fungal component and other large particles, while leaving indigenous bacteria in the slurry), hereafter Invaded-Soil Bacterial Slurry (ISBS); 0.22 μM filtered soil slurry (to remove fungi and indigenous bacteria), then supplemented with the five previously isolated *S. halepense*-associated N_2 -fixing-bacteria, (hereafter *Sorghum* N_2 -fixing Slurry, SN_2FS); and a 0.22 μM filtered soil slurry (to remove fungi and indigenous bacteria) with no further amendment, hereafter Sterile Soil Slurry (SSS). Bacterial concentrations in slurries from the ISBS and SN_2FS treatments were determined by DAPI-based microscopic enumeration using standard techniques and ranged $10^6 - 10^8$ cells mL^{-1} (data not shown). Applications of ISBS,

SN₂FS, and SSS were applied twice weekly during seedling germination (21 d) at a volume of 250 mL of each treatment at each application. Throughout the remainder of the study (69 d) plants were watered weekly with filter-sterilized liquid fertilizer supplemented with either high N or devoid of N (as described above). Thus, experimental treatments consisted of three microbial slurry amendments (ISBS, SN₂FS, SSS) and two fertilizers (+N, -N; n=30, N = 180).

Growth-chamber conditions / plant measurements: Plants in all treatments were grown in a growth chamber where temperature was maintained between 22-26°C and 65-70% RH on a 12 h light/12h dark cycle. Growth (measured as total height) was monitored daily throughout the course of the study on all plants until harvest, when a subset of plants in the microbial slurry amendments (ISBS/+N, SN₂FS/+N, SSS/+N; ISBS/-N, SN₂FS/-N, SSS/-N; n = 15, N = 90), and all plants in the sterile RAH medium / tetracycline treatments (+E/+N, +E/-N, -E/+N, -E/-N; n = 10, N = 40) were dried (3 d, 60°C), their total weight recorded, as well as allocation into above- and below-ground biomass.

Nitrogenase activity associated with *S. halepense* rhizomes: A portion of the remaining plants in the microbial soil-slurry treatments (n = 9, N = 54) was used for determination of nitrogenase activity using the acetylene reduction assay (ARA) using the method of Rout and Chrzanowski (2009). Briefly, rhizomes (0.25 – 0.75 g wet weight) were harvested, surface sterilized (as above), and assayed for ARA rates (Shimadzu GC-2014). Controls (in triplicate per treatment) consisted of tubes containing rhizomes without acetylene to account for endogenous ethylene production.

Isolation and analysis of bacterial DNA from *S. halepense* rhizomes: 16S rRNA gene-based denaturing gel electrophoresis (16S-DGGE) was used to compare the bacterial community signatures associated with the remaining plants in the microbial soil-slurry

treatments (n = 6, N = 36) and assess whether they were capable of recruiting bacteria from the physical substrates in which they were growing. Rhizomes (0.25 – 0.75 g) were harvested and surface sterilized (as described above), after which their outer surfaces were removed (see Rout & Chrzanowski 2009) and the remaining tissue ground in 1 mL of sterile phosphate buffered saline (PBS, pH 7) using a sterile mortar and pestle. Bacterial genomic DNA was extracted from ground rhizome tissue, in duplicate, using the Ultra Clean Microbial DNA Isolation Kit (MoBio). Amplicons (~371 bp) of the 16S rRNA gene of bacteria present in the tissue were amplified using the generally conserved primer pair 536f and 907r (Feris et al. 2003). The amplicons from the duplicate extraction of each rhizome sample were pooled, then analyzed in triplicate by DGGE to generate plant-associated bacterial community fingerprints for each rhizome sample. Triplicates were tested for similarity using pattern-matching software before being compiled into one representative fingerprint per sample for comparative analyses between treatments (see *Statistical analyses* below).

In-vitro bacterial physiology analyses

To assess effects of *S. halepense* bacterial endophytes on plant growth and soil biochemical parameters and via what mechanisms, a suite of biochemical and physiological tests were performed on the five bacterial isolates described previously. These are described in the sections below.

Phosphate solubility: The ability of each bacterial strain to solubilize phosphate was determined using a modification of the method of Nautiyal (1999). Briefly, bacteria were grown in yeast extract, phosphate salts broth (YP, Young et al. 1996) at room temperature with shaking (180 rpm, New Brunswick Scientific, USA Model 4230) to an OD₆₀₀ of 0.5, after which 20 µL of

each strain was individually inoculated (in triplicate) into 125 mL Erlenmeyer flasks containing 20 mL of National Botanical Research Institute's phosphate growth medium without yeast extract (NBRIP; Nautiyal 1999). These cultures were incubated for 2 d at 30°C with shaking, after which the cells were pelleted by centrifugation (13,000 x *g* for 10 min at 4°C, Sorvall RC-5B). An aliquot (10 mL) of the supernatant was collected for spectrophotometric determination of soluble reactive phosphate (SRP) using a Shimadzu, Model UV-1601 (Columbia, MD) according to the method of Fiske and Subbarow (1925). Uninoculated sterile NBRIP medium served as a negative control and blank for these assays.

Iron siderophore production: All glassware was carefully rinsed with 6M HCl and then triple rinsed with sterile Milli-Q water prior to use. Trace iron was removed from the media (deferration) by gently stirring for 1 h with Chelex 100 resin (5g 100 mL⁻¹, BioRad, Hercules, CA). Resin was subsequently removed by filtration (0.22 µm acetate filter), after which the media was dispensed into acid-washed growth flasks and sterilized by autoclaving. Two different approaches were used to detect siderophore production; the universal chrome azurol S liquid assay (CAS, Schwyn & Neilands 1987), and an overlay-based modification, the O-CAS method, which is reportedly more sensitive than the liquid CAS approach (Perez-Miranda et al., 2007).

CAS liquid assay: Disposable plastic 50 mL conical tubes containing 25 mL of deferrated YP broth were inoculated with individual bacterial colonies growing on deferrated YP agar and incubated for 3 d at 30°C with shaking (as above). Cells were pelleted by centrifugation (as above) and an aliquot of supernatant collected for determination of siderophore production. Siderophore production was considered positive if color developed in tubes containing equal volumes of supernatant and CAS solution. Positive controls were created using

+/- catechin hydrate (catechol + control yields a purple-pink color) and hydroxamic acid (hydroxamate + control yields a yellow-orange color), each at $500 \mu\text{g mL}^{-1}$ dissolved in 100% methanol. Assays were conducted in triplicate and individual isolates inoculated onto non-deferrated medium served as negative controls.

O-CAS overlay assay: The agar overlay was prepared using methods of Perez-Miranda et al. (2007). The overlay agar contained Chrome azurol S (CAS) 60.5 mg, hexadecyltrimethyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mM HCl 10 mL, and agarose (0.9%, w/v) as gelling agent. The overlay (10 mL) was applied to bacterial colonies that had been growing on deferrated YP agar for 3 d at 30°C and incubated for an additional 24 h. Siderophore production was assessed and considered positive if color developed in the agar overlaying colonies. Assays were conducted in triplicate and individual isolates inoculated onto non-deferrated medium served as negative controls.

Indole-3-acetic acid (IAA) production: Bacterial production of IAA was determined by the colorimetric analysis developed by Sawar and Kremer (1995) and modified by Grube et al. (2009). IAA medium was inoculated by 1:250 dilution of bacterial cultures previously grown at 30°C in YP broth to an OD_{600} of 0.5 with shaking. The IAA cultures were subsequently incubated with shaking for 3 d in the dark at room temperature. Cells were pelleted by centrifugation (as above) and an aliquot of supernatant collected for determination of IAA production. Supernatant was mixed with Salkowski reagent (50.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 35% (v/v) HClO_4) at a ratio of 3:2 and incubated for 30 min in the dark, after which IAA concentration was determined at 530 nm (Hewlett Packard Instruments, Model 1100). All assays were conducted

in triplicate. Uninoculated sterile IAA medium served as negative controls and blanks for this assay.

Reporting: For quantitative assays, data are reported as the mean concentration of SRP or IAA following 48 h (SRP) or 72 h (IAA) incubations, where values determined for negative controls were subtracted from values for the experimental samples. Variability among replicates is reported as standard deviation. Iron siderophore production was determined qualitatively by visual examination of plates following 24 h of growth.

Statistical analyses

Soil biogeochemistry data and all plant measures (growth, biomass, ARA) were analyzed separately with Repeated Measures Multivariate or Univariate Analyses of Variance (ANOVA), as appropriate. For soils, main effects of soil source (native, transition, and invaded prairie) and time (month) were used in the model. For plant growth measures from RAH medium experiments, main effects of endophyte active state (+E, -E) and fertilizer (+N, -N) were used; main effects of microbial slurry amendments (ISBS, SN₂FS, SSS), fertilizer (+N, -N) and time (day) were used in ANOVA on plants in the soil-slurry treatments. Normality and homogeneity of variances were examined and data were transformed when appropriate. Kruskal–Wallis ANOVA on ranks was applied when data failed normality or equal variance tests. Post hoc means comparisons were conducted using Tukey’s HSD (ANOVA) or by Dunn’s method (Kruskal–Wallis). DGGE pattern matching analysis was performed using BioNumerics v4.6 software (Applied Maths, Kortjik, Belgium). Since visual examination of gel patterns is subjective and prone to human bias, digital images of DGGE gels were captured and analyzed using the pattern matching algorithms supplied with the software. Detrended Correspondence

Analysis (DCA) was used to analyze DGGE banding patterns. All statistical analyses were conducted using SPSS v19 software (IBM, Somers, NY).

RESULTS

Soil nutrient analyses

Soils invaded by *S. halepense* had much higher concentrations of all nutrients except calcium (Ca) throughout the 46 month sample period compared to soils in transition and native communities (Figs. 1, 2; Table S1). For all nutrients but copper (Cu), F values for the effect of community types (invasion) were much greater than those for time, indicating that the effect of *S. halepense* on soil biogeochemistry is stronger than that of seasonality (Figs. 1, 2). Total Ca had a overall mean of $3,458 \pm 522 \text{ mg kg}^{-1}$ in invaded soils vs. $4,227 \pm 1009 \text{ mg kg}^{-1}$ and $4,387 \pm 535 \text{ mg kg}^{-1}$ in transition and native soils, respectively. The mean pH over the duration of the study was 7.54 ± 0.03 in native soil, 7.14 ± 0.09 in transition soil, and 6.91 ± 0.04 in invaded soil. These patterns remained consistent throughout the study period.

Plant-associated analyses: N₂-fixation, S. halepense growth and resource allocation

Experiments with microbial soil-slurries in sterile sand: Based on growth responses and ARA measurements under sterile conditions, we found bacteria capable of N₂-fixation inhabiting *S. halepense* seeds, (Fig. 3A, B respectively). Plants grown from surface sterilized seeds under N-deprivation (-N) in Sterile Soil Slurry (SSS) showed similar growth (Fig. 3A) and rates of acetylene reduction (Fig. 3B) compared to those amended with *Sorghum* N₂-fixing bacterial Slurry (SN₂FS) or Invaded-Soil Bacterial Slurry (ISBS). Repeated measures ANOVA revealed N-fertilization level had a significant effect on plant growth (see Fig. 3A; $F = 311.734$, $p < 0.0001$). Microbial slurry amendment also had a significant effect on growth over time ($F =$

16.885, $p < 0.0001$), but there was no significant interaction between N-fertilization and microbial slurry amendment ($F = 0.410$, $p = 0.665$). The mean height of all plants at harvest (41 d) in +N fertilization treatments ranged from 69.80 – 72.00 cm, while plants in –N treatments ranged from 23.75 – 34.40 cm. The majority (75%) of plants grown under –N conditions tested positive for ARA in all substrates; interestingly, so did many (33% of the combined total) of those grown in high +N conditions (data not shown). ANOVA showed that the microbial slurry amendment did not have a significant effect on ARA rates ($F = 0.127$, $p = 0.881$), while N fertilization did ($F = 7.532$, $p = 0.009$). There was also not a significant interaction between microbial slurry amendment and N fertilization on ARA rates ($F = 0.499$, $p = 0.611$). The mean ARA rate for plants under N-deprivation (-N) was almost three times higher than those under high N fertilization ($0.734 \pm 0.19 \text{ nmol g}^{-1}$, $2.12 \pm 0.37 \text{ nmol g}^{-1}$ respectively).

These results suggest that bacterial endophytes were present in plant tissues and confirmed the necessity for experimental inhibition of bacterial activity (via tetracycline application) from surface-sterilized seeds upon germination.

Experiments in sterile agar: Mortality rates for bacteriostatic plants (tetracycline-treated, endophyte restricted –E) grown in RAH medium devoid of N were high (-E-N mortality was 67%; data not shown), while those not receiving tetracycline and retaining seed-source endophytes in their active state (+E) had a low mortality rate (11%; data not shown). All plants in the -E- N treatments also had red pigmentation of the leaves, an observation that has been reported to be indicative of N_2 -fixing bacterial endophytes in sugar cane (Baldani et al. 1996). All plants were used to calculate the growth (Fig. 4) and biomass (Fig. 5); biomass was collected either upon plant death, or at the termination of the study (60 d), whichever came first.

The growth of *S. halepense* was increased by active endophytes (+E), regardless of N-availability (Fig. 4A, B). In an overall model using repeated measures ANOVA on height of *S. halepense* comparing the effects of endophyte and fertilizer, a significant interaction was noted ($F_{(1, 36)} = 4.428$, $p = 0.042$). To explore this interaction, repeated measures ANOVAs were run individually to separate the effects of endophyte activity status from N fertilization on growth of *S. halepense*. When looking only at plants grown under +N fertilization, endophyte status had a significant effect on plant height ($F_{(1, 18)} = 5.273$, $p = 0.034$); mean heights at the end of the study were 73.10 ± 10.14 cm for +N -E plants and 94.40 ± 7.04 cm for +N +E. In contrast, plants growing in -N fertilization were not significantly affected by endophyte activity status ($F_{(1, 18)} = 2.881$, $p = 0.107$), even though at the end of the study there was a significant difference in the mean heights (4.90 ± 1.37 cm for -N-E and 7.60 ± 1.46 cm for -N +E). Not surprisingly, N fertilization had highly significant effects on plant heights in both endophyte treatments (+E $F_{(1, 18)} = 655.270$, $p < 0.0001$; -E $F_{(1, 18)} = 105.762$, $p < 0.0001$). ANOVA on biomass at plant harvest (separated into root, shoot, and total biomass) showed significant effects of endophyte treatment for all categories; N fertilization had highly significant effects on all biomass categories (data not shown).

Root:shoot ratios for +E plants was similar, regardless of N fertilization. Ratios for +N and -N treatments were 1.718 ± 0.227 and 1.658 ± 0.269 respectively; these ratios were at least double those for -E plants (Fig. 5). ANOVA revealed a significant effect of endophyte activity status (tetracycline treatment) but not N fertilization on root:shoot ratio ($F = 36.282$, $p < 0.0001$; $F = 0.214$, $p = 0.647$ respectively); this effect was driven by a significant decline (>5.5 fold) in below-ground biomass in -E compared to +E plants (0.33 ± 0.12 g, 1.83 ± 0.56 g respectively).

Plant-associated analyses: bacterial community structure in rhizomes

Bacterial community fingerprints from plants in the microbial slurry amendments (SN₂FS, ISBS, SSS) suggest that *S. halepense* is also capable of recruiting bacterial partners from its external environment. DCA conducted on the DGGE fingerprints showed that internal plant tissues from replicate plants within each soil-slurry treatment had bacterial endophyte fingerprints more similar to each other, regardless of N treatment, with 53% of the total variance explained (Fig. 6).

In-vitro bacterial physiology analyses

Phosphate solubility: The NBRIP medium for detection of phosphate-solubilizing bacteria contains insoluble Ca₃(PO₄)₂ as the only source of P. All five strains grew well in this medium, producing visibly turbid cultures with distinct cell pellets upon centrifugation, indicating that all were capable of mobilizing insoluble P. However, three of the five strains (FWNCR-*Pseudomonas*, FWNCR-*Sphingobium*, and FWNCR-*Xanthomonas*) mobilized P in excess of that required for growth and liberated SRP into the medium (Table 1). The FWNCR-*Pseudomonas* isolate was particularly effective at liberating P, producing 13,501 ± 111 µM SRP in 48 h. SRP could not be detected in cultures of FWNCR-*Agrobacterium* and FWNCR-*Caulobacter*.

Iron siderophore production: Iron siderophores are low molecular weight molecules often characterized as hydroxamates or catechols (Rodriguez & Fraga 1999; Sterner & Elser 2002). Both types of siderophores can be detected by the O-CAS assay. The production of yellow to yellow-orange pigment is indicative of the presence of hydroxamates, whereas orange to red pigments are indicative of the presence of catechols (Schwyn&Neilands 1987; Perez-Miranda et al. 2007). The liquid CAS assay is less discriminatory. Based on the liquid CAS

assay, two of the five isolates (FWNCR-*Pseudomonas* and FWNCR-*Xanthomonas*) produced iron siderophores likely to be hydroxamates (Table 2). The O-CAS assay indicated that each of the five isolates produced hydroxamates and that the FWNCR-*Sphingobium* isolate also produced catechols (Table 2).

IAA production: Each of the five isolates produced IAA, although there was considerable variability in IAA production after 72 h of growth (Table 1). The isolates FWNCR-*Caulobacter*, FWNCR-*Pseudomonas*, and FWNCR-*Sphingobium* produced low levels of IAA ($\sim 0.20 \mu\text{g mL}^{-1}$) compared to FWNCR-*Agrobacterium* ($13.01 \pm 0.163 \mu\text{g mL}^{-1}$) and FWNCR-*Xanthomonas* ($\sim 3 \mu\text{g mL}^{-1}$).

DISCUSSION

Sorghum halepense often grows in densely-packed monocultures which may reach densities of >90 ramets (individual members of a clonal plant) m^{-2} , with individual plants exceeding 2 m in height and producing 60 – 90 m of rhizomes y^{-1} (Holm et al 1977; McWhorter 1981). Rout and Chrzanowski (2009) found that soils invaded by *S. halepense* were characterized by elevated levels of N, P, Fe, and several other elements, compared to soils associated with native communities. Our results support these prior findings and indicate that the effects of *S. halepense* on biogeochemical cycles have the potential to be caused in part by the physiologies of the endophytic bacteria inhabiting rhizomes of this invasive grass. Furthermore, these results demonstrate that endophytic bacteria have strong enhancing effects on the growth and biomass allocation of *S. halepense*, likely through mechanisms other than increased N supply through symbiotic N_2 -fixation, as the results obtained within this study were consistently documented under excessive N fertilization.

All five endophytic bacterial isolates tested have the capacity to mobilize insoluble P *in vitro*. Thus, in addition to fixing N₂, these endophytes have the capacity to supply *S. halepense* with an additional critical nutrient. Increased P supply may be particularly important for a plant with high N acquisition because as N supply increases, P-deficiency can limit plant growth (Stevenson & Cole 1999). This scenario is supported for *S. halepense* by our results showing decreased total Ca and increased PO₄³⁻ in *S. halepense*-invaded soils (Figs. 1A, 2B). Results from many terrestrial ecosystems suggest that symbiotic N₂-fixing plants hold an advantage in P acquisition from soils, although the mechanisms for this are likely varied (reviewed by Houlton et al. 2008). Excess soluble P was only detectible in the isolates FWNCR-*Pseudomonas*, FWNCR-*Sphingobium*, and FWNCR-*Xanthomonas*. However, since growth was observed for all five isolates, this suggests all are capable of phosphate solubilization from insoluble forms to support their own growth. Thus, the liberated P appeared to be sequestered and utilized by the isolates FWNCR-*Agrobacterium* and FWNCR-*Caulobacter* to sustain bacterial growth, rather than released into the media.

Iron (Fe) chelation is another common metabolic feature among bacteria (Stevenson & Cole 1999). Fe³⁺ pools were very high in *S. halepense*-invaded soils compared to native and transition soils throughout the study period (Fig. 2 C) and we found that endophytic bacteria had the potential to contribute to this pattern. All five bacterial endophyte isolates produced Fe siderophores *in vitro* (Table 2), potentially liberating insoluble Fe into bioavailable forms in acidic soils, which occurred with *S. halepense* invasion (Fig. 2D). Fe³⁺ concentrations reached levels in invaded soils that are toxic to many crops (Stevenson & Cole 1999) and this might also have negative effects on native species, yet how *S. halepense* tolerates these high concentrations is unknown.

In addition to these direct physiological findings, other evidence from the current study suggests that plant-microbe associations are playing a role in the shifting soil biogeochemical parameters that have been documented in invaded soils. DCA analysis of bacterial fingerprints from *S. halepense* rhizomes indicated that this plant can recruit bacterial partners from the physical matrix in which it grows (Fig. 6). The extent of this capability and the identities of potential bacterial recruits (e.g. symbionts, PGPB, pathogens to competitors) are currently unknown and warrant further inquiry. Regardless, the significant overall effect of microbial slurry amendments on repeated measures of growth (see Results, Fig. 3A) supports the possibility that *S. halepense* can recruit at least some bacterial endophytes from the physical substrate. This finding further emphasizes the role of the bacterial community in invaded soils on the positive feedback observed in the field on *S. halepense* density (Rout et al. *accepted*), and hints at a number of mechanisms that help explain the increased soil biogeochemical parameters observed for invaded soils in this study.

The type of soil microbial amendments that plants were exposed to had a significant effect on the growth of *S. halepense*, but the primary determinant of plant height was exogenous N supply (, as indicated by the overall statistical model and the grouped distributions of mean plant heights at the end of the study (see Results, Fig. 3A). The growth of *S. halepense* in sterile conditions without supplemental N (SSS/-N) was not significantly different from growth in N-depleted substrate supplemented with known N₂-fixing bacterial endophyte isolates (SN₂FS/-N; $p = 0.384$, Tukey's HSD). In fact, the survival and growth data for the SSS/-N treatments indicates these plants were accessing N from a source other than the substrate, strongly suggesting N₂-fixation from endophytic bacteria transferred vertically via seeds. This was confirmed with the positive results for ARA on plants in the SSS/-N treatment (Fig. 3B), since

these plants were grown aseptically from surface sterilized seeds. Additionally, ARA rates were approximately equal for all plants growing under N-deprivation (Fig. 3B); this was substantiated by the lack of a significant effect of soil microbial amendments in the ANOVA. From these findings, we conclude that *S. halepense* seeds contain N₂-fixing bacteria, confirming the capacity for vertical transmission.

This study confirms that bacterial endophytes significantly enhance the growth and resource allocation of *S. halepense*, and these advantages were eliminated when bacterial endophytes were restricted from growth (-E, see Figs. 4,5). These results are not an artifact of the tetracycline, and this latter point is illustrated by a key finding regarding N fertilization. There was a significant decline in heights of *S. halepense* plants grown under both N-fertilizations, including high-N treatments (Fig. 4B). This indicated that *S. halepense* relies on these bacterial partners for added growth benefits, even when exogenous N is available in the environment. Based upon destructive growth response variables (see Fig. 5), it appears that *S. halepense* below-ground biomass is significantly reduced when endophyte growth is restricted. The significant decline in root:shoot ratio was similar for tetracycline-treated plants, irrespective of exogenous available N. This suggests that these bacteria are essential for below-ground biomass of *S. halepense*, and that this is not related to bioavailable N. In light of previous research that estimated below-ground growth at astonishing rates of 60 – 90 m yr⁻¹ for an individual plant (McWhorter 1981), our findings suggest these bacterial endophytes play a substantive role in resource allocation for this invasive grass. Due to the nature of our experimental designs, we are uncertain of the mechanism(s) utilized by these bacterial endophytes that might explain these significant effects on below-ground growth. Nevertheless, we were particularly interested in the potential of the N₂-fixing bacteria previously isolated from

S. halepense rhizomes to produce plant growth-promoting substances like IAA, perhaps contributing to this finding.

The plant growth hormone IAA appears to be synthesized by approximately 80% of rhizosphere bacteria (Loper & Schroth 1986). When secreted at low levels, IAA promotes root growth (Patton & Glick 1996; Glick 1999), while high levels, a characteristic of plant pathogens, inhibits plant growth and causes developmental perturbations (Sarwar & Kremer 1995). Thus, IAA promotes plant growth under some circumstances, yet is inhibitory under others. Each bacterial isolate was capable of producing IAA; FWNCR-*Pseudomonas*, FWNCR-*Sphingobium* and FWNCR-*Caulobacter* produced IAA at levels known to induce root growth among various plant species; while FWNCR-*Xanthomonas* and FWNCR-*Agrobacterium* produced IAA at levels typical of plant pathogens (Table 1). It is not possible to determine if the *in vitro* rates of IAA production are typical of what might be expected *in situ*, but it seems likely that bacterial production of IAA might contribute to the growth and below ground biomass of *S. halepense* observed in this study (see Fig. 4). Bacterial and other biotic and abiotic environmental factors might regulate plant hormonal pathways, including IAA and ethylene production, which represent integrated pathways leading to plant growth and senescence (Hardoim et al. 2009). The bacterial contribution to hormone signaling pathways in *S. halepense* remains to be determined.

Based upon these collective findings, we propose a revision to the conceptual model initially proposed by Rout and Chrzanowski (2009). This revised model (Fig. 7) shows both the direct and indirect actions of bacterial endophytes that might contribute to the higher soil PO_4^{3-} and Fe^{3+} , and lower total Ca levels, in these invaded soils (Rout & Chrzanowski 2009, this long-term study). The *in vitro* production of the plant growth hormone IAA suggests an additional

feedback loop into this system, creating more rhizomatous growth supporting additional bacterial endophyte loads, and potentially exacerbating the above direct and indirect effects, originally referred to as Microbially Enhanced Competitive Ability (MECA, Rout & Chrzanowski 2009).

Conclusions

Our results provide evidence for endophytic bacterial populations that possess multiple mechanisms, both direct and indirect, for promoting plant growth and biogeochemical perturbation in an invaded ecosystem. We also suggest that the physiological activities of bacterial endophytes in this (and possibly other) invasive species may play a larger and more direct role in the biogeochemical perturbations that are often observed in plant invasion systems. For example, it is known that plant invasions, on average, cause an increase in NPP as well as increased concentrations of soil nutrients (Liao et al. 2008; Rout & Callaway 2009). It seems appropriate to ask whether bacterial endophytes, as exemplified by these five isolates from *S. halepense*, may also be characteristic, or at least indicative of activities conveyed by endophytes of other invasive plant species. If so, it may be reasonable to consider that invasive plants affect microbially-mediated soil processes in ways that are quantitatively different than those of plants in their native ranges

The most striking feature of our results is that a number of different microbially-mediated mechanisms (MECA) are described and documented that help to explain the prolific growth of *S. halepense*, and the substantive perturbations to several soil biogeochemical cycles that are observed during invasion. Presumably, these are mediated by multiple simultaneous plant:microbe interactions of both vertically transmitted and horizontally acquired endophytes—all in this single invasive plant system. To our knowledge, this represents the most diverse

repertoire of microbially enhanced invasive traits described for a single plant. However, other microbially mediated activities are certainly likely to occur in plant tissues and rhizospheres that remain to be discovered. The role of indigenous or exotic bacterial endophytes in plant invasions is an intriguing, yet largely underexplored aspect of exotic plant invasion.

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TABLE 1. Phosphate and 3-indole acetic acid (IAA) production by bacteria isolated from the rhizomes of *Sorghum halepense*.

Bacterium	Phosphate production (μM)	IAA production (mg L^{-1})
FWNCR- <i>Agrobacterium</i>	0	13.01 ± 0.16
FWNCR- <i>Caulobacter</i>	0	0.10 ± 0.00
FWNCR- <i>Pseudomonas</i>	$13,501 \pm 117$	0.22 ± 0.07
FWNCR- <i>Sphingobium</i>	$6,904 \pm 681$	0.18 ± 0.07
FWNCR- <i>Xanthomonas</i>	229 ± 60	2.69 ± 0.40

TABLE 2. Production and classification of iron siderophores by bacteria isolated from the rhizomes of *Sorghum halepense*.^a

Bacterium	Liquid CAS response ^a	O-CAS halo ^a	Siderophore type	
			Hydroxamate	Catechol
FWNCR- <i>Agrobacterium</i>	none	yellow-orange	+	-
FWNCR- <i>Caulobacter</i>	none	orange	+	-
FWNCR- <i>Pseudomonas</i>	yellow	yellow	+	-
FWNCR- <i>Sphingobium</i>	none	orange, pink	+	+
FWNCR- <i>Xanthomonas</i>	yellow-orange	orange	+	-

^a Classifications based upon criteria described in (Perez-Miranda et al. 2007) and (Schwyn & Neilands 1987).

FIGURE LEGENDS

Fig. 1. Mean concentrations of soil micronutrients and trace metals from 4 replicate 1m² plots within each of native, transition, and invaded prairie (n =4, N = 12) sites at the FWNCR field site (see text). Soils were sampled quarterly November 2006 – March 2010, with a total of 12 sampling points spanning 46 mo. Compared to native and transition plots, invaded soils contained the highest concentrations for metals throughout the study at all sampling intervals, with the exceptions of total Ca (Panel A) which was lowest in invaded soils and Mn²⁺ (Panel E), which was not significantly different than in transition soils, but generally higher in transition and invaded soils than in native soils.

Fig. 2. Mean concentrations of soil macronutrients and pH from native, transition, and invaded prairie plots described in Fig.1. Invaded soils contained the highest concentrations of NO₂⁻ (Panel A), PO₄³⁻ (Panel B), and K⁺ (Panel C) and lower pH (Panel D) compared to native soils at all sampling points throughout the study.

Fig. 3. Repeated measures of growth (height), (Panel A); and acetylene reduction assay (ARA) rates, (Panel B) for plants growing aseptic conditions exposed to one of three microbial slurry amendments: Sterile Soil Slurry (SSS); Invaded-Soil Bacteria Slurry (ISBS); and *Sorghum*-N₂-fixing bacteria Slurry (SN₂FS). Each of these slurry treatments was exposed to two complete fertilizer regimes, only differing in the levels nitrogen (+N = 1000 ppm NO₃-N, -N = 0 ppm NO₃-N).

Fig. 4: Repeated measures of growth (height) of *S. halepense* grown in sterile RAH medium devoid of N (A; 0 ppm NO₃-N) or supplemented with high-N (B; 1000 ppm NO₃-N). Surface-sterilized seedlings were aseptically transferred into receptacles at 7d when growth was repeatedly measured as the extent of green tissue. Antibiotics (tetracycline 12.5 mg mL⁻¹; -E,

open symbols) or sterile water (+E, solid symbols) were injected into the substrates on two occasions (17, 32 d) indicated by the arrows on the graph.

Fig. 5. Root : shoot ratios for plants grown under sterile conditions in RAH medium with or without tetracycline antibiotic (-E, +E, respectively) and treated with two complete fertilizer regimes, only differing in the levels of nitrogen (+N = 1000 ppm NO₃-N, -N = 0 ppm NO₃-N).

Fig. 6. Detrended Correspondence Analysis (DCA) of bacterial fingerprints from within *S. halepense* rhizomes. Each symbol corresponds to an individual plant grown in aseptic conditions and exposed to one of three microbial slurry amendments: Sterile Soil Slurry (SSS); Invaded-Soil Bacteria Slurry (ISBS); and *Sorghum*-N₂-fixing bacteria Slurry (SN₂FS). DNA was extracted in duplicate from each rhizome and pooled, then each pooled sample was analyzed in triplicate by denaturing gradient gel electrophoresis (DGGE).

Fig. 7. A revised conceptual model of microbial processes occurring in the rhizosphere of *S. halepense*. In this scenario, rhizosphere N₂ is fixed by endophytic nitrogen-fixing bacteria within rhizomes. NH₄⁺ is shuttled into plant biomass and dhurrin (plant N-containing defense compound in *S. halepense* leaves) synthesis. Some NH₄⁺ is lost to the rhizosphere and converted rapidly into NO₃⁻, the preferred form of external N for *S. halepense* by nitrifying bacteria in the rhizosphere and surrounding soil. NH₄⁺ and NO₂⁻ metabolism acidifies soils, promoting dissolution of hydroxyapatite and thereby indirectly resulting in increased available PO₄³⁻, Ca²⁺, and Fe³⁺ for plant uptake and growth. In addition, endophytic bacteria previously isolated from *S. halepense* rhizomes contribute directly to these latter processes (this study) by solubilizing PO₄³⁻ and chelating Fe³⁺.

FIGURES

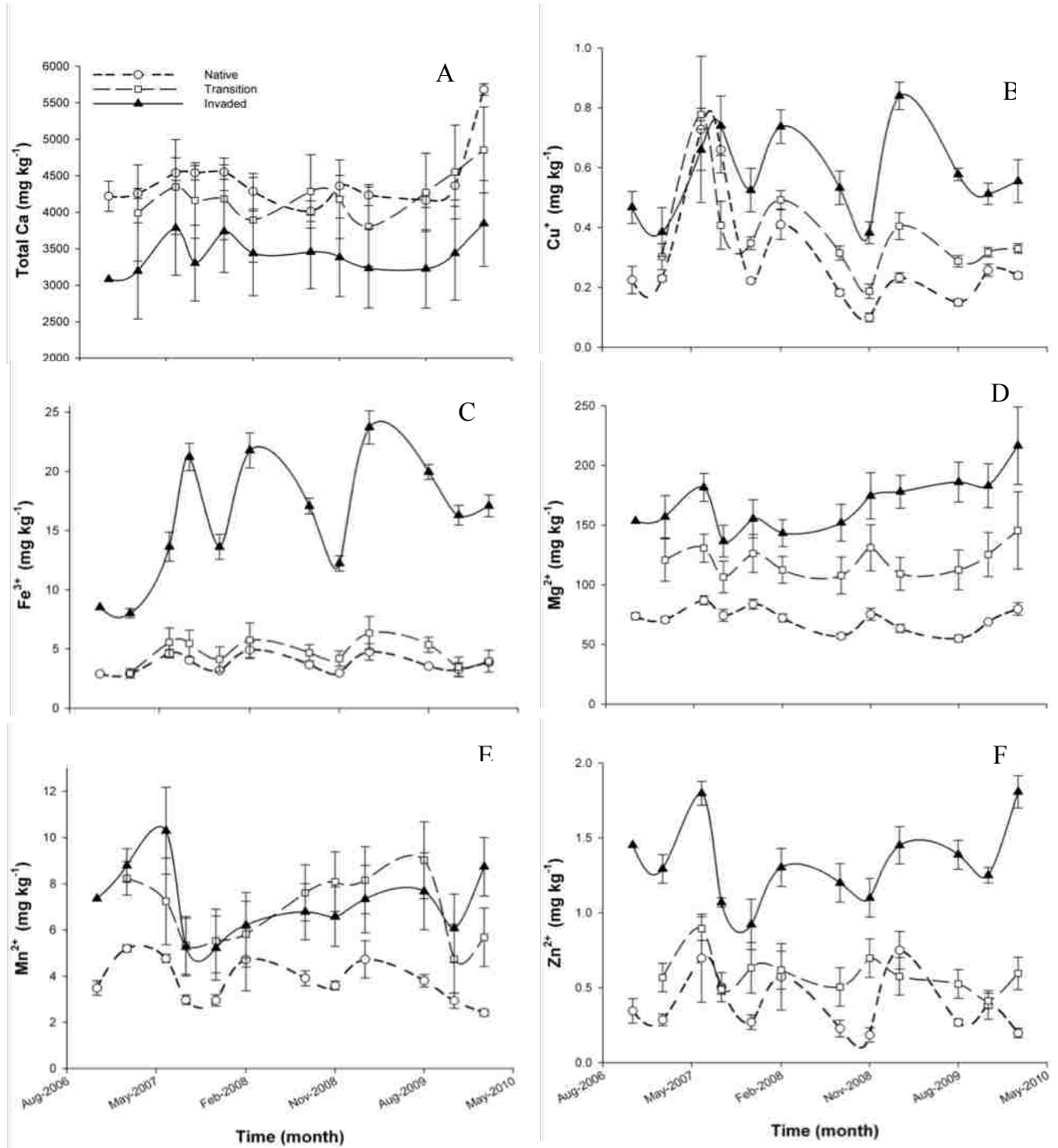


FIGURE 1

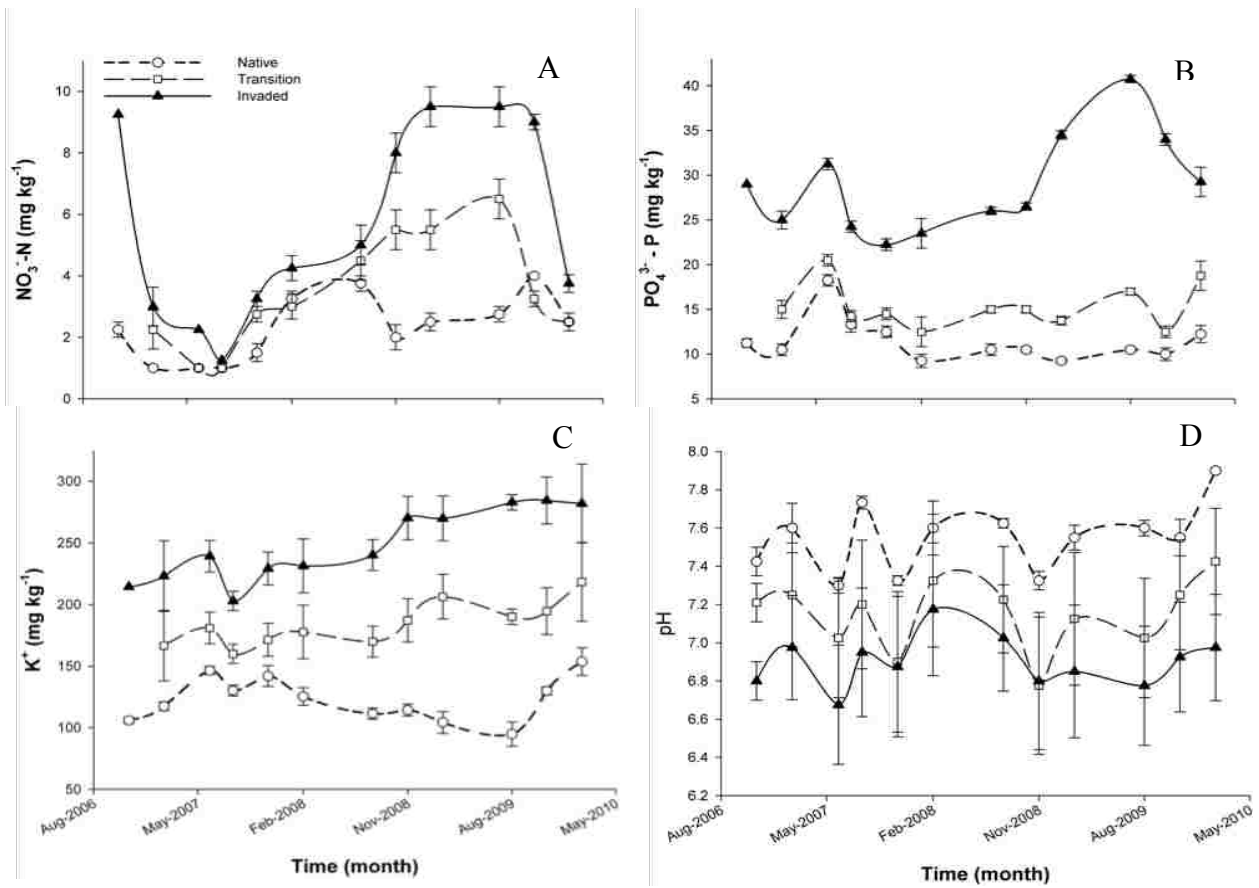


FIGURE 2

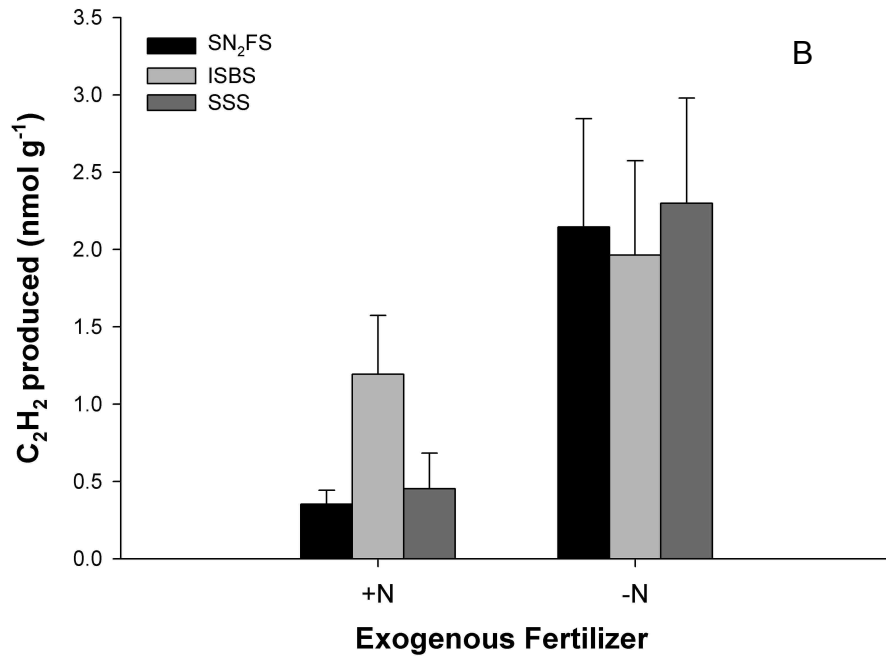
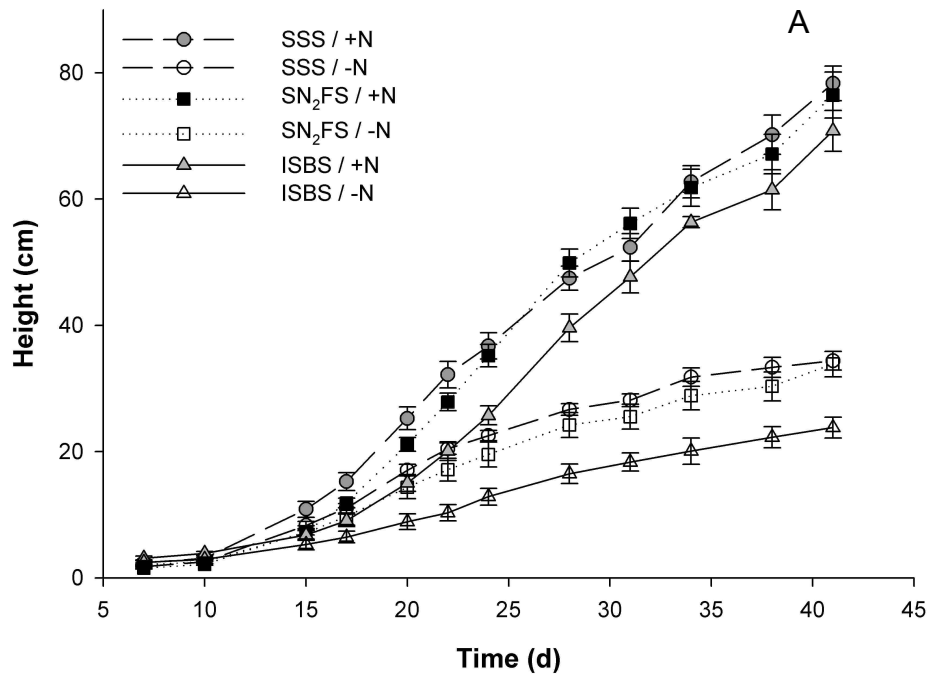


FIGURE 3

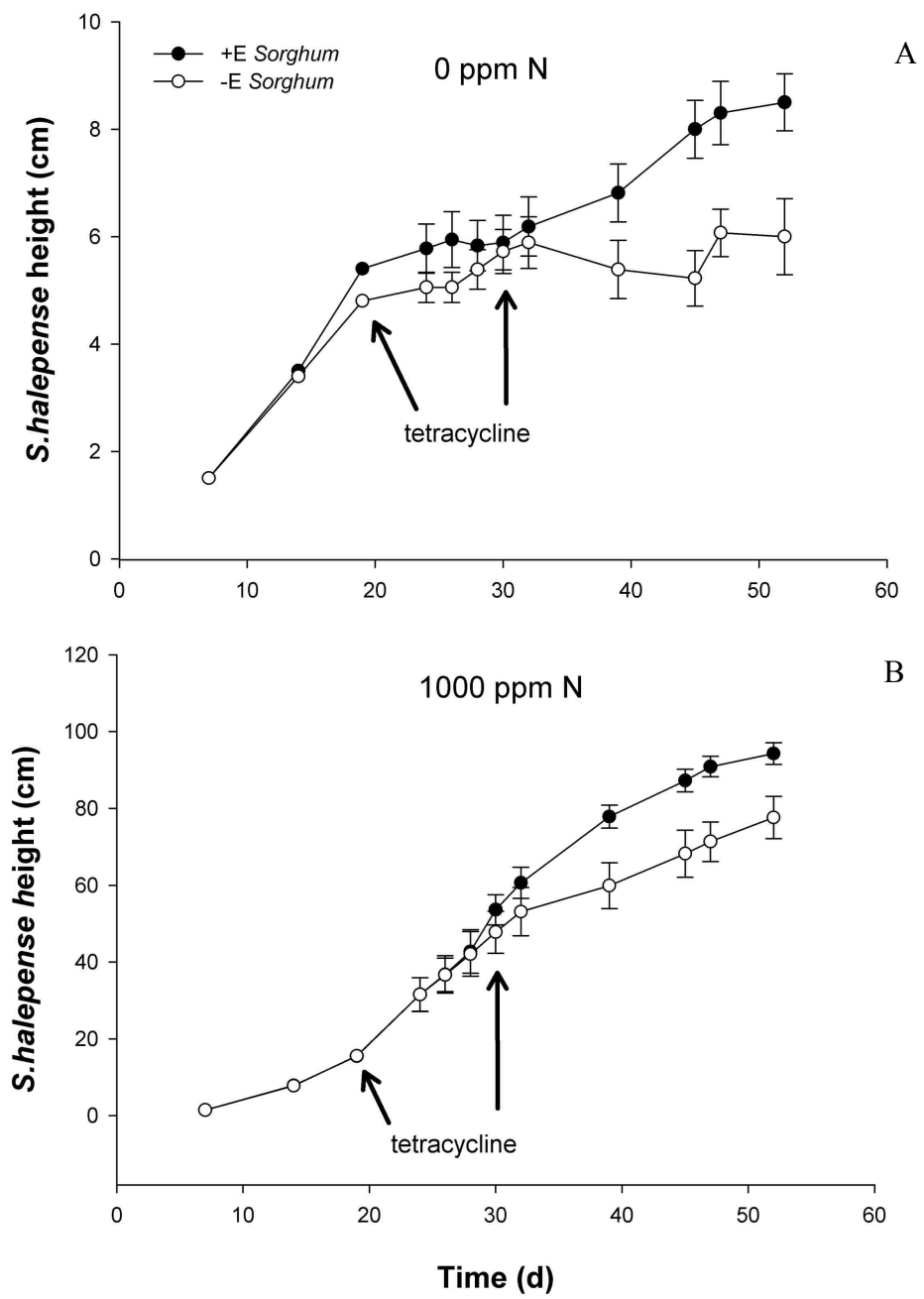


FIGURE 4

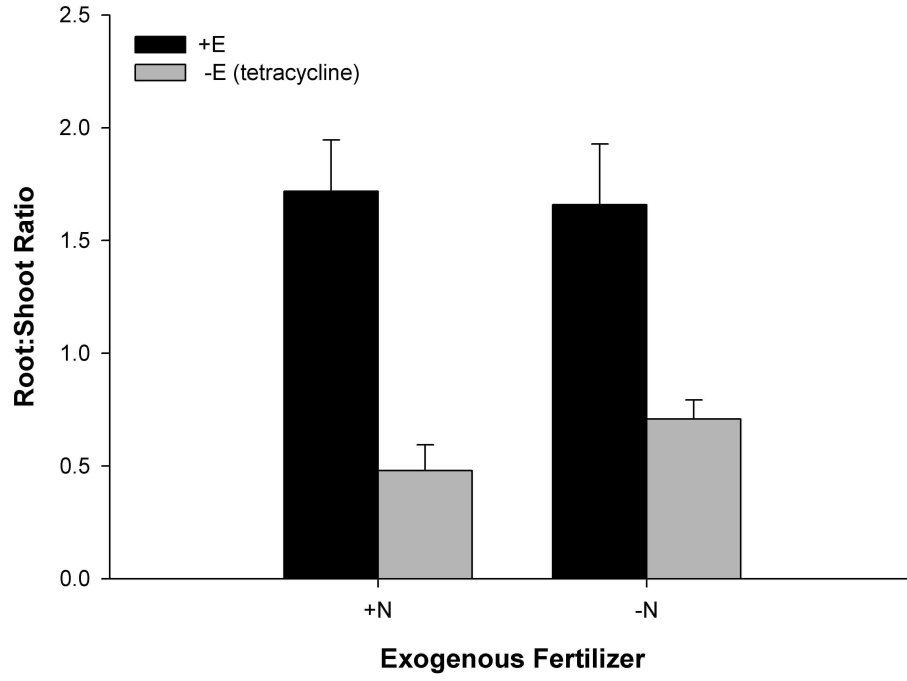


FIGURE 5

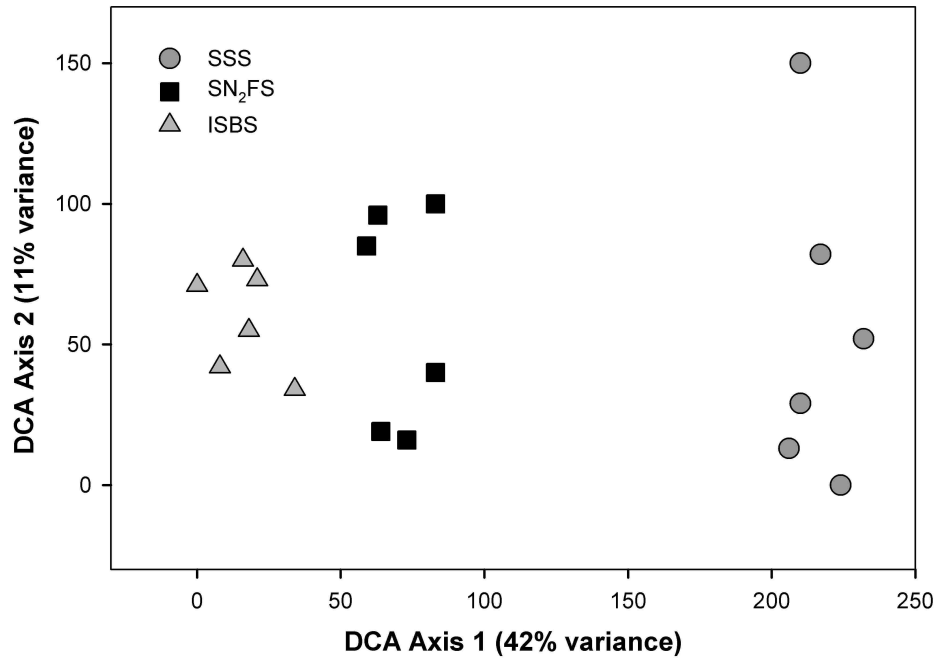
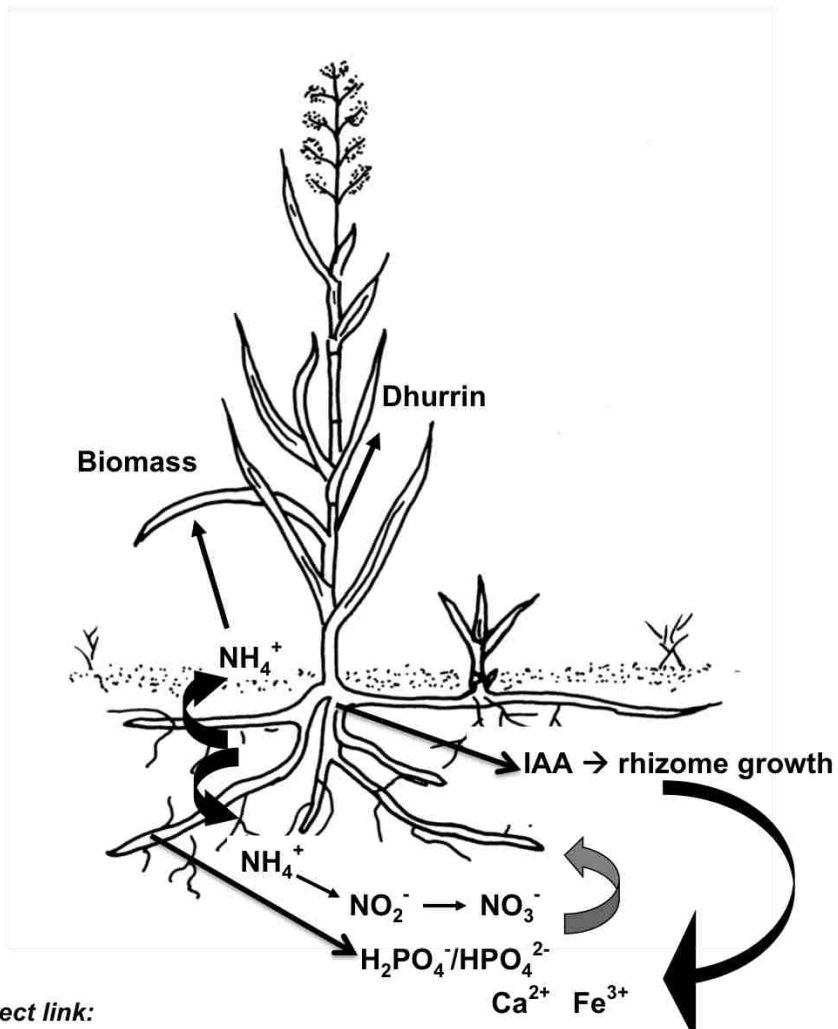


FIGURE 6



Direct link:
 rhizomatous phosphate-solubilizing & iron-chelating bacteria:
 mobilizes PO_4^{2-} , Ca^{2+} , Fe^{3+}

Indirect link:
 acidification of calcareous soils from N_2 -fixation:
 mobilizes NO_3^- , PO_4^{2-} , Ca^{2+} , Fe^{3+}

FIGURE 7

SUPPLEMENTAL DATA

TABLE S1. Repeated Measures Multivariate Between-Subjects ANOVA for soil biogeochemistries from native, transition and invaded soils.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig. (p)
Corrected Model	NO ₃	780.797	34	22.965	29.948	<0.0001
	PO ₄	9440.920	34	277.674	17.322	<0.0001
	K	449833.116	34	13230.386	10.959	<0.0001
	Ca	36049745.567	34	1060286.634	1.870	.008
	Mg	252740.880	34	7433.555	11.67	<0.0001
	Fe	5788.725	34	170.257	4.388	<0.0001
	Zn	27.841	34	.819	9.977	<0.0001
	Mn	557.755	34	16.405	4.487	<0.0001
	Cu	10.241	34	.301	9.807	<0.0001
Intercept	NO ₃	1823.944	1	1823.944	2378.560	<0.0001
	PO ₄	46862.942	1	46862.942	2923.371	<0.0001
	K	4612674.936	1	4612674.936	3820.890	<0.0001
	Ca	2.230E9	1	2.230E9	3933.638	<0.0001
	Mg	1958161.312	1	1958161.312	3074.980	<0.0001
	Fe	9506.325	1	9506.325	245.018	<0.0001
	Zn	81.229	1	81.229	989.718	<0.0001
	Mn	4669.752	1	4669.752	1277.263	<0.0001
	Cu	28.664	1	28.664	933.240	<0.0001
Community (native, transition, invaded)	NO ₃	203.684	2	101.842	132.810	<0.0001
	PO ₄	7058.671	2	3529.335	220.164	<0.0001
	K	331996.794	2	165998.397	137.504	<0.0001
	Ca	22004241.542	2	11002120.771	19.405	<0.0001
	Mg	196936.434	2	98468.217	154.629	<0.0001
	Fe	4593.636	2	2296.818	59.199	<0.0001
	Zn	19.888	2	9.944	121.162	<0.0001
	Mn	272.522	2	136.261	37.270	<0.0001
	Cu	.928	2	.464	15.104	<0.0001
Time (month)	NO ₃	397.973	11	36.179	47.181	<0.0001
	PO ₄	809.134	11	73.558	4.589	<0.0001
	K	43972.922	11	3997.538	3.311	.001
	Ca	10069900.191	11	915445.472	1.615	.105
	Mg	20390.768	11	1853.706	2.911	.002
	Fe	521.569	11	47.415	1.222	.282
	Zn	3.504	11	.319	3.881	<0.0001
	Mn	158.208	11	14.383	3.934	<0.0001
	Cu	6.628	11	.603	19.618	<0.0001
Community * Time	NO ₃	163.818	21	7.801	10.173	<0.0001
	PO ₄	1107.442	21	52.735	3.290	<0.0001
	K	38713.394	21	1843.495	1.527	.084
	Ca	3661031.045	21	174334.812	.307	.998
	Mg	20542.231	21	978.201	1.536	.081
	Fe	439.498	21	20.928	.539	.947
	Zn	2.843	21	.135	1.650	.052
	Mn	98.839	21	4.707	1.287	.201
	Cu	2.529	21	.120	3.921	<0.0001

CHAPTER 6

Bacterial endophytes increase establishment, growth, competitive effects, and herbivore defense of the invasive grass *Sorghum halepense*

ABSTRACT

Many plant species depend on mutualists for growth, fitness, and survival. Mutualists can also play important roles in plant invasions, but we know little about how mutualists might directly affect key ecological processes leading to invasion: disproportionately strong competitive effects of invaders and disproportionately weak effects of native consumers. Antibiotics and different nitrogen (N) supply rates were used to explore the effects of endophytic N₂-fixing bacteria on these ecological interactions for the globally invasive grass, *Sorghum halepense*. With *S. halepense* grown from seed under sterile conditions, we used the antibiotic tetracycline (inhibitory to known endophytes of *S. halepense*) to restrict bacterial endophytes at two time periods during seedling development. Establishment of *S. halepense* was inhibited when bacterial endophytes were restricted early in development (7 d) and growth was significantly increased by bacterial endophytes. In competition experiments, *Schizachyrium scoparium*, a native grass frequently excluded by *S. halepense* in tallgrass prairie, was highly suppressed by the invader. This competitive suppression was eliminated when the physical growth substrates were treated with tetracycline. *Schizachyrium scoparium* had significantly weaker competitive effects on conspecific plants than did endophyte-infected *S. halepense* on *S. scoparium*; but tetracycline did not affect the growth or intraspecific competition of the native grass. When feeding on *S. halepense* leaves from plants without tetracycline treatment, the generalist insect herbivore (*Tricoplusia ni*) did not grow and experienced almost 100% mortality. Tetracycline

treatment of *S. halepense* dramatically increased the growth and survival of *T. ni*, and these effects corresponded with the virtual elimination of the defense compound dhurrin in leaves of plants grown in substrate amended with tetracycline. These results indicate that microbial mutualists might be obligatory for establishment, growth, competitive impact, and herbivore defense of *Sorghum halepense*.

INTRODUCTION

The symbiosis between plants and N₂-fixing bacteria is one of ecology's most important mutualisms (Vitousek & Walker 1989), primarily since nitrogen (N) is the most limiting nutrient in terrestrial ecosystems (Stevenson & Cole 1999). Studies of this plant-microbe symbiosis have focused almost entirely on nodulating species, such as legumes. However, there is far less known regarding the ecology of non-nodulating, but endophytic, N₂-fixing bacteria, even though these microorganisms can also have strong effects on the growth of many plant species (particularly grasses) that harbor these bacteria inside of their tissues rather than in specialized structures (nodules). Examples of these include *Miscanthus sacchariflous* (Silver banner grass), *Pennisetum purpureum* (elephant grass), *Saccharum spontaneum* (sugar cane), and *Sorghum bicolor* (grain sorghum) (Baldani et al. 1996; James et al. 1997; Kirchhof et al. 2001). What additional roles these N₂-fixing bacterial endophytes may play in the ecology of their non-nodulating hosts, including resource allocation, herbivore and pathogen defense, and competitive interactions are virtually unknown.

Exotic plant species frequently depend on mutualisms in their non-native ranges for pollination, dispersal, resource acquisition, and success in their interactions with other species (Marler et al. 1999; Richardson et al. 2000; Reinhart & Callaway 2006). However, most such studies have focused on the symbiosis between mycorrhizal fungi and plants and the potential effects of N₂-fixing mutualisms with non-nodulating invasive plants remains poorly characterized (but see Callaway et al. 2011). Recently, Rout & Chrzanowski (2009) discovered that the highly invasive grass, *Sorghum halepense*, harbors N₂-fixing bacterial endophytes in its rhizomes. *Sorghum halepense* is a C₄ grass that is globally invasive and listed as one of the world's worst weeds (Holm et al. 1977). Once thought to be native to the Mediterranean region,

this species is actually a spontaneously-arisen hybrid (allopolyploid) between an annual species, *S. bicolor*, and a perennial species, *S. propinquum* (Paterson et al. 1995). Several traits of *S. halepense* are thought to contribute to its invasive character, including the clonal production of very extensive rhizomes, from 60-90 meters per plant⁻¹ yr⁻¹ (McWhorter 1981; Knopf & Scott 1990). Further, this invasive grass also produces the constitutive anti-herbivory defense compound dhurrin, a cyanogenic glycoside (Nielsen & Moller 1999) in its leaves, and exudes the highly allelopathic compound sorgoleone from root hairs (Czarnota et al. 2001). Both of these compounds are novel to the genus *Sorghum*.

Here we examined the impacts of several N₂-fixing bacterial endophytes associated with *S. halepense* on three ecological characteristics of the invader: 1) plant establishment and growth, 2) competitive effects on other plants, and 3) herbivore defense.

MATERIALS & METHODS

Seed sterility: To determine whether plant-associated, N₂-fixing, endophytic bacteria contribute to plant growth, competitive effects, and herbivore defense, it was necessary to ensure seeds used in these experiments were free of externally harbored bacterial and fungal communities. Thus, seeds were surface sterilized and confirmation of sterility was confirmed prior to use in experimental manipulations as described below.

Seeds from 20 populations of *S. halepense* were collected from the south-central U.S. from the states of AR, LA, MS, OK, and TX in June 2007 and stored at 4°C prior to use. *Schizachyrium scoparium* is a C₄ perennial caespitose grass (Diggs et al. 1999) and was chosen as the focal species for competition experiments with *S. halepense* due to its historical dominance among vegetation of the tallgrass prairie ecosystem at the primary test site,

specifically the Fort Worth Prairie (Dyksterhuis 1946). Seeds of *Schizachyrium scoparium* were collected in June 2007 from a remnant tallgrass prairie that has been undergoing extensive invasion by *S. halepense* inside the Fort Worth Nature Center and Refuge (FWNCR; 32° 84' N, 97° 47' W, ~200 m elevation).

To obtain sterile seeds for each plant species, randomly selected subsets of seeds were surface-sterilized, separately, by soaking in sterile Milli-Q water ,for 5 min, followed by sequential submersion in: 1% Chloramine-T detergent solution (5 min), 95% ethanol (1 min), and 1.6% hypochlorite (5 min); the seeds were rinsed 3x with sterile Milli-Q water between each step. Assessments of surface sterilization were conducted by randomly selecting 18 seeds, which were extensively streaked (without tearing open the seed coat) across the surface of several standard microbiological media agar plates in triplicate and incubated at 37°C for ~2 weeks in order to observe any potential bacterial or fungal growth. Six different media were tested, namely JNF agar (Baldani et al. 1996), R2 agar (Reasoner et al. 1979), Minimal Agar, Nutrient Agar, Mycobiotic Agar, and Potato Dextrose Agar (the latter three media were prepared as described by Atlas 2004).

Endophyte effects on *S. halepense* establishment and growth: Surface-sterilized seeds of *S. halepense* were allowed to germinate on sterile Whatman #1 filter paper moistened with 2 mL of sterile Milli-Q water in sterile glass Petri dishes (90 mm diameter) . Each dish contained 20 seeds, sealed with parafilm, and incubated in the dark at room temperature. Upon germination (7 d), individual seedlings were aseptically transferred into aluminum-foil-covered tissue-culture grade Magenta boxes (250 mL volume; Fisher Scientific) filled with 200 mL of Rout's Agar Hydroponic (RAH) medium. RAH medium is a semi-solid inert agar comprised of 5 g L⁻¹ of

Difco Noble agar (Becton-Dickenson, Sparks, MD) and 12 mL of filter-sterilized liquid fertilizer (per 1 L from 1M stock solutions: 2 mL micronutrients; 2 mL Fe-EDTA; 2 mL MgSO₄; 1 mL KH₂PO₄) and supplemented with nitrogen (50 ppm NO₃-N L⁻¹) in the form of KNO₃ and Ca(NO₃)₂. A 1 cm diameter hole was placed through the lids of the Magenta boxes prior to sterilization and served as the means by which seedlings were transplanted.

Antibiotic application was used to restrict endophytic bacterial growth (hereafter -E). Tetracycline, the antibiotic used in this study, is a broad-spectrum bacteriostatic antibiotic that was previously demonstrated to be effective against the N₂-fixing bacterial isolates originally described by Rout & Chrzanowski (2009) at the minimal inhibitory concentration (12.5 mg mL⁻¹, data not shown). Two different antibiotic application times were used, where a subset (n = 10) were exposed to tetracycline additions at day 7 post transplanting (EARLY -E), while another subset (n = 10) were exposed to tetracycline additions at day 18 post transplanting (LATE -E). Individual seedlings were allowed to grow in the RAH medium until antibiotic injection application (EARLY -E or LATE -E), at which point 1 mL of tetracycline was injected once into the agar during the 60-day experiment. Controls consisted of 1 mL injections of sterile water into the RAH medium, timed with either the EARLY (+E, n = 10) or LATE (+E, n = 10) antibiotic applications. In addition, plants from the LATE treatments (-E and +E) were also used to collect leaf disks for analyses on dhurrin concentrations (detailed below).

Endophyte effects on *S. halepense* competition with *S. scoparium*: Surface-sterilized seeds of each species were allowed to germinate and incubated as described above, where each dish contained 20 seeds of a single species. Upon germination (7-10 d), individual seedlings were aseptically transferred into similar aluminum-foil-covered tissue-culture grade Magenta boxes

filled with 200 mL of RAH medium (as detailed above), where the lids were modified to have two 1 cm diameter holes spaced ~5 cm apart prior to sterilization to accommodate seedling transplantation. Seedlings of *S. scoparium* were grown in competition with *S. halepense* (n = 18) or with conspecifics (n = 18).

Seedlings of the native *S. scoparium* in competition with either conspecifics or *S. halepense* were allowed to grow for 18 d, at which point a subset (n = 9) in each competition regime was treated with 1 mL of tetracycline (12.5 ug mL⁻¹), which was injected into the RAH medium twice (at 18 and 32 days post transplanting) during the 60-day experiment. Controls consisted of 1 mL injections of sterile water into the RAH medium in each competition regime. Thus, the experiment included two endophyte (E) treatments (tetracycline amended, -E; or sterile water amended, +E), and two species pairings (*S. scoparium* vs. conspecific or *S. scoparium* vs. *S. halepense*; n = 9, N = 36).

Endophyte effects on *S. halepense* defense: Leaf tissues from *S. halepense* plants grown from surface-sterilized seeds were used to test the effects of N₂-fixing bacterial endophytes on the production of the defense compound dhurrin using herbivore feeding trials and through direct quantification of dhurrin concentrations collected from *S. halepense* leaf disks. Plant consumption was tested using the generalist insect herbivore, *Tricoplusia ni* (Cabbage looper). This insect is a member of the Noctuidae family and is found throughout North America.

Insect herbivory assays: For *T. ni* feeding experiments, *S. halepense* plants were grown in the same conditions described above for the establishment and growth experiment, with the exception of timing of antibiotic application. Plants were grown for 18 d at which point the RAH medium was amended with 1 mL of tetracycline (12.5 mg mL⁻¹, -E, n = 20), while

controls were injected with the same amount of sterile water (+E, n = 20). These treatments were applied again at 32 d. Leaves of *S. halepense* were clipped (2-3 cm) after the second tetracycline amendment (7 d post-amendment) and fed to *T. ni* in two separate feeding trials. In the first trial, individual insects were placed in sterile Petri dishes lined with sterile filter paper and fed repeatedly with *S. halepense* leaves from the same plant. Biomass of individual *T. ni* was recorded daily for the duration of the study (7 d). In the second feeding trial, five *T. ni* were placed in each of 20 Petri dishes and grown for 5 d on lettuce leaves, at which point their diet was changed to either +E *S. halepense* leaves (n = 10) or -E *S. halepense* leaves (n = 10). The mean survivorship in each Petri dish was recorded over the duration of the experiment (7 d).

Dhurrin / CN assays: Growth conditions of surface-sterilized *S. halepense* seeds were manipulated to separate the effects of N-fertilization levels from endophyte infection source on dhurrin concentrations. Dhurrin concentrations in leaf-disks (1-2 cm diameter) of *S. halepense* grown in different physical substrates, endophyte infection sources, and N-fertilization levels (see below, *Assessing vertical and horizontal modes of endophyte acquisition*) were quantified spectrophotometrically by measuring cyanide concentrations (CN assay) after Gleadow et al. (2010). The CN assay involved rupturing the plant cell walls with repeated freeze-thaw intervals to convert the dhurrin into HCN in the presence of 1M NaOH to create a 'test suspension'. The assay involved sequential addition of the following: test suspension (50 uL); 2M acetic acid (125 uL); Succinimide reagent [*N*-chlorosuccinimide (0.5 g L⁻¹)/ succinimide (5 g L⁻¹); 125 uL]; and Pyridine reagent (30 g barbituric acid in 300 mL pyridine in a total volume of 1L). Preparations were incubated for 20 min, and then absorbance read at 595 nm (BioTek Instruments, PowerWave Microplate Spectrophotometer). Dhurrin concentrations were determined based on standard curves constructed using known concentrations of cyanide. Leaf-disks were collected

from two different leaves from each *S. halepense* individual, immediately stored at -80 °C until analysis, and each assay was conducted in analytical triplicate.

Assessing vertical and horizontal modes of endophyte acquisition: Surface-sterilized seeds were grown under one of three N-fertilization regimes in either triple-autoclaved silica sand (exposed to either high-N or no-N-fertilizations), or in RAH sterile agar medium (low-N-fertilization, detailed above). Plants from these experiments were monitored for growth rates and used to collect leaf hole punches used for the dhurrin / CN assays.

Treatments in sterile-sand were conducted on plants grown from seeds planted into thrice-autoclaved (on 3 separate days) silica sand in sterile receptacles (autoclave-sterilized plastic cups, ~946 mL, ~9 cm diameter, Kroger brand) in the following substrates: 1.0 uM-filtered invaded-soil-slurry made in a 10:1 sterile Milli-Q water-soil dilution (to remove the fungal component and other large particles, while leaving indigenous bacteria in the slurry; hereafter invaded soil substrate); 0.22 uM filtered invaded-soil-slurry (to remove fungi and indigenous bacteria), then supplemented with the five previously isolated *S. halepense*-associated N₂-fixing-bacteria (hereafter N₂-fixing culture substrate). Bacterial concentrations in these slurries were determined by DAPI-based microscopic enumeration using standard techniques and ranged 10⁶ – 10⁸ cells mL⁻¹ (data not shown). Substrate applications of invaded soil and N₂-fixing culture were applied twice weekly during seedling germination (21 d) at a volume of 250 mL each treatment. Throughout the remainder of the study (60 d) plants were watered weekly with a filter-sterilized liquid fertilizer supplemented with either high-N (+N = 1000 ppm NO₃-N L⁻¹) or no N (-N = 0 ppm N; contents of both fertilizers are described in Chapter 5).

Treatments conducted in RAH medium enabled experimental manipulation of

endophytes inherent in seeds, through tetracycline antibiotic applications administered into the agar (described above). Thus, experimental treatments consisted of three substrates (invaded soil, N₂-fixing culture, agar) and three fertilizers (high-N, low-N, -N) under the following treatment combinations: +E_{invaded soil} / high-N, +E_{invaded soil} / -N (n = 10, N = 20); +E_{N₂-fixing culture} / high-N, +E_{N₂-fixing culture} / -N (n = 10, N = 20); and +E_{agar} / low-N, -E_{agar} / low-N (n = 5, N = 10).

Growth-chamber conditions / plant measurements: Plants in all treatments were maintained in a growth chamber at 22-26°C and 65-70% RH on a 12 h light/12h dark cycle. Growth (measured as total height) was monitored daily throughout the course of the study on *S. halepense* plants in experiments testing the effect of endophytes on establishment and growth, and also on *S. scoparium* plants in the competition experiments. At harvest, all plants were dried for 3 d at 60°C and then weighed. Biomass was compartmentalized into above-ground (AG), below-ground (BG) total plant (T), and root:shoot ratio (data not shown) prior to statistical analyses.

Statistical analyses: All plant measurements (height, final biomass, final dhurrin concentration) and insect measurements (growth and mortality) were analyzed separately with Multivariate or Univariate Analyses of Variance (ANOVA), as appropriate. For repeated measurements over time, the main effects of time (day), and endophyte (+E, -E), or competitor when appropriate (*S. scoparium* vs. *S. scoparium*, *S. scoparium* vs. *S. halepense*) were used to assess plant growth (measured as height and biomass). Repeated measurements in herbivore experiments used main effects of time (d) and endophyte (+E, -E) to assess insect biomass and mortality. ANOVAs

used to analyze dhurrin concentrations were conducted separately for those performed in sterile-sand versus those performed in RAH agar medium. In each experimental analysis, the main effects of endophyte (+E_{invaded soil}, +E_{N2-fixing culture}; or +E_{agar}, -E_{agar}) were tested, and fertilizer when applicable (+N, -N), on plant growth rate (cm d⁻¹) and dhurrin concentration (uM CN g⁻¹). Normality and homogeneity of variances were tested and data were transformed when appropriate. Kruskal–Wallis ANOVA on ranks was applied when data failed normality or equal variance tests. All statistical analyses were conducted using SPSS v19 software (IBM, Somers, NY).

RESULTS

Endophyte effects on *S. halepense* establishment and growth:

Establishment of *S. halepense* from seed was significantly inhibited when bacterial endophytes were restricted early in the plant growth cycle (Fig. 1A). The growth of *S. halepense* was increased by the presence of active-state endophytic bacteria (+E), regardless of the timing of tetracycline applications (EARLY and LATE, Fig. 1A, B respectively). Tetracycline application in the EARLY treatments showed that endophyte status had a significant overall effect on plant height ($F_{(1, 18)} = 62.895$, $p < 0.0001$); whereas mean heights at the end of the study were 2.80 ± 0.58 cm for EARLY –E plants and 19.29 ± 1.60 cm for EARLY +E. Plants growing in LATE tetracycline applications were not significantly affected by endophyte status in the overall model ($F_{(1, 18)} = 1.225$, $p = 0.290$), even though at the end of the study there was a significant difference in the mean heights (11.57 ± 2.15 cm for LATE-E and 22.07 ± 2.04 cm for LATE +E). ANOVA for biomass at plant harvest (separated into root, shoot, total, and

root:shoot ratio) showed significant effects of endophyte treatment for all categories (data not shown).

Endophyte effects on *S. halepense* competition with *S. scoparium*: In competition experiments, the growth of the native *S. scoparium* when grown with *S. halepense* was significantly reduced in +E conditions, but growth of the native was not reduced under -E conditions (Fig. 2A). Additionally, growth of *S. scoparium* grown in competition with other *S. scoparium* was similar in both endophyte treatments, and was not significantly different from that of *S. scoparium* grown in competition with *S. halepense* in -E conditions (Fig. 2A). In an model using repeated measures ANOVA for height of *S. scoparium* comparing the effects of endophyte and competitor species, a significant interaction was found ($F_{(1, 36)} = 8.446$, $p = 0.006$). This interaction was explored by running repeated measures ANOVAs individually to separate the effects of endophyte and competitor species on growth of the native grass. When *S. scoparium* was in competition with other *S. scoparium*, endophyte status (+E, -E) had no significant effect on growth of the native grass ($F_{(1, 18)} = 0.030$, $p = 0.865$). In contrast, *S. halepense* affected the growth of the native *S. scoparium* only when endophyte status was in the active (+E) state ($F_{(1, 18)} = 30.294$, $p < 0.0001$). Competitor species significantly affected *S. scoparium* growth in +E conditions ($F_{(1, 18)} = 11.689$, $p = 0.003$), while in -E treatments there was no measurable effect of the competitor ($F_{(1, 18)} = 0.00$, $p = 1.000$). The final mean height of *S. scoparium* when grown in competition with *S. halepense* was 4.79 ± 0.32 cm in +E conditions, compared to 6.43 ± 0.28 cm in -E conditions. The final mean heights of *S. scoparium* grown with other *S. scoparium* were similar at the end of the experiment, regardless of antibiotic treatments (+E = 6.31 ± 0.54 cm, -E = 6.25 ± 0.27 cm; Fig. 2A).

Similar results to those detailed above on plant height were obtained for *S. scoparium* biomass at the end of the experiment (Fig. 2B). In an overall ANOVA model comparing biomass (separately as total, root, shoot, and root:shoot ratio) of *S. scoparium* and the effects of endophyte and competitor species, a significant interaction was noted ($F_{(1, 36)} = 8.79$, $p = 0.005$). To explore this interaction, ANOVAs were run separately to differentiate between the effects of endophyte and competitor species on biomass of *S. scoparium*. When growing with other *S. scoparium* plants, endophyte status had no significant effect on any biomass category of the native grass (total biomass $F_{(1, 18)} = 1.28$, $p = 0.273$). In contrast, *S. scoparium* in competition with invasive *S. halepense* showed significant effects of endophyte status on all biomass categories (total biomass $F_{(1, 18)} = 66.75$, $p < 0.0001$). Competitor species significantly reduced *S. scoparium* biomass in all categories in +E conditions (total biomass $F_{(1, 18)} = 15.00$, $p = 0.001$), while in -E treatments the effect of competitor was not significant for any category of biomass (total biomass $F_{(1, 18)} = 1.28$, $p = 0.273$). Mean total biomass of *S. scoparium* when grown in competition with *S. halepense* was 49.22 ± 13.62 mg in +E conditions, compared to 105.98 ± 17.24 mg in -E conditions. Mean total biomass of *S. scoparium* grown with other *S. scoparium* was similar at the completion of the experiment, regardless of antibiotic treatments (+E = 155.57 ± 85.75 mg, -E = 122.11 ± 37.62 mg; Fig. 2A). Together, these findings on growth and biomass indicated the native *S. scoparium* was negatively impacted by *S. halepense*, that tetracycline had no significant effects on growth of *S. scoparium*, and that tetracycline was relevant at alleviating the competitive suppression exhibited by *S. halepense* on *S. scoparium*.

Endophyte effects on *S. halepense* defense:

Insect herbivory assays: *Trichoplusia ni* caterpillars feeding on +E *S. halepense* were much smaller (Fig. 3A) and lower survivorship (Fig. 3B). Repeated measures ANOVA for biomass and survival of the insects showed a significant overall effect of endophyte ($F_{(1, 8)} = 7.601, p = 0.051$; $F_{(1, 18)} = 12.830, p = 0.002$ respectively). Herbivory experiments were terminated at the end of 7 d, due to almost complete mortality among of *T. ni* in the +E treatment.

Dhurrin / CN assays: Regardless of the manner in which *S. halepense* was grown (substrate and exogenous N-fertilizations), plants with endophytes (+E invaded soil, +E culture, +E agar) had the highest dhurrin concentrations (Fig. 4A, B). However, plant performance (growth rate) was not consistent across all treatments, thus, dhurrin concentrations were plotted for each individual plant as a function of growth rate (cm d^{-1}) to reveal the variation in performance among treatments (Fig. 5).

For plants grown in agar, ANOVA for dhurrin concentrations using growth rate as a covariate in the model, indicated that growth rate was not a significant predictor of dhurrin concentration ($F_{(2, 9)} = 0.110, p = 0.750$). Multivariate analysis showed that endophytic bacteria in active states (+E) had a significant effect on both dhurrin concentration and plant growth rate in agar substrates ($F_{(1,9)} = 6.815, p = 0.031$; $F_{(1, 9)} = 33.300, p < 0.0001$ respectively). Plants grown in sand were analyzed similarly using growth rate as a covariate in the model, and the results indicated again that growth rate was not a significant predictor of dhurrin concentrations in plants ($F_{(4, 38)} = 1.142, p = 0.293$). Additionally, there was a significant interaction between source of the endophytes (invaded soil or culture) and N-fertilization in the overall model ($F_{(4, 38)} = 4.319, p = 0.045$). Analysis of plants grown in -N conditions indicated that endophyte source

did not significantly affect dhurrin concentrations, or plant growth rates ($F_{(1, 19)} = 0.599$, $p = 0.450$; $F_{(1, 19)} = 4.022$, $p = 0.061$). Analysis of plants grown in +N conditions indicated the opposite effect; endophyte source was a significant driver of dhurrin concentration, but not plant growth rates ($F_{(1, 19)} = 4.533$, $p = 0.047$; $F_{(1, 19)} = 0.678$, $p = 0.421$). Average dhurrin concentrations were highest for plants grown with endophytes under high-N fertilization (+E_{N₂-fixing culture} / high-N, +E_{invaded soil} / high-N) and in those with seed-source endophytes under low-N fertilization (+E_{agar} / low-N). Dhurrin concentrations ranged between $2.4 - 5.1 \times 10^6 \mu\text{MCN g}^{-1}$ plant tissue (Fig. 5, solid symbols). Plants with endophytes but under N-deprivation (+E_{N₂-fixing culture} / -N, +E_{invaded soil} / -N) had reduced dhurrin concentrations (ranging between 1,205 – 1,728 $\mu\text{M CN g}^{-1}$ plant tissue, Fig. 5, open symbols). Dhurrin concentrations in plants without endophytes were very low and detectable only at the extreme limit of analytical resolution (indicated by the larger variation among analytical replicates, -E_{agar} / low-N; Fig. 5, gray triangles).

DISCUSSION

Our results indicate that bacteria harbored as endophytic mutualists significantly enhance several traits of *S. halepense* that are likely to be crucial to successful invasion. Endophytes significantly increased the establishment and growth of *S. halepense*, and when bacterial growth was restricted early in seedling development (7 d) plants were unable to establish. Restricting bacterial endophytes later in the growth cycle of *S. halepense* (18 d) reduced the height of *S. halepense* at the end of the study. This suggests that these endophytic bacteria are essential mutualists of *S. halepense*. However, the underlying mechanisms for these effects remain unknown. In another set of experiments (see Chapter 5), *S. halepense* grown with restricted

bacterial endophytes (-E) produced 5.5 times less rhizome biomass than *S. halepense* without endophyte suppression (+E), and endophytes stimulated plant growth even in the complete absence of exogenous N. Further, N fertilization did not affect the root:shoot ratio of *S. halepense*, but the presence of endophytes resulted in an increased allocation to belowground biomass by 2 to 4-fold. Taken together, these findings suggest that these bacterial endophytes are necessary for not only seedling establishment, but also for the extensive rhizomatous production well documented in *S. halepense* (McWhorter 1981).

The competitive effects of *S. halepense* on the native grass *S. scoparium* were eliminated when the competitors were treated with the antibiotic tetracycline, and *S. scoparium* plants in this treatment performed similarly to those grown with conspecifics (see Fig. 2A, B). We suspect that through shifts in resource allocation in +E plants, increased concentrations of the allelopathic root-exudate, sorgoleone, is produced. Further studies that measure sorgoleone exudates from +E and -E plants are necessary to answer this critical question and will provide insight into the mechanism(s) enabling the competitive effects observed in this study.

Regardless, these findings suggest that endophytes allow *S. halepense* to be a better competitor,

Endophytic bacteria also increase the production of the N-rich defense compound dhurrin. Plants for which the bacteria were suppressed by tetracycline (-E) were readily eaten by *T. ni*, whereas plants for which the bacteria were not suppressed (+E) were highly toxic to the generalist herbivore (see Fig. 3A, B). These inhibitory effects of E+ leaves on *T. ni* corresponded with a < 6-fold increase in dhurrin concentrations in +E compared to -E plants (Figs. 4, 5).

Bacterial endophytes provided different benefits to *S. halepense* under varying N-availability. Under N-deprivation, mutualistic bacteria significantly contributed to plant growth

but not plant defense; whereas under excess N-availability bacteria increased plant defense but not plant growth. Even more striking, when N supply was almost zero, bacteria increased plant growth and defense.

One possible mechanism accounting for these effects could be that endophytes, through fixed N₂ (see Rout & Chrzanoswki 2009) contribute to increased dhurrin concentrations. Alternatively, endophytes might be contributing to other precursor molecules of dhurrin (i.e., tyrosine), as is known to exist in ant-microbe associations (Zientz et al. 2006). It also appears that endophytic bacteria are essential for dhurrin production. Plants with minimal N-availability (50 ppm N: +E_{agar} / low-N) had similarly high dhurrin levels as plants supplemented with extremely high-N (1000 ppm N: +E_{N2-fixing culture} / high-N; +E_{invaded soil} / high-N; see Figs. 4, 5). This suggests that endophytes are essential for dhurrin defense production, and that high concentrations of dhurrin are possible, even under limited N-availability.

There are several findings from this work that have the potential to change our perspectives on mutualistic interactions among plants and microbes. First, bacterial endophytes in invasive *S. halepense* significantly contribute to plant establishment and growth, to competitive effects on the native *S. scoparium*, and to herbivore defense. These advantages were eliminated when bacterial endophytes were restricted. These results are not an artifact of the antibiotics themselves. In Chapter 5, there were no significant differences in growth and biomass of the native *S. scoparium* in the +E / -E conspecific treatments (see Fig. 2A, B), thus it is reasonable to conclude that artifact effects of tetracycline did not cause the effects reported here.

Previously, mutualisms involving invasive plants have been thought to “permit” invasion, but not “drive” invasion (Reinhart & Callaway 2006). My findings indicate that mutualistic

bacterial endophytes may drive invasion by *S. halepense*. Mutualistic endophytic bacterial communities that dramatically improve the competitive effects of an invasive plant on a native is substantially different than other roles attributed to mutualists in invasions (see Mack et al. 2000; Richardson et al. 2000; Levine et al. 2003; Hierro et al. 2005). Current approaches concentrate on escape from co-evolved organisms controlling invaders in home ranges, novel morphological traits expressed in invaded ranges bestowing an advantage to the invader, rapid evolution of invasive traits, or other forms of disrupted evolutionary relationships within communities. The idea that an invader can recruit growth-promoting bacterial mutualists introduces yet another layer of complexity to theoretical and empirical models for successful plant invasion and the effects of invaders on ecosystems (Rout & Callaway 2009). Thus, a key component to understanding factors regulating some plant invasions may reside, literally, within the plants themselves, through association with mutualistic microbial partners.

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FIGURE LEGENDS

Fig. 1: Repeated measures of growth (height) of *S. halepense* grown in sterile-agar substrates supplemented with the antibiotic tetracycline at 7d post transplanting (A; EARLY) or at 18d post transplanting (B; LATE). Surface-sterilized seedlings were aseptically transferred into receptacles at 7d after germinating when growth was repeatedly measured as the extent of green tissue. Tetracycline (12.5 mg mL^{-1} ; -E, open symbols) or sterile water (+E, solid symbols) were injected into the substrates once per treatment group.

Fig. 2: Measures of native *S. scoparium* growth (A) and biomass (B) grown in competition experiments conducted in sterile-agar substrates with low-N ($50 \text{ ppm NO}_3\text{-N}$). Repeated measures of growth (A) were recorded on plants grown in competition with *S. halepense* (triangles) or with conspecifics (squares). Surface-sterilized seedlings were aseptically transferred into receptacles at 7d when growth was repeated measured as the extent of green tissue. Antibiotics (tetracycline 12.5 ug mL^{-1} ; -E, open symbols) or sterile water (+E, solid symbols) were injected into the substrates on two occasions (17, 32 d) indicated by the arrows on the graph. At harvest, biomass of *S. scoparium* (B) was recorded from the competition experiments. Black bars represent sterile water (+E) treatments, gray bars represent antibiotic (-E) treatments.

Fig. 3: Repeated measures of biomass (A) and survivorship (B) of *T. ni* feeding on *S. halepense* grown in sterile-agar substrates supplemented with low-N ($50 \text{ ppm NO}_3\text{-N}$). Individual *S. halepense* leaves were repeatedly fed to the same replicate insects to calculate individual insect biomass (A). Survivorship (B) was calculated on replicate *T. ni*. ($n=5$, $N=50$) feeding on lettuce leaves 5d prior to the trial, at which point insects were fed +E leaves (solid symbols) or -E leaves (open symbols) treated with antibiotics or sterile water as described previously.

Fig 4: Concentration of dhurrin (log transformed) in *S. halepense* leaves, measured as $\mu\text{M CN g}^{-1}$ plant tissue, depicted as a function of substrate grown in (A) and exogenous N-fertilization (B). Substrates were sterile agar fertilized with low-N (50 ppm $\text{NO}_3\text{-N}$), or sterile sand fertilized with high-N or devoid of N (1000 ppm or 0 ppm $\text{NO}_3\text{-N}$, respectively). Seedlings grown in agar were supplemented with tetracycline or sterile water (-E, +E respectively; $n = 5$, $N = 10$). Seedlings grown in sand were supplemented with one of two bacterial slurries (N_2 -fixing culture or invaded soil; $n = 10$, $N = 40$). Error bars represent standard error of the means.

Fig. 5: Concentration of dhurrin (log transformed) in *S. halepense* leaves, measured as $\mu\text{M CN g}^{-1}$ plant tissue, log transformed as a function of plant growth rate (cm d^{-1}). Three +E endophyte sources grown in the presence of N (black symbols) and absence of N (open symbols) are represented: those from N_2 -fixing cultures added to substrate (N_2 -fixing culture); those from bacteria in invaded soils (invaded soil); and those from bacterial endophytes inherent in seeds grown in sterile-agar substrate (sterile agar). One -E endophyte source (gray triangles) depicts plants grown in sterile agar treated with antibiotics as described previously. Dhurrin concentrations were calculated on 2 leaf-disks plant^{-1} , analyzed in analytical triplicate. Growth rate was calculated from repeated measures of growth (height) over time from individual plant replicates used for the CN assays.

FIGURES

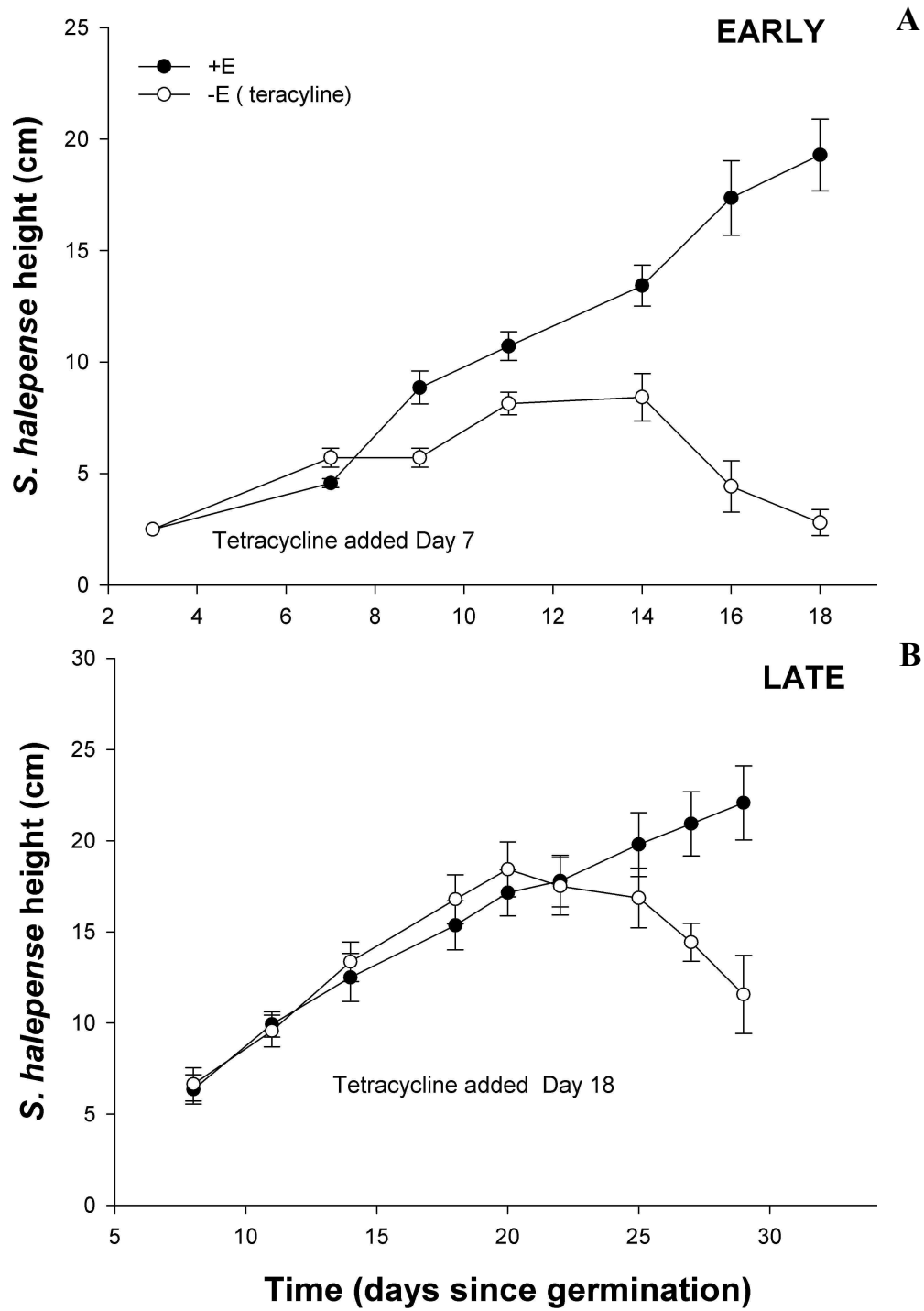


FIGURE 1

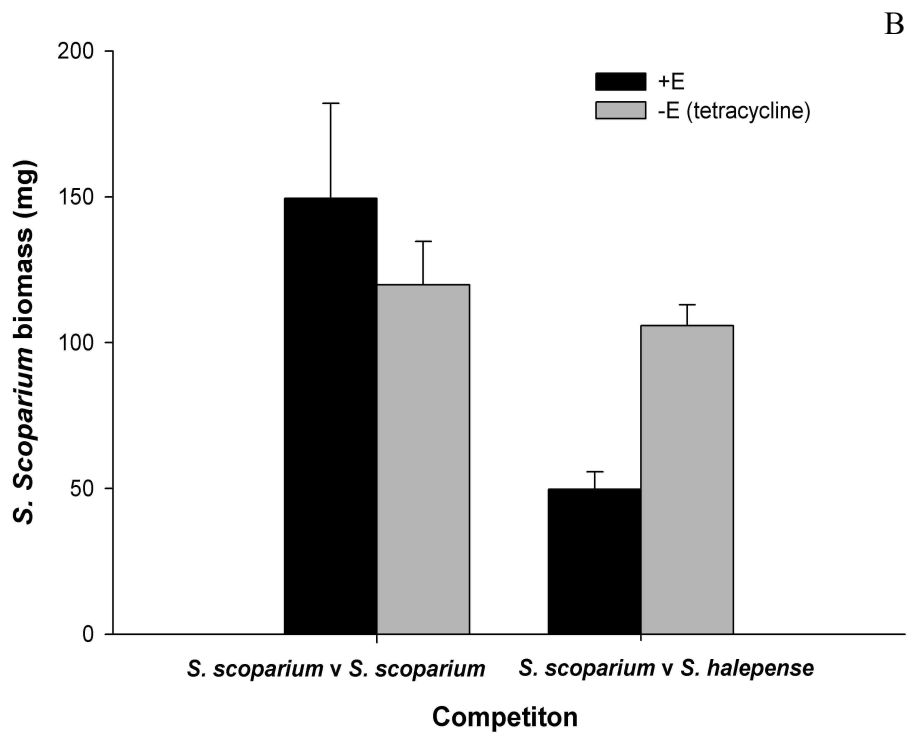
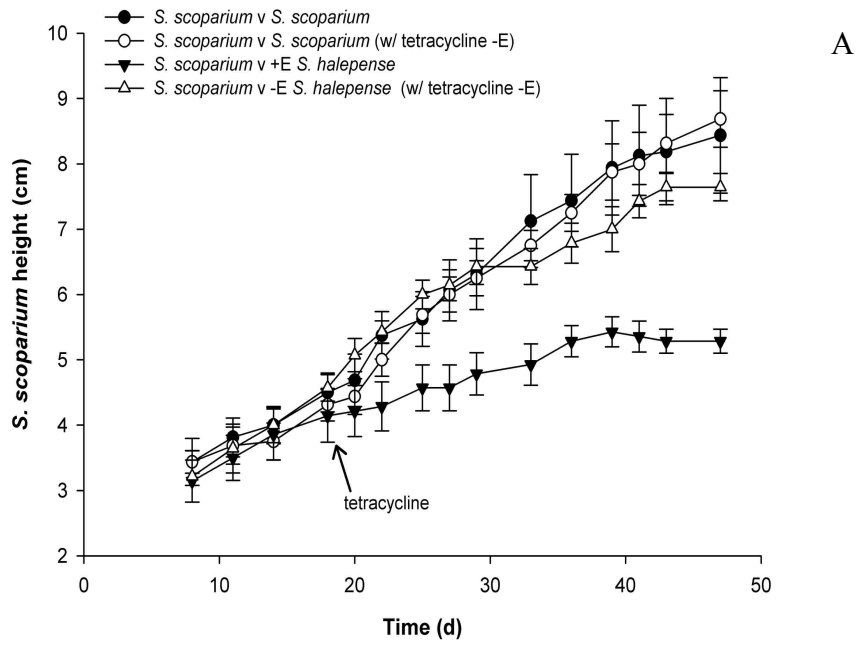


FIGURE 2

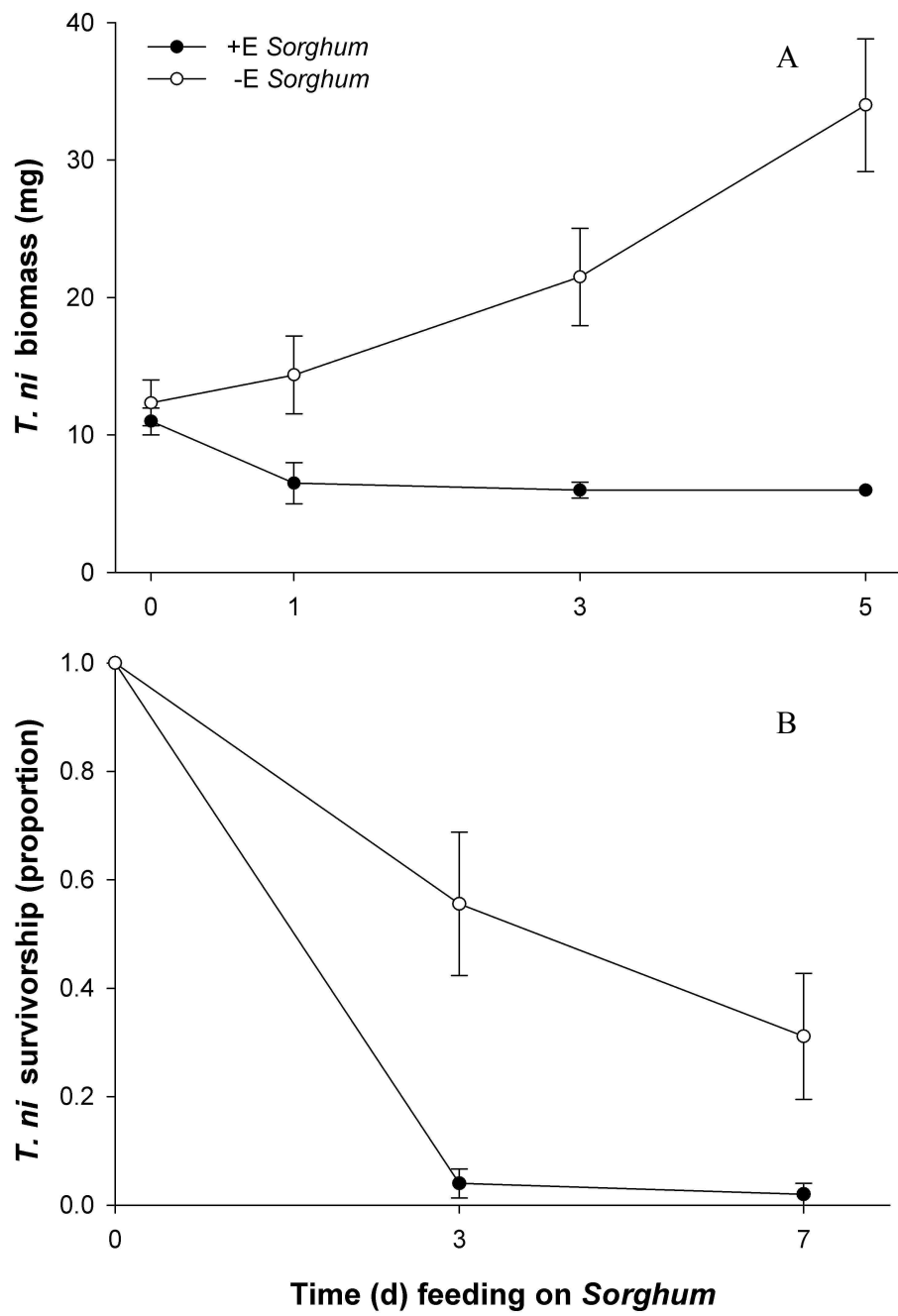


FIGURE 3

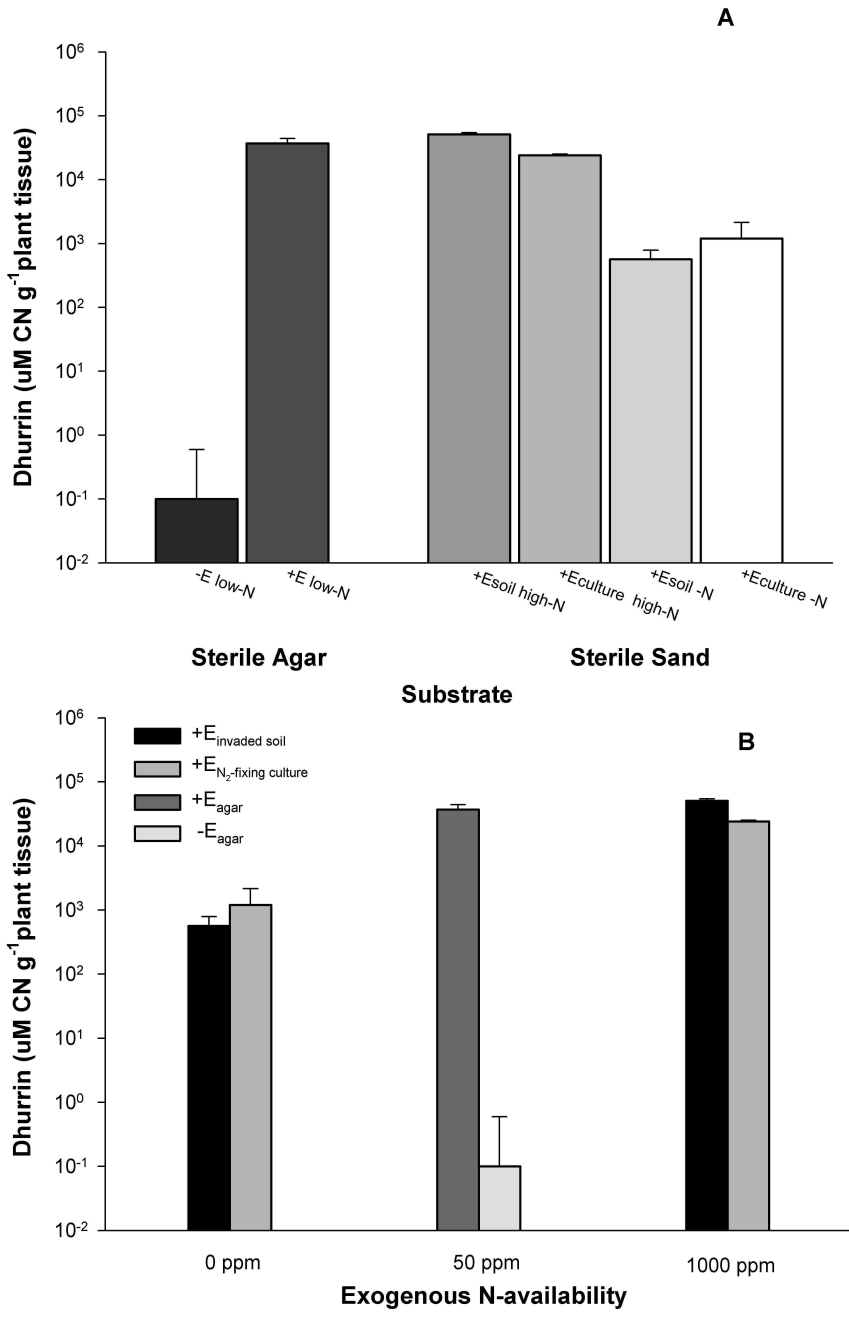


FIGURE 4

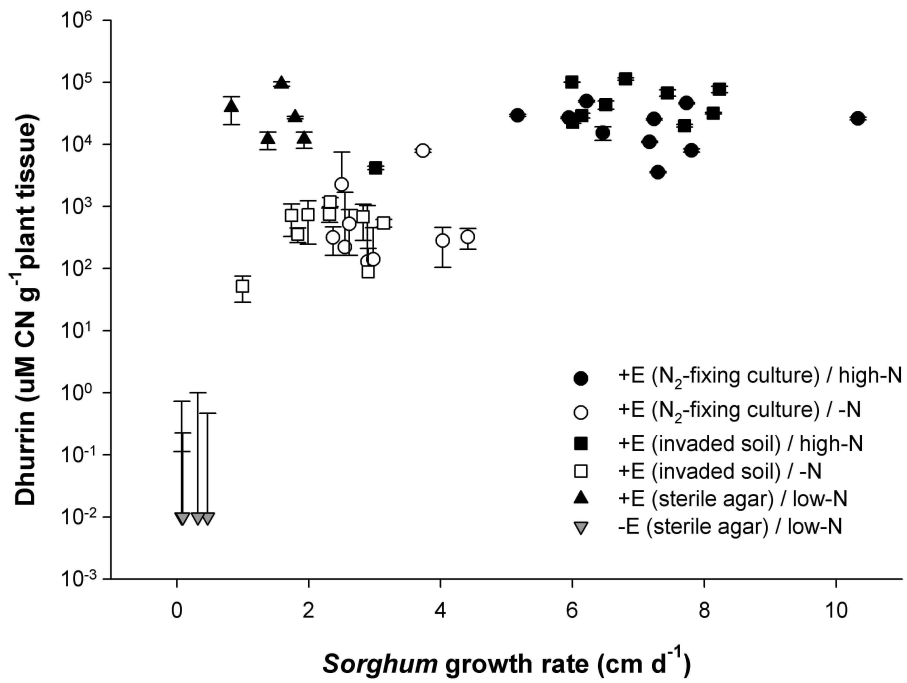


FIGURE 5