GARLIC MUSTARD AND ITS EFFECTS ON SOIL MICROBIAL COMMUNITIES IN A SANDY PINE FOREST IN CENTRAL ILLINOIS

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Abstract.—This study evaluated the impacts of garlic mustard (*Alliaria petiolata),* an invasive species, on soil microbial community dynamics in a pine plantation on sandy soils in central Illinois. In situ soil carbon dioxide efflux was significantly greater in invaded sites. Similarly, in vitro carbon mineralization was significantly greater for soils collected from invaded sites, but only early in the incubation period. Incubations with selective inhibitors showed a decrease in fungi relative to bacteria. Nitrogen-free selective agar plates inoculated with soil slurries supported greater numbers of bacterial colonies on invaded soils. Overall, our studies suggest that garlic mustard invasions have the potential to shift microbial community structure by selectively increasing some bacterial populations and decreasing fungal populations. Furthermore, garlic mustard invasions may significantly affect microbes involved in nitrogen turnover, suggesting that removal of this invasive species may not be sufficient to restore soil microbial community dynamics and ecosystem function.

INTRODUCTION

The number of invasive species has increased dramatically in nearly every habitat across the world (Baskin 2002). Invasive plant species may be introduced accidentally as seeds in foreign goods or via shipping vessels, or purposely for aesthetic, food, or medical purposes. Garlic mustard (*Alliaria petiolata*) is a herbaceous biennial plant of the Brassicaceae family that was introduced to North America by European settlers in the mid-1800s (Anderson et al. 2010). By 2009, garlic mustard (GM) had been documented in 37 states and 5 Canadian provinces (Natural Resources Conservation Service 2009). Studies have focused on understanding what predisposes areas to invasion and the impact that invasive species have on native ecosystem dynamics once established, with the ultimate goal of creating practical preventive and management strategies to reduce ecological and economic impacts alike (Kolar and Lodge 2001, Rejmánek and Richardson 1996).

Invasive plant species have been found to modify soil physiochemical properties as well as change biotic properties, including microbial community structure and function (Belnap and Phillips 2001, Ehrenfeld et al. 2001). Garlic mustard is no exception. Previous studies have suggested that GM alters soil nutrient conditions in its invaded range by increasing the amount of plant-available nitrogen (Morris et al. 2012, Rodgers et al. 2008), a macronutrient that, if present in only low concentrations, commonly limits plant growth. Additionally, GM is one of the few plant species that do not form mycorrhizal associations due in part to its ability to release allelochemicals as native species root exudates, resulting in reduced germination of mycorrhizal associations and colonization (Roberts and Anderson 2001). A survey of the presence or absence of mycorrhizae in >6,500 angiosperm species conducted by Cronquist (1981), found that 82 percent of species surveyed had

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mycorrhizal associations. Furthermore, GM can destroy fungal associations of native plant seedlings, which are necessary for growth, thus perpetuating its invasion (Stinson et al. 2006). In addition to this unique characteristic, allelopathic glucosinolates have been found to affect germination and growth of native plant species through inhibition of soil mycorrhizae (both ectomycorrhizal fungi and arbuscular mycorrhizal fungi) and rhizobial bacteria (Anderson et al. 2010, Murray et al. 2011, Rodgers et al. 2008). Another chemical defense is the release of cyanide from plant tissues (Cipollini and Gruner 2007).

The goal of this study was to examine the effects of GM on soil microbial community dynamics. Our research was conducted in forest soils with and without a history of GM invasion in a pine (*Pinus* sp.) plantation in central Illinois at Sand Ridge State Forest (SRSF). The sandy texture of the soil makes the area susceptible to nitrogen leaching, which can lead to decreased availability of nitrogen for plant uptake (Lee and Jose 2005). Many areas of SRSF have dense populations of GM, but our ongoing study of this site has confirmed that there are areas that are currently uninvaded (NGM). The ability of GM to change the local soil microbial community may be an important factor in understanding its invasibility, likely mediated through increases in the abundance of free-living nitrogen-fixing bacteria (Ehrenfeld et al. 2001, Wolfe and Klironomos 2005). This relationship may help explain why GM is able to establish itself in undisturbed, nitrogen-poor habitats such as those at SRSF. Because GM has been found to alter microbial community structure and nitrogen availability, GM invasion could potentially affect overall productivity and nutrient cycling in this managed forest.

METHODS

Sampling Site: Sand Ridge State Forest

Three study sites were chosen at SRSF near Forest City, IL, for soil sampling. This forest covers >7,500 acres and is the largest state forest in Illinois. Located in northern Mason County, it contains soils predominantly composed of sandy loam, which was formed at the end of the last ice age. In 1939, the Civilian Conservation Corps, managed by the Division of Forestry, oversaw 5,504 acres of the forest tract as a conservation and experimental area. During this time, 2,200 acres were cleared and converted into a series of pine plantations. Today, SRSF has 7,200 acres and is composed of 3,916 acres of dry land oak-hickory (*Quercus-Carya* spp.) woodlands, 2,492 acres of pine woodlands, and 792 acres of open fields and sand prairie (Illinois Department of Natural Resources 2013).

Three separate pine sites (1, 2, and 3) with similar characteristics were selected at SRSF. At each site, six 2-m^2 plots were randomly selected in GM-invaded and uninvaded areas (12 total plots per site). The three study sites chosen in the forest met the following criteria and were compatible with our sampling parameters: (1) pine overstory, (2) age >50 years, and (3) a minimum area of relatively uniform plant diversity comprising mainly pine and GM. Our study consists of several semi-independent experiments examining soil microbial communities at SRSF. Therefore, the three sites were used differently depending upon the parameters of each sampling method being tested.

In Situ Soil Carbon Dioxide Efflux

In situ soil carbon dioxide (CO_2) efflux was measured by using a portable infrared LI-8100 analysis machine (LI-COR Inc., Lincoln, NE) at all three sites. Ten-cm-diameter polyvinyl chloride collars were permanently placed in the center of each plot at a depth of 2.54 cm from the top rim and left

undisturbed for ≥24 hours before measurement. This "resting time" allowed the removal of any excess CO_2 released by roots damaged during collar placement.

The head used for measurements was a standard LI-COR® 20-cm head and the offset of the program was adjusted to 2.54 cm. A temperature probe was placed in the soil and allowed to stabilize before initiating the program. About 10 minutes before sampling, the collars were cleared of live vegetation and litter to reduce the possibility of disturbance effects. Accurate measurements require that the soil remain undisturbed to prevent the loss of CO_2 from the chamber during readings (LI-COR Biosciences 2012). Removal of the litter ensured that we measured CO_2 levels of the soil within the collars and not from CO_2 being emitted from leaf litter. Measurements from the LI-COR were set at the 10-ppm range. Three complete cycles were run per collar per plot and an average reading was recorded and analyzed by using the LI-8100A soil CO_2 flux system embedded instrument software (LI-COR Biosciences 2012). In situ soil CO_2 efflux was compared as a function of garlic mustard density in each plot.

In Vitro Catabolic Response Profiles

A modified version of the catabolic response profile (CRP) approach was used to measure the catabolic differences of the soil microbial community in plots with and without GM invasion. The method developed by Degens and Harris (1997) analyzes microbial communities' short-term respiratory response to simple organic substrates. In our study, nitrogen and carbon substrates were used to assess patterns of microbial communities at SRSF without the need to extract or culture organisms from the soil. The CRP technique is a functional measure of microbial response that is useful in comparing diversity between communities by examining metabolic response patterns of the organisms to the substrates within a sample (Degens and Harris 1997).

Bulked GM and NGM soils were collected randomly from plots within site 1 to a depth of 5 to 10 cm and divided into two sets of six different treatments with four replicates per treatment. The first set of GM and NGM soils was treated with glucose (3 mg/g) with or without the addition of nitrogen. The second set of treatments used sawdust (4 mg/g) with or without addition of nitrogen. Nitrogen was added as 0.0114 $NH_4^+NO_3^-$ mg/g soil following the protocol outlined by Brewer (2004). Deionized water was used in two treatments with no additional substrates to measure unamended soil respiration rates. The samples were stored in quart-sized jars at 25 °C and CO_2 released was measured after 3, 7, 10, 13, and 20 days of incubation on a Model LI-G252 CO_2 Analyzer (LI-COR).

Substrate-Induced Respiration Using Selective Inhibitors

Developed by Anderson and Domsch (1978), substrate-induced respiration (SIR) measures the amount of microbial respiration of soils after amending them with an excess amount of a soil nutrient, typically glucose, in order to trigger microbial activity (Aira and Dominguez 2010). Soil samples were collected randomly from six GM and six NGM plots at site 1 by using a soil corer 2 cm in diameter. Samples were spread out into two rows of four within a 2 m by 2 m plot frame. Each row of four samples was located about 0.75 m from the top and bottom of the plot frame, respectively. Soils were sieved and refrigerated until use. Before analysis soil samples were split into subsamples and weighed into incubation flasks. Moisture was maintained at field capacity at room temperature for 12 hours.

For each subsample, soils (25 g) were amended with glucose, glucose and cycloheximide (a eukaryotic inhibitor), glucose and streptomycin (a bacterial inhibitor), and glucose and the two inhibitors together. Samples measured for basal respiration were unamended. For glucose, the minimum amount necessary for maximum respiratory response was added to soil. For the inhibitors, the minimum amount necessary for maximum inhibition of respiration was added once the minimum glucose amount had been determined. These values were determined for the soils at SRSF by varying concentrations and measuring respiratory response. Inhibitors and glucose were added dry to soil samples. The soil was mixed by stirring, then immediately sealed. Headspace CO_2 was taken at 0, 1, and 5.5 hours. Glucose was added at a rate of 5.0 mg/g soil, cycloheximide at 8.0 mg/g soil, and streptomycin at 14.0 mg/g soil.

Selective inhibition was carried out as described by Bailey et al. (2002). One hour after the addition of inhibitors, glucose was added at the concentration to achieve maximal respiration (over 6 hours) for each soil sample. The incubation jars were sealed immediately after glucose addition and a CO_2 reading was taken before the jars were placed in the dark at 25 °C. Jars were wrapped in aluminum foil to further reduce any effects of light. Headspace CO_2 was withdrawn through a rubber septum from the jar with a gastight syringe for each treatment at 0 and 5.5 hours and measured on a Model LI-G252 CO_2 Analyzer (LICOR). The ratio of fungal to bacterial activity was calculated as the respiratory inhibition caused by the fungicide, divided by the respiratory inhibition caused by the bactericide at 5.5 hours.

Bacterial Isolation

Soils used to determine the number of culturable bacteria on nitrogen-free media were collected from GM and NGM plots on sites 1 and 2. Bacterial number was evaluated by inoculating soil slurries on nitrogen-free (Thompson-Skerman) agar plates. Serial dilutions were prepared to obtain plates with a countable number of colonies. Nitrogen-free plates consisted of 500 ml of nanopure water, agar (10 g), glucose (5 g), ferrous sulfate heptahydrate (5 mg $FeSO_4 \cdot 7H_2O$), calcium carbonate (2.5 g $CaCO_3$), dipotassium phosphate (0.45 g K_2HPO_4), calcium chloride dihydrate (50 mg $CaCl_2 \cdot 2H_2O$), potassium phosphate (50 mg KH_2PO_4), magnesium sulfate heptahydrate (5 mg $MgSO_4 \cdot 7\ H_2O$), sodium molybdate dihydrate (2.5 mg $NaMoO_4 \cdot 2H_2O$), and bromothymol blue (1.1 ml of 5 percent ethanol solution). Plates were incubated at 30 °C for 2 to 7 days and then colony counts were performed. Individual colonies that grew on nitrogen-free plates were reinoculated onto another nitrogen-free plate to ensure introduced nitrogen was not a factor in bacterial growth.

Statistical Analyses

Differences between NGM and GM basal and substrate-induced respiration with and without inhibition were determined by using one-way analysis of variance (ANOVA; PROC MIXED; SAS Institute Inc., Cary, NC). All data were evaluated for and met assumptions of ANOVA. In situ CO_2 flux was evaluated by using regression analysis for each site. In vitro respiration rates did not meet assumptions of ANOVA, so they were analyzed by using a nonparametric one-way ANOVA (PROC NPAR1WAY; SAS Institute Inc.) with mean separations determined from Bonferroni multiple comparisons based on ranked data. For bacterial plate counts on nitrogen-free media, colony-forming units were log transformed and plotted as a function of GM density. Linear regressions were performed for each site and then as a whole.

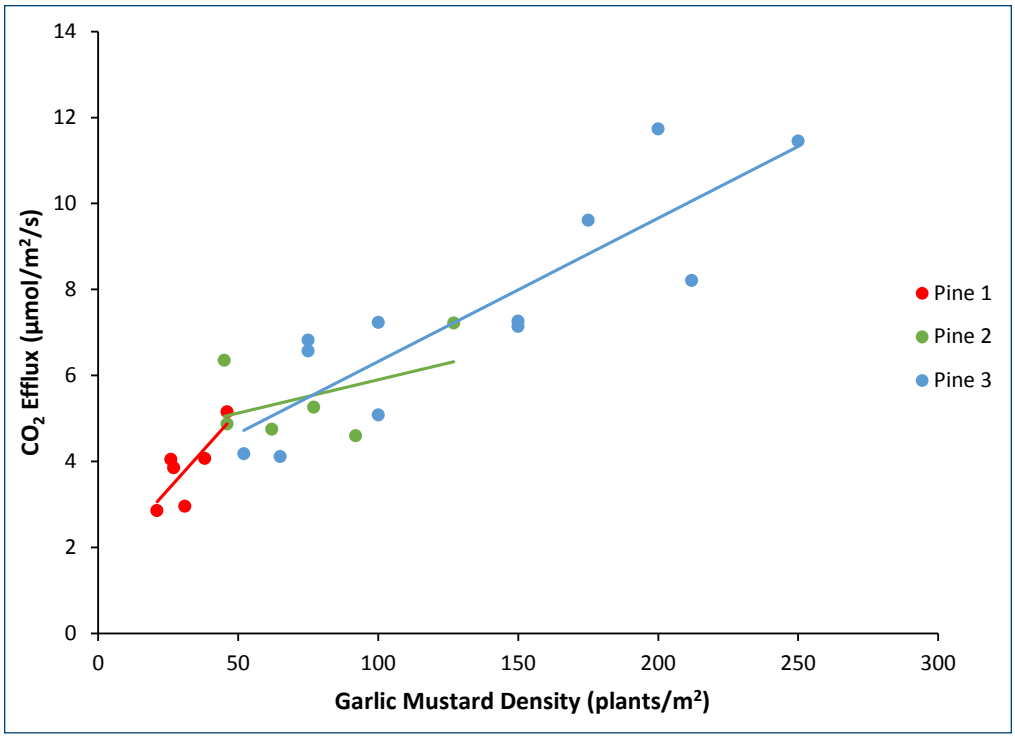

Figure 1.—In situ CO_2 efflux measurements at three different pine sites with varying garlic mustard population densities at Sand Ridge State Forest, Illinois. Pine Site 1: n=6, $R^2 = 0.6146$; Pine Site 2: n=6, $R^2 = 0.2148$; Pine Site 3: n=12, $R^2 = 0.7617$.

RESULTS

In Situ Soil CO_2 Efflux

There was an increase in soil CO_2 efflux as GM densities increased. At two of the three sites (1 and 3) more than 60 percent of the variation in soil CO_2 efflux was explained by the density of GM (Fig. 1).

In Vitro Catabolic Response Profiles

Soil CO_2 efflux was also measured in the laboratory, where the impacts of soil roots would be minimized. Although basal respiration did not differ between GM and NGM soils, soil respiration rates did differ when substrates were added with and without nitrogen. The NGM soils had soil respiration rates similar to GM soils early in the incubation when glucose was added, but only when nitrogen was added with the glucose. Carbon dioxide efflux was significantly greater for GM soils than for unamended NGM soils early in the incubation when we added either glucose with or without nitrogen or sawdust with or without nitrogen. Overall, GM and NGM soils amended with sawdust and sawdust with nitrogen had consistently higher rates of respiration compared to unamended soils, with respiration rates for all treatments declining over the 20-day incubation (Fig. 2). Soils treated with glucose had higher rates of respiration compared to those treated with sawdust (Figs. 2 and 3). Unamended GM and NGM soils did not differ throughout the incubation. Treatments with glucose had higher rates of respiration compared to unamended GM and NGM at all time points (Fig. 3). Early in the incubation (days 3 and 7) GM soils with glucose and with or without nitrogen had higher rates of respiration compared to NGM soils with added glucose. On day 3, NGM soils with added glucose and nitrogen had respiration rates significantly greater than NGM soils amended with only glucose (Fig. 3).

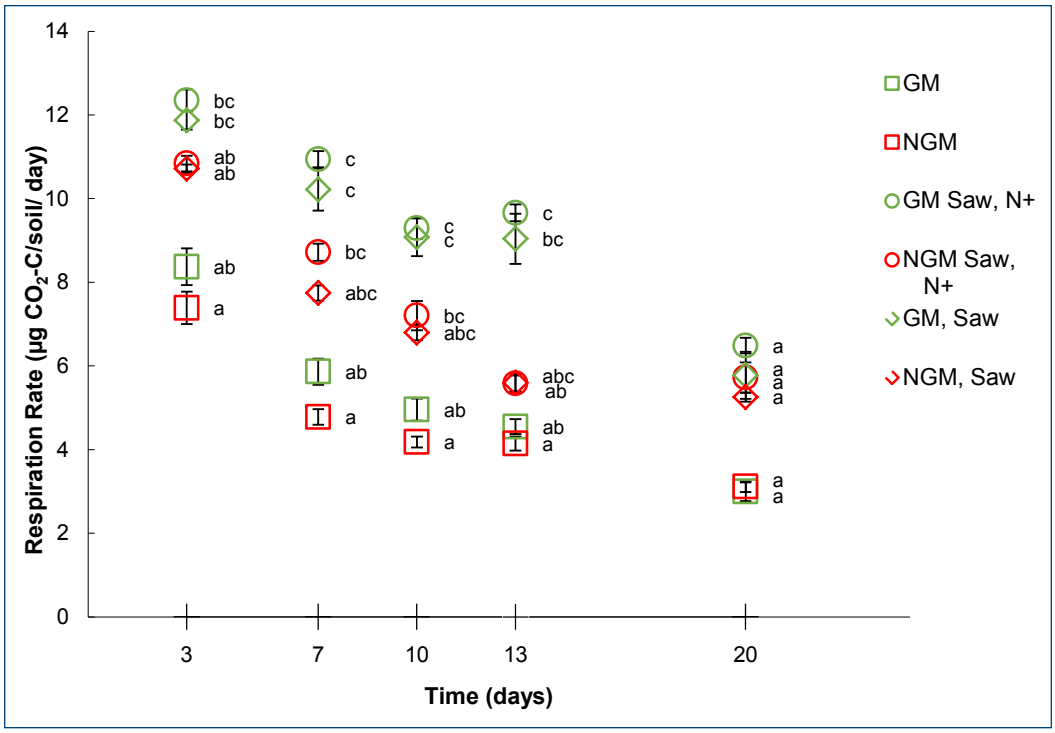

Figure 2. —In vitro respiration rates of garlic mustard and nongarlic mustard soils amended with nitrogen (N+) and sawdust (saw) at 0, 3, 7, 13, and 20 days of incubation at Sand Ridge State Forest, Illinois. Points labeled with the same letter represent means that are not significantly different at $p \leq 0.05$. Error bars represent mean±standard error (SE).

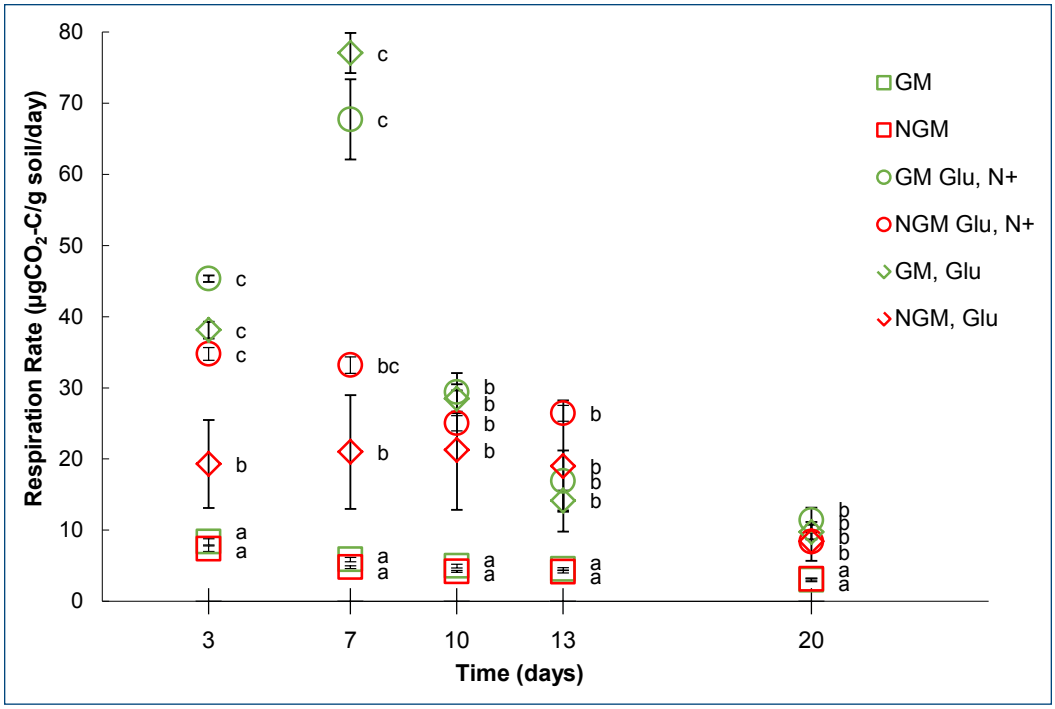

Figure 3.—In vitro respiration rates of garlic mustard and nongarlic mustard soils amended with nitrogen and glucose (glu) at 0, 3, 7, 13, and 20 days of incubation at Sand Ridge State Forest, Illinois. Points labeled with the same letter represent means that are not significantly different at $p \leq 0.05$. Error bars represent mean±SE.

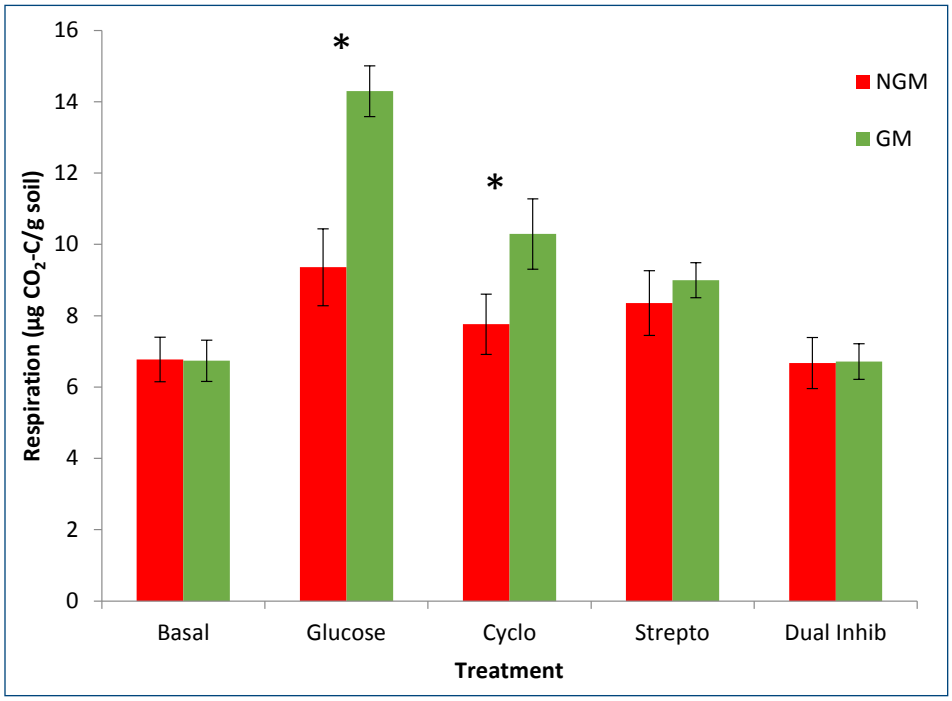

Figure 4.—Substrate-induced respiration after 5.5 hours of incubation in garlic mustard and nongarlic mustard soils at Sand Ridge State Forest, Illinois. Treatments represented are additions of water (basal respiration), glucose, cycloheximide (cyclo), streptomycin (strepto), and cycloheximide and streptomycin (dual inhib) added together. Histogram bars represent mean ± SE. Asterisks (*) indicate significant difference at $p \leq 0.05$.

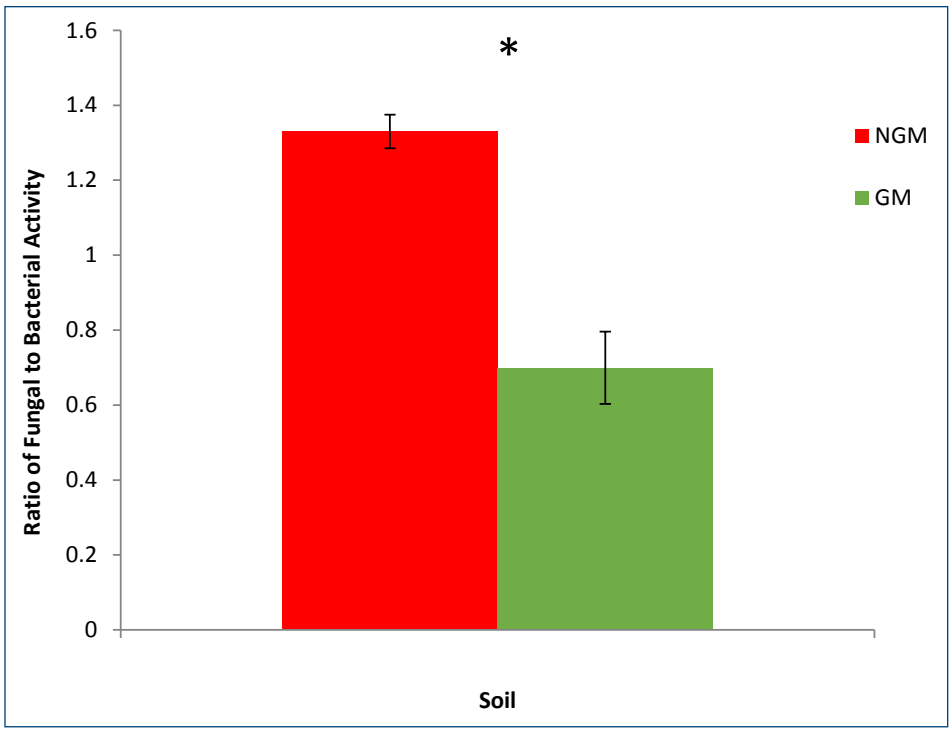

Figure 5.—Ratio of fungal to bacterial activity calculated from fungal inhibition with cycloheximide, and bacterial inhibition with streptomycin after 5.5 hours of incubation in garlic mustard and nongarlic mustard soils at Sand Ridge State Forest, Illinois. Histogram bar represents mean ± SE. Asterisk (*) indicates significant difference at $p \leq 0.05$.

Substrate-Induced Respiration Using Selective Inhibitors

Microbial respiration in soils treated with glucose was significantly greater for GM soils compared to NGM soils, with rates of about 14 µg CO_2-C/g soil and 9 µg CO_2-C/g soil, respectively (Fig. 4). After treatment of GM soils and NGM soils with cycloheximide, GM soils had significantly higher respiration rates. Respiration rates for GM and NGM soils did not differ between the other treatments. Based on these values, the calculated ratio of fungal to bacterial activity for NGM soils was significantly greater than for GM soils (Fig. 5).

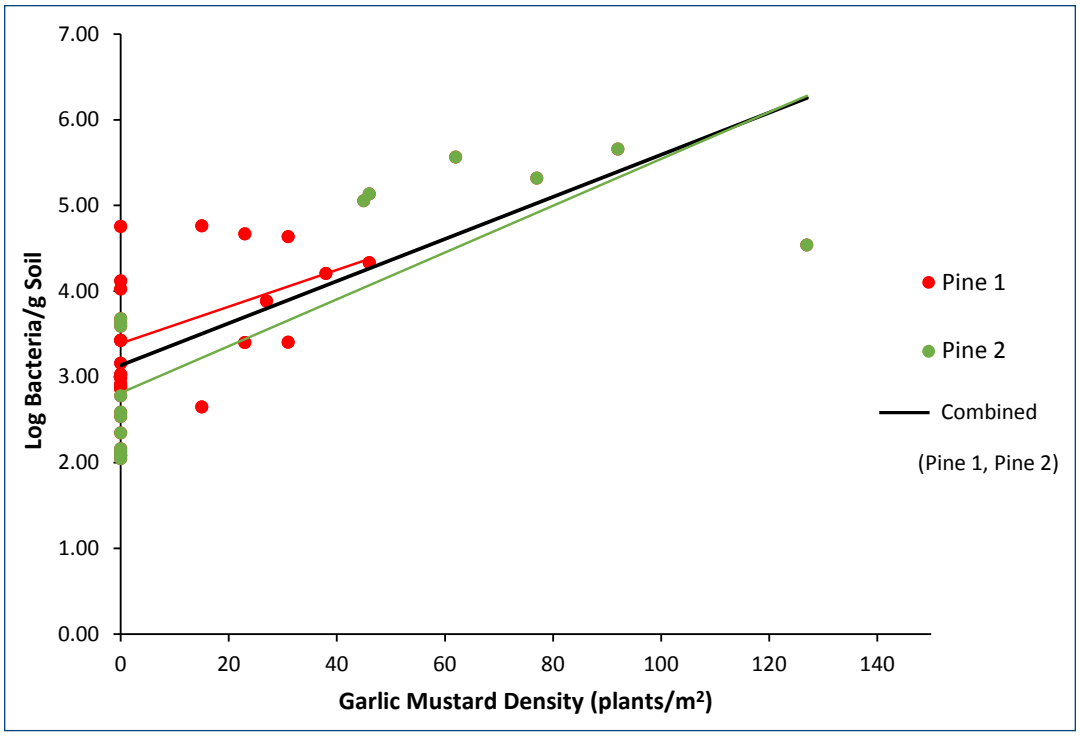

Figure 6.—Relationship of nitrogen-fixing bacteria to garlic mustard density in two selected pine sites at Sand Ridge State Forest, Illinois. Pine Site 1: n=21, R^2 = 0.2178; Pine Site 2: n=18, R^2 = 0.6391; Combined Pine Sites 1 and 2: n=39, R^2=0.4831.

Bacterial Isolation

As GM density increased, there was an increase in the number of bacteria able to grow on nitrogen-free media (Fig. 6). When the sites were analyzed together, nearly 50 percent of the variation in bacterial number was explained by GM density.

DISCUSSION

We hypothesized that GM invasion would alter microbial community dynamics. There are many approaches to evaluating microbial community dynamics. Our first measure at the largest scale was an estimate of soil CO_2 efflux in GM and NGM plots in the field. Soil CO_2 efflux increased as GM density increased across the three sites evaluated (Fig. 1). In situ soil CO_2 efflux reflects contributions from roots as well as from members of the soil microbial community (Hanson et al. 2000). Teasing apart the impacts of GM on the soil microbial community from the impacts of roots or through changes to root dynamics such as increased rhizosphere activity requires an examination of soil CO_2 efflux without the presence of roots.

Soil efflux measured in the lab provided evidence that soil respiration rates did differ when soil was amended with and without nitrogen. These data also support an ample nitrogen supply in the face of a limited carbon supply and an increase in available nitrogen in the presence of garlic mustard that was not observed in NGM soils. Morris et al. (2012) found increased rates of nitrogen mineralization in GM soils compared to NGM soils. These findings indicate that GM has the potential to increase nitrogen availability in soils and also provide a mechanism for the differences in response to carbon substrates in our data.

The response of the microbial community to substrate additions in the GM and NGM soils may also be the result of differences in the composition of the microbial communities. Bacteria typically decompose simpler substrates, whereas fungi decompose complex organic molecules like tannins and lignins (Wall and Moore 1999). This generalization is supported in many experiments that have examined how ratios of substrate carbon to nitrogen favor growth of bacteria compared to fungi, or vice versa (Rousk and Bååth 2007). Therefore, the large increase in respiration observed when soils were treated with glucose in both the CRP and SIR experimental samples suggests an increase in bacteria compared to fungi (Figs. 3 and 4). Alternatively, the defense compounds created and exuded from GM roots may have predisposed the microbial communities to favor glucose use. That the difference in response to substrate additions was a reflection of a change in the composition of the microbial community is also supported by results from the addition of cycloheximide. This addition led to a more significant reduction in respiration in NGM soils compared to GM soils (Fig. 4) and a resultant decrease in the ratio of fungal to bacterial activity in GM soils (Fig. 5). Therefore, there is evidence of changes in the microbial community with GM invasion.

The alteration of soil microbial interactions has also been thought to play an important role in the successful invasion of GM (Rodgers et al. 2008). Studies have found a decrease in the richness and diversity of native bacterial species in the presence of GM (Hammer 2009, Lankau et al. 2011). These changes in diversity may also reflect a change in microbial community function. Nitrogen-free plates allow for identification of bacterial colonies that are likely to fix nitrogen as their sole source of nitrogen. We found a strong relationship between the number of bacterial colonies observed (a proxy for the number of bacteria in the soil sample) and the density of GM on the site sampled (Fig. 6). Rodgers et al. (2008) suggested that GM increases soil nitrogen and that preexisting nitrogen conditions were not a factor in GM invasibility. Taken together with experimental results, their analysis suggests that GM may alter microbial community structure at least partly by increasing the number of free-living, nitrogen-fixing bacteria. These observations are also supported by the substrate addition data (Figs. 4 and 5), which suggest that respiration in GM soils is carbon limited rather than nitrogen limited.

Our results indicate that garlic mustard invasions can have significant impacts on microbial communities, particularly on those microbial guilds involved in nitrogen turnover. There is ample literature to support changes in plant community dynamics as a consequence of increased nitrogen availability in soils (Baer et al. 2004, Davis et al. 2000, De Schrijver et al. 2011). Forest managers charged with ensuring forest integrity should consider that removal of this invasive species may not be sufficient to restore soil microbial community dynamics and consequently ecosystem function. Understanding the effects of invasive species such as garlic mustard on soil microbial communities and subsequent impacts on nutrient cycling can increase our capacity to develop management strategies for addressing specific impacts of invasives on forest systems.

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