The Effect of *Azospirillum lipoferum* Inoculation on Microbial Abundance and Diversity of the Corn Soil Microbial Population after a Moderate-Intensity Fire

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High-intensity forest fires can negatively affect the soil rhizosphere by decreasing microbial populations and limiting ecological processes. After a high-intensity fire, the soil microbiome can take months or years to completely recover and resemble pre-fire conditions. However, both plant growth and an increase in microbial diversity can aid in the recovery process over time. Inoculation of the soil rhizosphere has induced positive effects in the soil microbiome in some studies with unburned soil. Studies have demonstrated that *Azospirillum lipoferum* inoculation significantly increases corn soil nitrogen and osmotic potential of corn plants in water-stressed environments. However, whether *Azospirillum lipoferum* also has a positive effect on the soil rhizosphere during fire recovery has not yet been studied. The goal of this study is to explore the possibilities of *Azospirillum lipoferum* inoculation in aiding corn growth and rhizosphere recovery after a high-intensity fire. Soil taken from the roots of corn plants was burned for six hours at temperatures above 800°C. After burning, both the unburned and burned soil were cultured. The burned soil had no culturable bacteria on any of the 10^{-4} dilution plates immediately post-fire. The post-burn greenhouse study found that soil inoculation with *A. lipoferum* led to an increase in microbial diversity and plant growth when compared to unburned soil.

Introduction

A rise in global temperatures due to greenhouse gases has led to increased wildfires worldwide[1]. In the United States, the largest area consumed by wildfires is crop land[2]. Crops with high yields including wheat and corn are destroyed, limiting the amount of food grown by farmers.

Much focus has been given to above-ground matter, yet, microorganisms including fungi and bacteria are also greatly affected by fires. However, different fire intensities have varying effects on soil microbial populations[3]. While controlled burns and low-intensity fires allow for the availability of minerals and compounds, higher-intensity fires such as forest fires lead to partial sterilization of soil and a large decrease in soil organic matter. This decrease in soil organic matter can have negative effects on soil microbial populations in the months or years after a fire, as organic matter provides a storage reservoir of nutrients, including inorganic ions essential for plant growth[4]. Soil organic matter also provides the main source of energy for soil microorganisms[5]. A healthy rhizosphere and soil community with diversity and an abundance of microorganisms is necessary for plant health and renewed growth after fire[6].

Plants and their rhizospheres are deeply intertwined. Plant roots exude chemicals that shape their surrounding microorganisms[7]. In the same strain, beneficial bacteria and fungi such as rhizobacteria and arbuscular mycorrhizae provide plants with important nutrients including fixed nitrogen. However, fire hinders this symbiotic relationship[8].

Azospirillum lipoferum, a nitrogen-fixing rhizobacterium, when used as an inoculant, has been found to increase osmolyte production in drought-stressed soil[9]. It is also known to produce phytohormones which play a role in plant growth[10]. *A. lipoferum*, has also been used to increase crop productivity through seed and root inoculation due to its nitrogen-fixing abilities[9-11]. The nitrogen-fixing abilities of this bacterium also have a positive effect on the surrounding microbes, increasing rhizosphere diversity[10].

After a high-intensity fire, partial sterilization can decrease rhizosphere diversity and cut off microbe access to plant roots[8]. *A. lipoferum*, as both a plant growth-promoting bacterium and nitrogen-fixing bacterium may decrease such drastic effects.

If successful, *A. lipoferum* will be able to limit the ramifications of fire on agricultural land and quicken the recovery of the soil microbial community. To study the possible effects of *A. lipoferum* on burned soil, the soil was taken from the roots and surrounding area of corn plants, burned, and inoculated with *A. lipoferum*. The first objective was to seek to understand the role *A. lipoferum* plays in microbial recolonization. The second objective was to determine whether any change will have a significant effect on plant growth.

Materials and Methodology

Pre-Fire Soil Preparation. Ten gallons of soil were collected from corn mazes at the Foster Family Farm in South Windsor, CT. Soil was collected from the area around the roots of 11 corn plants. Soil was shaken from the roots into two 5 gallon buckets. Soil was stored outdoors away from moisture and sunlight for 20 hours before the burn with a temperature around 6°C.

Fire. Five gallons of soil were burned for a total of 6 hours. The soil was placed in a circular rock enclosure that was surrounded by kindling, firewood, and old wooden fence sections. The fire was ignited with kerosene. Two hours after the fire began, the temperature of the soil was measured with a pyrometer (BTMeter BT-1500 infrared thermometer, Bestmeter Electronic Technology, Zhuhai, China) to ensure that the fire reached a temperature of at least 800°C[12]. At the two-hour mark, the soil reached a temperature of 865°. The wood temperature was taken with a non-contact infrared thermometer set to 90% emissivity. Soil was left to cool after 6 hours and was removed from the enclosure the next day. Both the unburned and burned soil was sifted through a 0.25-inch mesh and packed into plastic gallon containers and refrigerated at 10°C for one week.

Bacterial Growth. *Pre-plant growth.* Two days after the burn, pH was measured and soil samples were plated on agar plates. 1g of soil was placed in 9 ml NaCl (0.85%). The samples were vortexed for 30 seconds. 100 µl of the vortexed solution was placed in 900 µl NaCl for a 10^{-1} dilution. This mixture was vortexed for 10 seconds. 100 µl of the 10^{-1} dilution was added into a microtube of 900 µl. The new solution was vortexed for 10 seconds. This procedure was repeated until the mixture was at a 10^{-6} dilution. The 10^{-4}, 10^{-5}, and 10^{-6} dilutions were used for spread plating. 100 microliters of each dilution was spread on three replicate petri dishes. This was completed for both the unburned and burned samples. The petri dishes were parafilmed and incubated at 28°C for 48 hours. Since plate counts were not taken until one week following plating, petri dishes were refrigerated at 10°C after 48 hours of growth. *Post-plant growth.* After one month of growth, soil from 3 out of the 4 pots was randomly selected and plated using the same dilution process mentioned above.

PCR Analysis. Eight well-isolated colonies on petri dishes from each of the 4 groups were chosen for PCR analysis on post-growth soil. The desired bP length was 1400 since 16S rRNA, bacterial primers, primers that amplify the length of the gene between the 27th and 1492nd, were used[13]. Post PCR analysis was performed using the Nucleotide Blast through the National Center for Biotechnology Information.

Soil Preparation for Growth. The burned and unburned soils were divided into 2 groups each for a total of 4 groups (2 unburned groups and 2 burned groups). Each group had 4 pots for a total of 16 pots. One burned and one unburned group was inoculated. Corn plants were grown in each pot. One quart of burned soil and one quart of unburned soil were placed in 16 quart-sized plastic plant pots (8 unburned, 8 burned). After inoculation, each pot of burned soil was watered with 157 mL of water. Pots of unburned soil were watered with 42 mL of water. To replenish water after burning burned soil a larger quantity of water was required to restore soil moisture.

Inoculation Preparation. The *Azospirillum lipoferum* inoculation (Lot No. 211027A2, TerraMax, Easton, Minnesota) was prepared by adding 20 drops of the inoculum to one cup of burned soil and 20 drops of the inoculum to one cup of unburned soil. A quarter teaspoon of the inoculant mixture was placed in 4 of the unburned and 4 of the burned pots. Soil with the inoculant mixture was mixed thoroughly in a plastic container so that the inoculant was thoroughly distributed. Inoculant was added to the soil 6 weeks after the burn.

Plant Growth. Plant pots were placed in a greenhouse with a heat setpoint of 15.6°C and a cool setpoint of 26°C. Overhead lights were on for 15 hours (6:00 am - 9:00 pm). Initially, seeds were planted 1 inch below the soil surface. After a week of no germination in any of the pots, new corn seeds were planted at ½ an inch below the soil. Two seeds were planted in each pot to ensure the germination of at least 1 plant. Corn plants were grown for one month under greenhouse conditions.

Plant Weight. Plants were trimmed at the end of the stem and weighed after the growth period. Following the initial measurement, plants were placed in brown paper bags and dried in an oven at 90°C for 48 hours. The oven-dried plants were weighed again. To determine the weight of plants without water content, the oven-dried plant weight was subtracted from fresh plant weight.

Soil Chemical Analysis. To measure pH, soil was placed in water in a 2:1 water to soil ratio. The tube was vortexed for 30 seconds and was placed on the HulaMixer for 5 minutes. A pH meter was used to measure the pH. To determine total nitrogen, soil samples from each plant pot were oven-dried at 60°C for 48 hrs. Each sample was ground into a fine powder. Two replicates of each sample were placed in aluminum cups for analysis using a Leco Analyzer (Leco Corporation, St. Joseph, Michigan).

Statistical Analysis. A two-factor ANOVA test was used to analyze pH, total nitrogen, plant weight, and colony-forming units (CFUs) from plate counts ($P < 0.05$). A paired t-test was used to analyze statistical differences in pH and nitrogen values ($P < 0.05$).

Results

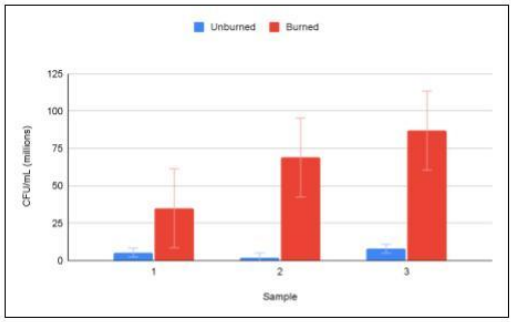

Fig 1. Viable colonies in uninoculated soil
Bars: Standard error of mean

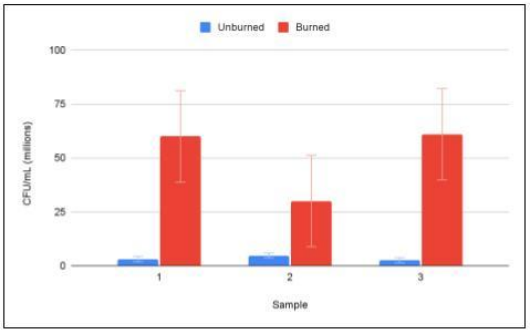

Fig 2. Viable colonies in inoculated soil
Bars: Standard error of mean

Microbial Abundance and Diversity

There was no noted significant difference in CFU counts between unburned soil with *A. lipoferum* and unburned soil without *A.lipoferum*. The same can be said for burned soil. However, there was a significant increase in the viable colonies of burned soil when compared with unburned soil with and without *A.lipoferum* inoculation (Fig. 1, Fig. 2). The average colony count of unburned soil without inoculum, 5.2×10^6 , and the average colony count of unburned soil with inoculum 3.46×10^6 do not differ significantly from the original colony count, 3.13×10^6 taken 3 days post-fire. The microbial abundance of burned soil went through the most change during these 3 months. While there was no bacterial growth on the agar plates of burned soil post-fire, by the second month, burned soil without inoculum had an average CFU count of 6.36×10^7, and burned soil with inoculum had an average CFU count of 4.5×10^7. Both of these values are ten-fold their unburned counterparts.

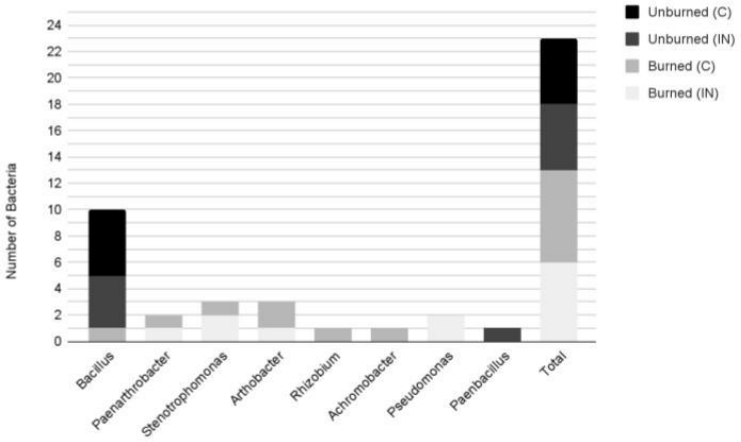

Figure 3. Bacterial species identified with PCR analysis. Percent identity was Stenotrophomonas sp. (94.47) Remaining samples had a minimum of a 97 percent identity. IN signifies soil with inoculum. C signifies soil without inoculum.

Samples taken from unburned soil, whether with or without inoculum, were mostly populated with *Bacillus* sp. (Fig. 3). Of the six successful PCR samples of unburned soil without inoculum, six were found to be in the *Bacillus* genera with at least a 99% identity. Unburned soil with inoculum samples had four out of five samples of *Bacillus* and one sample of *Paenibacillus*. Burned soil had almost no *Bacillus* present in sequenced samples. Burned soil without inoculum had only one *Bacillus* sample, the other six samples are split amongst *Paenarthobacter*, *Arthobacter*, *Stenotrophomonas*, and *Rhizobium*; this spread was the most diverse of the four groups. Burned soil with inoculum had two samples of *Pseudomonas* and no *Bacillus*. Similar to the burned soil without inoculum, sequenced samples contained *Stenotrophomonas*, *Arthobacter,* and *Paenarthrobacter*. While this is a diverse spread, it does not contain as much variation as the burned samples without inoculum. However, some failed PCR samples from the burned and unburned soil without inoculum were found to be fungi, which are more sensitive to fire[15].

	Uninoculated				
	Unburned		Burned		
	Mean	STD.	Mean	STD.	p. value
Nitrogen (mg)	0.2244	.006	0.1889	0.005	0.003

Table 1. Total nitrogen (mg) in uninoculated soil. Standard deviation was calculated using variance. The p value was found using a two-tailed t-test ($p < 0.05$)

	Inoculated				
	Unburned		Burned		
	Mean	STD.	Mean	STD.	p. value
Nitrogen (mg)	0.2281	0.004	0.1912	0.007	0.006

Table 2. Total nitrogen (mg) in inoculated soil. Standard deviation was calculated using variance. The p value was found using a two-tailed t-test ($p < 0.05$)

Soil Chemical Changes

Nitrogen and pH levels follow the same pattern as colony counts; there was no significant difference between burned and uninoculated and unburned and inoculated soil. The same can be said of unburned soil. However, there was a significant difference in total nitrogen (p=.003) between uninoculated burned and unburned soil (Table 1). Nitrogen levels of unburned and inoculated soil were also greater than that of burned and inoculated soil (p=.006) (Table 2). Inoculation with *Azospirillum lipoferum* made little difference in the total nitrogen content of the bulk soil surrounding the rhizosphere. pH values of unburned soil remained acidic after inoculation (Table 3). pH values of burned soil also remained basic even after inoculation (Table 4).

	Uninoculated				
	Unburned		Burned		
	Mean	STD.	Mean	STD.	p. value
pH	5.40	0.083	7.86	0.057	.000015

Table 3. pH values of uninoculated soil. Standard deviation was calculated using variance. The p value was found using a two-tailed t-test ($p < 0.05$)

	Inoculated				
	Unburned		Burned		
	Mean	STD.	Mean	STD.	p. value
pH	5.34	0.145	7.93	0.059	.000012

Table 4. pH values of inoculated soil. Standard deviation was calculated using variance. The p value was found using a two-tailed t-test ($p < 0.05$)

Plant Growth

Plant growth was the only measured variable that showed a significant difference in-group, between burned soil with inoculum (Fig. 4) and unburned soil with and without inoculum. (Fig. 5) (P=.00042). Burned soil without inoculum did not have any plant that weighed over 1g, yet plants grown in burned soil with inoculum weighed an average of 2.4g. A similar trend was observed in unburned soil. While the average weight of a plant grown in unburned and uninoculated soil was .60g, the average weight for plants grown in unburned and inoculated soil was 1.7g. Overall, plants grown in burned soil with inoculum weighed the most.

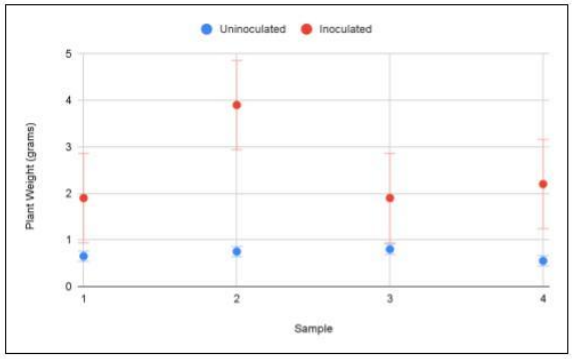

Figure 4. Plant Weight in burned soil
Error bars: calculated using variance

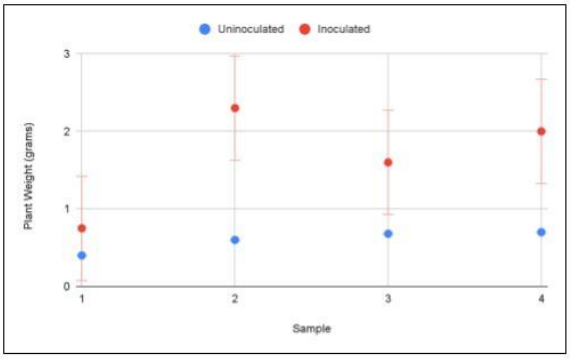

Figure 5. Plant Weight in unburned soil
Error bars: calculated using variance

Discussion

The increase in bacteria a few weeks or months following a fire is a common phenomenon[8,16]. It is possible that *Azospirillum lipoferum* did not have a strong enough effect on the microbial makeup of unburned soil to change the bacterial makeup. The bacterial samples used in the PCR analysis are classified as heterotrophic bacteria, which have been found to increase rapidly a few months after the fire due to an increased pool of organic nitrogen and other soluble compounds. An increase in microbial populations has also been noted when the pre-fire soil population was mostly made up of bacteria[16].

Unlike the majority of the sampled bacteria, *Azospirillum* is gram-negative. It is possible that it could not outcompete a large number of gram-positive bacteria. There was a large number of gram-positive bacteria in the sequenced bacterial samples. Unsurprisingly, gram-positive bacteria are also more resistant to heat and fire due to their thicker cell walls.

Due to ash production, burned soil increases its pH measurements[17]. While the burned soil with inoculum had a slight increase in pH most likely due to the rise in bacterial counts, *A. lipofeum* did not play a significant role. Nitrogen values in burned and unburned soil were far below the detectable amount. Nitrogen values in inoculated soil were slightly greater than their unburned counterparts and may increase over a longer growth period. Since this measurement was taken from the bulk soil surrounding the rhizosphere, nitrogen values are likely higher closer to the root; plants grown in unburned and burned soils with inoculant weighed more than plants grown in soil without the inoculum. It is important to take notice that an increase in plant growth after the fire due to available compounds is also common[4]. Despite no *A. lipoferum* found through 16S rRNA sequencing, differences in plant weight indicate the presence of *A. lipoferum*.

Conclusions

If corn growth is successful due to *A. lipoferum*, this might aid the soil microbial community in the long term. While total nitrogen in inoculated soil was insignificantly greater, this might change as the ephemeral rise in the bacterial population decreases over time. Inoculated soil did show a slight variation in bacterial species and may aid the soil microbial community over time. In the future, a similar study should be conducted to assess the efficacy of *A. lipoferum* over a longer time and in different environments.

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