Use of Microbial Biofertilizers in Coastal Restoration of Wetland Plants in Southeast Louisiana

Submitted By

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ABSTRACT

Microbes with beneficial effects to plant growth and health have been dubbed plant growth-promoting rhizobacteria (PGPR). PGPR has been extensively studied in crop plants; however, our study investigates the effects of PGPR on the wetland grass *Spartina alterniflora*. *S. alterniflora* is the dominant vegetation in coastal marshes and is often used in wetland restoration projects. Greenhouse raised *S. alterniflora* were subjected to three treatments: One of a consortia of microbes with freshwater, one of a consortia of microbes with 10 parts per thousand (ppt) saline water, and one with a pure culture and freshwater. Plant growth and soil nitrogen and phosphorus content were measured over 60 days and all plants were sacrificed at the end of the experiment to quantify biomass. Of the three treatments, the treatment receiving the consortia plus salt water had the most growth (41.1±4.4 cm) and greatest biomass (108.03 g) followed by the pure culture treatment with freshwater (34.9±3.2 cm, 96.25 g), the consortia treatment with freshwater (39.7±5.0 cm, 89.04 g), and lastly the control treatment (7.7±1.5 cm, 51.85 g). All treatments were significantly different from the control but not significantly different between each other. In consortia plus 10% saline water treatment, mean stem growth was almost six times greater, total biomass was doubled, and the number of additional stems was three times greater compared to the control. This study shows a positive relationship between microbial activity, soil nutrient cycling of nitrogen and phosphorus, and plant growth in greenhouse grown *S. alterniflora* inoculated with PGPR.

Keywords: Biorestoration, bioaugmentation, *Spartina alterniflora*, wetland, coastal erosion, salinity, nitrogen, and phosphorous.

1. **Introduction**

Coastal wetlands are some of the most vulnerable ecosystems on earth. Marshes and mangroves protect coastal regions from storms. Tidal wetland conversion to open water through sea-level rise is expected to accelerate. Large areas of marsh are being converted to open water in the Gulf of Mexico and especially the state of Louisiana in the US is losing land at an alarming rate due to coastal erosion. Although coastal wetlands have long been considered vulnerable to sea-level rise, many recent studies indicated feedback between plant growth and geomorphology allows wetlands to actively resist coastal erosion and land loss (Kirwan and Megonigal, 2013).

Coastal wetlands act as a transition zone from open-ocean to estuarine areas (Mitsch and Gosselink, 2007). Estuaries provide a host of ecosystem services including erosion protection, flood protection, breeding grounds for coastal birds, and habitat for many harvested species including fish, shrimp, and crabs (Gomes et al. 2010; Polidoro et al. 2010). Wetland plants are also important in nutrient cycling and biogeochemical processes of wetlands including carbon sequestration and mineralizing soil organics (Gomes et al. 2010; Polidoro et al. 2010; Miransari, 2011). Coastal wetlands are among the most productive ecosystems on earth and vegetation tends to stabilize their relative elevation. The growth of the grass *Spartina alterniflora* is positively correlated with inter-annual variations in sea level (Kirwan and Guntenspergen, 2012). Enhanced growth of *S. alterniflora* in coastal wetlands will protect the coastal land from erosion and land loss. Thus, it is imperative to find a natural means to enhance the growth of this most important coastal plant.

Kloepper and Schroth (1981) first coined the term “plant growth-promoting rhizobacteria” in a study investigating the effects of soil bacteria on the growth of radishes, sugar beets, and potatoes. In this study plant growth-promoting rhizobacteria (PGPR) are defined as free-living soil bacteria that can form close relationships within the rhizosphere of plants and can enhance plant growth and health. Although some microbial populations act as plant pathogens, PGPR benefits plants directly by increasing availability of essential plant nutrients like nitrogen and phosphorus (Glick et al. 1999) and by producing plant hormones such as indole acetic acid (IAA), which encourages

root growth (Patten and Glick, 2002). Various crop plants such as radishes and potatoes (Kloepper and Schroth, 1981), cotton, sweet corn (McInroy and Kloepper, 1995), cucumber (Wei et al. 1996; Raupach and Kloepper 1998) canola (Patten and Glick, 2002), soybean (Bai et al. 2002), and barely (Canbolat et al. 2006) have shown positive responses in growth and pathogen resistance when treated with microbial consortia or biofertilizers. Biofertilizers are defined as a microbial enrichment applied to the soil to enhance plant growth and health (Vessey, 2003). Fertilizers supply essential nutrients to plants such as nitrogen and phosphorus. Microbes can mobilize nitrogen and phosphorus trapped in soil making it available to plants (Glick et al. 1999). Bacteria transform organic nitrogen into ammonia (NH_3), nitrite (NO_3), and nitrate (NO_2), which can be utilized by plants and microorganisms (Glick et al. 1999; Mitsch and Gosselink, 2007). Phosphorus is typically abundant in nature but the mineral readily binds to clay, ferric iron, calcium, and aluminum making it insoluble and unavailable to plants (Mitsch and Gosselink, 2007). Some bacteria such as *Pseudomonas putida* and *Bacillus* spp. are known to solubilize phosphorus making it available to plants (Glick et al. 1999; Ahmad et al. 2008).

Increasing environmental concerns surrounding chemical fertilizer overuse such as the 13,080 km^2 "dead zone" in the Gulf of Mexico (Rabalais et al. 2010) make PGPRs an attractive alternative to traditional fertilizers and herbicides (Adesemoye and Kloepper, 2009; Miransari, 2011). Other concerns of traditional fertilizers use are changes in soil structure, pH, biogeochemical processes (Miransari, 2011), and the accumulation of herbicides and insecticides in the environment (Patten and Glick, 2002). Biofertilizers can help reduce the use of chemical fertilizer thereby alleviating the negative ecosystem issues they cause (Bashan and Holguin, 1993; Patten and Glick, 2002; Vessey 2003; Adesemoye and Kloepper, 2009; Miransari, 2011).

Interest in PGPR has largely been focused on crop plants. However, halo-tolerant coastal plant species that endure unique environmental stresses like flooding and salinity may host unique and potentially beneficial PGPR (Bashan and Holguin, 2002; Kathiresan and Selvam, 2006). There may also be benefit in microbial treatments of wetland restoration plants (Bashan and Holguin, 2002). Research conducted in the area of

mycorrhizae interactions with S*partina alterniflora* and *Spartina cynosuroides* resulted in a minimal plant growth response (McHugh and Dighton, 2004). However research by Gomes et al. (2010) using mangrove species demonstrated that nursery raised plants transplanted into the wild influence the resulting microbial community of the rhizosphere. Understanding the microbial ecology of coastal plants can help improve restoration efforts by improving stabilization of soil after restoration (Bashan and Holguin 2002; Kathiresan and Selvam 2006; Miransari, 2011).

The purpose of this study is to enhance the growth of *S. alterniflora,* through bioaugmentation process by adding natural bacterial amendments to the soil. *S. alterniflora* is a facultative halophyte that dominates intertidal coastal marshes along the Atlantic coast to Louisiana and Texas (Tiner, 1993; Bush, 2002). It is a perennial grass that can reach up to 2.5 meters with the shorter form in higher areas less affected by tidal events (Tiner, 1993). Bacterial consortia similar to those found in wild plants may harbor unique microbial communities, which can potentially be used as a biofertilizer for the production of restoration plants. Biofertilizers could potentially improve restoration efforts by producing healthier plants that would also increase soil health by introducing beneficial microbial communities to the restoration area thereby stabilizing the area. The major goal of this study is to test the effects of PGPR treatments on greenhouse raised *S. alterniflora* by measuring nitrogen and phosphorus in the soil and plant growth over the duration of the experiment.

2. Materials and Methods

2.1. Collection of Sample

Samples were collected from the rhizospheres of *S. alterniflora* in Elmer's Island, LA (Figure 1). Elmer's Island is located on the Louisiana coast, which is indicative of *Avicennia germinans* (black mangroves) and *S. alterniflora* habitats. The rhizospheric soil sample plot was located at N29° 11' 15.5", W90° 03' 58.9" and at N29° 11' 19.9", W90° 04' 00.2" (Fig. 1). Each site was chosen based on a visual assessment that 90% of the area was dominated by the target plant species to ensure collection of microbes that are specific to plant species. Samples were collected by digging up approximately 15 cm of the root system with the above ground biomass. Above ground biomass was collected

to ensure preservation of rhizosphere conditions as found at the collection sites. Samples were stored in plastic bags with the above ground biomass exposed on ice while in transit and upon returning to the lab samples were stored at 4°C in a walk-in cooler. At each site, soil temperature, pH, and salinity were recorded using an Aquaterr EC-300 salinity multimeter.

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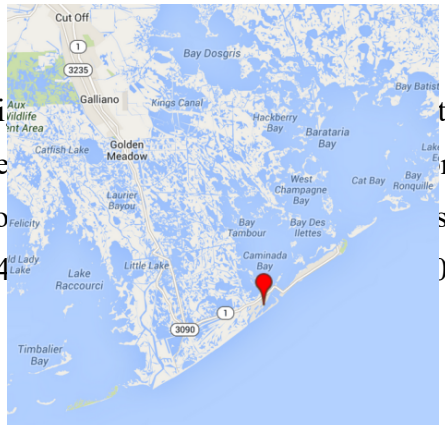

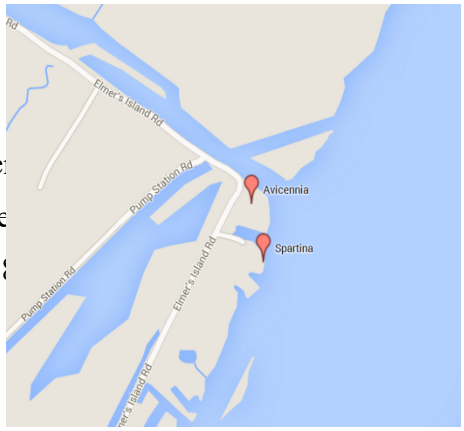

Table 1. Various treatments used in the study

Treatments	Description
T1	No bacterial inoculum/Freshwater application
T2	Bacterial consortium/Freshwater application
T3	Bacterial consortium/10 ppt saline water
T4	Pure culture of *P. putida*/Freshwater application

2.2. Isolation of Soil Bacteria

Bacteria were enriched and isolated on King's B medium and a halophilic rhizosphere medium. King's B medium was chosen because it is selective for *Pseudomonas putida* and *P. flourescens,* which are commonly found in the rhizosphere of plants and have known plant growth-promoting properties. *Pseudomonas* and *Bacillus* spp. have been reported as nitrogen transformers, phosphorus solubilizers, and siderophore producers, making it a good genera to investigate for plant growth-promoting properties (Glick et al. 1999, & Kumar et al. 2011, Ghodsalvi et al. 2013). The halophilic rhizosphere medium was selected to increase chances of isolating halophilic or salt tolerant microbial species since collected plants are found in brackish environments. Various rhizospheric soils were inoculated into the above-mentioned media for isolation of pure culture. Bacterial isolates were identified using the Biolog GEN III analyzer (BIOLOG Model GEN III, Hayward, CA, USA).

2.3. Propagation of S. alterniflora

The collected *S. alterniflora* were cut into small sections of 0.25 m x 0.25 m x 0.25 m. Individual stems were separated and roots were rinsed of soil before transplanting. Eighty stems were transplanted, entire stem with roots, were transplanted into ¾ Trade gallon pots (Classic 300S, Nursery Supplies, Inc.). Pots were filled one inch from the top of the pot with a 50/50 mix of potting soil (Hapigro®; Hope, AR) and top soil (Hapigro®; Hope, AR) that was first autoclaved for one hour at 15 psi and 121°C to kill pre-existing microbial communities. After initial transplantation, specimens were allowed to grow for two weeks before initial measurements were recorded and various

treatments were administered. Each treatment consisted of 15 plants. For the duration of the experiment plants were watered every three days. Treatments one, two, and four received freshwater and treatment three received a 10 ppt saline solution (Instant Ocean®). The sampling scheme was to measure stem heights every 10 days and to collect soil samples in duplicate every 10 days after an inoculation of bacterial consortia. There were three inoculations with one at the beginning of the experiment then one inoculation at 20 days and one inoculation at 40 days. At each inoculation event, a 5% (volume/weight) inoculum was used. After 60 days of growth plants were sacrificed.

2.4 Experimental Trials

Various bacterial pure cultures identified using BIOLOG assay include the following: *Bacillus subtilis, B. sonorensis*, *B. lichenformis, Pseudomonas putida*, *Actinobacillus capsulatus*, *A. germinans*, and *Paenibacillus zanthoxyli*. Most are common soil bacteria that have known plant growth-promoting properties especially *Pseudomonas* and *Bacillus* species. *P. putida* was selected as the pure culture inoculant because of its known plant growth-promoting properties like indole acetic acid (IAA) production, which increases root growth (Glick et al. 1999), increasing disease resistance (Kloepper and Schroth, 1981; Wei et al. 1996), increasing growth of seedlings and roots in barely (Canbolat et al. 2006) and phosphate solubilization (Ahmad et al. 2008). *Bacillus* species are also known to have plant growth-promoting properties. *B. lichenformis* has been shown to increase seedling and root length in barley (Canbolat et al. 2006). *Bacillus subtilis* increased weight in soybean plants (Bai et al. 2002). *Bacillus* species are also known to increase plant resistance to pathogens (Wei et al. 1996) and solubilize phosphate (Ahmad et al. 2008).

Two inoculants were prepared in Tryptic Soy Broth (TSB). First fresh tubes of cultures were grown for 24 hours and then large batches of TSB were inoculated with 10 mL of each selected bacterial species. Batch one consortia included all the above-mentioned species and batch 2 included only *P. putida*. Once media was inoculated, it was incubated for 48 hours. Various treatments used in the study are given in Table 1. Treatment one did not receive any inoculants and it received only freshwater, treatment two received the consortia and freshwater, treatment three received the consortia and 10

ppt saline water, and treatment four received a pure culture of *P. putida* and freshwater. In each treatment triplicate pots were maintained.

2.5 *Analytical Methods*

Periodically soil samples were analyzed from various treatments for phosphorus, ammonia, nitrite, and nitrate using HACH method (HACH Co., Loveland, CO, USA). The procedures for these analyses were given in HACH instruction manuals for inorganic analysis. Total heterotrophic bacterial counts were monitored using the method described by Brown (2005) using Tryptic soy agar media plates. Duplicate analyses were performed for each sampling event for each treatment.

2.6. Statistical Analysis

All chemical data were subjected to an analysis of variance (ANOVA) test ($p \leq 0.05$) followed by a tukey "*post hoc"* analysis when needed (SAS). Significant differences in biomass between treatments were determined by one-way ANOVA analysis.

3. Results and Discussion

3.1. Isolation and Identification of Bacteria

A total of 19 bacterial pure cultures were isolated. Out of these 19, seventeen pure cultures were isolated from King's B medium and two were isolated from halophilic rhizosphere medium. The BIOLOG Identification method identified seven pure cultures positively with known bacteria. The identified bacteria include *Bacillus subtilis, B. sonorensis*, *B. lichenformis, Pseudomonas putida*, *Actinobacillus capsulatus*, *A. germinans*, and *Paenibacillus zanthoxyli.* Understanding the microbial community of coastal plants can help develop new biofertilizers, improve restoration efforts, increase understanding of nutrient cycling and biogeochemical processes, and serve as a baseline microbial inventory for future studies. *Avicennia germinans* (black mangroves) and *S. alterniflora* are the dominant plant species (Tiner 1993) in coastal Louisiana and are increasingly being used in restoration projects (Craft et al. 1999; Osland et al. 2012). Biofertilizers have the potential to reduce the negative effects of chemical fertilizers in

agricultural processes (Gomes et al. 2010; Miransari, 2011). There is also a potential to use halo tolerant biofertilizers in arid areas to reduce salt stress in crops (Alizadeh & Parsaeimehr, 2011). Furthermore biofertilizers can help improve transplanting success of plants grown for restoration projects (Miransari, 2011). *A. germinans* and *S. alterniflora* are used widely in restoration projects with varying success but little research has been done to include the microbial communities typically associated with these plants. Microbes are also well known for their part in nutrient cycling and biogeochemical processes in wetlands (Mitsch & Gosselink, 2007). Although the microbes we are studying are associated with the roots of plants most are also found free living in the soil. Since most land is covered by some type of vegetation, rhizosphere bacteria may play an important role in the overall cycling of nutrients in wetland systems. The bacteria identified in this study such as *Pseudomonas, Paenibacillus,* and *Actinobacillus* are known to be common soil bacteria. *Bacillus* and *Pseudomonas* were two genera that are often used to produce house-hold plant soil enhancers or biofertilizers.

3.2. Plant Growth and Nitrogen and Phosphorous Content of Treated Soil

Soil total nitrogen and phosphorus content was lowest at the start of the experiment and peaked within first month of treatments with a corresponding trend in stem height. During the second month of treatment nitrogen, phosphorus, and growth started to decline. Treatment three and treatment four had the highest nitrogen and phosphorus but was closely followed by treatment two while the average increase in growth per stem increased about the same for all treatments except the control (Figure 2). It is also worth noting that during the last two weeks of the experiment temperatures were below 5°C at night which may have also slowed the growth.

Significant differences in biomass between treatments were determined by one-way ANOVA analysis and were tested using Tukey's honestly significant difference

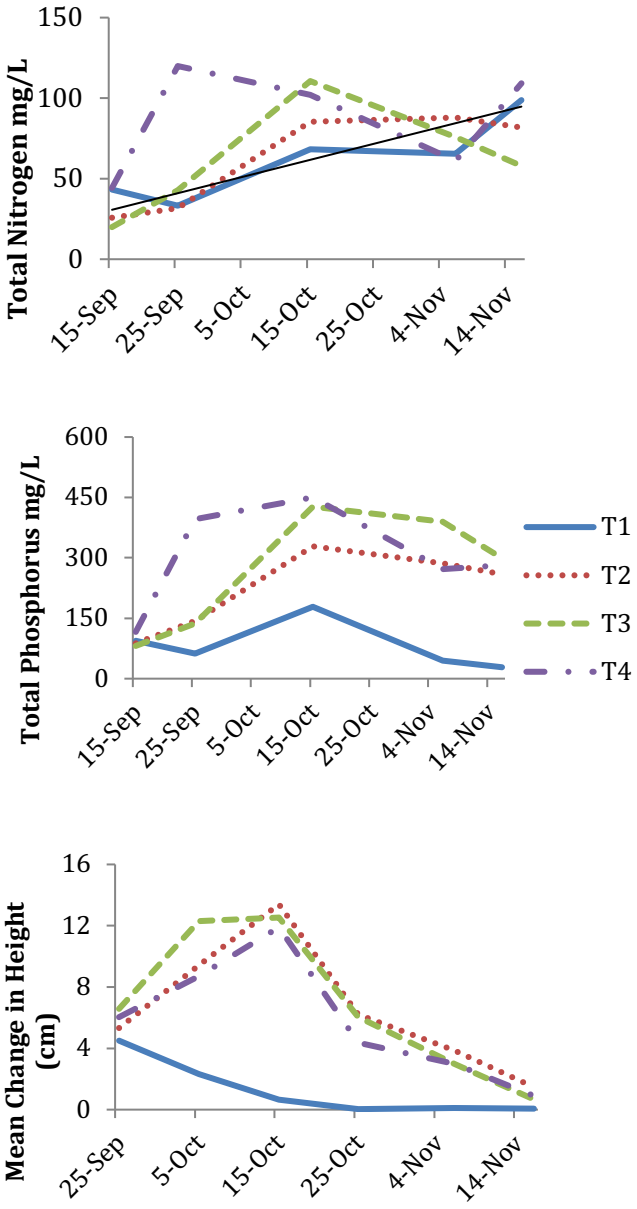

Figure 2. Relationship between soil nitrogen and phosphorous content and the mean change in height of main stems. Total nitrogen, total phosphorous and mean change in height.

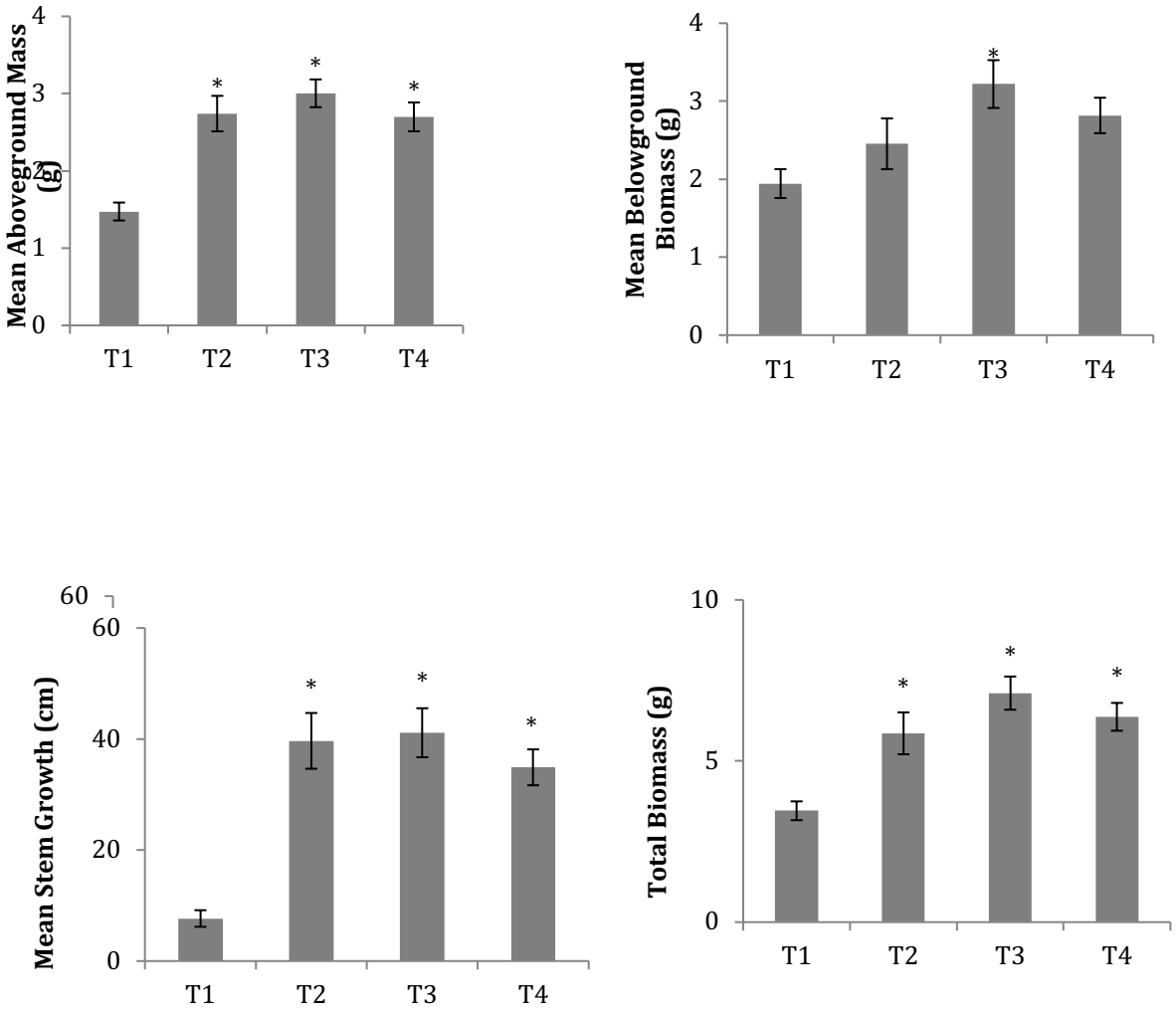

Figure 3. Comparison of the mean aboveground biomass, mean below ground biomass, mean stem growth, and mean total biomass. Asterisk (*) indicates a significant difference at $p<0.001$.

Table 2**.** Summary table of mean growth of main stem, number of additional stems, mean aboveground biomass, mean belowground biomass, aboveground biomass/belowground biomass ratio, and total biomass by treatment.

	Mean growth of main stem (cm)	Mean aboveground total weight (g)	Mean belowground total weight (g)	Total biomass (g)	Total Additional Stems	Ag/Bg ratio
T1	7.7±1.5	1.5±0.1	1.9±0.2	51.85	13	0.78
T2	39.7±5.0	2.7±0.2	2.5±0.3	89.04	32	1.1
T3	41.1±4.4	3.0±0.2	3.2±0.3	108.03	44	1.2
T4	34.9±3.2	2.7±0.2	2.8±0.2	96.25	47	1.3

posthoc test. There was a significant difference in mean ± SE in aboveground biomass per stem between treatment one (the control; 1.5±0.1 g) and treatment two (consortia and freshwater; 2.7±0.2 g), treatment three (consortia and 10 ppt saline water; 3.0±0.2 g), and treatment four (pure culture and freshwater; 2.7±0.2 g) with treatment three having the greatest aboveground biomass per stem. For mean ± SE belowground biomass there was only a significant difference between treatment one (1.9±0.2 g) and treatment three (3.2±0.3 g) with treatment three having the greatest belowground biomass per stem. Overall, there was a significant difference in total biomass between the control (51.85 g) and treatments two, three, and four (89.04 g, 108.03 g, and 96.25 g) but not between treatments two, three, and four with the greatest total biomass accumulating in treatment three. The mean ± SE stem growth also followed a similar pattern in which treatments two, three, and four (39.7±5.0 cm, 41.1±4.4 cm, 34.9±3.2 cm) were significantly different from treatment one but not significantly different between each other with treatment three having the greatest growth. Treatment three (consortia and 10 ppt saline water) had the maximum total biomass and greatest mean change in stem height. Conversely treatment four (pure culture and freshwater) had the second greatest biomass but a smaller change

in mean stem height than treatment two (consortia and freshwater). This difference may have been due to greater below ground biomass of treatment four (Figure 3).

Table 2**.** Summary table of mean growth of main stem, number of additional stems, mean aboveground biomass, mean belowground biomass, aboveground biomass/belowground biomass ratio, and total biomass by treatment.

Along with increases in biomass several plants also generated additional stems over the experimental period. Treatment three and treatment four had the most new stem growth at 44 and 47 additional stems respectively. Treatment one had an aboveground/belowground (Ag/Bg) ratio that was < 1 while treatments two, three, and four had a ratio of approximately one. Treatment three had greatest mass in above ground biomass, below ground biomass, and total biomass of all the treatments (Table 2).

3.3. Significance of the Study

This study demonstrates a positive relationship between rhizosphere inoculation with selected soil bacteria and an increase in soil nutrient cycling and plant growth. Bacterial consortia selected were originally isolated from wild wetland plants. Bacteria, particularly *Bacillus* and *Pseudomonas* species, used in the study are common soil species known to have plant growth-promoting properties. After the initial inoculation nutrient levels in the soil increased and peaked after the second inoculation. Plant growth also increased and peaked during this period. The leveling of nutrients may have been due to the limited starting amount of nutrients in the soil not being replenished from an external source. However the decrease in growth declined more sharply than soil nutrient levels, which are likely due to the leveling of nutrients and cooling temperatures.

Initial measurements of nitrogen and phosphorus in soils were lowest. Treatment one, the control, did not receive any microbial inoculation and it also had the poorest growth. Treatment one most likely had some bacteria from transplant but without refreshment of bacterial colonies it was not enough to keep up with plant demands. The top performer was treatment three, which received the bacterial consortia and 10 ppt saline water. Salt may be playing a secondary role such as inhibiting pathogens.

Additionally, salt may make nutrients, especially phosphorus more available to plants (Blomqvist et al. 2004). Treatment two, consortia with freshwater, was slightly outperformed by treatment four, which received the single species of bacteria. This was because treatment four had more belowground biomass but less aboveground growth than treatment two. *P. putida* is known to produce IAA, a root stimulating hormone (Patten and Glick, 2002), and the greater concentration of *P. putida* in the pure culture may have contributed to the increased belowground biomass of treatment four.

Microbial inoculations are effective biofertilizers to increase soil nitrogen and phosphorus content and to increase plant growth. This study demonstrated that without any additional chemical fertilizer microbes could support plant nutrient demands. To further test the effectiveness of biofertilizers, greenhouse raised plants treated with bacterial inoculations should be grown and then planted at a restoration site and monitored.

4. Conclusions

Several common soil bacteria from the plant rhizosphere were isolated that are known for promoting plant growth. The use of these organisms in a consortium and also as a pure culture was tested as biofertilizers for the growth of a common wetland grass*, S. alterniflora*. The growth of S. alterniflora was significantly improved in the consortium under saline conditions compared to freshwater. The plant growth was better when the bacterial consortium was used compared to pure culture conditions. The soil chemistry showed increased nitrogen and phosphorous concentrations in the treatment with consortium as biofertilizer compared to control and pure culture treatment. The plant above and belowground growth was significantly enhanced in the treatments with bacterial consortium. This study demonstrated the bioaugmentation potential of the rhizospheric bacteria, which may be applicable to restore the coastal wetlands in Louisiana.

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