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The Effects of an Applied Phyllosphere-Microbiome on Gas Exchange and Growth of Soybean Infected with Pseudomonas Syringae: Harnessing the Power of the Microbiome

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THE EFFECTS OF AN APPLIED PHYLLOSPHERE-MICROBIOME ON GAS EXCHANGE AND GROWTH OF SOYBEAN INFECTED WITH *PSEUDOMONAS SYRINGAE*: HARNESSING THE POWER OF THE MICROBIOME

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Charles Agbavor

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ABSTRACT

The microbiome is an important determinant of plant health, growth and resistance to stress. This study was conducted to determine the efficacy of the phyllosphere-microbiome on managing *Pseudomonas syringae pv. Glycinea (Psg)*, gas exchange and growth of *Glycine max* L. Merrill. A greenhouse study was conducted in the summer of 2019. A field isolated and cultured soybean phyllosphere-microbiome was applied to purposely infected and uninfected soybean. Gas exchange, *Psg* density, chlorophyll concentrations, and percent nitrogen and carbon in the leaves were measured during the V3-V6 stages of soybean. Not surprisingly, there was a two-fold reduction in copies of *Pseudomonas syringae pv. Glycinea* per leaf disc in the microbiome-*Psg* treated group. The microbiome had a significant positive effect on the dry shoot biomass. Surprisingly, mean dry shoot biomass of the microbiome treated group was 5% higher than the control group and this was significantly different. The microbiome had a significant effect on the fresh weight and the number of nodules. Mean chlorophyll concentration doubled in the microbiome augmented treated group. Percent nitrogen per leaf disc was significantly higher in the microbiome treated plants. Furthermore, transpiration rate was significantly increased in the microbiome treated plants. Interestingly, water use efficiency of the microbiome treated plants was significantly reduced, but not at a cost to overall carbon gain. Photosynthetic rate was reduced by the infection, with higher photosaturated photosynthetic rates (A_{max}) and ambient photosynthetic rates (Aamb) being significantly higher in the control, microbiome, microbiome +pathogen than the *Pseudomonas syringae pv. Glycinea* treatment. Overall, dry shoot biomass, root nodules, chlorophyll concentrations, percent nitrogen and photosynthetic activity were significantly increased by the presence of the microbiome on both infected and non-infected plants. Therefore, the phyllosphere-microbiome suggests the potential for increasing crop yield and plant probiotics against pathogens.

KEYWORDS: Microbiome, *Glycine max* L. Merrill, phyllosphere, *Pseudomonas syringae pv. Glycinea*, chlorophyll, probiotics.

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I am grateful to everyone who helped me during this project, and I hope you'll find joy in seeing this work published.

I dedicate this thesis to my **family.**

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INTRODUCTION

The presence of microbes on the leaves of plants usually suggest infections, but recent findings have shown the benefits of some of these microbes for plants [1]. An example is the protection conferred by the bacteria on the leaves of tomato plants against *Pseudomonas syringae* [2]. This association of plants and their microbes helps in protection against infections, and has been shown to increase nutrient acquisition and nitrogen fixation [3]. Berg (2009) and Copeland et al. (2015) have shown that several microbial cells (e.g. *Bacillus subtilis* and *Pseudomonas fluorescens*) inhabiting the phyllosphere (plant stem, leaves and flowers) increased the growth of agricultural crops such as apple, peach, pear, potatoes and vegetables [4, 5]. The microbial cells which colonize the phyllosphere includes bacteria, fungi and viruses [6, 7]

Bacteria species are the most abundant in the phyllosphere with colonial densities as high as one hundred million cells per square centimeter [8]. Additionally, most of these bacteria cells are non-pathogenic and they belong to a small class of phylogenetic groups including Alphaproteobacteria and Gammaproteobacteria [9]. The phyllosphere surface area represents about 10^9 km^2 of the earth and is twice as big as the land surface area. Furthermore, it provides a home for 10^{26} bacterial cells [9-11]. The phyllosphere therefore provides a major habitat for the development of solutions and innovations in agriculture that addresses increasing productivity and decreasing yield losses associated with plant diseases [10]. Moreover, discovering conditions that increase the success of beneficial microbes in this niche will advance the practical application of microbiomes in agriculture [12].

Pseudomonas syringae is a model bacterial pathogen for studying host-pathogen interactions and microbial ecology [13]. These genera of bacteria are opportunistic and can infect a wide spectrum of plants [14]. Approximately 50 pathogenic species have been identified and

they all infect economically important crop plants [13, 15]. For example, *Pseudomonas syringae pv. Glycinea* is the pathogen that causes bacterial blight in soybean [16], which is a very common bacterial disease of soybean in the United States [17-19]. The average soybean yield loss annually in the U.S. is about 11% (Allen et al., 2017). Phyllosphere microbiome research is also relevant to the rhizosphere. [20]. Much of the nitrogen available to crops is released by the symbiotic association of legumes and rhizobia. Out of the approximated 19 Tg of annual fixed nitrogen, soybean is responsible for 50% of the world's legume coverage [21].

Plants can resist infections in many ways. In most cases, normal microbial interaction on the host plant may confer this resistance [22]. Although little is known about microbial pathogen interactions on the leaves of plants, one recent study established dose-dependent protection for the tomato plant against *Pseudomonas syringae pathovar tomato* [2]. However, several questions on the importance of the microbiome of the phyllosphere remained unanswered. For example, how do we manipulate the plant microbiome to manage diseases? Does the microbiome affect plant growth and gas exchange? What are the effects of the microbiome and *Pseudomonas syringae pv. Glycinea* on the plant? Can the identification of biologically useful bacteria be employed in disease management and control of pathogens?

The goal of my research was to examine the interaction of soybean's microbiome and *Pseudomonas syringae pv. Glycinea*; and, study the effect of the pathogen and the microbiome on nitrogen fixation and physiology in soybean. Specifically, the research examines the interaction of soybean microbiome and *Pseudomonas syringae pv. Glycinea* and examines the effects of the microbiome on growth, nodulation and physiological characteristics of soybean. The results will address reducing yield losses by *Pseudomonas syringae pv. Glycinea* and

provide understanding about the microbiome of the phyllosphere and implications on biological nitrogen fixation in soybean.

METHODOLOGY

Experimental overview

Soybean *(Glycine* max L. Merrill.) were grown in a climate-controlled greenhouse at Missouri State University, Springfield, MO (37.1981° N, 93.2815° W). The soybean seeds (AG49X6) were purchased from the Associated Seed Growers, Inc. (Asgrow, MI, USA). The plants were maintained at 26–28 ˚C, 50–70% relative humidity for 14h light/10h each day in the greenhouse [23, 24]. The seeds of *Glycine Max* L. Merrill. were disinfected under the fume chamber using Clorox bleach-Hydrochloric acid solution, rinsed thoroughly in sterile water and later soaked in 0.8% of water agar for germination. The germinated seedlings were transplanted in autoclaved soils at pH of 6.4 and watered adequately each day [25-29]. The plants were put into four treatment groups: Control, Microbiome, Microbiome + *P. syringae* and *P. syringae;* with two of those treatments sprayed with or without *Pseudomonas syringae pv. Glycinea* (2e+7 cells/ml) and the microbiome inoculum. Bacterial densities were quantified using CFU count and OD_{600nm} using the BioMate™ 3S Spectrophotometer (ThermoFisher Scientific, Waltham, MA) after 20 and 35 days of growth. Plant growth (height, number of leaves) and gas exchange (photosynthetic rates, transpiration, stomatal conductance) were measured bi-weekly until harvest using the LI-6400xt Portable Photosynthesis System (LI-COR, Biosciences, Lincoln, NE).

Biosafety approval and pathogen culture

This study was approved by the Institutional Biosafety Committee (IBC) at Missouri State University on 4/15/2019. The *Pseudomonas syringae pv. Glycinea* was procured under the Plant Protection and Quarantine permit (P526P-19-01769) from the United States Department of

Agriculture's Animal and Plant Health Inspection Service (USDA-APHIS). The *Pseudomonas syringae pv. Glycinea* inoculum was streaked on nutrient media and incubated overnight at 30° Celsius for 24 to 48 hours [30, 31] and CFU densities determined by optical density at 600nm ($OD600_{nm}$) and CFU Count using the BioMate™ 3S Spectrophotometer (ThermoFisher Scientific, Waltham, MA).

Microbiome community isolation

Twenty healthy soybean plants in the V4-5 stage were sampled from two separate farms (Kindrick farms managed by the William H. Darr College of Agriculture and Buffalo area, MO.), with collected leaves and transported to the laboratory on ice. Three leaves from each plant were washed in 10 mM $MgCl_2$ buffer and 3 cm leaf discs were cut using a sterile cork-borer. The leaf discs were transferred into 500 mL Conical flasks containing 10mM $MgCl_2$ buffer and stirred for 20 minutes using the magnetic stirrer to wash out the microflora in the phylloplane from the leaf into the buffer. The solutions were further centrifuged at 1000 g to concentrate and harvest the microbial pellet. Serial dilutions up to 10^{-4} were prepared and 1 mL aliquots from the dilutions were transferred to sterile Tryptic Soy Agar medium (TSA) (three replicates per dilutions) and Nutrient Agar (three replicates per dilutions) plates. The plates were inverted and incubated at 30° C for 2-7 days. Colonies that appeared were counted using the colony counter. The bacteria density per cm^2 of leaf area was calculated using the formula: Bacteria/cm^2 = Total no. of colonies per ml/Total area of 50 leaf discs×2. Area of one leaf disc = πr^2 (where r is the radius of disc in cm). The bacterial isolates were purified by streaking on TSA and pure cultures were isolated. This protocol was adopted and modified from two published sources [2, 32]. The purified cultures were isolated and identified through pure cultures and Nucleotide Blast of the

16S rRNA. The isolated bacteria colonies were used to prepare community inoculation that was used to spray the soybean during the V4 stage.

Microbiome Identification: DNA extraction, 16S rRNA amplification and sequencing

Bacterial DNA from pure cultures were extracted using the DNeasy PowerLyzer Kit (Qiagen Qiagen, Hilden, Germany) following the manufacturer's protocol. The purity and yield of the DNA samples were measured with the nanophotometer and the samples were stored at -20ºC. The 16S rRNA gene from the bacteria were amplified with the 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') primers in a reaction volume of 25 µL containing 0.2mM dNTPS, 100ng/µl of the forward and reverse primers, 1× PCR buffer, 2 mM $MgSO_2$, 1 µL of template DNA, and 2 U of *Taq* DNA polymerase (GenScript, Piscataway,NJ). The PCR conditions includes denaturation for 7 minutes and 35 cycles of temperature cycling, 95°C for 45 s, 56°C for 30 s, and 72°C for 45 s) and a final extension at 72°C for 7 minutes. The resulted amplicons were visualized by electrophoresis using 1% agarose gel stained with Ethidium Bromide. The PCR product was cleaned with ExoSAP-IT (USB, Cleveland, OH) and the DNA was sequenced with the 8F and the 1492R primers using the 3730XL Genetic Analyzer (Life Technologies, Applied Biosystems).

Microbiome 16S rRNA gene identification

Bacterial 16S rRNA sequences (68) were trimmed using the Sequencher (version 4.5.6; Gene Codes Corp., Ann Arbor, MI). Resulting sequences were aligned and a consensus sequence

was created for all samples. The consensus sequences were identified using the National Centre for Biotechnology Information (NCBI) blast search tool [33, 34].

Sterilizing seeds and planting

Healthy soybean seeds were put in 1.5 mL Eppendorf® Safe-Lock tubes and placed on racks. In a 500 mL graduated beaker,100 mL of sodium hypochlorite solution (6%) was added and placed in a fume chamber. While under the fume chamber, 3 mL of concentrated Hydrochloric acid was added dropwise to the beaker containing bleach. The soybean seeds were kept under the hood for 3 hours. The sterilized soybean seeds were rinsed with sterile water, soaked in 0.8% of water agar for a week and the seedlings were transplanted into free draining pots (25 cm diameter by 40 cm height) containing potting media (Jolly Garden, Pro-Line C/20, Growing mix of Canadian sphagnum peat and perlite); filled ¾. Two treatments were grown in one greenhouse bay (Control, Microbiome) and two treatments in another (a door was the only connection between bays) (Microbiome + *P. syringae* and *P. syringae*). Plastic pots were positioned such that a space of 45 cm existed between them on the bench. The pots were completely randomized every week to reduce confounding factors due to microenvironment in greenhouse bays and to ensure uniform growth.

Plant growth

Soybean shoots and roots were harvested after 50 days of growth. The fresh weights were determined to the nearest 0.001 grams and the shoot samples were dried in a Quincy Lab 40AF-1 Forced Air oven (Quincy labs, Chicago, USA) for 24-48 hours at 100º C and the dry weights were determined using the Ohaus Explorer Analytical balance (Ohaus, New Jersey, USA). Root

nodules were carefully separated from the potting media, rinsed with water and allowed to air-dry. The root nodules were counted and put in petri dishes and weighed using the Ohaus Explorer Analytical balance (Ohaus, New Jersey, USA).

Measurement of chlorophyll

Chlorophyll concentrations were measured with a MC-100 Chlorophyll Concentration Meter (Apogee Instruments, Inc., Logan, UT, United States) after 30 days of growth. Measurements were done by clipping the sensor onto the leaf at three distinct points and the results were average by the instrument. All measurements were taken between the margin and the mid-rib of the leaf [35]. An area of 63.6 mm^2 (9 mm diameter), 19.6 mm^2 (5 mm diameter with reducer) of the leaves was measured by the instrument and the results were displayed as µmol of chlorophyll per m^2 of leaf surface. The relative chlorophyll concentrations were measured by measuring the absorbance at 653 nm and 931 nm. The chlorophyll concentration index was calculated by dividing the percent Transmittance (T) at 931 nm by T at 653 nm.

Gas exchange

Photosynthetic rates (µmol CO_2 m^{-2} s^{-1}), transpiration rates (mol H_2O m^{-2} s^{-1}), and stomatal conductance to H_2O (mol H_2O m^{-2} s^{-1}) were measured using a portable gas exchange system LI-6400XT (LI-COR Biosciences, Lincoln, NE, USA) after 20, 35 and 45 days of growth. The water use efficiency was calculated as the ratio of the net photosynthesis divided by the transpiration rate. The IRGA leaf chamber was set to a temperature of 24°C and the partial pressure of carbon dioxide was set at a reference point of 400 µmol CO_2/mol. The relative humidity was controlled at approximately 60% in the chamber to equal that in the greenhouse.

All gas exchange measurements were taken between 11am-2 pm and microclimatic conditions in the greenhouse were set to match those in the leaf chamber. Leaf area for measurements was 6 cm^2 and the midrib of the plants were centered in the leaf chamber.

Stable nitrogen and carbon isotopes

Leave disc samples were collected after 50 days and analyzed using the EA-Isolink elemental analyzer interfaced to a Delta V advantage (Thermo Electron, Bremen Germany). Samples were weighed into tin capsules prior to analysis and standards were loaded in a 49 place autosampler (Costech Zero Blank). The leave disc samples were combusted in the presence of a small amount of oxygen in a helium stream causing nitro oxides to be reduced to nitrogen and carbon dioxide. The carbon dioxide and nitrogen gases were separated on a 0.5-meter C/N column. The resulting gases were admitted into the Mass spectrometer via a ConFlo IV along with reference gas pulses.

Statistical analysis

All data were analyzed statistically using Minitab software version 19 (Minitab, LLC Pennsylvania, USA). The effects of the microbiome on the *Pseudomonas syringae pv. Glycinea* were analyzed using the one-way ANOVA. Tukey's pairwise comparison was used to analyze all statistically significant variables. The light response and CO_2 response curves (A_{ci}) curves were modeled using a modified R package for gas exchange variables [36]. All conclusions are based on the type-I-error rate of 0.05. Data are stored in the Microsoft OneDrive cloud database (Microsoft Corporation Redmond, Washington).

RESULTS

I identified 21 phyllosphere-microbiome species in this study (Table 1). The microbiome reduced the copies of *Pseudomonas syringae pv. Glycinea* per leaf disc by a factor of two compared to the *Pseudomonas syringae pv. Glycinea* treated group (Figure 1). Dry shoot biomass was significantly differrent across the treatment groups. The dry shoot biomass of the *Pseudomonas syringae pv Glycinea* treated group was significantly lower than the control. Moreover, the microbiome had a significant positive effect on the dry shoot biomass compared to the control (Figure 2), with its biomass being significantly higher than the other treatment groups. Surprisingly, the dry shoot biomass of the microbiome treated group was 5% higher than the control group (Figure 2). The dry shoot biomass of the *Pseudomonas syringae pv.Glycinea*+Microbiome treated group was significantly higher than the *Pseudomonas syringae pv. Glycinea* treated group. Contrary to my expectation, dry shoot biomass of the control and the *Pseudomonas syringae pv. Glycinea*+Microbiome treated group was not significantly different (Figure 2).

Interestingly, fresh weight of root nodules varies across the treatment groups with the *Pseudomonas syringae pv. Glycinea* treated group significantly lower compared to the control. However, the fresh weight of root nodules of the *Pseudomonas syringae pv. Glycinea*+Microbiome treated group was not significantly different from the control. Unexpectedly, the microbiome had a significant effect on the fresh weight of soybean nodules relative to the control (Figure 3). The fresh weight of the root nodules of the *Pseudomonas syringae pv. Glycinea*+Microbiome treated group was not significantly different from the *Pseudomonas syringae pv. Glycinea* treated group (Figure 3). However, the number of soybean root nodules was statistically different among the treatment groups. The number of nodules per

plant in the *Pseudomonas syringae pv. Glycinea* treated group was statistically lower compared to the control which was not surprising. Unexpectedly, the number of root nodules per plant was higher in the microbiome treated group compared to the *Pseudomonas syringae pv. Glycinea*+Microbiome treated group. Additionally, the number of soybean root nodules per plant was higher in the microbiome treated group compared to the control and this relation was statistically significant (Figure 4).

Nitrogen content (%) per leaf disc was significantly different among the treated groups (Figure 5). However, the *Pseudomonas syringae pv. Glycinea* treated group was not statistically different from the control. The nitrogen content (%) of the microbiome treated plants was statistically higher than the *Pseudomonas syringae pv. Glycinea* treated group. Furthermore, nitrogen content (%) in the *Pseudomonas syringae pv. Glycinea*+Microbiome treated group and the microbiome was also not statistically different. Nitrogen content (%) per leaf disc was significantly higher in the microbiome treated plants compared to the control group (Figure 5). The microbiome treated group recorded the highest percent nitrogen among the treated groups (Figure 5).

The chlorophyll concentrations differed significantly among the treatment groups (Figure 6). Surprisingly, chlorophyll concentrations doubled in the microbiome augmented treated group compared to the control and this relation is statistically significant. Unexpectedly, the chlorophyll concentrations of the control and the *Pseudomonas syringae pv. Glycinea*+microbiome treated groups were not significantly different and this was also true for the control and the *Pseudomonas syringae. pv. Glycinea* treated group. The chlorophyll concentration of the trifoliate leaflets was significantly different across all treated groups (Figure 7). In addition, the chlorophyll concentrations of the *Pseudomonas syringae. pv. Glycinea*

treated group was lower compared to the control. The microbiome treated group recorded a higher chlorophyll concentration of the leaflets compared to the control and this is statistically significant (Figure 7).

Net photosynthetic rates were statistically different among the treated groups (Figure 8). The net photosynthetic rate of *the Pseudomonas syringae pv. Glycinea* treated group was lower compared to the control and this was statistically significant. The net photosynthetic rates of *Pseudomonas syringae pv. Glycinea*+microbiome treated group was higher compared to the *Pseudomonas syringae pv. Glycinea* treated group and this was statistically significant. However, the net photosynthetic rates of the microbiome and the control groups were not statistically different while the net photosynthesis of the *Pseudomonas syringae pv. Glycinea* treated group and the control were statistically different (Figure 8).

Photosynthetic potential was significantly different among the treatment groups (Table 2). The rates of the maximum carboxylation and electron transport were significantly different among the treatment groups with the microbiome treated group higher compared to the control (Figure 9; Table 2). The rates of maximum carboxylation and electron transport were significantly reduced in the *Pseudomonas syringae pv. Glycinea* treatment group compared to the control. Photosaturated photosynthetic rate (A_{max}) and ambient photosynthetic rate (Aamb) were significantly different between the microbiome and the *Pseudomonas syringae pv. Glycinea* treatment groups (Table 3).

Transpiration rate was significantly different among the treated groups. The *Pseudomonas syringae pv. Glycinea* treated plants had a significantly lower transpiration rate compared to the control while the microbiome treated plants had higher transpiration rates compared to the *Pseudomonas syringae pv. Glycinea* +microbiome treated group and the control.

(Figure 10). Interestingly, the water use efficiency of the treatment groups varied significantly among the treatment groups. The *Pseudomonas syringae pv. Glycinea* treated plants recorded reduced water use efficiency compared to the control. The water use efficiency of the *Pseudomonas syringae pv. Glycinea*+microbiome treated group and the microbiome treated groups were also significantly different while the water use efficiency of microbiome treated plants was significantly reduced compared to the controls (Figure 11).

DISCUSSION

The microbiome is an important determinant of plant health and growth [37-42]. The results of my experiments further illustrate that augmenting the natural microbiome on agricultural plants can reduce pathogen disease, increase growth, increase leaf chlorophyll and nitrogen, and increase nodulation in a n-fixing crop species.

This study and several others [2, 43-46] have shown the microbiome's protection against plant pathogens, which are a constant major threat to the world's food security. The two-fold reduction in the copies of *Pseudomonas syringae pv. Glycinea* (Psg) illustrates protection provided by the microbiome (see Figure 1). The microbiome provides this protection via direct colonization of the phyllosphere environment, production of antibiotic products and induction of the plants innate immune system [47-51]. For example *Pantoea agglomerans* (see Table 1) was shown to directly inhibit the growth of *Erwinia amylovora* in vivo by the production of pantocin A and B [52]. Additionally, Psg colonizes the intercellular surface of the plant in a week and elicit the production of its phytotoxins coronafacic acid and coronamic acid through the type III secretion system (TTSS) leading to the pathogenesis [53-55]. This explains the appearance in my experiment of microscopic symptoms in about one week in the Psg treated group. About this time, Psg has entered the apoplast and multiplied within the soybean's tissues [56]. In this unfavorable, environment, Psg produces exopolysaccharides such as alginate that is resistant to toxic molecules, desiccation and production of water-soaked lesions [57-59].

The inoculation of the rhizosphere microbiome has shown remarkable improvement in the growth of the plants [42, 60-62]. There is abundant literature elucidating the functional roles of several microbial genes in enhancing plant growth [63-65]. An example is the ability of *Bacillus cereus* (see Table 1), a plant growth promoting bacteria to produce antioxidant enzymes

that increase growth and overall biomass of rice plants [66]. Many factors such as the production of PGPF, plant growth promoting bacterial endophytes (PGPBEs), and biofertilization enhance the growth of the plant [67-69]. Another example is the ability of *Pantoea agglomerans* (see Table 1) to promote the growth of rice plants and the allocation of photosynthates [70]. *Methylobacterium sp* (see Table 1) are majorly found in the phylloplane and they possess plant growth promoting substances [71]. The significant increase in the shoot biomass of the microbiome inoculated plants in this study suggest potential growth benefits. This observation may be due to the beneficial ability of the microbiome in promoting plant growth and the production of IAA [9, 72-75].

The phyllosphere microbiome has been implicated in biogeochemical processes such as carbon and nitrogen cycling [76-80]. One important function of leguminous plants (including soybean) is to fix atmospheric nitrogen by the symbiotic relationship with *Rhizobium* through nodule formation [81]. In fact, without nodulation, there is no biological nitrogen fixation in most legumes [82]. The number and the quality of the root nodules are important determinants of nitrogen fixation in nitrogen fixing plants [83, 84]. This is because nodule number and weight have been found to be positively correlated with the amount of nitrogen fixed in leguminous plants [85, 86]. Nodulation in legume plants is crucial for survival in nitrogen deficient soil [87]. The high nodule number and weight of nodules in the microbiome treated group (see Figure 3 and 4) suggests a direct or an indirect effect of the phyllosphere microbiome on nitrogen fixation. Additionally, the high nodulation may be due to the presence of *Paenibacillus peoriae*, a known nitrogen fixing microbe isolated in this study (see Table 1) [88]. Furthermore, a couple of studies have also shown the presence of nitrogen fixing bacteria in the phyllosphere, some of which have been identified [79, 89, 90]. In addition, efficient nodulation ensures a continuous conversion of

atmospheric nitrogen in the root nodules into ammonia; which is then assimilated into amino acids, nucleotides, and other cellular constituents for growth and development [91, 92].

Plants infected by pathogens induce a systemic cascade that leads to the production of jasmonic acids and innate immune effector proteins. This may have caused the reduced nodule number and weight of nodules in the *Pseudomonas syringae pv. Glycinea*-stressed plants since jasmonic acid is known to down-regulate the production of nodules in *L. japonicus* and *Medicago truncatula* [93, 94]. Additionally, pathogen-induced stress may also contribute to the reduced number of root nodules in the *Pseudomonas syringae pv. Glycinea* treated group. This is because the expression of *nodule inception* and *nodulation pectate lyase* symbiosis marker genes are inhibited by the inoculation of *Pseudomonas syringae pv. tomato strain* on *M. truncatula* [95]. Additionally, innate defense genes such as *MAPK3/6*, *RbohC, and WRKY33* have been shown to antagonistically reduce the number of root nodule infection threads in *M. truncatula* [95].

Nitrogen availability and uptake is directly related to crop productivity [96]. While nitrogen can be derived from mineralization of soil organic matter and nitrogen fertilizers, the major route of biological nitrogen for soybean is through biological nitrogen fixation [97]. The higher amount of nitrogen in the microbiome treated group could be due to the presence of *Paenibacillus sp* (see Table 1) since this bacteria are known for fixing nitrogen [75]. The reduced amount of apparent assimilated nitrogen in the leaves of the pathogen treatment (see Figure 5) may be due to its utilization of *Pseudomonas syringae pv. Glycinea* for its metabolism, production of the phytotoxin coronatine and other structural genes encoding the expression of coronamic acid [98]. *Pseudomonas syringae pv. Glycinea* cannot grow without an adequate source of carbon, nitrogen and phosphorus [56].

The highly significant increase in chlorophyll in the microbiome inoculated group has also been recorded in other studies [99, 100]. Chlorophyll concentrations have a direct relationship with the photosynthetic efficiency of the plant. This is because light reactions during photosynthesis are dependent on the light harvesting apparatus such as chlorophyll a and b [101]. The increased chlorophyll concentrations therefore suggest a high impact on the overall photosynthetic rate. Energy from the light reactions is used to produce electrons that increases the turn-over of NADPH and ATP [102]. Additionally, chlorophyll is highly rich in nitrogen, it represents 75% of total leaf nitrogen [99, 103]. There is a strong correlation between chlorophyll concentrations and leaf nitrogen, and this may explain why the mean chlorophyll concentrations doubled in the microbiome treated group compared to the control [104-106]. The observed results suggest optimal symbiotic nitrogen fixation and efficient assimilation of nitrogen to the leaves [107].

Gas exchange is directly related to crop productivity [108]. I found that photosynthetic rates were highly reduced in the *Pseudomonas syringae pv. Glycinea* treated plants (see Figure 8). This observation is not surprising because plant productivity is generally reduced by biotic (pathogens) and abiotic (temperature) stressors in the environment [108-110]. *Pseudomonas syringae pv. Glycinea* causes the formation of chlorotic halos on the leaves. This impairs the chlorophyll and its efficiency as the light harvesting apparatus [99]. *Pseudomonas syringae* pv. *tabaci* (*Pst*) has been shown to decrease the net photosynthetic rate and the carboxylation efficiency by reducing the maximum yield of photosystem II [111]. Furthermore, several photosystem II proteins have undergone modification during infection by *Pseudomonas syringae* [112]. In fact, many papers have recorded the downregulation of structural genes responsible for photosynthetic functions of plants infected with *Pseudomonas syringae* [111,

113-115]. D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the main enzyme that drives the photosynthetic process in plants [116, 117]. Although Rubisco is not 100% efficient, the increase in the rate of its action suggests the potential of improved efficiency by the phyllosphere-microbiome. The data from this study also suggests a faster CO_2 assimilation owing to the rapid ATP and NADPH generation in the microbiome treated plants shown in the photosynthetic rates.

Transpiration is related to transport of water and nutrients in plants [99], but the tradeoff with carbon gain is off great significance. Pathogens can reduce transpiration with concomitant decreases in carbon gain or direct water stress in leaves. This is because several environmental factors such as atmospheric carbon dioxide, water availability from the rhizosphere including infections by pathogens can modulate the opening and closing of the stomata [118-120]. Conversely, the microbiome may have the potential for improving the efficiency of transpiration in plants. Lindenthal et al., (2005) and others have shown that transpiration has a negative linear correlation with leaf temperature of cucumber of leaves corroborating how temperature can control the rate of transpiration [121].

Water is a key resource in increasing crop yield in agriculture [122]. Water use efficiency helps us to understand the amount of water lost to the amount of carbon fixed [123]. This important parameter underscores the need for solving water management problems and productivity in plants [124, 125]. The results of this study suggest the potential of increasing carbon gain without a corresponding increase in transpiration in plants by the inoculation of the phyllosphere microbiome. Notwithstanding, the role of phyllosphere-microbiome in improving water use in plants is majorly unexplored and the development of new approaches in the control of excessive water loss will be helpful in areas where there is drought and high salinity [126].

More evidence suggests that changes in the soil microbiome are influenced by water availability. Since phyllosphere microbiome resides in the stomata and the apoplast of plants, they may have a role in modulating the opening and closure of the stomata [9].

Interestingly, while photosynthetic rates were significantly higher in microbiome treatments (Figure 8), this did not result in an expected tradeoff with water use efficiency (Figure 11). This result combined with the research of [127] with a root fungal species indicates that microbiomes could increase crop yields without the need for more irrigation. Increasing crop yield without decreasing water use efficiency is a major goal of agricultural research and has been linked to microbiomes [128, 129].

CONCLUSION

The dry shoot biomass, root nodules, chlorophyll concentrations, percent nitrogen and photosynthetic activity were significantly increased by the presence of the microbiome. While large scale use of microbiome solutions on conventional crops production has so far been of limited success, it has potentially been because the applications have been to the rhizosphere and not the phyllosphere (i.e., utilizing the phytomicrobiome) (Singh and Trivedi, 2017 and see Compant et al., 2019). Therefore, the results of this soybean research suggest that methods can be used to develop a phyllosphere-microbiome as a plant probiotic against pathogens for the purpose of increasing yield in a suite of specialty crops.

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Table 1. Isolated phyllosphere members

Microorganism	Per. Identification (%)	Accession Number
Brevundimonas sp	99.07	MT160402
Bacillus subtilis	92.95	LT596053
Bacillus niacini	100	MT214114
Bacillus thuringiensis	99.74	MT102737
Bacillus mycoides	99.25	MT078667
Bacillus cereus	99.75	MT039631
Bacillus sp	99.2	MT254907
Cellulomonas sp	99.4	CP041203
Curtobacterium flaccumfaciens	98.75	MN989053
Microbacterium sp	96.6	MK398081
Methylobacterium sp	99.41	AB600003
Methylorubrum sp	99.21	MN893912
Pantoea agglomerans	99.88	MT184821
Paenibacillus sp	98.84	MT012248
Paenibacillus peoriae	95.75	MH569597
Pantonea dispersia	94.12	MT218355
Pseudomonas fluorescens	99.22	CP048408
Paenibacillus illinoisensis	99.45	HQ661869
Pseudomonas koreensis	98.93	MT192476
Pseudomonas sp	98.52	MH780493
Roseomonas hibiscisoli	99.14	NR_157795

Table 2. The rates of the maximum carboxylation and electron transport in the treatment groups

Treatment	V_{cmax}±SD	J_{max}±SD	Rd±SD
Control	101.94±5.56[a]	292.25±18.62[a]	17.65±1.52[a]
Microbiome	113.44±4.91[b]	401.89±35.42[b]	23.13±1.40[b]
Pseudomonas syringae pv. Glycinea+ Microbiome	89.96±4.60[a]	292.61±4.61[a]	13.75±1.39[a]
Pseudomonas syringae pv. Glycinea	53.19±5.54[c]	185.06±2.03[c]	7.96±2.04[c]

Data were expressed as Mean±SD (Standard Deviation) of the rates of V_{cmax}, J_{max} and Rd. The treatments that do not share the same letter are significantly different (alpha = 0.05, Tukey-adjusted).

Table 3. Measured photosaturated photosynthetic rate (A_{max}) and ambient photosynthetic rate (A_{amb}) in the treatment groups.

Treatment	A_{max} ±SD (µmol m^{-2} s^{-1})	A_{amb}±SD (µmol m^{-2} s^{-1})
Control	29.12±2.91[a]	15. 08±1.61[a]
Microbiome	30.57±2.38[a]	15.11±3.14[a]
Pseudomonas syringae pv. Glycinea+ Microbiome	21.67±3.54[b]	13.63±2.34[b]
Pseudomonas syringae pv. Glycinea	18.27±3.49[c]	11.21±2.53[c]

Data were expressed as mean±SD (Standard Deviation). Photosaturated Photosynthetic Rate (Amax) was measured at a PAR of 700 µmol m^{-2} s^{-1} and Ambient Photosynthetic Rate (A_{amb}) was measured at 700 µmol m^{-2} s^{-1}. The treatments that do not share the same letter are significantly different (alpha = 0.05, Tukey-adjusted).

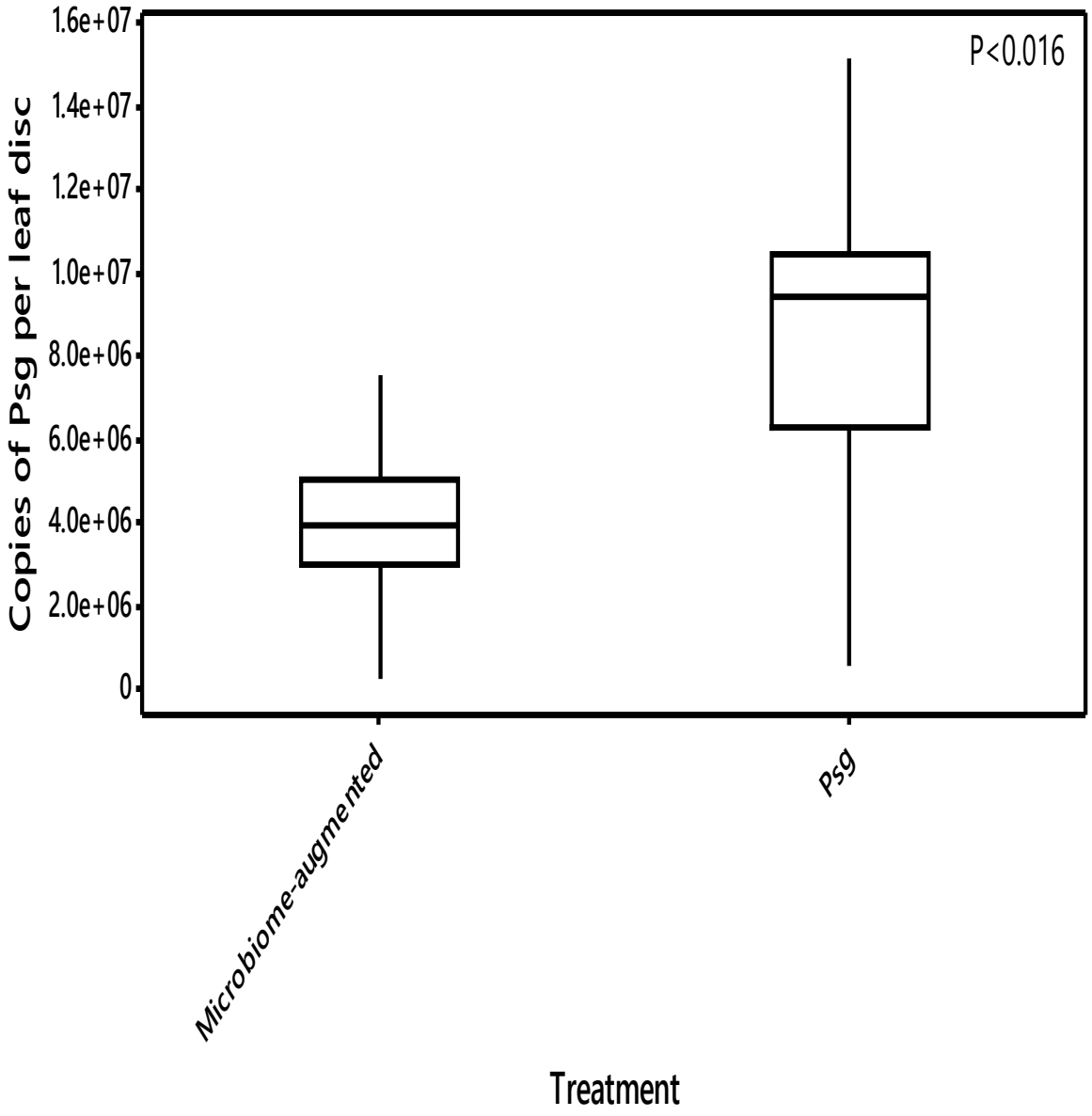

Figure 1**.** The density of *Pseudomonas syringae pv. Glycinea* as a function of treatment. Treatment had a significant effect on the Psg ($F = 6.40$; $P<0.016$). Psg here means *Pseudomonas syringae pv. Glycinea* in Figures 1-11) The Box-and-whisker plots show the median, upper and lower quartiles, and highest or lowest values that are within 1.5 IQR of the hinge, and IQR is the inter-quartile range. Figures 2-12 follow this same format.

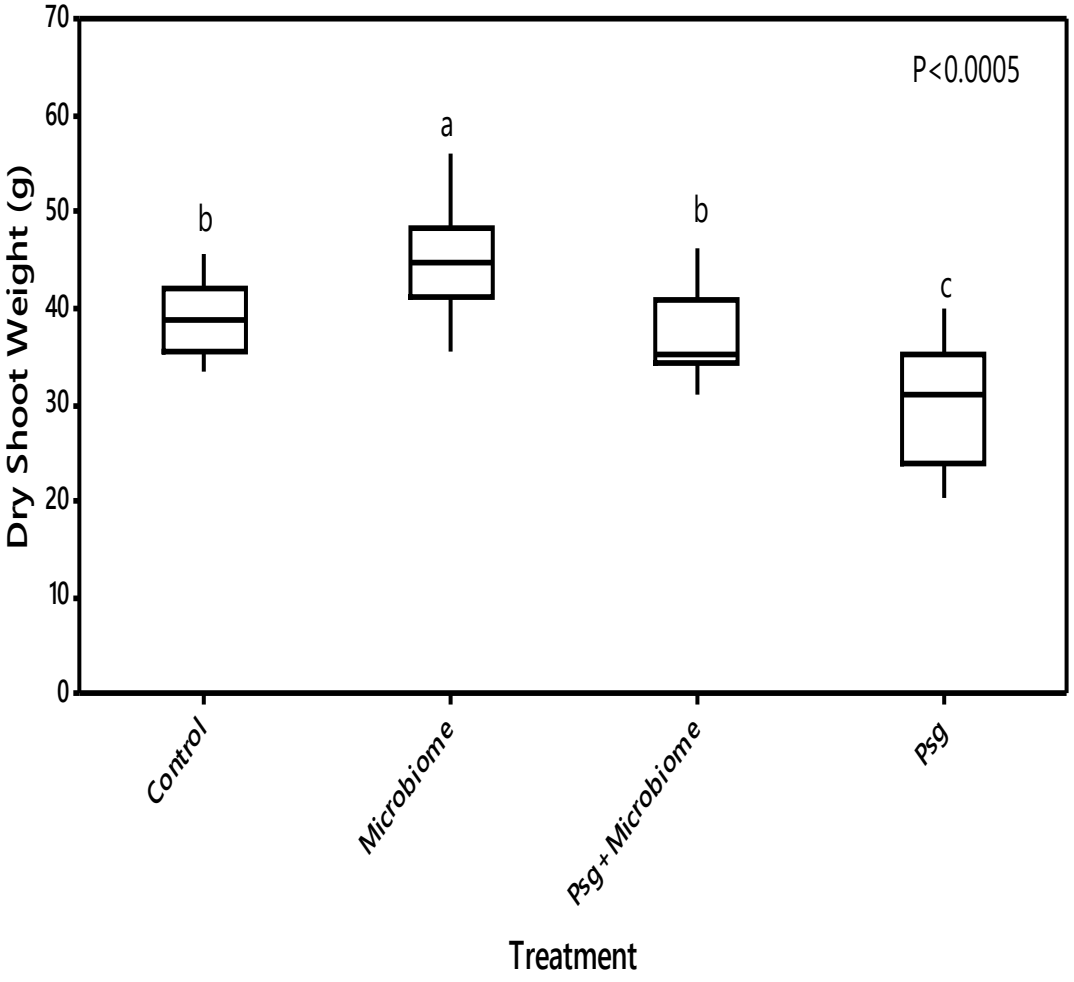

Figure 2**.** Dry shoot weight across the treatment groups. Twenty different plants (N=20) in the four different treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. Treatment had a significant effect on the dry weight ($F = 30.77$; $P<0.0005$). (See Figure 1 for description of symbols and box plot)

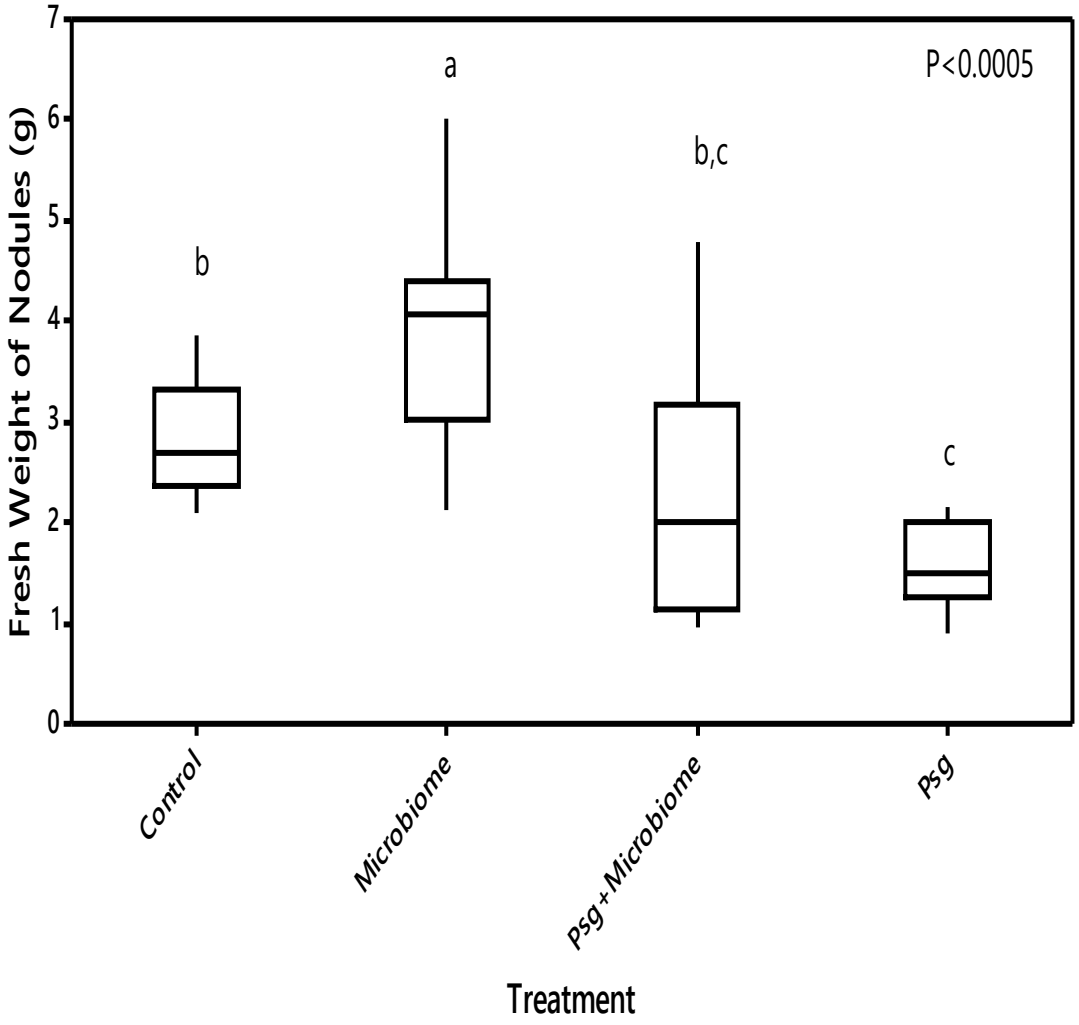

Figure 3**.** Fresh weight of nodules varies across the treatment groups. Ten different plants (N=10) in the four treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. Treatment had a significant effect on the fresh weight of the nodules ($F = 12.30$; $P<0.0005$). (See Figure 1 for description of symbols).

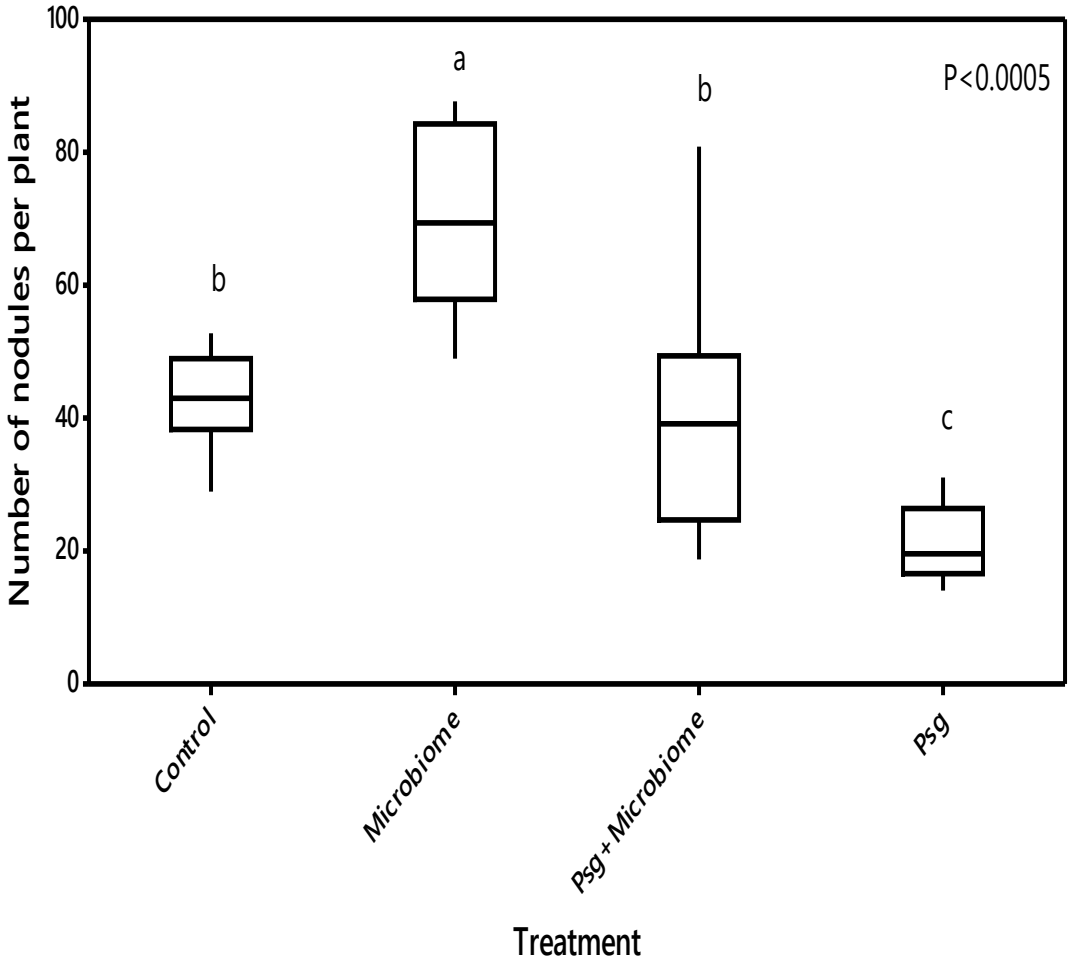

Figure 4**.** The number of root nodules as a function of the treatment groups. Ten different plants (N=10) in the four treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. Psg here means *Pseudomonas syringae pv. Glycinea*. Treatment had a significant effect on the number of the nodules per plant (F = 24.95; P<0.0005). (See Figure 1 for description of symbols).

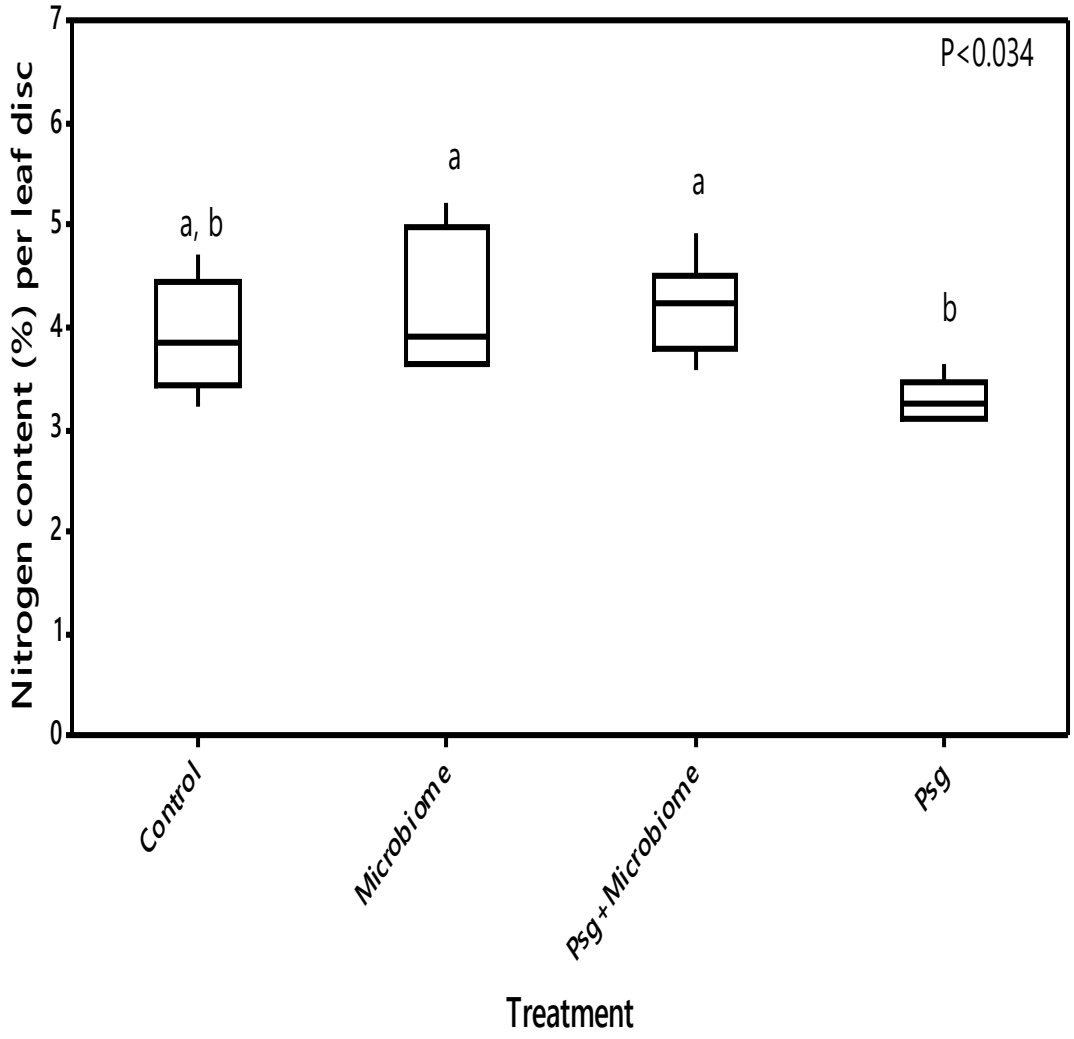

Figure 5**.** Nitrogen content (%) per leaf disc across the treatment groups. Ten different plants (N=10) in the four treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. Treatment had a significant effect on the nitrogen content (%) per leaf disc ($F = 3.59$; $P<0.034$). (See Figure 1 for description of symbols).

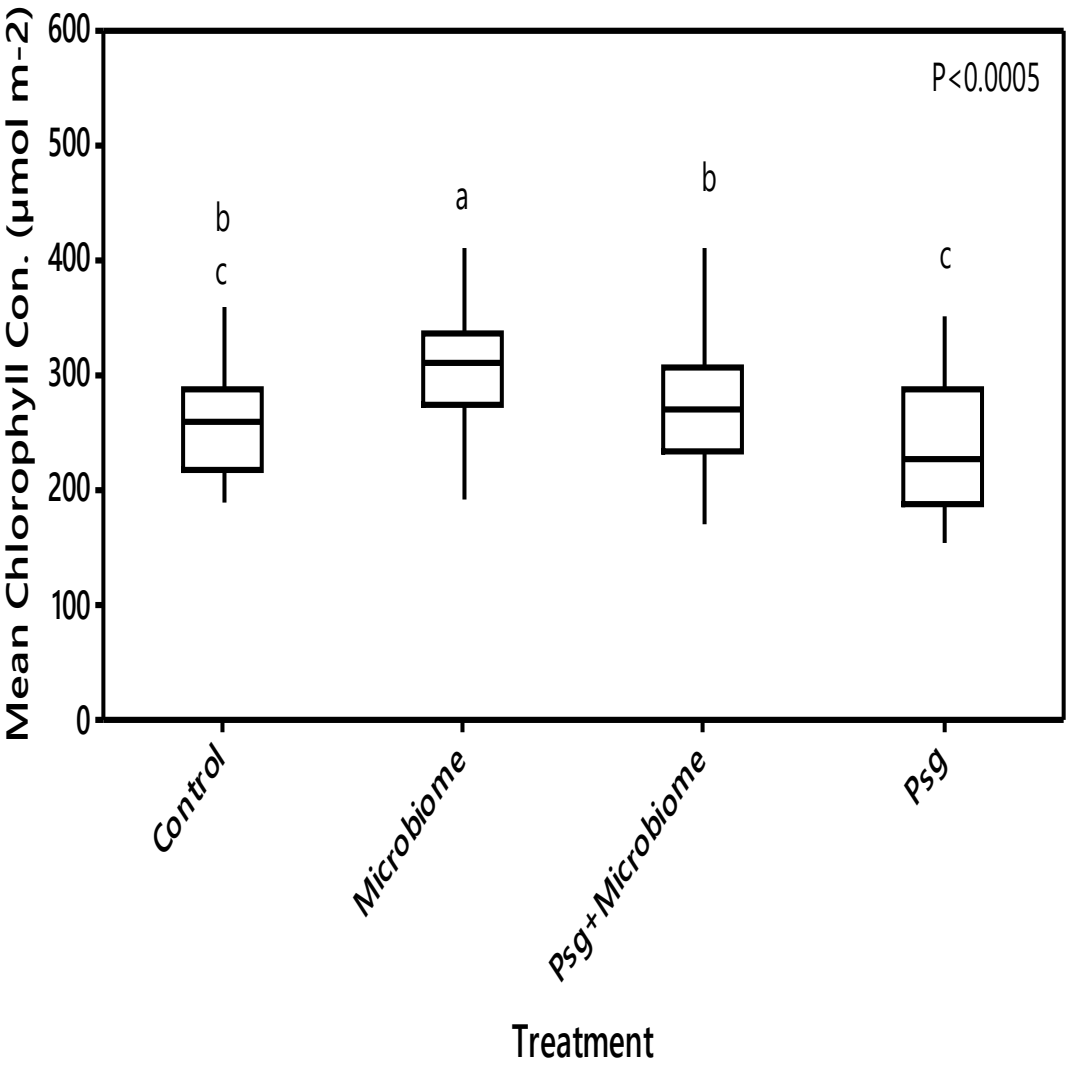

Figure 6**.** The mean chlorophyll concentration as a function of treatment group. Fifty different plants (N=50) in all the four different treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. The chlorophyll concentration of the trifoliolate leaves of the same age were measured and averaged per plant. Treatment had a significant effect on the mean chlorophyll concentrations ($F = 17.16$; $P<0.0005$). (See Figure 1 for description of symbols).

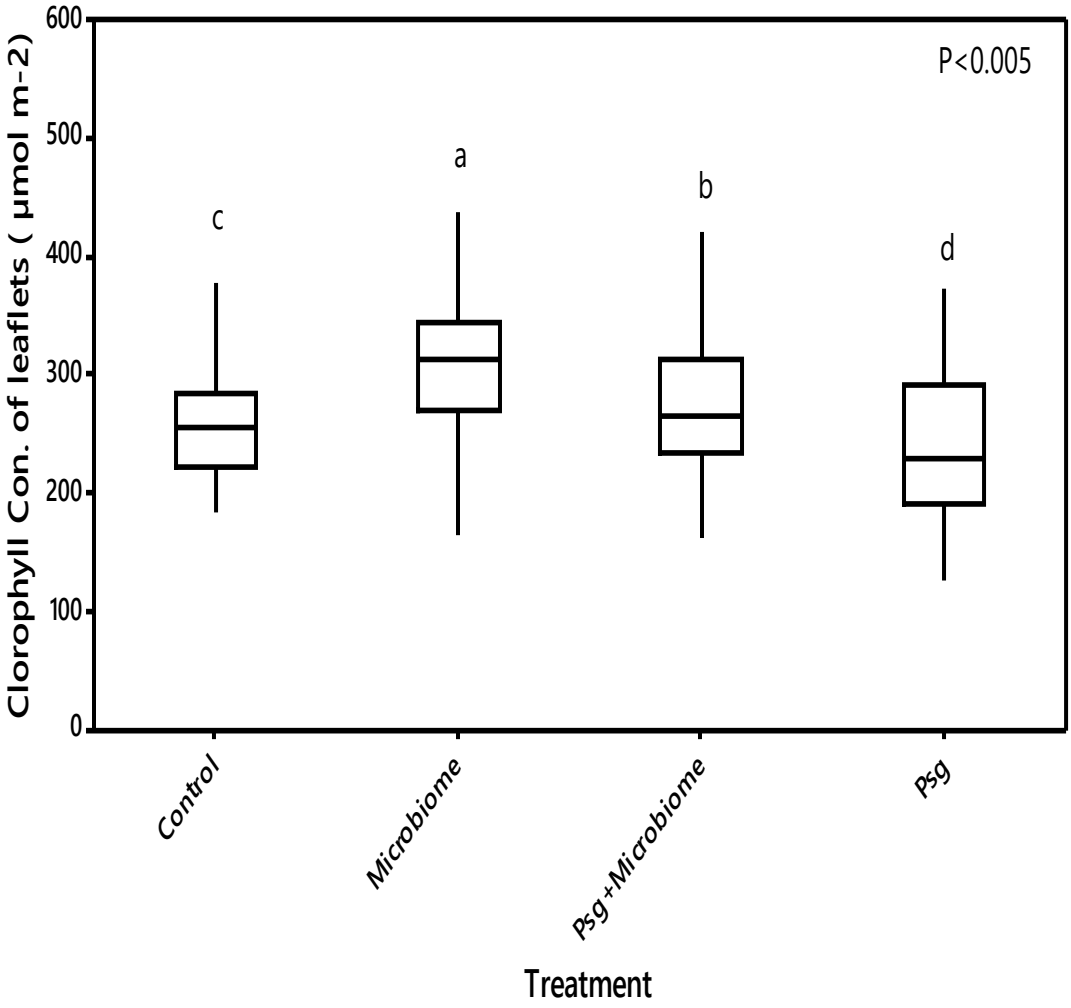

Figure 7**.** The Chlorophyll concentrations of the leaflets varies across the treatment groups. Fifty different leaflets (N=50) in the four different treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. Treatment had a significant effect on the chlorophyll concentrations in the leaflets ($F = 47.73$; $P<0.005$). (See Figure 1 for description of symbols).

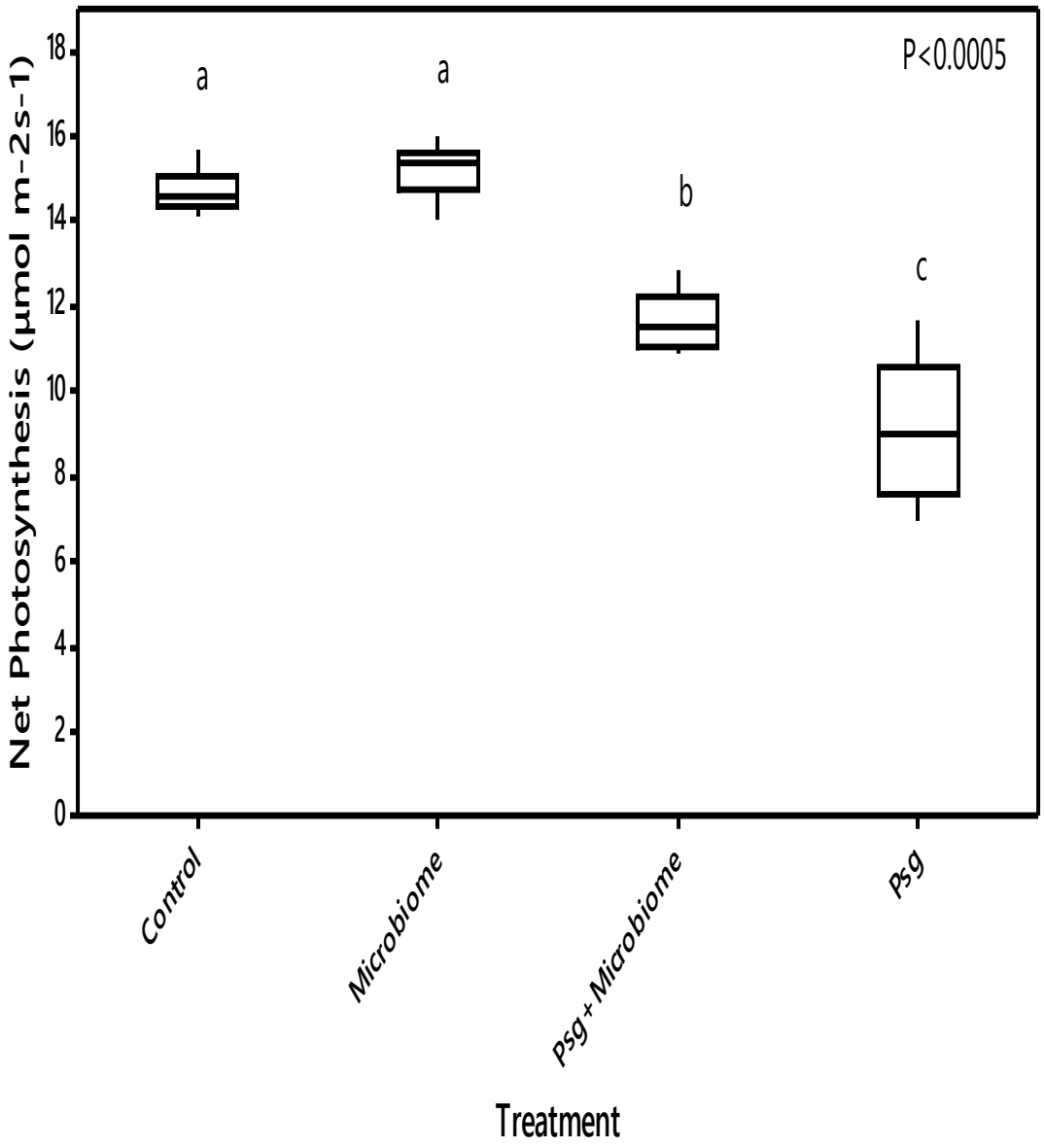

Figure 8**.** Net photosynthesis rate across the treatment groups. Ten different plants (N=10) in the four treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. Treatment had a significant effect on the net photosynthesis rate ($F = 92.95$; $P<0.0005$). (See Figure 1 for description of symbols

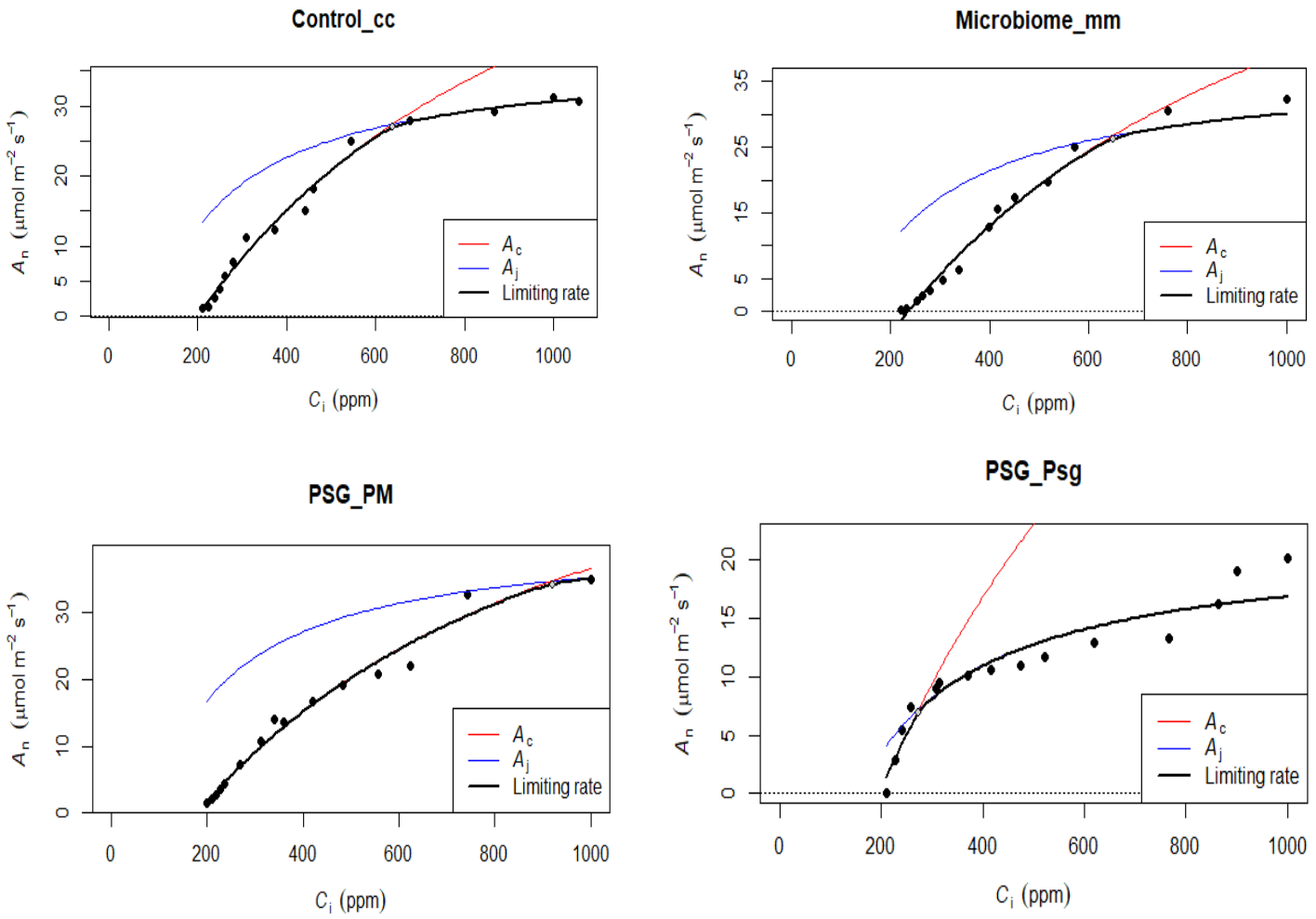

Figure 9. The ACi Curve of the control (control_cc), microbiome (microbiome_mm), *Pseudomonas syringae pv. Glycinea*±microbiome (PSG_PM) and *Pseudomonas syringae pv. Glycinea* (PSG_Psg) treated plants

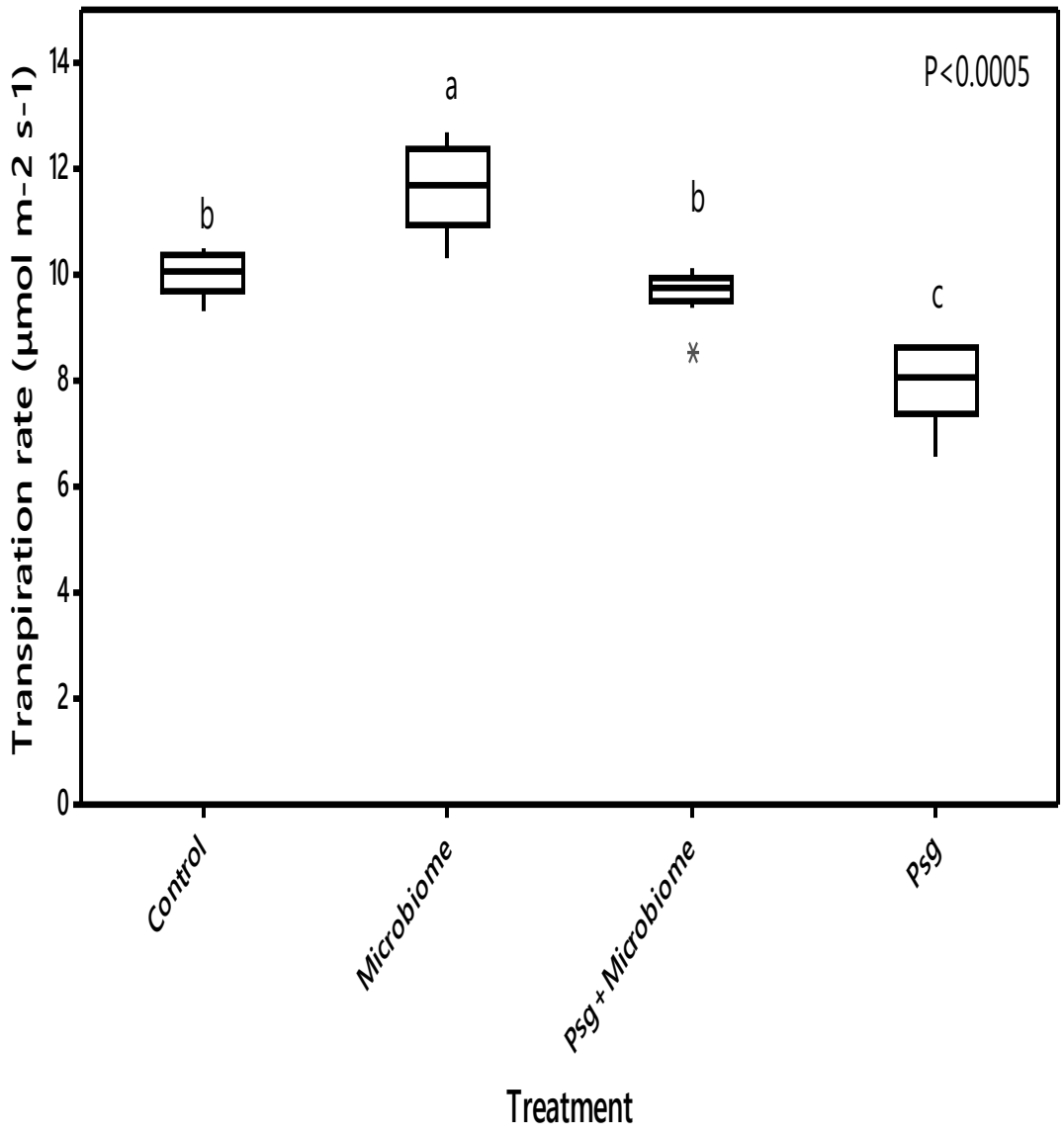

Figure 10**.** Transpiration rate as a function of the treatment groups. Ten different plants (N=5) in the four treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. Psg here means *Pseudomonas syringae pv. Glycinea*. Treatment had a significant effect on the transpiration rate ($F = 60.74$; $P<0.0005$). (See Figure 1 for description of symbols

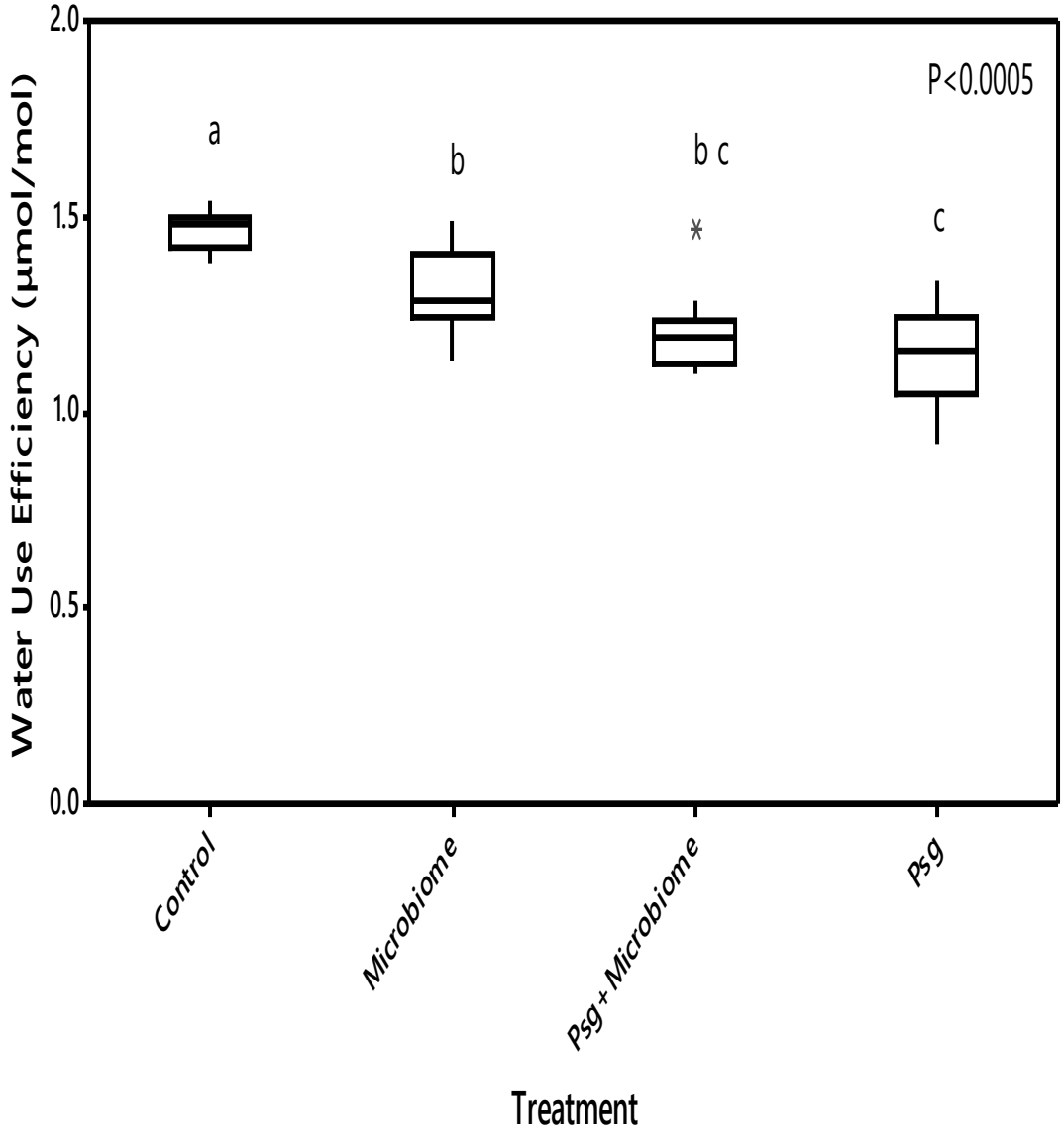

Figure 11**.** Water Use efficiency across the treatment groups. Ten different plants (N=10) in the four treatment groups (Control, Microbiome, Psg+Microbiome and Psg) were analyzed. Treatment had a significant effect on the water use efficiency (F = 20.64; P<0.0005). (See Figure 1 for description of symbols).