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2 *Burkholderia phytofirmans* strain PsJN advanced development and altered leaf level physiology
3 of switchgrass

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Abstract

Plant growth promoting rhizobacteria (PGPR) were reported to increase the productivity of switchgrass (*Panicum virgatum* L.), a model biofuel crop selected by the US Department of Energy (DOE). Physiological changes associated with the increased switchgrass productivity upon PGPR inoculation are unknown. Here we measured the photosynthetic rates, stomatal conductance, transpiration rates and water use efficiency weekly on both the upper canopy leaves and lower canopy leaves of switchgrass for 14 weeks and 8 weeks, respectively, on both *Burkholderia phytofirmans* strain PsJN inoculated switchgrass and control switchgrass. We also tracked changes in growth using a non-destructive photographic method. PsJN inoculation significantly enhanced early total leaf area of switchgrass by 107% ($p < 0.0001$). However, this beneficial effect linearly decreased with plant age. PsJN inoculation increased photosynthetic rates of upper canopy leaves by 14% ($p = 0.0047$). Nevertheless photosynthetic rates of lower canopy leaves were decreased by 8% ($p = 0.0393$). Accelerated leaf senescence and early flowering were also observed in PsJN inoculated switchgrass, which might contribute to slightly lower aboveground biomass at final harvesting as well as increased allocation to reproductive organs. In sum, this study verified advanced development of switchgrass due to PsJN inoculation and suggests that increased photosynthesis of newly developing foliage is related to enhanced productivity.

Key words

Switchgrass, *Burkholderia phytofirmans* strain PsJN, Photosynthesis, Water use efficiency, Photographic-based biomass assessment, Relative growth rates

1. Introduction

Switchgrass (*Panicum virgatum* L.) was selected as a model bioenergy crop through a plant screen trial from 1980s to 1990s in the U.S. [1]. First, as a native warm-season grass, switchgrass is well adapted across a large geographical area of North America from northern Mexico to southern Canada [2]. Therefore, it possesses the capability to be grown in large geographical areas without being considered ‘invasive’. Second, switchgrass is highly productive with relatively low water and nutrient requirements, which might be attributable to its C4 photosynthetic pathway and extensive root system [3]. Marginal lands unable to economically produce food crops may be suitable for cultivating switchgrass, avoiding the ‘food or fuel’ dilemma [4]. Finally, switchgrass is a deep rooted perennial grass species with potential for sequestering carbon, reducing soil erosion, capturing nutrients and improving wildlife habitat [5-7]. Nevertheless, knowledge of the symbiotic relationships between switchgrass and microbes is rather limited [8]. The fact that switchgrass was naturally associated with highly diversified fungi [9] and bacteria [10] suggests a great opportunity of using symbiotic microbes as ‘bio-fertilizers’ [11].

PGPRs are known as natural ‘bio-fertilizers’ working through mechanisms such as (1) nitrogen fixation, (2) manipulation of phytohormones, (3) solubilization of nutrients, and (4) production of siderophore and/or antibiotics (reviewed by [12]). Nevertheless, most of these studies investigated short-term effects of rhizobacteria [13-16]. How these bacteria benefit plant growth through an entire life cycle has not been widely studied. PGPR in *Arabidopsis* has been shown to accelerate flowering time [17] and change emissions of volatile organic compounds [18].

Burkholderia phytofirmans strain PsJN is a well-studied PGPR capable of enhancing the production of plants such as *Arabidopsis*, tomato, potato, grape, maize and switchgrass [13-15,

17, 19-22]. PsJN reduced host ethylene levels by excreting 1-aminocyclopropane-1-carboxylate (ACC) deaminase [23], which was necessary for its growth promotion effects [24]. In addition, PsJN possessed both production [25] and degradation pathways [21] of IAA, which interplays with the plant ethylene pathway [21]. Plants associated with PsJN displayed higher chlorophyll contents [13, 17], photosynthetic rates [13, 14], siderophore [24] and phenolic compounds [14]. PsJN has also modified plant morphology such as a longer root system, stronger stems, more root hairs, secondary roots and lignin deposits on vascular bundles [26, 27].

Knowledge of PGPR's long term effects on plants is not known except for *Arabidopsis* [17, 18, 28], though several studies have reported enhanced yields and establishment of switchgrass due to microbial association [8, 15, 29, 30]. However, physiological changes associated with PsJN inoculation have not been studied in switchgrass. Furthermore, leaf level physiology is known to vary with leaf age and position [31, 32], and previous gas exchange measurements on switchgrass were all on upper canopy leaves measured for short periods of time [33, 34]. Rhizobacteria are known to influence plant development [17, 28] so measuring leaf physiology over time as leaves mature is critical to our understanding of physiological mechanisms of PsJN's growth enhancement effects. Therefore, our objectives were to (1) study the long-term effects of PsJN on switchgrass growth using a non-destructive photographic technique; (2) study PsJN induced long-term leaf level physiological changes in both upper canopy and lower canopy leaves; and (3) assess PsJN's effect on long-term switchgrass development.

2. Materials and Methods

2.1. Plant material and seed sterilization

Switchgrass (*Panicum virgatum* L.) cv. Alamo seeds were surface-sterilized by treatment with 70% volume fraction ethanol for 2 min, followed by rinsing 3X with distilled water, immersed in 60% volume fraction H₂SO₄ with stirring for 30 min to de-husk, and then washed 3X with distilled water, sterilized with 0.4 mol L⁻¹ sodium hypochlorite containing 0.1% Triton 100 for 30 min followed by a 5X rinse with sterile, deionized, distilled water.

2.2. PsJN inoculation and plant growth conditions

Seeds were germinated on a wet filter paper while incubated at 25°C with a light cycle of 16h (light illumination of 67 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 3-5 days. *Burkholderia phytofirmans* strain PsJN-GFP derivative was obtained from Dr. Angela Sessitsch (Austrian Institute of Technology, Seibersdorf, Austria) and PsJN solution was prepared as described in [15]. Then half of these seedlings were soaked in PsJN solution for 1 min while the other half were soaked in PBS as a control. These treated seedlings were dried with a sterilized paper towel and planted in magenta boxes (76× 76 ×102 mm, Sigma-Aldrich) filled with 50 ml of Murashige and Skoog Basal Salt Mixture and vitamin, 30 g L⁻¹ maltose and 3 g L⁻¹ phytoGel at pH 5.8. Then they were incubated at the same condition as before for 2 weeks. After that they were transferred to 4-gallon pots filled with soil mix composed of 2/3 Miracle-Grow Potting Mix (Scotts Miracle-Gro Company, Marysville, Ohio) and 1/3 Arabidopsis growing media (Lehle Seeds, Round Rock, Texas) in a greenhouse at 25 °C with 16h photoperiod.

2.3. Leaf area and biomass measurements

108 Five pots were planted with PsJN inoculated Alamo seedlings and five pots were planted with
109 uninoculated control seedlings. There were five seedlings in each pot. Leaf area was estimated
110 every week for 15 weeks using a digital photograph taken from above the pot with Photoshop
111 CS5 to quantify (Adobe Systems Inc.). In each photo, a known area reference square was placed
112 at the height of the majority of the leaf area. Relative growth rate of leaf area was calculated
113 using following equation:

$$\text{RGR} = (\ln LA2 - \ln LA1)/(t2 - t1)$$

114 Where:

115 $t1$ =time one (in days)

116 $t2$ =time two (in days)

117 $LA1$ = leaf area of plant at time one (in cm^2)

118 $LA2$ = leaf area of plant at time two (in cm^2)

119 Photosynthetic rates (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration rates (E , $\text{mmol m}^{-2}\text{s}^{-1}$), stomatal conductance
120 (g , $\text{mmol m}^{-2} \text{s}^{-1}$) and water use efficiency ($\text{WUE}=A E^{-1}$, $\mu\text{mol mmol}^{-1}$) were measured after 4
121 weeks of growth with a LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE,
122 USA) at $3000 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD), $400 \mu\text{mol mol}^{-1}$ reference
123 CO_2 concentration, 25°C block temperature, and a flow rate of $385 \mu\text{mol sec}^{-1}$. One fully
124 expanded leaf was chosen from each pot and measured repetitively each week for 8 weeks.
125 These leaves were referred as lower canopy leaves. Lower canopy leaves were considered
126 senesced when leaves turned yellow with negative photosynthetic rates. The percentage of
127 plants in each treatment with senesced leaves was recorded at each measurement date. After five

weeks, a new fully expanded leaf was chosen from each pot and measured repetitively until the thirteenth week. At week 14, another set of newly expanded leaves was chosen and measured weekly because many of the previous chosen leaves had senesced. These leaves were referred as upper canopy leaves. After eighteen weeks, the aboveground biomass of all plants was harvested and immediately dried in an oven at 65 °C for 48 hrs. The percent change of all the biometric variables was defined as $(V_{\text{PsJN}} - V_{\text{Control}}) * 100 / V_{\text{Control}}$ (V indicates variable).

2.4. Experimental design and statistical analysis

A completely randomized design was used in this experiment with two treatments (PsJN inoculation and Control) and 5 replicates of each treatment. A t-test was performed to detect differences in growth and physiological parameters between treatments at $\alpha=0.1$ each day. An overall comparison of gas exchange parameters was also performed using a t-test by pooling all the measurement days together. Regression analysis was performed on physiological variables that are interdependent. All statistical analysis was performed with JMP Pro 10 (SAS Institute, Cary, NC)

3. Results

3.1. Leaf area and relative growth rate

During the first two months of growth, PsJN inoculated plants had significantly increased leaf area by 107% compared to controls ($p < 0.0001$, Figure 1). However, after two months, there was no statistical difference between PsJN inoculated plants and control plants and differences between treatments diminished. At the end of the study differences in leaf area between treatments were less than 10% ($p = 0.3529$, Figure 1). This changing growth pattern resulted in a significant interaction between plant age and PsJN inoculation. Significantly higher relative growth rate was found in PsJN inoculated plants through day 28 ($p = 0.0258$). However, control plants displayed slightly higher relative growth rates at 77 days old ($p = 0.0577$) and 98 days old ($p = 0.0911$, figure 1b).

3.2. Leaf level physiology

PsJN inoculated plants increased photosynthetic rates, stomatal conductance and transpiration rates in upper canopy leaves of switchgrass by 14% ($p = 0.0047$), 16% ($p = 0.0051$) and 13% ($p = 0.0084$), respectively, during the whole experiment (Figure 2). Lower canopy leaves in PsJN inoculated plants, however, had decreased photosynthetic rates, stomatal conductance and transpiration rates by 8% ($p = 0.0393$), 7% ($p = 0.0949$), and 10% ($p = 0.0157$), respectively. Water use efficiency of both upper and lower canopy leaves were not affected by PsJN inoculation. Therefore, PsJN inoculation induced differences in photosynthetic rates were attributable to altered stomatal conductance, which was significantly correlated with photosynthetic rates ($R^2 = 88\%$, $p < 0.0001$).

Leaf level physiology of lower canopy leaves was significantly influenced by leaf age ($P < 0.0001$, Table 1, Figure 3). Regression analysis indicated a faster decline of photosynthetic rates ($p = 0.0532$), transpiration rates ($p = 0.0393$) and stomatal conductance ($p = 0.0148$) in PsJN inoculated plants (Figure 3). PsJN increased photosynthetic rates ($p = 0.036$), transpiration rates ($p = 0.0594$) and stomatal conductance ($p = 0.0262$) of lower canopy leaves when plants were 49 days old (Figure 3). After that, lower photosynthetic rates were observed in the lower canopy leaves of PsJN inoculated plants at day 70 ($p = 0.0390$), day 84 ($p = 0.0782$), day 91 ($p = 0.0519$) and day 98 ($p = 0.0476$, Figure 3).

Photosynthetic rates, stomatal conductance, and transpiration rates of upper canopy leaves were not affected by plant age (Table 1). PsJN inoculation enhanced photosynthetic rates at day 112, day 126, and day 154 by 25% ($p = 0.0310$), 48% ($p = 0.0313$) and 40% ($p = 0.0345$), respectively (Figure 4a). Transpiration rates and stomatal conductance also followed similar patterns. Mean photosynthetic rates, transpiration rates and stomatal conductance were higher in the upper canopy leaves of PsJN inoculated plants for most days.

3.3. Flowering time, lower canopy leaf death and final harvest

PsJN inoculated plants flowered earlier than control plants (Figure 5). Similarly, PsJN inoculated switchgrass also had more leaf death than the control group (Figure 5), indicating faster senescence of old leaves. At the final harvest, PsJN inoculation slightly decreased the vegetative biomass (leaf and stem) by 14% ($p = 0.0568$, Table 2) and the total aboveground biomass by 14% ($p = 0.0868$, Table 2). However, the flower biomass was not affected by PsJN inoculation ($p = 0.9202$). The flower biomass linearly increased with higher vegetative biomass ($p = 0.006$, Figure 6).

Nevertheless, PsJN inoculated plants allocated more biomass to reproductive parts (flower) as compared to control plants ($p=0.0639$, Figure 6).

4. Discussion

PsJN inoculation initially (younger than 70 days old) enhanced growth of switchgrass Alamo plants. This is in agreement with a report that PsJN inoculated Alamo seedlings had significantly higher shoot biomass at every young age [35] or juvenile stage [15]. Similar results were also obtained in *Arabidopsis* [17], potato [27], grapevine [14] and maize [13] in early ontogeny. As a perennial grass, switchgrass establishment is challenged by annual weed competition[36], and this early growth enhancement could help switchgrass to outcompete weeds during plant establishment and improve field productivity [37].

Like most of these studies, the greenhouse study of PsJN's effects on switchgrass growth did not go beyond juvenile stages of development [15], and none investigated the leaf level physiology of PsJN inoculated switchgrass. In one study with *Arabidopsis* [17], biomass was assessed for the whole life cycle by analyzing rosette leaf area. Similar to this study, the beneficial effects of PsJN diminished with time in *Arabidopsis* [17]. The phenomena that PsJN's effects on growth in general decreased along the growing season might be attributable to pot environment and/or accelerated development.

PsJN enhances growth through a variety of mechanisms[21, 24, 38], including expanding root system [27] to reach larger volume of soil, thereby accessing more water and nutrient resources. However, the soil volume, water and nutrients were identical for all the treatments, which compromised PsJN's effects on enlarging the root system and might explain PsJN's gradually diminished beneficial effects on growth. However, this spatial limitation does not apply to the

field study, where PsJN inoculated plants could gain more water and nutrients by accessing larger volume of soil. Therefore, PsJN consistently increased biomass of switchgrass both at midseason and at the end of the growing season in our field trial over two years [39]. Nevertheless, even in the field trial, PsJN's effect was more pronounced at midseason (35.7%) than at the end of the growing season (12%)[39], suggesting that other mechanisms, such as accelerated development, came into play.

PsJN's accelerating development was evident in promoting growth and maturation in young seedlings [19, 35, 39], as well as inducing earlier senescence and flowering in adult plants [17, 40]. Therefore, PsJN's beneficial effects on growth were more evident when switchgrass was young, then gradually decreased because PsJN inoculated plants also aged earlier and showed relatively lower growth rates later in the growing season. In this study, faster decline of photosynthetic rates in aged leaves (lower canopy leaves) contributed to earlier leaf death of PsJN inoculated switchgrass, explaining PsJN's gradually diminished beneficial effects on growth. Accelerated flowering and earlier senescence were discovered in PsJN inoculated Arabidopsis and the expressions of the key genes that regulate flower time were up-regulated in PsJN inoculated Arabidopsis [17], which was in agreement with earlier flowering in PsJN inoculated switchgrass in this study, with lower vegetative biomass due to shortened vegetative growth. Although both PsJN inoculated switchgrass and control switchgrass had comparable flower and seeds biomass at final harvesting, the stem and leaf biomass of PsJN inoculated switchgrass was slightly lower than that of control plants. This was possibly the result of faster leaf senescence, earlier flowering or more allocation into below ground in PsJN inoculated plants. Although the positive effects of PsJN on growth diminished with time, this early growth advantage for switchgrass seedlings could benefit establishment and competitiveness of

switchgrass, a perennial species often challenged by annual weeds[36]. Competition with weeds did not occur in our pot study, but certainly will in field trials, which could be another reason for the consistently positive effects of PsJN on switchgrass throughout the growth season in field trials[39]. Besides, shortened vegetative growth might help more productive cultivars of southern types to move north which otherwise stay vegetative too late to survive winter [41]. Increased allocation to root biomass may also result in lower aboveground biomass [42]; however, this was not evaluated in this study.

There have been numerous reports on altered photosynthetic rate due to leaf ontogeny and leaf position in various plants [31, 32, 42-45]. For example, photosynthetic rates of winter wheat displayed a short term rise after ligule emergence, a plateau, and then a linear decline as leaves aged [44]. Nevertheless, previous studies on switchgrass only measured photosynthetic rates on upper canopy leaves once [33, 34, 46], which ignored the influence of leaf ontogeny. In our study, we monitored gas exchange of both upper canopy leaves and lower canopy leaves, correspondence to leaves in early ontogeny and leaves in later ontogeny, for 14 weeks and 8 weeks, respectively. The upper canopy leaves were still in the plateau stage while the lower canopy leaves underwent senescence. Higher photosynthetic rates, transpiration rates and stomatal conductance were observed in upper canopy, young leaves of PsJN inoculated switchgrass, which may have contributed to PsJN's growth enhancement. This is in agreement with studies on PsJN inoculated grapevine [14] and maize [13] which also found increased photosynthetic rates. The higher photosynthetic rates in PsJN inoculated switchgrass mostly resulted from higher stomatal conductance since no difference was found between water use efficiency of PsJN inoculated switchgrass and control switchgrass.

The photosynthetic rates of lower leaves declined in both PsJN and control plants as switchgrass aged, which agreed with previous reports on other crops and trees [31, 32, 43-45]. However, faster senescence was observed in lower leaves, indicated by faster decline of photosynthetic rates, transpiration rates and stomatal conductance of PsJN inoculated switchgrass, which supported the prediction that leaf life span was negatively related to initial photosynthetic rates [47]. Our study is the first to investigate physiological changes coupled with endophyte induced early leaf senescence. Faster senescence of leaves in PsJN inoculated Arabidopsis has been found but leaf level gas exchange was not measured [17]. In addition, this study is also the first attempt tracking whole life growth change of switchgrass using non-destructive photographic method.

5. Conclusions

Our study showed that PsJN inoculation advanced development of switchgrass Alamo plants. This included enhanced early growth rate, higher photosynthetic rates of younger leaf, accelerated leaf senescence and earlier flowering. Aboveground biomass was not increased by PsJN inoculation at the final harvest. However, PsJN promoted earlier growth which could be advantageous for switchgrass seedling establishment in the field. Further, a shortened life cycle could allow more productive southern switchgrass cultivars to be used further north where the growth season is shorter.

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Table 1. Effect of PsJN inoculation and plant age on leaf level physiological variables for lower canopy leaves and upper canopy leaves of switchgrass A, photosynthesis, E, transpiration, g, stomatal conductance, WUE, water-use efficiency

		A ($\mu\text{mol s}^{-1}\text{m}^{-2}$)	E ($\text{mmol s}^{-1}\text{m}^{-2}$)	g ($\text{mmol s}^{-1}\text{m}^{-2}$)	WUE ($\mu\text{mol mmol}^{-1}$)
lower canopy leaves	Age	p<0.0001	p<0.0001	p<0.0001	p<0.0001
	PsJN	p=0.1608	P=0.0798	p=0.2603	p=0.9579
	Age $\times$ PsJN	p=0.0532	P=0.0393	p=0.0148	p=0.8519
Upper canopy leaves	Age	p=0.3114	p=0.5235	p=0.2734	p=0.0075
	PsJN	p=0.0332	P=0.0721	p=0.0337	p=0.8485
	Age $\times$ PsJN	p=0.6549	P=0.8952	P=0.7872	P=0.5370

Probabilities are given for the results of a Two-Way analysis of variance for each parameter.

Table 2 Effect of PsJN inoculation on stem and leaf biomass, flower biomass and total aboveground biomass of switchgrass

	Stem and leaf (g)	Flower (g)	Aboveground (g) ⁴⁴⁹	
Control (n=5)	167.4±8.8	9.4±1.3	176.8±9.8	450
PsJN (n=5)	143.5±6.2	9.2±1.6	152.5±7.6	
p value	p=0.0568	p=0.9202	p=0.0868	451 Probab

ilities are given for the results of a One-Way analysis of variance for each parameter.

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475 Figure 1 Effect of PsJN inoculation on leaf area and relative growth rate (RGR) of inoculated
476 and non-inoculated switchgrass seedlings. Each value is the mean of five plants. Error bars are
477 $\pm$ SE of the means. * means significant difference at $\alpha=0.1$ level. ** means significant difference
478 at $\alpha=0.01$ level and *** means significant difference at $\alpha=0.001$ level between PsJN and control
479 using student T-test

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481 Figure 2 Effect of PsJN inoculation on physiological variables for lower canopy leaves and
482 upper canopy leaves of switchgrass A, photosynthetic rate, E, transpiration, g, stomatal

conductance, WUE, water-use efficiency. Each value is the mean of 40 measurements (lower canopy leaves) or 70 measurements (Upper canopy leaves) pooled together. Error bars are $\pm$ SE of the means. * means significant difference at $\alpha=0.1$ level and ** means significant difference at $\alpha=0.01$ level between PsJN

Figure 3 Leaf physiological variables of lower-canopy leaves in PsJN inoculated and control switchgrass seedlings. Each value is the mean of five plants. Error bars are $\pm$ SE of the means. * means significant difference at $\alpha=0.1$ level between PsJN and control using student T-test. A, photosynthesis, E, transpiration, g, stomatal conductance, WUE, water-use efficiency

Figure 4 Leaf physiological variables of upper-canopy leaves in PsJN inoculated and control switchgrass seedlings. Each value is the mean of five plants. Error bars are $\pm$ SE of the means. * means significant difference at $\alpha=0.1$ level between PsJN and control using student T-test. A, photosynthesis, E, transpiration, g, stomatal conductance, WUE, water-use efficiency

Figure 5 Flowering plants (%) and lower canopy leaf death (%) of PsJN inoculated switchgrass and Control

Figure 6. Flower biomass (g) vs stem and leaf biomass (g) at final harvest as influenced by PsJN inoculation. Open circles and dashed lines indicates PsJN-inoculated Switchgrass. $P_{\text{PsJN}} < 0.1$ indicate significantly change in slope due to PsJN inoculation

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