1 Early growth promotion and leaf level physiology changes in *Burkholderia*
2 *phytofirmans* strain PsJN inoculated Switchgrass

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Abstract

Switchgrass (SG) is one of the most promising next generation biofuel crops in North America. Inoculation with bacterial endophytes has improved growth of several plant species. Our study demonstrated that *Burkholderia phytofirmans* strain PsJN, a well-studied plant growth promoting rhizobacterium (PGPR) significantly increased both aboveground and belowground biomass (DW) and promoted elongation of root, stem and leaf within 17 days following inoculation. Furthermore, the enhanced root growth in PsJN inoculated plants lagged behind the shoot response, resulting in greater allocation to aboveground growth ($p=0.0041$). Lower specific root length (SRL, $p=0.0158$) and higher specific leaf weight (SLW, $p=0.0029$) were also observed in PsJN inoculated seedlings, indicating changes in development. Photosynthetic rates (Ps) were also significantly higher in PsJN inoculated seedlings after 17 days (54%, $p=0.0016$), and this occurred initially without increases in stomatal conductance resulting in significantly greater water use efficiency (WUE, 37.7%, $p=0.0467$) and lower non-stomatal limitation (LNS, 29.6%, $p=0.0222$). These rapid changes in leaf level physiology are at least partially responsible for the growth enhancement due to PsJN.

Highlights

- PsJN enhanced growth and Ps of SG by day 17 after inoculation
- PsJN inoculated SG allocated more DW aboveground, increased LSW and decrease RSL
- PsJN inoculated SG had decreased LNS to Ps resulting in increased WUE

Keywords

Switchgrass; PsJN; Plant growth promoting rhizobacteria (PGPR); Photosynthesis; Water use efficiency; Enhanced growth

1. Introduction

Human society's consumption of energy is heavily dependent on fossil fuels, which made up 86.8 % of total primary energy consumed in 2012 [1]. The finite and environmental unfriendly nature of fossil energy as well as the ever increasing demands for primary energy contributed to current food crop-based renewable energies such as corn, sugarcane, and rapeseed oil [2], which constituted 1.9% of the total primary consumption in 2012 [1]. The US government has suggested 30% displacement of present petroleum consumption with biofuels, requiring 910 million tonnes of biomass [3]. In order to avoid competition between food and fuel, massive efforts were devoted to future cellulose biofuel crops such as switchgrass (*Panicum virgatum*), a 'model' biofuel crop with the capability of maintaining a relatively high and reliable production rate with relatively low water and nutrient requirements [4].

Endophytic bacteria are known to promote growth through increasing available nutrients, physiological changes and hormone regulations [5]. In addition, endophytic bacteria also provide environmental and economic benefits to sustainable biofuel crop systems by reducing fertilizer and pesticide use [5-8]. The use of endophytic bacteria as 'bio-fertilizer' has been studied in switchgrass [9, 10] and thirty-one endophytic bacteria were found to be naturally associated with switchgrass in Canada [11]. Inoculating switchgrass with endophytic bacteria increased its tiller number and tiller height, which contributes to higher yield in PsJN inoculated plants [9, 10]. *Burkholderia phytofirmans* PsJN, an endophytic bacterial strain originally isolated from onion roots [12], significantly promoted growth of potato [13, 14], grapevine [15, 16], maize [17], *Arabidopsis thaliana* [18, 19] and switchgrass [9]. PsJN's growth promotion effects have also been found to be genotype dependent [9].

A few studies have investigated the physiological mechanisms behind the growth promotion effects, including lowering ethylene level [20], producing and regulating IAA [21], accelerating development [19], increasing chlorophyll content [17, 19] and photosynthetic rate [15, 17] as well as altering root morphology and vascular tissue [22, 23]. However, none of these physiological mechanisms has been studied in switchgrass. Further, most studies only measured variables at the end of experiment and not

1 along with developmental changes over time. Therefore, we investigated the biomass allocation,
2 morphology change, leaf gas exchange and instantaneous water use efficiency at 9 different time points
3 from the day of inoculation to the 24th day after inoculation. Our specific objectives were to investigate in
4 switchgrass: 1) the early PsJN induced physiological changes that may lead to growth changes and 2) the
5 growth, morphology and allocation changes induced by PsJN inoculation.

2. Methods

2.1. Plant materials

Switchgrass (*Panicum virgatum* L.) cv. Alamo seeds (Warner Brothers Seed Co. Lawton, OK) were surface-sterilized by soaking in 70% ethanol for 2 min and followed rinsing 3X with distilled water. Seeds were de-husked for 30 min with 60% H₂SO₄ with stirring. After de-husking, these seeds were washed 3X with distilled water, sterilized with 0.4 M sodium hypochlorite containing 0.1% Triton 100 for 30 min followed by 5X rinse with sterile, deionized, distilled water. These seeds were germinated in 24 magenta boxes (76 mm× 76 mm×102 mm, Sigma-Aldrich) with vented lids filled with 50 ml of Murashige and Skoog Basal Salt Mixture and vitamins and 3 g/l phytogel, pH 5.8 and incubated at 24°C with a light-dark cycle of 16 h and 8 h (light illumination of 67 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 3 weeks.

2.2. PsJN Inoculation

The root tips of all seedlings were cut before PsJN inoculation to facilitate bacterial penetration [9]. Half of the seedlings were soaked in PsJN solution (0.5 of OD600) for 1 min and half of the seedlings (control) were treated with PBS buffer alone, then blot-dried with sterile paper towel, placed on switchgrass growth medium in Magenta boxes containing 50 ml of media and 6 seedlings per box. Magenta boxes were randomly placed in a growth chamber with a light-dark cycle of 16 h and 8 h (light illumination of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 24°C for 24 days.

2.3. Experimental measurements

Every 2 or 3 days, root surface area and leaf area were determined on one randomly selected Alamo seedling per magenta box by optical surface measurement with a flat-bed scanner and the program WinRhizo 5.0A software 212 (Regent Instruments, QC, Canada). Each seedling was rinsed and floated in trays filled with distilled water and these trays were scanned for image analysis. Roots and leaves were dried in an oven (48 h, 65 °C) and dry weight measured. Specific root length and root/shoot ratio were calculated based on these data.

Gas exchange was measured using a portable infrared gas analyzer (LI-6400 system, LI-COR Inc., Lincoln, NE, USA) under the following settings: 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD), 400 $\mu\text{mol mol}^{-1}$ reference CO_2 concentration, 25 °C block temperature, and a flow rate of 385 $\mu\text{mol sec}^{-1}$. Instantaneous water use efficiency was calculated as the photosynthetic rate/ transpiration rate. The small seedlings were gently removed from the magenta boxes, roots wrapped in wet tissue paper and gas exchange measured in just a few minutes. Initially water from the damp tissue paper influenced the water exchange data (leaf and stem surfaces inside the cuvette became damp) so the data from day 0-6 was excluded from the analysis of stomatal conductance, ci/ca and water use efficiency. Once seedlings were a bit larger, it was easy to avoid wetting leaves and only dry surfaces were in the cuvette.

2.4. Experimental design and Analysis

A completely randomized design with 6 replications with repeated measures was used in this experiment. Differences in biometric parameters between treatments were investigated using a T -test and ANOVA at $\alpha=0.1$. Both simple linear regression and polynomial regression were performed on physiological variables that are interdependent. Statistical analysis was performed with JMP Pro 10 (SAS Institute, Cary, NC).

3. Results

3.1. Growth response

Inoculation with *Burkholderia phytofirmans* strain PsJN significantly increased aboveground biomass and total biomass by day 17 (Figure. 1). Root biomass response lagged behind above ground growth but became significantly higher in PsJN plants on day 20 (Figure. 1). By the end of the study, above ground, root and total biomass were increased by 39.1%, 52.4% and 39.5% in Ps JN inoculated plants as compared to the controls (Figure. 1).

Figure. 1. Effect of *Burkholderia phytofirmans* Strain PsJN on aboveground biomass (A), root biomass (B) and total biomass (C) of switchgrass cv. Alamo. Each value is the mean of six plants. 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 and control using student T-test.

The response of leaf area and plant height to PsJN inoculation was similar to aboveground biomass (Figure. 2). Both leaf area and height were significantly increased and at the end of the study were 46% and 51% (Figure. 2) larger in PsJN inoculated plants, respectively.

Figure. 2. Effect of Burkholderia phytofirmans Strain PsJN on leaf area (A) and height (B) of switchgrass cv. Alamo. Each value is the mean of six plants. 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 and control using student T-test.

3.2. Root Length, Root Surface Area and Root average diameter

Root morphology variables were also altered upon PsJN inoculation (Figure. 3). Root length and surface area did not respond as rapidly to PsJN inoculation but by the end of the study was 32.6% and 74% larger in inoculated seedlings (Figure. 3). Root diameter was initially significantly lower in the PsJN inoculated seedlings; however, after day 6 this pattern reversed and by the end of the study root diameter was 64.5% greater in the PSJN inoculated seedlings (Figure. 3).

Figure. 3. Effect of Burkholderia phytofirmans Strain PsJN on root length (A), root surface area (B) and root average diameter (C) of switchgrass cv. Alamo. Each value is the mean of six plants. 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 and control using student T-test.

3.3. Biomass and morphology allocation

Allocation between aboveground biomass and root biomass was significantly increased by PsJN inoculation ($p=0.0041$, Figure. 4). A similar pattern was observed in switchgrass morphology (root length vs height, Figure. 4). For seedlings with same root length, PsJN inoculated seedlings were 21.7% taller ($p<0.0001$, Figure. 4). Specific leaf weight was also significantly increased ($p=0.0029$, Figure. 4) and specific root length decreased ($p=0.0158$, Figure. 4) by PsJN inoculation.

Figure. 4. Effect of *Burkholderia phytofirmans* Strain PsJN on aboveground and below ground biomass allocation (A), Shoot height/root length (B), specific leaf area (C) and specific root length (D), solid line indicates control and dashed line indicates PsJN inoculated Switchgrass

3.4. Leaf gas exchange

One day after inoculation, photosynthetic rates fell in both treatments likely due to the result of root tip removal. Photosynthetic rates in PsJN inoculated plants were reduced more and were significantly lower than the control plants ($p=0.0002$) at day 1 and photosynthetic rates did not fully recover until day 6 (Figure. 5). After day 17, PsJN inoculation significantly enhanced photosynthetic rates of switchgrass seedlings by 54% ($p=0.0016$, Figure. 5).

Figure. 5. Effect of *Burkholderia phytofirmans* strain PsJN on photosynthetic rate of switchgrass cv.

Alamo. Each value is the mean of six plants. 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 and control using student T-test.

Despite increased photosynthetic rates, stomatal conductance of PsJN inoculated seedlings was only significantly higher than controls on the last day of the experiment (56.9%, $p=0.0326$, Figure. 6).

Consistently lower c_i/c_a (-29.6%, $p=0.0222$, Figure. 6) and higher water use efficiency (37.7%, $p=0.0467$,

Figure. 6) were observed in PsJN inoculated switchgrass seedlings after day 11 and day 17 respectively. PsJN inoculation significantly changed the relationship of photosynthetic rate and stomatal conductance (Figure. 7, $p=0.0084$). Specifically photosynthetic rates were increased in PsJN inoculated plants at any given stomatal conductance ($p=0.0084$, Figure. 7).

Figure. 6. Effect of *Burkholderia phytofirmans* strain PsJN on stomatal conductance (A), C_i/C_a (B) and water use efficiency WUE(C) of switchgrass cv. Alamo. Each value is the mean of six plants. 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 and control using student T-test.

Figure. 7. Photosynthetic rates vs stomatal conductance for PsJN inoculated switchgrass seedlings and control switchgrass seedlings

4. Discussion

Our results are in agreement with previous results that PsJN inoculation increased both aboveground biomass, belowground biomass, plant height and shoot/root length of switchgrass [9]. Additionally, we discovered that as soon as 17 days after inoculation, significantly higher aboveground biomass and aboveground morphology (height and leaf area) were observed in PsJN inoculated switchgrass seedlings. Although higher below ground biomass and altered root morphology, such as longer root length [9, 23], higher root surface area and average root diameter were found in PsJN inoculated seedlings, it occurred 3-6 days later than aboveground responses, indicating enhanced allocation of biomass to aboveground plant parts due to PsJN. This is similar to potato seedling response to PsJN [24], which also allocated more biomass to aboveground plant parts. PsJN inoculation also increased specific leaf weight and decreased specific root length, indicating advanced development of aboveground and belowground tissue [19]. The differences between PsJN inoculated seedlings and control seedlings generally increased with time. The changes in root morphology confirm earlier work with potato plantlets [22]. Previous reports showed that PsJN inoculated plants possessed more developed tissues, such as thicker cell walls [15], larger-diameter xylem [15], more chlorophyll content [17, 19], and more root hairs [19]. And these developmental changes, which are probably attributable to the changes in hormones such as ethylene and IAA [20, 21], might lead to higher specific leaf weight and lower specific root length of PsJN inoculated switchgrass in this experiment.

We also found that PsJN inoculation increased photosynthetic rate just 17 days after inoculation. Similar results were also found in grapevine and maize, of which the photosynthetic rates were increased by 70.2% [15] and 50% [17] respectively due to PsJN inoculation. PsJN inoculation initially lowered the photosynthetic rate one day after inoculation. This is likely the result of endophyte induced plant systematic resistance (reviewed by [25, 26], and host defense reactions were also obtained in PsJN inoculated grapevines [16]. Changes in stomatal conductance [17] did not follow the same pattern as photosynthesis, resulting in significantly higher water use efficiency in PsJN inoculated switchgrass

1 seedlings at day 17. This suggests more developed photochemical machinery in PsJN inoculated
2 switchgrass seedlings. In accordance with water use efficiency, consistent lower C_i/C_a was found in
3 PsJN inoculated seedlings, implying higher percentage of stomatal resistance in PsJN inoculated
4 switchgrass. Analysis of photosynthetic rate and water use efficiency vs stomatal conductance confirmed
5 that even at the same stomatal conductance , PsJN inoculated switchgrass seedlings still had higher
6 photosynthetic rate and water use efficiency. Therefore, PsJN inoculation increased photosynthetic rate
7 not only by increased stomatal opening, but more importantly, through better developed photochemical
8 machinery and water use efficiency, agreeing with previous studies, which showed that PsJN inoculation
9 increased maximum photochemical efficiency and chlorophyll content in maize and *Arabidopsis* [17, 19].

5. Conclusion

This study showed that PsJN inoculation very rapidly increased biomass and plant elongation, both above and belowground. Allocation of biomass was also altered by PsJN inoculation, with more biomass allocated to aboveground plant parts. Furthermore, more developed leaves and roots were observed in PsJN inoculated seedlings having the same biomass as control seedlings. Photosynthetic rates were also increased by PsJN inoculation likely through more developed photochemical machinery, which increased water used efficiency. Most significant changes developed after day 17 and roots responded 3-6 days later than aboveground plant parts. Most of these PsJN effects increased with time.

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Figure captions

Figure. 1. Effect of *Burkholderia phytofirmans* Strain PsJN on aboveground biomass (A), root biomass (B) and total biomass (C) of switchgrass cv. Alamo. Each value is the mean of six plants. 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 and control using student T-test.

Figure. 2. Effect of *Burkholderia phytofirmans* Strain PsJN on leaf area (A) and height (B) of switchgrass cv. Alamo. Each value is the mean of six plants. 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 and control using student T-test.

Figure. 3. Effect of *Burkholderia phytofirmans* Strain PsJN on root length (A), root surface area (B) and root average diameter (C) of switchgrass cv. Alamo. Each value is the mean of six plants. 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 and control using student T-test.

Figure. 4. Effect of *Burkholderia phytofirmans* Strain PsJN on aboveground and below ground biomass allocation (A), Shoot height/root length (B), specific leaf area (C) and specific root length (D), solid line indicates control and dashed line indicates PsJN inoculated Switchgrass

Figure. 5. Effect of *Burkholderia phytofirmans* strain PsJN on photosynthetic rate of switchgrass cv. Alamo. Each value is the mean of six plants. 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 and control using student T-test.

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2 Figure. 6. Effect of Burkholderia phytofirmans strain PsJN on stomatal conductance (A), Ci/Ca (B) and
3 water use efficiency WUE(C) of switchgrass cv. Alamo. Each value is the mean of six plants. Error bars
4 are $\pm$ SE of the means. * means significant difference at $\alpha=0.1$ level and ** means significant difference at
5 $\alpha=0.01$ level between PsJN and control using student T-test.

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7 Figure. 7. Photosynthetic rates vs stomatal conductance for PsJN inoculated switchgrass seedlings and
8 control switchgrass seedlings

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