

***Burkholderia Phytofirmans* Strain PsJN Effects on Drought Resistance,  
Physiological Responses and Growth of Switchgrass**

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*Panicum virgatum*, advanced development, drought resistance

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## **ABSTRACT**

To decrease dependency of fossil fuels and avoid direct competition with food crops, massive research efforts are investigating next-generation cellulose biofuel crops such as switchgrass (*Panicum virgatum*). A low-input, sustainable switchgrass production could be achieved by reducing traditional management practices though applying plant growth promoting rhizobacteria (PGPR), of which our understanding is still rather limited.

To elucidate physiological mechanisms behind PGPR's beneficial effects, we inoculated switchgrass seedlings with *Burkholderia phytofirmans* strain PsJN. Two experiments were conducted to determine the initial and long-term responses of switchgrass to PsJN inoculation by tracking growth and leaf physiology. In a third experiments, we tested the effects of PsJN on growth and leaf-level physiology of switchgrass under a moderate pre-drought conditioning and a successive severe drought stress.

PsJN inoculation increased biomass and promoted elongation of shoots within 17 days following inoculation. 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 ( $p=0.0158$ ) and higher specific leaf weight ( $p=0.0029$ ) were also observed in PsJN inoculated seedlings, indicating advanced development. Photosynthetic rates (Ps) were higher in PsJN inoculated seedlings after 17 days (54%,  $p=0.0016$ ), which were related to higher stomatal conductance, greater water use efficiency, and lower non-stomatal limitation of Ps.

These rapid changes in leaf physiology are at least partially responsible for switchgrass growth enhancement from PsJN treatment. The early growth enhancement in PsJN inoculated switchgrass linearly decreased with plant age. PsJN inoculation increased Ps of upper canopy leaves by 13.6% but reduced Ps of lower canopy leaves by 8.2%. Accelerated leaf senescence and early flowering were observed in PsJN-inoculated switchgrass, which might contribute to slightly lower aboveground biomass at final harvesting. Drought preconditioning increased Ps of PsJN-inoculated switchgrass during a later severe drought; whereas, control switchgrass only benefited from drought preconditioning when leaf water potential dropped below -1 MPa.

This study verified early growth enhancement and accelerated development of switchgrass due to PsJN inoculation. Rapid improvement in leaf physiology is related to enhanced productivity. PsJN inoculation also improve drought tolerance of switchgrass.

## Dedication

To my father

who encouraged me to pursue graduate study across the Pacific Ocean

gave me the deepest love in the world

Have a good rest in heaven

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# Chapter 1 Introduction

## 1.1. Justification

Human society's consumption of primary energy has tripled during the last four decades, with heavy reliance on fossil energy, which constituted 86.8% of overall primary energy (12.48 billion tons oil equivalent) consumed in 2012 (Petroleum 2013). The finite and environmentally unfriendly nature of fossil energy leads to the growing interests in developing renewable and sustainable bioenergy. There were 60.22 million tons oil equivalent biofuel produced in 2012, while the renewable energy consumption reached 237.4 million tons oil equivalent (Petroleum 2013). The Billion Ton report suggested that 30% of current petroleum consumption could be replaced by biofuels by 2030 (Perlack et al. 2005).

The existing biofuel production (first generation biofuels) is mainly based on food crops such as corn, wheat, sugarcane, canola, sunflower and rapeseed (Solomon 2010, Ajanovic 2011). In 2011, 10,368 million gallons of ethanol was produced in the U.S. and 99.9% of the feedstock used for ethanol production was corn. This first-generation biofuel production had a modest contribution (3%-30%) of the food price increase in 2008 (Mueller et al. 2011). In order to avoid the competition between food and fuels, second generation biofuels (e.g. cellulosic ethanol) will be based on non-food residual biomass or non-food crops (Karp and Richter 2011, Mueller et al. 2011). The U.S. Energy Independence and Security Acts (EISA) mandated the use of  $6.06 \times 10^{10}$  advanced biofuels, primarily cellulosic ethanol, by 2022 (Haque and Epplin 2012). In the updated Billion Ton report, Perlack et al. proposed 16 billion gallons per year (BGY) of cellulosic biofuel production and 4 BGY advanced biofuel production by 2022 (Perlack and Stockes 2011).

Today, switchgrass (*Panicum virgatum* L.) is considered one of most promising bioenergy crops in the US because it is capable of maintaining a relatively high growth rate with relatively low water and nutrient requirements. Marginal lands which are usually too nutrient poor and dry to economically produce food crops may be suitable for cultivating switchgrass, reducing the competition between biofuel and food crop production (Wright and Turhollow 2010). Further, switchgrass is a deep rooted perennial grass species with potential for increasing soil carbon sequestration and minimizing soil erosion (Sanderson et al. 1996, Hartman et al. 2011). Furthermore, switchgrass cultivation benefits marginal and degraded land conservation by improving soil quality, increasing water infiltration with permanent cover, capturing nutrients and improving wildlife habit (Parrish and Fike 2005, Hartman et al. 2011). Due to these advantages, this lignocellulosic crop will likely be a large component of an economically profitable and environmentally friendly biofuel industry in North America. Improving stress tolerance for planting switchgrass on marginal lands would help prevent direct competition with food crops for fertile land.

*Burkholderia phytofirmans* strain PsJN is a well-studied endophyte that can improve production and enhance temperature stress tolerance of grapevines and potato plants (Bensalim et al. 1998, Barka et al. 2006, Fernandez et al. 2012b). However, the physiological mechanisms of PsJN's effect on switchgrass growth and drought tolerance are unclear. Studying the relevant plant physiological changes with PsJN inoculation would build a solid foundation in developing a low input and sustainable switchgrass production system.

## 1.2. Literature Review

### 1.2.1. A “Model Biofuel Crop”-Switchgrass

Switchgrass is a perennial warm-season, C4 grass adapted across large geographical areas of North America from northern Mexico to southern Canada (McLaughlin and Walsh 1998b). Its economic value in soil conservation, prairie restoration, and as forage has long been recognized (Porter 1966, Casler et al. 2007). Switchgrass was selected as a ‘model’ biofuel crop through a plant screening trial from 1980s to 1990s by the U.S. Oak Ridge National Laboratory(ORNL) (Wright and Turhollow 2010). In seven independent herbaceous crop screening trials of ORNL’s Biofuels Feedstock Development Program, six studies recommended switchgrass as the next generation biofuel crop (Wright and Turhollow 2010). Switchgrass did not achieve highest biomass production in every single trial, but it maintained relatively high, reliable production over various site conditions.

There are several studies on factors that influence switchgrass productivity and stress tolerance including germplasm ( cultivars, ecotypes, latitudinal and longitudinal origin) (Sanderson et al. 1999, Casler et al. 2004, Casler et al. 2007) and management (fertilization, tillage and harvesting systems) (Muir et al. 2001, Berdahl et al. 2005, Fike et al. 2006, Guretzky et al. 2011). Switchgrass production varies among cultivars which are broadly grouped into ‘upland’ and ‘lowland’ ecotypes or “cytotypes”(Porter 1966). Upland ecotypes occur in upland areas and typically are of more northern origin; lowland ecotypes occur on flood plains and lowlands and more are of southern origin (Vogel 2004). In general lowland switchgrass cultivars have a later heading date and are taller with larger and thicker stems compared with upland plants (Parrish and Fike 2009). Lowland ecotypes are more productive and resistant to moisture stress compared to upland ecotypes (Barney et al. 2009). All lowland ecotypes are tetraploids,

while upland ecotypes can be tetraploids, hexaploids or octaploids (Hultquist et al. 1997). Further, the latitude of origin of switchgrass is negatively correlated with its productivity (Sanderson et al. 1999). Southern-origin ecotypes (mainly lowland ecotypes) are characterized by later maturity, more rapid stem elongation rate, longer retention of photosynthetic tissue, and high biomass yield potential; conversely, northern-origin ecotypes (mainly upland ecotypes) exhibit increased cold tolerance, which contribute to higher survival rate and sustained biomass yields at more northern locations (Casler et al. 2004). Longitudinal effects also have been observed with mesic-adapted material not as productive in humid environs and vice versa (Hopkins et al. 1995, Casler and Boe 2003). Casler et al.(2007) suggested that switchgrass used for biofuel production should not be moved more than one hardiness zone north or south from their origin, but some can be moved east or west of their origin(Casler et al. 2007).

### 1.2.2. Switchgrass Production and Management

Successful establishment of switchgrass seedlings and appropriate follow up management are crucial for economical biofuel crop production. Competition from weeds in the first seedling year very often causes stand failure. Avoiding N fertilization to switchgrass during the first year helps switchgrass compete with weeds and facilitates its establishment (Parrish and Fike 2009). Following the first year, spraying herbicides is only needed every five years or ten years after establishment(Mitchell et al. 2010).

Additionally, in order to maintain long-term productivity, the nitrogen removed in the biomass need to be returned to the soil by fertility amendment (Fike et al. 2006). Although the best N application rates varied among different studies, consensus was reached on the effect of harvesting frequency and time on the N removal: a two-cut system removes more N than a one-



cut system; summer harvest removes more N than the fall harvest when plants have senesced (Reynolds et al. 2000, Thomason et al. 2004, Parrish and Fike 2005). The production and transportation of petrochemical-derived fertilizers and pesticides rely on fossil energy, which add extra economic cost and energy consumption to current switchgrass production. Low-input, sustainable, alternatives are required to reduce input costs and maintain or increase yields (Farrar et al. 2014).

High productivity of switchgrass with limited nutrient inputs is suggestive of biological nitrogen fixation (Parrish and Fike 2005). Bacterial endophytes were recovered from switchgrass (*Panicum virgatum* L.) with demonstrated capacity to influence switchgrass growth (Ker et al. 2012, Xia et al. 2013). Switchgrass is largely undomesticated, thereby opening to opportunity to harness beneficial plant–microbe relationships that may have been lost through intensive crop breeding (Farrar et al. 2014). Using beneficial bacterial endophytes and other microbial symbionts may be an economic and environmentally viable way to improve switchgrass productivity as well as enhance stress resistance (Parrish and Fike 2009, Mei and Flinn 2010), but studies on interactions between switchgrass and potentially beneficial symbiotic microbial communities are rare (Brejda et al. 1998, Ker et al. 2012, Kim et al. 2012). Hetrick et al. (1988) suggested that switchgrass co-evolved with mycorrhizal fungi and as the species migrated north from its tropical origins it continues to depend fungi to maintain its production (Hetrick et al. 1988). Highly diversified endophytic fungi and bacteria were also found to be indigenously associated with switchgrass (Ghimire and Craven 2011, Kleczewski et al. 2012, Gagne-Bourgue et al. 2013). Eighteen orders of endophytic fungi were found in plants in Oklahoma (Ghimire et al. 2011); eleven orders of endophytic fungi were found in shoot tissue in the Midwest USA (Kleczewski et al. 2012); thirty one endophytic bacteria were found in plants

in Canada (Gagne-Bourgue et al. 2013). Switchgrass seedlings inoculated with its rhizosphere microflora exhibited enhanced nutrient uptake (N and P) as well as increased aboveground and belowground biomass compared with control seedlings (Brejda et al. 1998). One eighth of the endophytic fungi in shoot tissue significantly improved switchgrass biomass production (Kleczewski et al. 2012) and nutrient uptake (Hetrick et al. 1990) likely through mobilizing mineral nutrients (Koslowsky and Boener 1989), helping switchgrass to withstand acid soil induced stress (Clark et al. 1999) and other unclear mechanisms. Several indigenous bacterial strains promote switchgrass growth through mechanisms such as nutrient solubilization (e.g., inorganic P) as well as by excreting phytohormones and toxic lipopeptides (which have antimicrobial activities) (Gagne-Bourgue et al. 2013). Ker et al. (2012) isolated nitrogen fixing bacteria and other plant growth promoting rhizobacteria (PGPR) capable of mobilizing P and/or producing auxins, from switchgrass rhizomes that were grown in stands without N fertilization for ten years (Ker et al. 2012). This PGPR-inoculum increased switchgrass growth in a growth chamber without N input and under field conditions with low N input (Ker et al. 2012). Exogenous symbiotic fungi and bacteria also enhance productivity and drought stress tolerance of switchgrass (Ghimire and Craven 2011, Kim et al. 2012).

Clearly, symbiotic associations with mycorrhizae or rhizobacteria improve switchgrass establishment, reduce chemical fertilizer input and enhance stress tolerance. However, economic analyses to determine the value of fungal and bacterial symbionts have not been reported. Estimated breakeven price of ethanol from switchgrass ranges from \$ 0.44-\$0.72/L based on several studies, which included the cost of land rent, biomass production, biomass harvesting, storage and transportation (Haque and Epplin 2012). Utilizing PGPR may be a promising way of reducing these costs.

### 1.2.3. The effects of *Burkholderia phytofirmans* strain PsJN on crops

*Burkholderia phytofirmans* strain PsJN, which was isolated from *Glomus vesiculiferum* infected onion roots (Frommel et al. 1991, Sessitsch et al. 2005), is a gram negative, rod shaped, non-sporulating, motile bacterium. It can colonize the rhizosphere (Bordiec et al. 2011) and various organs (Compant et al. 2008, Compant et al. 2011) of many plant species. It was visualized colonizing the root surface, endorhiza and inflorescence stalks, pedicels and immature berries of grapevine, but was not inside flower buds and flowers (Fernandez et al. 2012a). PsJN enhances root and shoot growth of potatoes and grapevines plantlets (Bensalim et al. 1998, Barka et al. 2006, Fernandez et al. 2012a). Bacterization also enhances leaf hair formation, secondary root branching, and total lignin content (Bensalim et al. 1998). Photosynthetic capacity of grapevine seedlings was also changed by PsJN inoculation (Barka et al. 2006, Fernandez et al. 2012a). However, Barka et al. (2006) found PsJN inoculation enhanced photosynthetic activity of grapevine seedlings at 26 °C while Fernandez et al. (2012) reported that PsJN inoculation lowered photosynthetic rate of grapevine seedlings by 25% at 26 °C. Mechanisms related to these growth enhancement effects of PsJN include producing quinolinatephosphoribosyltransferase (QAPRTase), which may play a role in the signal pathway for promotion of plant growth (Wang et al. 2006, Hardoim et al. 2008) and down regulating plant ethylene levels by secreting 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Hardoim et al. 2008). A PsJN strain deficient in ACC deaminase synthesis was unable to promote root elongation of canola seedlings (Sun et al. 2009).

PsJN also significantly improved potato and grapevine performance under extreme temperature stress through a series of biochemical and physiological changes (Bensalim et al. 1998, Barka et al. 2006, Fernandez et al. 2012a, Fernandez et al. 2012b). Potatoes inoculated

with PsJN had greater tuber number and tuber weight under heat stress (Bensalim et al. 1998); PsJN inoculation also enhanced root growth and plantlet biomass at 4 °C by 10.7 fold and 2.2 fold, respectively (Barka et al. 2006). Bacterized grapevine plantlets displayed a carbohydrate balance favorable to cold tolerance (Fernandez et al. 2012a, Fernandez et al. 2012b) and had significantly enhanced levels of starch, proline and phenolics, which are correlated to enhanced cold tolerance. In addition, PsJN inoculation helps plants to defend against the grey mold diseases by inducing systemic resistance (Bordiec et al. 2011). After addition to grapevine cell culture suspensions, PsJN induced a transient and monophasic extra cellular alkalization but no accumulation of reactive oxygen species; some defense genes upregulated by PsJN challenge also participated in salicylic acid and jasmonic acid signaling pathways (Bordiec et al. 2011). The complete genome sequence of PsJN exhibited existing aminocyclopropane-1-carboxylate (ACC) deaminase gene and IAA synthesis pathway (Weilharter et al. 2011). However the genome does not indicate nitrogen fixation activity or antibiotic production (Weilharter et al. 2011).

Although the growth promoting effects of PsJN on young seedlings of many species are well documented, the physiological mechanisms under which PsJN improves productivity are unclear. Furthermore, it is not known if these growth promoting effects persist in mature plants since most work focused on young seedlings. In addition, PsJN's beneficial effect is genotype-specific. It increased the switchgrass biomass production of the lowland cultivar 'Alamo' by 50%, but no promotive effects on the upland cultivar 'Cave-in- Rock' was observed (Kim et al. 2012). This inconsistency hampers PsJN's application in the field. Understanding these mechanisms could help unravel why the beneficial effects of PsJN are inconsistent among different switchgrass cultivars. PsJN has been found to improve temperature stress tolerance in

potato and grapevine (Bensalim et al. 1998, Barka et al. 2006, Fernandez et al. 2012a); it may also improve switchgrass drought tolerance through a similar mechanism. This hypothesis seems plausible since many stress responses share the same pathway and mechanisms. Furthermore, many other ACC deaminase-containing plant growth-promoting bacteria enhanced plant growth under drought stress, salt stress and flooding stress (Glick 2004). Identifying these mechanisms would not only further our understanding of the interaction between switchgrass and PsJN, but could also have practical significance towards building a low input and sustainable switchgrass production system.

### 1.3. Objectives

The purpose of this study was to examine the effects of PsJN inoculation on switchgrass growth, physiology and drought tolerance. Finding the physiological explanations for enhanced growth will help to more efficiently utilize this bacterium in production systems. We expect higher photosynthetic rates in PsJN-inoculated switchgrass possibly resulting from changes in water use efficiency and stomatal conductance. We also expect better drought tolerance and faster recovery after rehydration of switchgrass with PsJN. Osmotic adjustment, stomatal conductance and water use efficiency might play a role in this process.

Our specific objectives were to: (1) examine the effects of PsJN inoculation on young switchgrass growth and development; (2) determine the physiological mechanisms related to advanced PsJN growth and development effects; and (3) evaluate the influence of PsJN inoculation on drought tolerance of switchgrass.

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## Chapter 2 Early growth promotion and leaf level physiology changes in *Burkholderia phytofirmans* strain PsJN-inoculated switchgrass

### 2.1. Abstract

Switchgrass 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 experiment demonstrated that *Burkholderia phytofirmans* strain PsJN, a well-studied plant growth promoting rhizo-bacterium (PGPR) significantly increased both aboveground and belowground biomass 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 morphological 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.

### 2.2. 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 (Petroleum 2013). 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 (Solomon 2010), which constituted 1.9% of the total primary consumption in 2012 (Petroleum 2013). The US government has suggested 30% displacement of present petroleum consumption with biofuels, requiring 910 million tonnes of biomass (Downing et al. 2011). 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 (Wright and Turhollow 2010).

Endophytic bacteria are known to promote growth through increasing available nutrients, physiological changes and hormone regulations (Mei and Flinn 2010). In addition, endophytic bacteria also provide environmental and economic benefits to sustainable biofuel crop systems by reducing fertilizer and pesticide use (Mehnaz and Lazarovits 2006, Mei and Flinn 2010, Bakker et al. 2012, Weekley et al. 2012). The use of endophytic bacteria as ‘bio-fertilizer’ has been studied in switchgrass (Ker et al. 2012, Kim et al. 2012) and thirty-one endophytic bacteria were found to be naturally associated with switchgrass in Canada (Gagne-Bourgue et al. 2013). Inoculating switchgrass with endophytic bacteria increased its tiller number and tiller height, which contributes to higher yield than uninoculated plants (Ker et al. 2012, Kim et al. 2012). *Burkholderia phytofirmans* PsJN, an endophytic bacterial strain originally isolated from onion roots (Pillay and Nowak 1997), significantly promoted growth of potato (Nowak et al. 2007, Da et al. 2012), grapevine (Compant et al. 2005b, Barka et al. 2006), maize (Naveed et al. 2014), *Arabidopsis thaliana* (Josefina Poupin et al. 2013, Poupin et al. 2013) and switchgrass (Kim et al. 2012). PsJN’s growth promotion effects have also been found to be genotype dependent (Kim et al. 2012).

A few studies have investigated the physiological mechanisms behind the growth promotion effects, including lowering ethylene level (Sun et al. 2009), producing and regulating IAA (Zuniga et al. 2013), accelerating development (Poupin et al. 2013), increasing chlorophyll content (Poupin et al. 2013, Naveed et al. 2014) and photosynthetic rate (Barka et al. 2006, Naveed et al. 2014) as well as altering root morphology and vascular tissue (Frommel et al. 1991, Nowak et al. 1998). However, none of these physiological mechanisms has been studied in switchgrass. Further, most studies only measured variables at the end of experiment and ignored developmental changes over time. Therefore, we investigated the biomass allocation, morphology change, leaf gas exchange and instantaneous water use efficiency at 9 different time points from the day of inoculation to the 24<sup>th</sup> day after inoculation. Our specific objectives were to investigate in switchgrass: 1) the early PsJN induced physiological changes that may lead to growth changes and 2) the growth, morphology and allocation changes induced by PsJN inoculation.

## 2.3. Methods

### 2.3.1. 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% H<sub>2</sub>SO<sub>4</sub> 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.

### 2.3.2. PsJN Inoculation

The root tips of all seedlings were cut before PsJN inoculation to facilitate bacterial penetration (Kim et al. 2012). Half of the seedlings were soaked in PsJN suspension (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.

### 2.3.3. 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<sub>2</sub> 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,  $c_i/c_a$  (non-stomatal limitation) 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.

#### 2.3.4. 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).

### 2.4. Results

#### 2.4.1. Growth response

Inoculation with *Burkholderia phytofirmans* strain PsJN significantly increased aboveground biomass and total biomass by day 17 (Figure 2.1). Root biomass response lagged behind above ground growth but became significantly higher in PsJN plants on day 20. By the end of the study, above ground, root and total biomass were increased by 76.6%, 55.5% and 72.0 % in Ps JN inoculated plants as compared to the controls (Figure 2.1).

The response of leaf area and plant height to PsJN inoculation was similar to aboveground biomass (Figure 2.2). Both leaf area and height were significantly increased and at the end of the study were 46% and 51% larger in PsJN-inoculated plants, respectively.

#### 2.4.2. Root Length, Root Surface Area and Root average diameter

Root morphology variables were also altered upon PsJN inoculation (Figure 2.3). Root length and surface area did not respond as rapidly to PsJN inoculation but by the end of the study were 32.6% and 74% larger in inoculated seedlings. 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 2.3).

#### 2.4.3. Biomass and morphology allocation

Allocation between aboveground biomass and root biomass was significantly increased by PsJN inoculation ( $p=0.0041$ , Figure 2.4). A similar pattern was observed in switchgrass morphology (root length vs height). For seedlings with the same root length, PsJN-inoculated seedlings were 21.7% taller ( $p<0.0001$ ). Specific leaf weight was also significantly increased ( $p=0.0029$ ) and specific root length decreased ( $p=0.0158$ ) by PsJN inoculation.

#### 2.4.4. 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 2.5). From day 17, PsJN inoculation significantly enhanced photosynthetic rates of switchgrass seedlings by 54% ( $p=0.0016$ ).

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 2.6). Consistently lower  $c_i/c_a$  (-29.6%,  $p=0.0222$ ) and higher water use efficiency (37.7%,  $p=0.0467$ ) were observed in PsJN-inoculated switchgrass seedlings from day 11 and day 17 respectively. PsJN inoculation significantly changed the relationship of

photosynthetic rate and stomatal conductance (Figure 2.7,  $p=0.0084$ ). Photosynthetic rates increased with higher stomatal conductance in PsJN-inoculated plants ( $R^2=32.3\%$ ,  $p=0.0009$ ) while no correlation was found between photosynthetic rates and stomatal conductance in control plants ( $R^2=0.001$ ,  $p=n.s.$ ).

## 2.5. Discussion

Our results are in agreement with previous studies, demonstrating that PsJN inoculation increased both aboveground biomass, belowground biomass, plant height and shoot/root length of switchgrass (Kim et al. 2012). Additionally, we discovered that 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, altered root morphology (such as longer root length (Frommel et al. 1991, Kim et al. 2012)), higher root surface area and average root diameter were found in PsJN-inoculated seedlings, these changes occurred 3-6 days later than the aboveground responses, indicating enhanced allocation of biomass to aboveground plant parts due to PsJN. This is similar to potato seedling response to PsJN (Bensalim et al. 1998), 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 (Poupin et al. 2013). The differences between PsJN-inoculated seedlings and control seedlings generally increased with time. The changes in root morphology confirm earlier work with potato plantlets (Nowak et al. 1998). Previous reports showed that PsJN-inoculated plants possessed more developed tissues, such as thicker cell walls (Barka et al. 2006), larger-diameter xylem (Barka et al. 2006), more chlorophyll content (Poupin et al. 2013, Naveed et al. 2014), and more root hairs (Poupin et al. 2013). These developmental changes, which are probably

attributable to the changes in hormones such as ethylene and IAA (Sun et al. 2009, Zuniga et al. 2013), might have led to the higher specific leaf weight and lower specific root length of PsJN-inoculated switchgrass in this experiment.

PsJN inoculation also 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% (Barka et al. 2006) and 50% (Naveed et al. 2014) 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 (Glick et al. 2007, Loon 2007), and host defense reactions were also obtained in PsJN-inoculated grapevines (Compant et al. 2005b). Changes in stomatal conductance (Naveed et al. 2014) did not follow the same pattern as photosynthesis, resulting in significantly higher water use efficiency in PsJN-inoculated switchgrass seedlings at day 17. This suggests more developed photochemical machinery in PsJN-inoculated switchgrass seedlings. In accordance with water use efficiency, consistent lower  $C_i/C_a$  was found in PsJN-inoculated seedlings, implying higher stomatal resistance in PsJN-inoculated switchgrass. Analysis of photosynthetic rate and water use efficiency vs stomatal conductance confirmed that even at the same stomatal conductance. PsJN-inoculated switchgrass seedlings still had higher photosynthetic rate and water use efficiency. Therefore, PsJN inoculation increased photosynthetic rate not only by increased stomatal opening, but more importantly, through better developed photochemical machinery and water use efficiency, agreeing with previous studies, which showed that PsJN inoculation increased maximum photochemical efficiency and chlorophyll content in maize and *Arabidopsis* (Poupin et al. 2013, Naveed et al. 2014).

## 2.6. 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.

Figures/Tables

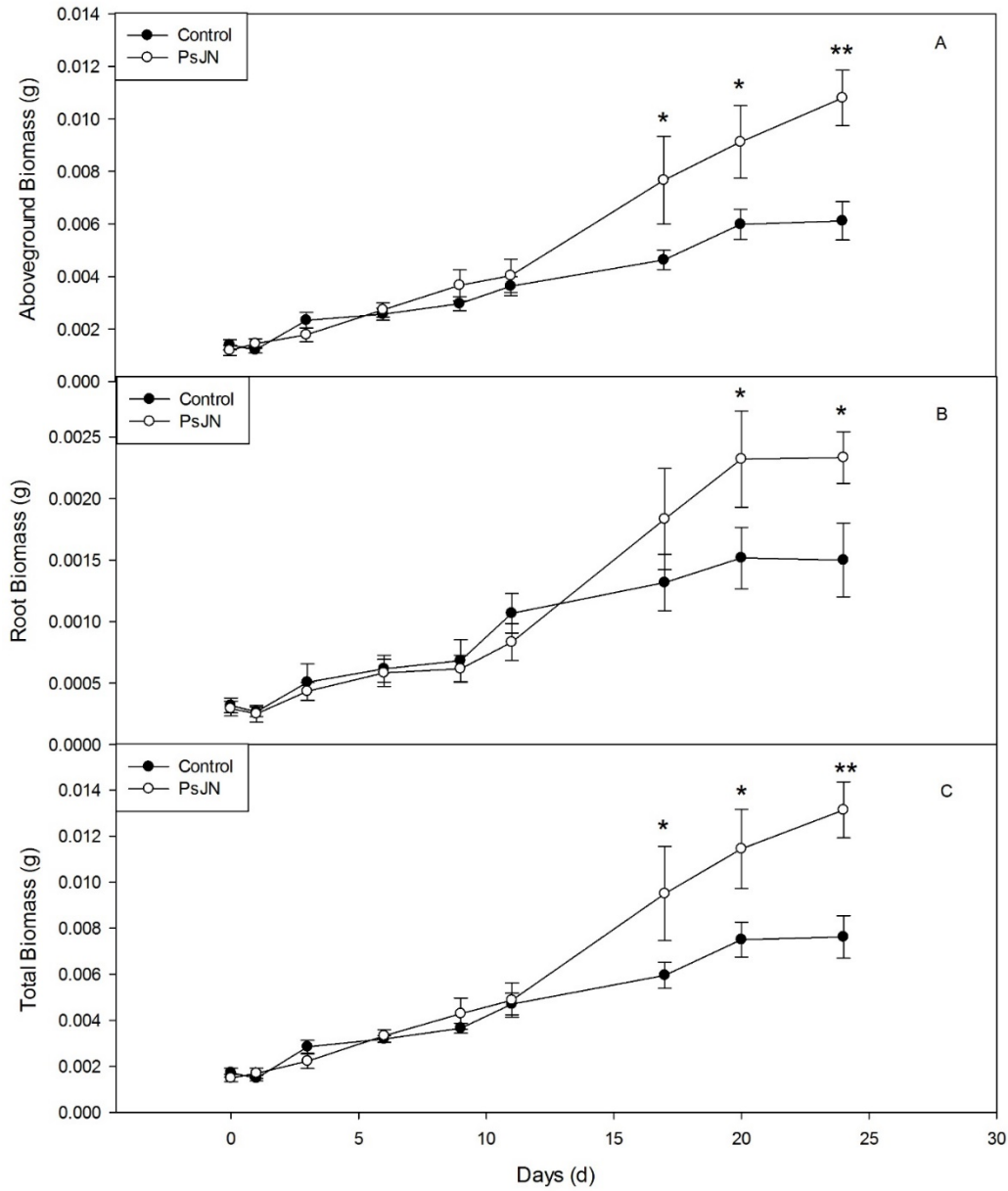


Figure 2.1 Effect of *Burkholderia phytofirmans* strain PsJN on aboveground biomass (A), root biomass (B) and total biomass (C) of switchgrass cv. Alamo. Each value is the mean of six plants. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ; \*\* $p < 0.01$ ).

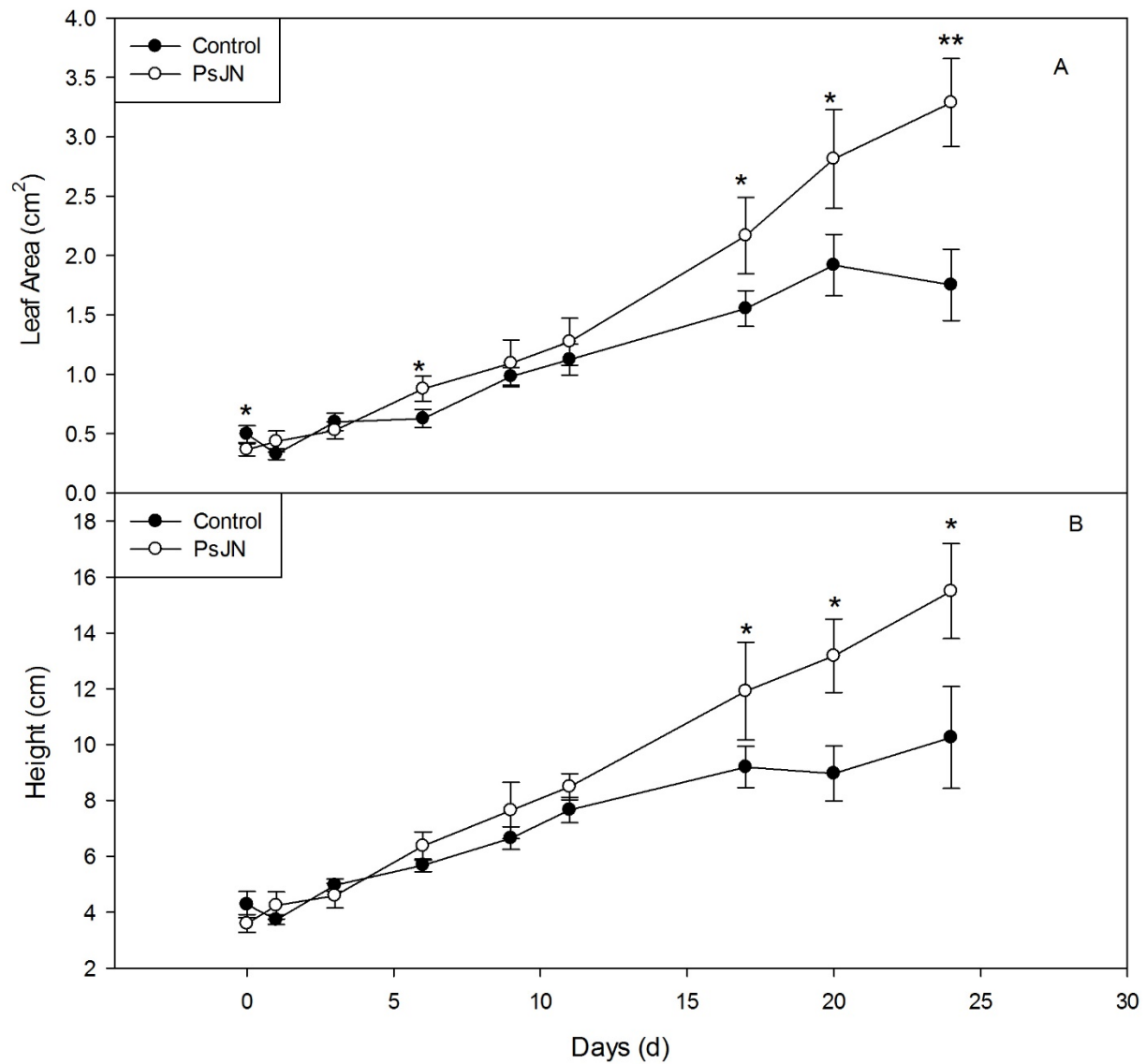


Figure 2.2 Effect of *Burkholderia phytofirmans* strain PsJN on leaf area (A) and height (B) of switchgrass cv. Alamo. Each value is the mean of six plants. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ; \*\* $p < 0.01$ ).



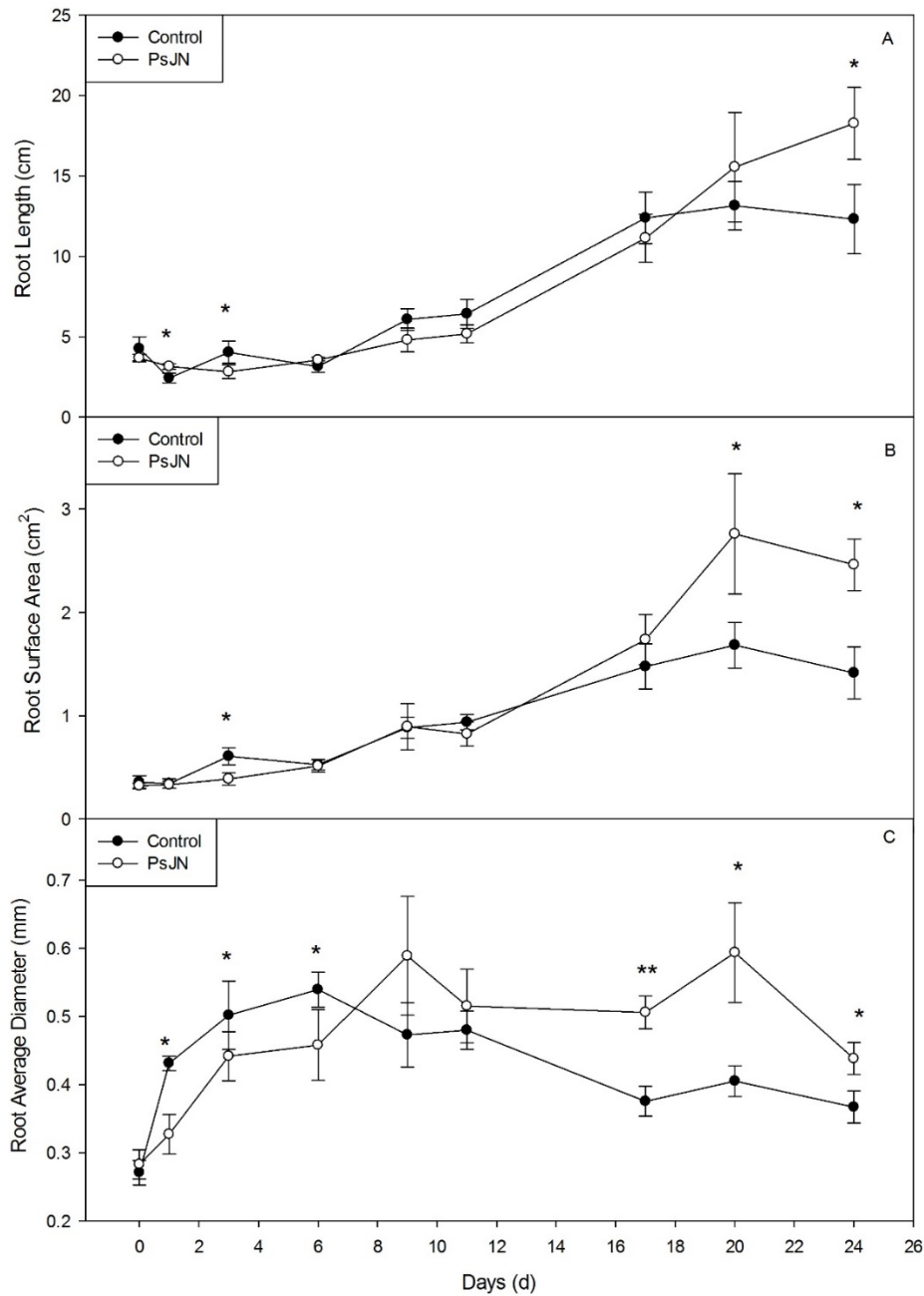


Figure 2.3 Effect of *Burkholderia phytofirmans* strain PsJN on root length (A), root surface area (B) and root average diameter (C) of switchgrass cv. Alamo. Each value is the mean of six plants. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ; \*\* $p < 0.01$ ).

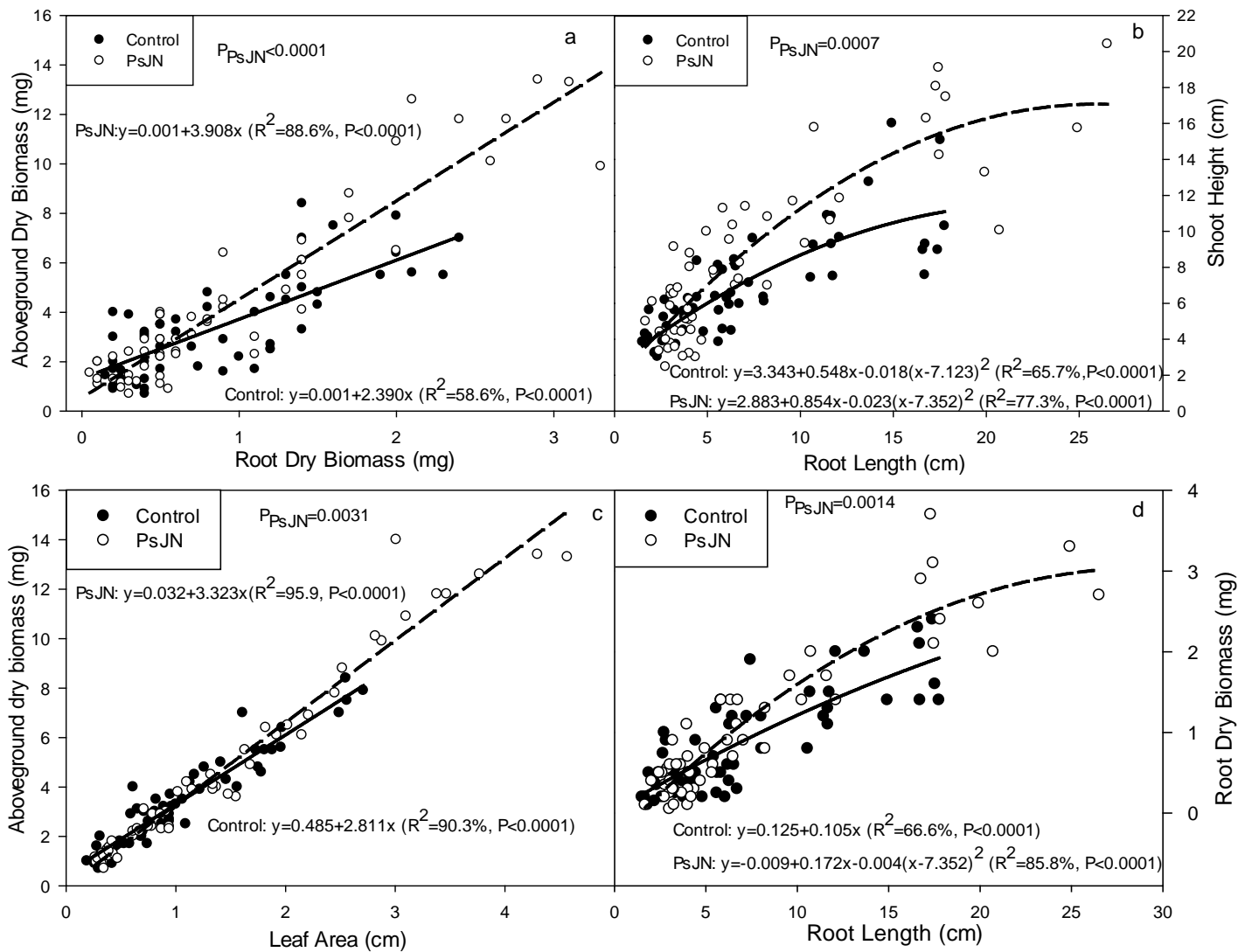


Figure 2.4 Effect of *Burkholderia phytofirmans* strain PsJN on aboveground and below ground biomass allocation (A), Shoot height/root length (B), specific leaf area (C) and specific root length (D), closed circles and solid lines indicate control, open circles and dashed lines indicates PsJN-inoculated Switchgrass.  $P_{PsJN}<0.1$  indicate significantly change in slope due to PsJN inoculation

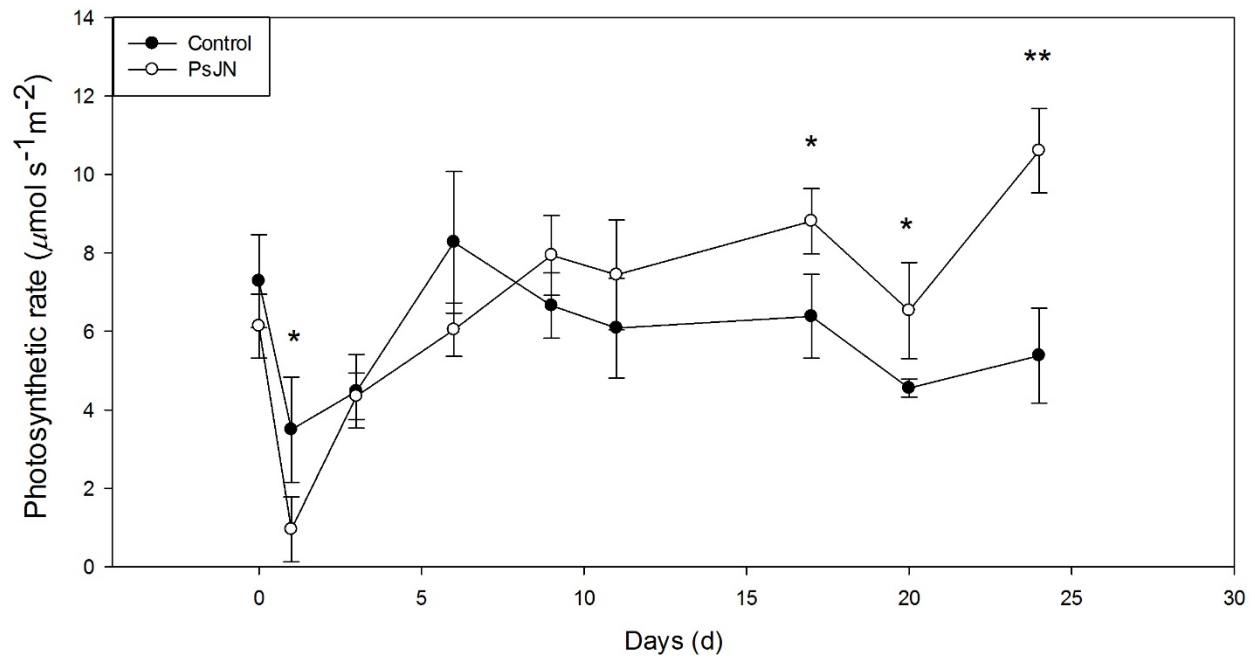


Figure 2.5 Effect of *Burkholderia phytofirmans* strain PsJN on photosynthetic rate of switchgrass cv. Alamo. Each value is the mean of six plants. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ; \*\* $p < 0.01$ ).

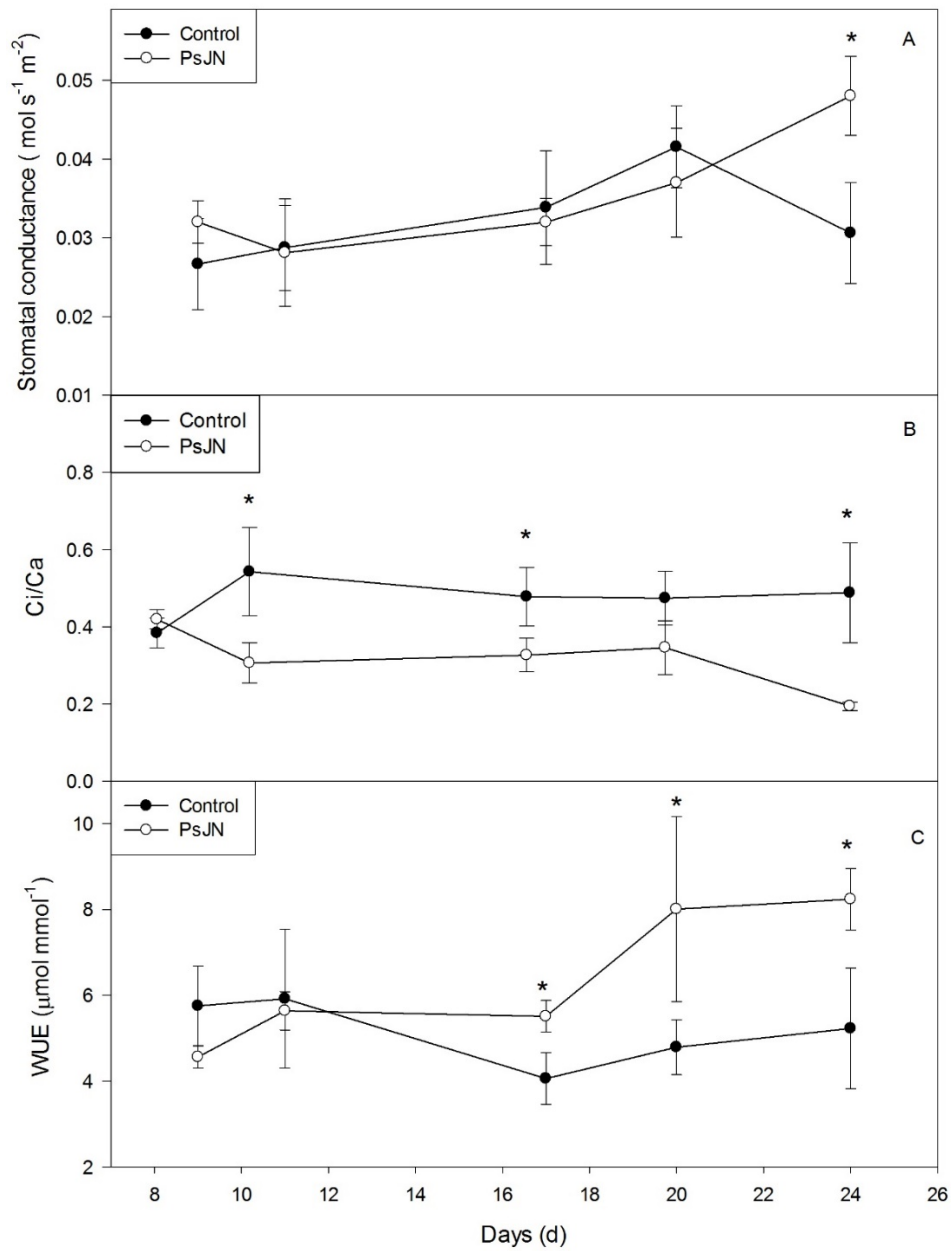


Figure 2.6 Effect of *Burkholderia phytofirmans* strain PsJN on stomatal conductance (A), Ci/Ca (B) and water use efficiency WUE(C) of switchgrass cv. Alamo. Each value is the mean of six plants. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ).

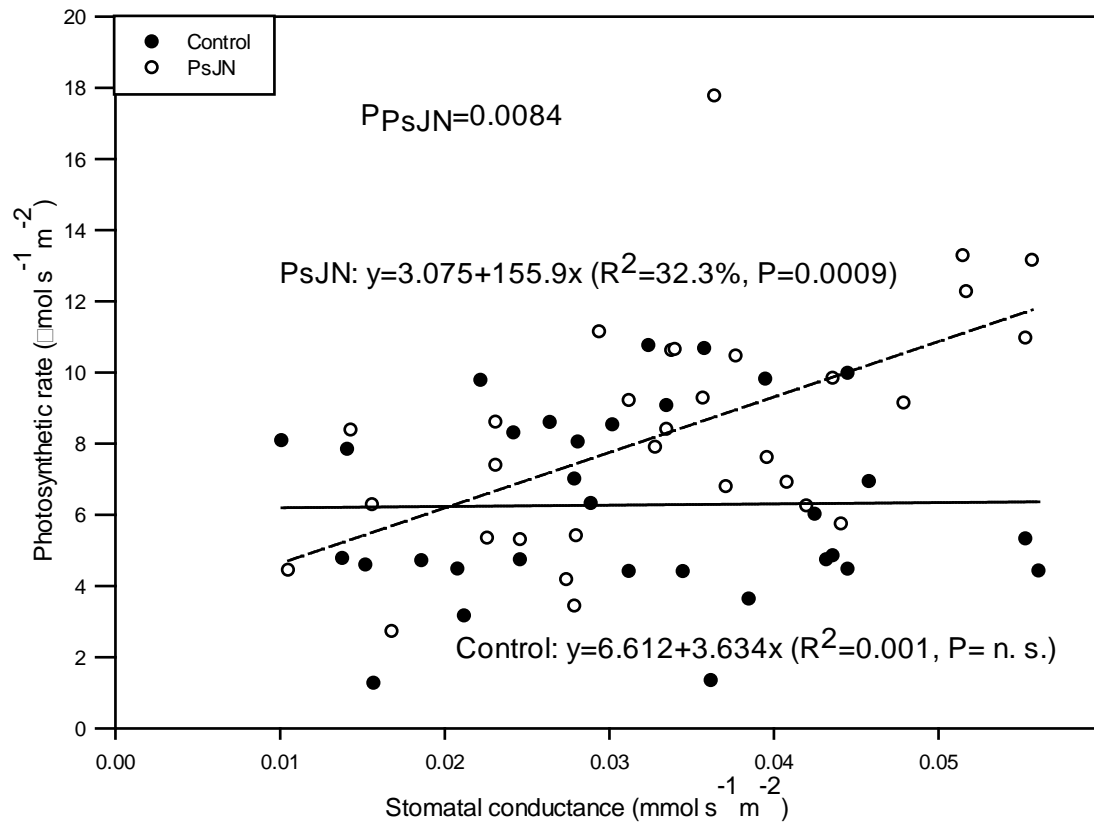


Figure 2.7 Photosynthetic rates vs stomatal conductance for PsJN-inoculated switchgrass seedlings and control switchgrass seedlings, open circles and dashed lines indicates PsJN-inoculated Switchgrass.  $P_{\text{PsJN}} < 0.1$  indicate significantly change in slope due to PsJN inoculation

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

### 3.1. Abstract

Plant growth promoting rhizobacteria (PGPR), have been reported to increase the productivity of switchgrass (*Panicum virgatum* L.), a model biofuel crop. 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 over 18 weeks on both the upper-canopy leaves and lower-canopy leaves of switchgrass inoculated with *Burkholderia phytofirmans* strain PsJN. We also tracked changes in growth using a non-destructive photographic method. PsJN inoculation significantly enhanced early growth of switchgrass by up to 125.2% ( $p=0.0011$ ). However, this beneficial effect linearly decreased with plant age. PsJN inoculation increased photosynthetic rates of upper-canopy leaves by 13.6%. Nevertheless photosynthetic rates of lower-canopy leaves were decreased by 8.2%. 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.

### 3.2. 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. (Wright and Turhollow 2010). 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 (McLaughlin and Walsh 1998a). 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 C<sub>4</sub> photosynthetic pathway and extensive root system (McLaughlin et al. 1999). Marginal lands unable to economically produce food crops may be suitable for cultivating switchgrass, avoiding the 'food or fuel' dilemma (Graham-Rowe 2011). Finally, switchgrass is a deep rooted perennial grass species with potential for sequestering carbon, reducing soil erosion, capturing nutrients and improving wildlife habitat (Sanderson et al. 1996, Parrish and Fike 2005, Hartman et al. 2011). The fact that switchgrass was naturally associated with highly diversified fungi (Kleczewski et al. 2012) and bacteria (Gagne-Bourgue et al. 2013) may play a role in the adaptation of switchgrass. Nevertheless, knowledge of the symbiotic relationships between switchgrass and microbes is rather limited (Ker et al. 2012), affecting the utilization of symbiotic microbes as 'bio-fertilizers' (Vessey 2003).

Plant growth promoting rhizobacteria (PGPR) are known as natural 'bio-fertilizers' working through mechanisms such as: (1) nitrogen fixation, (2) manipulating phytohormones, (3) solubilizing nutrients, and (4) producing siderophore and/or antibiotics (reviewed by Loon 2007). Nevertheless, most of these studies investigated short-term effects of rhizobacteria (Boddey 1995, Barka et al. 2006, Kim et al. 2012, Naveed et al. 2014). How these bacteria benefit plant growth through an entire life cycle has not been extensively studied. PGPR in *Arabidopsis* has been shown to accelerate flowering time (Poupin et al. 2013) and change emissions of volatile organic compounds (Xie et al. 2009).

PsJN is a well-studied PGPR capable of enhancing the production of plants such as Arabidopsis, tomato, potato, grape, maize and switchgrass (Bensalim et al. 1998, Sharma and Nowak 1998, Barka et al. 2006, Fernandez et al. 2012b, Kim et al. 2012, Poupin et al. 2013, Zuniga et al. 2013, Naveed et al. 2014). PsJN reduced host ethylene level by excreting 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick et al. 2007), which was necessary for its growth promotion effects (Sun et al. 2009). In addition, PsJN possessed both production pathways (Weilharter et al. 2011) and degradation pathways (Zuniga et al. 2013) of IAA, which interplays with the plant ethylene pathway (Zuniga et al. 2013). Plants associated with PsJN displayed higher chlorophyll contents (Poupin et al. 2013, Naveed et al. 2014), photosynthetic rates (Barka et al. 2006, Naveed et al. 2014), siderophore (Sun et al. 2009) and phenolic compounds (Barka et al. 2006). PsJN has also modified plant morphology such as longer root systems, stronger stems, more root hairs, secondary roots and lignin deposits on vascular bundles (Frommel et al. 1991, Nowak et al. 1998).

PGPR's long term effects on plants have not been studied in plant other than Arabidopsis (Xie et al. 2009, Bresson et al. 2013, Poupin et al. 2013). Although several studies have reported enhanced yields and establishment of switchgrass due to microbial association (Brejda et al. 1998, Ghimire et al. 2009, Ker et al. 2012, Kim et al. 2012, Lowman et al. 2014), physiological changes associated with PsJN inoculation have not been studied. Furthermore, leaf level physiology is known to vary with leaf age and position (Constable and Rawson 1980, Kitajima et al. 2002), and previous gas exchange measurements on switchgrass were all on upper-canopy leaves measured for short periods of time (Wullschleger et al. 1996, Dohleman et al. 2009). Rhizobacteria are known to influence plant development (Bresson et al. 2013, Poupin et al. 2013) 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.

### 3.3. Methods

#### 3.3.1. Plant material and seed sterilization

Switchgrass (*Panicum virgatum* L.) cv. Alamo seeds were surface-sterilized by treatment with 70% ethanol for 2 min, followed by rinsing 3X with distilled water, immersed in 60% H<sub>2</sub>SO<sub>4</sub> with stirring for 30 min to de-husk, and then washed 3X with distilled water, sterilized with 0.4 M sodium hypochlorite containing 0.1% Triton 100 for 30 min followed by a 5X rinse with sterile, deionized, distilled water.

#### 3.3.2. PsJN inoculation and plant growth conditions

Seeds were germinated on 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. Then half of these seedlings were soaked in PsJN suspension (Kim et al. 2012) for 1 min while the other half were soaked in PBS for 1 min as a control. These treated seedlings were dried with a sterilized paper towel and planted in magenta boxes (76 mm× 76 mm×102 mm, Sigma-Aldrich) filled with 50 ml of Murashige and Skoog Basal Salt Mixture and vitamin, 30 g/l maltose and 3 g/l phytogel at pH 5.8. Then they were incubated at the same condition as before for 2 weeks. After that they were transferred to ten 4-gallon pots filled with a 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) and kept in a greenhouse at 25 °C with a 16-h photoperiod.

### 3.3.3. Leaf area and biomass measurements

Five pots were planted with PsJN-inoculated Alamo seedlings and five pots were planted with uninoculated control seedlings, with five seedlings in each pot. Leaf area was estimated every week for 15 weeks using a digital photograph (Canon IXUS 60) taken from above the pot with Photoshop CS5 to quantify (Adobe Systems Inc.). In each photo, a known area reference square was placed at the height of the majority of the leaf area. Relative growth rate of the leaf area was calculated using the following equation:

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

Where:

t1=time one (in days), t2=time two (in days), LA1= leaf area of plant at time one (in cm<sup>2</sup>)

LA2= leaf area of plant at time two (in cm<sup>2</sup>)

Photosynthetic rates ( $A$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), transpiration rates ( $E$ ,  $\text{mmol m}^{-2}\text{s}^{-1}$ ), stomatal conductance ( $g$ ,  $\text{mmol m}^{-2} \text{s}^{-1}$ ) and water use efficiency ( $\text{WUE}=\text{AE}^{-1}$ ,  $\mu\text{molCO}_2\text{mmol}^{-1} \text{H}_2\text{O}$ ) were measured after 4 weeks of growth with a LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) at  $3000 \mu\text{mols m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD),  $400 \mu\text{mol mol}^{-1}$  reference  $\text{CO}_2$  concentration,  $25 \text{ }^\circ\text{C}$  block temperature, and a flow rate of  $385 \mu\text{mol sec}^{-1}$ . One fully expanded leaf was chosen from each pot and measured repetitively each week for 8 weeks. These leaves were referred to as lower-canopy leaves. Lower-canopy leaves were considered senesced when leaves turned yellow and had negative photosynthetic rates. The percentage of plants in each treatment with senesced leaves was recorded at each measurement date. After 5 weeks, a new fully expanded leaf was chosen from each pot and measured repetitively until the 13<sup>th</sup> week. At week 14, another set of newly expanded leaves was chosen and measured weekly

because many of the previously-chosen leaves had senesced. These leaves were referred to as upper-canopy leaves. After 18 weeks, the aboveground biomass of all plants was harvested and immediately dried in an oven at 65 °C for 48 h. The percent change of all the biometric variables was defined as  $(V_{\text{PsJN}} - V_{\text{Control}}) * 100 / V_{\text{Control}}$  (V indicates variable).

#### 3.3.4. Experimental design and statistical analysis

A completely randomized design was used in this experiment with two treatments (PsJN-inoculated 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)

### 3.4. Results

#### 3.4.1. Leaf area and relative growth rate

During the first two months of growth, PsJN-inoculated plants had significantly increased leaf area (86.4% -125.2% greater) compared to controls (Figure 3.1). 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% (Figure 3.1). 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 significantly higher relative growth rates at 77 days old ( $p=0.0577$ ) and 98 days old ( $p=0.0911$ , Figure 3.1).

### 3.4.2. Leaf physiology

PsJN-inoculated plants had significantly greater photosynthetic rates, stomatal conductance and transpiration rates in upper-canopy leaves (13.6%, 15.7% and 13.1%, respectively, Figure 3.2). Lower-canopy leaves in PsJN-inoculated plants, however, had decreased photosynthetic rates, stomatal conductance and transpiration rates (8.2%, 6.5%, and 10.1%, 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 ( $R^2 = 87.9\%$ ,  $p < 0.0001$ ).

Leaf level physiology of lower-canopy leaves was significantly influenced by leaf age ( $p < 0.0001$ , Table 3.1, Figure 3.3). 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 (Table 3.1). 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. After that, lower photosynthetic rates were observed in the lower-canopy leaves of PsJN-inoculated plants at day 70, day 84, day 91 and day 98 (Figure 3.3).

Photosynthetic rates, stomatal conductance, and transpiration rates of upper-canopy leaves were not affected by plant age (Table 3.1). PsJN inoculation significantly enhanced photosynthetic rates at day 112, day 126, and day 154 (Figure 3.4). 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.



### 3.4.3. Flowering time, lower canopy leaf death and final harvest

PsJN-inoculated plants flowered earlier than control plants (Figure 3.5). Similarly, PsJN-inoculated switchgrass also had more leaf death than the control group (Figure 3.5), indicating early senescence of old leaves. At the final harvest, PsJN inoculation decreased the vegetative biomass (leaf and stem) by 14.4% ( $p=0.0568$ , Table 3.2) and the total aboveground biomass by 13.7% ( $p=0.0868$ , Table 3.2). 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 3.6). Nevertheless, PsJN-inoculated plants allocated more biomass to reproductive parts (flower) as compared to control plants ( $p=0.0639$ , Figure 3.6).

## 3.5. Discussion

PsJN inoculation initially (younger than 70 days old) enhanced growth of switchgrass Alamo plants. This is in agreement with the report that PsJN-inoculated Alamo seedlings had significantly higher shoot biomass (Kim et al. 2012). Similar results were also obtained in *Arabidopsis* (Poupin et al. 2013), potato (Nowak et al. 1998), grapevine (Barka et al. 2006) and maize (Naveed et al. 2014) in early ontogeny. As a perennial grass, switchgrass establishment is challenged by annual weed competition (Parrish and Fike 2009), and this early growth enhancement could help switchgrass to outcompete weeds during plant establishment and improve field productivity (Lowman et al. 2014).

Most of these reported PsJN studies did not go beyond juvenile stages of development. In one study with *Arabidopsis* (Poupin et al. 2013), 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* (Poupin et al. 2013). Although both PsJN-inoculated switchgrass and control switchgrass had comparable flower and seed biomass at final harvest, 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. Earlier senescence was observed in lower-canopy leaves of PsJN-inoculated plants, indicated by faster decline of photosynthetic rates and earlier leaf death. 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* (Poupin et al. 2013). In our study, earlier flowering was also found in PsJN-inoculated switchgrass, which shortened the vegetative growth and might lead to lower vegetative biomass. Nevertheless, shortened vegetative growth might help more productive cultivars of southern types to move north which otherwise stay vegetative too late to survive winter (Vogel 2004). PsJN enhanced chilling tolerance of grapevine plantlets and potato. If this mechanism also works in switchgrass, PsJN will further facilitate the adaptation of southern cultivar in northern regions. Early flowering and early senescence may transfer more nutrients to belowground, which may help sustainability in biomass production in a long term. Increased allocation to root biomass may also result in lower aboveground biomass (Rogers et al. 2012); which may need to study in the future.

There were numerous reports on altered photosynthetic rate due to leaf ontogeny and leaf position in various plants (Jewiss and Woledge 1967, Constable and Rawson 1980, Rawson et al. 1983, Kitajima et al. 2002, Escudero and Mediavilla 2003, Rogers et al. 2012). 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 (Rawson et al. 1983). Nevertheless, previous studies on switchgrass only measured photosynthetic rates on upper-canopy leaves once (Ku et al. 1978, Wullschleger et al. 1996, Dohleman et al. 2009), which ignored the influence of leaf ontogeny

and leaf position. In our study, we monitored gas exchange of both upper-canopy leaves and lower-canopy leaves for 14 weeks and 8 weeks, respectively, discovering that 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 in 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 (Barka et al. 2006) and maize (Naveed et al. 2014) which also expressed 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 (Jewiss and Woledge 1967, Constable and Rawson 1980, Rawson et al. 1983, Kitajima et al. 2002, Escudero and Mediavilla 2003). 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 (Kikuzawa 1991). 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 (Poupin et al. 2013).

### 3.6. Conclusion

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 under our greenhouse conditions. However, PsJN promoted earlier growth which could be advantageous for switchgrass seedling establishment. Furthermore, a shortened life cycle could allow more productive southern switchgrass cultivars to be used further north where the growth season is shorter.

Figures/Tables

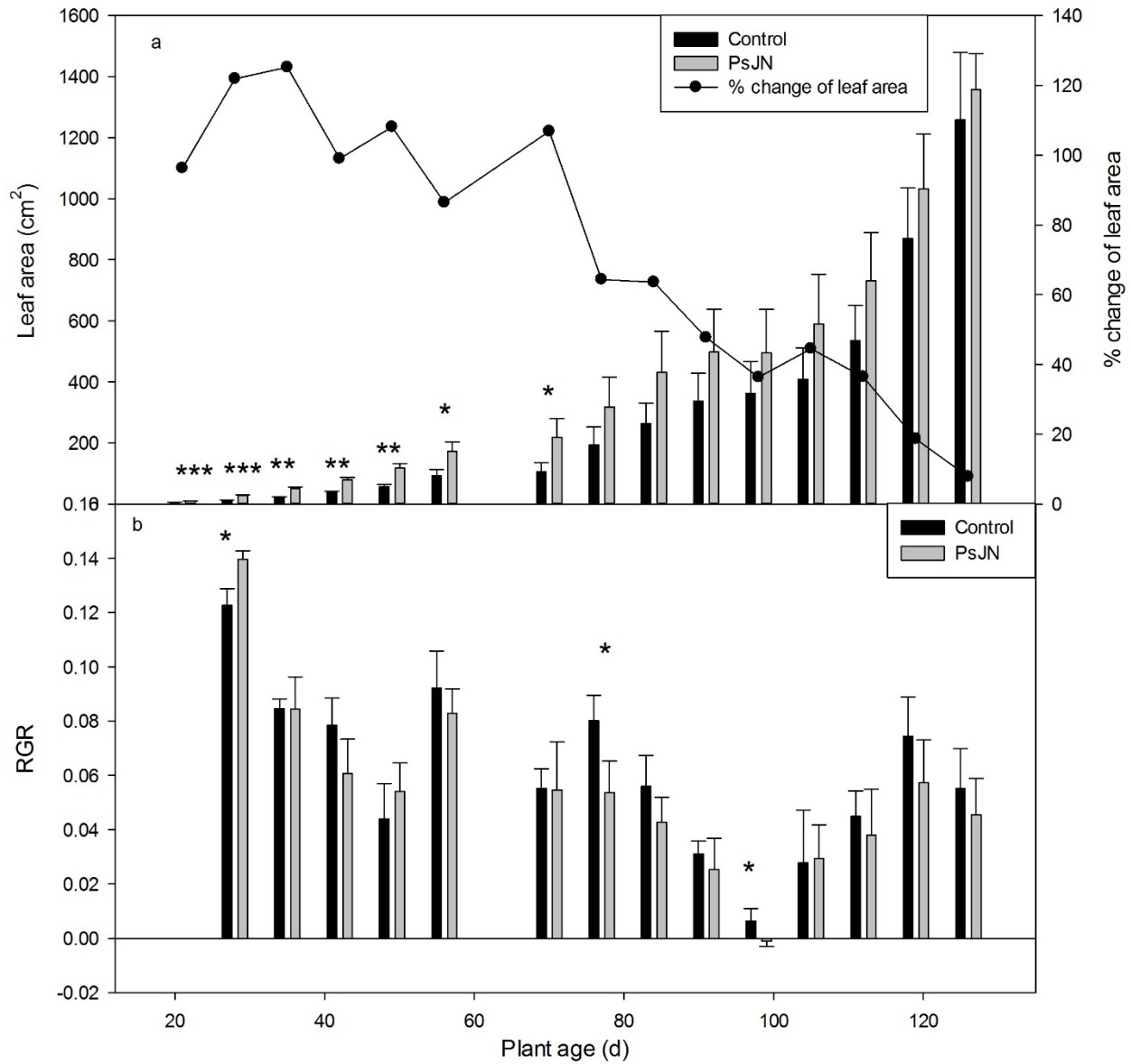


Figure 3.1 Effect of PsJN inoculation on leaf area and relative growth rate (RGR) of inoculated and non-inoculated switchgrass seedlings. Each value is the mean of five plants. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

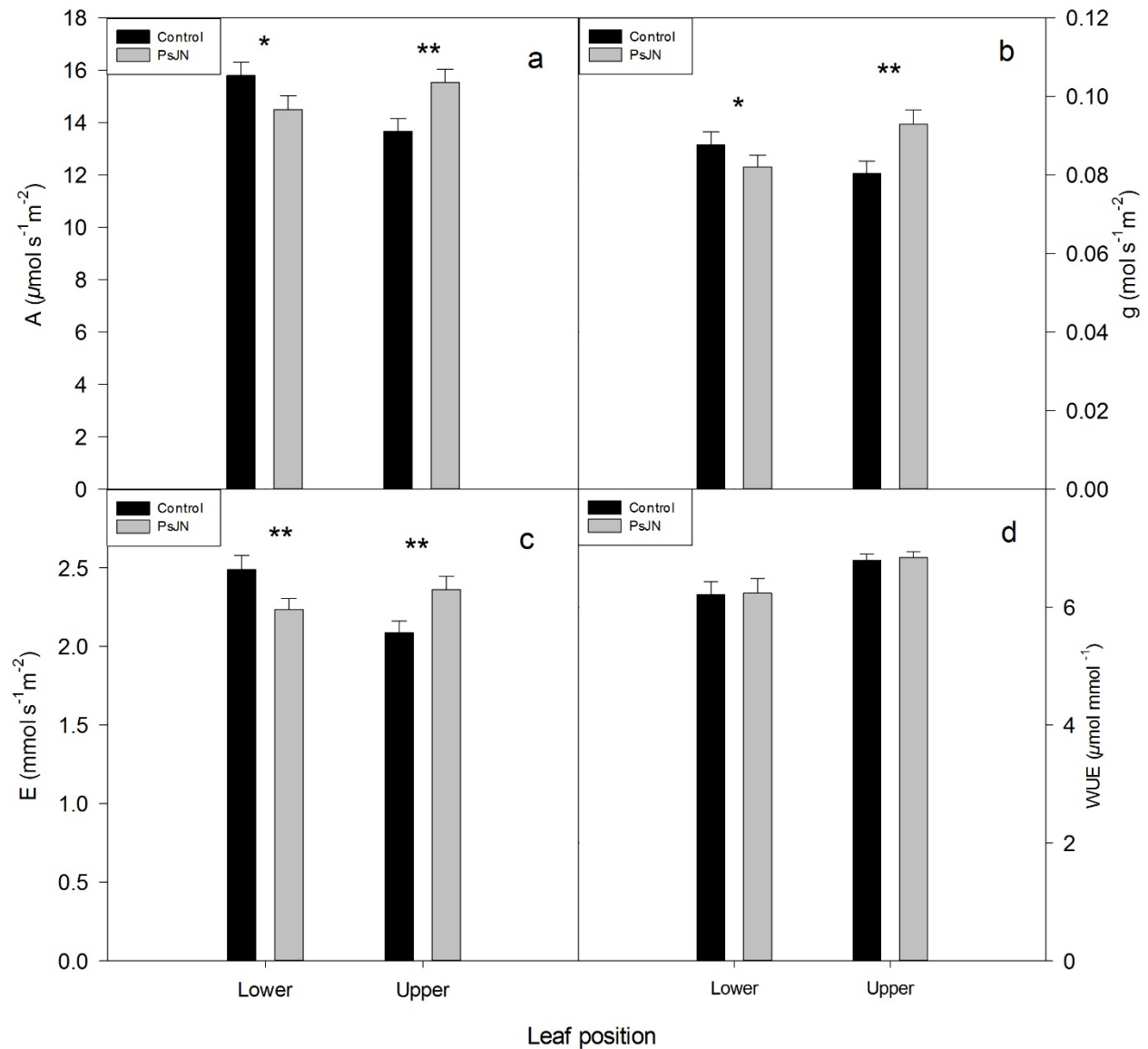


Figure 3.2 Effect of PsJN inoculation on physiological variables for lower-canopy leaves and upper-canopy leaves of switchgrass A, photosynthetic rate, E, transpiration, g, stomatal conductance, WUE, water-use efficiency. Each value is the mean of 40 measurements (lower-canopy leaves) or 70 measurements (upper-canopy leaves) pooled together. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control using student T-test (\* $p < 0.1$ ; \*\* $p < 0.01$ ).

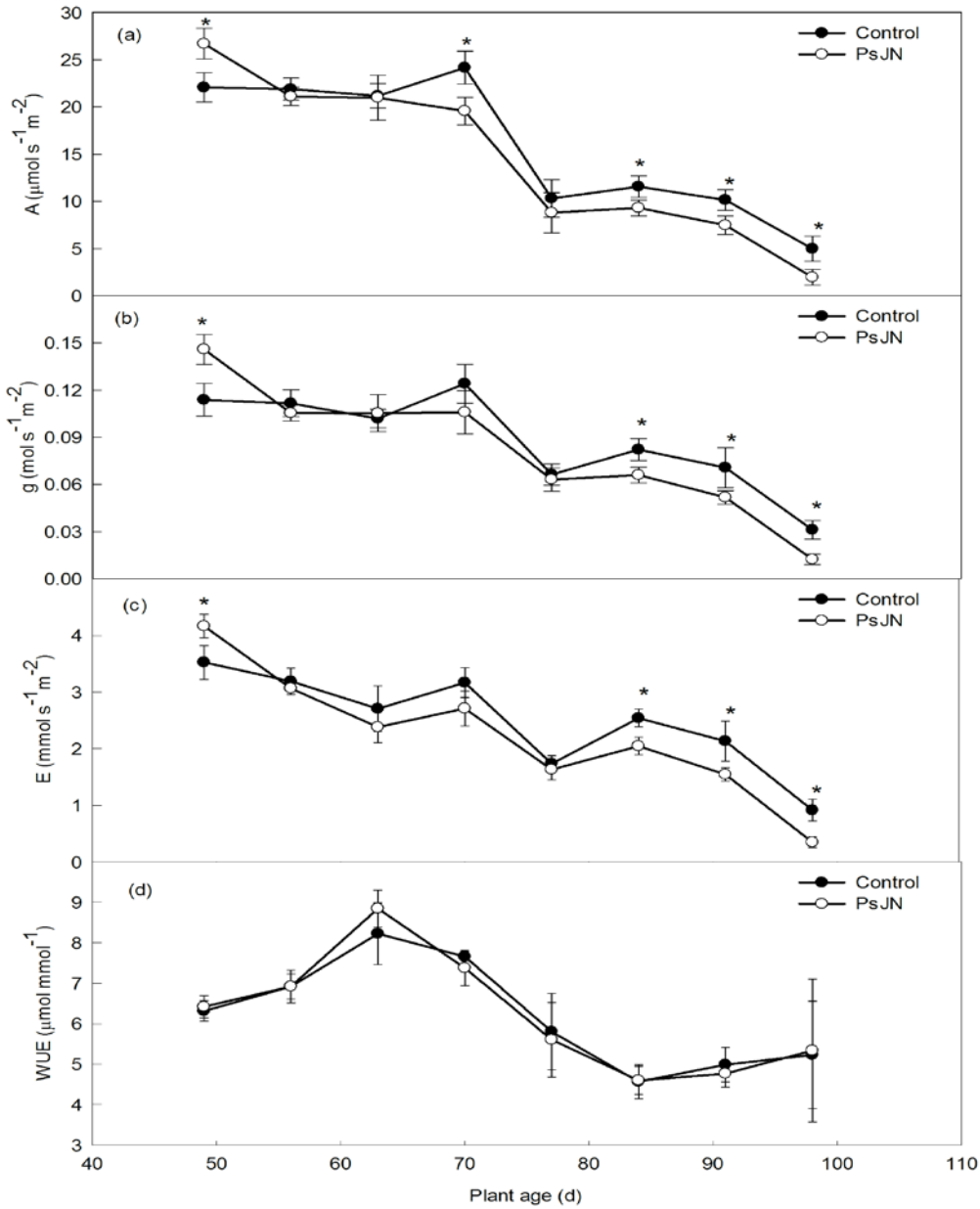


Figure 3.3 Leaf physiological variables of lower-canopy leaves in PsJN-inoculated and control switchgrass seedlings. Each value is the mean of five plants. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ). A, photosynthesis, E, transpiration, g, stomatal conductance, WUE, water-use efficiency

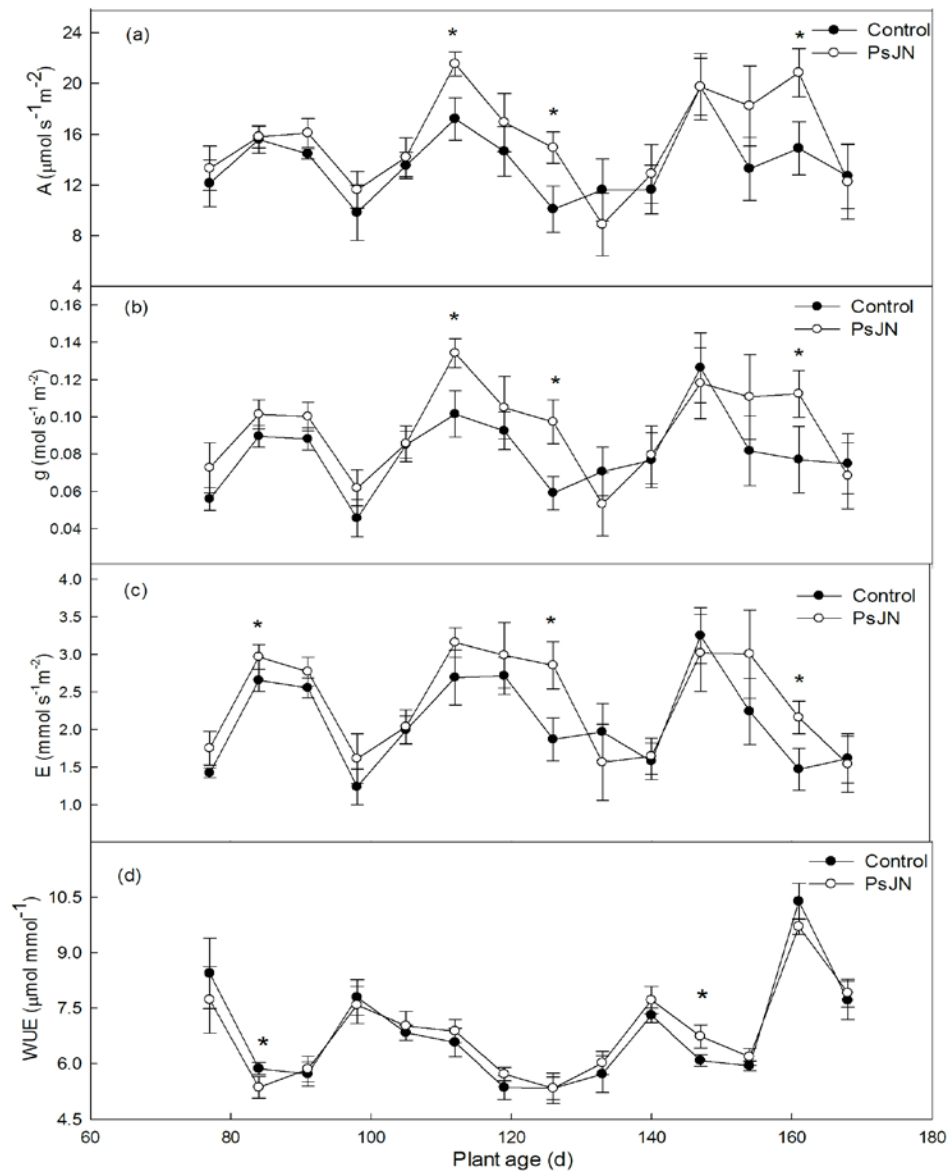


Figure 3.4 Leaf physiological variables of upper-canopy leaves in PsJN-inoculated and control switchgrass seedlings. Each value is the mean of five plants. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ). A, photosynthesis, E, transpiration, g, stomatal conductance, WUE, water-use efficiency



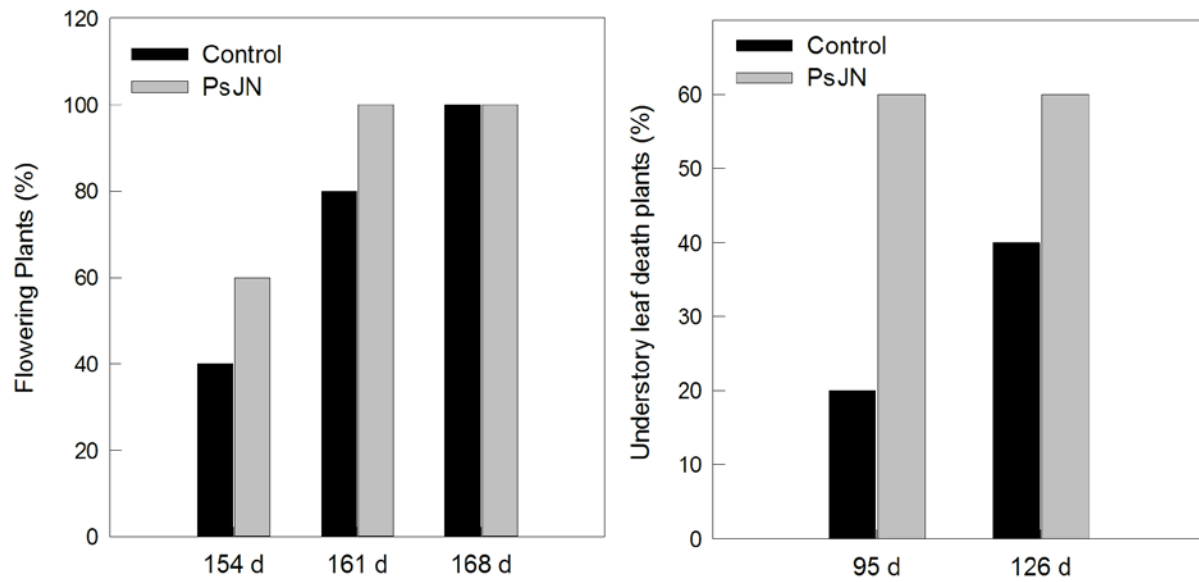


Figure 3.5 Flowering plants (%) and lower canopy leaf death (%) of PsJN-inoculated switchgrass and Control

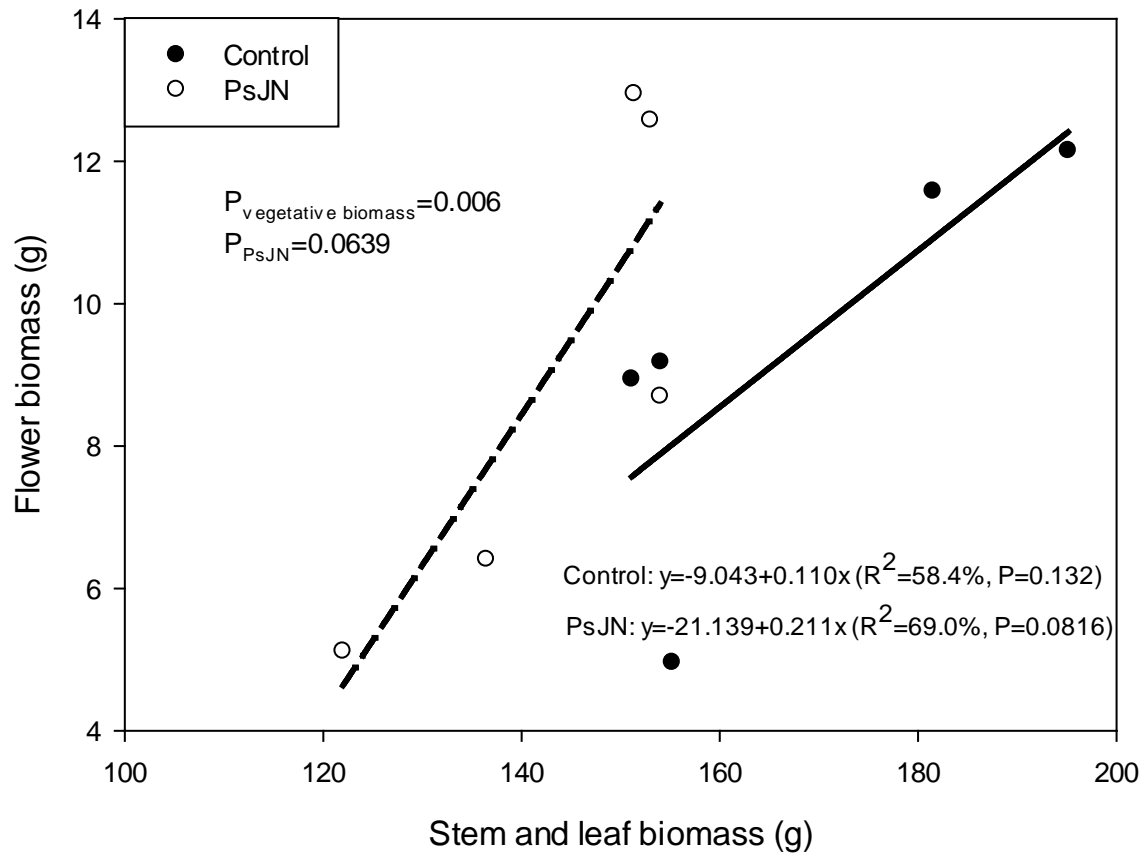


Figure 3.6 Flower biomass (g) vs stem and leaf biomass (g) at final harvest as influenced by PsJN inoculation. Open circles and dashed lines indicates PsJN-inoculated Switchgrass.  $P_{\text{PsJN}} < 0.1$  indicate significantly change in slope due to PsJN inoculation

Table 3.1 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	<0.0001	<0.0001	<0.0001	<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

A, E, g and WUE are regressed on switchgrass plant age. Linear regressions were performed

with JMP Pro 10 (SAS Institute, Cary, NC)

Table 3.2 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)
Control (n=5)	167.4±8.8(5)	9.4±1.3(5)	176.8±9.8(5)
PsJN (n=5)	143.5±6.2(5)	9.2±1.6(5)	152.5±7.6(5)
p value	p=0.0568	p=0.9202	p=0.0868

Each value is presented as mean±SE (n). Probabilities are given for the results of student T-test for each parameter. Student T-tests were performed with JMP Pro 10 (SAS Institute, Cary, NC)

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## Chapter 4 *Burkholderia phytofirmans* strain PsJN affected growth and leaf level physiology of switchgrass under two drought cycles

### 4.1. Abstract

Plant growth-promoting rhizobacteria (PGPR) increase productivity and induce stress tolerance, which could contribute to developing a low input and sustainable bioenergy crop production system. We tested for the first time, the effects of PGPR on growth and leaf-level physiology of switchgrass under a moderate drought preconditioning and a successive severe drought stress. *Burkholderia phytofirmans* strain PsJN-inoculated switchgrass plants were 23.6 % taller ( $p < 0.0001$ ) and possessed 116% more tillers ( $p < 0.0001$ ) during the moderate drought. Pre-drought conditioned PsJN-inoculated switchgrass had higher Ps and instantaneous water use efficiency at all levels of water stress; whereas, control switchgrass only benefited from pre-drought when leaf water potential (LWP) dropped below -1 MPa. Both PsJN inoculation and drought stress accelerated the leaf senescence and promoted tillering/height ratio, which indicated advanced development. In sum, PsJN's effects on enhancing drought tolerance of switchgrass were confirmed in this study.

### 4.2. Introduction

Global consumption of energy tripled during the last 4 decades with heavy reliance on unsustainable fossil energy, which gave birth to current bioenergy crops, which were mainly based on food crops (Solomon 2010) and future biofuel crops (e.g. switchgrass, Miscanthus and poplar). US government policy is to displace 30% of present petroleum consumption with 910 million tons of biomass creating a sustainable biofuel scenario (Downing et al. 2011).

Switchgrass (*Panicum virgatum*) maintains a relatively high growth rate with low water and

nutrient requirements, which enables it to be grown in marginal land where it is too barren for farming, thereby avoiding direct competition with food crops on fertile land. A variety of biotic and abiotic stresses have adversary effects on plant growth and crop yields. Drought stress is expected to impact more than 50% of arable lands by 2050 (Vinocur and Altman 2005) and has a direct effect on forage production worldwide (Frank et al. 1996). Improving productivity and drought stress tolerance will help to economically produce switchgrass, which will positively affect the biofuel industry.

Endophytes promote growth and improve stress resistance through increasing available nutrients, physiology changes and hormone regulations (Mei and Flinn 2010), with plant growth promoting rhizobacteria (PGPR) colonizing the root surfaces and rhizosphere being the most widely studied group (Compant et al. 2005a). In addition, PGPR provided environmental and economic benefits to sustainable biofuel crop system by reducing fertilizer and pesticide use (Mehnaz and Lazarovits 2006, Mei and Flinn 2010, Bakker et al. 2012, Weekley et al. 2012). Since switchgrass is naturally associated with a diverse group of fungi (Kleczewski et al. 2012) and bacteria (Gagne-Bourgue et al. 2013), utilizing PGPR as ‘bio-fertilizer’ and ‘bio-pesticide’ would be a natural and promising way to improve switchgrass productivity. A few studies reported enhanced growth of switchgrass due to endophyte association (Ghimire and Craven 2011, Ker et al. 2012, Kim et al. 2012, Kleczewski et al. 2012), but physiological mechanisms behind these endophyte-induced growth benefits were not studied.

The well-studied endophyte *Burkholderia phytofirmans* strain PsJN can improve production and/or enhance stress tolerance in a variety of crops such as *Arabidopsis* (Sun et al. 2009, Poupin et al. 2013, Zuniga et al. 2013), potato (Bensalim et al. 1998, Nowak et al. 1998), tomato (Sharma and Nowak 1998), grapevine (Barka et al. 2006, Fernandez et al. 2012b), maize

(Naveed et al. 2014) and switchgrass (Kim et al. 2012) by manipulating plant hormones, root growth, photosynthetic rates (Ps) and carbohydrate metabolism. The presence of a 1-aminocyclopropane-1-carboxylate (ACC) deaminase, IAA metabolic pathway and quorum sensing are necessary for PsJN's plant growth promotion effects (Sun et al. 2009, Zuniga et al. 2013). PsJN enhanced chilling resistance, drought stress resilience and verticillium wilt resistance in grapevine (Barka et al. 2006, Fernandez et al. 2012b), maize (Naveed et al. 2014) and tomato (Sharma and Nowak 1998), respectively. Higher biomass, Ps, chlorophyll content and photochemical efficiency were found in PsJN-inoculated grapevine and maize under chilling stress and drought stress (Barka et al. 2006, Naveed et al. 2014), respectively. Trehalose metabolism also participated in PsJN induced chilling tolerance of grapevine (Fernandez et al. 2012b). Previous results demonstrated that PsJN increased 'Alamo' switchgrass seedling biomass production by 50% (Kim et al. 2012). However, the effect of PsJN on drought tolerance of switchgrass has not been studied. How endophyte inoculation influences the relationship between Ps and leaf water potential or growth and development during drought is not known. Studying physiological changes associated with PsJN inoculation under drought stress would help develop a low input and sustainable switchgrass production system (Mei and Flinn 2010).

In this study, we investigated physiological and growth changes, including those associated with drought tolerance, in response to inoculation with the bacterial endophyte PsJN. We addressed the following specific questions. 1) Does PsJN inoculation enhance plant growth and biomass in drought-stressed switchgrass? 2) Do the PsJN inoculation and drought stress play a role in switchgrass development and leaf senescence? 3) Does a mild, drought preconditioning treatment prime switchgrass for later drought stress and how does PsJN inoculation influence 'priming' in terms of growth and physiological changes?

## 4.3. Material and Methods

### 4.3.1. Plant material and growth condition

Switchgrass (*Panicum virgatum* L.) cv. Alamo seeds were purchased from Warner Brother's Seed Co. (Lawton, OK). These seeds were surface-sterilized by soaking in 70% ethanol for 2 min and rinsing 3X with distilled water. Then they were de-husked by immersing in 60% H<sub>2</sub>SO<sub>4</sub> with stirring for 30 min, washed 3X with distilled water, and sterilized with 0.4 M sodium hypochlorite containing 0.1% Triton 100 for 30 min followed by 5X rinse with sterile deionized water.

Seeds were then germinated in petri-dishes in a growth chamber at 25°C, under white fluorescent light (67 μmol m<sup>-2</sup> s<sup>-1</sup>), 16 h photoperiod. After 3 days of germination, the roots of switchgrass seedlings were soaked in *Burkholderia phytofirmans* strain PsJN suspension (0.5 of OD<sub>600</sub>) and control seedlings were soaked in PBS buffer for one min.

After 3 weeks, plants were transferred to 4-gallon pots filled with a soil mix composed of 2/3 Miracle-Gro Potting Mix (Scotts Miracle-Gro Company, Marysville, Ohio) and 1/3 Arabidopsis growing media (Lehle Seeds, Round Rock, Texas) with 3 plants/pot in a greenhouse under temperature of 25°C and 16-h photoperiod conditions.

### 4.3.2. Two drought cycles

The drought stress experiment consisted of a mild, drought preconditioning cycle followed by a more severe drought cycle. After transplanting, each pot initially received 1300 ml water/week (simulating average growing season precipitation for central Virginia). The first drought cycle (mild preconditioning drought) started two-weeks after transplanting. Five pots of inoculated seedlings and five pots of non-inoculated seedlings were randomly chosen and

received 425 ml water/week while the rest of pots remained well watered. Following this 40-day water reduction all plants were rehydrated to field capacity for 10 days. The second severe drought cycle started after full hydration. All plants received no further water until photosynthesis (Ps) reached zero. Immediately, the pots were rehydrated to field capacity. The plants were harvested when the Ps rate recovered and became stable.

#### 4.3.3. Experimental measurements

During the first, mild drought preconditioning cycle, the height of plants in each pot was measured every 3 or 4 days. The average height of all plants in the pot was used in statistical analyses. Tiller number of each plant was recorded every 3-4 days from the 4<sup>th</sup> week of the experiment when PsJN-inoculated plants and control plants had 1 and 0.5 tillers/ plant respectively. At the end of the experiment, the height of each plant was also recorded and averaged for each pot. Leaf senescence was measured by counting the number of dead leaves in each plant from the end of first drought preconditioning cycle to the beginning of second drought stress.

During the second, severe drought cycle, gas exchange was measured every day on a fully expanded leaf at the top of the canopy. Gas exchange was measured with a LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) at ambient relative humidity and the following settings: 3000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD), 400  $\mu\text{mol mol}^{-1}$  reference  $\text{CO}_2$  concentration, 25 °C block temperature, and a flow rate of 385  $\mu\text{mol sec}^{-1}$ . During the gas exchange measurement, a leaf from the same pot was excised at the leaf collar and plant water potential was measured immediately using a pressure chamber (PMS Instrument Co., Corvallis,OR). At the end of rehydration, aboveground plant parts were harvested and dried in an oven at 65 °C for 48 h.

#### 4.3.4. Experimental design and Analysis

A completely randomized design with two factors and repeated measures was used in this experiment. Differences in biometric parameters between different treatments were investigated with a Student T-test and considered significantly different when  $p < 0.1$  at each date. Biomass indexes was analyzed using repeated measurements ANOVA (analysis of variance), where treatments were the between subjects effects and time was the within subject effect. ANCOVA (analysis of variance with covariant) was used to investigate the effects of treatment on leaf gas exchange using leaf water potential as a covariant. All statistical analyses were performed with JMP Pro 10 (SAS Institute, Cary, NC).

#### 4.4. Results

##### 4.4.1. Growth and yield

Prior to the start of drought preconditioning, PsJN inoculation had significantly increased leaf area, leaf number and plant height by 32% ( $p < 0.0001$ ), 25% ( $p < 0.0001$ ) and 50% ( $p < 0.0001$ ), respectively (Table 4.1).

PsJN-inoculated plants continued to have greater height growth during the mild drought preconditioning regardless of water treatment ( $p < 0.0001$ , Table 4.2, Figure 4.1). However, there was a significant interaction ( $p = 0.0764$ ) between PsJN and drought preconditioning where the growth enhancement benefits of PsJN inoculation were more evident in the drought preconditioning treatment: PsJN-inoculated seedlings were 31.9% taller than control seedlings under drought conditioning and only 24.3% taller than control seedlings when well watered (Table 4.2). Drought stress increased the height of PsJN-inoculated plants by 6.4% but did not affect the height of control plants. Drought preconditioning began to arrest the height growth of control seedlings by 14.1% (Table 4.2) since the first rehydration while the growth enhancement



benefits of PsJN inoculation on height vanished ( $p=0.4930$ ). The negative effects of the drought preconditioning on height of control plants lasted to the 2<sup>nd</sup> drought cycle ( $p=0.0031$ ), during which all treatments had water withheld. Nevertheless, the height of PsJN-inoculated switchgrass was not reduced by drought preconditioning, since no difference in height was found during 1<sup>st</sup> rehydration and 2<sup>nd</sup> drought stress period.

Relative growth rates of height gradually decreased as switchgrass aged ( $p<0.0001$ , Table 4.2). PsJN-inoculation decreased relative growth rate by 20.2% lower ( $p=0.012$ ) during the drought preconditioning treatment; however, PsJN-inoculated switchgrass were still larger. After the 1<sup>st</sup> rehydration, PsJN inoculation did not affect relative growth rate. Preconditioning increased the relative growth rates of switchgrass by 120% ( $p=0.0923$ ).

PsJN-inoculated switchgrass had 84.2% more tillers than control plants during drought preconditioning cycle ( $p<0.0001$ , Table 4.2, Figure 4.1). However, this effect of PsJN inoculation on tiller number did not extend to the following rehydration ( $p=0.6709$ ) and 2<sup>nd</sup> drought cycle ( $p=0.8678$ ). The preconditioning drought increased tiller number of PsJN-inoculated plants by 23% ( $p<0.0001$ ) but had no effect on tiller number ( $P=0.6008$ ) of control plants during first drought stress.

Because PsJN's growth promotion benefits disappeared by the end of the study and pre-drought conditioned switchgrass caught up during the second drought cycle, there was no difference between any treatments at final harvest in terms of height, tiller number and aboveground biomass (Figure 4.2Figure 4.2 ).

#### 4.4.2. Development and senescence

Tiller number was strongly correlated with plant height ( $p < 0.0001$ , Figure 4.3) and PsJN inoculation ( $p = 0.0446$ ) and drought preconditioning ( $p = 0.0388$ ) modified the correlation between height and tiller number. For switchgrass at the same height, PsJN-inoculated plants had 18.2% more tillers than non-inoculated plants and drought pre-conditioned plants had 16.7% more tillers than pre-watered plants on average. There was no interaction between pre-drought treatment and PsJN inoculation ( $p = 0.9175$ ). Nevertheless, PsJN's beneficial effects on tiller number/height ratio were more evident when switchgrass was shorter but gradually disappeared when switchgrass grew taller (Figure 4.3). In contrast, drought preconditioning did not affect tiller number/height ratio at the beginning but gradually came into effect as switchgrass grew taller (Figure 4.3).

Both drought treatment ( $p < 0.0001$ ) and PsJN inoculation ( $p < 0.0001$ ) increased leaf senescence and this effect lasted to the following rehydration (Table 4.2, Figure 4.4). There was significant interaction between drought stress and PsJN inoculation ( $p < 0.0001$ ) during the first drought preconditioning cycle. Leaf senescence was greatest (2.7 leaves per plant) when plants were inoculated with PsJN under drought preconditioning. Following the second severe drought stress there were no difference in number of senesced leaves due to PsJN inoculation or previous drought hardening.

#### 4.4.3. Gas exchange and leaf water potential

At the beginning of the final, severe drought cycle, at leaf water potentials above -1.0 MPa there was no relationship between leaf water potential and photosynthesis ( $p = 0.5064$ , Figure 4.5), indicating that switchgrass was not under drought stress. Above leaf water

potentials of -1.0 MPa, drought preconditioning increased Ps of PsJN-inoculated switchgrass by 26.4% ( $p=0.0024$ ) but preconditioning had no effect on the control seedlings (Figure 4.5).

At leaf water potentials below -1 MPa, there was a strong correlation between Ps and leaf water potential ( $R^2=82.6\%$ ,  $p<0.0001$ , Figure 4.5). Pre-conditioned switchgrass maintained higher Ps ( $p=0.0045$ ) under lower leaf water potentials (Figure 4.5). PsJN inoculation did not induce significant changes in Ps ( $p=0.3784$ ) but higher mean Ps were observed. There was no interaction between PsJN inoculation and drought preconditioning at leaf water potentials below -1 MPa.

#### 4.5. Discussion

PsJN has promoted the productivity of crops such as potato, grapevine and maize under stresses (Bensalim et al. 1998, Barka et al. 2006, Fernandez et al. 2012a, Naveed et al. 2014). During the preconditioning period, PsJN inoculation enhanced switchgrass cv. Alamo growth of both drought pre-conditioned and well watered switchgrass seedlings, which is consistent with the results of Kim et al. (2012) that PsJN inoculation increased the biomass of young switchgrass. During the preconditioning period, 2/3 reduction of watering did not inhibit height growth and tillering of control seedlings until the end of the drought cycle, probably because this reduction was too mild to impact the growth since the water consumption of the small seedlings was low. This is similar to reports on crop yield under mild drought (Erice et al. 2010). The demand for water rose as plants grew larger and the preconditioning treatment started to slow the growth of control switchgrass at the beginning of 1<sup>st</sup> rehydration and this negative effect extended into next drought stress cycle when all the treatments were under severe drought. The relative growth rates were higher in the preconditioning group during the final severe drought cycle, which might be attributable to the priming effects of the preconditioning treatment. PsJN

inoculation reduced the negative effects of 1<sup>st</sup> drought on growth, which might result from the priming effects of PsJN before drought stress. Enhanced height and tiller growth of endophyte inoculated switchgrass were observed in the pre-drought stress group compared to control under the same stress, especially at a younger stage. PsJN interplayed with drought stress, possibly through manipulating IAA and other hormones (Sun et al. 2009, Zuniga et al. 2013), to regulate branching (tiller) in grasses (McSteen 2009). However, similar to the whole life cycle analysis of PsJN's effects on *Arabidopsis* (Poupin et al. 2013), the growth advancement effects of PsJN gradually vanished with time as the plants grew larger and the relative growth rate of PsJN-inoculated switchgrass became lower than that of the control group. Thus, the aboveground biomass was not enhanced by PsJN inoculation at final harvesting. In some previous studies of PGPR's effects on crops, the aboveground biomass was also not influenced by endophytes but the underground biomass was increased (Rogers et al. 2012). The root growth promoting effects by PsJN helped plants to access more water and nutrient resources, which played an important role in the symbiosis relationship. In a greenhouse study, this benefit may be limited by the size of containers. Although the growth promotion effects vanished at the end, PsJN induced early growth of switchgrass may help switchgrass to compete with weeds and facilitate establishment, which is a bottleneck for switchgrass production. Better productivity of PsJN-inoculated switchgrass has been observed in a field trial (Lowman et al. 2014).

Although much attention has been devoted to the growth benefits brought by PGPR, rarely are its effects on development measured (Poupin et al. 2013, Lowman et al. 2014). Increased leaf senescence (Poupin et al. 2013) and advanced growth stage (Lowman et al. 2014) were associated with PsJN inoculation, which implied advanced development. In this study, both PsJN inoculation and drought stress accelerated the leaf senescence and functioned

independent of each other. Although previous studies showed increased tillering due to endophytes (Clay 1987, Ker et al. 2012, Kim et al. 2012), none investigated if the increase in tiller number was simply due to increased plant size or altered development. However, even for switchgrass at the same height, both PsJN and drought preconditioning changed tiller number/height ratio, which indicated altered development and allocation of biomass.

Several studies investigated the response of woody species (Zwiazek and Blake 1989, Ladjal et al. 2000, Ruiz-Sánchez et al. 2000, Guarnaschelli et al. 2003, Vilagrosa et al. 2003) and herbaceous species (Agnew and Carrow 1985, Elmi and West 1995, Jiang and Huang 2000, 2001) to drought preconditioning, which improved resistance of plants against freezing (Hoffman et al. 2012), heat shock (Jiang and Huang 2000, Ladjal et al. 2000, Jiang and Huang 2001), transplanting shock (Duryea and McClain 1984, Landis et al. 1999) and drought stress (Ruiz-Sánchez et al. 2000, Vilagrosa et al. 2003). Pre-conditioned plants maintained higher water status, higher Ps, stomatal conductance and root dry weight during the subsequent stresses (Jiang and Huang 2000, Vilagrosa et al. 2003), which probably resulted from greater osmotic adjustment (Zwiazek and Blake 1989, Elmi and West 1995, Jiang and Huang 2001). However, none studied the relationship between Ps and leaf water potential of pre-conditioned plants. During the final severe drought when all the switchgrass were completely cut-off from water, Ps rates were not correlated to LWP when LWP was above  $-1$  MPa. However, once LWP dropped below this threshold, Ps significantly decreased and was strongly correlated with lower leaf water potential. This threshold LWP ( $-1$  MPa) was very close to the leaf osmotic potential at full turgor ( $-1.04$ MPa) of well watered switchgrass we measured in another study (data not shown), which indicates that osmotic potential and turgor loss plays a role in the decline of Ps in response to water stress. At lower LWP, preconditioned and PsJN plants maintained higher Ps. This may

have been the result of osmotic adjustment in pre-conditioned plants. Previous studies also reported osmotic adjustment in Kentucky bluegrass which was associated with accumulation of ion solutes and water soluble carbohydrates (Jiang and Huang 2001). Both preconditioning drought (Clay 1987) and endophyte inoculation (Elmi and West 1995) adjusted osmotic potential in grass, therefore osmotic adjustments might contribute to the interplay of PsJN inoculation and drought stress on Ps.

#### 4.6. Conclusion

Our studies showed that PsJN inoculation enhanced height and tiller growth of switchgrass during a drought preconditioning and later severe drought stress. Drought preconditioning also primed switchgrass for the following severe drought stress, and this priming effect was magnified in PsJN-inoculated switchgrass. PsJN inoculation also advanced development and increased photosynthetic rates under both well watered and stressed condition, which might participate in PsJN induced drought stress tolerance of switchgrass.

Figures/Tables

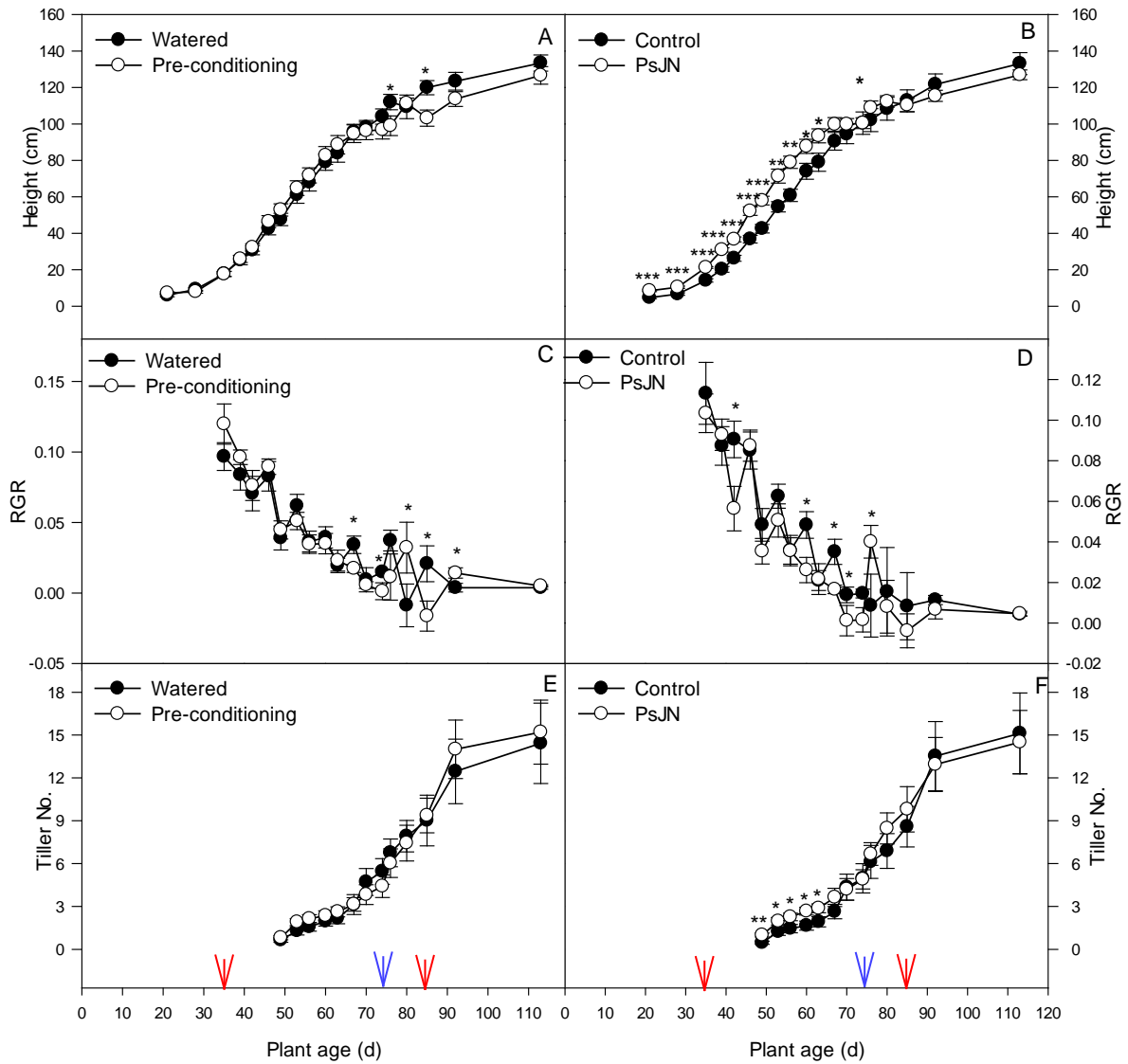


Figure 4.1 Effects of PsJN inoculation and mild drought preconditioning on height, relative growth rates (RGR) and tiller number of switchgrass. Symbols indicate significant differences between PsJN and control at each time period using student T-test (\* $p < 0.1$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). Red arrows indicate the beginning of drought stress and blue arrow indicates the beginning of rehydration

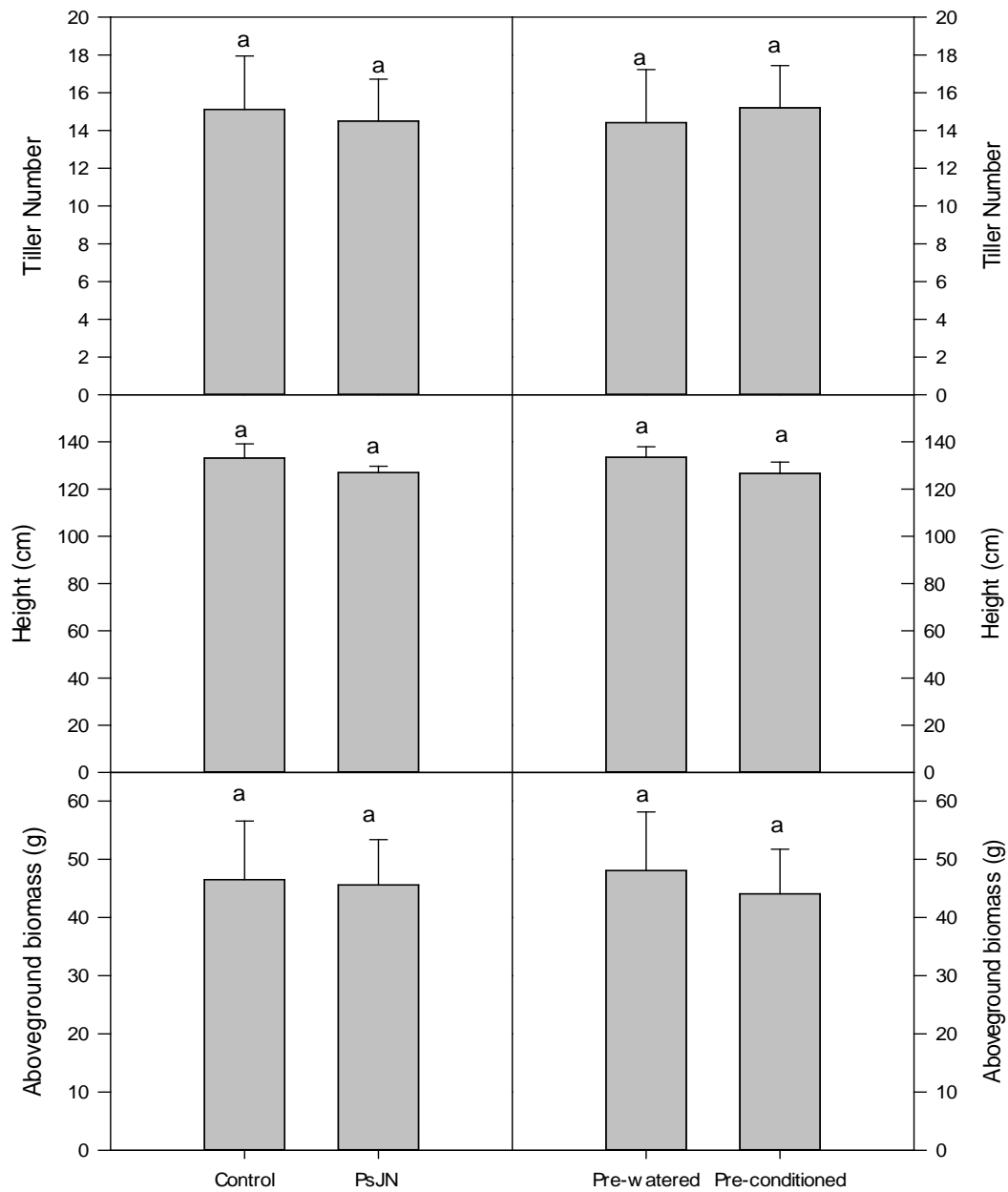


Figure 4.2 Height, tiller No. and aboveground biomass at final harvest influenced by PsJN inoculation and two drought stress cycles. Error bars are  $\pm 1$  SE and different letters indicate significant differences ( $p < 0.10$ ) between inoculation and pre-drought hardening treatment combinations



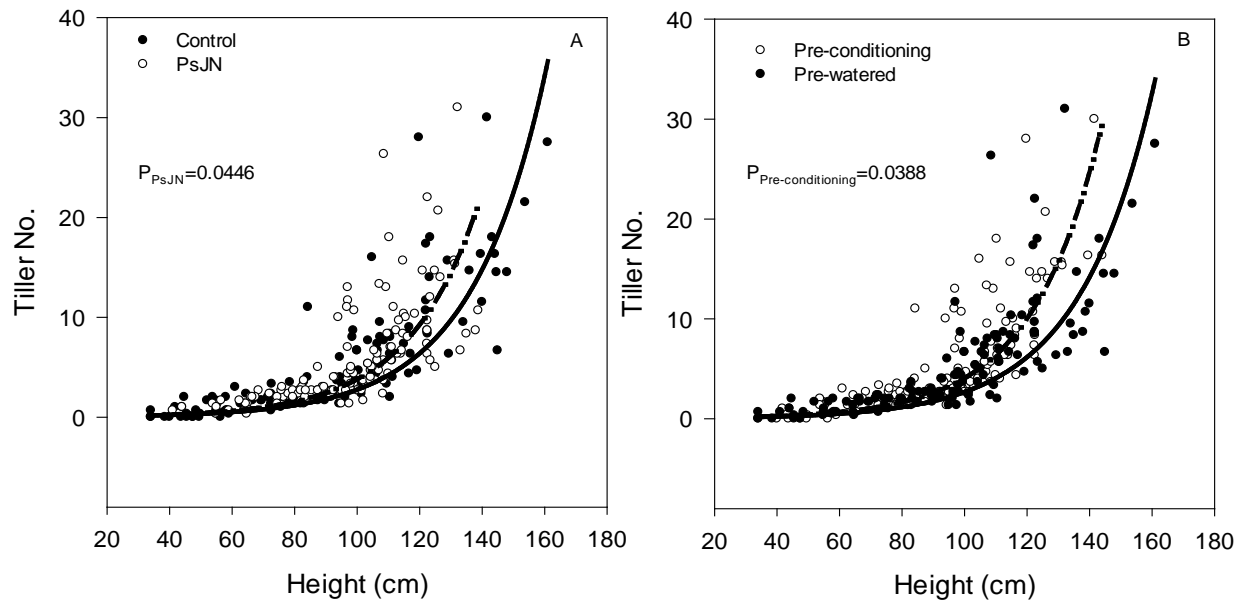


Figure 4.3 Tiller number related to height (cm) as influenced by PsJN inoculation and two drought stress cycles

Panel A models: Control (closed circles and solid lines)  $\text{Log}(y) = -2.663 + 0.040x$  ( $R^2 = 76.6\%$ ,  $p < 0.0001$ ), PsJN (open circles and dashed lines)  $\text{Log}(y) = -1.93 + 0.034x$  ( $R^2 = 68.6\%$ ,  $p < 0.0001$ ).

$P_{\text{PsJN}} < 0.1$  indicate significantly change in slope due to PsJN inoculation.

Panel B models: Pre-watered (closed circles and solid lines)  $\text{Log}(y) = -2.260 + 0.036x$  ( $R^2 = 76.4\%$ ,  $p < 0.0001$ ), Pre-conditioning (open circles and dashed lines)  $\text{Log}(y) = -2.749 + 0.043x$  ( $R^2 = 74.2\%$ ,  $p < 0.0001$ ).

$P_{\text{Pre-conditioning}} < 0.1$  indicate significantly change in slope due to drought pre-conditioning

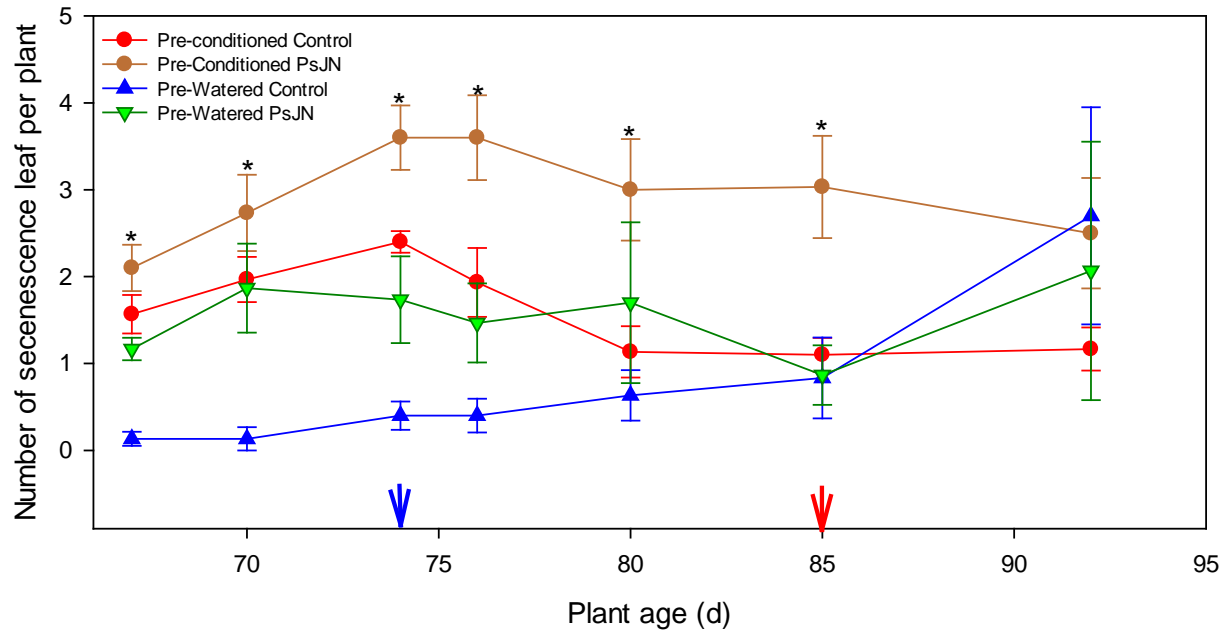


Figure 4.4 The effects of PsJN inoculation and preconditioning on leaf senescence. Blue arrow indicates the start of rehydration, and red arrow indicates the start of drought stress. Error bars are  $\pm$ SE of the means. Symbols indicate significant differences between treatments at each time period using ANOVA (\* $p < 0.1$ ).

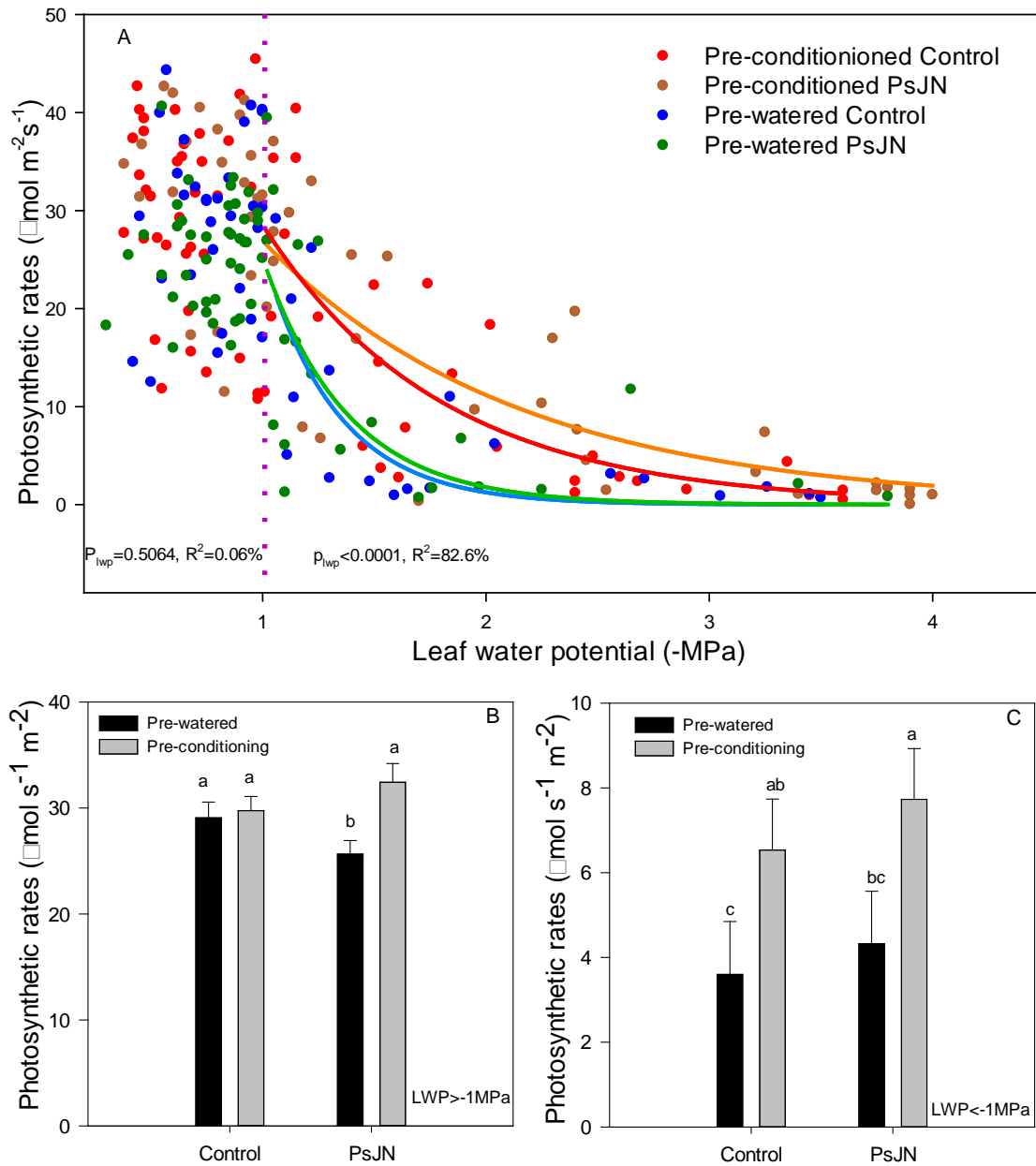


Figure 4.5 Effect of PsJN inoculation and drought preconditioning on the relationship between photosynthetic rate and leaf water potential during second drought cycle. Error bars are  $\pm 1$  SE and different letters indicate significant differences ( $p < 0.1$ ) between inoculation and pre-drought hardening treatment combinations

Table 4.1 Effect of Ps JN endophyte inoculation before the start of mild drought preconditioning on switchgrass leaf area (cm<sup>2</sup>), leaf number and height (cm)

	Leaf Area (cm <sup>2</sup> )	Leaf No.	Height (cm)
Control	3.1± 0.1(10)	4.0± 0.1(10)	14.2± 0.9(10)
PsJN	4.1± 0.1(10)	5.0± 0.1(10)	21.3± 0.5(10)
p-value	<0.0001	<0.0001	<0.0001

Each value is presented as mean±SE (n). Probabilities are given for the results of student T-test for each parameter. Student T-tests were performed with JMP Pro 10 (SAS Institute, Cary, NC)

Table 4.2 Effect of PsJN endophyte inoculation and drought stress on height, relative growth rate (RGR), tiller and leaf senescence of switchgrass during two drought cycles

		Height (cm)	RGR	Tiller	Death leaf
1 <sup>st</sup> Drought (Pre-conditioning)	Pre-conditioned Control	57.7±1.6 (60) <sup>c</sup>	0.043±0.003(60) <sup>ab</sup>	1.3±1.1(40) <sup>b</sup>	1.9±0.1(15) <sup>b</sup>
	Pre-conditioned PsJN	76.1±0.9 (60) <sup>a</sup>	0.037±0.003(60) <sup>b</sup>	2.8±1.1(40) <sup>a</sup>	2.7±0.1(15) <sup>a</sup>
	Pre-watered Control	57.5±1.4 (60) <sup>c</sup>	0.048±0.003(60) <sup>a</sup>	1.4±1.1(40) <sup>b</sup>	0.1±0.1(15) <sup>d</sup>
	Pre-watered PsJN	71.5±1.4 (60) <sup>b</sup>	0.038±0.003(60) <sup>b</sup>	2.2±1.1(40) <sup>a</sup>	1.5±0.1(15) <sup>c</sup>
	Days	<0.0001	<0.0001	<0.0001	p =0.0005
	Days <sup>2</sup>	<0.0001	p=0.0007		
	PsJN	<0.0001	p=0.0120	<0.0001	<0.0001
	Preconditioning	p=0.2008	p=0.3680	p=0.5808	<0.0001
	PsJN* Preconditioning	p=0.0764	p=0.5619	p=0.2028	<0.0001
1 <sup>st</sup> Rehydration	Pre-conditioned Control	96.2±5.1(15) <sup>b</sup>	0.011±0.013(15) <sup>a</sup>	5.4±1.0(15) <sup>a</sup>	1.8±0.2(15) <sup>b</sup>
	Pre-conditioned PsJN	104.0±2.6(15) <sup>ab</sup>	0.023±0.009(15) <sup>a</sup>	6.7±0.6(15) <sup>a</sup>	3.4±0.3(15) <sup>a</sup>
	Pre-watered Control	112.8±4.1(15) <sup>a</sup>	0.002±0.012(15) <sup>a</sup>	6.8±0.9(15) <sup>a</sup>	0.5±0.1(15) <sup>c</sup>
	Pre-watered PsJN	108.8±3.0(15) <sup>ab</sup>	0.010±0.007(15) <sup>a</sup>	6.4±0.8(15) <sup>a</sup>	1.6±0.4(15) <sup>b</sup>
	days	p=0.5403	p =0.0323	p=0.0064	p =0.1312
	PsJN	p =0.4930	p =0.7829	p=0.6709	<.0001
	Preconditioning	p =0.0138	p =0.1404	p=0.4001	<.0001
		PsJN* Preconditioning	p =0.3407	p =0.2739	p=0.3972
2 <sup>nd</sup> Drought & Rehydration	Pre-conditioned Control	107.8±5.8(10) <sup>c</sup>	0.101±0.024(5) <sup>a</sup>	11.1±2.4(10) <sup>a</sup>	1.2±0.2(5) <sup>a</sup>
	Pre-conditioned PsJN	109.2±3.0(10) <sup>bc</sup>	0.099±0.049(5) <sup>a</sup>	12.2±1.0(10) <sup>a</sup>	2.5±0.6(5) <sup>a</sup>
	Pre-watered Control	126.7±4.6(10) <sup>a</sup>	0.058±0.019(5) <sup>a</sup>	11.0±1.9(10) <sup>a</sup>	2.7±1.2(5) <sup>a</sup>
	Pre-watered PsJN	118.6±3.6(10) <sup>ab</sup>	0.032±0.020(5) <sup>a</sup>	10.5±2.3(10) <sup>a</sup>	2.1±1.5(5) <sup>a</sup>
	Days	p =0.0675		p =0.0398	
	PsJN	p =0.4302	p =0.6474	p =0.8678	p =0.7384
	Preconditioning	p =0.0019	p =0.0923	p =0.6181	p =0.6006
		PsJN* Preconditioning	p =0.2671	p =0.6953	p =0.6808

Means followed by the same letter (s) in a column for each parameter do not differ significantly at  $\alpha=0.1$ . Data are means  $\pm$  standard error (replicates number). Probabilities are given for the results of a Two-Way ANCOVA for each parameter

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## Chapter 5 Synthesis

### 5.1. Synthesis of Results

The objective of this study was to identify some of the physiological mechanisms of the growth promoting effects of PsJN inoculation on switchgrass cv. Alamo throughout the plant entire life cycle and to determine if PsJN affected the drought tolerance of switchgrass. The growth chamber experiment was used to determine the initial response of young seedlings to PsJN inoculation. One of the greenhouse studies was used to determine the growth response to PsJN inoculation throughout its whole life cycle as well as the physiological modifications due to PsJN. The second greenhouse experiment was devoted to studying the effects of drought stress on PsJN-inoculated switchgrass. The major results are summarized as follows.

1. Enhanced growth and allocation in PsJN-inoculated switchgrass seedling

PsJN significantly enhanced growth within 17<sup>th</sup> day of inoculation. Both aboveground growth and belowground growth were stimulated by PsJN inoculation. However, the growth enhancement of belowground biomass lagged behind that of aboveground biomass, which resulted in increased biomass allocation to aboveground in PsJN-inoculated switchgrass seedlings. Morphological changes of both aboveground and belowground were in agreement with the biomass enhancement due to PsJN. Leaf area, plant height, root length, root surface area and root diameter were all increased in PsJN-inoculated seedlings. The growth beneficial effects of PsJN inoculation were also evident when switchgrass seedlings started to tiller. PsJN-inoculated plants initiated tillers earlier and had more tillers than the control group. Based on the photographic analysis, the total interception leaf area was also enlarged by PsJN inoculation. However, PsJN's growth benefits gradually diminished after two months, which was consistent

in all of the greenhouse experiments. Therefore, no significant differences in biomass and morphological features were found in PsJN-inoculated switchgrass at final harvest.

The increased photosynthetic rates in PsJN-inoculated seedlings were observed after 17 days of inoculation. The initially increase in photosynthetic rate resulted in increased water use efficiency several days later. In the growth chamber experiment with young plants, photosynthetic rates were correlated with stomatal conductance in PsJN-inoculated young seedlings. In contrast, no correlation was found between photosynthetic rates and stomatal conductance in control plants. This contrast indicated better control of stomatal conductance in PsJN inoculated plants.

As switchgrass matured, PsJN inoculation still increased photosynthetic rates in younger, upper-canopy leaves throughout the whole life cycle of the plant. However, this benefit in photosynthetic rates was not observed in all of the sampling dates. The younger leaves and upper-canopy leaves in PsJN-inoculated switchgrass showed higher stomatal conductance, which explained the increased photosynthetic rates in PsJN-inoculated switchgrass. In contrast to the study with the very young plants, there were no difference in water use efficiency between PsJN-inoculated switchgrass and control switchgrass.

## 2. Advanced development due to PsJN inoculation

PsJN inoculation not only enhanced growth of switchgrass, but also modified its development. Switchgrass inoculated with PsJN displayed lower root/shoot ratio, higher specific leaf weight as well as lower specific root length, even when compared to control plants of similar root biomass, root length, and leaf area. Therefore, these changes were not caused by the plant “size” effects of PsJN.

Accelerated development also contributed to faster senescence of leaves and earlier flowering. Those PsJN induced growth enhancements in early ontogeny gradually diminished, which were in line with lower relative growth rate in PsJN-inoculated plants.

Faster senescence of PsJN-inoculated leaves was observed in two greenhouse experiments.

Although the photosynthetic rates of PsJN-inoculated switchgrass were higher at the beginning, faster drop of photosynthetic rates was also observed in PsJN-inoculated switchgrass as leaves matured. Therefore, the photosynthetic rates of the older leaves in PsJN-inoculated switchgrass were lower than controls. Faster decrease of photosynthetic rates in PsJN-inoculated switchgrass indicated faster leaf senescence. And this conclusion was consistent with a higher senesced leaf number in PsJN-inoculated switchgrass. Accelerated development also resulted in earlier flowering in PsJN-inoculated switchgrass. Therefore, the flower biomass/aboveground biomass ratio was higher in PsJN-inoculated plants, though there was no difference in flower biomass between PsJN-inoculated and non-inoculated plants.

### 3. Enhanced drought resistance

Mild drought preconditioning stimulated height growth of PsJN-inoculated switchgrass but did not affect control plants. During the following severe drought, there was no difference between pre-conditioned plants and pre-watered plants. However, for control plants, those receiving drought preconditioning were shorter than the pre-watered ones after the drought preconditioning. Thus, better drought preconditioning effects were observed in PsJN-inoculated switchgrass in terms of height growth. During the conditioning, drought increased leaf death of control switchgrass and PsJN-inoculated switchgrass by 1.8 leaves per plant and 1.2 leaves per plant, respectively. There was a significant interaction between PsJN inoculation and drought, indicating that PsJN ameliorated the negative effect of drought conditioning on leaf senescence.

At the beginning of the severe drought cycle, drought preconditioning increased Ps of PsJN-inoculated switchgrass by 26.4% ( $p=0.0024$ ) but preconditioning had no effect on the control seedlings. Later in the severe drought cycle at leaf water potentials below -1 MPa, Ps correlated with leaf water potential ( $R^2=82.6\%$ ,  $p<0.0001$ ). Pre-conditioned switchgrass maintained higher Ps ( $p=0.0045$ ) under lower leaf water potentials. PsJN inoculation did not induce significant changes in Ps.

## 5.2. Novel Contributions to the Current Body of Knowledge and Implications

Our research contributed to the current body of knowledge in a number of ways. First, the growth chamber experiment greatly improved our knowledge on the initial physiological response of switchgrass to PsJN inoculation. Although the effects of endophytes on plants were investigated in a number of studies (Barka et al. 2006, Sun et al. 2009, Kim et al. 2012, Naveed et al. 2014), the initial physiological responses upon endophyte inoculation were ignored, probably due to the difficulty in measuring photosynthetic rates in young, small leaves. Therefore, the few studies on the initial response of plants to endophyte mainly focused on the genetic response (Sun et al. 2009, Poupin et al. 2013). This research is the first study measuring physiological response on just inoculated seedlings and reveals some of the initial physiological and morphological changes including: increased water use efficiency, higher stomatal conductance, decreased root/shoot ratio, increased specific leaf weight and decreased specific root length.

Second, we tracked the whole life cycle growth of switchgrass using a photographic method on a weekly basis. Although similar technique was applied to trees (Seiler and McBee 1992, Stovall et al. 2013) and Arabidopsis (Arvidsson et al. 2011), we applied this method to grass for the first time to indirectly determine the growth of PsJN-inoculated switchgrass.

Previous studies on the relationship between endophyte and plants were usually conducted within a short period of time or had 1-3 harvests (Ker et al. 2012, Rogers et al. 2012, Naveed et al. 2014). Our study provided a holistic view for the consecutive growth responses of switchgrass to PsJN inoculation. We found that PsJN inoculation initially increased interception leaf area, but this PsJN induced growth enhancement gradually diminished over time. This result was consistent with height growth and tiller number changes in PsJN-inoculated switchgrass in another greenhouse experiment.

Third, we found advanced development and faster leaf senescence in PsJN-inoculated switchgrass. The majority of researches were devoted to studying the growth promotion effects (Ker et al. 2012, Kim et al. 2012, Lowman et al. 2014). Only a few papers discussed the effects of endophytes on development and senescence in *Arabidopsis* (Poupin et al. 2013). The older leaves of PsJN-inoculated switchgrass showed faster senescence in one greenhouse study. Advanced development was also confirmed by more senescing leaves in PsJN-inoculated switchgrass and earlier flowering. The initial accelerated growth and advanced senescence in PsJN-inoculated switchgrass forward our understanding behind PGPR's effect on plants, which lead to a hypothetic scheme (Figure 5.1). The maximum biomass depends the size of container and available resources.

Fourth, the gas exchange in both upper- and lower-canopy leaves were measured on a weekly basis throughout the whole life cycle, which provided a holistic scenario on PsJN's effects on photosynthetic rates. Although PsJN only enhanced photosynthetic rates in upper-canopy leaves at several sampling dates, the mean Ps in PsJN-inoculated plants were always higher. This also suggests that PsJN inoculation enhances the development of plants since lower leaves appear to age more rapidly.



Finally, the gas exchange of PsJN-inoculated switchgrass under severe drought was studied after a mild drought preconditioning. Although previous studies also discussed the effects of drought preconditioning and drought stress on endophyte inoculated plants (Klar et al. 1978, Ladjal et al. 2000, Hoffman et al. 2012), none analyzed Ps as water potential decreased. This method adjusted the bias introduced by different plant size and thereby different drought stress level. We found that the threshold water potential of Ps downslide seems to be the leaf osmotic potential. Both leaf level physiology data and morphology data supported the conclusion that drought preconditioning appeared to improve PsJN-inoculated plant performance more than that of control plants.

### 5.3. Future work

Further investigations are necessary to provide explanations for the mechanism of PsJN's growth promotion and drought resistance effects on switchgrass.

1. Effects of PsJN on the anatomy and biochemical properties of young switchgrass seedlings

Switchgrass seedlings inoculated with PsJN produced a heavier leaf than control plants, which might indicate changes in leaf anatomy and biochemical properties. Relevant researches on leaf thickness, vascular bundle structure, epidermis thickness, chlorophyll content and soluble sugar of PsJN-inoculated young seedlings, are necessary for understanding the earlier growth promotion effects.

2. Effects of PsJN on hormone regulations of switchgrass throughout the whole life cycle

PsJN represents a group of PGPR that possessed ACC deaminase activity (reviewed in (Glick et al. 2007)). Manipulating ethylene and IAA production is necessary for PsJN's growth promotion effects (Sun et al. 2009, Zuniga et al. 2013). Nevertheless, these studies on hormone control of PsJN were conducted on Arabidopsis seedlings. Since the growth promotion effect of PsJN on switchgrass gradually diminished, it would be interesting to study if the corresponding hormone regulation in PsJN-inoculated switchgrass also gradually diminished with time.

### 3. Study the physiological mechanisms of the interaction between PsJN and switchgrass in field trials

Most mechanism studies on PsJN's growth beneficial effects were conducted in a growth chamber or a green house. Whether similar results could also be obtained from field trials is unclear. For example, the biomass accumulation in greenhouse experiments is limited by the pot size. The earlier root growth in PsJN-inoculated seedlings could benefit aboveground growth by accessing more water and mineral nutrients allowing switchgrass to be more competitive with weed species. However, this advantage is largely compromised by pot experiments where no interspecific competition occurs. Field trials also confront more environmental variability, which might antagonize or facilitate PsJN's growth enhancement effects on switchgrass.

Lowman et al.'s field trial showed that PsJN-inoculated switchgrass still showed higher biomass at the final harvest (Lowman et al. 2014), which might be attributed to some of those reasons.

The physiological response of switchgrass to PsJN inoculation may also be influenced by all of these factors and show a different story.

### 4. Effects of PsJN on establishment and competitiveness of switchgrass

Switchgrass establishment is a bottle neck for sustainable switchgrass production system. As a perennial grass, switchgrass puts more effort on developing root system compared with the annual weeds, which in turn lead to its less competitiveness. Increased allocation to aboveground, as well as advanced development in PsJN-inoculated switchgrass might facilitate switchgrass establishment by increasing survival rates and competitiveness of young seedlings. Understanding the early competition between PsJN-inoculated switchgrass and other weeds would greatly facilitate our understanding of PGPR's beneficial effects.

5. Understand the effects of PsJN inoculation on drought stressed young seedlings and use a different way to simulate drought stress

Studies evaluating drought tolerance are usually conducted on older plants. Sometimes this is simply because we need enough mature leaves for leaf water potential and leaf level physiology measurements. As a result in the older switchgrass many PsJN induced growth benefits may have already vanished. We speculated that PsJN would have more evident effects on drought resistance at an early age similar to the effect on growth enhancement. This hypothesis could be tested by leaf physiology and biochemical property measurements. Drought stress level could be controlled by Polyethylene glycol (PEG) solution.

In our study, we simulated drought stress using a 2/3 reduction in irrigation compared with a well-watered control. Nevertheless, PsJN-inoculated switchgrass was still larger than control during the first two month, which contributed to higher water consumption. Therefore, the same amount of water reduction would cause more severe drought for PsJN-inoculated young seedlings. In natural ecosystems, the increased consumption of water in PsJN-inoculated switchgrass could be compensated by a larger root system and more access to water, while in a greenhouse the below ground advantage of larger root system is limited by pot size. Therefore,

other drought stimulate methods should be employed to maintain the drought stress of PsJN-inoculated switchgrass and control switchgrass at the same level. Alternatively a size control would be necessary but then these plants likely would not be the same age.

#### 6. Understand the mechanism of PsJN's genotype specific effect

The effects of PsJN on switchgrass were genotype specific. Different cultivars showed positive, neutral or negative response to PsJN inoculations (Kim et al. 2012). Also some plant species or cultivars are more susceptible than others. What are the drivers for these differences, environmental cues or genetic determinations? Understanding the mechanisms that affect the genotype specificity fills in the blank of our current body of knowledge and makes PsJN more applicable as a bio-fertilizer in sustainable biofuel production.

#### 7. The niche of PsJN in natural microbial community

Most studies on the effects of PsJN on switchgrass ignored the competition of microbial communities from natural soil. Would the endophyte still have the same effect on plant growth when they are in a natural environment or would the organism be outcompeted? What determines its destiny?

Answering these questions will unravel the complex interactions between PsJN and switchgrass under the natural environment. Some of these questions focus on fundamental physiological mechanisms that contribute to advancing our knowledge of the symbiosis relationship between plants and endophytes, while other questions have direct influence on efficient switchgrass biofuel production. Because of the complexity of the symbiosis relationship and natural environment, collaborative efforts across different disciplines as well as

more advance analysis should be devoted to forwarding our understanding of these questions and providing guidance for building a sustainable switchgrass production system.

## Figures/Tables

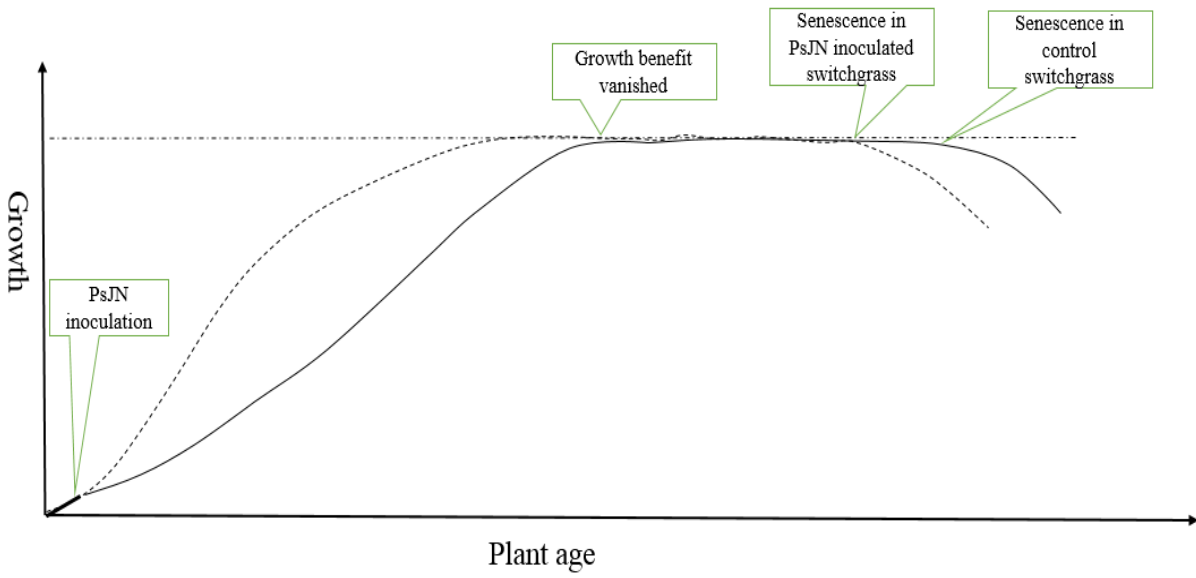


Figure 5.1 The hypothetical scheme of PsJN's effects on growth

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