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A microbial endophyte enhanced growth of switchgrass under two drought cycles improving leaf level physiology and leaf development

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ABSTRACT

Beneficial microbial endophytes 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 microbial endophytes on growth and leaf-level physiology of switchgrass (*Panicum virgatum*) 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 photosynthetic rates (Ps) 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 the strain promoted tillering/height ratio, which indicated more rapid development. In sum, enhancement of drought tolerance of switchgrass by PsJN was indicated by this study.

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1. Introduction

Global consumption of energy tripled during the last 4 decades with heavy reliance on unsustainable fossil energy. This led to interest in bioenergy crops, which are mainly based on food crops (Solomon, 2010) and future biofuel crops (e.g. switchgrass, *Miscanthus* and poplar). The 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* L.) maintains a relatively high growth rate with low water and nutrient requirements, which enables it to be grown on marginal lands unsuitable for farming, thereby avoiding direct competition with food crop production on fertile land. A variety of biotic and abiotic stresses have negative effects on plant growth and crop yields. Drought stress has been predicted 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 and positively affect the biofuels industry.

Beneficial symbiotic microbes promote growth and improve stress resistance of plants through increasing available nutrients, physiology changes and hormone regulations (Compant et al., 2010, 2005a; Glick, 2004; Mei and Flinn, 2010). In addition, these symbionts provide environmental and economic benefits to sustainable biofuel crop system by reducing fertilizer and pesticide use (Bakker et al., 2012; Compant et al., 2005a; Mehnaz and Lazarovits, 2006; Mei and Flinn, 2010; 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 beneficial microbial 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 symbiotic association (Ghimire and Craven, 2011; Ker et al., 2012; Kim et al., 2012; Kleczewski et al., 2012), but physiological mechanisms behind these symbiotic microbes induced growth benefits were not studied.

The well-studied bacterial endophyte *Burkholderia phytofirmans* strain PsJN was originally isolated from onion roots, which also inhabited the vegetative organs of grapevine (Compant et al., 2005b), switchgrass (Kim et al., 2012) and maize (Naveed et al., 2014). *B. phytofirmans* can improve production and/or enhance stress tolerance in a variety of plants such as *Arabidopsis* (Poupin et al., 2013; Sun et al., 2009; 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

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(Ps) and carbohydrate metabolism. The presence of a 1-amino-cyclopropane-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, photosynthetic rates, 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 photosynthetic rates 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 changes, including those associated with drought tolerance, in response to inoculation with the bacterial endophyte PsJN. Our hypothesis are: (1) PsJN inoculation enhances drought tolerance in switchgrass by inducing more rapid development and leaf senescence. (2) A mild, drought preconditioning treatment primes switchgrass for later drought stress by modifying development and leaf-level physiology, and (3) PsJN inoculation increases drought tolerance of switchgrass by allowing a maintenance of photosynthesis to lower potentials.

2. Materials and methods

2.1. Plant material and growth condition

Switchgrass (*P. 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_2SO_4 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. Finally the sterilized seeds were then germinated in petri-dishes in a growth incubator at 25 °C, under white fluorescent light (67 $\mu mol\, m^{-2}\, s^{-1}$) and a 16 h photoperiod. After 6 days of germination, switchgrass seedlings were soaked in *B. phytofirmans* strain PsJN suspension (0.5 of OD_{600}) and control seedlings were soaked in PBS buffer for 1 min.

After 3 weeks, plants were transferred to 20 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 a temperature of 25 °C and a 16-h photoperiod on April 2nd 2012. Ten pots were planted with PsJN inoculated Alamo seedlings and ten pots were planted with uninoculated control seedlings.

2.2. Bacterial endophyte culture conditions

B. phytofirmans strain PsJN-GFP was obtained from Dr. Angela Sessitsch (Austrian Institute of Technology, Seibersdorf, Austria). PsJN-GFP cultures were streaked on King's B (KB) solid medium as previously described in (Pillay and Nowak, 1997). Inoculum was produced by transferring one loop of PsJN from 2-day-old cultures to 5 ml KB broth in a 15-ml culture tube, followed by incubation at 28 °C on a shaker (220 rpm) overnight. 5 ml of the overnight PsJN cultures were added to 45 ml KB broth in a 250-ml Erlenmeyer flask and grown to 0.7 OD_{600}. Bacterial cells were collected by centrifugation at 3500 rpm for 7 min at 4 °C, then re-suspended in PBS buffer (10 mM NaH_2PO_4 containing 0.8% NaC1, pH 6.5), after which the OD_{600} was adjusted with PBS buffer to 0.5.

2.3. 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 from April 2nd 2012 to April 16th 2012 (simulating average growing season precipitation for central Virginia). The first drought cycle (mild drought preconditioning) started two-weeks after transplanting. Five pots of inoculated seedlings and five pots of non-inoculated seedlings were randomly chosen and received only 425 ml water/week while the rest of pots remained well watered. Following this 40-day water reduction (April 16th 2012 to May 25th 2012) all plants were rehydrated to field capacity for 10 days (May 25th 2012 to June 4th 2012). The second severe drought cycle started after full hydration on June 4th 2012. All plants received no further water until photosynthesis (Ps) reached zero, which was monitored by a LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) each day at ambient relative humidity. Immediately, the pots were rehydrated to field capacity. The plants were harvested individually when the photosynthetic rates recovered and became stable. Harvesting of switchgrass was finished on July 3rd 2012.

2.4. 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. Relative growth rates (RGR) were calculated as the following equation: $RGR = \ln[(Height_{Dn+1} - Height_{Dn})/(D_{n+1} - D_n)]$. D_n and $Height_{Dn}$ indicated age and average height of switchgrass in the same pot at the n_{th} measurement, respectively. D_{n+1} and $Height_{Dn+1}$ indicated age and average height of switchgrass in the same pot at the $(n+1)_{th}$ measurement, respectively. Tiller number of each plant was recorded every 3–4 days from the 4th week of the experiment. 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 the first drought preconditioning cycle to the beginning of the 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 with a LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) at ambient relative humidity and the following settings: 3000 $\mu mol\, m^{-2}\, s^{-1}$ photosynthetic photon flux density (PPFD), 400 $\mu mol\, mol^{-1}$ reference CO_2 concentration, 25 °C block temperature, and a flow rate of 385 $\mu mol\, s^{-1}$. During the gas exchange measurement, a leaf from the same pot was excised at the leaf collar and plant water potential was determined 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.

2.5. Experimental design and analysis

A completely randomized design with two factors and repeated measures was used in this experiment. Five replicates were assigned to each treatment. Differences in biometric parameters between different treatments were investigated with a Student

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