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TECHNICAL ADVANCE

Inoculation of hybrid poplar with the endophytic bacterium *Enterobacter* sp. 638 increases biomass but does not impact leaf level physiology

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Abstract

Endophytic bacteria have been shown to provide several advantages to their host, including enhanced growth. Inoculating biofuel species with endophytic bacteria is therefore an attractive option to increase the productivity of biofuel feedstocks. Here, we investigated the effect of inoculating hard wood cuttings of *Populus deltoides* Bartr. × *Populus. nigra* L. clone OP367 with *Enterobacter* sp. 638. After 17 weeks, plants inoculated with *Enterobacter* sp. 638 had 55% greater total biomass than un-inoculated control plants. Study of gas exchange and fluorescence in developing and mature leaves over a diurnal cycle and over a 5 week measurement campaign revealed no effects of inoculation on photosynthesis, stomatal conductance, photosynthetic water use efficiency or the maximum and operating efficiency of photosystem II. However, plants inoculated with *Enterobacter* sp. 638 had a canopy that was 39% larger than control plants indicating that the enhanced growth was fueled by increased leaf area, not by improved physiology. Leaf nitrogen content was determined at two stages over the 5 week measurement period. No effect of *Enterobacter* sp. 638 on leaf nitrogen content was found indicating that the larger plants were acquiring sufficient nitrogen. *Enterobacter* sp. 638 lacks the genes for N_2 fixation, therefore the increased availability of nitrogen likely resulted from enhanced nitrogen acquisition by the 84% larger root system. These data show that *Enterobacter* sp. 638 has the potential to dramatically increase productivity in poplar. If fully realized in the production environment, these results indicate that an increase in the environmental and economic viability of poplar as a biofuel feedstock is possible when inoculated with endophytic bacteria like *Enterobacter* sp. 638.

Keywords: biomass, endophytic bacteria, *Enterobacter* sp. 638, leaf area, photosynthesis, poplar

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Introduction

The bottleneck limiting the full potential for the generation of cellulosic biofuels from biomass will likely shift toward feedstock productivity and sustainability as advances in feedstock quality, pretreatment and bioprocessing are made (Lynd *et al.*, 2008; Somerville *et al.*, 2010). Increased productivity will have several positive impacts on the biofuel industry. Improved productivity will reduce the encroachment of biofuels into natural ecosystems and reduce competition with food crops for arable land. The increased feedstock yield per unit land area will also reduce transport costs associated with the low energy density of lignocellulosic crops and significantly improve feedstock economics. (Heaton *et al.*, 2008; Lynd *et al.*, 2008). In short, increasing the productivity and sustainability of biofuel feedstocks is central to efforts to increase the environmental and economic viability of the biofuel industry.

Hybrid poplar, a short rotation energy crop that is typically managed using either coppice or single stem production systems, is a well-established, high yielding, cellulose and solid fuel, biofuel crop, and considerable

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effort has been made to improve the management and germplasm of this feedstock for biofuel production (Tuskan, 1998; Proe *et al*., 1999; Deckmyn *et al*., 2004; Karp & Shield, 2008; Di Nasso *et al*., 2010). One novel approach to increase productivity is to capitalize on the growth promoting effect of endophytic bacteria (Ryan *et al*., 2008; van der Lelie *et al*., 2009). Endophytic bacteria, which colonize the vascular tissue of the host plant, have been shown to have growth promoting effects in several species, including poplar (Ryan *et al*., 2008; Weyens *et al*., 2009b). The stimulation in growth is most commonly associated with improved access to nutrients facilitated by the endophytic bacterium, through either N_2-fixation, enhanced P-mobilization or Fe-chelation, or through the production of phytohormones, principally indole acetic acid (IAA), that increase root growth and proliferation and have also been implicated in stomatal regulation and drought tolerance (Ryu *et al*., 2003; Cho *et al*., 2008; Ryan *et al*., 2008; van der Lelie *et al*., 2009; Taghavi *et al*., 2009; Weyens *et al*., 2009a, b). These findings suggest that there is potential for improved productivity in biofuel species associated with endophytic bacteria and that this symbiosis may enable a more sustainable production of feedstocks on marginal soils where nutrient supply and periodic drought make land less suitable for agriculture (van der Lelie *et al*., 2009).

The endophytic bacterium *Enterobacter* sp. 638 was first isolated from the stem of a 10-year old hybrid poplar (*Populus trichocarpa* Torr. & Gray × *Populus deltoides* Bartr., clone H11-11). Inoculation with *Enterobacter* sp. 638 has been shown to confer marked growth promoting properties on the early development of hard wood cuttings of *Populus deltoides* Bartr. × *Populus nigra* L. clones DN-34 and OP367 (Taghavi *et al*., 2009). The genome of *Enterobacter* sp. 638 was recently sequenced (Taghavi *et al*., 2010). Unlike many other growth promoting endophytic bacteria, *Enterobacter* sp. 638 lacks the *nif* genes and is unable to fix nitrogen. However, the bacterium has the genes necessary for the production of the phytohormones IAA, acetoin and 2,3-butanediol. Previous experiments (S. Taghavi, unpublished results) have shown that *Enterobacter* sp. 638 is only able to produce low levels of IAA, <4 μg mL^{-1} after 48 h incubation with tryptophan under standard culture conditions (Schatz & Bovell, 1952; Glickmann & Dessaux, 1995). When grown in mineral media, *Enterobacter* sp. 638 does not produce acetoin or 2,3-butanediol, but when grown in the presence of plant extract or 0.2% sucrose, to simulate host photosynthate, both hormones were seen within 12 h. In addition, within 8 h of adding sucrose to cell cultures of *Enterobacter* sp. 638 a 200-fold and 70-fold increase in the abundance of transcripts encoding the enzymes responsible for acetoin and 2,3-butanediol synthesis was observed (Taghavi *et al*., 2010).

Recent investigation of the interaction between *P. deltoids* × *P. nigra* clones and *Enterobacter* sp. 638 have provided preliminary evidence for a stimulation of plant growth in the presence of the endophytic bacterium, and *in vitro* analysis has identified mechanisms that may underlie this response. These preliminary studies were conducted in hydroponic systems or on relatively young plants grown in small containers in a greenhouse (Taghavi *et al*., 2009). Small containers may result in a physical restriction of the rooting volume and lead to potential carbohydrate feedbacks that limit the positive growth response of the host plant to the endophytic bacterium (Arp, 1991). In addition, growth in a greenhouse uncouples the plant from its natural environment, potentially masking responses of stomatal conductance.

Here, for the first time, we have conducted an intensive physiological examination of gas exchange and a biomass harvest on poplar inoculated with *Enterobacter* sp. 638 grown outside in large (105 L) containers where physical restriction of the rooting volume would be minimized, and where the plant had a more natural coupling with its environment. We addressed the following three questions. (1) Does inoculation with *Enterobacter* sp. 638 enhance biomass in poplar? (2) If so, is increased C acquisition supported by improved physiology or increased leaf area? (3) Does *Enterobacter* sp. 638 alter photosynthetic water use efficiency in poplar?

Materials and methods

Plant material

Hardwood cuttings of *P. deltoides* Bartr. × *P. nigra* L., clone OP-367 were selected at random for inoculation with *Enterobacter* sp. 638. Two groups of seven nonsterile, ca. 23 cm hardwood cuttings were placed in 1 L of half-strength Hoagland's solution (Hoagland Basil Modified Salt Mixture; Phytotechnology Laboratories, Shawnee Mission, KS, USA), submerged to a depth of 12 cm and illuminated with fluorescent lighting (photosynthetic photon flux density was ca. 150 μmol m^{-2} s^{-1}) for 16 h each day for 8 days until roots were at least 1 cm in length. A flask containing 100 mL of 1 : 10 diluted Luria Broth (Sigma-Aldrich, St. Louis, MO, USA) was inoculated with *Enterobacter* sp. 638. The cultures were incubated overnight at 30°C at 150 rpm in an orbital shaker (3527 Environ Shaker; LabLine, Mumbai, India). The cultures were centrifuged at 5900 **g** for 10 min and resuspended in 2 mL of 10 mM $MgSO_4$. Cuttings were placed, in groups of seven, in plastic bags containing 400 mL of ½ strength Hoagland's solution and cells were added to reach a final concentration of 10^8 cfu mL^{-1}. After 3 days, the bacterial solution was replaced and plants were inoculated for an additional 3 days. Control plants were treated similarly, except no bacterial culture was added to the bags. Previous work with *gfp*-labeled strains of *Enterobacter* sp. 638 confirmed internal colonization of poplar roots, external colonization of the root surface

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was not detected (Taghavi *et al*., 2009). On May 4, 2010, following inoculation, cuttings had all but the leader shoot removed. Each cutting was transferred to a large 104.5 L container (Econo-Grip EG-10000; Griffin Greenhouse and Nursery Supply Inc., Brookhaven, NY, USA) filled with a general purpose peat based growing medium (Pro-Mix BX). Each container was supported on blocks to avoid potential cross contamination of containers with leachate. Seven control plants and seven plants inoculated with *Enterobacter* sp. 638 were distributed randomly within the unshaded footprint of an old hoop house and protected from grazing herbivores with fencing (n = 7 plants for each treatment). Plants were watered daily and fertilized with 4 L of a multipurpose fertilizer (Peter's 20-20-20 General purpose, mixed as directed) every 2 weeks. Most measurements were made on developing and mature leaves, leaves at leaf plastochron index (LPI) 6 and 11 were selected to represent developing and mature leaves, a reference length of 25 mm for LPI 0 was used to calculate plastochron index (Erickson & Michelini, 1957).

Biomass, leaf area, and leaf N content

The total leaf area in each plant was determined on August 30, 2010. Leaves were removed from the plants by cutting the petiole at the lamina. Bags of leaves were then transferred to the laboratory and total leaf area determined using a high throughput leaf area meter (LI-3100C; LI-COR, Lincoln, NE, USA). The remaining above ground biomass was removed at the soil surface, cut into ca. 5 cm sections, and transferred to a custom built forced-air drying oven. Over the next 3 days, root biomass was separated from the soil using a coarse screen (5 mm). Retrieved roots were washed and transferred to the drying oven. Leaves, shoots, and roots were dried to constant mass at 45°C before determination of dry mass. Leaf N content was determined by dry combustion with an elemental analyzer (PE 2400 Series II CHN analyzer; Perkin Elmer, Waltham, MA, USA) as described previously (Ainsworth *et al*., 2007).

Leaf gas exchange and fluorescence

Gas exchange measurements followed an approach that has been described previously (Rogers *et al*., 2004; Bernacchi *et al*., 2006). Leaf CO_2 uptake (A), stomatal conductance (g_s) and modulated fluorescence were measured using a portable open path gas exchange system with an integrated fluoremeter (LI-COR 6400XT; LI-COR). Prior to each measurement period, ambient temperature and photosynthetically active radiation (PAR) were measured using the leaf thermocouple and the external quantum sensor on the cuvette. Measured values were used to set the cuvette block temperature and the incident PAR. The CO_2 concentration inside the cuvette was set to 380 μmol mol^{-1}. The humidity of the air inside the chamber was not controlled and was dependent on ambient conditions. These parameters were held constant for the duration of a given set of measurements such that all leaves measured within a given time period were surveyed under the same conditions. Gas exchange and fluorescence parameters were recorded once A and g_s stabilized, usually within 60 s. Net CO_2 assimilation and g_s were determined as described previously (von Caemmerer & Farquhar, 1981). The analysis of the variation in A and g_s with LPI was carried out over 2 days during the middle 3 h of each day. All plants were measured but not all LPIs were able to be measured on every plant, for a given LPI n = 3–7, LPI 17 and 18 were omitted due to missing leaves from prior sampling and folivory. Diurnal measurements of gas exchange and fluorescence were conducted on four plants from each treatment selected at random for measurement at each time point. Midday survey measurements were made on developing (LPI 6) and mature (LPI 11) leaves at approximately weekly intervals between June 20th and July 22nd during the period 1 h either side of solar noon. The maximum quantum efficiency of photosystem II photochemistry (F_v/F_m) was measured in leaves that were dark adapted using clips designed for use with the LI-6400XT. Preliminary investigations determined that a 1 h dark adaption was sufficient to oxidize the quinone acceptor pool to an extent that F_v/F_m measurements made after 1 h were indistinguishable from measurements made after a 24 h dark adaptation.

Statistical analysis

Significant effects of inoculation on biomass were detected using a t-test. Effects of inoculation and LPI, and inoculation and time of day were detected using a 2-way ANOVA. Effects of inoculation and LPI over the 5 week measurement campaign were detected using a 2-way ANOVA with DOY considered a repeated measure. A power analysis revealed that the probability of a type II error was low. For example, there was an 80% chance of detecting a 15% difference in photosynthesis and a 90% chance of detecting a 10% difference in leaf N content when α = 0.05.

Results

Biomass, leaf area and leaf N content

Plants inoculated with *Enterobacter* sp. 638 had 55% greater total biomass ($t_{6(2)}$ = 2.45, P = 0.030) resulting from a significant 84% stimulation in root biomass ($t_{6(2)}$ = 2.62, P = 0.024), a nonsignificant 38% increase in shoot biomass, and a significant 48% increase in leaf mass ($t_{6(2)}$ = 2.40, P = 0.033, Fig. 1). The large increase in root biomass relative to shoot biomass resulted in a marginally significant 23% increase in root to shoot ratio ($t_{5(2)}$ = 1.90, P = 0.083, data not shown). There was no significant effect on leaf mass area (48 ± 2 and 50 ± 2 g m^{-2} for control and inoculated plants, respectively) and trends in leaf area followed leaf mass exhibiting a 39% increase in plants inoculated with *Enterobacter* sp. 638 ($t_{6(2)}$ = 2.65, P = 0.021, data not shown). Mean plant height was not significantly different (162 ± 6 and 176 ± 12 cm for control and inoculated plants, respectively). There was no correlation between transplanted fresh weight of the hardwood cuttings and

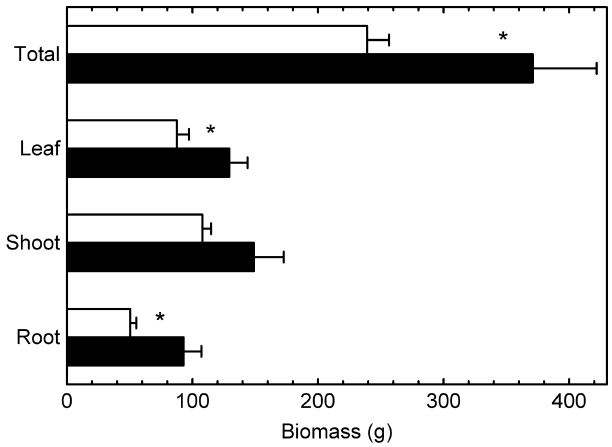

Fig. 1 Total biomass and component leaf, shoot and root biomass of *Populus deltoides* Bartr. × *Populus nigra* L., clone OP-367 grown outdoors in large (105 L) containers for one growth season. Prior to planting, hardwood cuttings were inoculated with *Enterobacter* sp. 638 (filled bars) and are compared with control plants (open bars) that were not inoculated. Bars show mean + SE ($n = 7$ plants). For control plants, total biomass does not equal the sum of leaf, shoot and root biomass due to missing data for one replicate root biomass sample ($n = 6$), therefore for the control plants $n = 6$ for total biomass. Significant (*$P < 0.05$) effects due to *Enterobacter* sp. 638 were identified using a *t*-test.

final biomass ($r^2 = 0.005$, data not shown). Leaf N content was markedly lower in older leaves ($F_{1,24} = 115$, $P < 0.0001$) and also significantly lower later in the growth season ($F_{1,24} = 14.5$, $P = 0.001$), but there was no effect of inoculation on either recently matured leaves or developing leaves ($F_{1,24} = 0.00$, $P = 0.985$, Supporting Information, Table S1).

Leaf gas exchange and fluorescence

A survey of photosynthetic parameters revealed expected age related effects on leaf development, i.e. significantly greater A and g_s in mature leaves (Fig. 2, panels a and b) but no significant effect of inoculation with *Enterobacter* sp. 638 was detected (Table S2). There was no significant effect of inoculation on photosynthetic water use efficiency (A/g_s) which varied by less than 2% between control and inoculated plants ($F_{1,117} = 0.189$, $P = 0.664$, data not shown). With the exception of very young leaves, the ratio of the CO_2 concentration inside the intercellular space of the leaf to the CO_2 concentration in the ambient air ($c_i : c_a$) was maintained at ca. 0.7 in all leaves and there was no effect of inoculation (Fig. 2, panel c), indicating that the stomatal limitation on photosynthesis was unaffected by the presence of the endophytic bacterium. There was a significant effect of leaf development on the maximum efficiency of photosystem II (F_v'/F_m'; Fig. 2, panel d, Table S2), but no effect of inoculation. There were no effects of leaf development or inoculation on the operating efficiency (F_q'/F_m') or efficiency factor (F_q'/F_v') of photosystem II (Fig. 2, panels e and f, Table S2). To assess whether inoculation with *Enterobacter* sp. 638 impacted diurnal dynamics, we conducted measurements over the course of a photoperiod. As expected, there was a significant effect of time of day on these parameters, that was dependent upon incident light levels. There was no effect of inoculation on leaf temperature, A, g_s, F_v'/F_m' or F_q'/F_m' (Fig. 3, Table S3). These trends were confirmed in a 5 week study of gas exchange and fluorescence in developing (LPI 6) and recently matured (LPI 11) leaves. Significant effects of measurement date and leaf development on A, g_s, F_v'/F_m' and F_q'/F_m' were detected (Supporting Information, Figs S1 and S2, Table S4). However, there was no significant effect of inoculation on any of these parameters, except A which showed a significant 49% reduction in developing leaves. In developing leaves, this 49% reduction was equivalent to an average reduction in A of <2 μmol m^{-2} s^{-1}. Such a small reduction in C acquisition in one cohort of leaves with a relatively low A is unlikely to negatively impact whole canopy C acquisition given the large increase in leaf area. There was also no effect of inoculation on photosynthetic water use efficiency ($F_{1,22} = 1.465$, $P = 0.239$, data not shown). During the fifth week of measurements a survey of dark adapted F_v/F_m was conducted. Similarly, no impact of inoculation was found (Table S5).

Discussion

Poplar inoculated with *Enterobacter* sp. 638 showed a marked increase in biomass supported by increased C acquisition from a substantially increased leaf area and improved nutrient acquisition from a dramatically increased root biomass. If fully realized in the production environment, these results indicate that poplar inoculated with *Enterobacter* sp. 638 will have greater productivity, markedly improving the environmental and economic viability of poplar as a biofuel feedstock.

No effects of inoculation on leaf level photosynthetic parameters, photosynthetic water use efficiency or leaf N content were detected. We examined developing and mature leaves over a 5 week measurement period and a diurnal time course. There was no evidence that leaves on plants inoculated with *Enterobacter* sp. 638 had any improved leaf level characteristics that would provide an advantage to inoculated plants. It was clear that increased capacity for C acquisition was entirely attributable to the increase in leaf area. Plants inoculated with *Enterobacter* sp. 638 had 48% more leaf mass, but had

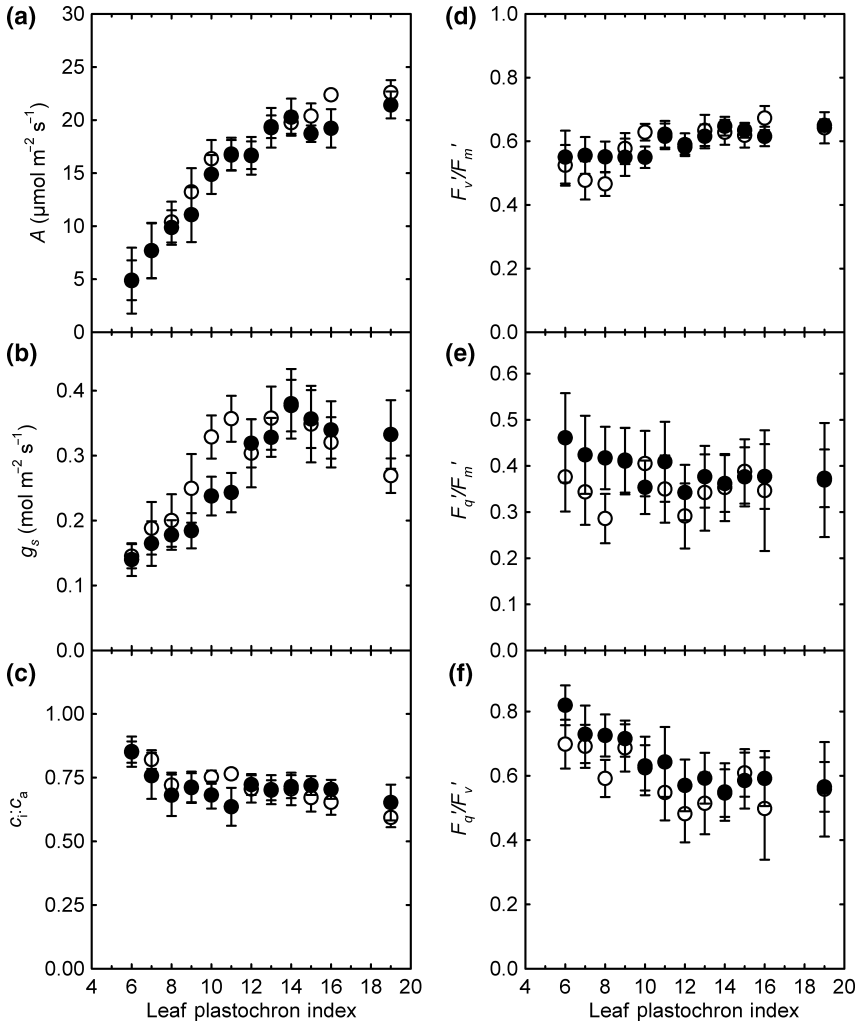

Fig. 2 CO_2 uptake (A, panel a), stomatal conductance (g_s, panel b), the ratio of the CO_2 concentration inside the intercellular space of the leaf to the CO_2 concentration in the ambient air ($c_i : c_a$, panel c), the maximum efficiency of photosystem II (F_v'/F_m', panel d), the operating efficiency of photosystem II (F_q'/F_m', panel e) and the efficiency factor of photosystem II (F_q'/F_v', panel f) in the plants described in Fig. 1. The nomenclature for fluorescence parameters follows (Baker, 2008). Open symbols show data from control plants and filled symbols show data from plants inoculated with *Enterobacter* sp. 638. Measurements were made on all plants on available leaves. Data show mean ± SE where n = 3–7 available leaves at each leaf plastochron index (LPI). Leaves denoted by LPI 17 and 18 were either missing or damaged and were not measured.

the same leaf N content as control plants, providing clear evidence that plants inoculated with *Enterobacter* sp. 638 had improved access to N and possibly other nutrients. The enhanced nutrient acquisition in plants inoculated with *Enterobacter* sp. 638 is most likely attributable to the 84% greater root biomass. Previous work in short-term studies on nutrient poor soil, and in hydroponics, had identified a significantly greater growth index in poplars inoculated with *Enterobacter* sp. 638 (Taghavi *et al*., 2009), our data supports these preliminary findings. Together, this previous work, and the data presented here, suggest that the plant growth promoting effects of *Enterbacter* sp. 638 may be realized over a wide range of soil types (Taghavi *et al*., 2009). In addition, we have provided new data on the effect of *Enterobacter* sp. 638 on root biomass that are consistent with mechanisms thought to underlie the growth promoting response of *Enterobacter* sp. 638, i.e. synthesis of acetoin and 2,3-butendiol that results in the stimulation of root development (van der Lelie *et al*., 2009; Taghavi *et al*., 2009).

There was no effect of inoculation on stomatal conductance, the stomatal limitation of photosynthesis or photosynthetic water use efficiency in mature and developing leaves, over a diurnal time course and over a 5 week measurement period. This strongly suggests

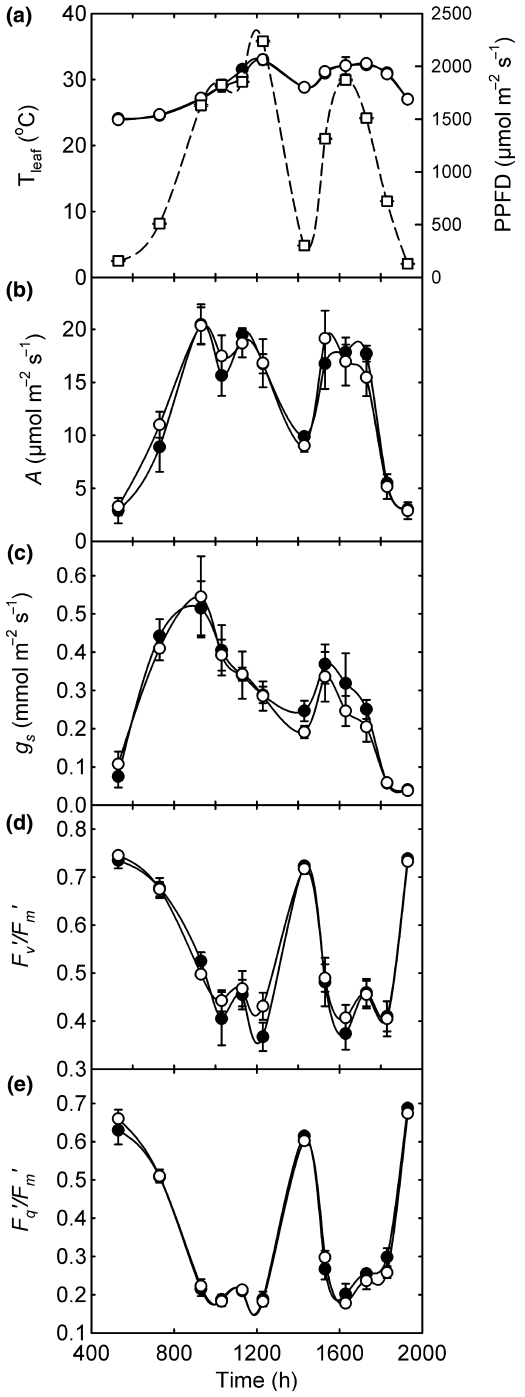

Fig. 3 Leaf temperature (T_{leaf}, panel a, solid line), photosynthetic photon flux density (PPFD, panel a, squares, broken line), CO_2 uptake (A, panel b), stomatal conductance (g_s, panel c), maximum efficiency of photosystem II (F_v'/F_m', panel d) and the operating efficiency of photosystem II (F_q'/F_m', panel e) in the plants described in Fig. 1, measured over a diurnal time course on DOY 203 on mature (LPI 11) leaves. Open circles show data from control plants and filled circles show data from plants inoculated with *Enterobacter* sp. 638. Data show mean ± SE where $n = 4$ plants. The nomenclature for fluorescence parameters follows (Baker, 2008).

that that whole plant water use would increase by approximately 40%, in parallel with the increase in leaf area. Therefore, it is likely that poplar inoculated with *Enterobacter* sp. 638 will require greater access to water to support the observed accelerated development. It is possible that the more developed root system in plants inoculated with *Enterobacter* sp. 638 may be able to meet this demand and access water available in deeper soil.

The markedly increased root growth in poplar inoculated with *Enterobacter* sp. 638, and the marginally significant increase in root to shoot ratio suggests that the endophyte may also enhance the potential for C sequestration under plantations which have been inoculated with *Enterobacter* sp. 638. A recent analysis of factors influencing C sequestration under no-till replanted poplar plantations indicated that below ground C allocation was an important control on soil C sequestration (Garten *et al.*, 2011). However, increased below ground biomass is only one component contributing to long-term C sequestration (Garten *et al.*, 2011) and further investigation of C sequestration under poplars inoculated with *Enterobacter* sp. 638 managed in field conditions over several harvest cycles would be required to resolve the question.

This short-term (17-weeks) experiment shows that there is great potential for endophytic bacteria to enhance the productivity of biofuels. Improved root proliferation and increased leaf area may markedly aid the establishment of new plantations and reduce the requirement for weeding and fertilization during the cost intensive establishment phase of short rotation energy crops (Buhler *et al.*, 1998; Bauen *et al.*, 2010; Di Nasso *et al.*, 2010). Enhanced establishment will shorten the time taken to reach maximum growth rate and potentially reduce the time taken to reach peak production. Both reduced establishment costs and accelerated initial development will have obvious economic benefits. It is unclear if the endophytic bacteria, or the traits they confer, will persist in the host for an extended period of time where they might be able to enhance productivity of over longer management cycles, or repeated coppice cycles. Evidence from phytoremediation studies suggests that long-term residence in the host plant is a possibility (Weyens *et al.*, 2009a). However, it is not clear what advantage the endophytic bacterium will confer on established plantations that already have closed canopies and/or well-developed root systems.

In summary, we have clearly demonstrated that *Enterobacter* sp. 638 increased biomass in poplar but did not alter leaf level physiology or photosynthetic water use efficiency. In this short-term study, young plants, under favorable growth conditions, demonstrated impressive

increases in biomass due to inoculation with *Enterobacter* sp. 638. However, it is not clear if these findings will translate to the production environment. Extensive field trials are required to provide the answer.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Leaf N content in plants inoculated with *Enterobacter* sp. 638.

Table S2. F-statistics and *P*-values from Fig. 2.

Table S3. F-statistics and *P*-values from Fig. 3.

Table S4. F-statistics and *P*-values from Figs S1 and S2.

Table S5. Maximum quantum efficiency of photosystem II photochemistry in plants inoculated with *Enterobacter* sp. 638.

Figure S1. CO_2 uptake and stomatal conductance in mature and developing leaves of plants inoculated with *Enterobacter* sp. 638 measured on four occasions over a 5 week period.

Figure S2. The maximum efficiency of photosystem II and the operating efficiency of photosystem II in mature and developing leaves of plants inoculated with *Enterobacter* sp. 638 measured on four occasions over a 5 week period.

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