



Growth enhancement and drought tolerance of hybrid poplar upon inoculation with endophyte consortia[☆]

Zareen Khan^a, Hyungmin Rho^a, Andrea Firrincieli^b, Shang Han Hung^a, Virginia Luna^c, Oscar Masciarelli^c, Soo-Hyung Kim^a, Sharon L Doty^{a,*}

^a School of Environmental and Forest Sciences, University of Washington, Seattle, WA 98195-2100, USA

^b Department for Innovation in Biological, Agro-food and Forest systems, University of Tuscia, Viterbo 01100, Italy

^c Laboratorio de Fisiología Vegetal, Departamento de Ciencias Naturales, Fac. de Cs. Exactas, Universidad Nacional de Río Cuarto, Ruta 36 Km 601, 5800 Río Cuarto, Argentina

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ABSTRACT

With increasing effects of global climate change, there is a strong interest in developing biofuels from trees such as poplar (*Populus* sp.) that have high C sequestration rates and relatively low chemical inputs. Using plant-microbe symbiosis to maximize plant growth and increase host stress tolerance may play an important role in improving the economic viability and environmental sustainability of poplar as a feedstock. Based on our previous research, a total of ten endophyte strains were selected as a consortium to investigate the effects of inoculation on commercial hardwood cuttings of *Populus deltoides* × *P. nigra* clone OP-367. After one and a half months of growth under non-stress conditions followed by one month under water stress, there was substantial growth promotion with improved leaf physiology of poplar plants in response to the endophyte inoculation. Furthermore, inoculated plants demonstrated reduced damage by reactive oxygen species (ROS) indicating a possible mechanism for symbiosis-mediated drought tolerance. Production of important phytohormones by these endophytes and identification of microbial genes involved in conferring drought tolerance suggests their potential roles in the modulation of the plant host stress response.

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1. Introduction

Hybrid poplars are increasingly being considered as the premier woody perennial candidate for bioenergy feedstock production because of their ability to produce a significant amount of biomass in a short period of time and their high cellulose and low lignin content [1–4]. Hybrid poplar tree farms are established where there is sufficient water as increased productivity is associated with adequate growing-season precipitation [5,6]. As a consequence, productivity closely depends on water availability and could seriously limit yields at plantation sites where water availability is insufficient. Climate change models suggest that more frequent drought events of greater severity and length can be expected in the coming decades. Consequently, commercial genotypes that have

high water use efficiency or increased drought tolerance, in addition to the traits such as high productivity, resistance to pests and insects, and improved wood quality are being used in poplar selection. However, this may not be simple to achieve because some of these beneficial traits may need to be compromised for others [7].

It has been demonstrated that in areas with abiotic stress factors, plants are more dependent on microorganisms that are able to enhance their ability to combat stress [8–12]. Among these plant-associated microbes, the role of endophytes (bacteria or fungi living inside plants) in stimulating plant growth and nutrition, in addition to increasing stress tolerance of their host plants is gaining more attention [13–16]. These microbial symbionts may confer benefits to their host plants via multiple mechanisms including biological nitrogen fixation [17,18] enhancing the bioavailability of phosphorous (P), iron (Fe) and other mineral nutrients [19], production of phytohormones including indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA), brassinosteroids (BR), jasmonates (JA), salicylic acid (SA) [20–23], generation of antioxidants [24–27] for increased plant productivity and tolerance to biotic or abiotic stresses. Another key factor may be microbial production of

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* Corresponding author.

E-mail address: sldoty@uw.edu (S.L. Doty).

1-aminocyclopropane-1-carboxylate (ACC) deaminase to decrease plant stress [10,28]. Although the interaction between endophytes and their host plants is not fully understood, several studies have demonstrated the positive effects of inoculation of endophytes to increase plant productivity and enhance drought tolerance as a result of multiple mechanisms [10,12,26,27,29]. Recently, the availability of genome sequences of important plant growth promoting endophytes is providing new insight into the biosynthetic pathways involved in plant-endophyte symbiosis, leading to optimization of this technology to increase plant establishment and biomass production.

The aim of the present study was to test the ability of an endophyte consortium to confer drought tolerance and to investigate the underlying mechanisms of endophyte-induced drought tolerance of a commercially-important hybrid poplar clone by monitoring physiological parameters, assaying for ROS activity and analyzing phytohormone production by endophytes in axenic medium. Finally, genome annotations of *Rhodotorula graminis* WP1, *Burkholderia vietnamiensis* WPB, *Rhizobium tropici* PTD1, *Rahnella* sp. WP5, *Acinetobacter calcoaceticus* WP19 and *Enterobacter asburiae* PDN3 allowed identification of genes known to be involved in improving plant growth under drought stress.

2. Materials and methods

2.1. Endophyte strains and inoculum preparation

9 bacteria and 1 yeast strain previously isolated from poplar and willow trees growing in stressful environments [30–32] were selected based on their plant growth promoting abilities under nitrogen-limitation and drought stress on a variety of plants and grasses [33–37]. These are as follows: WP1 (*Rhodotorula graminis*), WPB (*Burkholderia vietnamiensis*), PTD1 (*Rhizobium tropici*), WP19 (*Acinetobacter calcoaceticus*), WP5 (*Rahnella* sp.), WP9 (*Burkholderia* sp.), PDN3 (*Enterobacter asburiae*), WW5 (*Sphingomonas yanoikuyae*), WW6 (*Pseudomonas* sp.), and WW7 (*Curtobacterium* sp.). For inoculum preparation, each isolate was grown in 25 ml MG/L [38] and incubated at 30 °C under shaking conditions for 24 h. To prepare the inoculum, cells were harvested by centrifugation at 8000 rpm at 4 °C for 10 min, resuspended in half strength Hoagland's solution [39] and the cell density of each strain was adjusted to produce an inoculum with a final optical density (OD₆₀₀) of 0.1.

2.2. Plant materials, growth conditions, drought stress

Woody stem cuttings of *Populus deltoides* x *P. nigra* clone OP367 were obtained from the Boardman Research Site near Boardman, Oregon (GreenWood Resources Inc.). Two groups of cuttings (approx. 15 cm) were washed and soaked overnight in sterile water. The next day, twenty cuttings were transplanted into Sunshine Mix #4 soil (Steubers Inc. USA) and grown in the greenhouse under the following conditions: average temperature of 22.3 °C, average relative humidity of 61.42% and the average photosynthetic photon flux density (PPFD) of 290.9 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and 14/10-h light/dark photoperiod with supplementary high-pressure sodium light bulbs. After two weeks, 100 ml of the inoculum was poured at the base of the stem to one group (n = 10) of randomly selected plants. The control plants (n = 10) were mock-inoculated with 100 ml of half strength Hoagland's solution. After one and a half months of colonization, all the plants were subjected to drought by withholding water for one month after which they were harvested and separated into roots and stems. The samples were oven-dried at 70 °C, ground and weighed. For total nitrogen analysis, the oven dried root samples were ground by a plant grinder, passed through a 20 mesh screen

and analyzed on a PE 2400 series II CHN analyzer (University of Washington, SEFS Chemical Analysis Center).

2.3. In-vitro production of phytohormones by the endophytic isolates

The endophyte strains were grown in M9 minimal medium (with tryptophan added for the IAA analysis) to exponential growth phase (10E+9) and centrifuged separately at 8000 rpm at 4 °C for 15 min. Supernatants were acidified at pH 2.5 with acetic acid solution (1% v/v), and 50 ng of deuterated ²H₆-ABA, ²H₄-SA, ²H₂-GA₃, ²H₆-JA, and 2H5-AIA (OlChemIm Ltd, Olomouc, Czech Republic) were added as internal standards. Each sample in triplicate was partitioned four times with the same volume of acetic acid-saturated ethyl acetate (1%, v/v). After the last partition, acidic ethyl acetate was evaporated to dryness at 36 °C in a Speed-Vac concentrator. Dried samples were dissolved in 1500 μl methanol, filtered and resuspended in 50 μl methanol (100%), and placed in vials. Analysis was done by Liquid Chromatography with Electrospray Ionization (LC) (Waters Corp., New York, NY, USA). The instrumental parameters are described elsewhere [40].

2.4. Effects of endophytic colonization on Fv/Fm, chlorophyll and stomatal conductance

The following plant physiological parameters were recorded from fully expanded second or third youngest leaves of both irrigated and drought-stressed poplar cuttings at midday (between 12–1 pm) every 4–5 days before and after the drought stress treatment.

2.4.1. Photochemical efficiency of PSII (F_v/F_m)

Maximal photochemical efficiency is inversely proportional to damage to photosystem II (PSII) and this parameter was used to assess photosynthetic stress experienced by the poplar plants grown under drought stress. This was performed by using a portable fluorometer OS-30P+ (Opti-Sciences, Inc., Hudson, NH, USA). The samples were dark-adapted for 30 min before taking minimal fluorescence, F_0 , followed by illuminating a saturating light flash to gain maximal fluorescence, F_m . Variable fluorescence, $F_v = (F_m - F_0)$, was calculated by a built-in program to estimate maximal photochemical efficiency of PSII (F_v/F_m) [41].

2.4.2. Indirect measurement of chlorophyll content using SPAD

Leaf chlorophyll content in vivo was measured using a SPAD 502 (Konica Minolta Sensing Americas, Inc., Ramsey, NJ, USA) hand-held chlorophyll meter. The instrument measures 'greenness of leaves' which is tightly correlated with the in vitro chlorophyll content of samples [42].

2.4.3. Measurement of stomatal conductance (g_s)

A steady state leaf porometer SC-1 (Decagon Devices, Inc., Pullman, WA, USA) was used to measure stomatal conductance (g_s) of poplar leaves at the midday. This time point was chosen based on preliminary results that indicated that the inoculation effects on g_s were most remarkable from 12 p.m. to 3 p.m. (data not shown).

2.5. Reactive oxygen species (ROS) assay

Using a cork borer, 3 leaf disks (2 mm) were obtained from each of 3 plants from the inoculated or control group and incubated in a solution of 1 μM of the herbicide paraquat (*N,N'*-Dimethyl-4,4'-bipyridinium dichloride) and incubated at 22 °C under fluorescent lights [43,44]. After 48 h exposure to paraquat, leaf disks were pho-

tographed to document chlorophyll oxidation visualized by tissue discoloration. All assays were performed three times.

2.6. Genomic analysis

The poplar bacterial endophyte strains, PTD1, WPB, WP5, WP9, WP19 and PDN3 were grown on NL-CCM agar medium [45]. Five ml MG/L broth or TYC broth [25] were inoculated with the strains and genomic DNA was subsequently isolated and purified using protocols provided by the Joint Genome Institute (JGI). All bacterial endophyte strains were sequenced by the Joint Genome Institute (JGI) and were annotated using JGI's microbial annotation pipeline [46]. Annotations and comparative analysis of all genomes are available through the Integrated Microbial Genome system [47]. The WP1 yeast genomic DNA was isolated, sequenced, and analyzed as described [48]. The JGI annotated genomes were analyzed for genes that have been reported to have beneficial effects during plant growth under drought stress conditions and for tolerance against oxidative stress. In this context, the presence of the following genes: *acdS*, *pqqABCDEF*, *budABC* genes involved in the biosynthesis of ethylene, pyrrolo-quinolone quinine (PQQ) and 2R,3R-butanediol, respectively, were assessed [49,50]. Moreover, genes involved in the trehalose biosynthesis were also considered due to their importance in tolerance against desiccation and drought-like stress [51–53]. Potential annotation gaps were detected as described [54]. For the analysis of putative genes encoding for a functional ACC deaminase, the amino acid sequences computed from *acdS* genes and D-cysteine desulhydrase encoding genes were aligned in ClustalW2 software using the *acdS* gene from *Pseudomonas putida* strain UW4 as a reference (AY823987.1). For the detection of *pqqA* in *Acinetobacter calcoaceticus* WP19, the intergenic region of 396 bp upstream to *pqqB* was compared to all the genomes present in IMG database using BLASTX 2.2.26+ [55]. The annotated genome of *Rahnella aquatilis* HX2 (NCBI/RefSeq: CP003403; CP003404; CP003405; CP003406) and *Rahnella* sp. WP5 were compared to each other to identify genes without homologues in WP5. These genes of interest were compared against the WP5 genome by using TBlastn.

2.7. Statistical analysis

The effects of the drought stress and inoculation were evaluated using the paired *t*-test procedure for the time series data (*SPAD*, F_v/F_m , and g_s). Initial values measured before the drought treatment (0 days after drought stress, 0 DDS) were used to compare the drought effects on the variables at each time point. Control vs. Inoculated comparison at each time point was conducted using the same paired *t*-test procedure. The biomass allocation and N content data were collected only after the drought stress at harvest, so the simple two-tailed *t*-test procedure was used to test the inoculation effects on these variables. All the statistical analyses were applied to ten replicated samples per treatment. RStudio v.0.98.945 [56] was used for conducting the statistical analyses.

3. Results

3.1. Biomass, N content, wilting response

At harvest, the inoculated plants had a significant 28% ($P < 0.001$) increase in total biomass resulting from a 42% stimulation in root biomass ($P < 0.001$), 43% ($P < 0.001$) higher total plant nitrogen, and a 21% ($P < 0.001$) increase in shoot biomass (Fig. 1). The large increase in the root biomass relative to shoot biomass resulted in a significant 23% ($P < 0.001$) increase in root to shoot ratio. At 20 days of drought stress (DDS), the leaves of the control plants started browning whereas the leaves of the inoculated plants remained

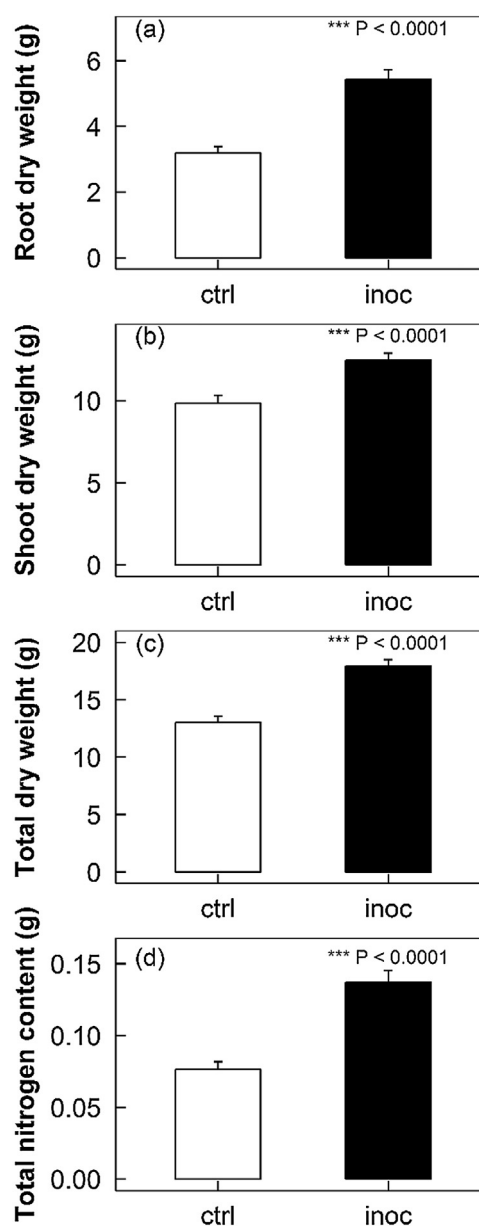


Fig. 1. Biomass and nitrogen content of the poplar plants in response to the drought stress treatment at harvest (31 days after the drought treatment). (a) Root dry weight; (b) shoot dry weight; (c) total dry weight; (d) total nitrogen. Open and closed bars represent the means of each response from the mock-inoculated control (ctrl) and the inoculated (inoc) samples, respectively. The bars represent means from ten replicated samples of each group along with error bars standing for standard errors of the means. The *P*-values for the *t*-statistics are presented where the differences are significant.

green. Complete wilting of all the mock-inoculated control plants was observed at 1 month of drought stress whereas most of the endophyte-treated plants had retained turgor (Fig. 2).

3.2. Analysis of culture filtrates for quantification of phytohormones

As seen in Table 1, all the selected endophyte strains produced IAA, GA₃, SA, ABA, JA and Brs in different concentrations. However, each microorganism varied in the type and quantity of the compound produced in our experimental conditions. The yeast endophyte-strain WP1 was the major producer of IAA and GA₃ while willow endophyte WW7 produced the most ABA. Isolate

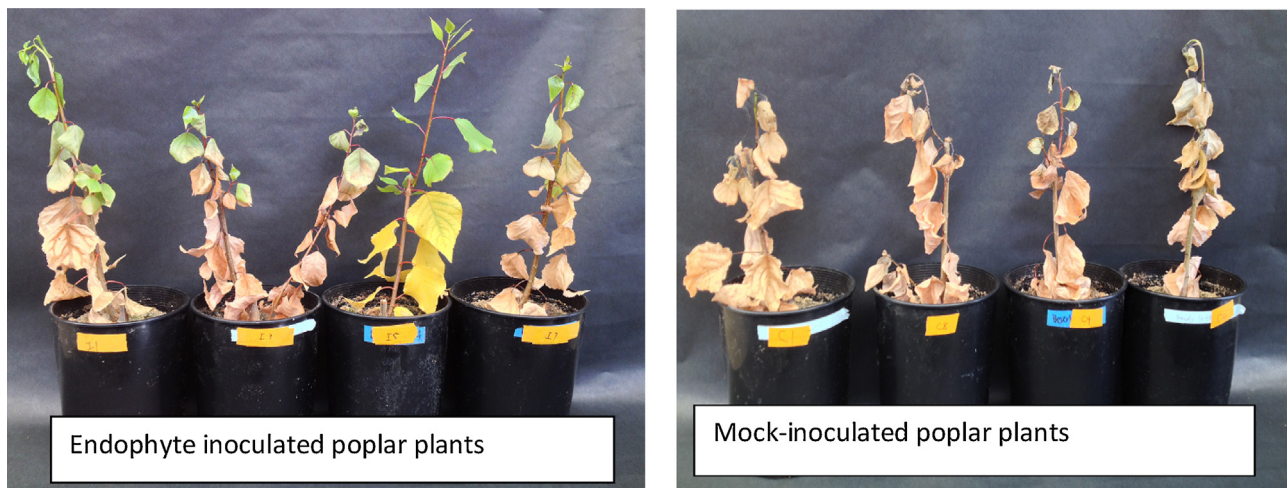


Fig. 2. Photo of four representative plants showing the effects of one month of drought stress in inoculated poplars (on the left) and mock-inoculated poplars (on the right) under greenhouse condition.

Table 1

Phytohormone production (SA, salicylic acid; ABA, abscisic acid; IAA, indole-3-acetic acid; JA, jasmonic acid; GA₃, gibberellins-3-acid; Brs, epibrassinolides) in exponential growth cultures of endophytes.

Endophyte strains and 16S rRNA match	SA	JA	IAA	ABA	GA ₃	Brs
WP1 (<i>Rhodotorula graminis</i>)	8.224 ± 1.12	0.613 ± 0.05	61.308 ± 1.34	0.435 ± 0.02	2.694 ± 0.25	4.306 ± 0.17
WPB (<i>Burkholderia vietnamiensis</i>)	1.729 ± 0.13	1.799 ± 0.23	3.293 ± 0.26	0.416 ± 0.02	0.729 ± 0.02	nd
PTD1 (<i>Rhizobium tropici</i>)	2.853 ± 0.18	2.469 ± 0.21	55.847 ± 2.02	0.436 ± 0.03	0.972 ± 0.03	7.699 ± 0.65
WP19 (<i>Acinetobacter calcoaceticus</i>)	9.76 ± 0.2	0.165 ± 0.02	17.727 ± 1.84	0.66 ± 0.03	2.275 ± 0.21	0.426 ± 0.04
WP5 (<i>Rahnella</i> sp.)	4.627 ± 0.37	0.171 ± 0.02	2.429 ± 0.26	0.405 ± 0.02	1.189 ± 0.25	4.563 ± 0.42
WP9 (<i>Burkholderia</i> sp.)	0.904 ± 0.009	0.462 ± 0.04	0.569 ± 0.02	0.405 ± 0.02	1.674 ± 0.16	2.26 ± 0.2
WW5 (<i>Sphingomonas yanoikuyae</i>)	48.519 ± 2.86	0.468 ± 0.04	5.627 ± 0.42	0.411 ± 0.02	0.562 ± 0.04	3.226 ± 0.32
WW6 (<i>Pseudomonas</i> sp.)	2.459 ± 0.46	0.194 ± 0.02	1.666 ± 0.2	0.404 ± 0.02	0.964 ± 0.03	8.849 ± 0.63
WW7 (<i>Curtobacterium</i> sp.)	1.378 ± 0.26	0.171 ± 0.02	9.141 ± 0.24	0.831 ± 0.03	1.045 ± 0.2	0.677 ± 0.05

Phytohormones are expressed in $\mu\text{g}/\text{ml}^{-1}$.

PTD1 produced significant amounts of IAA and JA and isolate WW5 produced high levels of SA.

3.3. Changes in plant physiology- greenness, chlorophyll fluorescence and stomatal conductance

Before withholding water from the poplar cuttings, indirect chlorophyll content measurements (SPAD) between the mock-inoculated control and inoculated poplar plants were comparable. After 19 DDS the difference of SPAD values of the control and inoculated plants began to increase, becoming largest at 31 DDS ($P=0.059$) (Fig. 3a and Table 2). The SPAD value of the inoculated cuttings was 66.0% higher than that of the control at 31 DDS. Up to 15 DDS there was no effect of the inoculation on potential quantum yield of photosystem II (PSII) (F_v/F_m) of the poplar leaves. At 19 DDS, however, the inoculated plants maintained the same level of F_v/F_m value compared to an abrupt decrease of F_v/F_m of the control plants. This resulted in 32.9% higher F_v/F_m in the inoculated poplars compared to the mock-inoculated control plants ($P=0.071$) (Fig. 3b and Table 2). Stomatal conductance (g_s) in the midday of both the control and inoculated groups showed a common response to water deficit; it steeply decreased 11 days after the imposition of the drought stress. However, it is noteworthy that the inoculated samples always had lower g_s throughout the drought period which was highlighted at 19 DDS up to 32.6% lower than the control (as the lowest $P=0.110$) (Fig. 3c and Table 2). This substantial decrease of g_s in the inoculated leaves coincided with the higher F_v/F_m values at 19 DDS.

3.4. Decreased ROS activity in response to inoculation

Chlorophyll bleaching of photosynthetic tissue by the herbicide paraquat is indicative of oxidation damage due to production of superoxide ions. When leaf disks were exposed to paraquat, after 48 h the tissues of endophyte inoculated plants remained green, indicating an absence of ROS generation whereas the mock-inoculated tissues no longer remained green, indicative of chlorophyll bleaching (Fig. 4).

3.5. Putative endophytic genes involved in drought tolerance

Genomic analysis revealed the presence of microbial genes characterized for having beneficial effects against host plant drought stress and improving tolerance to oxidative stresses (Table 3). The genome annotation of PTD1, WP1, WP19 and PDN3, revealed the presence of putative genes involved in the biosynthesis of (*R,R*)-butane-2,3-diol and acetoin. Putative genes predicted to encode ACC deaminase were detected in WP19, PDN3 and WP5. However, the amino acid sequences lacked residues Glu295 and Leu322 known to be important for ACC-deaminase activity, thus it likely has only D-cysteine desulphydrase activity as reported in other systems [57]. Only the gene in WPB predicted to encode ACC-deaminase showed a perfect match in amino acid residues (Supplementary Fig. S1). Genes required for PQQ biosynthesis (*pqqABCDEF*) that have been related to biological functions such as phosphate solubilization, antimicrobial activity and tolerance to oxidative stress were found in WP5 and WP19. In *Rahnella* sp. WP5, the *pqqA* gene that encodes for a small peptide of 25 amino acids was detected in the intergenic region upstream to the *pqqBCDE* operon (scaffold32: 29265–29660) by using the “Phylogenetic Pro-

Table 2
Paired *t*-test results of the means ± 1 S.E. (*n* = 10) of three plant physiological response variables (chlorophyll content, Fv/Fm, and *g*_s) over the drought stress period (DDS, days after drought stress). *P*-values of *t*-statistics of the drought effects are reported in a horizontal direction. (0 DDS vs. ~). *P*-values of the inoculation effects are reported in a vertical direction at each time point on the third rows (Control vs. Inoculated). See Section 2.7 for details. NA indicates that the measurements were not available due to seriously wilted leaf surfaces from the drought responses. Numbers in bold mean significant differences at *P* < 0.05 level.

Treatment	0 DDS	7 DDS	11 DDS	15 DDS	19 DDS	23 DDS	27 DDS	31 DDS
Chlorophyll content (SPAD)								
Control	28.53 ± 1.434	27.67 ± 1.057 (0.354)	26.93 ± 1.143 (0.062)	25.34 ± 0.881 (0.028)	21.60 ± 2.774 (0.029)	12.44 ± 2.914 (<0.001)	6.64 ± 1.523 (<0.001)	6.29 ± 1.079 (<0.001)
Inoculated	30.59 ± 1.647	30.18 ± 1.623 (0.535)	28.04 ± 1.460 (0.029)	27.40 ± 1.340 (0.002)	20.42 ± 3.924 (0.023)	14.83 ± 3.762 (0.004)	11.33 ± 3.101 (<0.001)	10.44 ± 1.665 (<0.001)
<i>P</i> > <i>t</i>	(0.295)	(0.240)	(0.571)	(0.2763)	(0.833)	(0.4031)	(0.211)	(0.059)
Fv/Fm (unitless)								
Control	0.754 ± 0.012	0.770 ± 0.006 (0.293)	0.774 ± 0.007 (0.154)	0.778 ± 0.007 (0.065)	0.596 ± 0.100 (0.165)	0.433 ± 0.177 (0.141)	NA	NA
Inoculated	0.754 ± 0.013	0.766 ± 0.013 (0.304)	0.770 ± 0.006 (0.256)	0.787 ± 0.004 (0.013)	0.792 ± 0.008 (0.061)	0.605 ± 0.147 (0.346)	NA	NA
<i>P</i> > <i>t</i>	(0.931)	(0.773)	(0.732)	(0.310)	(0.071)	(0.495)		
<i>g</i> _s (mmol H ₂ O m ⁻² s ⁻¹)								
Control	153.4 ± 21.97	229.3 ± 23.21 (<0.001)	251.1 ± 27.75 (0.002)	179.9 ± 22.92 (0.413)	110.8 ± 15.31 (0.253)	123.4 ± 25.34 (0.434)	67.6 ± 18.16 (0.024)	NA
Inoculated	129.0 ± 26.24	212.3 ± 19.79 (0.006)	233.9 ± 24.39 (0.001)	193.8 ± 25.42 (0.030)	75.7 ± 11.26 (0.112)	96.7 ± 23.54 (0.465)	74.9 ± 22.61 (0.198)	NA
<i>P</i> > <i>t</i>	(0.204)	(0.571)	(0.657)	(0.751)	(0.110)	(0.423)	(0.821)	

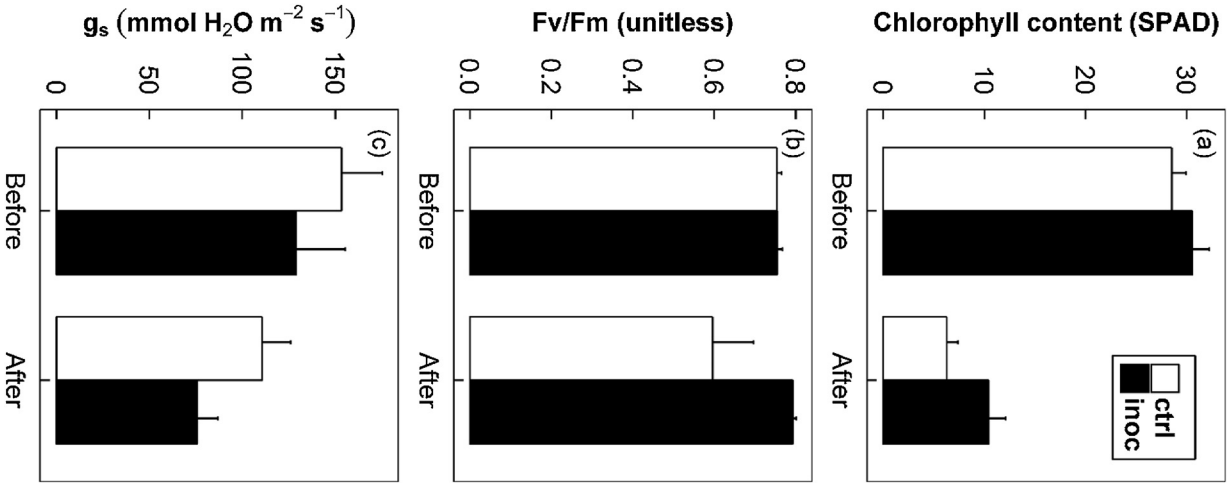


Fig. 3. Photosynthesis parameters of poplar leaves before and after the drought stress treatment. (a) Chlorophyll content; (b) potential quantum yield of PSII (Fv/Fm); (c) stomatal conductance (*g*_s). The After values for chlorophyll content was chosen at 31 days after drought stress, while as those for Fv/Fm and *g*_s were chosen at 19 days after drought stress because the rates of the physiological responses to drought differ by the process. The bars represent the means of each response from ten replicated mock-inoculated control (open bars) and inoculated groups (closed bars). Error bars stand for ± standard errors.

filer for Single Genes" tool. In a similar way *pqg4* was detected in the intergenic region of 396 bp (scaffold32: 29265–29660), upstream to *pqgB* in *A. calcoaceticus* WP19 (see Materials and methods). Finally, putative genes involved in the biosynthesis of trehalose via OtsA-OtsB from UDP-glucose and glucose 6-phosphate, were detected in WP1, PTD1, WP5, WP19 and PDN3 [58]. Two putative genes encoding for a malto-oligosyltrehalose trehalohydrolase (TreZ) and a malto-oligosyltrehalose synthase (TreY), respectively, were detected in WP5, PTD1 and PDN3. These genes are specific to the biosynthetic pathway TreY–TreZ which synthesizes trehalose from malto-oligosaccharides or alpha-1,4-glucans [59].

Table 3

List of putative drought tolerance genes in strains WP19, PDN3, WP5, PTD1, WPB and WP1.

Strain	Biological process	² Locus Tag	IMG Product Name
WP19	(R,R)-butane-2,3-diol Synthesis	EX32DRAFT_00261	acetolactate synthase, small subunit
		EX32DRAFT_00262	acetolactate synthase, large subunit
		EX32DRAFT_03428	threonine dehydrogenase and related Zn-dependent dehydrogenases
	Trehalose synthesis via OtsA-OtsB	EX32DRAFT_01928	trehalose-phosphatase (OtsB)
		EX32DRAFT_01927	trehalose-6-phosphate synthase (OtsA)
		EX32DRAFT_03479	coenzyme PQQ biosynthesis enzyme PqqE
	PQQ synthesis	EX32DRAFT_03480	coenzyme PQQ biosynthesis protein PqqD
		EX32DRAFT_03481	coenzyme PQQ biosynthesis protein C
		EX32DRAFT_03482	coenzyme PQQ biosynthesis protein B
		¹ EX32DRAFT_03482.1	coenzyme PQQ peptide PqqA
PDN3	Trehalose synthesis via OtsA-OtsB	EX28DRAFT_0655	trehalose-phosphatase (OtsB)
		EX28DRAFT_0656	alpha,alpha-trehalose-phosphate synthase (OtsA)
	Trehalose synthesis via TreY-TreZ	EX28DRAFT_1155	malto-oligosyltrehalose trehalohydrolase (TreZ)
		EX28DRAFT_1156	malto-oligosyltrehalose synthase (TreY)
		EX28DRAFT_2401	acetoin reductases (BudC)
		EX28DRAFT_2402	acetolactate synthase, large subunit (BudB)
	(R,R)-butane-2,3-diol and (R)-acetoin Synthesis	EX28DRAFT_2403	alpha-acetolactate decarboxylase (Buda)
		EX28DRAFT_3673	acetolactate synthase, large subunit
		EX28DRAFT_3674	acetolactate synthase, small subunit
		EX28DRAFT_3953	acetolactate synthase, small subunit
		EX28DRAFT_3954	acetolactate synthase, large subunit
		EX28DRAFT_4440	acetolactate synthase, large subunit
		EX28DRAFT_4441	acetolactate synthase, small subunit
		EX31DRAFT_01699	coenzyme PQQ biosynthesis probable peptidase PqqF
WP5	PQQ synthesis	EX31DRAFT_01700	coenzyme PQQ biosynthesis enzyme PqqE
		EX31DRAFT_01701	coenzyme PQQ biosynthesis protein PqqD
		EX31DRAFT_01702	coenzyme PQQ biosynthesis protein C
		EX31DRAFT_01703	coenzyme PQQ biosynthesis protein B
		¹ EX31DRAFT_01703.1	coenzyme PQQ biosynthesis protein A
	Trehalose synthesis via TreY-TreZ	EX31DRAFT_01717	malto-oligosyl trehalose synthase (TreY)
		EX31DRAFT_01718	malto-oligosyl trehalose hydrolase (TreZ)
		EX31DRAFT_02312	acetolactate synthase, small subunit
		EX31DRAFT_02313	acetolactate synthase, large subunit
	(R,R)-butane-2,3-diol and (R)-acetoin Synthesis	EX31DRAFT_02434	alpha-acetolactate decarboxylase
		EX31DRAFT_04636	acetolactate synthase, large subunit
		EX31DRAFT_04637	acetolactate synthase, small subunit
		EX06DRAFT_01128	trehalose-phosphatase (OtsB)
		EX06DRAFT_01129	alpha,alpha-trehalose-phosphate synthase (OtsA)
PTD1	Trehalose synthesis via TreY-TreZ	EX06DRAFT_01921	malto-oligosyltrehalose synthase (TreY)
		EX06DRAFT_01922	malto-oligosyltrehalose trehalohydrolase (TreZ)
	(R,R)-butane-2,3-diol and (R)-acetoin Synthesis	EX06DRAFT_05002	acetolactate synthase, large subunit
		EX06DRAFT_05003	acetolactate synthase, small subunit
	Trehalose synthesis via OtsA-OtsB	Ga0008009_10698	Putative trehalose-phosphatase (OtsB)
WPB	Trehalose synthesis via OtsA-OtsB	Ga0008009_10699	alpha,alpha-trehalose-phosphate synthase (OtsA)
		Ga0008009_10872	malto-oligosyltrehalose synthase (TreY)
	Trehalose synthesis via TreY-TreZ	Ga0008009_10874	malto-oligosyltrehalose trehalohydrolase (TreZ)
		Ga0008009_118101	acetolactate synthase, large subunit
	(R,R)-butane-2,3-diol and R-acetoin Synthesis	Ga0008009_118102	acetolactate synthase, small subunit
		Ga0008009_10299	Threonine dehydrogenase or related Zn-dependent dehydrogenase
	1-Aminocyclopropane-1-carboxylic acid degradation	Ga0008009_11518	1-aminocyclopropane-1-carboxylate deaminase
WP1	Trehalose synthesis via OtsA-OtsB	scaffold_19:208485-212329	Putative trehalose-phosphatase (OtsB)
		scaffold_9:870873-873744	alpha,alpha-trehalose-phosphate synthase (OtsA)
	(R,R)-butane-2,3-diol and (R)-acetoin Synthesis	scaffold_15:276834-278070	Putative acetolactate synthase, large subunit
		scaffold_5:1501147-1504056	Putative acetolactate synthase, small subunit
		scaffold_14:338156-339969	(R,R)-butanediol dehydrogenase

4. Discussion and conclusions

These endophytes either as single strain or multi-strain inoculum have previously exhibited mutualistic behavior when added to other plant species including grasses, corn, rice, Douglas-fir and a variety of crop plants [33–37,60]. Combined application of endophytes can result in larger effects than those possible with individual inoculations [34,35,61]. Rogers et al. [62] reported growth enhancements of hybrid poplar clone OP-367 using a single endophyte *Enterobacter* sp. strain 638 isolated from poplar growing at a phytoremediation field site. In our study, inoculation with a consortium of endophytes resulted in 28% higher biomass com-

pared to mock-inoculated controls. Some of these endophytes were isolated from wild poplar growing on rocks and gravel in their native riparian habitat. It can be expected that these trees have selectively recruited the most beneficial endophytes for their survival in that challenging environment, therefore these strains may harbor adaptive traits and have a superior potential to enhance host plant growth under stressful conditions. The inoculated plants also had a doubling of root biomass and this may reduce water requirements and increase survivability during the costly establishment phase of short rotation energy crops, thereby improving the economic viability of poplar as a feedstock for biofuel applications [63–66]. The auxin, IAA, is a well-known plant phytohormone that



Fig. 4. Effect of inoculation on paraquat induced oxidative stress (ROS) under laboratory conditions. After 48 h of exposure to paraquat, leaves from plants that were inoculated with endophytes remained green (left) while leaves from the mock-inoculated plants were photobleached (right). Leaf disks were sampled from leaf tissues of similar size, developmental age, and location for comparison.

is involved in multiple plant growth processes and stress responses [67,68]. Since the endophytes produced significant amounts of IAA in culture, it is possible that the enhanced root growth and drought stress resistance may be via auxin related mechanisms. Other stress responsive hormones such as GA₃, JA, SA and Brs [69–74] were also produced by these endophytes which may have led to the morphological changes in the host plant.

It has been well established that drought stress causes oxidative damage by producing reactive oxygen species such as O₂^{•−}, H₂O₂, and •OH [75–77] causing oxidative damage to DNA, lipids, and proteins. In this study we assessed tissue tolerance to ROS by exposing photosynthetic tissue to the herbicide paraquat (mimics endogenous ROS production) which is oxidized by molecular oxygen resulting in the generation of superoxide ions and subsequent photobleaching/discoloration of chloroplasts [44]. When exposed to stress, mock-inoculated plant tissues lost their greenness indicating ROS activity while the inoculated tissues remained green. It is likely that endophytes may be helping plants to cope with drought stress by either efficiently scavenging ROS or preventing ROS production under drought stress [78,79]. Production of pyrroloquinoline is correlated to ROS activity [80]. Interestingly, genes involved in PQQ synthesis were identified in WP5 and WP19 suggesting a possible direct involvement by reducing the oxidative stress in cells. Biochemical analysis of ROS activity during water stress and mutant analysis will aid in confirming this hypothesis.

Besides an improved survival of the host plant, the survival of a microorganism under water-limited conditions may also represent an important trait for a stable interaction with the host plant. A well-known osmolyte used by microorganisms and plants

during desiccation stress is trehalose, a disaccharide that protects biomolecules during osmotic stress [81]. The possible role of trehalose in beneficial plant-microbe interactions was explored in two studies involving engineered bacteria overexpressing trehalose biosynthesis genes. Bean plants inoculated with *Rhizobium etli* overexpressing *otsA* had increased drought tolerance, grain yields, and biomass [82]. In a similar study, the beneficial effect of trehalose against drought stress was assessed in maize plants inoculated with a genetically modified *Azospirillum brasilense* which overaccumulated trehalose through the overexpression of a chimeric trehalose biosynthetic gene as well as an exogenous copy of *otsA* from *Rhizobium etli* [83]. While only 40% of uninoculated maize survived the drought stress, 85% of those inoculated with the trehalose overexpressing strain survived. In our study the presence of trehalose biosynthesis genes in endophytic strains WP5, WP1, WPB, WP19 and PDN3 provide an opportunity to test its involvement in conferring drought stress tolerance.

Another suggested mechanism for microbially-conferred plant host drought tolerance is production of ACC deaminase that reduces host stress ethylene [10]. While ethylene is normally produced by plants at low levels, when exposed to biotic and abiotic stresses, production is significantly elevated, leading to reduced plant growth. Plants inoculated with ACC deaminase-producing bacterial strains have improved stress tolerance. For example, tomato and pepper plants exhibited increased drought tolerance when inoculated with the ACC deaminase producing strain ARV8 [84]. Genomic sequences of plant-associated microorganisms are commonly misannotated as having ACC deaminase, requiring a close inspection of the sequence encoding the active site of the enzyme [85]. Strain WPB does contain the putative ACC deaminase sequence, and likely does have this activity, although this must be confirmed biochemically. However, strains WP5, WP19, and PDN3, while annotated as having this enzyme, have substitutions that would likely make the resulting enzyme incapable of breaking the cyclopropane ring of ACC but would have D-cysteine desulhydrase activity [86]. These two different enzyme activities, interchangeable with two amino acid residue alterations, may both have roles in protection of the plant host. D-cysteine desulhydrase converts D-cysteine into pyruvate, hydrogen sulfide and ammonia, and may be involved in sulfur induced pathogen resistance [87].

The genomes of PDN3, WP19, PTD1, WP5, WPB and WP1 carried genes involved in synthesis of the volatile organic compounds (VOCs), acetoin and 2,3-butanediol (Table 3). Production of these VOCs by plant growth promoting bacteria was reported to increase systemic resistance and drought tolerance [88]. *Enterobacter* sp. 638, an endophyte isolated from poplar stems increased drought tolerance of hybrid poplar, and annotation of the genome revealed the presence of genes for acetoin and 2,3-butanediol synthesis [88]. The involvement of 2,3-butanediol in inducing stomatal closure and drought tolerance was shown by Cho, et al. [49]. Under drought stress, root colonization of *Arabidopsis* plants by *Pseudomonas chlororaphis* strain O6 increased plant survival and reduced stomatal aperture. Exogenous application of this volatile compound to the plants gave similar results suggesting that the bacterial volatile may be a key determinant in increased drought tolerance.

The genomic analysis reported here is the first step in beginning to decipher the microbial mechanisms for improving host plant drought tolerance. Identification of genes that may be involved in the process provided the necessary insight that is leading to functional analysis using a directed mutational approach.

We also evaluated the response of poplars to water deficit based on physiological traits such as chlorophyll content, photochemical efficiency of PSII, and stomatal conductance which have been used as indicators of plant stress [89–91]. Previous studies demonstrated an increase of F_v/F_m due to beneficial

microsymbionts under drought stress conditions [26,27,92]. Likewise, endophyte inoculation improved F_v/F_m in the present study. It is possible that the positive effects of endophyte inoculation may be linked to photosynthesis through production/stimulation of specific phytohormone(s) associated with leaf development [93]. Other physiological aspects that might also be influencing the system are water-use efficiency related to photosynthesis, xylem hydraulic conductivity, and partitioning of photoassimilates. The delay of the decreasing photosynthetic activities by the inoculated endophytes corresponded with the delay of degradation of chlorophyll determined by SPAD in the present study. Chlorophyll concentration is positively connected to photosynthetic activities of PSII, especially under water stressed conditions [94]. It was reported by Lawler et al. [95] that the drought-adapted ecotypes and cultivars of *Andropogon gerardii* had higher SPAD values in the drier habitats. Under severe drought stress conditions, the inoculated endophyte consortia may help the host plants preserve chlorophyll molecules in the leaves by providing fixed nitrogen and phytohormones which eventually increase the host plants' adaptation to the limited water environment.

Stomatal regulation is one of the fastest physiological mechanisms in plants responding to water deficit, limiting CO_2 diffusion and leading to decrease of the CO_2 assimilation rate of the plants [96]. Our results showed typical stomatal responses of plants after the drought stress imposed; at 15 DDS g_s of both control and inoculated groups started to rapidly decrease to 49.9% (Fig. 3c). Interestingly, however, even before the drought, g_s appeared to be decreased by the inoculation of the endophytes although the differences were not statistically significant. This response was highlighted at 19 DDS with the lowest $P=0.110$. The decrease of g_s by the inoculation coincided with the delay of reduced F_v/F_m values at 19 DDS as described above. These combined physiological responses indicate that the endophytes may trigger the host plants to close the stomata, thereby losing less water and yet maintaining the photosynthetic efficiency of PSII. ABA is a key signal for stomatal closure of plants induced by drought or salt stresses as discussed by Fricke et al. [97], Zeng et al. [98] and Park et al. [99]. Especially since *Populus* is an isohydric species, stomatal control is strongly dependent on water status of the plants [97]. Therefore, the role of ABA in stomatal control under drought stress becomes more crucial in the present study. The amount of endophyte-produced ABA in vitro in our hormonal profile assay is enough to signal the stomatal reactions when compared to the amount of endogenously produced ABA in planta under severe drought reported by Fricke et al. [97]. An intriguing hypothesis for endophyte-mediated drought tolerance related to stomatal closure is that the colonized host plants may be able to utilize CO_2 respired by the endophytes, enabling continued photosynthesis with closed stomata while avoiding the water losses normally incurred through open stomata. It was shown by Bloemen et al. [100] that root-respired CO_2 could be incorporated into photosynthetic CO_2 assimilation up to 2.7% in *P. deltoides*. Since we saw extensive intercellular colonization by the endophytes, it is possible that CO_2 released by endophytic microbial respiration in the intercellular spaces might be another source of the CO_2 assimilation, and this combined with phytohormone modulation and reduced ROS, could eventually increase the host plants' adaptation to the limited water environment.

In conclusion, the results presented here demonstrate that inoculation of a commercial hybrid poplar with a consortia of beneficial endophytes can significantly enhance plant growth and tolerance of water deficit stress under greenhouse conditions. Field trials are underway to test if these findings will translate into the production environment. Since poplar production systems must be optimized to produce stable high yields despite the increased stresses imposed by climate change, a better understanding of plant-microbe interactions could be a key to adapting plants to a

water limited environment. The availability of genomic sequences will greatly promote the progress of the research into the fundamental mechanisms of symbiosis and may yield ways to further increase biomass in an environmentally sustainable way.

5. Nucleotide sequence accession numbers

The sequence data (16S/28S rDNA) of the selected strains have been deposited in NCBI GenBank under accession numbers: EU563924 (WP1), JN634853(PDN3), EU563924(WPB), KT962907 (PTD1), KU523563(WP19), KU497675(WP5), KU523562(WP9), KT984987(WW5), KU557506(WW6), KU523564(WW7).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cpb.2016.08.001>.

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