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

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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.

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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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 vietnamensis* 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 (OD_{600}) 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 μmol m^2/s 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 $^2H_6$-ABA, $^2H_4$-SA, $^2H_2$-GA_3, $^2H_6$-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 12-1pm) 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_o, followed by illuminating a saturating light flash to gain maximal fluorescence, F_m. Variable fluorescence, $F_v = (F_m - F_o)$, 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-