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Improving a "Generation 1.5" biofuel feedstock crop: Colonization and growth enhancement of energy beet (*Beta vulgare* L. Beta 5833R) by inoculation with *Gluconacetobacter* spp.

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ABSTRACT

"Generation 1.5" biofuels are derived from biomass feedstocks that originated as food crops, but with selective breeding and modified production management, are more energy dense and have lower carbon footprints than their food crop progenitors. Energy beets, specialized genotypes of sugar beet (*Beta vulgaris* L.), grown under the proper low-carbon footprint conditions, is an example of such a Generation 1.5 feedstock crop. In this study, *Beta vulgaris* variety-Beta 5833R was tested for its responses to 14 different strains of the growth-promoting bacterium, *Gluconacetobacter* spp. in greenhouse studies at low (1 or 2 mM) and high (10 mM) levels of NO_3^- supplied to the plants. Using a β-glucuronidase (GUS)-labeled strain of the bacterium, confirmed that *G. diazotrophicus* is able to colonize the plant with root tips, root hairs and lateral root junctions being the major infection sites. Using ^{15}N-isotope dilution technique, the present work demonstrates for the first time that N_2 fixation associated with *Gluconacetobacter* spp. increased N accumulation and lead to increases of up to 110% in sugar beet biomass. However, biomass increases in sugar beet were still evident even at higher levels of NO_3^- supply even though N_2 fixation by the bacterium was quite low, suggesting that other mechanisms may also have been at work in the growth promotion of sugar beet by *Gluconacetobacter* spp.

1. Introduction

First-generation, or "conventional" biofuels commonly refers to liquid fuels derived from sugar, starch and vegetable oils. First-generation biofuel feedstock is often derived from agricultural annual crops, such as corn, wheat, and rape-seed/canola; crops which can also be harvested for food. Given the large energy and chemical inputs in the production of annual crops, there has been much concern in both the scientific and public domains in regard to the actual greenhouse gas (GHG) savings from first-generation biofuels as compared to petroleum-based fuels. Life-cycle analysis (LCA) of biofuels derived from first generation feedstock crops, such as corn, often show marginal GHG savings, while others, such as sugar cane, can show substantive decreases in GHG emissions compared to petroleum (Schubert, 2006). First-generation biofuels have also fueled the "food versus fuel" debate (Tomei and Helliwell, 2016) which includes issues of how the reallocation of land from the production of food to biofuel feedstocks can affect food security, and how policies incenting the production of biofuel feedstock crops can cause increases to food commodities prices.

Second-generation, or "advanced" biofuels are those derived from lignocellulosic feedstocks. These feedstocks may be derived from purpose-grown biomass crops (e.g. hybrid poplar, *Miscanthus* sp.), forestry and agricultural residues, and municipal solid waste. Being derived from non-food feedstock, second-generation biofuels avoid the food versus fuel debate. Also being derived from predominantly low-input, perennial crops and waste streams, they tend to have LCA with positive impacts on GHG emissions (Schubert, 2006). A drawback of second-generation biofuels is that the technology to convert these feedstock to liquid fuels is not as developed and is more costly than that for conventional biofuel production (Ren et al., 2016).

Madison (2012) used the term "Generation 1.5 Ethanol" to describe production of bioethanol which would meet United States revised Renewable Fuel Standard (RFS2) standards for advanced biofuels, but still utilizing feedstocks and processes that are more commonly associated with first-generation, conventional biofuels. These include "energy crops" – forms of traditional food crops that have been bred to increase their energy density and are grown specifically as biofuel feedstocks rather than food crops. Examples include sweet sorghum (Mullet et al., 2014) and energy beets (Shao et al., 2015).

Application of fertilizers to crop plants to increase productivity has

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been shown to have negative and unpredictable effects on the environment (Di and Cameron, 2002; Erisman et al., 2011). The use of plant growth promoting bacteria as biofertilizer has been considered as an alternative or a supplemental means of reducing applications of these chemical fertilizers (Vessey, 2003).

Gluconacetobacter diazotrophicus was originally isolated as an endosymbiont of sugarcane (Cavalcante and Döbreiner, 1988). Biological N_2 fixation by the bacterium has been indicated to contribute up to 60% of the nitrogen requirement for sugarcane growth (Boddey et al., 1991). The occurrence and survival of *G. diazotrophicus* has also been reported in other agricultural crops such as sweet potato (Paula et al., 1992), coffee (Jiménez-Salgado et al., 1997), finger millet (Loganathan et al., 1999), pineapple (Tapia-Hernández et al., 2000), tomato (Luna et al., 2012), sorghum (Luna et al., 2010; Yoon et al., 2016), common bean (Trujillo-Lopez et al., 2006), sugar beets (Madhaiyan et al., 2004) and rice (Muthukumarasamy et al., 2005; Rouws et al., 2010).

Previous investigations have documented the efficiency of N_2 fixation by *G. diazotrophicus* in sugarcane (Saravanan et al., 2008). In addition to N_2 fixation, other mechanisms (Sevilla et al., 2001) have been indicated for the enhancement of plant growth by this bacterium, including production of plant hormones (Bastián et al., 1998; Mehnaz and Lazarovits, 2006) and mobilization of minerals (Saravanan et al., 2007; Stephen et al., 2015).

Sugar beet is a type of *Beta vulgaris* cultivated for sugar production in temperate regions, particularly in the northern hemisphere. Global sugar beet production in 2013 was approximately 247 million tonnes, with 68% of the production in Europe. The world's largest sugar beet producers in 2013 were Russia (39 Mt), France (33 Mt), the United States (30 Mt), Germany (23 Mt) and Turkey (16 Mt). Fresh root yields of sugar beet range from 50 to 60 Mt ha^{-1}. Martin et al. (2006) reported that sugar concentrations of sugar beet average 18.7%, about 25% higher sucrose content compared to sugar cane. In 2009, sugar beets accounted for 20% of the world's sugar production (Food and Agriculture Organization of the United Nations, 2009).

Sugar beet is also one of major crops for ethanol production. van Walwijk (2005) reported that approximately 70% of France's ethanol production from was derived from sugar beets. It is a potential feedstock for ethanol production in US (Maung and Gustafson, 2011). However, sugar beet generally requires up to 200–250 kg N ha^{-1} to maximize yields and Carter et al. (1976) reported that the plant required 5.4 kg N $tonne^{-1}$ of fresh roots to maximize sucrose yields. Given that the production and use of fertilizer N is the single biggest contributor to GHG emissions in the production of energy crops (Camargo et al., 2013), the N requirement of the crop is a significant challenge in utilization of traditional sugar beet lines and production methods for its use as a sustainable biofuel feedstock.

Although there is a report describing isolation of *G. diazotrophicus* from sugar beet in India (Madhaiyan et al., 2004), there are no previous reports of enhanced productivity of sugar beet by inoculation with *Gluconacetobacter* spp. In this study, sugar beet plants were inoculated with 14 different strains of *Gluconacetobacter* spp. to test their ability to colonize the plant and to evaluate whether the bacteria can improve yield of sugar beets.

2. Materials and methods

2.1. Gluconacetobacter strains and culture

Fourteen strains of *Gluconacetobacter* spp. including 13 strains of *G. diazotrophicus* and 1 strain of *G. johannae* (Table 1). *Gluconacetobacter johannae* was originally isolated from rhizoplane of coffee tree roots, is phylogenetically closely related to *G. diazotrophicus*, and is also able to fix N_2 (Fuentes-Ramirez et al., 2001). All strains were cultured with a modified liquid LGI-P medium (Cavalcante and Döbreiner, 1988; Pan and Vessey, 2001) on a rotary shaker at 150 rpm and 30 °C until the broth OD value up to 0.6 at λ 600 nm. A β-glucuronidase marked *G. diazotrophicus* strain UAP5541/pRGS561 [constructed by Fuentes-Ramírez et al. (1999) and kindly provided by Dr. Jesus Caballero-Mellado, UNAM, Mexico] was cultured with the same medium and culture conditions as above, and also containing 45 mg l^{-1} streptomycin.

2.2. Colonization experiment

Seeds of sugar beet (*Beta vulgaris* L. variety-Beta 5833 R, from Betaseed Inc. ON, Canada) were rinsed with 70% ethanol for 10 s, then immersed in with 20% Javex® [10% (w/w) sodium hypochlorite] containing 0.05% Twin-20 for 5 min, then washed 5 times with sterilized distilled water. The seeds were transferred to Petri dishes lined with filter paper for germination in the dark at 22/18 °C (d/n). The germinated seeds were planted into small, 5-cm diameter pots containing 200g of silica sand. The pots (1 plant pot^{-1}) were placed in a growth chamber with a photoperiod of 16/8 h (d/n) and temperature regime of 22/18 °C (d/n). After one week of growth, the seedlings were inoculated with *G. diazotrophicus* strain UAP5541/pRGS561. The inoculant was prepared by centrifuge of the culture medium at 5000g for 10 min, then re-suspending the bacteria in PBS-buffer (pH 6) at 10^8 cfu ml^{-1}. One ml of the inoculant was applied onto the sand surface around the seedling. Negative control plants were not inoculated and positive controls were inoculated with wild type *G. diazotrophicus* PAL5T. Two 2 weeks after inoculation, the plants were harvested and washed carefully to separate sands from below-ground tissues. The seedlings were fixed, washed and stained based on the procedures from β-glucuronidase reporter gene staining kit (GUSS, Sigma-Aldrich, St. Louis, USA). Samples were observed directly and as hand-cut cross sections of the roots under a light microscope equipped a camera (INFINITY Lite, Lumenera Corp. Ottawa, Canada) using INFINITY CAPTURE software.

2.3. Sand culture experiment

Sugar beet seeds were germinated in Petri dishes lined with one layer of filter paper wetted with distilled water at room temperature in the dark. When seedlings reached about 2.5 cm in length, they were transferred into 3.7 l pots containing 3 kg of silica sand (1 plant pot^{-1}). Plants were grown in a greenhouse with the temperature set at 25/18 °C (d/n), the photoperiod at 16/8 h (d/n), and irradiance from the natural daylight supplement with 350–370 μmol m^{-2} s^{-1} by High Pressure Sodium lamps. Plants were watered with a nutrient solution twice a week and with tap water on other days. The volume of water was initially 50 ml pot^{-1} and increased to 100 ml, 200 ml and 300 ml pot^{-1} over the 4 months of growth. Throughout the experiment, pots were regularly rearranged on the benches to ensure plants were exposed to relatively even light intensity.

The nutrient solution was modified from Knop's solution (Mohr and Schopfer, 1995) containing $Ca(NO_3)_2$ 0.656 g l^{-1}, KNO_3 0.202 g l^{-1}, KH_2PO_4 0.250 g l^{-1}, $MgSO_4$ 0.120 g l^{-1}, H_3BO_3 2.86 mg l^{-1}, $MnCl_2{\cdot}4H_2O$ 1.81 mg l^{-1}, $ZnSO_4{\cdot}7H_2$ O 0.22 mg l^{-1}, 0.08 mg l^{-1}, $CuSO_4{\cdot}5H_2O$ 0.08 mg l^{-1}, $H_2MoO_4{\cdot}H_2O$ 0.02 mg l^{-1}, $FeSO_4{\cdot}H_2O$ 6.95 mg l^{-1}; pH was adjusted to 6.0 with 1% acetic acid solution. In the N treatments, N concentrations were 1 mM NO_3^- and 10 mM NO_3^-, respectively. Both nutrient solutions were enriched with 1% of $^{15}NO_3^-$ ($K^{15}NO_3$, 99 at%, Cambridge Isotope Laboratories, Inc. Andover, MA, US). In each N treatment, the plants were inoculated by applying 1 ml of broth (10^8 cfu ml^{-1}) of one of 14 strains *G. diazotrophicus* spp. onto the sand surface around the plant. Inoculation occurred at the two-leaf stage in all treatments. There were 5 replicates in each treatment.

The plants were harvested after 12 weeks of growth. The shoots, beets, and subtending fibrous roots were collected separately and were dried at 80 °C for 3 days to measure dry biomass. All samples were ground into fine powder for total N and ^{15}N analyses. The analyses were conducted at the Agriculture and Agri-Food Canada Research Center,