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Authors: Yang, Xuan, Allen Hill, Kathleen, Vessey, J. Kevin, and Tian, Lining

Source: Canadian Journal of Plant Science, 102(2) : 324-335

Published By: Canadian Science Publishing

URL: https://doi.org/10.1139/CJPS-2020-0143

Canadian Science Publishing

ARTICLE

Colonization of *Brachypodium distachyon* by *Gluconacetobacter diazotrophicus* and its effect on plant growth promotion

Xuan Yang, Kathleen Allen Hill, J. Kevin Vessey, and Lining Tian

Abstract: *Gluconacetobacter diazotrophicus* is a nitrogen (N) fixing bacterium originally isolated from sugarcane in Brazil. Understanding the interactions between this bacterium and plants is important to exploit the nitrogen-fixing feature of *G. diazotrophicus* in different crops. Research was conducted to establish, monitor and optimize methods for introducing *G. diazotrophicus* into *Brachypodium distachyon*, a model for molecular research involving monocot plants. Colonization was studied in three plant growth media including liquid, semi-solid, and solid media. The effect of mineral nitrogen on colonization was also studied in each of these three media. The colonization of *B. distachyon* by *G. diazotrophicus* was analyzed by a histochemical *β*-glucuronidase (GUS) assay, PCR using *G. diazotrophicus* specific primers and bacterial re-isolation. Colonization was established in all tested plant growth conditions. The highest level of colonization was achieved in the liquid media without mineral nitrogen. Microscopic observations showed that *G. diazotrophicus* colonizes *B. distachyon* through the lateral root emergence sites, root hairs, and root tips. The colonization was found to be limited to root tissues and not found in other organs. These results indicate that *B. distachyon* roots are the primary organ of interaction and colonization by the bacterium. Plant fresh weight and lateral root numbers were significantly increased in inoculated plants indicating that *G. diazotrophicus* can stimulate growth of *B. distachyon*. This study indicates that *B. distachyon* is a useful model to study the mechanisms of *G. diazotrophicus* colonization and growth enhancement in monocot plants.

Key words: Gluconacetobacter diazotrophicus, *Brachypodium distachyon*, colonization, plant growth promotion, nitrogen.

Résumé : *Gluconacetobacter diazotrophicus* est une bactérie fixatrice d'azote isolée au départ dans la canne à sucre, au Brésil. Il est important de comprendre les interactions de cette bactérie avec les plantes en vue de mieux exploiter ses propriétés fixatrices avec diverses cultures. Les auteurs ont élaboré, vérifié et perfectionné des méthodes pour introduire *G. diazotrophicus* dans *Brachypodium distachyon*, plante employée comme modèle pour la recherche moléculaire sur les monocotylédones. Ils ont examiné la colonisation de trois milieux de croissance pour les plantes, soit un liquide, un semi-solide et un solide, tout en étudiant les effets de l'azote minéral sur la colonisation de chacun d'eux. La colonisation de *B. distachyon* par *G. diazotrophicus* a été analysée par essai histochimique (GUS), par la réaction en chaîne à la polymérase avec des amorces spécifiques à *G. diazotrophicus* et par un nouvel isolement de la bactérie. Toutes les plantes ont été colonisées, quelles que soient les conditions de croissance. La colonisation la plus intense a été observée dans le milieu liquide, sans apport d'azote. L'examen au microscope révèle que *G. diazotrophicus* colonise *B. distachyon* par les sites d'émergence des racines latérales, les poils racinaires et l'extrémité des racines. La bactérie ne colonise que les tissus racinaires, car on ne la retrouve pas dans les autres organes. Selon ces résultats, les racines de *B. distachyon* constituent donc le principal organe d'interaction et de colonisation de la bactérie. Le poids frais et le nombre de racines latérales des plantes inoculées étaient sensiblement plus élevés, signe que *G. diazotrophicus* stimule la croissance de *B. distachyon*. Cette étude prouve que *B. distachyon* est un modèle utile pour étudier les mécanismes de colonisation de *G. diazotrophicus* ainsi que l'amélioration de la croissance des monocotylédones. [Traduit par la Rédaction]

Received 2 June 2020. Accepted 12 July 2021.

X. Yang. Department of Biology, The University of Western Ontario, London, ON N6A 5B7, Canada; London Research and Development Centre, Agriculture and Agri-Food Canada, London, ON N5V 4T3, Canada.

K. Allen Hill. Department of Biology, The University of Western Ontario, London, ON N6A 5B7, Canada.

J.K. Vessey. Department of Biology, Saint Mary's University, Halifax, NS B3H 3C3, Canada.

L. Tian. London Research and Development Centre, Agriculture and Agri-Food Canada, London, ON N5V 4T3, Canada.

Corresponding author: Lining Tian (email: lining.tian@agr.gc.ca).

Mots-clés : Gluconacetobacter diazotrophicus, *Brachypodium distachyon*, colonisation, accélération de la croissance des plantes, azote.

Introduction

Plants are in constant contact with different soil microbial populations. Studies focusing on plant–microbe interactions, especially beneficial plant–microbe interactions, have been receiving increasing attention in recent decades. Using plant growth promoting (PGP) microbes as biofertilizer and biocontrol agents to improve crop yield is more economically and environmentally sustainable compared to conventional farming practices (Morgan et al. 2005; Farrar et al. 2014; Trivedi et al. 2017; Gouda et al. 2018; Martínez-Hidalgo et al. 2019; Ferreira et al. 2019).

Gluconacetobacter diazotrophicus is an endophytic nitrogen (N) fixing bacterium which was first isolated from sugarcane in Brazil (Cavalcante and Dobereiner 1988) and later also found naturally associated with some other plant species, such as coffee trees (Jimenez-Salgado et al. 1997), tea plants (Madhaiyan et al. 2004), and wetland rice (Muthukumarasamy et al. 2005). A considerable amount of fixed nitrogen from *G. diazotrophicus* can potentially be transferred to and used by its plant host (Boddey et al. 1991; Boddey 1995; Sevilla et al. 2001). Nitrogen fixation in most nitrogen-fixing bacteria is inhibited by the presence of nitrogen compounds in their surrounding environment, whereas there is evidence that the nitrogen-fixing capability of *G. diazotrophicus* is not affected by the presence of low to medium levels of nitrate (Cavalcante and Dobereiner 1988). *Gluconacetobacter diazotrophicus* can also stimulate plant growth through production of phytohormones (Bastián et al. 1998; Lee et al. 2004), mineral nutrient solubilization (Saravanan et al. 2008; Muthukumarasamy et al. 2017; Delaporte-Quintana et al. 2020), plant pathogen control (Muthukumarasamy et al. 2000; Blanco et al. 2005; Mehnaz and Lazarovits 2006; Rodriguez et al. 2019), and by increasing plant tolerance to abiotic stress (Vargas et al. 2014; Vurukonda et al. 2016). *Gluconacetobacter diazotrophicus* is an attractive candidate for use as biofertilizer in monocot crop production because it was originally isolated from a monocot crop (i.e., sugarcane) and it appears that it has a broad host range (Cocking et al. 2006; Luna et al. 2012). Research has demonstrated that *G. diazotrophicus* can be introduced into several monocot plant species, including wheat (Luna et al. 2010), corn (Tian et al. 2009), and sorghum (Luna et al. 2010; Yoon et al. 2016). However, widespread use of *G. diazotrophicus* for nitrogen fixation and plant growth promotion in different crops has not been achieved. The underlying molecular mechanism of these interactions is still largely unclear (Zamioudis and Pieterse 2011; Farrar et al. 2014), which restricts research progress. Major factors that hinder progress in this research area are large genome sizes, genome complexity, long life cycle and lack of molecular biology tools for many monocot crops.

Brachypodium distachyon (L.) P. Beauv (hereafter referred to as *Brachypodium*) was first proposed as a new model species for monocot research by Draper et al. (2001) and the full genome sequence of *Brachypodium* (Bd21) was published in 2010 (International Brachypodium Initiative 2010). According to genome analysis, *Brachypodium* is closely related to most monocot crops as it diverged from wheat 32–39 million years ago, from rice 40–53 million years ago, and from sorghum 45–60 million years ago (International Brachypodium Initiative 2010). Compared to many other monocot crop species, it has a small stature of approximately 20 cm, a relatively small genome size of approximately 272 million base pairs, simple growth requirements, a short life cycle ranging from 12–14 wk, self-fertility and extensive natural variation in biological traits (Febrer et al. 2010; Vain 2011). Successful and efficient protocols for *Brachypodium* genetic transformation have been developed. A collection containing 23 649 T-DNA insertion mutant lines has been generated for researchers to use free of charge and the number of mutant lines will continue to increase (Vogel and Hill 2008; Thole et al. 2012; Hsia et al. 2017). Therefore, *Brachypodium* is an excellent choice as a model species for studies of *G. diazotrophicus* in monocot crops.

We hypothesize that *G. diazotrophicus* can colonize *Brachypodium* and also promote growth of this plant. The objective of this study is to evaluate microbe colonization under six plant growth conditions and develop an inoculation method that can effectively introduce *G. diazotrophicus* into *Brachypodium*. For this purpose, different plant growth media were tested for colonization. Microscopic observation of *Brachypodium* root tissue was performed to determine the pattern of colonization. The effect of *G. diazotrophicus* colonization on *Brachypodium* fresh weight and root development also was analyzed. This research provides an important base towards the study of nitrogen fixation of *G. diazotrophicus* in *Brachypodium*, and ultimately towards greater use of this beneficial microbe in other monocot crops.

Materials and Methods

Plant material and growth conditions

Brachypodium distachyon seeds were surface sterilized before germination. After removing the lemma, the seeds were soaked in 70% ethanol for 30 s and then washed with sterile deionized water three times. The seeds were gently shaken in 1.3% sodium hypochlorite solution for four minutes, followed by three thorough rinses with sterile deionized water and plated on sterile filter paper saturated with sterile water in a sterile Petri

dish. Seeds were vernalised in the dark at 4 °C for 3–4 d and then transferred to 25 °C under a 16 h photoperiod (300–350 μmol/m^2·s)/8 h dark phase. After germination, seedlings were grown on one of the six growth media described below under a 20 h photoperiod (250–300 μmol/m^2·s, 22 °C)/4 h dark phase (20 °C) for three weeks.

The plant growth media used in the experiment included a Murashige and Skoog (1962) solid medium (PhytoTechnology Laboratories, Lenexa, KS), an MS solid medium (containing 15g/L agar) without nitrogen (formulated in our laboratory based on the solid medium formulation above, but with NH_4NO_3 omitted and KNO_3 replaced by KCl), an MS semi-solid medium (as above but with agar content reduced from 15 g/L to 7.5 g/L), an MS semi-solid medium without N, a Hoagland solution (Hoagland and Arnon 1950), and a Hoagland solution without N ($NH_4H_2PO_3$, $Ca(NO_3)_2$ and KNO_3 were replaced by K_2HPO_3, KCl and $CaCl_2$). The media were autoclaved for sterilization and the pH for all media was adjusted to 5.8 before sterilization.

Bacterial culture and inoculation

The *G. diazotrophicus* strain used in this study was UAP 5541/pRGS561 which has a *β*-glucuronidase (GUS) reporter gene fused to the bacterial genome for the rapid detection of bacterial presence via a GUS staining assay (Fuentes-Ramírez et al. 1999). Bacteria were cultured in LGIP medium (Cavalcante and Dobereiner 1988) at 28 °C for 48 h. The bacterial cultures were centrifuged at 8000× *g* for 10 min, resuspended in 0.8% NaCl solution, and then serially diluted to a final concentration of ~10^8 CFU/mL (equivalent to $OD_{600} \approx 0.02$) for inoculation. To inoculate the plants, the roots of 7 to 9 d old *Brachypodium* seedling roots were soaked in the bacterial suspension for one hour. The roots of control plants were soaked in 0.8% NaCl solution for the same time period. After the inoculation, the roots of *Brachypodium* seedlings were rinsed with sterile water three times and then transferred into magenta boxes that contain 120 mL of a selected growth medium from the six different plant growth media described previously. To avoid damage to the roots, shallow incisions were made on the surface of the medium to fit the plant roots when transferring plants to the solid media. Colonization was examined weekly until 3 wk after inoculation. The inoculation study was conducted in a growth room and plants were grown under a 20 h light (250–300 μmol/m^2·S, 22 °C)/4 h dark cycle (20 °C). The experiment was repeated three times and 32 plants were sampled for each growth medium in each of the trial.

Histochemical GUS staining assays and optical microscopic analysis

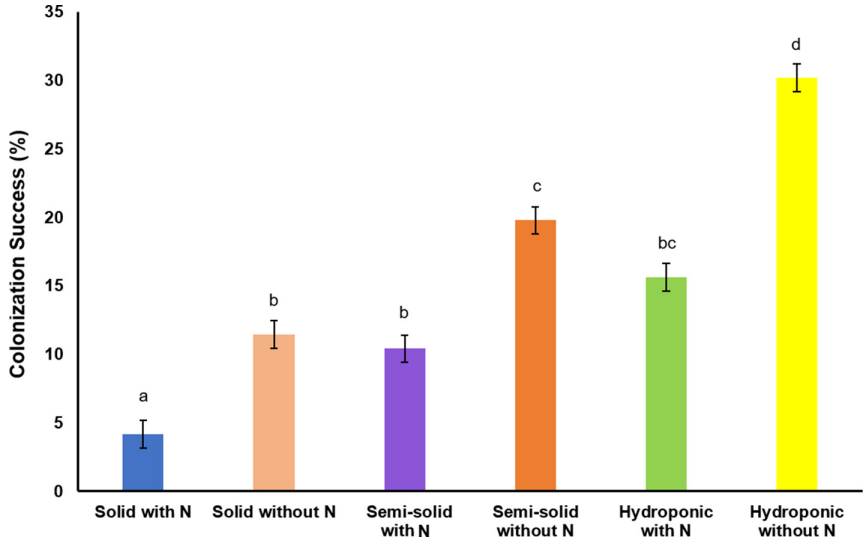
To visualize the colonization of *Brachypodium* by *G. diazotrophicus*, the seedlings were harvested for the GUS staining assay 21 d after inoculation. The seedlings were washed with sterile water three times and then immersed in a GUS staining solution (0.1 M Na_3PO_4 pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mM $K_3Fe(CN)_6$, 2 mM 5-brom-4-chloro-3-indolyl-glucuronide, 20% methanol). The samples were vacuum infiltrated for 30 minutes and then incubated at 37 °C overnight (Fuentes-Ramírez et al. 1999; Cocking et al. 2006). To remove chlorophyll from the tissues, the staining solution was removed and replaced with 75% ethanol. The 75% ethanol was changed at least three times. The incubation time was 12 h between each 75% ethanol change until the samples were completely clear. To examine the progress of colonization of *Brachypodium* by *G. diazotrophicus*, a separate set of 40 seedlings was inoculated and grown on Hoagland solution without mineral nitrogen. Ten plant samples per treatment group were collected every 7 d for GUS staining assay and microscopic observation.

After GUS staining, whole plant samples were observed using an optical microscope (Motic BA410 Elite, Motic Microscopes, Richmond, BC). The samples exhibiting GUS blue stain were cut into different segments based on the areas that contained the GUS stain. The segmented samples were fixed in 2% (*v*/*v*) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 24 h at 4 °C, dehydrated with ethanol and embedded in 5% (*w*/*v*) agarose solution. After the agarose solidified, the embedded samples were sectioned by a vibratome (Leica VT1000 S, Leica Biosystems, Buffalo Grove, IL). The thickness of the sections was approximately 30 μm. The sections were then examined and imaged using Invitrogen EVOS XL Core Cell Imaging System (Thermo Fisher Scientific Corporation, Waltham, MA). Three sections per plant sample were analyzed and three plants per treatment group were examined.

Re-isolation of *G. diazotrophicus*

Ten plant root samples per treatment group were harvested 21 d after inoculation. The root samples were washed under running tap water for 20 min to attempt to remove adhering bacteria. The root samples were then surface sterilized with 75% ethanol for 30 sec and rinsed with sterilized deionized water three times. Each root sample was homogenized in 2 mL of 0.8% NaCl solution. Three aliquots of 100 μL from each root extract were plated onto solid LGIP medium. After 5 d of incubation at 28 °C, three colonies were picked from each plate that showed bacterial growth for PCR analysis to confirm the identity of the bacteria. The primers used for colony PCR were specifically designed to detect the bacteria based on *G. diazotrophicus* 16S rDNA sequence (Franke-Whittle et al. 2005, GDI-F: 5′-TGAGTAACGCGTAGGGATCTG-3′, GDI-R: 5′-GGAAACAGCCATCTCTGACTG-3′). The thermocycling program used in colony PCR had an initial phase of 95 °C for 10 minutes, then 30 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s. This was followed by a final extension at 72 °C

Fig. 1. *Gluconacetobacter diazotrophicus* colonization under different plant growth conditions. The percent success of *G. diazotrophicus* colonization in *Brachypodium* is calculated as the percentage of plants being colonized (percent success = number of colonized plants/total number of inoculated plants × 100%) in different plant growth conditions. The different letters (a, b, c, d) indicate the significant differences among the groups (ANOVA, $p < 0.05$). The experiment was repeated three times and 32 plants were sampled for each condition in each of the trial. The error bars indicate the standard error of the mean for three replicates. [Colour online.]



for 5 min. The expected PCR amplification product was 878 bp (Franke-Whittle et al. 2005; Tian et al. 2009).

Fresh weight and lateral root measurement

Seven-day old *Brachypodium* seedlings were inoculated with *G. diazotrophicus*, and then transferred to the Hoagland solution without mineral nitrogen. For root measurement, thirty plants were harvested four weeks after inoculation and the number of lateral roots and total fresh weight were measured. The numbers of lateral roots were counted for the primary root and the two longest seminal roots for each plant. For fresh weight measurement, the surface of the plants was blot dried with filter paper to remove the excess Hoagland solution and the procedure was conducted carefully and consistently for each of the plants to ensure that all of the plants were equally blot dried. The whole plant fresh weight was then measured using an analytical balance (model AUW220, Shimadzu Scientific Instruments, Colombia, MD).

Statistical analysis

Student t-tests were conducted to assess the effect of *G. diazotrophicus* inoculation on plant fresh weight and number of lateral roots. An analysis of variance (ANOVA) with a post-hoc Tukey's test was used to assess the effect of plant growth condition on *G. diazotrophicus* colonization success. The significance level used for all statistical analyses was $\alpha = 0.05$ and all statistical analyses were performed using IBM SPSS Statistics for Windows software (version 21.0, IBM Corp, Armonk, NY).

Results

Different plant growth media affect colonization of *Brachypodium* by *G. diazotrophicus*

To establish *G. diazotrophicus* colonization of *Brachypodium*, six different plant growth conditions for inoculation were tested, including a MS solid medium, a MS solid medium without nitrogen, a MS semi-solid medium, a MS semi-solid medium without nitrogen, a Hoagland liquid solution, and Hoagland liquid solution without nitrogen. This line of research was to test and develop methods for introducing the microbe to *Brachypodium* rather than testing plant growth individual nutrients for *G. diazotrophicus* colonization. Colonization was visualized via the GUS staining assay and a plant with dark blue staining appearing at more than three different sites in the roots was counted as a colonized plant. Colonization rate was calculated as the percentage of plants in which the bacterium was found. The colonization percentage of plants in different plant growth conditions was: 4.17% in MS solid medium, 11.46% in MS solid medium without nitrogen, 10.42% in MS semi-solid medium, 19.79% in MS semi-solid medium without nitrogen, 15.63% in Hoagland liquid solution, and 30.21% in Hoagland liquid solution without nitrogen (Fig. 1). In the solid MS medium, only 4.17% of *Brachypodium* plants were colonized, which was the lowest among the six conditions (Fig. 1). The plants grown in the Hoagland liquid solution without nitrogen showed a significantly higher percentage (30.21%) of colonization compared to all other culture conditions (Fig. 1). Within the same type of growth medium (solid, semi-solid, or liquid), the plants

in growth medium without nitrogen supply showed a higher percentage of colonization compared to the plants in growth medium that contained nitrogen. The percentage of colonization in nitrogen free solid MS medium is 2.75-fold the percentage of colonization in nitrogen containing solid MS medium, the percentage of colonization in nitrogen free semi-solid MS medium is 1.90-fold the percentage of colonization in nitrogen containing semi-solid MS medium and the percentage of colonization in nitrogen free Hoagland liquid medium is 1.93-fold the percentage of colonization in nitrogen containing Hoagland liquid medium. In nitrogen free media, the percent success of colonization in liquid media is significantly higher than in solid and semi-solid media. A similar trend is observed when the medium contained nitrogen, but the difference between colonization success in liquid and semi-solid media is not significant. The colonization success in liquid media was only slightly higher than that in semi-solid medium.

Because *Brachypodium* grown in the liquid medium without nitrogen had the highest levels of colonization by *G. diazotrophicus,* this growth medium was selected to test *G. diazotrophicus* inoculation on promotion of the plant growth.

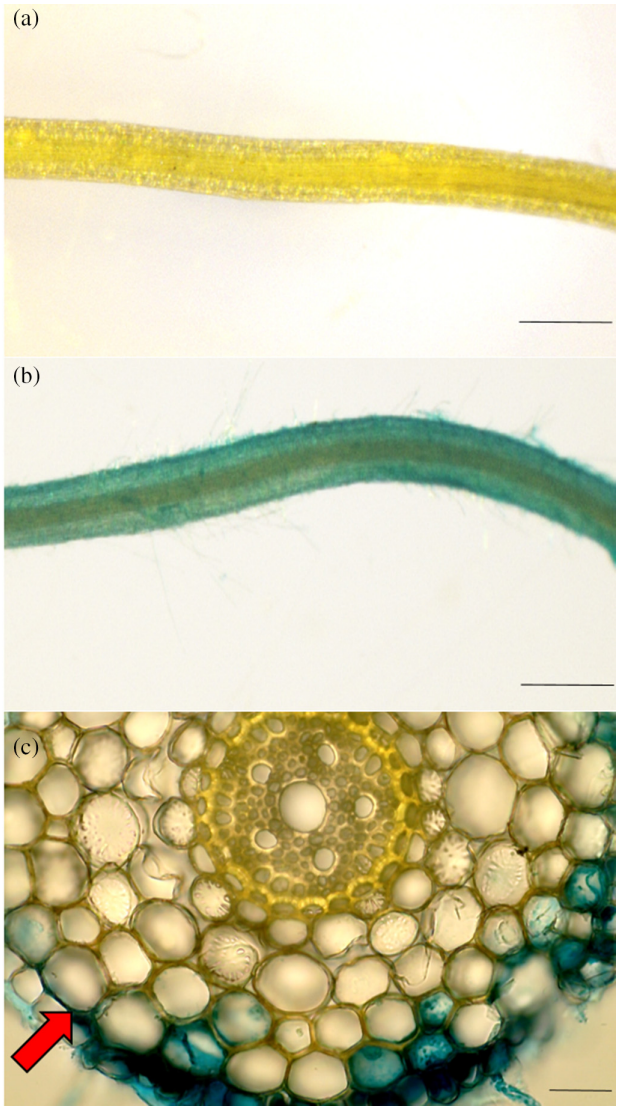
The patterns of *G. diazotrophicus* colonization in *Brachypodium*

Plant samples were taken every week after inoculation to monitor the establishment of colonization. The results showed that the *G. diazotrophicus* colonization was established in root tissues by 3 weeks after inoculation (Figs. 2*a* and 2*b*). Cross sections of inoculated root samples showed the colonization of *Brachypodium* by *G. diazotrophicus* appeared within root tissues (Fig. 2*c*). No GUS activity was observed in stems and leaves.

In the 7 d post inoculation root samples, a few patches of dark blue-stained *G. diazotrophicus* were found around and inside of interface where lateral roots emerge from the primary root (Fig. 3*a*). *G. diazotrophicus* bacteria were also found at the edge and base of the root tip openings (Fig. 3*b*). At 14-day post inoculation, the bacteria were also clearly visible in the root hair of *Brachypodium* (Fig. 3*c*). Twenty-one days after inoculation, the inside of root tissues was colonized more extensively by *G. diazotrophicus* through lateral root emergence sites and the root hair. The colonization of *Brachypodium* by *G. diazotrophicus* appeared to be saturated at 3 weeks after inoculation because the extent of *G. diazotrophicus* colonization as assayed by GUS satin is very similar in *Brachypodium* plants between 3-wk and 4-wk post inoculation.

To further confirm the colonization by *G. diazotrophicus* in *Brachypodium*, colony PCR analysis was conducted. Bacterial colonies re-isolated from the surface of sterilized inoculated root samples were subjected to PCR assays. An 898 bp product was amplified from the colonies using *G. diazotrophicus* specific primers. This further verified the bacterial colonization and confirmed that *G. diazotrophicus* colonized the inside of root tissues (Fig. 4*b*). In the uninoculated control *Brachypodium* root samples, no dark blue-stained bacterial colonies were observed. No bacteria were re-isolated from the surface sterilized uninoculated control root samples (data not shown).

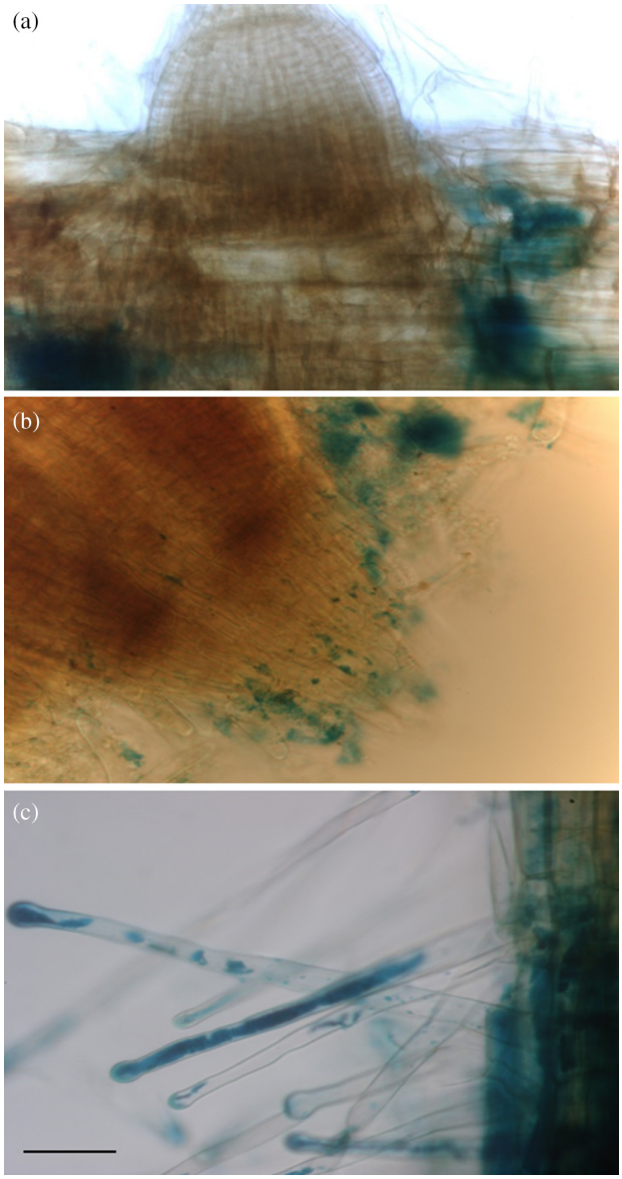
Fig. 2. GUS stained *Brachypodium* root samples 21 days after inoculation. (*a*) Control root sample after GUS staining assays. No blue stain was found on the control root sample. Scale Bar = 2 mm. (*b*) Colonization of *G. diazotrophicus* was established in *Brachypodium* root. The colonization showed as blue stain after GUS staining assays. Scale Bar = 2 mm. (*c*) Cross section of a *G. diazotrophicus* colonized *Brachypodium* root. The red arrow shows *G. diazotrophicus* colonization in the intercellular space between epidermal cells and cortical cells. Scale bar = 20 μm. [Colour online.]



Plant growth is promoted by *G. diazotrophicus*

To study the effect of *G. diazotrophicus* inoculation on *Brachypodium* growth and development, the fresh weight

Fig. 3. Patterns of *G. diazotrophicus* colonization in *Brachypodium* roots. (*a*) *G. diazotrophicus* entered *Brachypodium* from a lateral root emergence site. (*b*) *G. diazotrophicus* colonized *Brachypodium* from the opening at root tips. (*c*) GUS staining is apparent within root hairs in *Brachypodium*. Scale bar = 50 μm (*a*–*c*). [Colour online.]



and lateral root numbers for both control and inoculated plants were measured. No disease symptoms were observed in the inoculated *Brachypodium* plants, while promotional effects on plant growth were evident (Fig. 5*a* shows representative plants respectively). At 28 days after inoculation, the inoculated plants accumulated a higher fresh weight than the uninoculated plants (Fig. 5*b*). The average fresh weight of inoculated plants was 55.2 ± 3.3 mg, which is significantly higher than the average fresh weight of uninoculated plants at 42.5 ± 2.4 mg (*t*-test, $p < 0.01$). As plants were still small and the dry weight was minimal, dry weight measurements could not be taken accurately at this stage. The inoculated plants also developed more lateral roots than the uninoculated plants. The number of lateral roots was 13.1 ± 2.6 and 4.6 ± 1.2 for inoculated and uninoculated plants, respectively (Fig. 5*c*) and the difference is significant (*t*-test, $p < 0.01$).

Discussion

This study marks the first documented study of an interaction between the monocot model *Brachypodium* and the nitrogen-fixing bacterium *G. diazotrophicus*. The experiment was designed to test six plant growth conditions and to identify a condition that is more suitable for introducing *G. diazotrophicus* to *Brachypodium*. This study showed that *G. diazotrophicus* can colonize *Brachypodium*, but the level of colonization varies under different plant growth conditions. Current understanding of the interaction between nitrogen-fixing bacteria and their hosts shows that the success of colonization is greatly influenced by some environmental factors, such as soil texture, moisture, as well as nutrient status (Peoples et al. 2012; Li et al. 2015; Hassan et al. 2019; Rilling et al. 2019). The results from the six plant growth conditions in this study showed that the colonization by *G. diazotrophicus* was affected by the medium composition, solidity and the level of nitrogen content in the medium. The percent success of colonization increased with reduced medium solidity when an equivalent nitrogen level was present in the medium. Across medium solidity, the percent success of colonization was higher in the media that did not contain nitrogen. The highest colonization was seen in the liquid media without nitrogen (Fig. 1), at which, the colonization level was 30.21%. The roles of specific factors, such as medium solidity and nitrogen, on *G. diazotrophicus* colonization is not clear at this time. The colonization rate reported in this study can be practically useful for introducing *G. diazotrophicus* into *Brachypodium* for various studies, such as plant growth promotion as described in this report. In addition, we have used this inoculation method in a study that revealed *Brachypodium* genes and gene expression profiles in response to *G. diazotrophicus* colonization (Yang et al., unpublished study).

The trends found in this study are consistent with observations reported in previous studies. The decreased colonization rate from liquid to solid media could be associated with the reduced mobility and growth rate of bacteria in more solid media (Skandamis and Jeanson 2015; Tamar et al. 2016). When bacteria grow in solid media, the motility of bacteria may be restricted by higher agar content, so that the bacteria cannot move as freely as the bacteria grown in liquid and semi-solid media (Jeanson et al. 2015; Tamar et al. 2016). Therefore, bacteria would move along primary roots at a slower speed and may not have been able to access many lateral root emergence sites. Consequently, in solid media, *G. diazotrophicus* bacteria may only be able to start the

Fig. 4. Recovery of *G. diazotrophicus* from inoculated *Brachypodium* roots. (*a*) PCR analysis of the re-isolated *G. diazotrophicus* colonies from *Brachypodium* roots. A 898 bp PCR product is amplified from positive colonies using *G. diazotrophicus* specific primers from positive colonies. C1: colony #1, C2: colony #2, C3: colony #3, +: positive control. Sterile water was used as negative control (not shown in the picture). (*b*) The *G. diazotrophicus* colonies re-isolated from *Brachypodium* roots. The orange colour of colonies is a typical characteristic of *G. diazotrophicus* when grown in LGIP medium that contains bromothymol blue dye. [Colour online.]

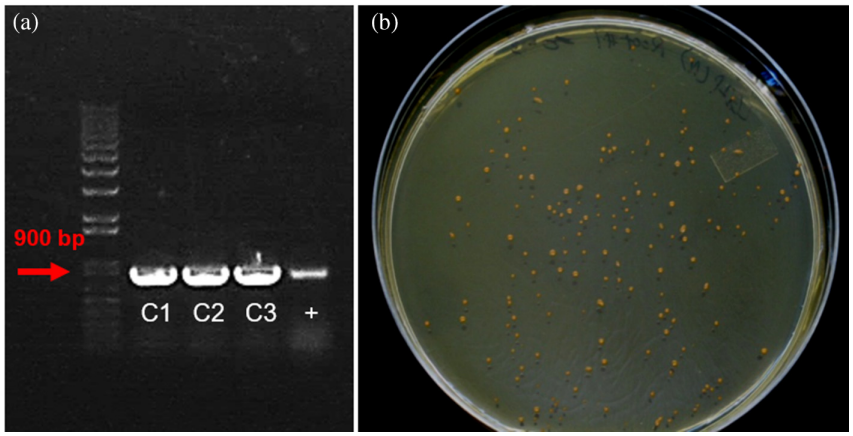
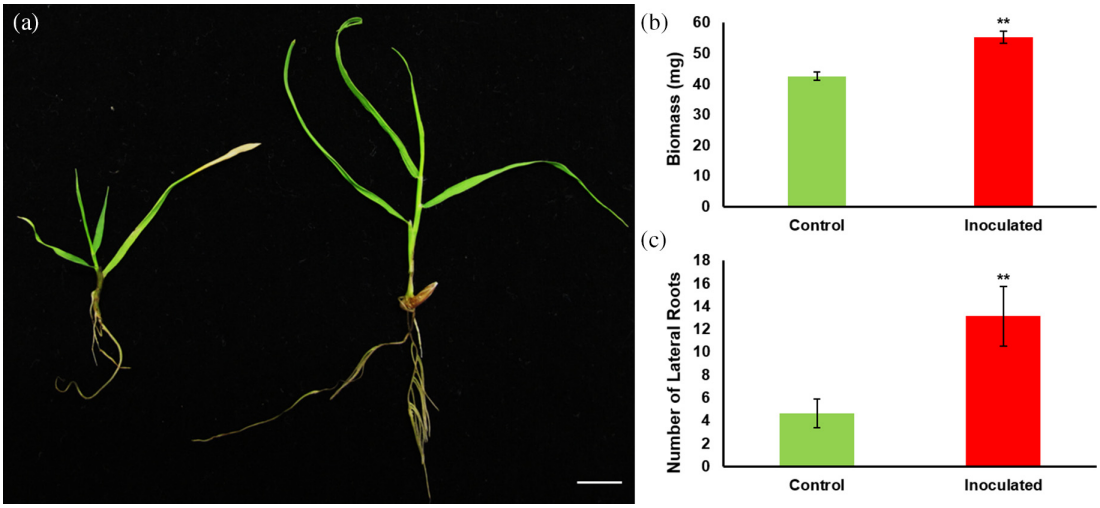


Fig. 5. Inoculation of *G. diazotrophicus* stimulated plant growth in *Brachypodium*. (*a*) Comparison of an uninoculated representative control plant and an inoculated representative plant; scale bar = 1 cm. (*b*) Comparison of the whole fresh weight between the uninoculated control plants and inoculated plants. For each treatment group, 30 plants were measured, and the experiment was repeated three times. ** indicates a significant difference between control and inoculated samples (*t*-test, $p < 0.01$), error bars indicate the standard error of the mean of three replicates. (*c*) Comparison of the number of lateral roots between the uninoculated control plants and inoculated plants. For each treatment group, 30 plant samples were measured. ** indicates a significant difference between control and inoculated samples (*t*-test, $p < 0.01$), error bars indicate the standard deviation of the number of lateral roots. [Colour online.]



colonization process through the lateral root emergence sites near their original adhering point on the primary roots. On the other hand, *G. diazotrophicus* could grow and move faster in semi-solid conditions, so more lateral root emergence sites could have been reached for colonization.

The nitrogen level in the environment showed an impact on the colonization of *Brachypodium* by *G. diazotrophicus*. With the solid MS media, the colonization rate in plants grown in the nitrogen-free-condition is significantly higher (2.75-fold) than the colonization success in plants grown in nitrogen containing solid medium. The same patterns were observed in the plants cultivated in the semi-solid MS media (1.90-fold) and hydroponic Hoagland solution (1.93-fold). This finding agrees with the results of the colonization experiments with some other nitrogen-fixing bacteria, such as *Azospirillum brasilense*, *Azospirillum lipoferum* and *Bradyrhizobium japonicum* (Abdel Wahab and Abd-Alla 1995; Fallik and Okon 1996; Tsagou et al. 2003;

Rodríguez-Blanco et al. 2015; Rodríguez-Andrade et al. 2015). It has been suggested that a high level of nitrogen does not negatively affect bacterial cells themselves. Instead, a high level of nitrogen triggers plant physiological changes that can subsequently affect the colonization by *G. diazotrophicus* (Fuentes-Ramírez et al. 1999; Rodríguez-Andrade et al. 2015). A higher environmental nitrogen concentration can change the uptake of NH_4^+ and NO_3^- in plants and then these nitrogen compounds act as signalling molecules to activate systemic resistance (Pieterse et al. 2014; Mur et al. 2017). As such, the colonization by *G. diazotrophicus* could be inhibited in *Brachypodium* plants grown in media that contain nitrogen. The effect of nitrogen availability on root growth and architecture could be another possible explanation for reduced *G. diazotrophicus* colonization in nitrogen containing media. It has been well documented that initiation of lateral root development can be induced under nitrogen limited conditions but is inhibited under higher levels of nitrogen supply (Walch-Liu et al. 2006; Kiba and Krapp 2016). More lateral roots provide more potential entry sites for *G. diazotrophicus* colonization. Thus, plant growth including size, biomass and architecture can be affected by nutrients such as nitrogen in the medium, which subsequently affects bacterial colonization to plants.

The presence or omission of nitrogen in growth media could affect *G. diazotrophicus* colonization indirectly by altering *G. diazotrophicus* exopolysaccharides (EPS) production. It has been demonstrated that EPS production is required for successful establishment and maintenance of plant colonization by *G. diazotrophicus* (Meneses et al. 2011). EPS plays an important role in attaching and anchoring *G. diazotrophicus* cells to plant roots at the initial phase of the colonization (Meneses et al. 2011) and protects *G. diazotrophicus* cells from reactive oxygen species that are produced by plant immune response (Meneses et al. 2017). Bacterial EPS production can be influenced by environmental conditions, such as nitrogen concentration, carbon source, and oxygen level (Valepyn et al. 2012; Rabha et al. 2012; Kaur et al. 2013; Ates 2015). Therefore, the variation of colonization observed in this study could potentially be the reflection of varying *G. diazotrophicus* EPS productions in different growth media due to the presence or absence of nitrogen and solidity of the medium. Along with the research progress in this area, deep understanding of the scientific questions about the different media and plant growth conditions on the colonization of *Brachypodium* by *G. diazotrophicus* will be revealed.General observation showed that the colonization of *Brachypodium* by *G. diazotrophicus* started mainly from the "crack entry" at the emergence sites of the lateral roots (Fig. 3*a*). The colonization of *Brachypodium* root tissues by *G. diazotrophicus* displayed a "crack entry" pattern, meaning *G. diazotrophicus* bacteria may enter *Brachypodium* plants at the lateral root emergence and at the openings in root tips. Lateral roots originate from the pericycle, located in the central part of a root called the stele. As they develop, lateral roots break through the endodermis, cortex, and epidermis then finally emerge from primary roots (Péret et al. 2009). The development of lateral roots appears to form passages for *G. diazotrophicus* to enter plants and expand the colonization in the different layers of root tissues. Other sites for *G. diazotrophicus* colonization include root tips and root hair (Figs. 3*b* and 3*c*). Based on hand-made cross section samples, it appeared that some colonization occurred intercellularly (Fig. 2*b*), which agrees with some previous studies (Luna et al. 2010; Fei et al. 2017). The entry and location of *G. diazotrophicus* in *Brachypodium* plant tissues during and after colonization is not yet fully understood.

In sugarcane, *G. diazotrophicus* can establish colonization in different parts of plants including root, stem and leaf (James et al. 1994; James et al. 2001). However, the *G. diazotrophicus* colonization pattern in *Brachypodium* is more similar to the patterns seen in rice, sorghum, wheat, and maize (Muthukumarasamy et al. 2005; Tian et al. 2009; Luna et al. 2010; Yoon et al. 2016), where the root tissues are the major sites for the colonization by *G. diatrophicus* in *Brachypodium*. In fact, most facultative endophytic bacteria were found to colonize only root tissues, and only a few non-pathogenic endophytic bacteria can spread colonization from roots to aerial vegetative parts of plants (Compant et al. 2010). In order to move from roots to upper vegetative tissues, endophytic bacteria need to pass through the endodermis, penetrate into xylem vessels, and then move up to aerial parts of the plant via transpiration. Passage through the endodermis can be achieved by active penetration of the cell layer via secretion of cell wall degrading enzymes such as cellulase, pectinase, and xyloglucanases; or, more passively, entering the passage created by lateral root eruption (Sessitsch et al. 2011; Compant et al. 2010). However, penetration of xylem vessels is difficult due to the degradation resistance of lignin in secondary walls (de Gonzalo et al. 2016). As a result, through this path the majority of endophytes are not able to further colonize their host plants beyond the roots. The restricted colonization in roots could also be explained by the activation of defense response in *Brachypodium*. It is well documented that mild plant defense response can be triggered and modulated to control the extent of endophyte colonization with respect to beneficial endophyte-plant host interactions (Iniguez et al. 2005; Bordiec et al. 2011; Liu et al. 2017).

Gluconacetobacter diazotrophicus colonization did not cause any disease symptoms in *Brachypodium*. We measured the fresh weight and lateral root number to evaluate plant growth promotion by this bacterium. While dry weight is a better measurement for quantification of biomass growth, due to some tissue handling limitations, only fresh weight measurements of the biomass are available in this study. However, in taking the fresh

weight measurements, procedures were taken to get as accurate measurements as possible, including careful and consistent blot-drying of all tissues in both the treatment group and the control group. The fresh weight of plants with *G. diazotrophicus* inoculation was higher than the control (Fig. 5). Also, the number of lateral roots in the inoculated plants was higher than in the uninoculated, control group (Fig. 5). Thus, overall, results show that *G. diazotrophicus* is beneficial to *Brachypodium* growth (Fig. 5). It has been reported that *G. diazotrophicus* can promote plant growth and yield in a several plant species (Luna et al. 2012; Lopes et al. 2019, Pace et al. 2020; Pagnani et al. 2020; Fei et al. 2020). Nevertheless, growth promotion associated with this bacterium in a vast majority of plant species has not been yet investigated and reported and its universal growth promotion has not been proven. The current study demonstrated plant growth promotion of *Brachypodium* by *G. diazotrophicus*. The results can be useful to study the mechanisms of plant growth promotion of this microbe using a monocot model plant.

The growth promoting effect of *G. diazotrophicus* on plants can be both direct and indirect. The capability of N-fixation and phytohormone production allows *G. diazotrophicus* to stimulate growth directly by supplementing nitrogen to plant and modulating the development of roots and shoots (Santos et al. 2019; Silva et al. 2020). *G. diazotrophicus* could also promote plant growth indirectly by increasing mineral nutrient availability to plants via acid production (Crespo et al. 2011; Muthukumarasamy et al. 2017; Delaporte-Quintana et al. 2020) and alleviating biotic and abiotic stress through alteration in metabolite production and phytohormone regulation (Canellas et al. 2019; Kudoyarova et al. 2019; Rodriguez et al. 2019). Additional studies of nitrogen fixation and phytohormone production by *G. diazotrophicus* within *Brachypodium* is warranted to further the understanding of the physiological mechanisms underlying the enhancement of plant growth by this bacterium.

This study supports the use of *Brachypodium* as a model plant to study the interaction of *G. diazotrophicus* and monocot plants. Further research in this area may lead to greater use of *G. diazotrophicus* to enhance the growth and productivity of numerous monocot crops.

Author Contributions

L.T. formulated and designed the research. X.Y. designed and performed the experiments. X.Y. and L.T. analyzed the data and results. X.Y., K.H. and L.T. wrote the manuscript. K.V. provided the bacterial strain, participated in research discussions during the study and helped edit the manuscript.

Acknowledgements

This research was supported by Agriculture and Agri-Food Canada and Natural Science and Engineering Research Council of Canada Discovery Program. We would like to thank Dr. Gang Tian for helping with the inoculation protocol modification.

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