

Article

The Isolation of Free-Living Nitrogen-Fixing Bacteria and the Assessment of Their Potential to Enhance Plant Growth in Combination with a Commercial Biostimulant

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Abstract: The development of microbial-based biostimulants to enhance the growth of crops and support a healthy and sustainable soil requires the isolation and large-scale industrial culture of effective microorganisms. In this study, work was undertaken to isolate and characterize free-living nitrogen-fixing bacteria capable of acting as biostimulants alone or by incorporation into and/or supplementation with a current commercial crop biostimulant for farmers. Free-living bacteria were isolated from soil, sugar cane mulch, and plant roots following preliminary culture in a nitrogen-free media that targeted specific groups of known diazotrophs. Following the identification of each isolate by 16S rDNA sequence analysis, isolates selected for further study were identified as most closely related to *Priestia megaterium*, *Sphingobium yanoikuyae*, and *Burkholderia paludis*. Each isolate was investigated for its capacity to promote plant growth in nitrogen-free media. Wheat seedlings were inoculated with the isolates separately, together as a consortium, or in combination with the commercial biostimulant, Great Land Plus[®]. Compared to no-treatment control plants, the fresh weights were higher in both the shoots (183.2 mg vs. 330.6 mg; $p < 0.05$) and roots (320.4 mg vs. 731.3 mg; $p < 0.05$) of wheat seedlings inoculated with *P. megaterium*. The fresh weights were also higher in the shoots (267.8 mg; $p < 0.05$) and roots (610.3 mg; $p = 0.05$) of wheat seedlings inoculated with *S. yanoikuyae*. In contrast, the fresh weight of the shoot and root systems of plants inoculated with *B. paludis* were significantly lower ($p < 0.05$) than that of the no-treatment control plants. Moreover, when Great Land Plus[®] was supplemented with a consortium of *P. megaterium* and *S. yanoikuyae*, or a consortium of *P. megaterium*, *S. yanoikuyae*, and *B. paludis* no promotion of plant growth was observed.

Keywords: nitrogen fixation; plant growth promotion; diazotrophs; biostimulant



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1. Introduction

Global agriculture faces significant challenges today and into the future. With the current global population projected to increase by over 1.5 billion people in less than 30 years, reaching 9.6 billion people by 2050, the need for sustainable food production is clear [1]. On the agricultural lands of many countries, decades and sometimes centuries of intensive farming practices have produced soils that are either depleted of, or overburdened with, minerals crucial to soil and plant health. This is particularly true for the status of nitrogen in many agricultural soils, where the ongoing application of nitrogen fertilizers and animal manures have created problems, with excessive nitrogen levels adversely affecting soil health [2,3]. In addition, the amounts of nitrogen fertilizers that are applied to the soil are often in excess of the amount the crops require and are capable of taking

up into their tissues. This practice often leads to the accumulation of nitrogen in the soil and the leaching of that nitrogen into local water bodies, which, in turn, can lead to the eutrophication of those receiving waters. The reliance on, and questions surrounding the environmental sustainability of, synthetic nitrogen fertilizers have agriculturalists searching for alternatives to support the nutritional requirements of crops and the management of nitrogen cycling on-farm.

To counteract the overexploitation of chemical fertilizers, improve nutrient use efficiency, and implement environmentally sustainable practices, there is a growing interest in the application of biofertilizers and biostimulants for plant health and management [4]. The role and application of microorganisms in the management of soil and plant health is at the forefront of thinking in securing a sustainable future for agriculture [5]. It is widely acknowledged that microbes play crucial roles in plant mineral nutrition, whether they act on the gross mineral content of soils or via specific plant–microbe interactions in the rhizosphere, as endophytes or epiphytes. In particular, plant growth is highly dependent on nitrogen availability [6]. Modern agriculture has up until now benefited from the availability of cheap and abundant fertilizers, primarily via industrial chemical processes meeting the demands of agriculture and a growing world population. For example, the design and implementation of the Haber–Bosch process has been the single most important contribution to providing nitrogen fertilizers. However, the exploitation of these industrial processes to meet the mineral nutrient requirements of agriculture has led to adverse environmental impacts that require rethinking of the ways in which agricultural crops will source nitrogen [7,8].

The implementation of microbial inputs is being increasingly seen as part of the future practices of farming for crop management. Relevant bacteria and fungi, either alone or as part of consortia, have been studied with a view to their commercialization in global agriculture markets. Indeed, by using microorganisms to provide plants with the necessary nitrogen required, the use of chemical fertilizers could be reduced, positively impacting the soil and soil microbiome [9,10].

The most familiar plant–microbe interaction in agriculture is nitrogen fixation that occurs between legume plants and host-specific bacteria. Symbiosis between the host plant and associated bacteria, most notably the group collectively called rhizobia, takes place in specialized plant organs, namely, root nodules [11]. This symbiosis is observed primarily within the legume plant group, which limits the potential to extend symbiotic nitrogen fixation to non-legume crops. However, bacteria that have the capacity to fix nitrogen as free-living organisms can be found in environments including soil, and as plant epiphytes and endophytes [12–14]. To reduce chemical fertilizer use and better manage nitrogen input and losses from soil, the application of free-living diazotrophs to soil is seen as a potential alternative [13,15]. However, while it is widely accepted that there is a diverse range of bacteria that exist as free-living nitrogen fixers in soils and as plant endophytes and epiphytes, there is some debate as to whether or not they contribute agronomically significant amounts of fixed nitrogen to resident plants [16,17].

By isolating and characterizing free-living nitrogen-fixing bacteria, we are a step closer to developing sustainable methods that would improve nutrient use efficiency in agricultural crops. Some of these organisms can be safely and effectively used in combination with existing natural products such as biofertilizers or biostimulants, considerably decreasing the need to use synthetic industrial chemicals in soil and the associated adverse impacts of their manufacture and application. Thus, the major objectives of this study were (i) to isolate candidate free-living nitrogen-fixing bacteria with potential to culture at commercial scale; (ii) to determine if the isolated free-living nitrogen-fixing bacteria could act as plant-growth-promoting bacteria in the case of plants growing in nitrogen-depleted substrates; and (iii) to determine the size, if any, of

growth promoting effect(s) when the isolated free-living nitrogen-fixing bacteria are used alone or in combination with a commercial biostimulant.

2. Material and Methods

2.1. Preparation of Environmental Samples and Isolation of Bacteria

The four media used in the initial stage of this study, designated as NFB, JNFB, JMV and LGI [18], were used for the isolation of nitrogen-fixing bacteria and for the sub-culturing of each isolate into pure cultures. NFB and LGI are media that have been designed to support the growth of *Azospirillum* spp., while JMV and JNFB have been designed to support the growth of *Burkholderia* spp. and *Sphingomonas* spp., respectively.

The environmental samples for the isolation of free-living nitrogen-fixing bacteria were obtained from sites located in the Sunshine Coast region, in the state of Queensland, Australia (26.52° S, 153.06° E). Randomly selected samples were collected from the rhizosphere soil of maize and bean, sugarcane mulch, compost, and the root tissue of bean and clover. The roots of legumes (bean and clover) and the soils in which they grew were chosen as sampling sites for the strong likelihood of isolating diazotrophs, and therefore sites to validate the methodology employed in this study. The soil, compost, and mulch were prepared by weighing 2 g of each sample, mixing separately in 10 mL of sterile 0.85% NaCl, and homogenizing by vortexing for 30 s. From the homogenized samples, 100 µL was then diluted in 3 mL of sterile 0.85% NaCl, of which 10 µL was inoculated into vials of semi-solid media. The bean roots were rinsed with sterile reverse osmosis H₂O and cut into pieces 2–5 mm in length using a sterile scalpel blade. The cut pieces were ground with a mortar and pestle in 2 mL of sterile 0.85% NaCl. The crushed root pieces were left soaking in the NaCl solution for between 30 min and 1 h to allow the bacteria to migrate from the root tissues to the liquid suspension. The mixture was then blended with a vortex for 30 s, and 10 µL was used to inoculate semi-solid media. The clover roots were rinsed with sterile reverse-osmosis H₂O before sterilization in 1% bleach for 5 min. The roots were next washed with 70% ethanol and then rinsed 3 times with sterile reverse-osmosis H₂O. The liquid from the last rinse was collected and plated on nutrient agar to confirm that the root surfaces were sterile. Next, the clover roots were processed as described above for the bean roots. The bean roots were not sterilized as the clover roots to enable the isolation of diazotrophs other than the symbiotic rhizobia normally associated with legume root tissues.

For the initial isolation of bacteria, sterile semi-solid media (1.8 g/L agar) were prepared in 5 mL McCartney bottles and the environmental samples added to the media (Figure 1). Bromothymol blue (0.1 g/L of a stock solution of 5 g/L in 0.2 N KOH) was added to each medium as a pH indicator to substantiate growth. When growth was also observed as a pellicle located at the surface or subsurface of the media, 10 µL of that pellicle was inoculated into a fresh semi-solid vial of the same medium. If growth was confirmed on the second passage, then a loopful of the new pellicle was streaked onto the same medium but on solid agar plates (15 to 25 g/L, depending on the medium recipe recommendation). Subsequent passages were carried out on the same solid medium, enriched with yeast extract (50 mg/L), or on rich media, including nutrient agar and potato-P agar [19], when enhanced bacterial growth was necessary for preparing the glycerol stocks of each isolate.

Azotobacter vinelandii ATCC 478 was used as a positive control for the isolation of free-living nitrogen-fixing bacteria. *A. vinelandii* was grown in ATTC medium 12, which comprised (per L) (1) base medium (1 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.2 g NaCl, and 5.0 mg FeSO₄), (2) soil extract (38.5 g African violet soil, 0.1 g Na₂CO₃, and 100 mL de-ionized H₂O water; boiled for 1 h and filter-sterilized), and (3) mannitol solution (20 g mannitol and 100 mL de-ionized H₂O; filter-sterilized). The pH of the medium was adjusted to 7.6 before autoclaving at 121 °C for

30 min. In the experiments described in this study, the soil extract was omitted from ATTC medium 12.

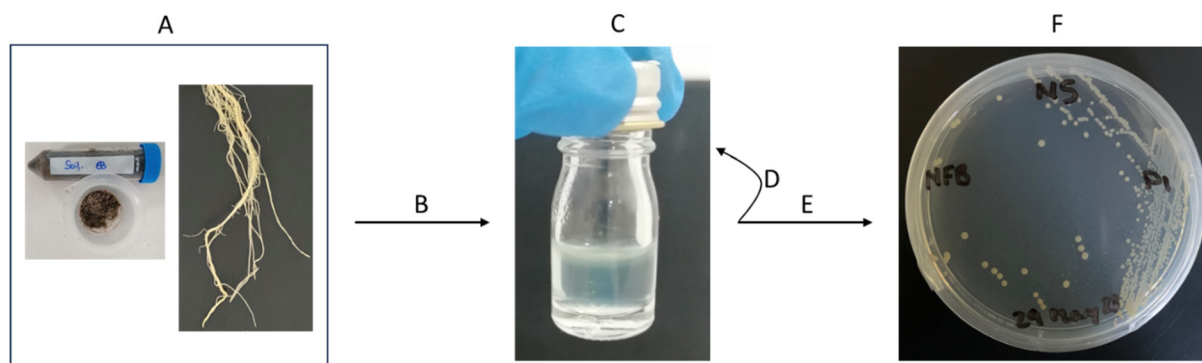


Figure 1. Isolation process from environmental samples. (A) Soil, compost, roots, and mulch were harvested from the local environment. (B) Each sample was inoculated into a semi-solid nitrogen-free medium. (C) Each McCartney bottle was filled to 50% with a nitrogen-free semi-solid medium and inoculated with 10 μ L of sample. A pellicle of growth can be observed on the surface of the medium. (D) In total, 10 μ L of the pellicle was inoculated into a new bottle containing the same nitrogen-free medium and, subsequently, (E) a loopful of the new pellicle was plated onto the same nitrogen-free medium as C but with agar (15–25 g/L). (F) Nitrogen-free solid medium plate showing the growth of single colonies.

2.2. Characterization of Isolates

The identification of each of the bacterial isolates was determined by DNA sequence analysis of PCR-amplified 16S rDNA. Genomic DNA was extracted from single colonies using the DNeasy PowerFood Microbial Kit (QIAGEN; Clayton, Melbourne, Australia). PCRs were performed using the universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') [20] under the following conditions in a total volume of 20 μ L: 25 mM of each primer, 50 ng genomic DNA, and 0.75 units of Taq DNA polymerase (REDTaq; Sigma Aldrich, Castle Hill, Melbourne, Australia); 35 cycles of denaturation at 94 $^{\circ}$ C for 1 min, annealing at 55 $^{\circ}$ C for 1 min, and extension at 72 $^{\circ}$ C for 2 min. The cycles were preceded by an initial denaturation at 94 $^{\circ}$ C for 4 min and completed by a final extension at 72 $^{\circ}$ C for 10 min. To verify that fragments of the appropriate size were amplified (~1498 bp), agarose gel electrophoresis was carried out, running each sample in 1% agarose gels in 1 $\times$ TBE buffer.

The PCR products were sent to the Australian Genome Research Facility (AGRF; Brisbane, Australia) for DNA sequencing. The DNA sequence data were used in blastn analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; accessed on 6 June 2023) to determine the identity of each isolate and/or the closest relative. The rRNA/ITS databases were searched and optimized for highly similar DNA sequences with megablast. Gram staining and light microscopy were also performed to determine the Gram morphology of each isolate and confirm that it coincided with the identification by 16S rDNA sequence analysis.

2.3. Plant–Microbe Interactions

An in-house bioassay was developed to determine the response of plants to the application of selected microorganisms and biostimulants. The aim of this bioassay was to enable the study of plant–microbe interactions in otherwise sterile media over a relatively short period of time, with sufficient development of plant shoot and root systems to allow the easy harvest of plants and the subsequent measurement of plant characteristics to efficiently determine the effectiveness of plant additives.

Two experiments were conducted to determine the plant growth promoting potential of the bacteria isolated in this study. These experiments used wheat as the plant host. The first experiment assessed the plant growth promotion potential of the three candidate nitrogen-fixing bacteria, individually and as a consortium. The second experiment re-assessed the plant growth potential of the nitrogen-fixing bacteria that showed a positive impact from the first experiment, adjusting the composition of the consortium accordingly. The bacterial isolates were grown in 20 mL of veggie peptone (Oxoid™, Melbourne, Australia) at 32 °C for 18 h. The objective was to obtain a final inoculant concentration for each isolate of approximately 1×10^8 cfu/mL live bacteria. The wheat seedlings were grown in 100 mL clear PET bottles (40 mm diameter $\times$ 115 mm height), with one plant per bottle. The medium used to test the capacity of the bacterial isolates to promote plant growth was nitrogen-free and contained the following (final concentrations): 0.5 mM KH_2PO_4 , 0.5 mM MgSO_4 , 1.25 mM KCl, 0.5 mM CaCl_2 , 25 μM H_3BO_3 , 12.5 μM Fe-EDTA, 4.5 μM MnCl_2 , 4 μM ZnSO_4 , 0.15 μM Na_2MoO_4 , 100 μM CuSO_4 , and 0.75% bacteriological agar. Seeds were sterilized with 80% ethanol for 3 min, and then rinsed with sterile de-ionized water. One seed was placed on the surface of the growth medium in each bottle. The plants were grown at ambient room temperature under LED lights (Spiderfarmer Model SF-2000, Sydney, Australia; 200 W; 660–665 nm/730–740 nm; PPF: 553.9 $\mu\text{mol/s}$; PPE: 2.7 $\mu\text{mol/J}$; 36,346 Lm $\pm$ 5%; Shenzhen Meizhiguang Technology Company Ltd., Beijing, China) on a 16 h light–8 h dark cycle for the number of days specified below.

The ability of the bacterial isolates to promote plant growth was assessed separately (as individual isolates) and when used as a supplement to the commercial microbial-based biostimulant, Great Land Plus® (Terragen Biotech; <https://www.terragen.com.au/great-land-plus/> (accessed on 20 January 2025); Coolumb Beach, Melbourne, Australia). Great Land Plus® contains the following live bacteria and yeast: *Lactocaseibacillus casei*, *Lentilactobacillus buchneri*, *Lentilactobacillus parafarraginis*, *Lentilactobacillus rami*, *Acetobacter fabarum*, and *Candida ethanolica*. The bacterial isolates and Great Land Plus® were applied to the plants, on the surface of the media, five days after sowing and then at weekly intervals. The treatment groups included each of the selected bacterial isolates (overnight liquid culture in veggie peptone), Great Land Plus®, combinations thereof, or 0.1 M KNO_3 . For each treatment group, 250 μL of the relevant component (i.e., live bacteria, KNO_3 , or Great Land Plus®) was applied per application. A no-treatment control group (NTC) was also included in this study. There were from 5 to 10 plants for each treatment and/or control group. Plants were harvested at 28 days (experiment 1) and 31 days (experiment 2) after sowing. The wheat seedlings were extracted from the growth media, and the fresh weight of the root and shoot tissue was determined for each plant.

2.4. Statistical Analysis

The data collected from the plant experiments (root and shoot weights for each treatment group) were analyzed with Microsoft Excel using the ANOVA single factor test, followed by Tukey's post hoc test to determine significant differences (<https://www.statskingdom.com/180Anova1way.html> (accessed on 20 January 2025); Melbourne, Australia). We used $p \leq 0.05$ as the limit to determine significant differences between treatment groups. Standard error for the mean of each group was calculated by dividing the standard deviation by the square root of the sample size for each group.

3. Results

3.1. Isolation of Bacteria

The four distinct nitrogen-free media used in this study enabled the isolation of bacteria considered candidate free-living nitrogen fixers from local and easily accessible

sources. The growth of these putative free-living nitrogen-fixing bacteria in semi-solid nitrogen-free media was seen as a distinct pellicle, either on the surface of the media or suspended below the surface, indicating the isolation of aerobic bacteria (surface pellicle) and microaerophilic/facultative anaerobic bacteria (sub-surface pellicle). The isolates designated as NFB1, NFB2, NFB4, NFB5, D1, and D2 were initially isolated on NFB medium. Similarly, LGI4 and LGI5 were isolated on LGI, JMV4 was isolated on JMV, and JNFB1 was isolated on JNFB. To enable the isolation of pure cultures, the ten candidates were next sub-cultured through the initial isolation medium and then nutrient-rich media (nutrient agar or potato-P agar) before further characterization.

3.2. Characterization and Identification of the Isolates

Genomic DNA extraction, followed by the PCR amplification and DNA sequence analysis of the 16S rDNA, allowed the identification of the ten putative nitrogen-fixing bacteria (Table 1). All ten isolates were shown via blastn analysis to be greater than 99% similar to their closest relative, and therefore from the same designated species. Based on the literature search of their closest relatives, some of the isolates were deemed to have the potential of being beneficial nitrogen-fixing bacteria, while a few isolates were identified with species not expected to be associated with free-living nitrogen-fixing bacteria. Using the approach presented in this study, it was not surprising that some of the isolates, in this case, D2 and JNFB1, were identified as members of the *Rhizobium* genus. Three isolates, JMV, LGI5, and NFB1, were also identified as members of the *Agrobacterium* genus. Two of the isolates, LGI4 and NFB4, were highly similar (>99%) to *Burkholderia paludis* and *Burkholderia contaminans*. As a cautionary observation for this type of study, isolate D1 was most closely identified with *Xanthomonas translucens*, which is noteworthy as a plant pathogen [21]. Of greatest interest to this study were the two isolates, NFB2 and NFB5, which were most closely related to *Priestia megaterium* and *Sphingobium yanoikuyae*, respectively. Based on the identification via DNA sequence analysis, the isolates identified as *P. megaterium*, *S. yanoikuyae*, and *B. paludis* were selected for further experiments to determine if they were capable of plant growth promotion.

Table 1. Description of the most relevant properties of each isolate from this study, including the source of each isolate, the closest relatives of each isolate based on 16S rDNA sequence BLAST analysis, the size of the 16S rDNA fragments used in the identification of each isolate, and the corresponding accession number of each 16S rDNA sequence.

Isolate	Environmental Sample	Closest Relative (% Similarity)	Size of DNA Fragment (bp); Accession Number
D1	Clover roots	<i>Xanthomonas translucens</i> (>99)	1243; OR378553
D2	Clover roots	<i>Rhizobium</i> sp./ <i>Agrobacterium pusense</i> (>99)	1401; OR378554
JMV4	Compost	<i>Agrobacterium rhizogenes</i> (100)	1393; OR378555
JNFB1	Maize rhizosphere	<i>Rhizobium</i> sp./ <i>Agrobacterium pusense</i> (>99)	1401; OR378556
LGI4	Compost	<i>Burkholderia paludis</i> / <i>Burkholderia contaminans</i> (>99)	1439; OR378557
LGI5	Bean roots	<i>Agrobacterium tumefaciens</i> (>99)	1402; OR378558
NFB1	Maize rhizosphere	<i>Agrobacterium tumefaciens</i> (>99)	1396; OR378559
NFB2	Sugarcane mulch	<i>Priestia megaterium</i> (>99)	1466; OR378560
NFB4	Compost	<i>Burkholderia paludis</i> / <i>Burkholderia contaminans</i> (>99)	1445; OR378561
NFB5	Bean roots	<i>Sphingobium yanoikuyae</i> (>99)	1395; OR378562

3.3. Plant–Microbe Interactions

The first plant bioassay experiment, using wheat as the host plant, involved the application of *P. megaterium*, *S. yanoikuyae*, and *B. paludis* separately and combined, as well as separate groups treated with Great Land Plus® and Great Land Plus® supplemented with all three putative nitrogen-fixing species (Figure 2). A ‘no-treatment’ negative control group (NTC) and a positive control group of KNO_3 application were also included in this study. The plant bioassay medium did not contain a readily metabolizable carbon source, and hence the treatments were applied weekly to the plants to ensure that viable microorganisms were available for interaction with the plants.

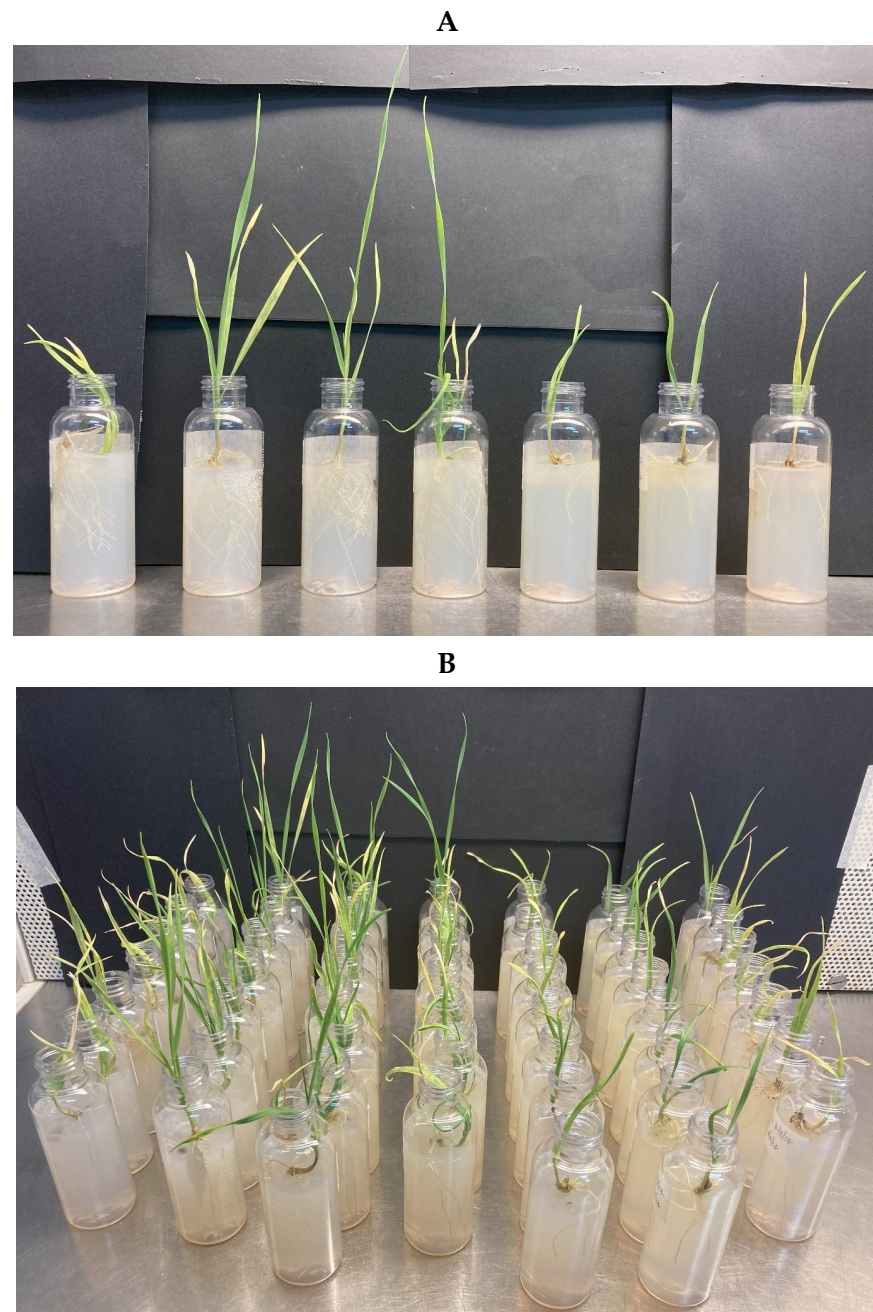


Figure 2. Wheat plants from the first bioassay experiment, testing the putative nitrogen-fixing isolates, immediately prior to harvest. Image (A) shows individual representatives from each control and treatment group, while image (B) shows all plants. In both images, the plants are, from left to right, no-treatment control, KNO_3 positive control, plus *P. megaterium*, plus *S. yanoikuyae*, plus *B. paludis*, plus all three nitrogen-fixing isolates, plus Great Land Plus®.

The measurement of both the root and shoot fresh weights showed some statistically significant differences (Figure 3A). The root fresh weights of the NTC plants (320.4 ± 46.0 mg) were significantly different to the plants treated with KNO_3 (610.3 ± 97.1 mg) and *P. megaterium* (731.3 ± 52.5 mg). The root fresh weights of plants treated with *S. yanoikuyae* (610.3 ± 128.1 mg) were greater than those of the NTC plants but not significantly different at $p \leq 0.05$. The root fresh weights of plants treated with *B. paludis* (142.0 ± 11.8 mg), a combination of the all the putative nitrogen-fixing isolates (165.2 ± 14.2 mg), and Great Land Plus® (302.5 ± 24.5 mg) were lower than those of the NTC plants but not significantly different at $p \leq 0.05$. While the *B. paludis* isolate is capable of fixing nitrogen by virtue of its capacity to be grown in nitrogen-free media, it did not promote the growth of the wheat plants in this bioassay. This might explain why the combination of all three putative nitrogen fixers did not promote plant root system development. Moreover, the root system of plants treated with either *P. megaterium* or *S. yanoikuyae* were not significantly different to the KNO_3 -treated positive control plants. A similar set of results was seen with the shoot fresh weights (Figure 3B). The shoot fresh weights of the NTC plants (183.2 ± 16.6 mg) were not significantly different to those of the plants treated with *B. paludis* (138.5 ± 6.3 mg), Great Land Plus® (153.8 ± 10.5 mg), or the combination of all putative nitrogen-fixing isolates (159.3 ± 5.1 mg). The plants treated with either *P. megaterium* (330.6 ± 14.5 mg) or *S. yanoikuyae* (267.8 ± 24.6 mg) had shoot fresh weights that were not different to those of the KNO_3 -treated plants (329.0 ± 28.4 mg).

A second wheat bioassay experiment was undertaken to determine if the elimination of *B. paludis* could lead to plant growth promotion when *P. megaterium* and *S. yanoikuyae* were used in combination, and if the combination of these two bacteria could supplement the growth promotion benefits of Great Land Plus®. While similar outcomes were observed for the treatment groups that were included in the first bioassay experiment, there were some unexpected outcomes in this second experiment. Firstly, for the root system data (Figure 4A), the KNO_3 treatment group (747 ± 137.1 mg) was the only group that was significantly different to the NTC group (238.4 ± 39.7 mg; $p < 0.05$). While the *P. megaterium* (378.41 ± 98.6 mg) and *S. yanoikuyae* (428.9 ± 116.1 mg) groups had larger root systems, they were not significantly different to the NTC group. The *P. megaterium* and *S. yanoikuyae* groups were also not significantly different to the KNO_3 treatment group. The combination of *P. megaterium* and *S. yanoikuyae* (155.0 ± 33.0 mg) and the supplementation of Great Land Plus® with these two species (183.2 ± 18.5 mg) did not lead to an enhanced root system.

The results obtained for the shoot systems are shown in Figure 4B. The shoot fresh weights of the KNO_3 group (213.2 ± 25.2 mg) were the only ones that were significantly greater than those of the NTC group (139.1 ± 11.7 mg). The shoot fresh weights of plants treated with *P. megaterium* (169.5 ± 15.9 mg), *S. yanoikuyae* (199.5 ± 15.0 mg), a combination of *P. megaterium* and *S. yanoikuyae* (179 ± 35.5 mg), Great Land Plus® (135.5 ± 13.9 mg), and a combination of *P. megaterium*, *S. yanoikuyae*, and Great Land Plus® (178.8 ± 15.9 mg) were not significantly different to those of either the NTC or KNO_3 groups.

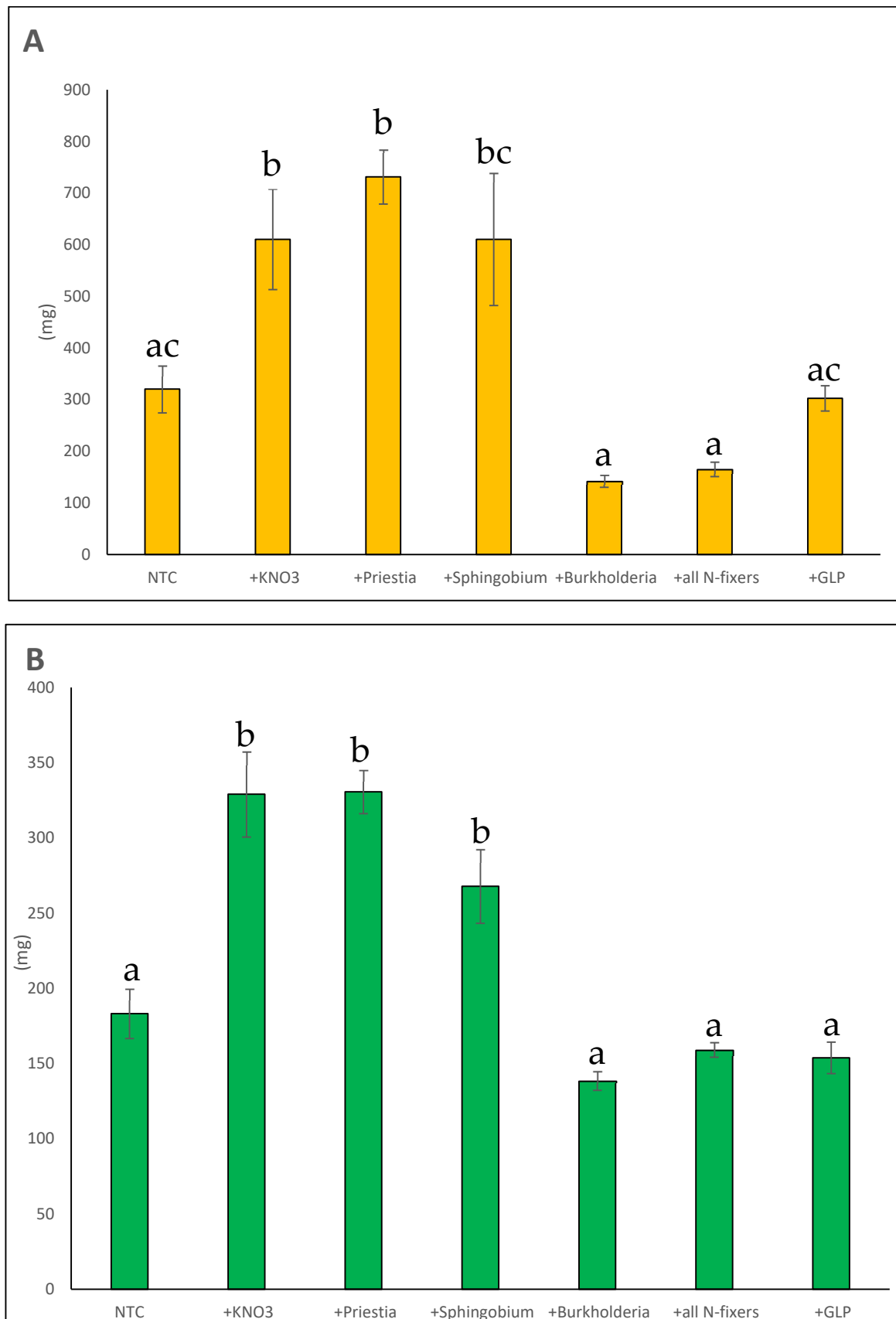


Figure 3. Root (A) and shoot (B) fresh weights of wheat plants from the first bioassay experiment. Groups sharing the same letter above a histogram column are not significantly different at $p \leq 0.05$.

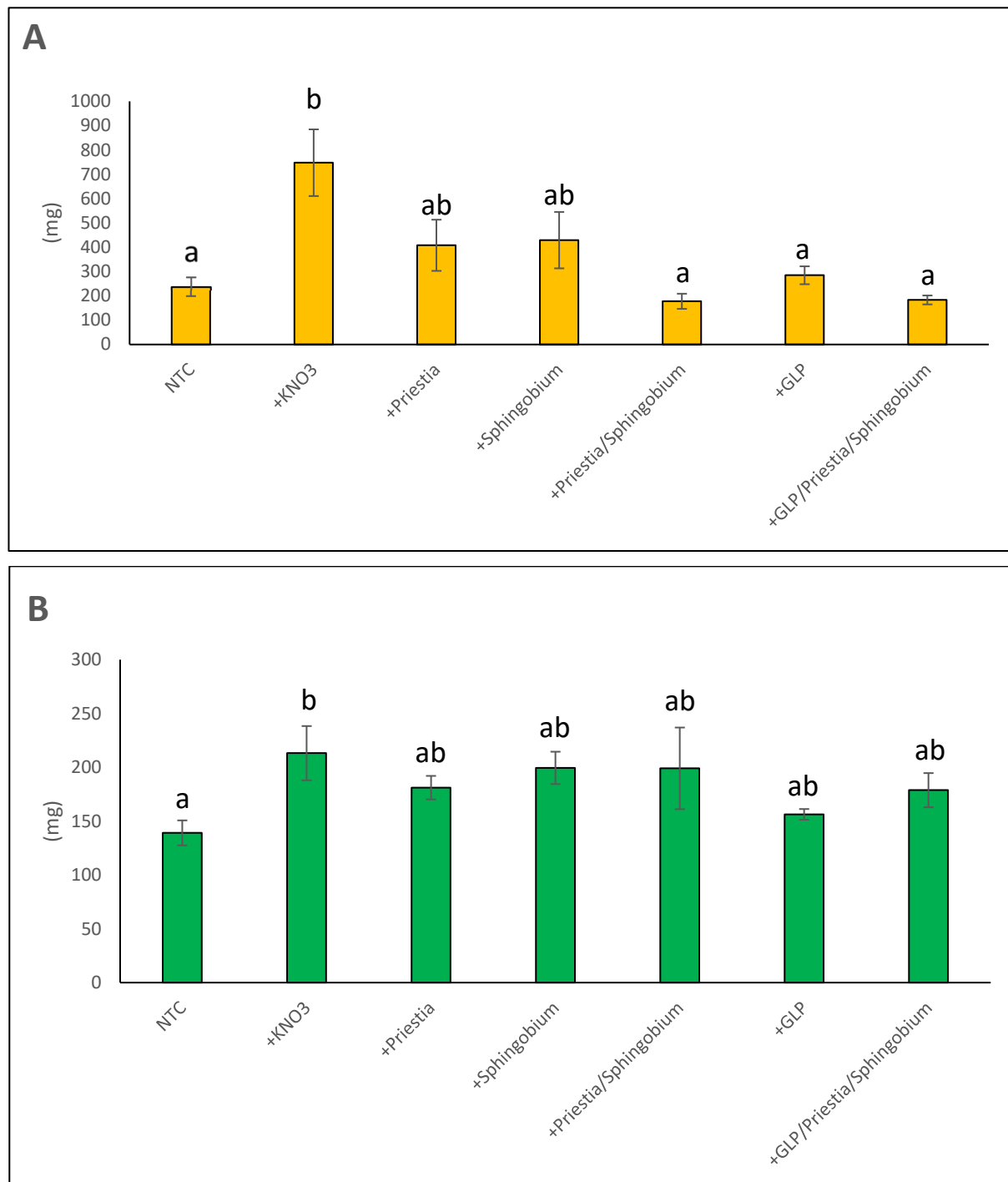


Figure 4. Root (A) and shoot (B) fresh weights of wheat plants from the second bioassay experiment. Groups sharing the same letter above a histogram column are not significantly different at $p \leq 0.05$.

4. Discussion

The exploitation of microorganisms to promote and support the growth of crops and pastures has seen the recent arrival of new companies and the expansion of established agricultural biotechnology companies towards the manufacture of a wide range of bio-stimulants, inoculants, and biofertilizers (<https://www.mixingbowlhub.com/landscape/2023-ag-biologicals-landscape> (accessed on 20 January 2025); Menlo Park, CA, USA). The microbial composition of these products is diverse, with an extensive range of bacterial, fungal, and microalgal species being employed [22]. The mechanisms by which these products operate are similarly diverse, with microbes present that are capable of improving

nutrient acquisition (including nitrogen fixation), plant hormone production and regulation, protection from abiotic and biotic stress, and improvements in the soil microbiome.

This study demonstrated the isolation and plant growth promoting potential of candidate diazotrophs from local sources following their growth on nitrogen-free media. A diverse group of ten distinct isolates was identified by 16S rDNA sequence analysis. Expectedly, some of these isolates were not deemed to be relevant targets of this study, in particular, representatives from the rhizobia and agrobacterium groups. *Agrobacterium tumefaciens* has previously been described as a diazotroph [23]; however, its role as the agent in crown gall disease eliminated the corresponding isolates from further analysis in this study. The three species of most interest from this study were *P. megaterium*, *S. yanoikuyae*, and *B. paludis*. Members of the genus *Burkholderia*, including *B. contaminans*, have been noted as both nitrogen fixers and plant growth promoters [24,25]. *Priestia megaterium* (formerly *Bacillus megaterium*) is known to promote plant growth, has the capacity for nitrogen fixation [26], and has the properties of bioremediation and tolerance to high salinity [27]. Similarly, the genus *Sphingobium* contains some species with plant growth promoting, biodegradation, and bioremediation properties [28,29]. Two of these three isolates, *P. megaterium* and *S. yanoikuyae*, proved to be capable of promoting the growth of wheat seedlings in nitrogen-free media when compared to the NTC group. The bioassay system developed in-house proved to be effective at demonstrating the positive impacts of *P. megaterium* and *S. yanoikuyae* in a relatively short period of time and in the absence of impacts from other microorganisms. Interestingly, each of these bacteria were capable of promoting plant growth when applied individually, but not when applied in combination. This final observation suggests that there may be some antagonistic metabolites being produced by one or both of these bacteria that may negatively impact on their ability to support microbial viability and/or plant growth in nitrogen-free media. It also provides a cautionary note for the formulation of multispecies biostimulants. As it is likely that not all plant growth-promoting microbes can be combined to exert an additive or synergistic positive impact, studies of combinations of microbes should be undertaken during the formulation of new products to demonstrate a beneficial impact.

It is important to recognize the limitations of any study employing an in vitro assay to assess plant–microbe interactions, and the assay developed for this study is no exception. The medium used to support the growth of plants was nitrogen-free in order to show that the nitrogen-fixing bacteria were the only route to supply nitrogen to the plants. Further, the lack of any carbon source to support the growth of the nitrogen-fixing bacteria or the microorganisms in Great Land Plus® may have limited the potential benefit that these inoculants may have provided to the plants. Finally, the impacts of the applied microorganisms on host plants in the context of resident soil microbiome are not addressed with an in vitro bioassay such as the one used in this study.

Nonetheless, the positive plant growth promotion results from this study follow previously reported positive outcomes for *P. megaterium* on the growth of maize in greenhouse and field studies [30]. Further, in another study, *P. megaterium* was shown to improve the growth of the herbaceous plant *Centella asiatica* [31]. In the absence of quantitative measurements for nitrogen fixation activity, it is difficult to categorically conclude that the growth promotion observed in this study was singularly due to nitrogen fixation. It is known that many nitrogen-fixing bacteria are capable of plant growth promotion via other mechanisms (e.g., the biosynthesis of plant hormones), and so the positive impacts on plant growth may be due to an accumulation of effects. In particular, the benefits seen with the development of the root systems of plants treated with either *P. megaterium* or *S. yanoikuyae* are likely due to the metabolites synthesized by these bacteria [32,33]. A recent extensive study, also using wheat as the host plant, isolated bacteria on nitrogen-free media,

and assessed their capacity to dissolve phosphate, demonstrate antifungal activity, and produce ammonium, indole-3-acetic acid, and siderophores [34], with isolates exhibiting plant growth promotion via one or more of these activities.

By adding the isolated diazotrophs to an existing commercial biostimulant, Great Land Plus[®], we could hypothesize that there would be an additive effect in growth promotion (that is, in excess of any stimulation achieved by the commercial biostimulant alone). In fact, the combination of Great Land Plus[®] with both *P. megaterium* and *S. yanoikuyae* failed to see growth of wheat seedlings beyond that seen in NTC plants. It is also worth noting that, in this study, the application of Great Land Plus[®] did not result in growth promotion. This would indicate that the plant growth promoting capabilities of Great Land Plus[®] are not a result of nitrogen fixation. Further studies will be required before any of the diazotrophs isolated in this study are used alone as biostimulants or incorporated into products such as soil conditioners or biostimulants. Before any field studies are performed on a range of crops and pastures to support the positive impacts on plant growth reported here, it will be important to establish that any candidate diazotrophs are capable of being cultured at a commercial scale and are compatible with any other microorganisms deemed appropriate to include in any candidate consortia.

The absence of an additive effect of *P. megaterium* and *S. yanoikuyae* with or without the supplementation of Great Land Plus[®] is of interest, but the design of this study cannot help explain this finding. It is possible that these two species are not compatible and/or their mechanisms of plant growth promotion are counteractive. Additional research is required to elucidate a possible mechanism. For example, the plant bioassay should be repeated and plants inoculated with Great Land Plus[®] supplemented separately with *P. megaterium* and *S. yanoikuyae*, to determine if these nitrogen-fixing bacteria individually can add to the growth promotion of Great Land Plus[®]. It is widely recognized that positive impacts on plants observed through in vitro glasshouse studies are not always automatically translated to field studies. Now that it has been demonstrated that *P. megaterium* and *S. yanoikuyae* can positively impact plant growth, it will be valuable to assess their plant growth promotion potential in field studies.

5. Conclusions

In conclusion, this study demonstrated the relative ease of isolating bacteria capable of growth in nitrogen-free media and demonstrating the capacity for two of these isolates, *P. megaterium* and *S. yanoikuyae*, to promote the growth of wheat seedlings in a simple bioassay in nitrogen-free media. This study also showed that any growth promotion benefit that could be attributed to each of these bacteria when applied to plants was not maintained or manifested as a positive additive effect to an already established biostimulant, providing a cautionary note to the manufacturers of new products.

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Data Availability Statement: The nucleotide sequences of 16S rDNA for the bacteria isolated in this study are freely available via the accession numbers in Table 1. All other data are freely available upon request to the corresponding author.

Conflicts of Interest: All three authors were employees of Terragen at the time the experimental work in this study was performed. M.S. was a consultant with Martin Soust & Co Pty Ltd. and GR International Pty Ltd. during the preparation of the manuscript.

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