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Rhizosphere Bacteria Biofertiliser Formulations Improve Lettuce Growth and Yield under Nursery and Field Conditions

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Abstract: Rhizosphere bacteria can provide multiple benefits to plants, including increased nutrient supply, pathogen/disease control, and abiotic stress tolerance, but results from pot trials do not always translate to field conditions. This study tested whether rhizosphere biocontrol bacteria can also provide plant growth promotion and how benefits can be provided at a commercial farm. Commercial lettuce seeds and plants were treated with rhizosphere biocontrol bacteria *Bacillus velezensis* UQ9000N, *B. amyloliquefaciens* 33YE, *Brevibacillus laterosporus* 4YE, and *Pseudomonas azotoformans* UQ4510An. 33YE increased the head diameter, plant height, and fresh weight of the Green Moon cultivar, while 33YE, UQ4510An, and UQ9000N increased the fresh and dry weight of Liston, a more heat-tolerant cultivar, via a single seed treatment or repeat root treatments under nursery and field conditions across different inoculation schedules and growth stages. Significant growth promotion was also demonstrated when inoculating field plants after transplanting (in particular for 33YE). Applications of these microbial biostimulants to lettuce seeds or plantlets potentially enable earlier transplanting and earlier harvests. Repeat inoculations using irrigation water and long-lasting formulations may further advance the benefits of these biostimulants as microbial biofertilisers for plant growth promotions in the field.

Keywords: *Bacillus*; *Brevibacillus*; biostimulant; field trial; *Lactuca sativa*; microbial biofertiliser; PGPR; plant growth promotion; *Pseudomonas*

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1. Introduction

Lettuce (*Lactuca sativa* L.) is a major leaf vegetable that is extensively cultivated worldwide [1,2]. It is an annual and cool-season crop that is particularly grown in temperate and subtropical regions [1,2]. In 2020, global production of lettuce was 28 million tonnes (combined with chicory), with China producing 52% of the world's total (14.3 million tonnes), followed by the USA with 16% and India with 4% [3]. Lettuce cultivars can be divided into six main types based on the shape and size of the leaf, stem type, and head formation, including (1) crisphead or iceberg (most commonly cultivated), (2) romaine or cos, (3) butterhead, (4) Latin, (5) leaf or cutting, and (6) stem or stalk [1,2]. Lettuce is usually consumed raw and is a good source of fibre, folate, iron, and other health-beneficial compounds [2].

Globally, the standard method of cultivation of lettuce is transplant production, which has many advantages over direct seeding in the field [4,5]. Advantages include lower cost,

earlier crop maturation and harvesting, improved usage of land, and enhanced control of weeds [4–6]. This method of cultivation requires strict control of several factors for optimal growth and yield of crops, such as frequency of irrigation, fertilization rates, the addition of biological amendments, characteristics of the growth medium, and greenhouse growth parameters [5,7–10]. One of the key parameters is the availability of macronutrients and micronutrients during vegetable transplant production for its subsequent health and development in the field [5,11]. Typically, inorganic chemical fertilisers are supplied to lettuce seedlings in nursery greenhouses to improve their vigour and increase their growth and yield after transplanting, followed by more fertiliser applications [2,5,10,12]. However, overuse of chemical fertilisers can result in nutrient runoff, nitrogen pollution, and leaching losses, leading to harmful effects on the environment, biodiversity, and human health [5,12–14]. Nitrate contamination of water can lead to severe adverse effects on human health; for example, thyroid disease, colorectal cancer, and neural tube defects [2,15,16].

Plant growth-promoting rhizobacteria (PGPR) are a sustainable alternative strategy as biostimulants for growth enhancement and improved yield of plants compared to chemical fertilisers [13,14,17]. The main advantages of PGPR-based biofertilisers over chemical fertilisers include relatively low or no direct impact on the environment, biodiversity, and human health [5,13,18,19]. PGPR strains from the genera *Bacillus* spp. are some of the best-studied and utilised in microbial-based biofertilisers and biopesticides [19–21]. Vetrano et al. [5] reported that the treatment of lettuce seedlings of the Meraviglia d'inverno cultivar with *Bacillus* spp. promoted plant growth and improved yield and nitrate content under variable nutritional levels in greenhouse trials and after transplanting in the field. Kröber et al. [22] showed that the *B. amyloliquefaciens* strain FZB42 improved the growth of lettuce plants and had no major changes in the native rhizosphere microbiomes of lettuce under field conditions.

Many other bacterial genera are used as biofertilisers [13]. Trinh et al. [2] showed that the *Pseudomonas nitroreducens* strain PnIHB enhanced the growth of lettuce plants under field conditions by stimulating cell development and nitrate absorption. Similarly, Cipriano et al. [23] demonstrated that two *Pseudomonas* strains, *P. cremoricolorata* IAC-RBcr4 and *P. fluorescence* IAC-RBru1, promoted the growth of lettuce cultivars Veronica and Sakata under greenhouse and tropical field conditions. They also altered the rhizosphere microbiome, specifically bacteria belonging to the Planctomycetes and Actinobacteria phyla. Several studies have also shown the great potential of *P. azotoformans* strains as biofertilisers [24–30]. The commercial biofertiliser product Amase contains *P. azotoformans*, which has been used in Sweden [31–33]. Silambarasan et al. [34] showed that *Stenotrophomonas rhizophila* CASB3 improved the growth of lettuce under salinity stress and also biodegraded the herbicide 1,1-dimethyl, 3-(3′,4′-dichlorophenyl) urea (Diuron) under greenhouse conditions.

We have previously shown that the rhizobacteria, *Bacillus velezensis* UQ9000N, *Bacillus amyloliquefaciens* 33YE, *Brevibacillus laterosporus* 4YE, and *Pseudomonas azotoformans* UQ4510An produce antimicrobial compounds and inhibit a range of plant pathogens by direct antagonism or through induced plant defence responses [35,36]. While the potential of these bacteria as microbial biopesticides has been established, the current study sought to test their potential to act as biostimulants for plant growth promotion. Significant growth promotion was observed in commercial lettuce plants for all bacteria used under pot, nursery, and field conditions, suggesting their potential as microbial biofertlisers.

2. Materials and Methods

Seven plant trials were conducted in this study, which included one laboratory experiment and six trials at a commercial lettuce farm (Koala Farm, Lake Clarendon, Queensland, Australia). Table 1 summarises (1) the three lettuce cultivars used, namely Green Cos, Green Moon, and Liston, (2) four bacterial isolates and treatments, including *B. amyloliquefaciens* (33YE), *B. laterosporus* (4YE), *B. velezensis* UQ9000N, and *P. azotoformans* UQ4510An, and (3) plant parameters measured in the trials.

Table 1. Summary of lettuce trials used in the present study.

Trial Number	Experiment Type	Field Row Number	Plant Cultivar	Number of Plants (Per Treatment)	Bacteria Name	Bacteria Volume (OD 600 nm of 0.1)	Treatment Type	Treatment Time	Harvest/Measure Time	Measured Parameters
Potting trial	Preliminary experiment	NA	Green Cos	30	33YE; UQ9000N	2 mL per plant	1st treatment: Seed treatment	Week 0	Week 2	Surface area
							2nd treatment Soil treatment	Week 4	Week 6	Fresh weight, dry weight
Preliminary trial	Preliminary experiment	NA	Green Moon	60	33YE	2 mL per plant	Seed treatment	Week 0	Week 2	Surface area
								Week 4	Week 4	Plant height, fresh weight, chlorophyll content
Trial 1	Nursery experiment	NA	Green Moon	144	33YE; UQ9000N; YEP	2 mL per plant	1st treatment: Soil treatment	Week 1	Week 6	Plant height
							2nd treatment Soil treatment	Week 3		
Trial 2	Field experiment, transplant at week 6	68L	Green Moon	144	33YE; UQ9000N; YEP	2 mL per plant	1st treatment: Seed treatment	Week 0	Week 8	Head diameter
							2nd treatment: Soil treatment	Week 2	Week 10	Fresh weight
Trial 3	Field experiment, transplant at week 6	71L	Green Moon	144	33YE	5 mL per plant	Soil treatment	Week 5	Week 7	Head diameter
									Week 9	Head diameter
									Week 12	Fresh weight
Trial 4	Nursery experiment	NA	Liston	20	33YE; UQ9000N; UQ4510A; 4YE; YEP	2 mL per plant	Seed treatment	Week 0	Week 2	Surface area
									Week 4	Plant height, fresh weight, dry weight
Trial 5	Field experiment, transplant at week 6	74L	Liston	60	33YE; YEP; water	5 mL per plant	Soil treatment	Week 6	Week 8	Head diameter
Trial 6	Field experiment, transplant at week 6	74L	Liston	60; 10	33YE(pip); 33YE(Po); UQ9000N; 4YE; YEP; water	2 mL per plant	Seed treatment	Week 0	Week 8	Head diameter, fresh weight
							Soil treatment	Week 6		

Shaded blocks indicate that the measurements were obtained from fields.

2.1. Plant and Soil Preparations

In the laboratory lettuce trial, commercial seeds (cv. Green Moon) were obtained from Mr. Fothergills (New South Wales, Australia). Seeds were sown in UQ23 potting mix (70% composted pine bark (up to 5 mm), 30% cocoa peat, mineral fertiliser) and incubated in a growth chamber at 18/20 °C, 10 h/14 h day/night. Two commercial lettuce cultivars, Green Moon and Liston (Boomaroo Nurseries, Lara, Victoria, Australia), were used in the field trials in collaboration with Koala Farms, Lake Clarendon. Seeds were germinated in Lithuanian sphagnum peat moss covered by vermiculite during the nursery phase and transferred to fields at Lockyer Valley, Gatton, Queensland, Australia at week 6 after sowing, and the seeds were subsequently fertilised and irrigated as needed. The soil type in the field was clay loam soil (Dermasol at Lockyer Creek Levee). The trial took place from July to October 2020, when day average temperatures ranged between 15 and 25 °C, while night temperatures were between 10 and 15 °C.

2.2. Microbial Inoculum Preparation

B. amyloliquefaciens (33YE), *B. laterosporus* (4YE), *B. velezensis* UQ9000N, and *P. azotoformans* UQ4510An were isolated from clayey soil populated by oleander and longan tree roots collected from Tennyson, QLD, Australia (GPS coordinates 27°31′37.0″ S 152°59′51.7″ E) [35,36]. The isolates were pre-cultured from −80 °C glycerol stocks in yeast extract peptone (YEP) broth (10 g/L bactopeptone, 10 g/L yeast extract, 5 g NaCl) overnight on a flat shaker incubator (100 rpm) at 28 °C in 50 mL Falcon tubes with 25 mL of medium in each tube. After 24 h, 1 mL aliquots obtained from these pre-cultures were added into a fresh YEP broth medium in 50 mL Falcon tubes and again incubated overnight under the same conditions. The suspensions were then diluted in YEP broth to an OD 600 nm of 0.1. This served as the main inoculum culture of all experiments. Water and YEP were used as negative controls for the inoculations.

2.3. Pot Trials under Controlled Laboratory Conditions

For laboratory pot trials, Green Cos seedlings were kept in temperature-controlled growth chambers at 18 h of light, 22 °C day/15 °C night, and 75% humidity, with a light intensity of 200 µmol photons m^{-2} s^{-1} (white fluorescent lamps). Plants were treated with 33YE or UQ9000N twice; first at the seed stage (seeds were placed on wet filter paper and after 24 h soaked in a bacterial culture of OD 600 nm of 0.1 for 1 h before planting into the soil), and a subsequent soil treatment around the plant stem was conducted at week 4 after sowing. The latter consisted of providing 2 mL of bacterial solution (OD 600 nm of 0.1) via pipetting to the soil at the base of plant stems. The control group only received water. Each group included 30 plants. The surface area was measured at week 2 after sowing, and the fresh and dry weight was measured at week 6.

2.4. Lettuce Trials at Commercial Farm

Commercial iceberg cultivars Green Moon and Liston were used in nursery and field experiments at Lockyer Valley at Koala Farm, Lake Clarendon, Queensland, Australia. A random block design was used for both nursery and field trials. Trial 1 was carried out in the nursery, while trials 2, 3, 5, and 6 were conducted in the field, with plants transplanted from the nursery into the field 6 weeks after sowing. Two negative controls were used in trials 4, 5, and 6: a sterile YEP growth medium (formulation) and water-only (business-as-usual) treatment.

2.5. Preliminary Trials at the Nursery

In a preliminary trial, Green Moon lettuce seeds were treated with 33YE one day after sowing. Each seed in the soil received 2 mL of the bacterial solution at OD 600 nm of 0.1 in phosphate-buffered saline (PBS), and each treatment included 60 plants. A repeat treatment was carried out at week 4. The surface area of plants was recorded at week 2, while the fresh weight, plant height, and chlorophyll content were measured at week 6.

2.6. Nursery and Field Trials with the Green Moon Cultivar

Three experimental trials (trials 1, 2, and 3) were conducted using the Green Moon cultivar, including one nursery and two field trials. All three trials used 144 plants or seeds in each group. Trials 1, 2, and 3 included different treatment times and/or inoculations. Trial 1 was conducted with 144 lettuce seedlings per treatment with 33YE or UQ9000N or YEP formulation only, which were inoculated 1 week after germination (week 1) and again 2 weeks later (week 3) by sprinkling formulations onto seedling trays. Untreated control plants received water only. This trial had two objectives: (1) to determine whether a more user-friendly application of formulation (by sprinkling) can be used on lettuce seedlings (rather than seeds) in a commercial setting and (2) whether YEP formulation-only treatments would still achieve growth promotion compared to water-only (business-as-usual) controls.

Trial 2 was conducted with 144 lettuce plants per treatment with bacterial strains 33YE or UQ9000N or YEP formulation only, which were applied to seeds sowed in pots and again 2 weeks after sowing. This trial was conducted to further test whether plant growth differences can also be observed at later plant stages after transplanting to the field. Each plant received 2 mL inoculum in the soil where seeds were sown (seed treatments) or on the soil around plant stems (plant treatments). Six-week-old plants were transferred into the field and continued to be monitored until plants were thirteen weeks old (close to harvesting). The last Green Moon trial, trial 3, used only the 33YE strain. A total of 5 mL bacterial inoculum was added to 5-week-old nursery-grown lettuce seedlings before transplanting to the field in week 6 after sowing. The plant head diameters were recorded on 7-week-old and 9-week-old plants (2 weeks and 4 weeks post-inoculation), and the plant fresh weight was measured on 12-week-old plants.

2.7. Nursery and Field Trials with the Liston Cultivar

Experiments with the Liston variety of iceberg lettuce included one nursery trial (trial 4) and two combined nursery/field trials (trials 5 and 6). Trials 4 and 6 tested whether a single bacterial inoculation was sufficient before harvesting.

In trial 4, 2 mL inoculations with a 33YE, UQ9000N, 4YE, UQ4510An, or YEP formulations medium were applied to Liston seeds in 144-cell trays immediately after sowing (week 0). Leaf surface area was recorded for 2-week-old seedlings, and plant height, fresh weight, and dry weight were recorded for 4-week-old nursery plants. In trial 5, only 33YE was used to inoculate 6-week-old lettuce at the time of transplanting to the field. Each plant was treated with 5 mL of formulations containing 33YE, YEP, or water added to the soil at the base of each plant. The plant head diameters were measured and compared at 8 weeks after transplanting (14-week-old plants). In trial 6, formulations containing 33YE, UQ9000N, 4YE, or YEP were applied to seeds. This trial included an alternate inoculation method where continuous pouring (33YE Po) and pipetting (33YE pip) methods were compared, while only the pipetting method was used for the other trials. Each plant received 5 mL inocula at the seed stage (week 0) and at the time of transplanting from nursery to the field (week 6). An equivalent amount of water was also added to untreated control plants after field transplanting.

2.8. Measurements and Statistical Analysis

Plant height, shoot height, fresh weight, and dry weight were recorded, and graphs were prepared using Microsoft Excel 2007 (Redmond, WA, USA), while imaging analysis was carried out using ImageJ version 1.8.0 software (National Institutes of Health, Bethesda, MD, USA). Chlorophyll was measured using a hand-held SPAD-502 meter (Spectrum Technologies, Aurora, IL, USA) that rapidly measures leaf chlorophyll concentrations accurately and non-destructively. For statistical analyses, two-tailed Student's t-tests with unequal variance were applied for data with two populations, and ANOVA with Tukey's post hoc tests based on Bonferroni and Holm corrections were applied using the www.astatsa.com (accessed on 31 August 2022) online tool (by Navendu Vasavada), for data with more than two populations at significance levels of $p < 0.05$.

3. Results

3.1. Rhizosphere Bacterial Treatments under Controlled Pot Trial Conditions Demonstrated the Growth Promotion of Lettuce Plants

The laboratory pot trial conducted on Green Cos lettuce showed significant increases in leaf surface area for treatments with 33YE at 2 weeks post-emergence of seedlings ($p < 0.01$, Figure 1). The inoculant UQ9000N did not result in any significant difference in leaf surface area compared to the YEP formulation-only control. Both treatments, 33YE and UQ9000N, achieved similar results on plant fresh weight improvement, with significant increases by approx.30% ($p < 0.01$) compared to YEP controls.

3.2. Plant Growth Promotion Was Confirmed in a Nursery Trial at a Commercial Lettuce Farm

To test whether plant growth promotion can also be achieved in a commercial setting, bacterial formulations were tested in a preliminary nursery pot trial at Koala Farms Pty Ltd., a large-scale commercial vegetable grower. After 2 weeks post-inoculation of Green Moon lettuce seeds, the inoculant 33YE showed 25% (1.2 cm^2 to 1.5 cm^2) higher leaf area per plant compared to untreated (water-only/business-as-usual) control plants (Figure 2A). Significant growth promotion was also recorded for plant heights at week 6 (11 cm compared to 7.9 cm), fresh weights (24.7 g/plant compared to 24.0 g/plant), and chlorophyll content (38 μmol/m^2 compared to 38 μmol/m^2) ($p < 0.001$, Figure 2B–D).

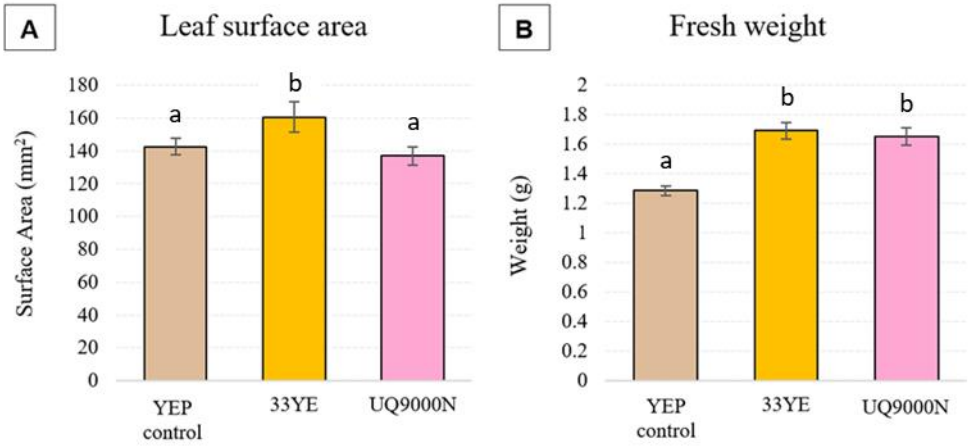

Figure 1. Phenotypic analysis of lettuce plants (*L. sativa*; cv. Green Cos) post-inoculation with *B. amyloliquefaciens* 33YE and *B. velezensis* UQ9000N in a laboratory pot trial under controlled conditions. Shown are mean values ± SE (n = 30 plants per treatment) of (**A**) leaf surface area and (**B**) fresh weight 2 weeks after treatments with PGPR isolates compared to control plants treated with YEP formulation only. Different small letters indicate significant ($p < 0.05$) differences between treatments.

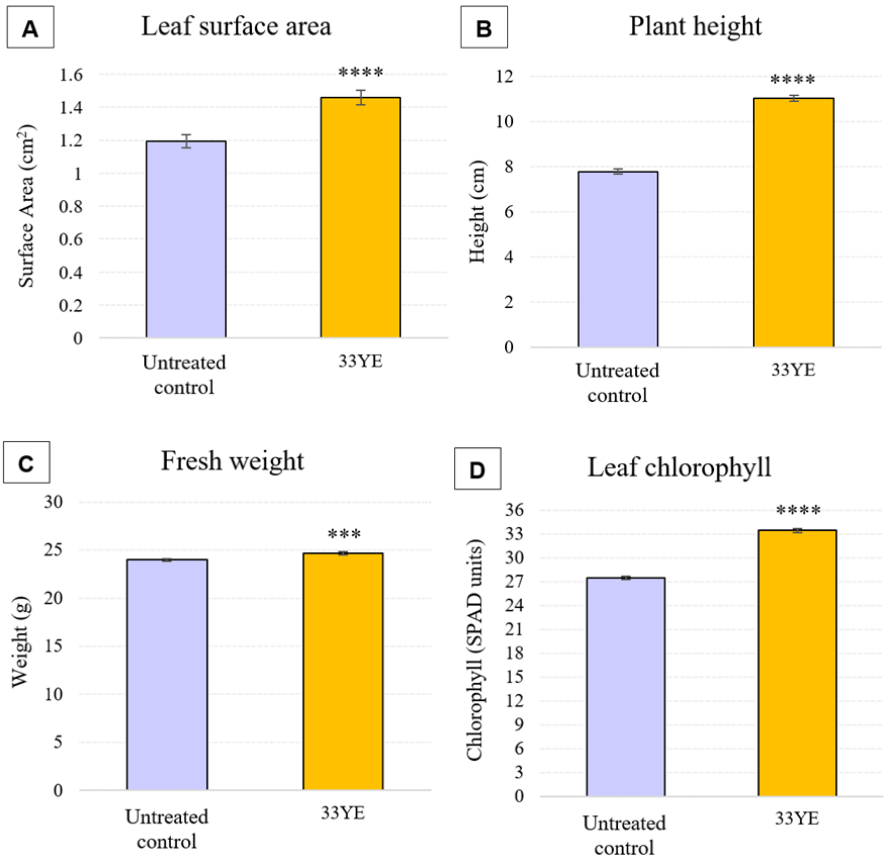

Figure 2. Phenotypical assessments of lettuce plants (*L. sativa*; cv. Green Moon) post-inoculation with *B. amyloliquefaciens* 33YE in a preliminary trial at Koala Farms nursery. Shown are mean values ± SE (n = 60 plants per treatment) of (**A**) leaf surface area at 2 weeks after seed treatments, as well as (**B**) plant height, (**C**) fresh weight, and (**D**) leaf chlorophyll content at 6 weeks after treatments with PGPR isolate compared to untreated (water-only) control plants. Statistical significance was determined by Student's *t*-test, and black asterisks show significant differences to the untreated control plants with *** $p \leq 0.001$ and **** $p \leq 0.0001$.

3.3. Sprinkling of Microbial Biofertilisers Was Adequate to Achieve Plant Growth Promotion in Nursery Trials

This trial (trial 1) had two objectives: (1) to determine whether a more user-friendly application of formulation (by sprinkling) can be used on lettuce seedlings (rather than seeds) in a commercial setting and (2) whether bacterial formulations would still achieve growth promotion compared to YEP formulation-only treatments. Hence, inoculations were carried out by sprinkling on 1-week-old nursery seedlings followed by a repeat treatment 2 weeks later (week 3), and plants were monitored for growth performance at the time of transplanting (week 6). Plants treated with 33YE and UQ9000N exhibited significantly higher plant heights by 20% and 4%, respectively, on week 6 compared to YEP formulation-only treated control plants (Figure 3A). Plants that received 33YE by sprinkling also looked markedly taller and stood out compared to the untreated control plants in the nursery (Figure 3B), suggesting that plants could possibly be transplanted earlier to the field when receiving this treatment.

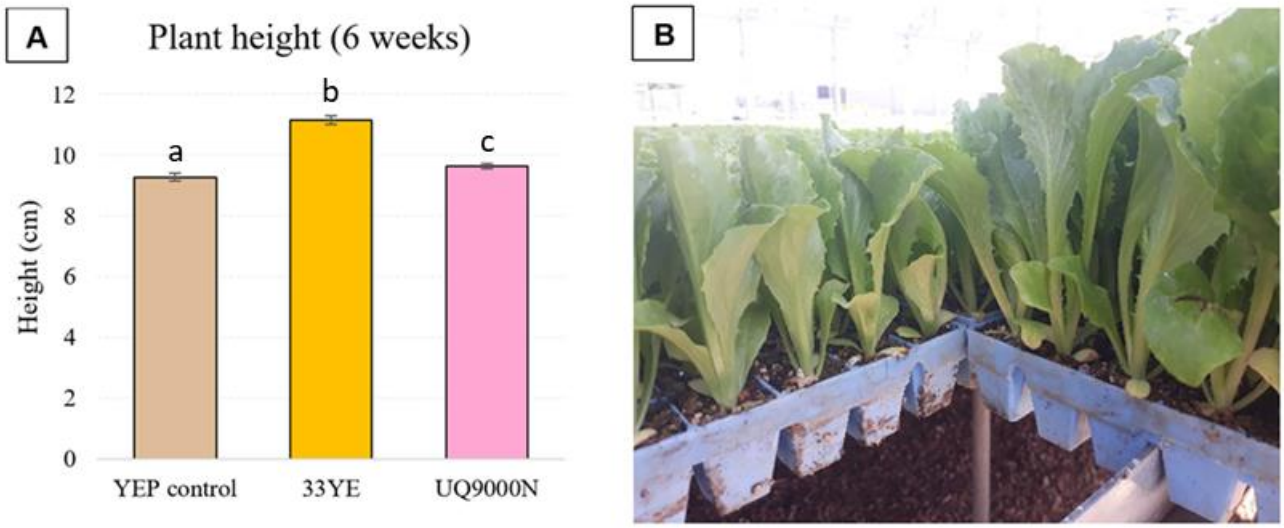

Figure 3. Phenotypical assessments of lettuce plants (*L. sativa*; cv. Green Moon) post-inoculation with *B. amyloliquefaciens* 33YE and *B. velezensis* UQ9000N in trial 1 at Koala Farms nursery. Shown are mean values ± SE (n = 144 plants per treatment) of plant height (**A**) and (**B**) a photo showing phenotypical difference at 2 weeks post-inoculation (second inoculation) with PGPR isolates (**right**) compared to control plants (**left**) treated with a YEP medium. Different small letters indicate significant ($p < 0.05$) differences between treatments.

3.4. Plant Growth Promotion Effects Continued from the Nursery to Field Stage

Trial 2 was conducted to further test whether plant growth differences can also be observed at later plant stages after transplanting to the field. Bacterial treatments with 33YE, UQ9000N, or YEP formulation only were applied on freshly sown lettuce (day 0), and repeat treatments were applied on 2-week-old plants. The treatment with 33YE led to significantly higher lettuce head diameters, from 15.8 cm to 17.2 cm, compared to the YEP formulation control in 8-week-old plants ($p < 0.0001$), while UQ9000N treatment showed no significant difference at this stage (Figure 4). Both strains improved the fresh weight of 10-week-old plants, especially the 33YE treatment, which led to a 50% higher yield compared to the YEP formulation control. Finally, the 13-week-old plants showed a positive trend for improved yields at harvesting that were 9% for 33YE (1.29 kg compared to 1.18 kg) and 11% for UQ9000N (1.44 kg compared to 1.29 kg), although this was not significant ($p > 0.05$).

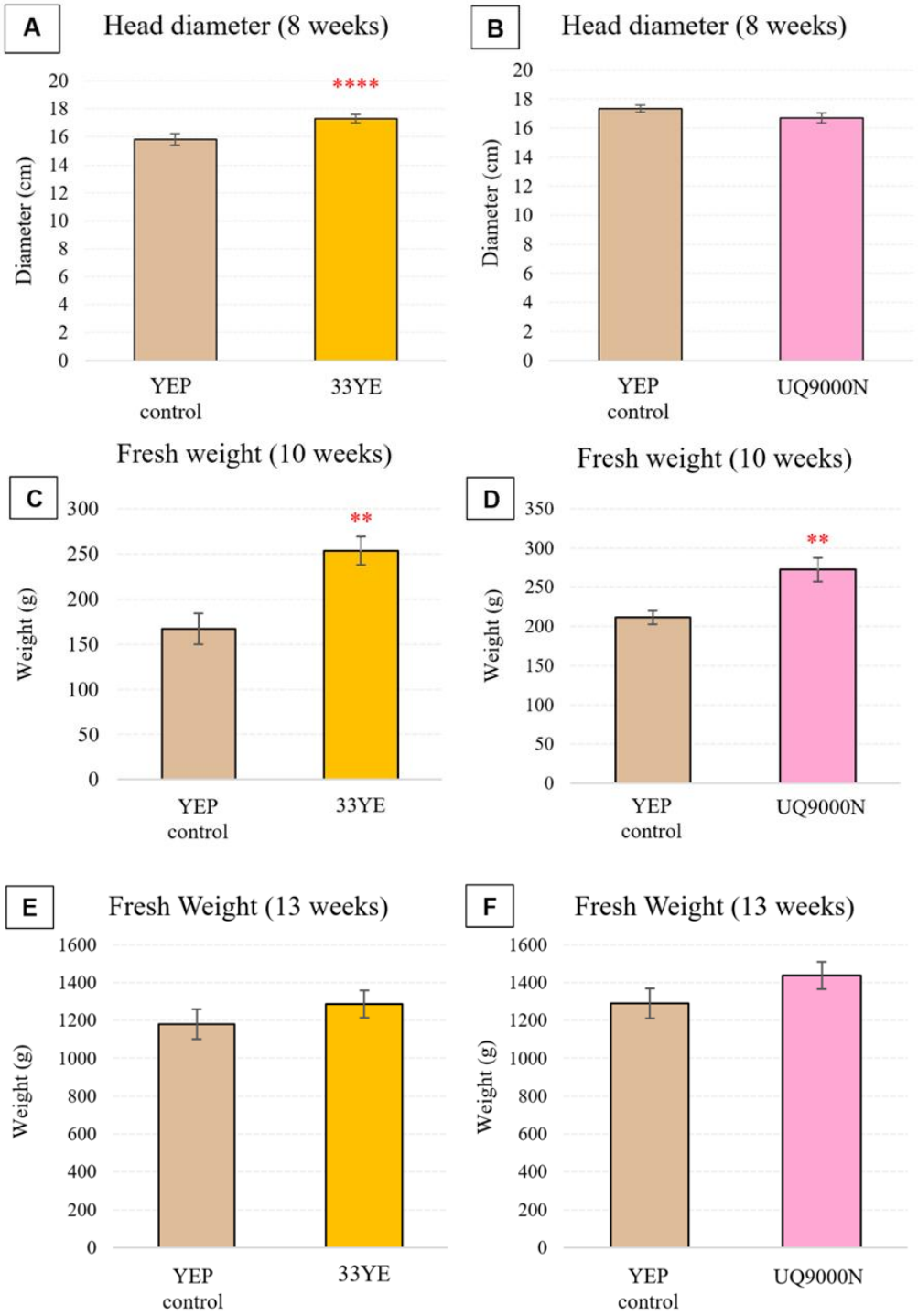

Figure 4. Phenotypical assessments of lettuce plants (*L. sativa*; cv. Green Moon) inoculated with *B. amyloliquefaciens* 33YE or *B. velezensis* UQ9000N in trial 2 at Koala Farms. Shown are mean values ± SE (n = 144 plants per treatment) of the (**A**,**B**) head diameter at 6 weeks post-inoculation (8-week old seedlings, 2 weeks after transplanting to the field), the (**C**,**D**) fresh weight at 8 weeks post-inoculation (10-week-old seedlings), and the (**E**,**F**) fresh weight 11 weeks post-inoculation (13-week-old seedlings) after treatments with PGPR isolates compared control plants treated with a YEP medium. The statistical significance was determined by Student's t-test, and red asterisks show significant differences to the YEP control plants with ** $p \leq 0.01$ and **** $p \leq 0.0001$.

3.5. A Single Treatment before Transplanting Was Sufficient for Growth Promotion in the Field

A single treatment with 33YE was tested on 5-week-old nursery Green Moon plants (trial 3) to test whether growth promotion can be achieved just before transplanting to the field, which occurred when plants were 6 weeks old. At 2 weeks after treatments, the 7-week-old plant seedlings showed significantly increased head diameters compared to untreated (business-as-usual) control plants (15.9 cm compared to 12 cm), and this also included transplanting stress ($p < 0.0001$, Figure 5A). Transplanting stress can lead to reduced growth, which may have been minimized for 33YE-treated plants. At 4 weeks post-inoculation, the 9-week-old plants still showed a trend of larger head diameters (23 cm compared to 21.6 cm, $p > 0.05$; Figure 5B), and this trend was also observed when young plants were harvested on week 12 (fresh weights of 370 g/plant compared to 320 g/plant, $p > 0.05$; Figure 5C).

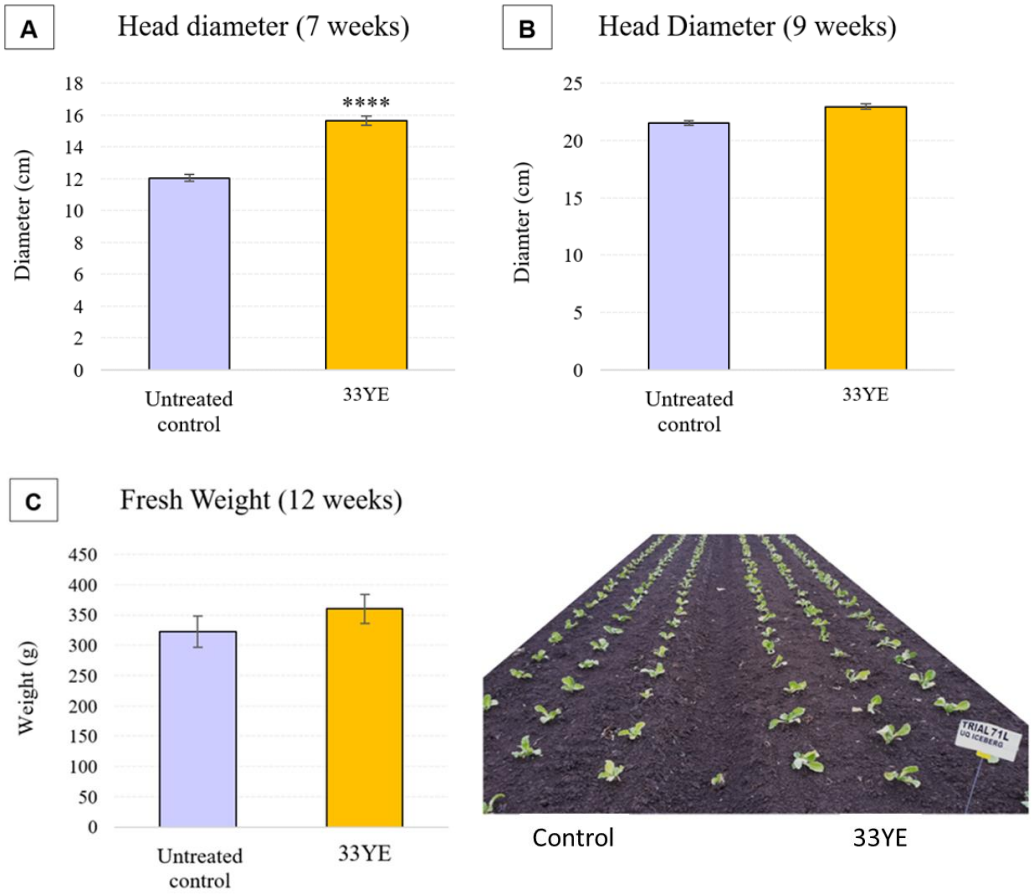

Figure 5. Phenotypical assessments of lettuce plants (*L. sativa*; cv. Green Moon) inoculated with *B. amyloliquefaciens* 33YE in trial 3 at Koala Farms. Shown are mean values ± SE (n = 144 plants per treatment) of the (**A**) head diameter at 2 weeks post-inoculation and 1 week after transplanting (7-week-old seedlings), the (**B**) head diameter at 4 weeks post-inoculation (9-week-old seedlings; see photo), and the (**C**) fresh weight at 7 weeks post-inoculation (12-week-old seedlings) after treatments with the PGPR isolate compared to untreated (water-only) control plants. The statistical significance was determined by Student's t-test, and black asterisks show significant differences to the untreated (business-as-usual) control plants with **** $p \leq 0.0001$.

3.6. Microbial Biofertiliser Treatments Were also Effective on Heat-Tolerant Cultivars and Liston Cultivars

Trial 4 was carried out to test whether bacterial treatments also influenced the growth of the more heat-tolerant Liston lettuce cultivar, and two additional strains were included, 4YE and UQ4510An. Surface areas of seed-inoculated 2-week-old Liston seedlings were insignificantly ($p > 0.05$) larger for 33YE-treated plants (30% higher compared to YEP

formulation-only treatments. Trends for positive effects were also found for 4YE (20%) and UQ9000N (20%, $p > 0.05$; Figure 6A). Plants were then measured again at 4 weeks post-inoculation, and plant fresh weight, dry weight, and plant height were recorded. All bacterial treatments significantly increased the plant weight from 1.6 g for control plants to 2.6 g for 33YE-treated plants ($p < 0.01$, Figure 6B), and similar results were recorded for dry weights (Figure 6C) and plant heights (Figure 6D). However, compared to the YEP formulation control, only 33YE showed a significant increase in fresh weight ($p < 0.001$, ~17%) (Figure 6B).

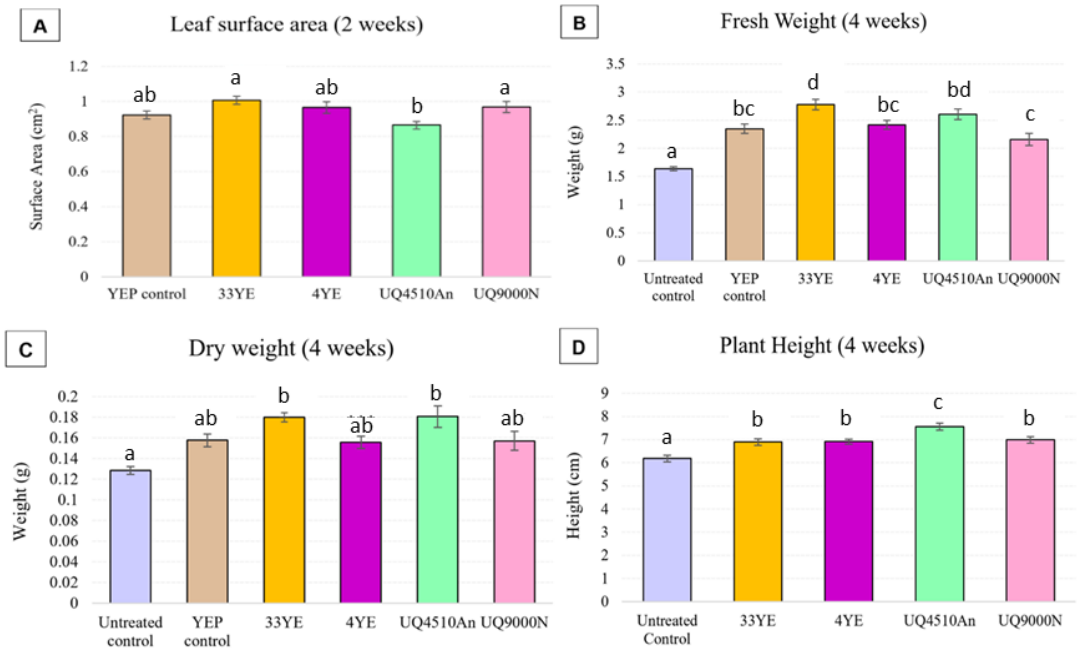

Figure 6. Phenotypical assessments of lettuce plants (*L. sativa*; cv. Liston) post-inoculation with *B. amyloliquefaciens* 33YE, *B. laterosporus* 4YE, *P. azotoformans* UQ4510An, and *B. velezensis* UQ9000N in trial 4 at Koala Farms nursery. Shown are the means ± SE (n = 20 plants per treatment) of (**A**) leaf surface area at 2 weeks after seed treatments with PGPR isolates compared to control plants treated with a YEP medium, as well as (**B**) fresh weight, (**C**) dry weight, and (**D**) plant height at 4 weeks after seed treatments with PGPR isolates compared to (1) untreated (water-only) control plants and (2) control plants treated with a YEP medium. Different small letters indicate significant ($p < 0.05$) differences between treatments.

3.7. A Single Application with Microbial Biofertiliser Formulations at Transplanting Was Sufficient for Growth Promotion

Trial 5 was carried out to investigate whether a single inoculation at the time of transplanting (6-week stage) was sufficient to observe growth promotion. Inoculating the bacterial strain 33YE in the field immediately after transplanting led to significantly increased head diameters compared to untreated or YEP-treated controls ($p < 0.01$, Figure 7A). Fresh weights were significantly increased compared to the water (business-as-usual) control, both for the YEP medium treatments and 33YE treatments ($p < 0.01$, Figure 7B). No significant fresh weight difference was observed between the YEP medium control and the 33YE treatment.

3.8. The Pouring of Microbial Fertilisers and Repeat Inoculations Were Effective for Growth Promotion

Trial 6 was conducted to compare inoculation methods for more efficient implementation in farm operations. Bacterial inoculation by pouring was compared to the previously used work-intensive pipetting method using 33YE in the bacterial treatment. In addition, following seed inoculations, a repeat bacterial inoculation was used (seeds, 2-week-old and

6-week-old seedlings), as previous trials suggested that benefits from bacterial inoculations may fade over time (Figures 4 and 5). As shown in Figure 8A,B, the pouring of 33YE, rather than pipetting, led to significantly higher fresh weights in 8-week-old plants, with 33YE (35%, $p < 0.01$).

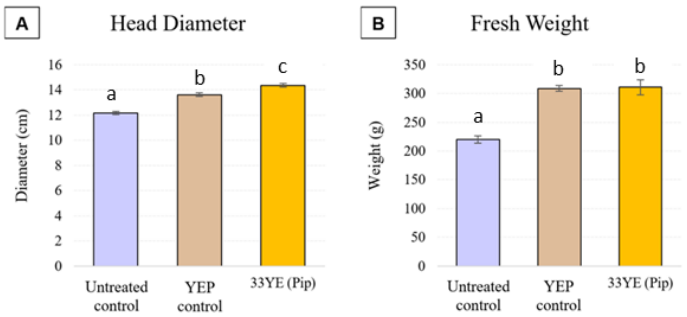

Figure 7. Phenotypic assessments of lettuce plants (*L. sativa*; cv. Liston) inoculated with *B. amyloliquefaciens* 33YE in trial 5 at Koala Farms. Shown are the means ± SE (n = 60 plants per treatment) of the (**A**) head diameter and (**B**) fresh weight of lettuce seedlings inoculated once via pipetting (Pip) with the PGPR isolate at the 6-week seedling stage measured at 2 weeks after transplanting (8-week-old plants). Different small letters indicate significant ($p < 0.05$) differences between treatments.

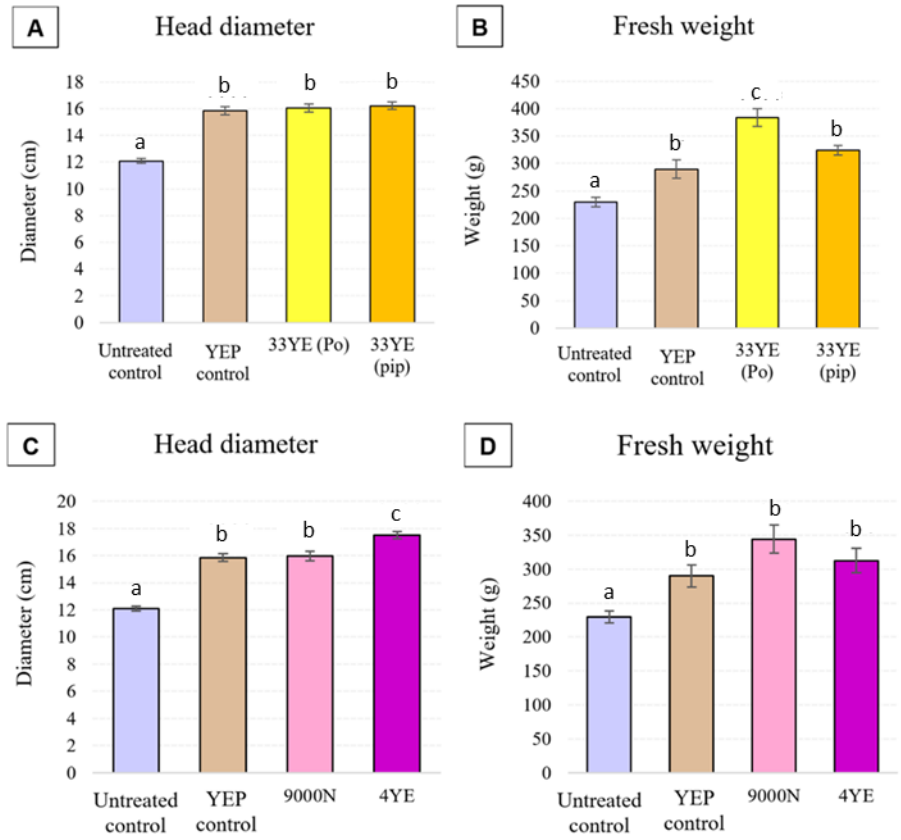

Figure 8. Phenotypic assessments of lettuce plants (*L. sativa*; cv. Liston) inoculated with *B. amyloliquefaciens* 33YE, *B. velezensis* UQ9000N, and *B. laterosporus* 4YE in trial 6 at Koala Farms. Shown are the means ± SE (n = 60 plants per treatment) of (**A**,**C**) the head diameter of 8-week-old plants and (n = 10 plants per treatment) the (**B**,**D**) fresh weight of 11-week-old plants after application methods, such as pouring (Po) vs. pipetting (pip), at the seed stage and repeat inoculations at the time of transplanting to the field (6-week stage) with PGPR isolates compared to (1) untreated (water-only/business-as-usual) control plants and (2) formulation-only control plants treated with a YEP medium. Different small letters indicate significant ($p < 0.05$) differences between treatments.

By comparison, no differences were observed in head diameters for the previous trial on Green Moon (Figure 5). Similar to results shown in younger plants (Figure 5), repeat treatments of all bacteria led to growth promotion in older plants compared to untreated (water-only) control plants (Figure 8A–D), but only the 4YE treatment showed improved growth compared to the YEP medium control (Figure 8C). The head diameter of 4YE-treated plants (17 cm) was significantly higher compared to water and the YEP control (42% and 8%, respectively, $p < 0.05$, Figure 8C).

4. Discussion

This study showed the potential of four PGPR strains to be used as biostimulants for plant growth promotion, namely *B. velezensis* UQ9000N, *B. amyloliquefaciens* 33YE, *B. laterosporus* 4YE, and *P. azotoformans* UQ4510An. Trials were conducted to test their ability to enhance the growth and yield of different commercial lettuce cultivars as transplant production in nursery-protected cropping and subsequently under field conditions in Queensland,, Australia. Results are comparable with other studies that investigated plant growth promotion of different *Bacillus* and *Pseudomonas* spp. under nursery greenhouse and field conditions [2,5,22,23]. Notably, success from PGPR inoculations varies from farm to farm and is dependent on soil properties, environmental factors, and plant cultivars [37–40]. The inconsistencies between different studies are believed to be caused by differences in climate, temperature, edaphic factors (e.g., soil structure, pH, salinity, etc.), native soil microbiome, and plant species and cultivars [36–42]. Hence, the current study used a customised approach to determine PGPR applications that not only provide growth promotion but can also be integrated into the standard farming practices of a major vegetable grower in Australia (Koala Farm Pty Ltd., Lake Clarendon, Queensland Australia). The new findings in this study include (1) the successful use of rhizosphere bacteria with biocontrol functions as growth-promoting biostimulants and (2) the successful practical application of these bacteria at a commercial farm. Different species and even strains of the same species can have very different functions, and often successful pot trials do not translate to success under field conditions where conditions vary widely from farm to farm. This study has shown the usefulness of a customised approach to identifying different strains, formulations, and application methods that provide biofertiliser benefits at a specific farm, which may not be provided with untested commercial products.

Six trials were conducted to optimise strategies for bacterial inoculations on lettuce, which included single or multiple inoculations, different inoculation times, different inoculation methods, and different lettuce cultivars. The results show that strains 33YE and UQ9000N can significantly ($p < 0.05$) promote the growth of different lettuce genotypes (Green Moon and Liston) in the commercial nursery and the field compared to the formulation-only treated control plants. This included significant differences for leaf surface area, plant height, fresh weight, and head diameter for 33YE, as well as plant height and fresh weight for UQ9000N. Growth differences were significant up to the 10-week stage for Green Moon (Figure 4) and the 11-week stage for Liston (Figure 7), while later stages (13-week stage for Green Moon, Figure 4) only showed non-significant ($p > 0.05$) trends for growth promotion. This could be caused by other microbes outcompeting the microbial biofertilisers after several weeks or months, and repeat inoculations at later growth stages may further improve yields at the harvesting stage. The use of a preferred carbon substrate in the formulation could potentially overcome the need for repeat inoculations. UQ4510An also significantly increased the plant height and fresh and dry weight of Liston in the commercial nursery. Differences in the effectiveness of the different microbes are likely to do with the range of beneficial attributes they provide, as well as different host specificities and competitiveness in the microbiome.

These results are in line with numerous other studies that show the potential of PGPR belonging to *Bacillus*, *Brevibacillus,* and *Pseudomonas* genera as biofertilisers [24,28,29,43–52]. *Bacillus* spp. are some of the best studies of PGPR, particularly the *B. subtilis* species complex containing *B. amyloliquefaciens* and *B. velezensis,* which exhibit a wide range of plant

growth promotion mechanisms [53–55]. These plant growth-promoting abilities include N fixation, P and K solubilisation, and the production of auxins, cytokinins, and gibberellic acids [44,46,48–51,56–58]. For example, CK-producing *B. subtilis* and GA-producing *B. methylotrophicus* improved the growth of lettuce plants [44,48]. Similarly, Wang et al. [52] reported that *B. laterosporus* AMCC100017 promoted the growth of apple rootstock *Malus robusta* plants by synthesising IAA, increasing photosynthetic efficiency and affecting the plant through multiple metabolic pathways. Astorga-Eló et al. [45] reported that *B. laterosporus* DSM 25 exhibited ACC deaminase activity in vitro. Also, several studies reported that *P. azotoformans* strains possess various growth promotion mechanisms, including N fixation, P and K solubilisation, the production of IAA, ammonium, and siderophores, and the exhibition of ACC deaminase activity [24,26–29,43,47,59]. We previously found that *B. amyloliquefaciens* 33YE and *Brevibacillus laterosporus* 4YE produce a range of antimicrobial compounds, including antimicrobial peptides [35]. We recently also showed that *B. velezensis* UQ9000N and *P. azotoformans* UQ4510An possess the ability to suppress a broad range of soil-borne pathogens by direct inhibition or by assisting plant defence responses in tomatoes [36]. Although studies were carried out in tomatoes and not lettuce [36], defence gene repression in healthy plants at 3 days after UQ4510An inoculation suggested that suppressions of the oxidative burst and the jasmonic acid pathways enable beneficial plant–microbe interactions. The current study demonstrates that these bacteria also have the ability to promote plant growth. Plant pathogen suppression may have contributed to this under field conditions, but the effect observed during early plant development under nursery conditions may solely be attributed to plant growth-promoting attributes independent of pathogens.

Interestingly, all bacterial treatments, and also the YEP formulation control treatments, resulted in plant growth promotion compared to untreated or water-treated "business-as-usual" plants. This suggests that YEP formulation-only soil amendments could play an important role in lettuce growth promotion that should be further investigated. As YEP is essentially a type of organic fertiliser, it can be envisaged that additional nutrients are provided to the plants, in the form of direct uptake and through the action of modified soil and rhizosphere microbiomes [60,61]. The results from the present study further emphasise the importance of formulations, and future work may focus on developing formulations that are lower in cost than YEP. In addition, a rhizosphere microbiome study (e.g., by 16S rDNA and ITS amplicon sequencing) following YEP formulation soil treatments on the lettuce rhizosphere with and without bacterial inoculants at different times would reveal the microbiome dynamics, how long bacterial inoculants persist, and whether other potential PGPRs are encouraged to grow. These studies could also be used to optimise the application of rhizosphere bacteria by testing whether various carbon substrates in the formulation may increase the persistence of the applied beneficial bacteria in the soil, which would limit the number of repeat inoculations under field conditions.

Irrespective of formulation, plant developmental stage, and application method, the results from the present study show that *B. amyloliquefaciens* 33YE could be a highly suitable biofertiliser for lettuce cultivation. Seed inoculation and later soil treatments (before or after) transplanting to the field were successful at promoting lettuce plant growth. However, several experiments indicated that this was a transient effect (e.g., Figure 5) and, although the values of treated plants were still higher than untreated plants, these results were not statistically significant ($p > 0.05$). For example, plants treated with 33YE or UQ9000N early in the nursery (at the seed and 2-week stage) in trial 1 still showed a trend towards higher yields at 13 weeks at the time of harvesting fully-grown plants with 9% and 11% yield increases, respectively, for 33YE and UQ9000N treatments (Figure 4E,F).

The production of healthy and vigorous lettuce transplants in nursery greenhouses requires the strict control of many factors, including the availability of nutrients, particularly nitrogen [5,7,8]. Therefore, careful management of inorganic fertiliser and the addition of PGPR-based biofertilisers can lead to healthier, more vigorous lettuce seedlings in nursery greenhouses and improved establishment in the field after transplantation, while

also minimising the harmful impact to the environment and human health compared with synthetic agrochemicals [2,5,12–14]. Vetrano et al. [5] showed that a combination of *Bacillus* spp. with different levels of nutrients improved the growth, health, and yield of lettuce seedlings after transplantation in the field. They also noted that lettuce transplants with higher weight and leaf number are better parameters than plants with increased length, which could be detrimental after transplantation in the field (e.g., weaker and more susceptible to shock transplant and diseases) [5,7]. The current study shows that lettuce seedlings reach the critical plant stage for transplanting earlier when treated with microbial biofertilisers. Plants (Green Moon or Liston) at the 6-week transplanting stage at this farm are typically 8 cm tall (Figure 2B). When plants were treated with microbial biofertilisers in the nursery, this stage could already be reached at 4–5 weeks (Figure 6D), potentially freeing up valuable space in the nursery.

Fresh weight was found to be the most relevant parameter to measure growth, as this is also how lettuce heads are sold, while leaf surface area, plant height, and head diameter are related to plant architecture that is influenced by growth density. Although pipetting was initially used to provide accurate dosage to each plant, the more farm-friendly applications of sprinkling and pouring of liquids were also adequate to achieve growth promotion (Figures 3 and 8), although an edge effect was clearly visible (Figure 3B). Pouring was even significantly ($p < 0.05$) more effective for fresh weight gain compared to pipetting (Figure 8B). A better-distributed reservoir of beneficial bacteria in the soil may be more effective than bacteria concentrated around the stem/root area that, when overdosed, may lead to undesirable plant defence responses.

There are several reports that demonstrate increased nutritional profiles of plants treated with PGPR [62,63]. Trinh et al. [2] showed that *P. nitroreducens* PnIHB enhanced the growth of lettuce plants under field conditions by stimulating cell development and nitrate absorption while also maintaining a low level of nitrate in plants (which can be harmful to human health). Nitrate was not measured in the current study, but increased chlorophyll contents were found in 33YE-treated plants compared to untreated control plants (Figure 2D). Further studies may perform metabolic profiling of PGPR-treated lettuce plants to test whether improved nutritional values can be obtained.

To optimise the use of PGPR-based biofertilisers, it is paramount to obtain consistent and reproducible results under field conditions [23]. This was attempted in the present study at Koala Farms, which uses a uniform, standardised cultivation system with consistent nursery, transplanting, and field growth regimes. Furthermore, it is important to understand the interactions between the PGPR isolates with the host plant holobiont [22,23,64–66]. Kröber et al. [22] reported that *B. amyloliquefaciens* FZB42 had no major effects on endogenous lettuce rhizospheric microbiomes. However, Cipriano et al. [23] showed that *P. cremoricolorata* IAC-RBcr4 and *P. fluorescence* IAC-RBru1 favoured the enrichment of different bacterial phyla, including Planctomycetes, Actinobacteria, Chloroflexi, Bacteroidetes, and Firmicutes.

Other important factors include agricultural management, chemical composition, and the type of soil, and endogenous endosphere, phylosphere, and rhizosphere microbiomes [5,23,61,67]. Finally, the activity of biofertilisers may be improved using microbial consortia composed of microbial strains from the same or different taxonomic groups, e.g., combinations of bacterial strains from the present study could be used to complement their actions. Taken together, this suggests that a range of microbial biofertilisers should be available to farmers that must be tested and customised for their conditions. The current study indicates that *B. velezensis* UQ9000N, *B. amyloliquefaciens* 33YE, *B. laterosporus* 4YE, and *P. azotoformans* UQ4510An show potential as biofertilisers for commercial lettuce production, especially 33YE and UQ9000N during the early nursery and transplanting conditions of the Green Moon cultivar up to the 10-week stage and the Liston cultivar up the 11-week stage.

5. Conclusions

The present study aimed at testing the ability of biocontrol bacteria to provide a farm-friendly, customised approach to plant growth promotion at a specific farm, Koala Farms, without having to alter farm practices. The results are encouraging (especially for 33YE), demonstrating that plants treated in the nursery show a growth promotion effect that would allow transplanting to the field 10–14 days earlier, resulting in a faster turnover by freeing up space for additional plants. Another encouraging result came from the data obtained when plants were treated just before transplanting (5-week stage, 1 week before transplanting) or at the time of transplanting (6-week stage), which resulted in improved growth under field conditions, even when pouring (mimicking irrigation) was used as the application method. Repeat bacterial treatments under field conditions using the existing irrigation regimes for bacterial applications should be carried out to establish that plants at harvesting still show significant growth promotion benefits or that plants can be harvested earlier, freeing up fields for other cropping. Further research potential also exists for the development of long-lasting or slow-release formulations to ensure an adequate supply of these microbial biofertilisers throughout plant development.

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