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Application of PGPB Combined with Variable N Doses Affects Growth, Yield-Related Traits, N-Fertilizer Efficiency and Nutritional Status of Lettuce Grown under Controlled Condition

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Abstract: Nitrogen (N) fertilization is a crucial agricultural practice for boosting production traits in vegetables. However, N synthetic fertilizers—commonly adopted by farmers—have several counterproductive effects on the environment and on humans. The research was performed to assess the combined influence of plant growth promoting bacteria (PGPB) (*Azospirillum brasilense* DSM 1690, *A. brasilense* DSM 2298 and *Pseudomonas* sp. DSM 25356) and various N fertilization doses (0, 30, 60 or 120 kg ha^{-1}) on growth, yield, quality and nitrogen indices of lettuce in protected cultivation. Plant height, root collar diameter, number of leaves and fresh weight were enhanced by *A. brasilense* DSM 2298 inoculation and N at 30 or 60 kg ha^{-1}. Overall, soluble solids content (SSC), ascorbic acid, total phenolics, carotenoids, total chlorophyll and total sugars were augmented by the combined effect of *A. brasilense* strains and 30, 60 or 120 kg N ha^{-1}. Furthermore, PGBP inoculation improved potassium (K) and magnesium (Mg) concentrations in leaf tissues. PGPB inoculation increased N leaf concentration; however, it hastened N indices. These results suggest that the PGPB tested can be considered an eco-friendly tool to improve lettuce yield, particularly when combined with N at 30 or 60 kg ha^{-1}.

Keywords: *Azospirillum brasilense* strains; *Pseudomonas* sp.; N fertilization rate; 'Canasta' lettuce; nutritional features; functional components; NUE

1. Introduction

Lettuce (*Lactuca sativa* L.) is a cool-season leafy vegetable grown in all regions [1,2]. Currently, more than a million hectares are cultivated with a production of more than 22 million tons [3]. Lettuce is mainly consumed as a fresh-cut, raw salad vegetable and provides a notable source of vitamins (A, B9, C and E), carotenoids, flavonoids, minerals and phenolic compounds [4–9].

Vegetable production systems need specific agricultural practices [10–12] and depend on high quantity of mineral nutrients (especially N, P and K) to enhance growth, yield and quality [13–15]. However, the incessant use of artificial fertilizers may pose threats on the ecosystem survival. In this scenario—in line with the European Green Deal strategies—plant biostimulants, including plant growth promoting bacteria (PGPB), are considered eco-friendly tools to hasten growth and development of vegetable crops [13,14,16–18]. PGPB consist of a group of microorganisms characterized by the ability to colonize roots, rhizosphere, and interior plant tissues [19,20]. PGPB can elicit plant development by influencing several processes such as nitrogen fixation, nitrate reductase activity [21], hormones synthesis (auxins, cytokinins, gibberellins, and ethylene) [22–24], solubilization of phosphate [25], and biological control of pathogens [26]. Nowadays, a wide range of PGPB genus was recognized, involving *Pseudomonas*, *Burkholderia*, *Bacillus*, *Bradyrhizobium*, *Rhizobium*, *Gluconacetobacter*, *Herbaspirillum*, and *Azozpirillum* [27–29]. Among them, *Azospirillum* and *Pseudomonas* are two free-living genus commonly found all over the world and are generally used in the agriculture sector [21,30].

As reported by Broadley et al. [31], N availability influences phenotypic and physiological plant parameters, which in turn affect marketability features and visual quality traits. However, the high nitrogen fertilization rate—commonly adopted by farmers—bring an upsurge in terms of nitrate content in plant tissues, causing harmful effects on the ecosystem (N leaching) and on human health. In this respect, the application of PGPB could be helpful to boost lettuce yield due to their aptitudes to enhance NUE, eliciting mineral uptake and utilization efficiency. Nevertheless, since plant response to PGPB and N dose are influenced by genotype and growing conditions, a detailed study is required to appraise methods and doses. To the best of our knowledge, the literature lacks information on the interaction between the PGPB tested and N dose in lettuce and on its effects on quantitative and qualitative traits.

Starting from the aforesaid premise, the aim of the present research was to appraise the influence of three PGPB (*Azospirillum brasilense* DSM 1690, *A. brasilense* DSM 2298 and *Pseudomonas* sp. DSM 25356) under four nitrogen fertilization levels (0, 30, 60, 120 kg ha^{-1}) on 'Canasta' lettuce yield, as well as nutritional and functional compounds. This research offers information on the function of three different PGPB and on their influence on lettuce N assumption.

2. Materials and Methods

2.1. Experimental Site and Material

The investigation was accomplished during the 2021 winter-spring period at the field facilities of the Department of Agricultural, Food and Forestry Sciences of the University of Palermo (SAAF), Palermo (latitude 38°12′ N, longitude 13°36′ E, altitude 65 m), located in a sub-urban area. Plants under investigation were grown in a polyethylene-covered tunnel. The tunnel was equipped with a drip irrigation system for water and nutrients distribution. A data logger was placed inside the tunnel to record microclimate data (daily maximum and minimum temperature) (Figure 1).

On 5 February 2021, 720 *Lactuca sativa* L. 'Canasta' (Syngenta Seed, Basel, Switzerland) plug plants at 3–4 true leaves stage were transplanted adopting an inter-/intra-row spacing of 0.25 m and obtaining a density of 16 plant m^{-2}. On 6 April 2021, all plants were harvested by cutting the collar, and then the external damaged leaves were removed. Throughout the whole cultivation cycle, all plant needs (water, nutrients, and cultivation practices) were covered, as recommended by Tesi [32]. The soil was a medium-textured soil at pH 7.1, containing 1.7% total nitrogen and 3.1% organic matter.

2.2. Design and Procedure

Four N levels (0, 30, 60, or 120 kg ha^{-1}) were combined with four microorganism treatments (not inoculated or inoculated with *A. brasilense* DSM 1690, *A. brasilense* DSM 2298, and *Pseudomonas* sp. DSM 25356) in a two factorial experiment rendering 16 treatments. The treatments were set in a complete randomized block design, and they were replicated three times. Each experimental block enclosed 15 lettuce plants for a total of 720 plants (16 treatments × 3 blocks × 15 plants).

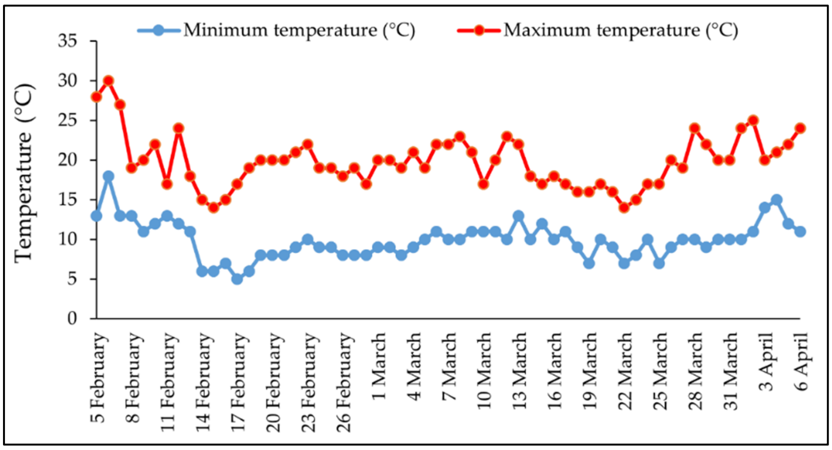

Figure 1. Maximum and minimum temperatures inside the tunnel from 5 February 2021 to 6 April 2021.

The nitrogen fertilization was accomplished using Nitrosol 34® (Mugavero fertilizers, Italy) and it was applied weekly starting one week after transplant. This nitrogen fertilizer contained 34% of nitrogen in the form of ammonium nitrate (NH_4NO_3), distributed via a fertigation system.

The PGPB strains adopted for this study are deposited in the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). Each strain was grown in nutrient agar plate. All Pre-cultures were obtained inoculating pure single colonies in 100-mL Erlenmeyer flasks at 28 °C with 10 mL of nutrient broth (NB), containing 5.0 g L^{-1} of peptone and 3.0 g L^{-1} of meat extract, spinning at 150 rpm in a centrifuge for 24 h (*Azospirillum brasilense*) and 48 h (*Pseudomonas* sp.).

After the incubation time, each bacterial culture was collected in a 50 mL tube and centrifuged at 4000 rpm for 10 min, then the supernatant was removed, and the pellet resuspended in 0.8% NaCl. The optical density was corrected up to 1.0 (540 nm) for about 10^9 CFU/mL. A volume of 2 mL of this suspension was inoculated in 500-mL flasks with 120 mL of nutrient broth.

Growth was established optically at 600 nm (Beckman DU730 spectrophotometer). Finally, CFU per millilitre was assessed by multiplying the number of colonies by the dilution factor.

Each of the three bacteria was inoculated to the plants during the transplanting phase by soaking the roots for 2 min in a solution containing 10 mL L^{-1} of microorganism suspension; in addition, 15 days after transplant, the inoculation was repeated in the soil using 100 mL of solution. Control plants only received water treatment.

2.3. Measurements

Determinations on agronomic and colorimetric traits were performed on six samples, casually chosen from each replicate. Once 'Canasta' plants were harvested, head fresh weight, number of leaves, collar diameter and plant height values were collected. CIELab colour coordinates were appraised via a colorimeter (Chroma-meter CR-400, Minolta corporation Ltd., Osaka, Japan).

To appraise head dry matter content, the samples were put in a thermo-aerated oven at 105 °C until constant weight. The dry matter value was expressed as percentage using the following formula: dry weight (g)/fresh weight (g)*100.

All analysis on plant nutritional and bioactive traits were appraised on six samples, arbitrarily chosen from each biological replicate. Soluble solids content values (SSC) were obtained through a refractometer (MTD-045 nD, Taipei, Taiwan) and the results were presented as °Brix.

Plant ascorbic acid content was determined by employing a Reflectometer RQflex10 Reflectoquant® (Sigma-Aldrich, Saint Louis, MO, USA) and Reflectoquant Ascorbic Acid

Test Strips (Merck, Darmstadt, Germany). Total phenolic values were appraised by the Folin-Ciocalteu method [33]. Total sugar concentration was evaluated following the method described by Serna et al. [34].

Total chlorophyll and carotenoid contents were assessed spectrophotometrically following the procedure of Costache et al. [35]. Both pigments values were expressed as mg 100 g^{-1} of fresh weight. Total nitrogen (N) was evaluated via the Kjeldahl method. Phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg) concentrations were appraised following the Fogg and Wilkinson method [36] and the Morand and Gullo method [37]. The entire mineral profile data were reported as mg g^{-1} dry weight.

N indices (nitrogen use efficiency and nitrogen uptake efficiency) were calculated as follow: Nitrogen use efficiency (NUE) = yield (t)/nitrogen application rate (kg), nitrogen uptake efficiency (UE) = plant nitrogen content (kg)/nitrogen application rate (kg).

2.4. Statistical Analyses

All collected data were analysed (StatSoft, Inc., Chicago, IL, USA) through two-way analysis of variance (ANOVA) by the SPSS software v. 20 (StatSoft, Inc., Chicago, IL, USA), setting nitrogen doses and microorganisms as main factors. For means separation, Tukey's honestly significant difference (HSD) test at $p \leq 0.05$ was used. Data expressed as percentages were subjected to arcsin transformation before ANOVA analysis ($\varnothing = \arcsin(p/100)^{1/2}$).

3. Results

3.1. Implications of PGPB and Nitrogen Dose on Yield and Yield-Related Features

Plant height, root collar diameter, number of leaves, head fresh weight and head dry matter percentage were influenced by PGPB application and nitrogen dose (Table 1). Moreover, ANOVA indicated a significant effect of the interaction PGPB × N (Table 1).

Table 1. Interaction effect of plant growth promoting bacteria (PGPB) (*A. brasilense* DSM 1690, *A. brasilense* DSM 2298 and *Pseudomonas* sp. DSM 25356) and nitrogen dose (0, 30, 60 and 120 kg ha^{-1}) on plant height, root collar diameter, number of leaves, head fresh weight and head dry weight of 'Canasta' lettuce.

Treatments		Plant Height (cm)		Root Collar Diameter (mm)		Number of Leaves (No. plant^{-1})		Head Fresh Weight (g plant^{-1})		Head Dry Matter (%)	
N dose (kg ha^{-1})	PGPB										
0	Non-inoculated	24.4	g	16.8	i	24.0	d	292.9	l	12.5	g
	A. brasilense DSM 1690	27.9	d	20.1	h	28.3	b	633.4	h	14.2	c
	A. brasilense DSM 2298	30.2	c	28.5	a	25.7	c	637.4	h	13.8	d
	Pseudomonas sp. DSM 25356	25.2	f	12.8	j	23.7	e	457.0	k	13.3	e
30	Non-inoculated	28.4	d	28.5	a	24.7	cd	565.0	j	12.7	g
	A. brasilense DSM 1690	31.5	b	22.9	f	25.3	c	751.3	c	14.7	c
	A. brasilense DSM 2298	32.8	a	24.1	e	24.7	cd	835.3	a	14.0	c
	Pseudomonas sp. DSM 25356	28.0	d	21.8	g	30.0	a	653.5	g	13.6	de
60	Non-inoculated	29.0	c	28.9	a	28.7	ab	631.1	h	13.1	f
	A. brasilense DSM 1690	32.0	a	23.8	ef	30.3	a	603.5	i	14.9	b
	A. brasilense DSM 2298	26.9	e	25.3	d	26.0	c	675.3	e	14.3	c
	Pseudomonas sp. DSM 25356	30.2	c	27.1	c	25.7	c	725.6	d	13.8	d
120	Non-inoculated	30.0	c	26.0	d	26.7	bc	630.1	h	13.5	e
	A. brasilense DSM 1690	25.8	f	28.0	b	27.7	b	658.1	f	15.5	a
	A. brasilense DSM 2298	29.8	cd	24.2	e	26.3	c	816.3	b	15.1	b
	Pseudomonas sp. DSM 25356	30.3	c	22.7	f	29.7	a	829.4	a	13.8	d
Significance											
Nitrogen (N)		***		***		***		***		***	
PGPB		***		***		***		***		***	
N × PGPB		***		***		***		***		***	

Means followed by the same letter for treatments are not significantly different accordingly to Tukey's honestly significant difference (HSD) test at $p \leq 0.05$. Significance levels: *** significant at $p \leq 0.001$.

The highest plant height was observed in plants from the combination 30 × *A. brasilense* DSM 2298 and the combination 60 × *A. brasilense* DSM 1690, followed by those supplied with 30 kg N ha^{-1} and treated with *A. brasilense* DSM 1690. Control plants (0 kg N ha^{-1} × non-inoculated) displayed the lowest values (Table 1).

Both plants supplied with *A. brasilense* DSM 2298 and treated with 0 kg N ha^{-1} and those non-inoculated and supplied with 30 or 60 kg N ha^{-1} had the highest root collar diameter, followed by plants subjected to the highest N level and inoculated with *A. brasilense* DSM 1690 (Table 1). Lettuce plants from the 0 × *Pseudomonas* sp. DSM 25356 combination had the lowest root collar diameter.

The highest number of leaves was appraised in the combinations: 30 × *Pseudomonas* sp. DSM 25356, 60 × *A. brasilense* DSM 1690 and 120 × *Pseudomonas* sp. DSM 25356, followed by those with N at 60 or 120 kg N ha^{-1} and *A. brasilense* DSM 1690 (Table 1). Nevertheless, plants from non-inoculated plots and treated with 60 kg N ha^{-1} did not significantly diverge neither from plants from the combinations 30 × *Pseudomonas* sp. DSM 25356, 60 kg × *A. brasilense* DSM 1690 and 120 × *Pseudomonas* sp. DSM 25356 nor from those supplied with 60 or 120 kg N ha^{-1} and inoculated with *A. brasilense* DSM 1690. The lowest number of leaves was observed in plants treated with 0 kg N ha^{-1} and inoculated with *Pseudomonas* sp. DSM 25356 (Table 1).

Plants fertigated with 30 kg N ha^{-1} and treated with *A. brasilense* DSM 2298 and those exposed to 120 × *Pseudomonas* sp. DSM 25356 combination had the highest head fresh weight, followed by plants from plots fertigated with the 120 kg N ha^{-1} and inoculated with *A. brasilense* DSM 2298, which in turn revealed higher values than plants from the combination 30 × *A. brasilense* DSM 1690 (Table 1). Control plants had the lowest head fresh weight.

The greatest head dry matter percentage was found in plants fertigated with the highest nitrogen dose and inoculated with *A. brasilense* DSM 1690, followed by those exposed to 120 kg N ha^{-1} and inoculated with *A. brasilense* DSM 2298 (Table 1). The lowest head dry matter percentage was observed in control plants and in non-inoculated plants fertigated with 30 kg N ha^{-1}.

3.2. Implications of PGPB and Nitrogen Dose on Leaf Colour, Nutritional and Functional Components and Mineral Concentrations

ANOVA showed that SSC and CIELab colour parameters were significantly influenced by PGPB and nitrogen dose treatments and by their interaction (Table 2).

Plants from the 30 × *A. brasilense* DSM 1690, 60 × *A. brasilense* DSM 2298 and 120 × *Pseudomonas* sp. DSM 25356 combinations had the highest SSC (Table 2), whereas, non-inoculated plants fertigated with 30 kg N ha^{-1} had the lowest SSC.

Plants supplied with 30 kg N ha^{-1} and treated with *A. brasilense* DSM 2298 had the highest a* values, whereas non-fertigated plants inoculated with *A. brasilense* DSM 1690 had the lowest a* values. Plants from the combinations 60 × *A. brasilense* DSM 1690 had the highest b* (Table 2), while, non-fertigated plants inoculated with *brasilense* DSM 2298 had the lowest b*.

The highest L* value was observed in plants fertigated with 120 kg N ha^{-1} and treated with *Pseudomonas* sp. DSM 25356, followed by that detected in plants not fertigated and exposed to *Pseudomonas* sp. DSM 25356 (Table 2). The lowest lightness value was recorded in plants treated with *A. brasilense* DSM 1690 and not fertigated with N.

As regard total sugars, ANOVA did not show a significant effect of the interaction N × PGPB (Figure 2). Averaged over PGPB, total sugars level was not affected by nitrogen dose (Figure 2).

Contrariwise, when averaged over nitrogen dose, sugar content was significantly affected by inoculation. Plants inoculated with *A. brasilense* DSM 1690 revealed the highest values, followed by those inoculated with *A. brasilense* DSM 2298, which in turn showed a higher total sugar concentration than those treated with *Pseudomonas* sp. DSM 25356 (Figure 2). Non-inoculated plants had the lowest total sugar content.

Table 2. Interaction effect of plant growth promoting bacteria (PGPB) (*A. brasilense* DSM 1690, A. brasilense DSM 2298 and *Pseudomonas* sp. DSM 25356) and nitrogen dose (0, 30, 60 and 120 kg ha^{-1}) on soluble solid content (SSC), a*, b* and L* of 'Canasta' lettuce.

Treatments		SSC (°Brix)		a*		b*		L*	
N dose (kg ha^{-1})	PGPB								
0	Non-inoculated	4.1	b	−19.32	c	26.87	c	46.50	cd
	A. brasilense DSM 1690	4.1	b	−20.67	d	28.30	c	41.70	e
	A. brasilense DSM 2298	4.0	c	−17.09	b	22.58	e	45.39	d
	Pseudomonas sp. DSM 25356	4.1	a	−19.40	c	32.46	a	53.10	b
30	Non-inoculated	3.2	e	−17.26	b	24.85	d	52.17	b
	A. brasilense DSM 1690	4.4	a	−19.34	c	32.61	a	44.65	d
	A. brasilense DSM 2298	4.2	b	−11.30	a	24.39	d	44.36	d
	Pseudomonas sp. DSM 25356	4.1	b	−17.96	b	33.34	a	52.11	b
60	Non-inoculated	3.7	d	−17.87	b	30.55	b	52.96	b
	A. brasilense DSM 1690	4.2	b	−20.74	d	33.67	a	47.36	c
	A. brasilense DSM 2298	4.3	a	−18.82	c	25.11	d	44.67	d
	Pseudomonas sp. DSM 25356	4.0	c	−19.63	c	31.15	b	47.60	c
120	Non-inoculated	4.2	b	−16.81	b	25.74	d	45.50	d
	A. brasilense DSM 1690	4.1	b	−17.13	b	25.49	d	48.05	c
	A. brasilense DSM 2298	4.1	b	−20.22	d	23.18	e	44.18	d
	Pseudomonas sp. DSM 25356	4.5	a	−17.00	b	26.17	c	54.31	a
Significance									
Nitrogen (N)		*		***		***		***	
PGPB		***		***		***		***	
N × PGPB		***		***		***		***	

Means followed by the same letter for treatments are not significantly different accordingly to Tukey's honestly significant difference (HSD) test at $p \leq 0.05$. Significance levels: * significant at $p \leq 0.05$; *** significant at $p \leq 0.001$.

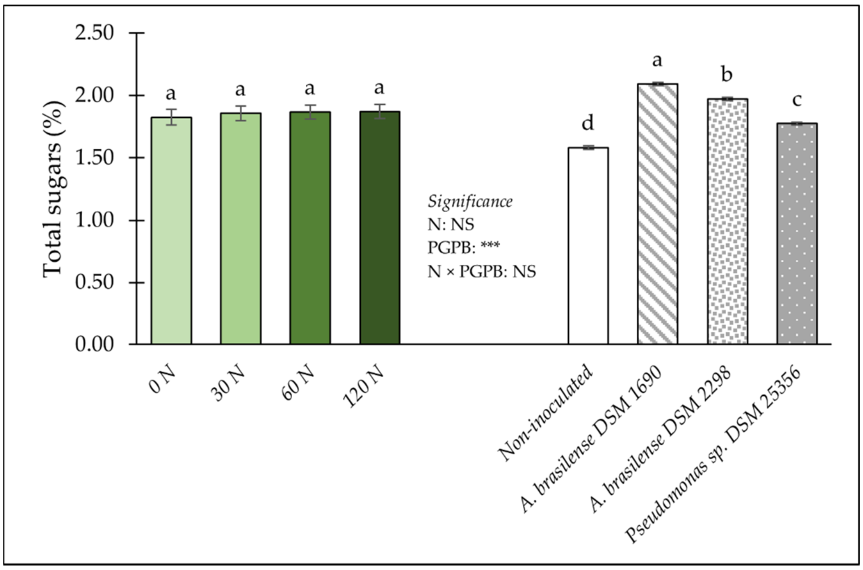

Figure 2. Impact of plant growth promoting bacteria (PGPB) (*A. brasilense* DSM 1690, *A. brasilense* DSM 2298 and *Pseudomonas* sp. DSM 25356) and nitrogen dose (0, 30, 60 and 120 kg ha^{-1}) on total sugars of 'Canasta' lettuce. Means followed by the same letter for treatments are not significantly different accordingly to Tukey's honestly significant difference (HSD) test at $p \leq 0.05$. Significance levels: NS non-significant; *** significant at $p \leq 0.001$. Bars indicate standard error. 0 N, 30 N, 60 N and 120 N represent 0, 30, 60 and 120 kg N ha^{-1}, respectively.

As regard ascorbic acid, total phenolics, carotenoids, total chlorophyll and nitrogen, ANOVA showed a significant effect of the interaction between nitrogen dose and PGPB application (Table 3).

Table 3. Interaction effect of plant growth promoting bacteria (PGPB) (*A. brasilense* DSM 1690, *A. brasilense* DSM 2298 and *Pseudomonas* sp. DSM 25356) and nitrogen dose (0, 30, 60 and 120 kg ha^{-1}) on ascorbic acid, total phenolics, carotenoids, total chlorophyll and nitrogen (N) of 'Canasta' lettuce.

Treatments		Ascorbic Acid (mg g^{-1} fw)		Total Phenolics (µg g^{-1} fw)		Carotenoids (mg 100 g^{-1} fw)		Total Chlorophyll (mg 100 g^{-1} fw)		N (mg g^{-1} dw)	
N dose (kg ha^{-1})	PGPB										
0	Non-inoculated	28.28	f	47.90	d	14.94	g	33.47	h	25.66	i
	A. brasilense DSM 1690	39.06	a	55.38	a	17.52	e	38.32	c	30.27	f
	A. brasilense DSM 2298	36.84	b	51.15	b	17.23	e	36.74	f	28.98	g
	Pseudomonas sp. DSM 25356	35.65	c	49.76	c	16.70	f	35.81	g	25.56	i
30	Non-inoculated	28.14	f	47.52	d	15.59	g	35.37	g	28.67	h
	A. brasilense DSM 1690	39.18	a	55.65	a	20.96	b	40.50	b	33.46	d
	A. brasilense DSM 2298	36.51	b	51.51	b	18.67	d	37.67	d	30.18	f
	Pseudomonas sp. DSM 25356	34.94	d	49.80	c	17.20	ef	36.96	e	29.43	g
60	Non-inoculated	27.78	f	47.52	d	15.52	g	35.45	g	30.46	f
	A. brasilense DSM 1690	39.35	a	55.49	a	22.88	a	40.43	b	35.47	b
	A. brasilense DSM 2298	36.46	bc	51.55	b	19.52	c	37.86	d	31.55	e
	Pseudomonas sp. DSM 25356	34.44	d	49.61	c	17.23	e	36.88	e	31.69	e
120	Non-inoculated	27.22	g	45.54	e	15.48	g	35.41	g	34.36	c
	A. brasilense DSM 1690	37.27	b	52.01	b	22.79	a	42.53	a	38.59	a
	A. brasilense DSM 2298	34.99	d	50.26	c	19.48	c	38.76	c	34.70	c
	Pseudomonas sp. DSM 25356	30.98	e	48.18	d	17.36	e	37.28	e	35.52	b
Significance											
Nitrogen (N)		***		***		***		***		***	
PGPB		***		***		***		***		***	
N × PGPB		***		**		***		***		***	

Means followed by the same letter for treatments are not significantly different accordingly to Tukey's honestly significant difference (HSD) test at $p \leq 0.05$. Significance levels: ** significant at $p \leq 0.005$; *** significant at $p \leq 0.001$.

Plants inoculated with *A. brasilense* DSM 1690 and fertigated with 0, 30 or 60 kg N ha^{-1} had a higher ascorbic acid concentration than those treated with *A. brasilense* DSM 2298 and fertigated with 0, 30 or 60 kg N ha^{-1} (Table 3). The lowest ascorbic acid concentration was detected in non-inoculated plants fertigated with the highest nitrogen dose. Data on total phenolics followed the trend described for ascorbic acid (Table 3).

Plants inoculated with *A. brasilense* DSM 1690 and fertigated with 60 or 120 kg N ha^{-1} had a higher carotenoids content (Table 3) than those fertigated with 30 kg N ha^{-1} and treated with *A. brasilense* DSM 1690. Regardless N dosages, non-inoculated plants revealed the lowest carotenoids concentrations.

Lettuce plants supplied with the highest N dosage and inoculated with *A. brasilense* DSM 1690 had a higher total chlorophyll concentration (Table 3) than those inoculated with the same PGPB strain and fertigated with 30 or 60 kg N ha^{-1}. The lowest values were detected in control plants.

Plants exposed to 120 kg N ha^{-1} and inoculated with *A. brasilense* DSM 1690 had the highest N concentration (Table 3), followed by those fertigated with 60 kg N ha^{-1} and inoculated with *A. brasilense* DSM 1690. Control plants and those from non-fertigated plots and inoculated with *Pseudomonas* sp. DSM 25356 had the lowest N concentration values.

ANOVA for P, K, Ca and Mg did not reveal a significant effect of the interaction N × PGPB (Figure 3).

Regardless of the PGPB application, plants fertigated with 60 or 120 kg N ha^{-1} had the highest P concentration (Figure 3A), whereas plants not supplied with nitrogen showed the lowest P concentration values. Conversely, when averaged over nitrogen fertigation, PGPB application did not significantly affected P concentration.

Nitrogen fertigation level did not significantly affect K concentration (Figure 3B). Contrariwise, PGPB significantly affected the aforementioned parameter. The highest K concentration was observed in plants inoculated with *A. brasilense* DSM 1690, followed by that detected in plants treated with the other strain of *A. brasilense* (DSM 2298). The lowest K concentration was recorded in non-inoculated plants.

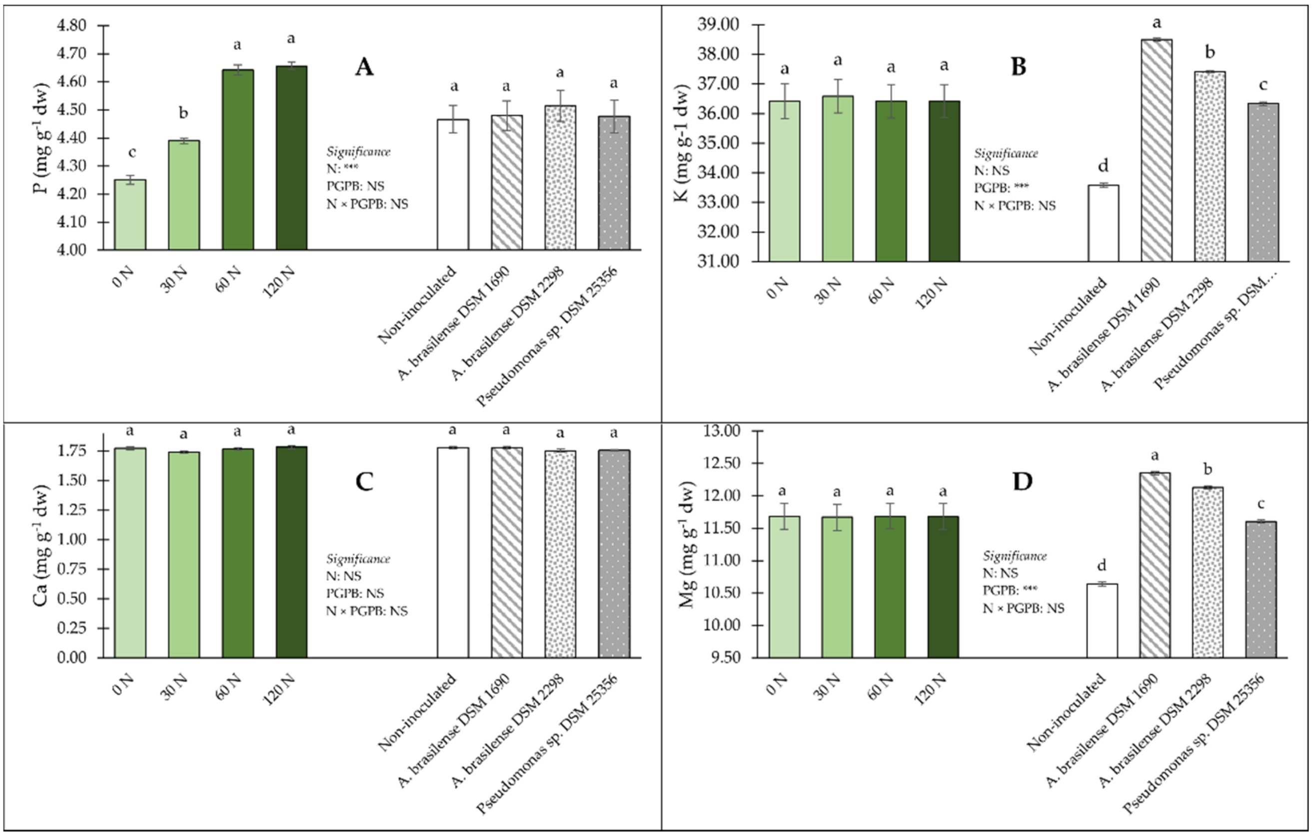

Figure 3. Impact of plant growth promoting bacteria (PGPB) (*A. brasilense* DSM 1690, *A. brasilense* DSM 2298 and *Pseudomonas* sp. DSM 25356) and nitrogen dose (0, 30, 60 and 120 kg ha^{-1}) on phosphorous (P) (**A**), potassium (K) (**B**), calcium (Ca) (**C**) and magnesium (Mg) (**D**) of 'Canasta' lettuce. Means followed by the same letter for treatments are not significantly different accordingly to Tukey's honestly significant difference (HSD) test at $p \leq 0.05$. Significance levels: NS non-significant; *** significant at $p \leq 0.001$. Bars indicate standard error. 0 N, 30 N, 60 N and 120 N represent 0, 30, 60 and 120 kg N ha^{-1}, respectively.

Nitrogen dose and PGPB did not significantly affect Ca concentration (Figure 3C).

Data on Mg concentration supported the trend presented for K concentration (Figure 3D).

3.3. Nitrogen Indices

As regard NUE and UE, ANOVA revealed a significant effect of the interaction N × PGPB (Table 4).

Lettuce plants from the combination 30 × *A. brasilense* DSM 2298 displayed the highest NUE value, followed by those fertigated with 30 kg N ha^{-1} and inoculated with *A. brasilense* DSM 1690 (Table 4). The lowest NUE was observed in non-inoculated plants fertigated with 120 kg N ha^{-1}.

Plants treated with *A. brasilense* DSM 1690 and fertigated with 30 kg N ha^{-1} had the highest UE values, followed by those inoculated with *A. brasilense* DSM 2298 and supplied with 30 kg N ha^{-1}, which in turn revealed a higher UE value than those inoculated with *Pseudomonas* sp. DSM 25356 and fed with 30 kg N ha^{-1} (Table 4). The lowest UE values were recorded from non-inoculated plants and exposed to the highest N level.

Table 4. Interaction effect of plant growth promoting bacteria (PGPB) (*A. brasilense* DSM 1690, *A. brasilense* DSM 2298 and *Pseudomonas* sp. DSM 25356) and nitrogen dose (30, 60 and 120 kg ha^{-1}) on nitrogen use efficiency (NUE) and nitrogen utilization efficiency (UE) of 'Canasta' lettuce.

Treatments		NUE (t kg^{-1})		UE (kg kg^{-1})	
N dose (kg ha^{-1})	PGPB				
30	Non-inoculated	3.01	d	11.00	d
	A. brasilense DSM 1690	4.01	b	19.66	a
	A. brasilense DSM 2298	4.45	a	18.87	b
	Pseudomonas sp. DSM 25356	3.49	c	13.92	c
60	Non-inoculated	1.68	g	6.70	f
	A. brasilense DSM 1690	1.61	h	8.50	e
	A. brasilense DSM 2298	1.80	f	8.13	e
	Pseudomonas sp. DSM 25356	1.94	e	8.44	e
120	Non-inoculated	0.84	j	3.91	h
	A. brasilense DSM 1690	0.88	j	5.26	g
	A. brasilense DSM 2298	1.09	i	5.69	g
	Pseudomonas sp. DSM 25356	1.11	i	5.43	g
Significance					
Nitrogen (N)		***		***	
PGPB		***		***	
N × PGPB		***		***	

Means followed by the same letter for treatments are not significantly different accordingly to Tukey's honestly significant difference (HSD) test at $p \leq 0.05$. Significance levels: *** significant at $p \leq 0.001$.

4. Discussion

Nitrogen supply is a fundamental agronomic practice to guarantee prime growth, development, and yield of any crop. However, the improper use of nitrogen can determine environmental repercussions. Concomitantly, the nitrogen supply and its accumulation in plant tissues are a major issue, especially in vegetables classified as nitrogen iper-accumulators, such as lettuce. Concomitantly, contemporary agriculture must afford the dual mission of sustaining the global population and reducing the ecological effect of the vegetable production sector [38,39]. An innovative agronomic practice to face these challenges, is the application of biostimulants, including PGPMs, such as plant growth promoting bacteria (PGPB), which offer an attractive way to substitute chemical fertilisers [40].

Our study highlighted that both plants inoculated with *Azospirillum brasilense* and fertigated with 30 or 60 kg N ha^{-1} and those inoculated with *Pseudomonas* sp. and grown with 120 kg N ha^{-1}, showed the best results in terms of plant vigour traits, yield and head dry matter percentage. These findings are in accord with those reported by Gravel et al. [41], who, by studying the effect of two PGPB (*Pseudomonas* and *Trichoderma* genus) on growth and yield of tomato cultivated in greenhouse, found that plants treated with PGPB had higher shoots and roots fresh weight, plant length and fruit yield. Our outcomes are also in agreement with those by Bhattacharyya and Jha [42], who reported that PGPB application significantly enhances plant vigour-related traits and dry matter production in various crops, such as potato and tomato. Furthermore, Singh et al. [43] revealed a beneficial effect of *Azospirillum* PGPB application on broccoli plant growth features and yield. As reported by Mantelin and Touraine [44], overall, an implementation in N supply leads to a greater plant N status, corresponding in a low plant N request, which limits both the $NO^{-}{}_3$ transporters and plant development. The same authors [44] stated that the effects of PGPB on N absorption and plant growth are comparable to those of low N availability. However, our data on yield and yield-related traits showed that plants treated with *A. brasilense* (DSM 1690 and DSM 2298 strains) had the best performance when fertigated with the mild N dosages. Thus, we may assume that the highest N regime inhibited the *A. brasilense* activity. In this respect, we must point out that, currently, the exact mechanisms by which PGPB elicit plant growth and development are not totally understood. However, Parewa et al. [45]

reported that PGPB have a direct effect on plant growth via several mechanisms, such as, nitrogen fixation, solubilisation of inorganic phosphate and the ability to synthesize plant key hormones.

This study showed that both PGPBs tested exerted a positive effect on SSC. In particular, plants treated with *A. brasilense* had a higher SSC when subjected to mild N levels (30 or 60 kg ha^{-1}), whereas plants inoculated with *Pseudomonas* sp. revealed the highest SSC when fertigated with 120 kg N ha^{-1}. These findings agree with those of Ordookhani and Zare [46] who, investigating on the influence of PGPMs on intrinsic fruit quality parameters in tomato, found a positive effect of the microorganism on fruit SSC. Our data are also in line with those of Katsenios et al. [47], who studied the impact of different PGPB strains on growth, yield and quality of industrial tomato. Generally, our results underlined that SSC parameter is interactively modulated by PGPB application and nitrogen dose.

Vegetable colour is a visual aspect which influences product appeal, as it is directly related to the consumer's perceived quality [48]. Thus, agronomic practices causing colour changes should be taken into consideration, particularly for leafy green vegetables. It is well documented that leaf colour is chiefly related to its chlorophyll concentration, which in turn it is affected by nitrogen availability [49]. However, our data showed that plants cultivated without N supply had a greener leaf colour than plant fertigated with N. This is related to the fact that 'Canasta' lettuce leaves are characterized by an anthocyanin pigmentation and, consequently, N dose does not reflect the conventional trend reported for leafy green vegetables.

Our findings indicate that ascorbic acid and total phenolics in lettuce plants were boosted through PGPB inoculation, particularly in plants inoculated with *A. brasilense* DSM 1690. This finding is sustained by Parewa et al. [45], who stated that PGPB enhance secondary metabolites production. Similarly, Cappellari et al. [50] found that three PGPR genus, including two *Pseudomonas* and one *Bacillus*, elicit phenolic biosynthesis of *Mentha piperita*. Kloepper [51], Van Loon [52] and Babalola [53] reported that numerous PGPR strains trigger plant tolerance to phytopathogens via modification of the secondary metabolism, biosynthesizing phenolic compounds. Data on ascorbic acid and total phenols are in accordance with those of Ottaiano et al. [54] who, by appraising the impact of biostimulant supply under different N regimes on yield and quality of lettuce, found that ascorbic acid and total phenols contents decrease as N level increases. Our findings are also supported by Di Mola et al. [55] who, investigating on the interactive effect of seaweed extract application and N doses, found that ascorbic acid concentration is reduced by a high N level.

Results revealed that plants treated with *A. brasilense* DSM 1690 combined with 60 or 120 kg N ha^{-1} had the highest carotenoids concentration. Similarly, plants treated with *A. brasilense* DSM 1690 and fertigated with the highest N level showed the highest total chlorophyll. Our data fully agree with previous studies by Radhakrishnan and Lee [56] who, evaluating the influence of PGPB (*Bacillus methylotrophicus* KE2) on growth and nutritional metabolites of lettuce, found that PGPB application enhances leaf pigments (total chlorophyll and carotenoids) concentrations. Pinto et al. [57] underlined that chlorophyll and carotenoid concentrations are directly associated to mineral elements in plant. In line to the assumption of Radhakrishnan and Lee [56], the higher pigments concentration in plants treated with *A. brasilense* DSM 1690 could be linked to the profuse amount of Mg in plant tissues, since Mg is a core element in chlorophyll.

Outcomes pointed out that plants colonized by *A. brasilense* DSM 1690 and associated with the highest N level (120 kg ha^{-1}) displayed the highest N leaf concentration. The macronutrients, like N, can directly and/or indirectly affect cellular formations. The increase of plant N concentration by PGPB inoculation is extensively reported [56]. In this regard, de Santi Ferrara et al. [58] stated that nitrogen fixation process of microbes in soil is the main reason for this increase. Particularly, Hungria et al. [59] demonstrated that two strains of *A. brasilense* (Ab-V5 and Ab-V6), had similar *nif* and *fix* genes that induce the ability to fix atmospheric N.

Results showed that PGPB application did not influence P concentration. This finding is coherent with that of Hungria et al. [60] who, assessing the effect of different strains of *A. brasilense* and *A. lipoferum* on yield and mineral profile of leaves and grains in maize and wheat, found that PGPB does not significantly affect P concentration in leaves tissue. Data also showed that plants fertigated with 60 or 120 kg N ha^{-1} had the highest leaf P concentration. This agrees with precedent studies evaluating the influence of the combined treatment of *Trichoderma virens* and biostimulant on lettuce grown under various N regimes [61]. Findings on K and Mg concentrations revealed that PGPB elicit their accumulation in plant tissues. These results are totally in accord with those of Radhakrishnan and Lee [56], who associated these results to a catalysed metabolism of proteins, enzymes, lipids and nucleic acids. Furthermore, our data displayed that Ca leaf concentration was not affected neither by PGPB action nor by N level. These findings completely agree with those reported by Hungria et al. [60] and by Rouphael et al. [61].

Data revealed that plants colonized with PGPB had a higher total sugar concentration than non-inoculated plants. In this respect, our results are in accord with those of Sandhya et al. [62] who, by studying the effect of PGPB (*Pseudomonas* spp.) on compatible solutes, antioxidant status and plant growth of maize under drought stress, found that PGPB colonization enhances total soluble sugars. Furthermore, in agreement with our outcome, Pirlak and Köse [63] reported that PGPR improved total sugars in strawberry fruits. However, our data highlighted that N dose did not significantly influence total sugars. These results are coherent with those of Bénard et al. [64] who reported that N availability does not significantly influence tomato fruit sugars concentration.

Data revealed that the PGPB tested increased NUE indices (NUE and UE) under N deficit. These data are fully corroborated by Zeffa et al. [65], who found that *A. brasilense* increases NUE of maize genotypes through an increased plants growth and development. The beneficial effect of *A. brasilense* on lettuce, under low N regime, emphasise the importance of PGPB to overcome plant developmental limits under suboptimal-growth conditions.

5. Conclusions

The use of PGPB to enhance crop yield under optimal or suboptimal growth conditions is acquiring importance in the sector of leafy vegetable production due to PGPB capacity to increase the business profit and, concomitantly, reduce environmental impact of the conventional cultivation practices adopted by growers. The inoculation of PGPB, particularly *A. brasilense* DSM 2298, boosted plant growth and productivity mainly under low N doses. The application of both *A. brasilense* strains was efficient in sustaining better nutritional and functional status of lettuce in terms of the SSC, ascorbic acid, total phenolics, carotenoids, total chlorophyll and total sugars. Remarkably, K and Mg concentrations can be enhanced by PGPB, expressly by *A. brasilense* DSM 1690. *A. brasilense* increased N concentration in lettuce leaves; however, it also enhanced NUE and UE by 47.8% and 78.7%, respectively, compared with the control. The findings of the present study underline the advantage of applying PGPB in 'Canasta' lettuce plants to increase crop yield and quality under optimal and suboptimal N levels. This information can be beneficial both to the growers and the ecosystem sustainability.

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