

Article

Gluconacetobacter diazotrophicus Inoculation of Two Lettuce Cultivars Affects Leaf and Root Growth under Hydroponic Conditions

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Abstract: The growth-promoting effects of *Gluconacetobacter diazotrophicus* inoculation on the leaf lettuce (*Lactuca sativa* L.) cultivars “Black Seeded Simpson” and “Bibb/Limestone” were investigated. Plants of each cultivar were grown hydroponically in Kratky jars in a growth chamber-controlled environment in a completely randomized factorial design with three or four replications. Each experiment was repeated once. Factors were (1) with or without inoculant and (2) seven levels of nitrogen (N) fertilization ranging from deficient (37.5 mg L⁻¹ N) to excessive (172.5 mg L⁻¹ N). The shoot, root, and total biomass accumulation, nitrogen density, and carbon/nitrogen (C/N) ratios were measured for each variety. Black Seeded Simpson demonstrated a shifting of production towards aerial tissues, with significantly greater shoot production and reduced root production. The observed increase in shoot biomass was greatest at the slightly deficient N rate of 105 mg L⁻¹ N where inoculated plants produced 14.8% more than uninoculated plants. Lower N density and higher C/N ratios in inoculated shoot tissues indicate greater N use efficiency. Bibb/Limestone responded to inoculation with an average increase of 10.9% in shoot production and with greater root biomass. Bibb/Limestone also exhibited lower N density in inoculated shoot tissues with a corresponding increase in the C/N ratio. For growers looking to maximize lettuce yields, *G. diaz* inoculation may present a beneficial additive to the growing system by increasing leaf yields while not increasing N fertilizer requirements.



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1. Introduction

Lettuce (*Lactuca sativa* L.) is one of the most important vegetable crops sown in the United States, ranking third in total acreage (106,027 hectares) and first in economic value (USD 3.493 billion) in 2019 [1]. Over 97% of domestic production is concentrated in California and Arizona where environmental conditions allow multiple crops per season and year-round production [1,2]. Several impediments to the intensification of field-grown lettuce production have arisen in recent years, including limited acreage of ideally suited farmland, high nitrogen (N) requirements for optimal production, risk of fecal bacterial contamination when manures are used as an N source for organic lettuce production, and significant irrigation demands. These water demands are increasingly placing a burden on lettuce production, with chronic drought and increased salinity hindering yields [2,3].

Exogenous N applications exacerbate production concerns as leaching and run-off impairs water quality, reduces yields, and increases costs. Large-scale operations in these

states have also been impacted by food safety recalls over contaminated produce, leading to millions of dollars of losses and erosion of public trust [1]. The sustainability of such geographically constrained production can be questioned, especially when coupled with the negative environmental impacts of cross-country transportation. Unfortunately, environmental conditions outside of these growing areas are unsuitable for year-round production.

Controlled environmental agriculture in the form of hydroponic cultivation has arisen to localize and intensify lettuce production in areas otherwise unsuitable for continual, field-based sowing. In hydroponic production, light, temperature, and nutrient fertilization are all monitored and adjusted to maximize the crop output and can be implemented regardless of climate or latitude. Turnaround time from planting to harvest can be reduced by half under these conditions and yields can increase 11-fold per unit area, increasing the yields per annum and helping to offset the cost of hydroponic production [4,5].

Additionally, hydroponic systems can be located near centers of consumption to minimize transportation costs, environmental impacts, and loss of nutritive composition during shipping and transport. Hydroponic production also tightly controls water salinity and can recycle and/or reuse irrigation water, thereby, improving their water use efficiency. One costly consideration of hydroponic lettuce production is the need to provide 100% of the fertilization requirements in the form of nutrient salts.

Nitrogen salts are often provided in the form of synthetically sourced fertilizers whose production has significant energy and environmental costs. If microbe-assisted crop production strategies [6] can be incorporated into the lettuce production system to promote increased yield and/or fix atmospheric N, it may help alleviate some of the need for exogenous fertilizer additions.

Gluconacetobacter diazotrophicus (*G. diaz.*) is an obligate endophyte that was originally identified in sugarcane [7,8]. The significance of this discovery is that, in sugarcane, *G. diaz.* can fix up to 200 kg N ha⁻¹ year⁻¹ meeting nearly half of the crop's N requirement [9,10]. *G. diaz.* was subsequently found to be naturally associated with coffee [11], finger millet [12], banana, pineapple [13,14], tea, mango [8,15], and wetland rice [16]. Research has shown that inoculation with *G. diaz.* can result in non-pathogenically infected crop species across numerous plant families [17], including sweet potato, sugarcane, maize [18,19], tomato [20], sweet sorghum [21,22], carrot, beet [23,24], snap bean [25], and casava [26].

Unlike most N-fixing bacteria, *G. diaz.*'s nitrogenase is not wholly suppressed by agricultural levels of environmental N and can tolerate especially high nitrate levels as it lacks nitrate reductase genes [9,27]. However, the colonization of sugarcane by *G. diaz.* is partially inhibited by high N-fertilization [28], and the effect was shown to be related to the plant physiology [29]. In addition to its N-fixing capability, *G. diaz.* produces indole-3-acetic acid (IAA) and gibberellins A1 and A3, which are important plant growth-regulating hormones [30,31].

G. diaz. also produces a bacteriocin against *Xanthomonas albilineans*, a sugar cane pathogen [32,33]. Drought stress has been shown to be mitigated by *G. diaz.* colonization in sugarcane and rice [34,35]. In a survey of 50 tropical and subtropical plants in southern India, *G. diaz.* was found in association with the rhizosphere in ten plants [16]. This is of potential benefit as *G. diaz.* has been shown to solubilize and enhance the availability and uptake of zinc and iron [36,37].

The objective of this study was to determine the influence of *G. diaz.* inoculation on root and leaf plant growth as well as the variance between cultivars in two hydroponically grown lettuces.

2. Materials and Methods

2.1. Experimental Design and Randomization

Two cultivars of lettuce were chosen for the study based on similar days to maturity, compact stature, and disease resistance. The plants were grown in Kratky hydroponic jars under temperature-, light-, and humidity-controlled conditions in growth chambers (Convion MTR30, Convion Environmentals Limited) that housed a maximum of 44 jars

for the duration of the experiment [38]. The experiment was conducted as a two-factor fixed-effect completely randomized design (CRD) with three or four replicates.

The first factor was the presence/absence of *G. diaz* from the initial seed soaking solution. The second factor was different levels of nitrogen fertilization ranging from deficient (37.5, 60, 82.5, and 105 mg kg⁻¹ N) to sufficient (127.5 and 150 mg kg⁻¹ N) and hyper-sufficient (172.5 mg kg⁻¹ N). Placement of the Kratky jars was randomized via the “RAND” function in Microsoft Excel (2013), with re-randomization occurring once per week. The experiment was conducted two times for each of the two cultivars in the study.

2.2. Plant and Bacterial Material

The two cultivars of lettuce selected for this trial, Black Seeded Simpson (BSS) and Bibb/Limestone (BIB), exhibit different leaf growth patterns but similar overall structure and suitability for hydroponic production. Both varieties had an estimated days to maturity of 43–45 days after planting and were sourced from commercial seed companies. *G. diaz* was provided by Azotic Technologies, Ltd. (Nottingham, United Kingdom) and is an intracellular, endophytic strain of the bacterium developed for delivery through their commercialized ‘N-Fix’ product (Azotic-na.com 2019). Pure strain stocks of the bacteria were maintained in 25% glycerol at –80 °C until inoculum preparation.

2.3. Inoculant Preparation

G. diaz was cultured from glycerol preserved stock in a modified MESMA/ATGUS medium (2.7 g L⁻¹ glucose; 4.8 g L⁻¹ dipotassium phosphate (K₂HPO₄); 0.65 g L⁻¹ monopotassium phosphate (KH₂PO₄); 1.8 g L⁻¹ mannitol; 4.4 g L⁻¹ 2-(N-morpholino) ethanesulfonic acid (MES hydrate); 2.7 g L⁻¹ yeast extract) [39]. Nutrients were combined in ASTM Type II, deionized water (>1 MΩ·cm) and autoclaved prior to aseptic bacterial introduction to ensure a pure, single-strain culture.

Inoculated medium and bacteria-free controls were incubated in a positive-pressure, enclosed shaker table (30 °C at 120 rpm) for five days. Colony forming units (CFU) per milliliter solution were determined by serial dilution and standard plate counts on semi-solid ATGUS medium (same as above, plus 0.8% wt/vol agar) in triplicate, corroborated with OD₆₀₀ based estimation (NanoDrop One, Thermo Scientific) against standard curves. The achieved CFUs in concentrate were 4.0 × 10⁸ mL⁻¹ for BSS trial #1, BSS trial #2, and BIB trial #1, and 1.8 × 10⁹ mL⁻¹ for BIB trial #2. Differences in CFU were attributed to random variations inherent in bacterial culturing.

Liquid medium for the seed soak inoculation was prepared from a proprietary blend of nutritive and adhesive adjuvants in deionized water, as described by an operating protocol provided by Azotic Technologies, Ltd. (Internal document dated 2016) and reported on the safety data sheet (Azotic Technologies, Ltd.; Lancashire, United Kingdom; 2017). For each trial, 50 mL portions of medium were aseptically aliquoted into each of two sterilized flasks. One flask then received a 50 mL portion of cultured bacterial concentrate, and the second flask received a 50 mL portion of deionized water.

The portion containing bacterium, now at $\frac{1}{2}$ cultured CFU mL⁻¹, became the inoculation treatment (INOC), while the adjuvant and water only solution became the uninoculated control (NON). Both treatment stocks were assessed for equal pH and EC to ensure that the seeds soaked would experience identical conditions save for the bacterial presence. Thus, BSS trials #1 and #2 (BSS1, BSS2), and BIB trial #1 (BIB1) received 2.0 × 10⁸ CFU mL⁻¹ on seed, and BIB trial #2 (BIB2) received 9.0 × 10⁸ CFU mL⁻¹ on seeds at inoculation.

2.4. Seed Inoculation, Planting, and Growing Conditions

For seed treatment application, 130 seeds each were distributed to two 60 × 15 mm sterile petri dishes, and 250 µL of solution (INOC or NON) was added via pipette to each dish. Dishes were immediately sealed and gently agitated. After five minutes, the solutions were pipetted out, and a second 250 µL portion of the same treatment was applied as before

to ensure saturation. After a second five-minute soak, all residual solution was removed. Seeds were then stored for 10–30 min at 4 °C and 65% relative humidity until planting.

Three seeds were sown in 4 cm rockwool cubes (Grodan A036/40; Roxul Inc., Milton, ON, Canada) that were nested into 5 cm circular net cups (Hummert International) and placed into foil-wrapped Kratky hydroponic jars (Figure 1) [38]. Jars were prefilled with 800 mL of nutrient solution formulated to provide a full and balanced measure of all essential nutrients required for growth of a lettuce plant to maturity except for N, which was added according to the N treatments described above.

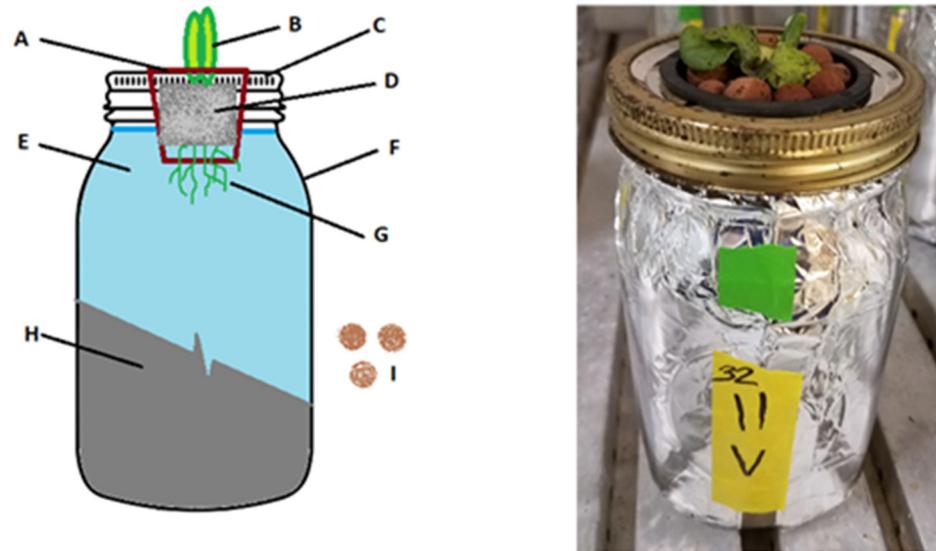


Figure 1. The Kratky soilless hydroponic growing method [38].

For all nutrients other than N, the nutrient content in mg L^{-1} matched that of the Jack's Hydroponic (5-12-26) + calcium nitrate recipe [40]. The bottom of the rockwool was immersed to allow for nutrient solution wicking onto the seeds. Extruded horticultural clay pebbles (Hydroton 8–16 mm, Hummert International) were placed on top of the rockwool to reduce evaporation and limit algal growth. No further nutrient solution was added after the beginning of the trial.

- A. 2" circular net cup used to suspend the plant above the nutrient solution through a hole cut in the center of the Mason jar lid.
- B. Growing lettuce shoots.
- C. Wide-mouth quart jar metal lid and collar. Pre-drilled 7/16" hole in lid center allows net cup to rest inside on the top lip.
- D. A036/40 rockwool used as seedling and plant support media. Inert, pH neutral, high wicking growth media with no nutrient content. Bottom 1/4" of rockwool extends into the nutrient solution to allow wicking.
- E. Nutrient solution, 800 mL in a 946 mL (1 qt) jar.
- F. 946 mL (1 qt) wide-mouth Mason jar.
- G. Roots growing into the media solution.
- H. Aluminum foil completely wrapping the sides and bottom of jar to prevent exposure to light.
- I. Hydroton clay pebbles placed atop the rockwool to limit evaporation and block light.

Growth chamber conditions were a 16/8 h day/night cycle under metal halide and high-pressure sodium lamps providing a photosynthetic photon flux density of $210 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 23/20 °C. Relative humidity was maintained at 70%. Plants were re-randomized once per week by operator-blind removal and redistribution back into the chamber concurrent with jars being topped off with deionized water to a minimum of 400 mL solution. Each jar had 0.11 m^2 of growing area and was thinned to a single plant

in trials BSS1, BSS2, and BIB1. Due to access restrictions stemming from the COVID-19 pandemic, BIB2 seedlings were not thinned.

2.5. Harvesting and Analysis

After 30/31 days, when the lettuce was still in a rapid growth stage, all plant material was harvested, sectioned, and dried at 60 °C until stable weights were achieved to yield shoot dry matter (SDM) and root dry matter (RDM). The dry weight of root material that could not be removed from the rockwool cubes was determined by subtracting the pre-recorded mass of the dried cubes. After all dry matter masses were recorded, the roots and shoots were finely ground to 0.5 mm, and elemental combustion analysis was performed (Elementar, Thermo Fisher Scientific) to determine the nitrogen and carbon content. The N content in tissues was calculated by multiplying the total tissue mass by the determined N%.

2.6. Statistical Analysis

Data were analyzed using SAS 9.4 software for Windows (Copyright 2016 by SAS Institute Inc., Cary, NC, USA). Observations across all N levels for INOC were pooled and statistically compared to the pooled observations for NON allowing for 28 degrees of freedom. All significance tests were performed at $\alpha < 0.05$ using PROC MIXED and PROC GLM for multi-factor ANOVA with type 3 tests of fixed effects and Tukey–Kramer HSD for the least squares mean differences. For dependent variables data exhibiting violations of Studentized and/or Pearson residual plots, PROC TRANSREG was used to generate BOX-COX lambdas for data normalization. For BIB2, where jars were not thinned, the number of plants present was included in the analysis as a discrete covariate in ANCOVA.

3. Results

3.1. Black Seeded Simpson (BSS) Trials

3.1.1. Biomass Accumulation

For the BSS cultivar, plant biomass responded curvilinearly to nitrogen fertilization in the shoot dry matter (SDM), root dry matter (RDM), and total dry matter (TDM) across both trials in accordance with typical luxury consumption trends (Table 1). In BSS1, inoculation affected a significant increase (INOC; 235 ± 106 mg, $p \leq 0.0345$) in the shoot biomass, an increase of 8.19%, and a significant decrease (INOC; -425 ± 137 mg, $p \leq 0.0140$) in the root biomass. The growth response did not vary by N-level, and the total plant biomass was not significantly affected in BSS1. In BSS2, there were no significant differences for any of the biomass measures.

3.1.2. Carbon Nitrogen Ratios and Nitrogen Accumulation

For the BSS cultivar, the N content responded curvilinearly to nitrogen fertilization in SDM and RDM across both trials in accordance with typical luxury consumption trends (Table 2). In BSS1, inoculation affected a significant decrease in SDM nitrogen density from 2.209% to 1.977% (INOC; -0.233 ± 0.659 %N, $p \leq 0.0015$), but there was no difference in root tissues. C/N ratios followed a downward curvilinear trend with INOC treatment in shoot tissue higher than NON (INOC; 2.223 ± 0.730 , $p \leq 0.005$). Root tissue exhibited no differences. In BSS2, no significant differences emerged for C/N ratios.

Table 1. Effects of inoculation and nitrogen fertilization on Black Seeded Simpson Biomass.

Trial and Measure	Fertilizer Treatment Provided Nitrogen Concentration (ppm)						
	37.5	60	82.5	105	127.5	150	172.5
BSS1							
Shoot Dry Matter (g) *	LSD: 0.235 s.e.: 0.1057 df = 28 $p \leq 0.0345$						
INOC	2.2843	2.7927	2.499	3.524	3.4793	3.416	3.7387
NON	2.224	2.645	2.3493	3.0683	3.241	3.2527	3.3087
Difference	0.0603	0.1477	0.1497	0.4557	0.2383	0.1633	0.43
Root Dry Matter (g) **	LSD: -0.4251 s.e.: 0.1366 df = 28 $p \leq 0.0042$						
INOC	0.602	0.1067	0.207	1.3737	0.7867	0.4473	0.7597
NON	0.7243	0.7117	0.639	1.913	1.1987	1.4433	0.629
Difference	-0.122	-0.605	-0.432	-0.539	-0.412	-0.996	0.1307
Total Dry Matter (g)	n/s						
INOC	2.8863	2.8993	2.706	4.8977	4.266	3.8633	4.4983
NON	2.9483	3.3567	2.9883	4.9813	4.4397	4.696	3.9377
Difference	-0.062	-0.457	-0.282	-0.084	-0.174	-0.833	0.5606
BSS2							
Shoot Dry Matter (g)	n/s						
INOC	-	4.7867	5.7825	5.705	6.3525	6.6475	6.2067
NON	-	4.47	6	5.6875	7.0975	5.715	6.7767
Difference	-	0.3167	-0.218	0.0175	-0.745	0.9325	-0.57
Root Dry Matter (g)	n/s						
INOC	-	1.78	2.0675	2.135	2.0425	2.2175	2.2333
NON	-	1.89	1.92	2.0525	2.285	1.885	2.13
Difference	-	-0.11	0.1475	0.0825	-0.243	0.3325	0.1033
Total Dry Matter (g)	n/s						
INOC	-	6.5667	7.85	7.84	8.395	8.865	8.44
NON	-	6.36	7.92	7.74	9.3825	7.6	8.9067
Difference	-	0.2067	-0.07	0.1	-0.988	1.265	-0.467

** = significant at $\alpha < 0.01$, * ≤ 0.05 , n/s = not significant. Note: Bolded main effect level shows which treatment was significantly greater. '-' indicates that this fertilization level was not used in this experiment.

Table 2. Effects of inoculation and nitrogen fertilization on the Black Seeded Simpson N density and C/N ratios.

Trial and Measure	Fertilizer Treatment Provided Nitrogen Concentration (ppm)						
	37.5	60	82.5	105	127.5	150	172.5
BSS1							
SDM Nitrogen % **	LSD: -0.2328 s.e.: 0.06591 df = 28 $p \leq 0.0015$						
INOC	1.290	1.465	1.930	1.898	2.268	2.596	2.390
NON	1.337	1.828	2.326	2.110	2.276	2.648	2.940
Difference	-0.047	-0.363	-0.396	-0.212	-0.008	-0.052	-0.550
SDM C/N Ratio **	LSD: 2.2234 s.e.: 0.7305 df = 28 $p \leq 0.0050$						
INOC	30.495	26.496	20.144	20.821	17.370	15.439	16.513
NON	29.260	21.143	16.466	18.783	17.419	15.060	13.583
Difference	1.236	5.353	3.678	2.038	-0.049	0.379	2.930
RDM Nitrogen %	n/s						
INOC	1.191	1.322	2.078	1.360	1.560	2.507	2.382
NON	1.339	1.475	1.589	1.475	1.656	2.193	2.404
Difference	-0.148	-0.153	0.489	-0.116	-0.096	0.314	-0.022
RDM C/N Ratio	n/s						
INOC	31.839	25.611	16.846	19.224	17.898	14.019	14.047
NON	27.854	22.803	18.133	18.065	17.514	14.886	13.412
Difference	3.985	2.808	-1.287	1.159	0.384	-0.867	0.635
BSS2							
SDM Nitrogen %	n/s						
INOC	-	0.981	1.099	1.282	1.416	1.455	1.670
NON	-	0.989	1.051	1.303	1.283	1.722	1.619
Difference	-	-0.008	0.048	-0.021	0.133	-0.267	0.051

Table 2. Cont.

SDM C/N Ratio	Fertilizer Treatment Provided Nitrogen Concentration (ppm)						
	n/s						
INOC	-	40.344	36.258	31.033	28.425	27.444	23.917
NON	-	39.789	37.960	30.498	30.977	23.460	24.860
Difference	-	0.555	-1.701	0.535	-2.552	3.983	-0.943
RDM Nitrogen %	n/s						
INOC	-	1.135	1.092	1.537	1.690	1.800	1.748
NON	-	1.099	1.320	1.380	1.326	1.985	1.737
Difference	-	0.036	-0.228	0.158	0.364	-0.185	0.011
RDM C/N Ratio	n/s						
INOC	-	32.169	35.669	25.131	24.014	21.337	22.515
NON	-	36.039	29.351	28.021	28.078	21.438	22.466
Difference	-	-3.871	6.318	-2.891	-4.064	-0.101	0.049

** = significant at alpha < 0.01, n/s = not significant. Note: Bolded main effect level shows which treatment was significantly greater. '-' indicates that this fertilization level was not used in this experiment.

3.2. Bibb/Limestone (BIB) Trials

3.2.1. Biomass Accumulation

For the BIB cultivar, the plant biomass responded curvilinearly to nitrogen fertilization across both trials in accordance with typical luxury consumption trends (Table 3). Inoculation affected a significant increase in SDM (10.9%) and TDM in BIB1 (10.0%), and a significant increase in SDM (16.2%) and RDM (18.2%) in BIB2. TDM in BIB2, therefore, also significantly increased in response to inoculation (INOC; 906 ± 294 mg, $p \leq 0.0041$).

Table 3. Effects of inoculation and nitrogen fertilization on the Bib/Limestone biomass.

Trial and Measure	Fertilizer Treatment Provided Nitrogen Concentration (ppm)						
	37.5	60	82.5	105	127.5	150	172.5
BIB1							
Shoot Dry Matter (g) *	LSD: 0.5296 s.e.: 0.2387 df = 32 $p \leq 0.0338$						
INOC	-	4.280	6.048	5.535	5.378	5.825	5.377
NON	-	4.217	4.805	5.025	5.250	5.188	4.780
Difference	-	0.063	1.243	0.510	0.128	0.638	0.597
Root Dry Matter (g)	n/s						
INOC	-	1.833	1.958	2.083	1.905	1.908	1.557
NON	-	1.637	1.805	2.000	1.863	1.680	1.563
Difference	-	0.197	0.153	0.083	0.043	0.228	-0.007
Total Dry Matter (g) *	LSD: 0.6655 s.e.: 0.3015 df = 37 $p \leq 0.0336$						
INOC	-	6.113	8.005	7.618	7.283	7.733	6.933
NON	-	5.853	6.610	7.025	7.113	6.868	6.343
Difference	-	0.260	1.395	0.592	0.170	0.865	0.590
BIB2							
Shoot Dry Matter (g) *	LSD: 0.6526 s.e.: 0.2482 df = 34 $p \leq 0.0127$						
INOC	-	4.457	6.381	4.825	3.908	3.676	3.843
NON	-	3.937	4.588	3.672	3.526	3.594	3.949
Difference	-	0.519	1.793	1.153	0.382	0.082	-0.106
Root Dry Matter (g) **	LSD: 0.2534 s.e.: 0.07679 df = 34 $p \leq 0.0023$						
INOC	-	1.930	2.047	1.862	1.246	1.144	1.416
NON	-	1.580	1.671	1.467	1.111	1.138	1.099
Difference	-	0.351	0.376	0.394	0.135	0.006	0.317
Total Dry Matter (g)**	LSD: 0.9060 s.e.: 0.2942 df = 34 $p \leq 0.0041$						
INOC	-	6.387	8.428	6.687	5.154	4.820	5.258
NON	-	5.517	6.259	5.140	4.637	4.733	5.047
Difference	-	0.870	2.169	1.547	0.517	0.088	0.211

** = significant at alpha < 0.01, * < 0.05, n/s = not significant. Note: Bolded main effect level shows which treatment was significantly greater. '-' indicates that this fertilization (FERT) level was not used in this experiment.

3.2.2. Carbon Nitrogen Ratios and Nitrogen Accumulation

For the BIB cultivar, the N content responded curvilinearly to nitrogen fertilization in SDM and RDM across both trials in accordance with typical luxury consumption trends (Table 4). In BIB1, inoculation had no effect on the N density or C/N ratio. In BIB2, inoculation affected a 20.6% decrease in nitrogen density in SDM and a corresponding increase in the C/N ratio, but RDM was not affected.

Table 4. Effects of inoculation and nitrogen fertilization on the Bib/Limestone N density and C/N ratios.

Trial and Measure	Fertilizer Treatment Provided Nitrogen Concentration (ppm)						
	37.5	60	82.5	105	127.5	150	172.5
BIB1							
SDM Nitrogen % **	n/s						
INOC	-	1.078	1.167	1.525	1.813	1.853	2.275
NON	-	1.095	1.284	1.612	1.729	2.059	2.122
Difference	-	-0.017	-0.118	-0.087	0.085	-0.207	0.153
SDM C/N Ratio **	n/s						
INOC	-	36.990	34.444	26.534	22.670	21.712	17.938
NON	-	36.442	31.337	25.226	23.529	19.697	19.051
Difference	-	0.548	3.107	1.308	-0.860	2.015	-1.114
RDM Nitrogen %	n/s						
INOC	-	0.929	1.016	1.396	1.661	1.712	2.293
NON	-	0.961	1.119	1.265	1.583	1.693	2.090
Difference	-	-0.031	-0.103	0.131	0.079	0.018	0.204
RDM C/N Ratio	n/s						
INOC	-	38.137	37.768	29.106	23.449	22.393	17.555
NON	-	36.593	30.946	29.356	23.147	17.578	17.169
Difference	-	1.544	6.822	-0.250	0.303	4.815	0.385
BIB2							
SDM Nitrogen % *	LSD: -0.2767 s.e.: 0.1311 df = 29 p ≤ 0.0436						
INOC	-	0.821	0.525	1.347	1.944	2.578	2.711
NON	-	1.166	1.018	1.794	2.266	2.517	2.598
Difference	-	-0.345	-0.493	-0.447	-0.321	0.061	0.113
SDM C/N Ratio *	LSD: 4.3860 s.e.: 1.8028 df = 29 p ≤ 0.0214						
INOC	-	46.711	54.038	34.718	21.985	18.160	15.702
NON	-	45.598	42.960	24.143	20.420	16.329	15.546
Difference	-	1.113	11.078	10.575	1.565	1.830	0.155
RDM Nitrogen %	n/s						
INOC	-	0.976	1.086	1.711	3.172	3.247	2.854
NON	-	1.602	1.470	1.926	2.681	2.901	2.901
Difference	-	-0.627	-0.384	-0.215	0.491	0.346	-0.047
RDM C/N Ratio	n/s						
INOC	-	37.729	30.613	25.942	14.957	16.632	16.155
NON	-	29.565	28.208	22.692	18.673	15.365	16.032
Difference	-	8.164	2.405	3.250	-3.717	1.267	0.123

** = significant at alpha < 0.01, * ≤ 0.05, n/s = not significant. Note: Bolded main effect level shows which treatment was significantly greater. '-' indicates that this fertilization (FERT) level was not used in this experiment.

4. Discussion

4.1. Black Seeded Simpson (BSS) Trials

For BSS1, the observed increase in shoot biomass was greatest at the slightly deficient N rate of 105 mg L⁻¹ N. At this N rate, INOC outperformed NON by 456 mg dry matter, an increase of 14.8% (Figure 2a). This corresponds to the best conservative fertilization rate, because the harvested aerial biomass was greater at this combination of *G. diaz* and fertilization than that for all NON plants fertilized at an equivalent or higher rate. By inoculating with *G. diaz*, a grower could decrease the amount of N fertilizer use by 42.8% (150 mgL⁻¹ reduced to 105 mgL⁻¹) for the same biomass yield, which would represent a considerable savings on the cost of fertilizer input.

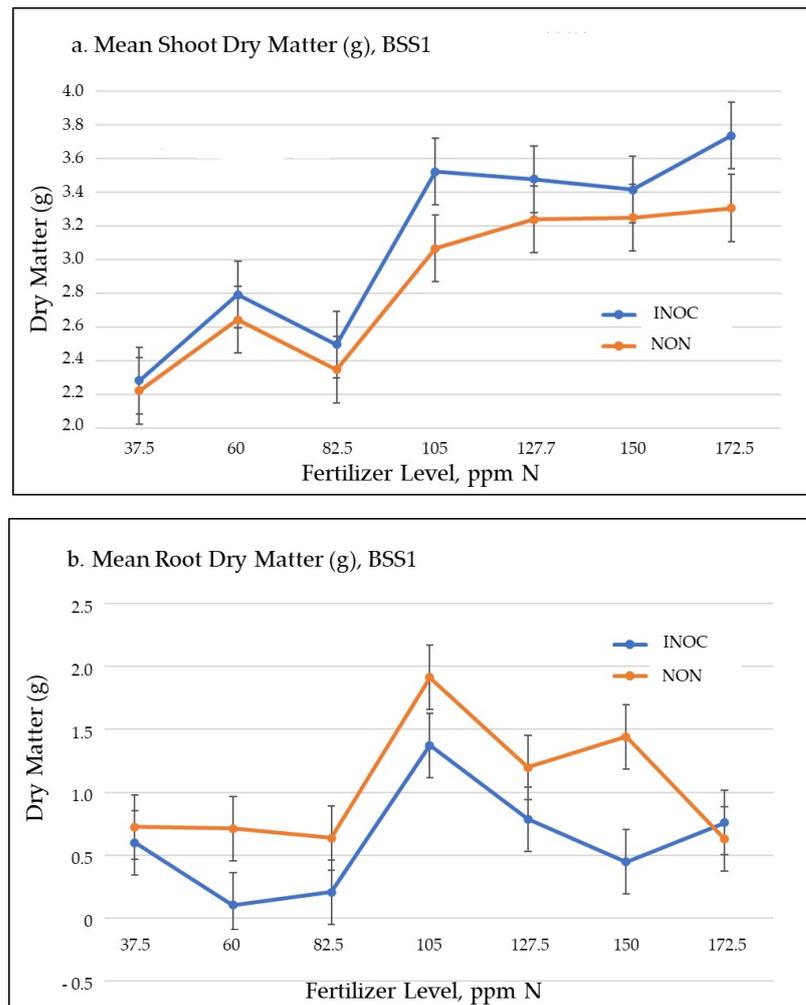


Figure 2. The mean shoot (a) and root (b) dry matter, Black Seeded Simpson1.

At the same time, the root biomass decreased by 425 mg dry weight, or 41.0% (Figure 2b), with no significant difference in total biomass accumulation in the whole plant (TDM). Inoculated plants shifted biomass synthesis away from root tissues and into shoot tissues. Since root material is of no value in hydroponic lettuce production, there is no detriment due to reduced root biomass production under hydroponic growth conditions. A previously observed response of lettuce to high levels of IAA in aerial tissues [41] possibly indicates *G. diaz* contributions to the plant available IAA. Exogenous gibberellins at moderate levels can also produce this phenotype response [42], which would be another potential explanation for the differences but this would require quantitative testing.

The total amount of N contained in the INOC shoot tissue was not significantly different from NON plants. Therefore, the plants were producing more shoot biomass with an equivalent amount of N incorporation as in the control. This indicates increased nitrogen use efficiency (NUE) [43,44]. The result also results in significantly lower N density in INOC SDM tissues compared with NON (1.97% INOC vs. 2.21% NON) and a higher C/N ratio in inoculated SDM (21.04 INOC vs. 18.82 NON). Both hormone families produced by *G. diaz* have been implicated in increased NUE in other plant-microbe synergisms [45]. Increased NUE was most significant at the lower levels of N fertilization (Figure 3). At these lower levels, N would be the limiting growth factor. The greater biomass, greater carbon sequestration, and static N presence indicates considerable added value to producers.

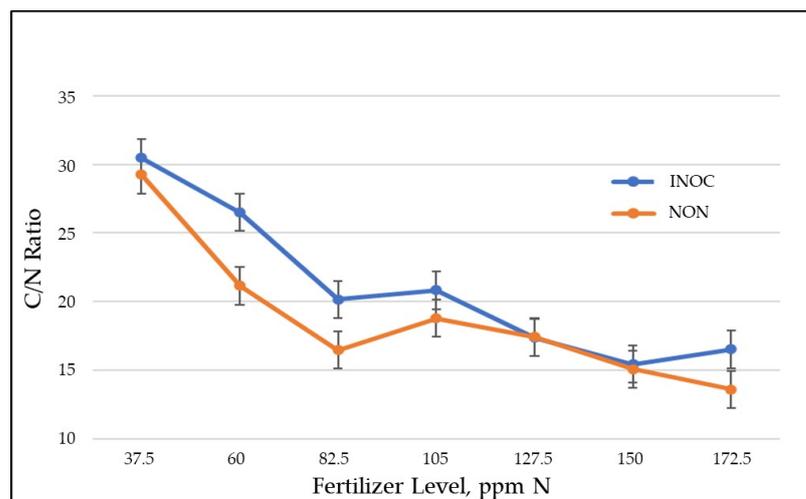


Figure 3. The C/N ratio in shoot dry matter, Black Seeded Simpson 1.

The reason for a lack of response in the second trial is not clear. Conditions were consistent between trials, and seed treatments contained the same bacterial CFU concentration. Seeds for the second trial were held over from the first trial but maintained in the dark at 4 °C and 70% relative humidity for the five months between BSS1 and BSS2. Storage conditions exclude the possibility of heat-induced thermal dormancy. The seeds were commercially sourced, and thus the age could not be determined prior to receipt. Therefore, the age of the seeds may still have been a factor affecting the observed plant response.

4.2. Bibb/Limestone (BIB) Trials

For Bibb lettuce, both trials exhibited similar responses to inoculation, with greater SDM and TDM in both trials and an increase in RDM in trial 2. As has been exhibited in other multi-plant cultivar *G. diaz* experiments [18,46,47], the response to inoculation was not consistent between even closely related varieties, as is seen here in comparing BSS to BIB. Both cultivars exhibit an improvement in aerial tissue production under inoculation, but root response was reversed (i.e., lower RDM in BSS1 and higher RDM in BIB2).

For the BIB trials, inoculated BIB1 saw an average SDM increase of 10.86% (5.407g INOC vs. 4.877 g NON), and for BIB2 this was +16.16% (4.690 g INOC vs. 4.038 g NON) compared to +8.19% for BSS1 (3.105 g INOC vs. 2.870 g NON). Even with the significant reduction in root mass in BSS1, this was not able to match the percent increases in either BIB trial. BIB2 outperformed both its replicate and BSS1 in that it also significantly increased in root biomass by 18.73% (1.606 g INOC vs. 1.353 g NON) over the controls. Whereas the total biomass for BIB1 and BIB2 was also significantly improved by inoculation, Bibb/Limestone lettuce biomass responded to inoculation with a greater total biomass and did not employ any root/shoot tradeoffs as seen in BSS.

This may be important under non-hydroponic growing conditions, such as smallholder or low-input systems, where inconsistent irrigation would favor the survival of plants with more prolific root systems. In BIB1, the nitrogen density and C/N ratios were not affected by inoculation. BIB2 did exhibit a lower N density in INOC shoot tissues with a corresponding increase in the C/N ratio. As in BSS1, this indicates increased NUE in aerial tissues.

4.3. Cross Experiment Observations and Implications

Although determination of the causes and effects of the plant growth response to inoculation is beyond the scope of this study, certain observations suggest possible mechanisms. At high N levels, lettuce often exhibits luxury consumption with an accumulation of nitrate that is categorized as an anti-nutrient [48]. Previous studies on *Poa pratensis*, which shares

a similar luxury consumption trait, demonstrated that, concurrent with inoculation, high levels of N fertilization can still increase crop physiological responses [49].

Conversely, under nitrogen deprivation conditions where lettuce yields are significantly reduced, bacterial nitrogen fixation may alleviate some of the nutrient deficiency impairing growth, as has been demonstrated in aquaponic lettuce inoculated with N-fixing *Rahnella* [50]. Endophyte-sourced plant growth-regulating hormones can increase the production of both above ground and below ground biomass as has been shown in carrot leaf and taproot [23] as well as beetroot [24]. Specific to lettuce, IAA imbalances have been implicated in altering root and shoot formation, with increased concentrations in aerial tissues reducing root formation [42].

Gibberellins at low concentrations have been shown to increase lettuce leaf production; however, at high concentrations, they impede growth [42]. In this study, the shift from RDM growth to SDM growth in BSS with no overall increase in TDM suggests a plant growth-regulating hormone effect. However, greater SDM and TDM in both BIB trials and an increase in RDM in BIB trial 2 suggests a possible N-fixation effect with or without a possible plant growth-regulating hormone effect in combination.

5. Conclusions

For growers looking to maximize lettuce yields on a static agricultural footprint, *G. diaz* inoculation may present a beneficial additive to the growing system. The ability to thrive under oxygenated conditions and avoid suppression in a high nitrogen presence make it especially suitable for hydroponic and/or aeroponic setups. For the two cultivars tested in this study, the harvestable biomass was increased in all but one of the trials, with increases from +8.19% in Black Seeded Simpson to +16.16% in Bibb/Limestone. For BIB lettuce, the highest biomass yield was realized with inoculation and an N fertilization level well below the established requirements, potentially allowing growers to reduce their N fertilizer requirements and still maintain production.

There is seemingly a significant bacterium × cultivar interaction when it comes to root production, allowing growers to tailor their use of *G. diaz* or their selection of lettuce variety to match their cultivation needs. Luxury consumption trends were not seen in this study, partly because of the non-replenishment fertilizer regime employed in Kratky jars. As this is the first reported case of *G. diaz* inoculation of lettuce, further research is needed to elucidate the exact mechanisms behind the plant responses. Whether from PGRH production, N fixation, NUE, or alteration in the shuttling of free N *in planta*, *G. diaz* has the potential to increase the yield of this valuable crop.

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