Kula Bio Efficacy Data

INTRODUCTION & VERIFICATION

Kula Bio has two microbial biofertilizer products in their portfolio, Kula-N and Kula-Next. *Xanthobacter autotrophicus*, the active ingredient in both products, fixes atmospheric nitrogen, resulting in increased soil fertility. Internal and external vegetable research trials were conducted to support these claims. The first two studies support the claim "nitrogen-fixing bacteria" and the last two studies support the claim "increase soil fertility".

The biological mode of action of these products is crop agnostic since it delivers nitrogen at the root zone. Therefore, we expect efficacy across multiple crop types, soil types, and geographies. In addition, our recommendation application rate of 2-8 oz per acre per pound nitrogen desired further accommodates for variation in crop and soil type.

SUPPORTING DATA

Study 1: *In vitro* Nitrogen-Fixation – Internal $^{15}N_2$ Incorporation Trial

I. Introduction: In this report, we investigate the nitrogen (N) fixing activity of *Xanthobacter autotrophicus* under laboratory culture conditions. *X. autotrophicus* has been characterized as a non-symbiotic diazotrophic (or nitrogen-fixing) bacteria (Wiegel 2015), but few studies have directly assessed rates of N-fixation by this bacteria species. Here, we first confirm the N-fixing capability of *X. autotrophicus in vitro* using cells grown in liquid, pure culture.

II. Materials & Methods: *Xanthobacter autotrophicus* is the key bacterial species present in Kula Bio's products. In this study, *Xanthobacter autotrophicus* 7c (NCMA B104) was used for all experiments described below.

$^{15}N_2$ incorporation is considered the gold standard method for verifying and quantifying nitrogen fixation activity. We used this method to assess rates of N-fixation in culture incubations of *X. autotrophicus* following a procedure previously described by Smercina *et al* (2019) with adaptations for liquid culture incubations. Briefly, 20 mL glass vials equipped with PTFE/silicon septa (Supelco, St. Louis, MO) were filled with 9.5 mL of *X. autotrophicus* culture plus 0.5 mL of sterile water. Six replicate vials each of *X. autotrophicus* and *E. coli* were prepared. *E. coli* is a non-nitrogen fixing bacteria and was used as a negative control in this study. From the six replicate vials per microbe, three replicates were used for natural abundance (e.g. unenriched) and three replicates were incubated with ^{15}N-enriched gas. For enriched incubation, 6 mL of headspace were removed using a syringe and replaced with 98 atom% $^{15}N_2$ (Sigma-Aldrich, St. Louis, MO), generating an incubation atmosphere of 49 atom% ^{15}N. All vials were incubated for 3 days at 30°C on an orbital shaker at 100 rpm. At harvest, vial volumes were transferred to 15 mL tubes. Tubes were centrifuged at 2500 *g* for 10 minutes to pellet cells, supernatant was removed and discarded, and pellets were dried at 60°C for 48 hours until completely dry. Samples were prepared and analyzed via Isotope Ratio Mass Spectrometry (IRMS) following standard procedures at University of Arkansas Stable Isotope Laboratory (Fayetteville, AR). IRMS results produce delta ^{15}N signatures (in per mil, ‰) which are then used to calculate N-fixation rates as follows: Total N fixed = $AE_i / AE_{atm} \times TN_i$ where AE_{sample} = ^{15}N signature measured by IRMS, $AE_i = AE_{sample}$ – Average(Natural Abundance AE_{sample}), TN_i = Total cell dry weight x (%N / 100) and AE_{atm} = atom% of incubation vessel atmosphere after $^{15}N_2$ addition.

III. Results and Discussion:
The purpose of this experiment was to observe the ability of *X. autotrophicus* to fix nitrogen *in vitro*. *X. autotrophicus* cells under natural abundance conditions (no $^{15}N_2$) had an average delta ^{15}N signature of -1.7 ± 0.4, while *X. autotrophicus* cells exposed to $^{15}N_2$ had an average delta ^{15}N signature of 552.9 ± 108.5 indicating significant incorporation of ^{15}N into bacterial cells via N-fixation. In comparison, the non-N-fixing bacteria *E. coli* showed an average delta ^{15}N signature of 4.0 ± 0.4 when under natural abundance conditions and an average delta ^{15}N signature of 4.1 ± 0.5 when exposed to $^{15}N_2$ demonstrating no significant incorporation of ^{15}N into *E. coli* cells. Using ^{15}N signatures to calculate rates of N-fixation (as described above), we determined that *X. autotrophicus* fixed an average of 21.2 ± 3.5 µg N g^{-1} cell dry weight day^{-1} into cellular biomass (FIG. 1). Using the same approach, N-fixation rates by *E. coli* would be on average 0.1 ± 0.1 µg N g^{-1} cell dry weight day^{-1}, essentially zero. Using a t-test, we confirm that N-fixation rates by *X. autotrophicus* are significantly greater than the negative control *E. coli* ($p = 0.0004$, $t = 10.66$, $df = 4$). These findings confirm *X. autotrophicus* to is able to fix meaningful amounts of nitrogen from the atmosphere.

IV. Conclusions: *In vitro*, *X. autotrophicus* demonstrated its ability to fix nitrogen, incorporating 21.2 (± 3.5) µg N g^{-1} cell dry weight day^{-1} into cell biomass.

V. Figures & Tables:
Table 1. Results of *In vitro* ^{15}N Experiment

Treatment	Replicate	Enrichment	Delta δ15N (‰)	µg N fixed per g dry cell weight per day
E. coli	1	Unenriched	4.2	----
E. coli	2	Unenriched	3.6	----
E. coli	3	Unenriched	4.2	----
E. coli	1	Enriched	3.64	0.00
E. coli	2	Enriched	4.05	0.02
E. coli	3	Enriched	4.57	0.15
Kula product	1	Unenriched	-1.3	----
Kula product	2	Unenriched	-2.0	----
Kula product	3	Unenriched	-1.9	----
Kula product	1	Enriched	650.8	18.9
Kula product	2	Enriched	571.7	19.7
Kula product	3	Enriched	436.3	25.2

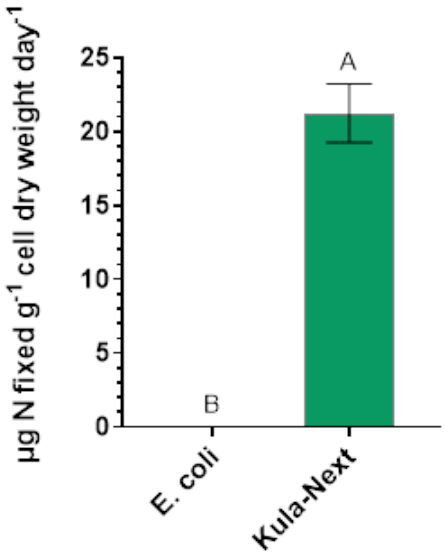

Figure 1: Quantification of N-fixation by *X. autotrophicus* in liquid culture compared to a non-N-fixing *E. coli* in liquid culture. Values are reported in µg N fixed g^{-1} dry cell weight day^{-1} ± standard error (n = 3). Letters indicate significant differences between treatment groups at $p < 0.05$

Study 2: *In planta* Nitrogen-Fixation – Internal $^{15}N_2$ Incorporation Trial

I. Introduction: In the previous experiment, we confirmed the diazotrophic capability of *X. autotrophicus* in liquid culture conditions. However, a key aspect of a successful nitrogen (N) fixing biofertilizer is the ability to transfer fixed nitrogen to nearby plants. In this experiment, we investigated the potential of *X. autotrophicus* as a biofertilizer to provide biologically fixed N to plants. Previous work identified this microorganism's unique potential to combine carbon dioxide (CO_2) reduction and N_2 reduction using hydrogen (H_2) oxidation to fuel these activities and in the process generating renewable electricity and active microbial cells (C. Liu et al., 2017). C. Liu et al. (2017) found that the resulting active microbial cells could be applied to cropping systems as a N-fixing biofertilizer and were able to significantly increase crop biomass, in some cases by over 1400%. Here we build on this work, empirically demonstrating *X. autotrophicus* can effectively deliver fixed N to plants.

II. Materials & Methods: *Xanthobacter autotrophicus* is the key bacterial species present in Kula Bio's products. In this study, *Xanthobacter autotrophicus* 7c (NCMA B104) was used for all experiments described below.

Romaine lettuce seedlings (*Lactuca sativa var. salivus*, Johnny's Selected Seeds, Winslow, ME) were sown into germination trays filled with Sunshine® Mix #1 (Sungro Horticulture, Agawam, MA). Trays were placed in a growth chamber with 16-hour day/8-hour night light cycles, temperatures at 25°C during the day and 22°C at night, and humidity maintained at 50%. Seedlings were grown for 21 days in trays before transplanting them into glass jars. Glass jars (32 oz, Ball Corp., Broomfield, CO, USA) were filled with a single layer of glass marbles topped with 100 g of moist coconut coir (CANNA Coco Brick, CANNA, Arcadia, CA). After transplant, lettuce plants were returned to the growth chamber for an additional 21 days, including a 7-day $^{15}N_2$ incorporation incubation. During the first 14 days, jars remained unsealed and open to the air. Plants received weekly applications of nitrogen-free Hoagland's solution for micronutrients, nitrogen fertilizer as calcium nitrate (YaraLiva, Tampa, FL) equivalent to 75% of grower

standard practice (GSP), and either Kula product containing *X. autotrophicus* (treated plants, n = 6) or sterile water (untreated plants, n = 6). 75% GSP for the growth period of this experiment was equivalent to 36 lbs. N ac^{-1} and created conditions through which plants could rely on nitrogen fixation by *X. autotrophicus* as a supplemental nitrogen source. Kula product was applied weekly to plants, for a total of 3 applications, at a rate of 3.33e9 CFU per application for a total of 1e10 CFU per plant. Applied Kula product is from bioreactor cultures after harvest. Following the last application of Kula product in week 3, jars were sealed with standard lids equipped with butyl rubber septa. Immediately after sealing, 50 mL of headspace was removed via syringe and replaced with 98 atom% $^{15}N_2$, creating an atmosphere enriched to 14.4 atom% ^{15}N. In addition to enriched jars that received $^{15}N_2$, an additional set of 75% GSP untreated jars were incubated under natural abundance (no $^{15}N_2$) conditions. Sealed jars were incubated in the growth chamber for 7 days during which CO_2 levels were monitored daily using a Licor LI-7000 CO_2/H_2O analyzer and replenished via syringe using UHP CO_2 as needed. After 7 days, the aboveground leaf material was harvested. Samples were dried at 60°C for 48 hours until completely dry. Samples were prepared and analyzed via Isotope Ratio Mass Spectrometry (IRMS) following standard procedures at the Marine Biological Laboratory Stable Isotope Laboratory (Woods Hole, MA).

III. Results and Discussion:
The purpose of this experiment was to confirm and quantify transfer of fixed N from Kula product to target crops. A one-way ANOVA with Tukey's post-hoc confirmed there was a significant increase in the delta ^{15}N signature ($\delta^{15}N$) of leaf tissue when Kula product was present compared to when it was not (FIG. 1A, $p < 0.0001$, $F = 82.60$), indicating that nitrogen fixation directly contributed to the uptake of nitrogen enriched with ^{15}N in the lettuce plants. The δ15N values and leaf tissue nitrogen content were used to calculate the nitrogen derived from atmosphere (Ndfa) in the leaf tissue (Warembourg et al. 1993, Weaver, et al 1994). Ndfa is calculated as $AE_i / AE_{atm} \times TN_i$ where AE_i is the atom percent excess of the enriched sample, AE_{atm} is the atom percent enrichment of the incubation atmosphere, and TN_i is the total nitrogen in the enriched sample. On average, the Ndfa in the experimental system when Kula Bio products were present was 243.0 ± 156.1 µg N over 7 days, while when Kula Bio products were not present the Ndfa was only 8.5 ± 5.0 µg N (FIG. 1B). A t-test confirmed the Nfda results from Kula Bio product application was significantly greater than without application ($p = 0.0043$, $t = 3.677$, $df = 10$). Assuming the rate of N incorporation into plants was consistent over the entire growth period, the uptake of nitrogen fixed by *X. autotrophicus* met ~12% of the plant nitrogen deficit. This is the first demonstration of direct transfer of recently fixed N from *X. autotrophicus* to a nearby plant and is a critical validation of this microbe's potential as a biofertilizer.

IV. Conclusions: We confirm *in planta* transfer of fixed N from Kula product to nearby plants. In this trial, Kula Bio products contributed ~12% of the plant's nitrogen deficit – marking a significant step toward validating its role as a biofertilizer.

V. Figures & Tables:
Table 1. Results of *In planta* 15N Experiment

Treatment	Replicate	Total Leaf N (mg)	Delta δ15N (‰)	Ndfa (µg N)
75% GSP	1	46.6	43.61	12.3
75% GSP	2	48.2	46.37	16.1
75% GSP	3	37.9	37.15	3.8
75% GSP	4	36.6	39.24	5.6
75% GSP	5	52.7	36.22	4.0

75% GSP	6	47.4	40.95	9.3
75% GSP + Kula product	1	38.8	267.36	230.2
75% GSP + Kula product	2	48.9	174.95	175.4
75% GSP + Kula product	3	34.1	158.06	107.7
75% GSP + Kula product	4	89.6	275.71	550.1
75% GSP + Kula product	5	41.1	209.75	183.9
75% GSP + Kula product	6	49.1	202.78	210.8
Natural Abundance	1	47.6	32.45	----
Natural Abundance	2	49.3	33.80	----
Natural Abundance	3	48.8	31.99	----
Natural Abundance	4	47.6	32.29	----
Natural Abundance	5	46.5	34.18	----
Natural Abundance	6	42.7	34.45	----

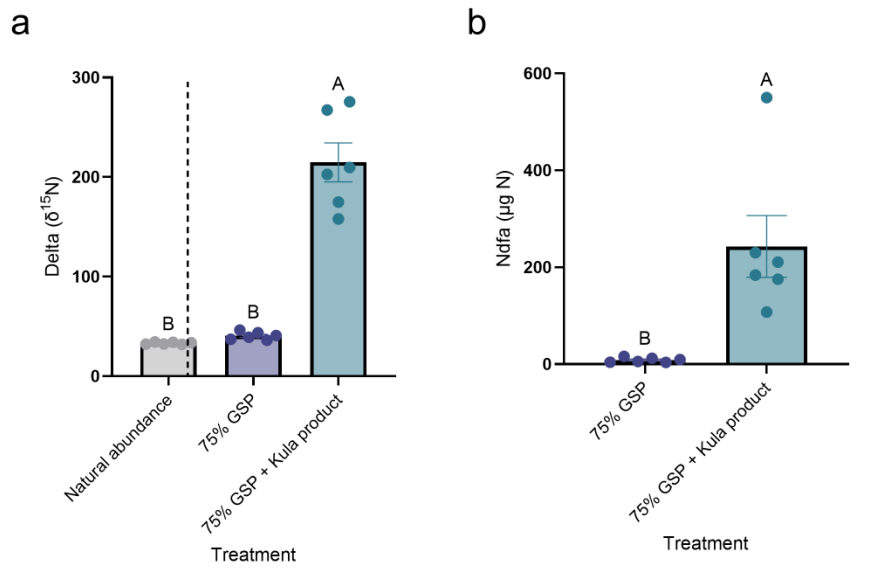

Figure 1: Quantification of transfer of fixed nitrogen from Kula product to lettuce. (a) Delta ^{15}N values for leaf tissue from *in planta* ^{15}N incubation experiment using lettuce grown under 75% grower standard practice (GSP) N. Values are reported in per mil ± standard error (n = 6). (b) Nitrogen derived from the atmosphere (Ndfa) during a 7-day incubation measured in leaf tissue of lettuce. Values are reported in µg N ± standard error (n = 6). Letters indicate significant differences between treatment groups at $p < 0.05$.

Study 3: Increased Soil Fertility – Internal Lettuce Trial

I. Introduction: In this report, we investigate the ability of Kula Bio's products to enhance soil fertility while under nitrogen-deficient conditions. Previous work that the resulting active *X. autotrophicus* cells could be applied to cropping systems as a N-fixing biofertilizer and were able to significantly increase crop biomass, in some cases by over 1400% (C. Liu et al. 2017). Here we build on this work, investigating yield responses of lettuce under nitrogen deficiency to the application of Kula product.

II. Materials & Methods: To investigate the impact of Kula product on lettuce yield under nitrogen deficiency, a replicated pot trial was conducted. Romaine lettuce seedlings (*Lactuca sativa var. salivus*, Johnny's Selected Seeds, Winslow, ME) were sown into germination trays filled with Sunshine® Mix #1 (Sungro Horticulture, Agawam, MA). Trays were placed in a growth chamber with 16-hour day/8-hour night light cycles, temperatures at 25°C during the day and 22°C at night, and humidity maintained at 50%. Seedlings (n = 12 per treatment) were grown for 21 days in trays before transplanting into 4" pots containing ~100g of moist coconut coir (CANNA Coco Brick, CANNA, Arcadia, CA). Twelve replicate plants were transplanted per treatment. After transplant, lettuce plants were grown for an additional 28 days before harvest. Plants received weekly applications of nitrogen-free Hoagland's solution for micronutrients, nitrogen fertilizer as urea ammonium nitrate (UAN-32, Simplot, Boise, ID) equivalent to 80% or 100% of grower standard practice (GSP), and either Kula product containing *X. autotrophicus* (treated plants) or sterile water (untreated plants) according to their treatment (Table 1). 100% GSP nitrogen for this growth period of this experiment was equivalent to 48 lbs. N ac^{-1}, while 80% GSP was equivalent to 38 lbs. N ac^{-1}. Kula product was treated at a rate of 8 oz ac^{-1} per lb. N equivalent. Total Kula product application was applied either as a weekly dose or every two weeks according to Table 1 below. After 28 days, plants were harvested for yield measured as aboveground fresh biomass and leaf nitrogen content used to determine nitrogen utilization efficiency (NUE_{crop}). NUE_{crop} is calculated as total N in leaf tissue divided by total N provided as fertilizer.

III. Results and Discussion: Using a one-way ANOVA with Tukey's post-hoc, we found there we significant differences between treatment groups in this trial ($p < 0.0001$, $F = 11.14$). First, we confirmed there was a significant difference in lettuce yield between 80% GSP and 100% GSP controls (FIG 1, $p < 0.0001$) demonstrating the experimental design successfully induced nitrogen limitation. Application of Kula product, whether weekly ($p = 0.002$) or bi-weekly ($p = 0.1122$), increased yield over the 80% GSP control (FIG 1). However, only weekly applications of Kula product were found to significantly improve yield, increasing aboveground fresh biomass by 17.5% over the 80% GSP while bi-weekly applications increased yield by only 8.7%. Weekly applications were also statistically similar to the 100% GSP ($p = 0.9809$) indicating weekly applications of Kula product were able to provide nitrogen equivalent to 20% of the total nitrogen program. This is similarly reflected in the tissue nitrogen data where lettuce receiving weekly Kula product applications had on average 25.4 mg more N compared to the 80% GSP control ($p = 0.0918$) and had similar levels of tissue N as the 100% GSP control ($p = 0.2844$). Ultimately, weekly applications of Kula product application resulted in highest NUE_{crop} across all treatments demonstrating that Kula product application increased soil fertility.

IV. Conclusions: These findings demonstrate the effectiveness of Kula product in improving soil fertility through observations of increased lettuce yield and NUE_{crop} under nitrogen-deficient conditions. Weekly applications of Kula product increased yield by 17.5% over the nitrogen-deficient control (80% GSP), matching the performance of the full nitrogen program (100% GSP) and providing nitrogen equivalent to 20% of the total program. Additionally, weekly applications enhanced tissue nitrogen content and achieved the highest nitrogen use efficiency (NUE_{crop}) among all treatments, validating yield increases resulted from enhanced soil fertility and confirming Kula Bio as an effective biofertilizer.

V. Figures and Tables:

Table 1. Description of treatments.

Treatment	Nitrogen	Kula-Next	Application Timing
80% GSP	80% (38 lbs. N)	-----	----

80% GSP + Kula-Next Weekly	80% (38 lbs. N)	8 oz/ac	Weekly
80% GSP + Kula-Next Bi-weekly	80% (38 lbs. N)	8 oz/ac	Every other week
100% GSP	100% (48 lbs. N)	----	-----

Table 2. Summary of Results

Treatment	Yield (g)	Total Leaf N (mg)	NUE_{crop}
80% GSP	124.1 ± 11.5	188.9 ± 24.6	0.76 ± 0.10
80% GSP + Kula-Next Weekly	145.9 ± 10.4	214.3 ± 22.3	0.87 ± 0.09
80% GSP + Kula-Next Bi-weekly	135.8 ± 13.2	207.8 ± 23.6	0.80 ± 0.12
100% GSP	147.7 ± 5.9	232.9 ± 25.8	0.75 ± 0.08

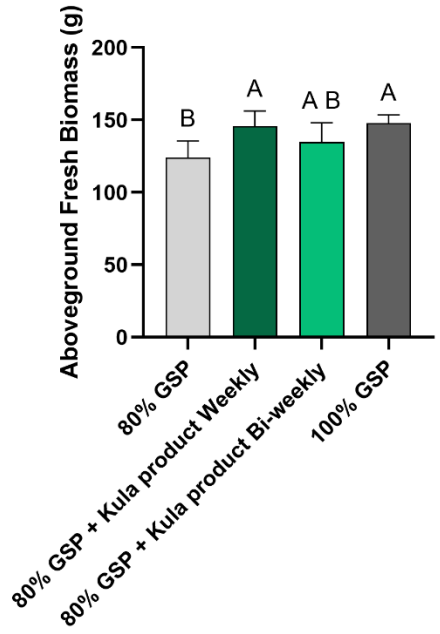

Figure 1: Lettuce yield measured as aboveground fresh biomass. Values are reported in g fresh biomass ± standard error (n = 12). Letters indicate significant differences between treatment groups at $p < 0.05$.

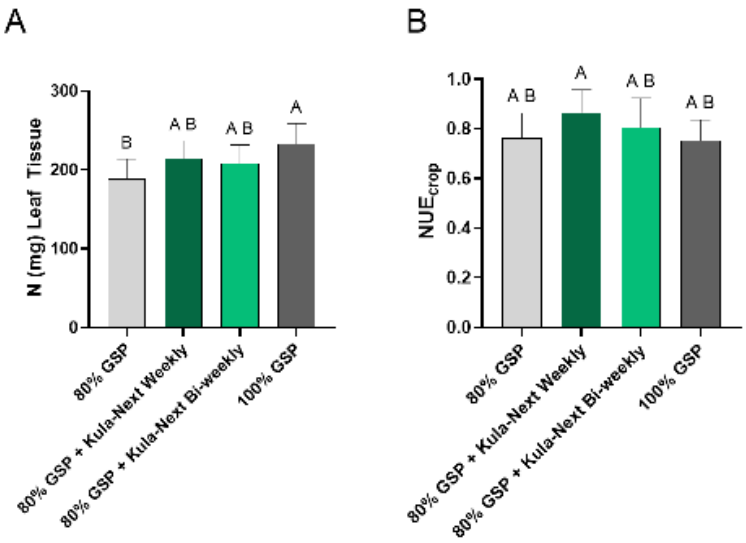

Figure 2: (A) Total leaf tissue nitrogen measured in lettuce aboveground biomass. Values are reported in mg nitrogen ± standard error (n = 12). (B) Nitrogen utilization efficiency (NUE_{crop}) calculated for lettuce aboveground biomass. Values are reported as ratios of total leaf N (mg) divided by total N applied as fertilizer (mg) ± standard error (n = 12). Letters indicate significant differences between treatment groups at $p < 0.05$.

Study 4: Increased Soil Fertility – Research Matters Celery Trial

I. Introduction:

A replicated small-plot celery trial was conducted by Research Matters, LLC at a research farm with sandy loam soil in Fillmore, CA from November 2023 until April 2024. This region experienced temperatures ranging from 47.7°F to 71.6°F during the trial period. This experiment aimed to prove the soil fertility benefits of Kula Bio's products in nitrogen (N) boost and reduction settings.

II. Materials & Methods:

This trial used a completely randomized block design with 6 replicates per treatment. Experimental blocks were 20-ft long x 32-in wide, with two lines of plants spaced 7-in apart. Fertility rates were determined using grower standard practice (GSP). When used as a boost, the application rate was calculated using 20% of the GSP N (53.2 lbs). When used as an N reduction, the application rate was calculated using the 40% N reduction (106.4 lbs). Treatment details are shown below (Table 1). Analysis of variance (ANOVA) and Student's t-test were conducted at a 0.05 significance level using R and JMP software.

III. Results & Discussion:

Plant height (vigor), yield, and stem diameter were analyzed in this experiment. The 100% GSP control and 60% GSP control showed statistical separation, demonstrating that decreasing nitrogen negatively impacts plant growth. The addition of Kula product significantly increased plant growth when applied on top of grower standard, as well as when applied in a reduced nitrogen environment, demonstrating that it increases soil fertility.

Application of Kula product as a boost (Treatment 3) significantly ($p=0.0226$) increased plant height by 7.9 cm (37%), significantly ($p=0.0014$) increased yield by 168 g (35.1%), and significantly ($p=0.0006$) increased stem diameter by 11 mm (16.4%). In this treatment, plants also demonstrated higher uniformity (Table 2).

Kula product used as a partial N replacement (Treatment 4) numerically (p = 0.0526) increased plant height by 6.3 cm (11.2%), significantly (p=0.0467) increased yield by 98 g (25.1%), and numerically (p=0.3233) increased stem diameter by 5 mm (8.3%). (Figures 1-3).

IV. Conclusion:

When used as a boost to the standard nitrogen program, Kula Bio's products show statistically significant increases in plant vigor, yield, and stem diameter. Increases in these metrics demonstrate that Kula Bio's products increase soil fertility. When used for partial nitrogen reduction, Kula Bio's products are equivalent or better than the 100% GSP control, suggesting nitrogen replacement potential. Statistical separation between 100% and 60% GSP controls confirmed nitrogen deficiency when the rate was reduced, supporting Kula Bio's product claims. These results demonstrate that Kula Bio's products are a viable option for farmers to increase soil fertility and can be used on top of the standard fertility program as a boost or as a partial replacement at nitrogen reductions up to 40%.

V. Figures & Tables:

Table 1. Treatment list with total amount of nitrogen and Kula product applied.

#	Treatment	Total Nitrogen (lbs/acre)	Total Kula Product (oz/acre)
1	100% GSP N Control	266	0
2	60% GSP N Control	160	0
3	100% GSP N + Kula @ 2 oz/lb N	266	106
4	60% GSP N + Kula @ 2 oz/lb N	160	212

Table 2. Plant height (cm) in the 100% treatments (1 and 3).

Statistical Parameter	100% GSP N Control	100% GSP + Kula
Mean	60.6	68.5
Low Value	50.0	60.5
High Value	68.5	74.0
Range	18.5	13.5

Table 3. Plant height (cm) in the 60% treatments (2 and 4).

Statistical Parameter	60% GSP N Control	60% GSP + Kula
Mean	56.3	62.6
Low Value	51.0	58.5
High Value	60.0	67.0
Range	9.0	8.5
Mean Comparison to 100%	-4.3	2

Table 4. Yield (g) in the 100% treatments (1 and 3).

Statistical Parameter	100% GSP N Control	100% GSP + Kula
Mean	479	647
Low Value	411	531
High Value	547	855
Range	136	324

Table 5. Yield (g) in the 60% treatments (2 and 4).

Statistical Parameter	60% GSP N Control	60% GSP + Kula
Mean	391	489

Low Value	320	397
High Value	494	565
Range	174	168
Mean Comparison to 100%	-88	10

Table 6. Stem diameter (mm) in the 100% treatments (1 and 3).

Statistical Parameter	**100% GSP N Control**	**100% GSP + Kula**
Mean	67	78
Low Value	64	70
High Value	69	87
Range	5	17

Table 7. Stem diameter (mm) in the 60% treatments (2 and 4).

Statistical Parameter	**60% GSP N Control**	**60% GSP + Kula**
Mean	60	65
Low Value	54	59
High Value	66	70
Range	12	11
Mean Comparison to 100%	-7	-2

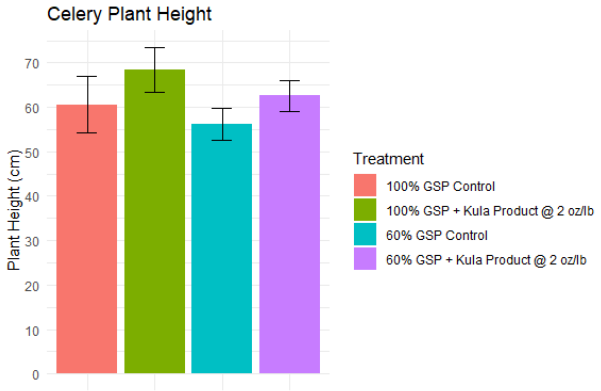

Figure 1. Plant height.

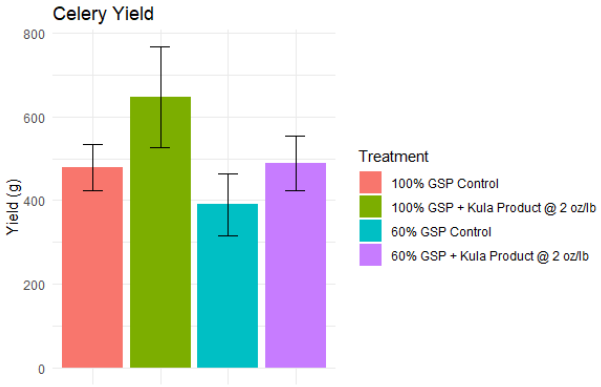

Figure 2. Celery yield

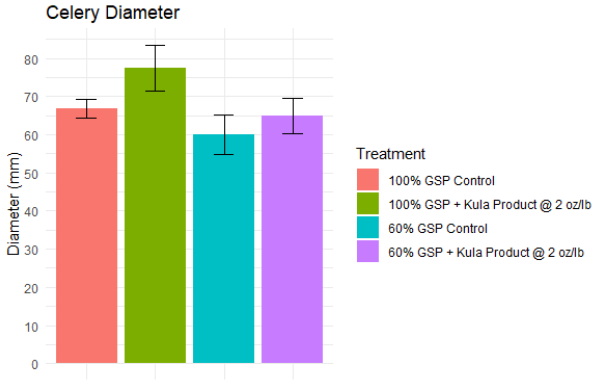

Figure 3. Stem diameter.

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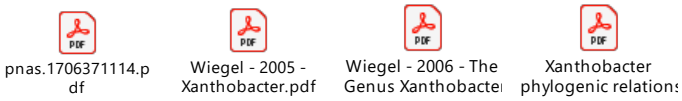

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