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Compilation of Original Research Efficacy Data Generated in Support of Abiotic Stress and Root Growth Claims for BioLiNE® Gold Fulvic Acids

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Product Introduction and Verification

1.1 Product Introduction

BioLiNE® Gold (BLG) is a purified fulvic acid that has been in the market in many U.S states and across Canada since 2018. Field trial work on the product began in 2015. The product has recently (2023) received CE certification under the PFC 6.B (Non-microbial Plant Biostimulants) based on the efficacy data dossier submitted.

1.2 Product Claims

The plant biostimulant product claims are:

- Improved Abiotic Stress Tolerance
- Improved Root Development

1.3 Target Crops and Crop Grouping

This report contains three field trial results providing high quality data, using scientifically validated protocols with strong statistical probability that treatment with BioLiNE® Gold improves a plant's ability to tolerate abiotic stress and improve root growth. The biostimulant claims associated with the use of BLG are not specific to any genus, family, order, or class of plants (taxonomic grouping); growing media; or country. The modes of action involved in the abiotic stress amelioration caused by application of BLG are universal to all classes and most phyla of plants.

A review of published literature provides strong evidence that the modes of action resulting in the abiotic stress tolerance, claimed for BLG are not limited to the crop, soil-type or particular country. These modes of actions are universal and occur in all angiosperm plants. The dossier provides data on the following physiological and biochemical responses to support the abiotic stress tolerance improvement claims:

1. Physiological Response:
    a. Improved root structure and development (root mass growth)
    b. Improved vegetative growth (plant weight)
    c. Improved number of pods or fruit set
    d. Improved yield
2. Biochemical Response:
    a. Increase in concentration of glutathione (GSH), an important antioxidant.
    b. Increased radical (ROS) scavenging activity as measured by quantifying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity.
    c. Reduced lipid peroxidation as estimated by Malondialdehyde (MDA) reduction.
    d. Increased concentration of catalase (CAT) an important antioxidant enzyme.
    e. Increased concentration of proline (osmoprotectant).
    f. Increase concentration of soluble proteins.

Table 1 below provides a summary of the trials included in this report. Details of protocols, data collection, analysis, and findings are included for each trial in the subsequent sections of this report.

Table 1: Summary of Original Research Data on BLG

Trial #	Crop	Abiotic Stress	Trial Type	GSH / ASA	CAT	MDA	DPPH	Sol. Prot.	Proline	Root wt.	Plant wt.	Yield
1	Canola	Drought	CEC	**GSH 42% p=0.08** **ASA 17% p=0.05**	No Data	No Data	No Data	No Data	No Data	**45% p=0.06**	**9% p=0.13**	No Data
2	Canola	Drought	CEC	**10% p=0.00**	**14% p=0.02**	**-50% p=0.00**	**4% p=0.00**	**13% p=0.00**	**42% p=0.00**	No Data	No Data	No Data
3	Corn	Drought	CEC	**30% p=0.00**	No Data	**-36% p=0.03**	No Data	No Data	No Data	**48% p=0.06**	**32% p=0.07**	No Data

1.4 *Statistical Analysis Principals Applied*

Statistical analysis used in this report are based on the recommendation made in the "US Biostimulant Industry Recommendations to Assess Efficacy, Composition, and Safety of Plant Biostimulant Products", published in the Journal of Regulatory Sciences, jointly by the Biological Products Industry Alliance (BPIA) and The Fertilizer Institute (TFI) Biostimulant Council (2022). According to the referenced document, "the main objective of the data analysis is to estimate the **magnitude of the difference** between the various treatments and provide a **measure of the variability** of those estimates. The decisions of acceptability or rejection of the treatment should not be based on p-value alone. P-values should be taken as a continuous measure of evidence against the null hypothesis, and p-values greater than 0.05 may be accepted depending on the study objectives."

In examining the significance of the efficacy data collected through field-trials, the principal criterion is that the product must produce **biological benefit to the crop or economic value to the grower.** The purpose of the statistical analysis is to establish that there was sufficient magnitude of difference resulting from treatment in relation to the variability within the dataset. This analysis is not arbitrary, and no single cut-off for p-value can be used across all efficacy trials. **One shoe will never fit all.** Researchers must take into consideration the experimental design, the type and number of datapoints collected in the determination of the acceptance or rejection of the null hypothesis. We hope that more researchers will adopt these principles and consult with statistician in the design of their experiments. This will increase the probability that they will employ the appropriate statistical analysis in their efficacy trials, rather than the use of an arbitrary cut-off p-value of 0.05 as the sole contributing factor to the acceptance or rejection of the null-hypothesis. Our preferred approach to statistical analysis of efficacy field-trials starts with the first step of obtaining the descriptive statistics for each of the measurements. Single-Factor Anova test were ran comparing treatments to control for key measurements in the trials including yield. The p-values for the results of the analysis are reported as calculated. We do not use any arbitrary p-value cut-off as the sole consideration for the acceptance and rejection of treatment effect.

Improving Drought Tolerance in Canola with BioLiNE® Gold Fulvic Acids

Report #1: Improving Drought Tolerance in Canola with BioLiNE® Gold Fulvic Acids

Introduction:

Fulvic acids reduce reactive oxygenated species (ROS) via radical scavenging and supporting the production of antioxidant enzymes and antioxidants like ascorbate and glutathione. The smallest size molecules, with the highest concentration of oxygenated functional groups (carboxylic acid, phenolic, hydroxyl) have been shown to have the greatest crop benefits. Canola is the second largest crop by acres grown in Canada with more than 8.8 million hectares (21.6 million acres) planted annually. Seasonal drought conditions are common to many regions where canola is grown in Canada. We began experimenting with growing Canola (*brassica napus*) in a controlled environment chamber (CEC) starting in July of 2021. We tried several approaches to inducing drought in the plants and characterizing a few biochemical markers. In this study we report on data from the first of a series of trials that investigate the impact reduced moisture has on Canola. This study also evaluated the efficacy of treatment with BioLiNE® Gold fulvic acids to improve abiotic stress tolerance of the crop.

Trial Conditions:

Growing Season: 2021

Growing media: Pro-Mix General Purpose Growing Medium in Tree Pods

Number of plants per treatment: 16 plants per treatment randomly located

Experiment Design: Completely randomized design (CRD)

Date of germination: October 21st, 2021

Date of Initiation of Drought Stress: November 16th, 2021

Soil Analysis:

Table 2: Soil Analysis of Growing Media Used in Trial

Sample	OM	P Bicarb. (ppm)	P Bray (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	pH	CEC (meq/100g)
Full	60.6	38.5 M	90 G	158 H	162 M	1305 M	42.5 H	5.9	10.3

Zn (ppm)	Mn (ppm)	B (ppm)	Saturation %P	Al (ppm)	Saturation %Al	K/Mg	ENR
2.9 M	6.5 L	0.7 M	9 M	34.5	0.05 G	0.3	112

% Base Saturation				
%K	%Mg	%Ca	%H	%Na
4	13.3	63.9	17.1	1.8

Application of the Product:

Fertility program:

All four treatments had the same soil nutrient profile. Aqua Vega Solutions A&B were used to create a nutrient enriched soil mix for all 64 germinated plants. The soil was made in single mix.

Trial Treatments: 16 plants per treatment for a total of 64.

Treatment #1: Well Watered Control

Treatment #2: Well Watered + BLG FA

Treatment #3: Drought Control

Treatment #4: Drought + BLG FA

BLG application rate:

Three foliar applications at 3,705 mL per hectare (1,500 ml/ac./application) equivalent

Treatment Dates:

Date of first BLG FA Application (Foliar): November 9th, 2021

Date of second BLG FA Application (Foliar): November 16th, 2021

Date of third BLG FA Application (Foliar): November 26th, 2021

Specimen Sample Collection Date:

December 7th, 2021

Lighting and Light Distribution:

The controlled environment chamber used four (2x) commercial grade, pro series ViparSepctra P2000, 200W Infrared Full Spectrum LED Growing Light with $0.84m^2$ coverage area each. The lighting maintains a uniform lux light intensity reading ranged between 734 to 826.

Light Readings (PAR)								
120				108				110
	154			134			131	
		177		160		160		
				131				
150	179	209	180	173	175	177	154	146
					142			
		174		143		162		
	142			129			138	
106				110				112

Figure 1: Report #1 – **Photosynthetically active radiation (PAR) distribution across the CEC**

Data Measurements Collected:

For this trial the following data was collected at harvest:

1. **Plant fresh weight (g)**

2. **Root fresh weight (g)**

3. **Chlorophyll** – Chlorophyll (Chl) content was measured using atLEAF CHL Plus chlorophyll meter (FT Green LLC, USA). We use the correlation recommended by the manufacturer of the reader to convert atLEAF reading values to chlorophyll content (µg/cm^2). The correlation was developed by Zhu, Tremblay, and Liang (2012), who studied the comparison of SPAD and atLEAF values with total chlorophyll content measured by lab assays in six crops including canola, wheat, barley, potato, and corn. The equation for the correlation is y=52.4x +28.1 (R^2=0.78), where Y is the atLEAF values and X is leaf chlorophyll content.

4. **Ascorbic Acid (ASA) –** Homogenize 200 mg of fresh plant tissue with 5 mL 5% (w/v) trichloroacetic acid (TCA). Centrifuge the homogenate at 3,900 g for 15 min at 4°C. Mix 0.1 mL of the supernatant with 0.3 mL of 200 mM NaH_2PO_4. To this mixture add 0.5 mL of 10% (v/v) TCA, 0.4 mL of 42% (v/v) H_3PO_4, 0.4 mL of 4% (w/v) bipyridyl (dissolved in 70% alcohol) and 0.2 mL of 3% $FeCl_3$ (w/v). Incubate the mixture at 42°C for 15 min. Immediately afterwards, measure the absorbance at 524 nm. The method described is adapted from Chen and Wang (2002) Guide to plant physiological experiments.

- **Glutathione (GSH)** - To determine glutathione (GSH) the method of Chen and Wang (2002) was followed. 0.5 g of fresh plant tissue was homogenized in 10 mL of 5% trichloroacetic acid (w/v). The homogenate was centrifuged at 3900 g for 15 minutes and the supernatant was collected. To the supernatant, 2.6 mL of 150 mM sodium phosphate buffer (pH 7.0) and 0.18 mL of 3 mM 5,5-dithio-bis (2-nitrobenzoic acid) (DNTB) in 100 mM phosphate buffer was added. Reaction mixture was kept for 5 minutes before being read on the UV-vis spectrophotometer and absorbance recorded at 412 nm. Reduced glutathione standard was purchased from Sigma Aldrich to generate a standard curve.

Trial Results:

Canola plants were germinated into a nutrient enriched potting mix. The moisture level was maintained at 70% to 79% from the germination date until we induced drought stress. For the drought control and the drought BLG plants we stopped adding water and allowed the soil moisture to drop to about 30%. Soil moisture for well watered plants was maintained between 70% to 76% by adding about 20ml of water per day per plant. In total, the drought plants received 519 milliliters of water per plant throughout the trial while the well watered plants received 855ml. During the moisture deficient phase of the trial, the drought (D) plants received 34ml of moisture including treatments and fertility. The well watered (WW) plants received 370ml.

Table 3 provides a summary of the results from this trial. The table compares the well-watered control canola results and the well-watered BLG treated. The table includes the percent difference resulting from the treatment and provides the corresponding p-values for each data point. Similarly, results of the drought control are compared with drought BLG treated.

Table 3: Report #1 - Summary of the results with the corresponding p-values for this trial

	WW Control	WW BLG	% Diff.	p-value	D Control	D BLG	Diff. (%)	p-value
Plant Weight (g)	10.02	10.76	7.4%	0.35	5.427	5.934	9.4%	0.13
Root Weight (g)	1.287	1.690	31.3%	0.03	0.757	1.094	44.6%	0.06
Chlorophyll (μg/cm^2)	0.419	0.393	-6.2%	0.53	0.458	0.499	9.0%	0.47
ASA (μM/g DW)	6.53	7.10	8.8%	0.22	7.1	8.3	16.5%	0.05
GSH (μM/g DW)	9.68	19.00	96.3%	0.07	28.5	40.4	42.1%	0.08

Average fresh weight (FW) per plant:

For the well-watered canola plants the average fresh weight per plant increased by 7.4% (p=0.35), however due to the variability in the data the standard deviation for the WW control was 2.31 g (23%) and for the BLG treated was 1.89 g (17.6%). The average plant weight substantially reduced because of the drought conditions from about 10 grams per plant for the WW control to 5.4 grams for the drought (D) control. Treatment with BLG fulvic acids resulted in a substantial increase in plant weight to 5.9 grams. The P value for this 9.4% increase was 0.13 showing good statistical probability of treatment effect. Even though the amplitude of the difference between drought control and drought BLG treated samples was not extremely high (<10%), due to the severe stunting of growth resulting from the drought, the standard deviations for the control was 0.78 and for BLG 0.98. The much smaller standard deviation across the 16 samples measured resulted in a much-improved statistical analysis for the drop response. Overall, it could be said that in this trial a decent plant response was observed resulting from treatment effect.

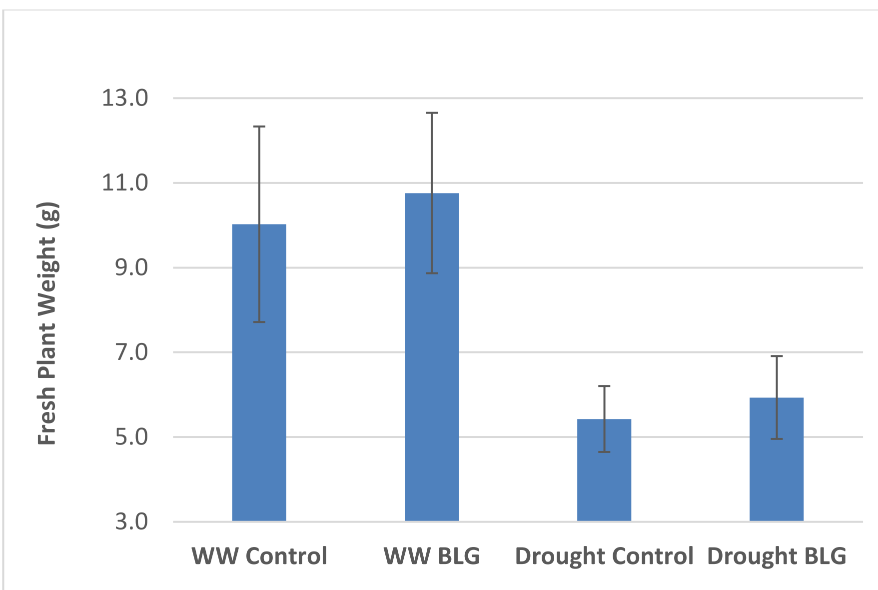


Figure 2: Report #1 - Plant weight measurement for all four treatments

Table 4: Report #1 - Descriptive statistics for plant weight measurements for all four treatments

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	10.02	10.76	5.43	5.93
Standard Error	0.60	0.49	0.20	0.25
Median	10.54	11.16	5.43	5.68
Standard Deviation	2.38	1.95	0.80	1.01
Sample Variance	5.68	3.82	0.65	1.02
Kurtosis	-1.09	0.07	0.21	0.05
Skewness	-0.43	-0.98	-0.07	0.62
Range	7.18	6.46	3.22	3.63
Minimum	6.27	6.93	3.82	4.52
Maximum	13.4	13.4	7.0	8.1
Sum	160	172	87	95
Count	16	16	16	16
Confidence Level (95.0%)	1.27	1.04	0.43	0.54

Average Root fresh weight (FW) per plant:

When it came to the measurement of the root weights the difference between treated and control was remarkable. Under WW conditions BLG treatment resulted in a 31% increase in root weight from 1.3 grams for the control plants to 1.7 grams for the BLG treated. The p-value for this comparison was 0.03 equating to a strong response. Similarly, under drought conditions the average root weight for the 16 samples increased by 44.6% from 0.76 grams to 1.1 grams. The p-value for this was 0.06.

Table 5: Descriptive Statistics for root measurements for all four treatments

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	1.29	1.69	0.76	1.09
Standard Error	0.10	0.15	0.07	0.15
Median	1.29	1.45	0.64	0.90
Standard Deviation	0.38	0.57	0.26	0.61
Sample Variance	0.15	0.33	0.07	0.37
Kurtosis	-0.28	1.38	-0.91	1.03
Skewness	0.47	1.47	0.62	1.32
Range	1.34	1.88	0.82	2.00
Minimum	0.69	1.18	0.38	0.51
Maximum	2.0	3.1	1.2	2.5
Sum	19	24	11	18
Count	15	14	14	16
Confidence Level(95.0%)	0.21	0.33	0.15	0.32

Ascorbic Acid (ASA):

Five samples per treatment were also analyzed for reduced ascorbate (ASA) concentration. For both ASA and GSH plant tissue was homogenized to generate five biological replicates per treatment. ASA and GSH are two well researched plant antioxidants. Studies have shown that plants increase production of these antioxidants in response to prolonged exposure to abiotic stress such as drought. The main function of these antioxidants is to reduce the concentration and accumulation of reactive oxygen species (ROS) and thereby reduce the potential for oxidative stress to kill plant cells. Fulvic acids have been shown in numerous studies to behave like antioxidants and scavenging for ROS as well as helping the plant to increase production of antioxidants such as ASA and GSH. In this study, we observed an 8.8% increase in ASA concentration accumulated in canola leaves of WW plants (p=0.22). **Figure 3** below provides the standard curve for the ASA standard used to quantify ASA in the canola specimen.

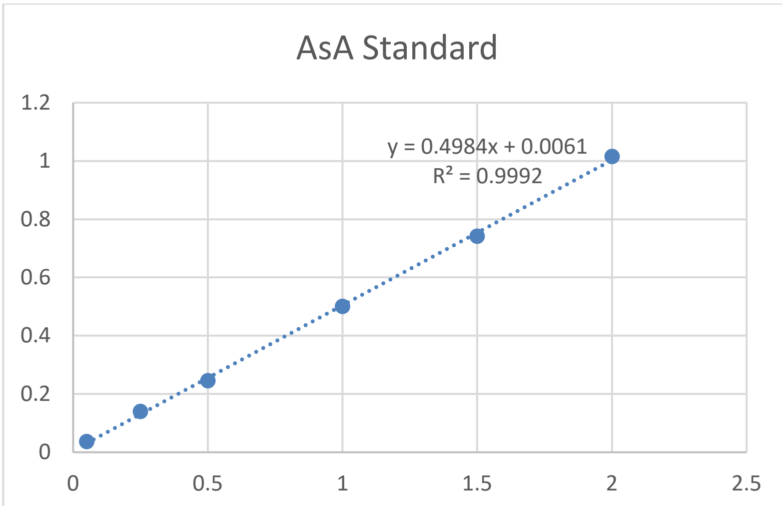


Figure 3: Report #1 - ASA standard calibration equation

For the drought stressed plants the treatment effect doubled, and the concentration of ASA increased by 16.5% for treated plants compared to control. The p-value for this comparison was 0.05 indicating a strong probability of treatment effect. **Table 6**, below provides the descriptive statistics for each of the five measurements of AsA.

Table 6: Descriptive Statistics for AsA measurements for all four treatments

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	6.53	7.10	7.10	8.27
Standard Error	0.18	0.39	0.25	0.44
Median	6.55	7.10	7.11	8.52
Standard Deviation	0.41	0.86	0.55	0.99
Sample Variance	0.17	0.75	0.30	0.98
Kurtosis	1.53	-1.61	1.97	-1.07
Skewness	0.31	-0.31	-0.92	0.31
Range	1.14	2.06	1.50	2.42
Minimum	5.99	5.95	6.24	7.21
Maximum	7.1	8.0	7.7	9.6
Sum	33	36	35	41
Count	5	5	5	5
Confidence Level(95.0%)	0.51	1.07	0.68	1.23

Glutathione (GSH):

Five samples per treatment were also analyzed for reduced glutathione (GSH) concentration. The GSH concentration measured increased by 96% in the WW canola plants from 9.7 µM/g DW to 19.0 µM/g DW (p=0.07). Comparing WW plants with drought plants for control treatment the GSH increased by more than 300% from 9.7 µM/g DW to 28.5 µM/g DW. The sharp increase in GSH was much higher than the ASA measurements. For the canola plants treated with BLG experiencing drought conditions the GSH further increased by 42% to 40.4 µM/g DW. The p-value for comparing the BLG treated canola plants with the control under drought conditions was 0.08 once again showing strong probability for treatment effect. This is the second canola study where we measured GSH.

Statistical Analysis:

We calculated standard deviations and standard deviation percentages along with averages for all the biochemical assays. For the plant weight, root weights we calculated the descriptive statistics and provided them in **table 4 and table 5**. We have also included the descriptive statistics for AsA in table 5. It should be noted that there were only five samples analyzed for ASA and GSH. For the plant and root weights we measured the quartiles and determined outliers. There were no outliers identified for the plant weights, for the root weights four out of the 64 weights measured were identified as outliers.

Single-Factor Anova test was run comparing each of the well-watered and drought FA treatments to control. The p-values for the results of the analysis are reported in the previous tables. We assigned an alpha-value of 0.15 to these efficacy trials and designed the experiment with the target of achieving a significance level lower than 0.15. In examining the significance of our trial data, the principal criterion is that the product must produce biological benefit to the crop in overcoming abiotic stress**.** When it comes to supporting root development, treatment with BLG improved root size under both well watered and drought conditions by 31.3% (p=0.03) and 44.6% (p=0.06), thus, clearly rejecting the null-hypothesis. Under drought conditions the 9.4% increase in plant weight (p=0.13) is also highly likely to be due to treatment effect and the data supports the rejection of the null-hypothesis. Similarly, under drought conditions both AsA and GSH concentrations are increased significantly with 16.5% (p=0.05) and 42.1% (p=0.08) respectively. Treatment with FA had no impact on the measured chlorophyll content due to the high p-values observed.

Conclusions:

In conclusion, the use of BioLiNE® Gold (BLG) fulvic acid treatment was effective in improving both plant and root weights for canola plants under drought conditions. Treatment with BioLiNE® Gold (BLG) fulvic acids increased plant weight by 9.4% (p=0.13) and root weight by 44.6% (p=0.06). BLG treatment also increased plant and root weights for canola under well-watered conditions, however only the increase in root weight provides strong evidence for treatment effect. The magnitude of the increase for well-watered plant weight was 7.4% (p=0.35) and 31.3% (p=0.03) for well-watered root weight. The chlorophyll content in the leaves of canola (*brassica napus)* plants did not show statistically significant response to BLG treatment. The chlorophyll content measured reduced by 6.2% (p=0.53) for the WW treated plants and increased by 9.0% (p=0.47) for the drought treated plants.

We analyzed two different antioxidants, reduced ascorbate (ASA) and reduced glutathione (GSH). The ASA concentrations increased by 8.8% (p=0.22) for the BLG treated plants under WW conditions and by 16.5% (p=0.05) for BLG treated plants under drought condition. The GSH concentrations also increased for canola plants treated with BLG under both well-watered and drought conditions. In this study we were able to show both physiological and biochemical treatment effect. The results from this trial show strong and statistically significant reduction in canola plant weight and root weight resulting from the reduction in moisture (drought conditions) induced in our controlled environment chamber. We also show statistically significant improvements in plant and root weight resulting from treatment with BLG under drought conditions. This is evidence of BLG overcoming drought stress and increasing the canola plants' tolerance to drought stress. We have also shown statistically significant increase in the production of plant antioxidants resulting from treatment with BLG as measured by the concentrations of ASA and GSH.

Report #2: Improving Drought Tolerance in Canola with BioLiNE® Gold Fulvic Acids

Introduction:

In the data reported in previous trials we observed the following physiological and biochemical responses to drought conditions:

1. Drought stress significantly reduced plant development including both root and plant weights. BLG treatment resulted in statistically significant improvement in root development.
2. Drought stress significantly increased the concentration of glutathione (GSH) and ascorbate (ASA) in canola plants. BLG treatment resulted in statistically significant increase in both antioxidants, key to reducing cellular damage resulting from ROS (reactive oxygenated species) accumulation caused by drought.

In this study, we look at investigating three timepoints for measurements of plant biochemicals and metabolites. Time point 1, when soil moisture content of 30% was achieved, time point 2, 7 days after time point 1, and time point 3, 14 days after time point 1. Soluble protein, GSH, CAT, proline, MDA, and DPPH were all measured in the study. The hypothesis is that some of the inconsistencies reported in peer-reviewed publication related to ROS reduction and measurement of soluble proteins, antioxidants, and antioxidant enzymes is related to the timepoint of sampling. These levels are constantly changing in the plant, and if the drought stress is allowed to continue long-enough eventually the plant dies regardless of the biostimulant used. These biostimulants prolong the survival of the plant and help maintain root growth in search of moisture. By measuring the levels over multiple timepoints, we aim to increase our understanding of what happens with these markers overtime during drought.

Trial Conditions:

Growing Season: 2023

Growing media: Pro-Mix General Purpose Growing Medium in Tree Pots

Number of plants per treatment: 22 plants per treatment randomly located

Experiment Design: Completely randomized design (CRD)

Date of germination: February 9th, 2023

Date of Initiation of Drought Stress: Mar 7th, 2023

Application of the Product:

Fertility program:

All four treatments had the same soil nutrient profile. Aqua Vega Solutions A&B were used to create a nutrient enriched soil mix for all germinated plants. The soil was made in single mix.

Trial Treatments: 22 plants per treatment for a total of 88 plants.

Treatment #1: Well Watered Control

Treatment #2: Well Watered + BLG FA

Treatment #3: Drought Control

Treatment #4: Drought + BLG FA

BLG application rate:

In-furrow at 1,235 mL per hectare (500mL/ac.) equivalent

Foliar at 3,705 mL per hectare (1,500ml/ac.) equivalent

Treatment Dates:

Date of First BLG FA Application (in-furrow): Feb 9th, 2023 (At Germination)

Date of Second BLG FA Application (Foliar): Mar 16th, 2023

Date of Third BLG FA Application (Foliar): Mar 24th, 2023

Harvest Date:

Apr 3rd, 2023.

Lighting and Light Distribution:

The controlled environment chamber used four (4x) commercial grade, pro series ViparSepctra P2000, 200W Infrared Full Spectrum LED Growing Light with 1.68m^2 coverage area each. The lighting maintains a uniform photosynthetically active radiation (PAR) light penetration of 165 µmol/m2/s. The lux light intensity readings ranged between 734 and 826.

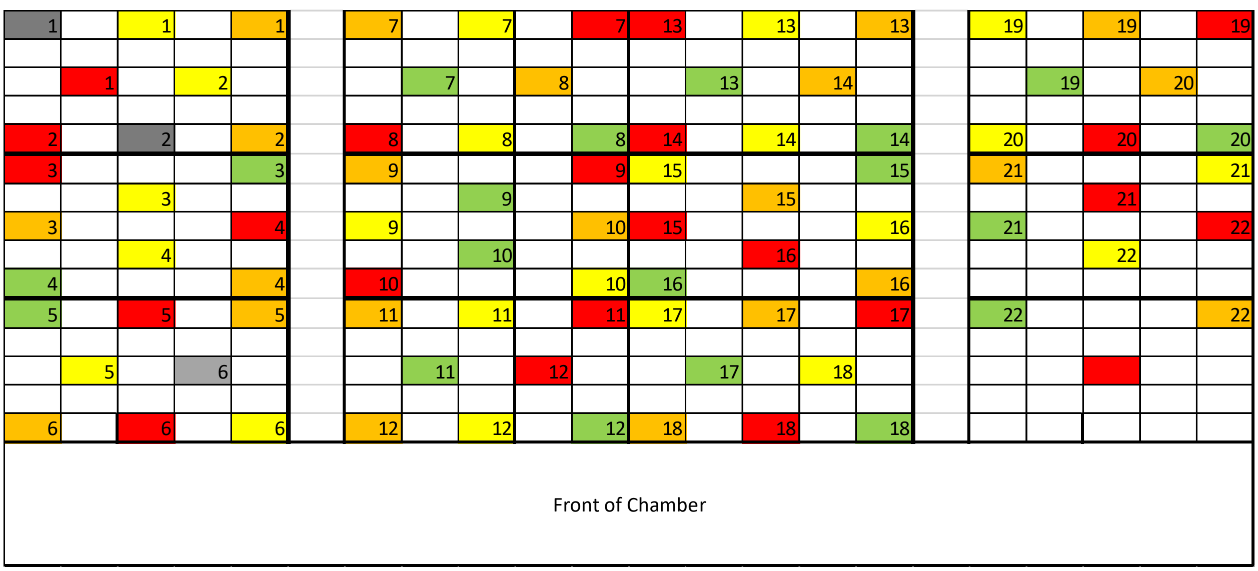


Figure 4: Report #2 - Random distribution of plant in the growing chamber

Data Measurements Collected:

For this trial the following data was collected at harvest:

- **Soluble protein** – Concentration of soluble protein was determined according to the method of Bradford. Briefly, 0.5 g of fresh leaf material was ground in 10 mL of a 50 mM sodium phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpyrrolidine-40 (PVP-40). The homogenate was centrifuged at 3900 g for 15 minutes and the supernatant was collected. 2 mL of Bradford solution was added to 300 µL of extract and absorbance was recorded at 595 nm. The standard curve was generated using bovine serum albumin (Sigma Aldrich) to determine concentrations.
- **Catalase (CAT) –** To determine antioxidant enzyme activity in the form of catalase (CAT), the method outline by Aebi (1984) was used. 0.3 g of fresh plant material was homogenized in 10 mL of 0.1 M potassium phosphate buffer (pH 6.8). The mixture was centrifuged at 3900 g for 15 minutes. 200 µL of enzyme extract was added to 2.85 mL of phosphate buffer in a test tube. Immediately prior to running on the UV-vis spectrophotometer, 100 µL of 0.7% hydrogen peroxide solution (H2O2) was mixed with the extraction solution and added to a cuvette. Absorbance was measured at 240 nm over a 90 second time interval to determine catalase concentration as a function of H2O2 disappearance. 1 Unit of catalase activity was defined as 0.01 decrease in absorbance at 240 nm per mg of protein per minute.
- **Glutathione (GSH)** - To determine glutathione (GSH) the method of Chen and Wang (2002) was followed. 0.5 g of fresh plant tissue was homogenized in 10 mL of 5% trichloroacetic acid (w/v). The homogenate was centrifuged at 3900 g for 15 minutes and the supernatant was collected. To the supernatant, 2.6 mL of 150 mM sodium phosphate buffer (pH 7.0) and 0.18 mL of 3 mM 5,5-dithio-bis (2-nitrobenzoic acid) (DNTB) in 100 mM phosphate buffer was added. Reaction mixture was kept for 5 minutes before being read on the UV-vis spectrophotometer and absorbance recorded at 412 nm. Reduced glutathione standard was purchased from Sigma Aldrich to generate a standard curve.
- **Proline** - Proline was quantified based on the method of Bates et al. (1973). 0.5 g of fresh leaf sample was homogenized in 10 mL of 3% (w/v) 5-sulfosalicylic acid and centrifuged for 15 minutes at 3900 g. Acid ninhydrin solution was prepared by combining 30 mL glacial acetic acid, 20 mL of 6 M phosphoric acid, and 1.25 g of ninhydrin and stirred on a hot plate until dissolved. 2 mL of homogenate was added in a test tube and combined with 2 mL of acid ninhydrin and 2 mL glacial acetic acid and heated for 60 min at 98 °C. The reaction mixture was cooled to room temperature and absorbance was measured at 520 nm.
- **Malondialdehyde (MDA)** – MDA content was determined to estimate the level of membrane damage caused by oxidation in the leaf samples according to Heath et al. (1968). 0.5 g of fresh plant tissue was homogenized in 5 mL of 5% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged for 15 minutes at 3900 g. 3 mL of 2-thiobarbituric acid in 20% TCA was added to 2 mL of homogenate prior to 10 minutes of heating at 98 °C. The samples were cooled in an ice bath and centrifuged for 10 minutes at 3900 g prior to measuring absorbance on the UV-visible spectrophotometer at 532 nm.
- **Radical Scavenging Quantification** – To determine radical scavenging activity, the **2,2-diphenyl-1-picrylhydrazyl (DPPH) assay** was conducted as per Brand-Williams et al. (1995). Briefly, a 0.1 mM methanolic solution of DPPH was prepared and stored in the dark at 4 °C. Fresh leaf samples were air dried, and 100 mg

of powder was mixed with 2 mL of methanol overnight. 1 mL of the solution was mixed with 3 mL of DPPH solution and placed into dark conditions for 30 minutes. Absorbance was read at 517 nm and a standard solution of ascorbic acid (Sigma Aldrich) was prepared and measured in the same manner to define DPPH Scavenging activity percentage as:

% Radical scavenging=(Ac-As)/Ac x 100

Where Ac = absorbance of ascorbic acid control

As = absorbance of plant extract sample

Trial Results:

The temperatures inside the controlled environment chamber were maintained between average daily lows of 20.5 degrees Celsius and average daily highs of 24.3 degrees Celsius. The humidity was maintained between a low of 32.2% and high of 52.1% on average. **Table 7** below provides a summary of the biochemical quantification results for all measurements at timepoint 3. The table compares the well watered control plants with the well water BLG treated plants and provides p-values for each data point. Similarly results of drought control are compared with drought treated.

Table 7: Report #2 - Summary of the results with the associated p-values for this trial

	Well Watered Control	Well Watered BLG	% Diff.	p-value	Drought Control	Drought BLG	Percent Diff. (%)	p-value
GSH (µM/g FW)	36.72	39.76	8.3%	0.00	45.0	49.5	10.1%	0.00
Sol. Protein (mg/ g DW)	5.78	6.33	9.4%	0.05	8.9	10.0	12.5%	0.00
Catalase (CAT) (U)	0.46	0.73	57.6%	0.00	1.0	1.1	14.0%	0.02
Proline (µg/g FW)	16.35	38.31	134.3%	0.00	129.7	184.5	42.3%	0.00
MDA (µmol/g FW)	1.86	1.70	-8.5%	0.02	3.0	1.5	-49.6%	0.00
DPPH (%)	59.4%	62.6%	5.4%	0.00	66.4%	69.2%	4.1%	0.00

Glutathione (GSH):

GSH levels across all treatments were measured at a baseline and 2 more times throughout the applied drought and displayed in **Figure 5**. Five samples per treatment were analyzed for GSH concentration. GSH content continuously increased in all 4 treatments and was highest at harvest. Drought treatments had a significantly higher GSH content than well watered controls, while fulvic acid treatments had a significantly higher GSH content than controls at all tested timepoints. DFA had the highest [GSH] with a 10% increase relative to the DC.

It is well known that prolonged exposure to abiotic stress in plants leads to increased production and accumulation of reactive oxygen species (ROS) and oxidative stress. To counteract this imbalance, plants produce higher concentrations of antioxidants and antioxidant enzymes to scavenge ROS. If the rate of ROS accumulation exceeds the rate of scavenging, then oxidative stress is increased which can lead to cell death.

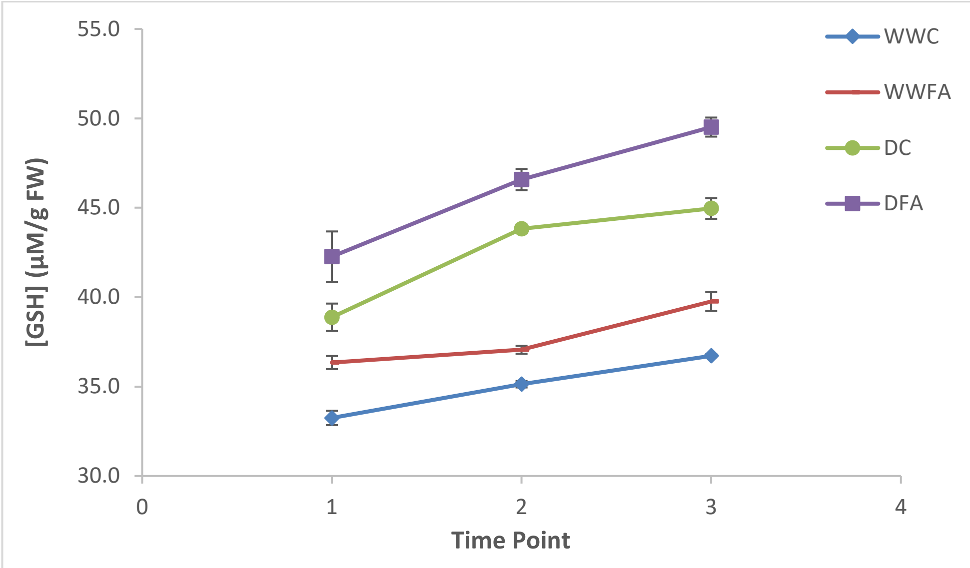


Figure 5: Report #2 - Changes in GSH from drought onset to harvest measured. Error bars displayed are SE.

The results of this study were in line with previous findings where we consistently found the foliar applications of fulvic acid further enhanced the production of GSH in both well-watered and drought conditions. GSH can directly scavenge ROS, as well as regenerate other antioxidants like ascorbate, which in turn scavenge ROS.

Soluble Protein:

Following the baseline measurement at timepoint 1 corresponding with the onset of drought, a week after 30% moisture was achieved in the drought treatments, soluble protein significantly increased followed by a sharp decline to return to levels slightly below baseline as shown in **Figure 6**. The trend was observed in all treatments with drought treatments having higher soluble protein content compared to well watered treatments, and fulvic acid applications having higher total soluble protein content compared to their control counterparts at all timepoints tested.

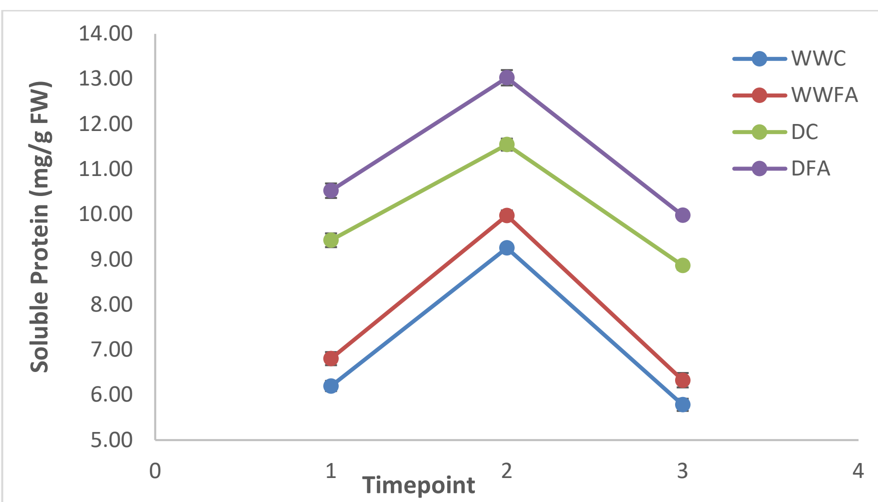


Figure 6: Report #2 - Changes in Soluble protein from drought onset to harvest measured. Error bars are SE.

The increase in soluble protein concentration ranged from 7.8% to 9.8% when comparing WWFA samples with WW, and 11.6% to 12.8% when comparing DFA with DC. for all time points and for both well watered and drought conditions the increase of soluble protein high p-values <0.05, indicating a strong probability of treatment effect.

Soluble proteins play important roles in many cellular processes, including enzyme function, metabolism, and signaling, and their accumulation may help to maintain these processes in the face of drought stress. Fulvic acid increasing the concentration of soluble proteins may be due to the ability of fulvic acid to enhance nutrient uptake and improve plant metabolism, which can lead to increased protein synthesis in the plant. As drought progresses and oxidative damage persists, soluble protein concentration decreased due to protein damage.

The results of this study for soluble protein show the importance of selecting the appropriate or optimal timing for sampling crops for these measurements. Ideally, samples from multiple time points would be analyzed to determine treatment effect. Plants protect themselves from abiotic stress through complex response mechanisms and metabolic pathways. This timepoint sensitivity from the onset of drought may be a contributing factor to the inconsistencies found in reported literature.

<u>Catalase (CAT):</u>

Catalase activity increased from the baseline measurement to the second time point following the same treatment trend as GSH with drought plants having higher catalase activity than well watered plants and fulvic acid treated plants having a higher catalase activity compared to untreated plants. As **Figure 7** shows, from timepoint 2 until harvest, the catalase activity in DC and DFA continued to slightly increase, whereas the WWC and WWFA catalase sharply dropped to below the baseline level.

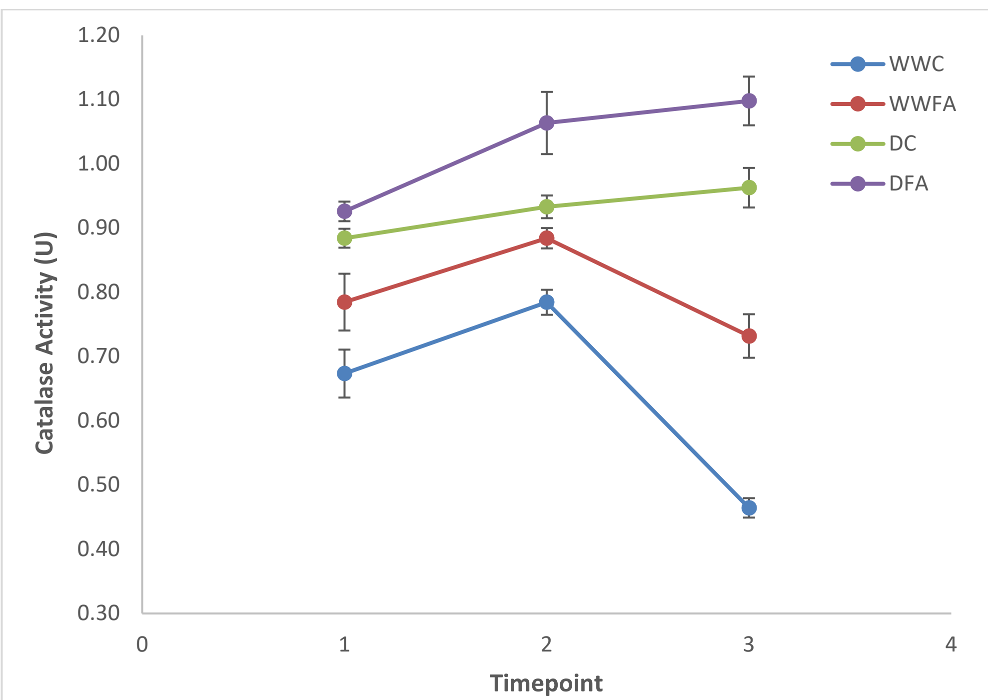


Figure 7: Report #2 - Changes in CAT from drought onset to harvest measured. Error bars displayed are SE.

Once again, at all three time points, and for both well watered and drought condition canola plants, the concentration of CAT increased due to treatment with BLG FA. The increases were statistically significant with p-values of <0.04 for time points 2 and 3, and around p=0.12 for timepoint 1.

Proline:

Proline content was measured at the harvest time point and found that FA treated plants had a significantly higher proline content than untreated plants, with the WWFA treatment having 3x increase over the WWC as shown in **Figure 8**. Similarly, drought stress was found to significantly increase the proline levels in the plants with an 11x increase over the WWC for the DC and a 16x increase over the WWC for the DFA. For both the WWFA to WWC and DFA to DC comparisons the p-values were near p=0.00.

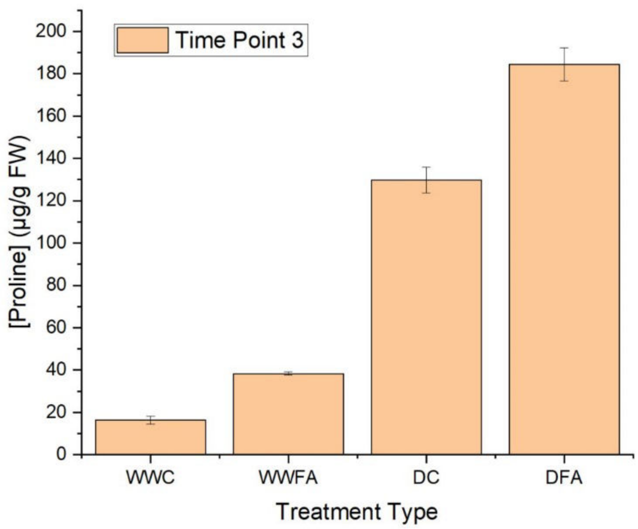


Figure 8: Report #2 - Proline measured in the canola plants at time of harvest. Error bars shown are SE.

Lipid Peroxidation as Measured by Malondialdehyde (MDA):

Malondialdehyde content was quantified to estimate the damage to lipids and was found to be at the highest concentration in the drought control. Treatment with BLG fulvic acids in the DFA plants reduce the MDA concentration of drought stressed plants by almost 50% when compared with DC. The p-value for the comparison was p=0.00 showing a strong treatment effect. Once again in this study we also show a reduction in MDA concentration when comparing WWFA with WWC of about 8.5% with p-value = 0.02.

Radical Scavenging Activity as Measured by DPPH:

Radical scavenging activity, as measured by the DPPH assay was estimated at time point 2 and timepoint 3 (harvest). There was a slight increase in radical scavenging between time point 2 and harvest across all treatments. The same trend observed in antioxidant and antioxidant enzyme activity was seen in the DPPH assay with drought stress causing increased radical scavenging activity, further enhanced by the application of fulvic acid for a consistent order of DFA > DC > WWFA > WC.

Comparing the radical scavenging activity (DPPH) measurements for WWFA to WWC the increase at timepoint 2 was 3.8% with a p-value of 0.03 and for timepoint 3 was 5.4% with a p-value of 0.00. Comparing the DPPH for drought stressed samples the concentrations for DFA versus DC increased by 6.9% at timepoint 2 with a p-value of 0.00 and 4.0% for timepoint 3 but also with a p-value of near 0.00. Thus it can be said that treatment with BLG fulvic acids increases the radical scavenging activity in canola crops for both well water and drought stressed plants in a statistically significant way.

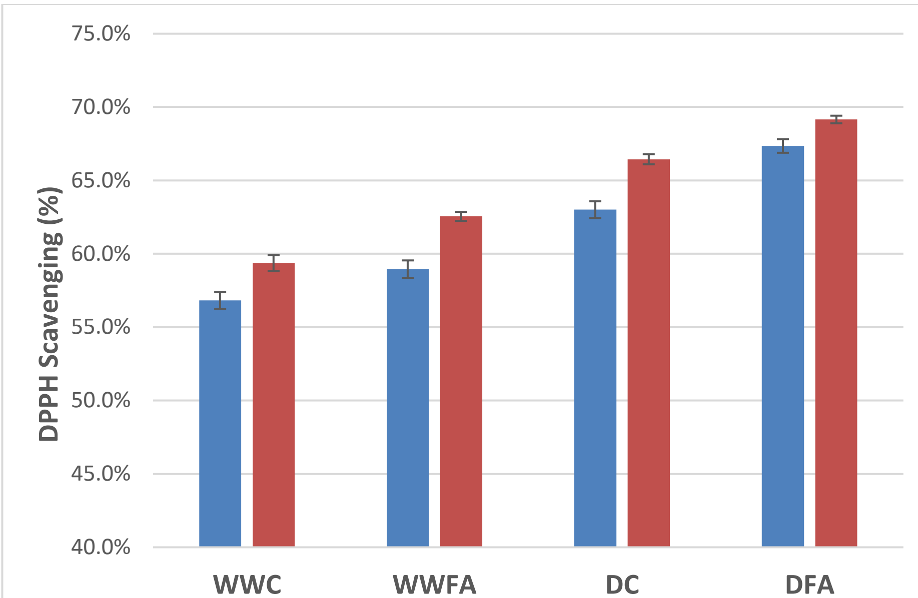


Figure 9: Report #2 - DPPH percentage for timepoints 2 and 3. Error bars displayed are SE.

Statistical Analysis:

The first step we took in analyzing the data was to obtain the descriptive statistics for each of the measurements. Due to the large number of biochemical assays at different timepoints, we have not included the descriptive statistics results in the main body of the report. The nine descriptive analyses are included in dossier under **Appendix A**.

Single-Factor Anova test was run comparing each of the well-watered and drought FA treatments to control. The p-values for the results of the analysis are reported in the previous tables. We assigned an alpha-value of 0.15. As depicted in **table 7** for all abiotic stress related measurements included in the trial which included glutathione (GSH), soluble protein, catalase (CAT), proline, lipid peroxidation (MDA), and radical scavenging activity (DPPH), treatment with BLG improvement was statistically significant under both well-watered condition and drought with the highest measured p-value of p=0.05 measured for soluble protein content comparing well-watered control and well-watered BLG treated plants.

Conclusions:

We found that foliar applications of BLG fulvic acids significantly enhanced the production of GSH and activity of CAT in both well-watered and drought conditions. CAT is responsible for breaking down H_2O_2 into H_2O and O_2, so it can be assumed that the increased CAT production following fulvic acid application helps reduce levels of oxidative stress by preventing overaccumulation of hydrogen peroxide. Similarly, the concentration of GSH in plant cells increased in drought stress. This increase is thought to be an adaptive response to the higher levels of ROS that are produced under stress conditions. GSH can directly scavenge ROS, as well as regenerate other antioxidants like ascorbate, which in turn scavenge ROS. Thus, the increase in GSH levels can help protect plants from oxidative damage caused by drought stress as found by several metabolic studies involving fulvic acid.

CAT activity decreased in the well watered treatment between time point 2 and harvest, which was similar to the decrease obtained by Anjum et al. (2021), across well watered and drought control and fulvic acid treatments in maize. Our findings were further validated through the DPPH results. DPPH radical scavenging activity was increased in drought conditions, and when fulvic acid was applied. Fulvic acid has been linked to the regulation of antioxidant metabolism in a variety of different plants in drought.

As ROS concentration increases, particularly in times of abiotic stress, damage to cell membranes can occur. As the peroxidation level increases, so does the accumulation of MDA which can accumulate and disrupt membrane fluidity and cause damage. Our study found a significantly higher amount of MDA in the DC treatment compared to the other treatments, and both FA treatments had the lowest amount of MDA, including the DFA treatment. The overall trend of drought increasing MDA and FA application lowering MDA remained consistent compared to other studies.

Previous studies have shown that proline is a key amino acid that accumulates in times of drought stress, particularly because of its speculative role as an osmoprotectant to maintain cell turgor and provide stability to reduce damage to important enzymes. Our research found that drought caused a substantial increase in proline accumulation in canola, and that BLG fulvic acids increased proline accumulation even further. The increase in proline followed by fulvic acid application has been seen in maize, soybeans, wheat, and other crops and potentially contributes to how fulvic acid is able to confer drought tolerance. Proline has also been linked to increased activity of antioxidant enzymes and partial stomatal opening to maintain photosynthesis in times of drought, which follows our observed trend of increased proline as well as antioxidant activity following fulvic acid application.

We have showed that both under drought and well-watered conditions, the application of fulvic acid caused an increase in the soluble protein concentration in canola. One possible explanation for the increase in soluble protein levels under drought stress is that it may reflect an adaptive response of the plant to maintain cell viability and function under stress. Soluble proteins play important roles in many cellular processes, including enzyme function, metabolism, and signaling, and their accumulation may help to maintain these processes in the face of drought stress. Fulvic acid increasing the concentration of soluble proteins may be due to the ability of fulvic acid to enhance nutrient uptake and improve plant metabolism, which can lead to increased protein synthesis in the plant. As drought progresses and oxidative damage persists, soluble protein concentration decreased due to protein damage. This shows the time sensitivity of sampling and analysis for soluble protein, that is unfortunately neglected by many authors in peer reviewed published research papers. Much of the inconsistency found in research publications can be attributed to neglecting this important factor. For many of these biochemical assays if the sampling is done too early the treatment effect hasn't taken hold and if it is done too late you may miss the response. As such multiple time points sampling and measurement is recommended to provide clearer picture of what is happening when plants are exposed to abiotic stress factors such as drought.

Improving Drought Tolerance in Green Peas with BioLiNE® Gold Fulvic Acids – CEC 2024

Report #3: Improving Drought Tolerance in Green Peas with BioLiNE® Gold Fulvic Acids

Introduction:

We have been carrying out experiments investigating the impact of drought stress on canola (*brassica napus)* and the modes of action for the amelioration of the negative impacts of the drought stress with the use of biostimulants. Our studies have focused on treatment with BioLiNE® Gold (BLG) fulvic acids as the tool for the amelioration of drought stress. In this study, we look at extending our drought experiments to green peas (*pisum sativum L*.).

Trial Conditions:

Growing Season: 2024

Growing media: Pro-Mix General Purpose Growing Medium in Tree Pots

Number of plants per treatment: 38 plants per treatment randomly located

Experiment Design: Completely randomized design (CRD)

Date of germination: January 5th, 2024

Date of first emergence: January 10th, 2024

Date of Initiation of Drought Stress: Jan 29th, 2024

Application of the Product:

Fertility program:

All four treatments had the same soil nutrient profile. Aqua Vega Solutions A&B were used to create a nutrient enriched soil mix for all germinated plants. The soil was made in single mix.

Trial Treatments: 38 plants per treatment for a total of 152 plants.

Treatment #1: Well Watered Control (WWC)

Treatment #2: Well Watered + BLG FA (WWBLG)

Treatment #3: Drought Control (DC)

Treatment #4: Drought + BLG FA (DBLG)

BLG application rate:

In-furrow at 1,235 mL per hectare (500mL/ac.) equivalent

Foliar at 3,705 mL per hectare (1,500ml/ac.) equivalent

Treatment Dates:

Date of First BLG FA Application (Soil): Jan 16th, 2024

Date of Second BLG FA Application (Foliar): Feb 7th, 2024

Date of Third BLG FA Application (Foliar): Feb 14th, 2024

Specimen Sample Collection Date:

Feb 27th, 2024.

Lighting and Light Distribution:

The controlled environment chamber used four (4x) commercial grade, pro series ViparSepctra P2000, 200W Infrared Full Spectrum LED Growing Light with 1.68m^2 coverage area each.

Data Measurements Collected:

For this trial the following data was collected at harvest:

- **Plant Fresh Weight**
- **Root Fresh Weight**
- **Glutathione (GSH)** - To determine glutathione (GSH) the method of Chen and Wang (2002) was followed. 0.5 g of fresh plant tissue was homogenized in 10 mL of 5% trichloroacetic acid (w/v). The homogenate was centrifuged at 3900 g for 15 minutes and the supernatant was collected. To the supernatant, 2.6 mL of 150 mM sodium phosphate buffer (pH 7.0) and 0.18 mL of 3 mM 5,5-dithio-bis (2-nitrobenzoic acid) (DNTB) in 100 mM phosphate buffer was added. Reaction mixture was kept for 5 minutes before being read on the UV-vis spectrophotometer and absorbance recorded at 412 nm. Reduced glutathione standard was purchased from Sigma Aldrich to generate a standard curve.
- **Malondialdehyde (MDA)** – MDA content was determined to estimate the level of membrane damage caused by oxidation in the leaf samples according to Heath et al. (1968). 0.5 g of fresh plant tissue was homogenized in 5 mL of 5% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged for 15 minutes at 3900 g. 3 mL of 2-thiobarbituric acid in 20% TCA was added to 2 mL of homogenate prior to 10 minutes of heating at 98 °C. The samples were cooled in an ice bath and centrifuged for 10 minutes at 3900 g prior to measuring absorbance on the UV-visible spectrophotometer at 532 nm.

Trial Results:

Following similar protocol as the trials conducted on treatment of Canola, the green pea plants were allowed to germinate and grow for about 4 weeks, from January 5th to January 29th, under well watered conditions, before the initiation of drought stress. Water addition to the drought stressed plants was reduced to 0 ml/day/plant on January 29th, 2024, resulting in a slow reduction of soil moisture down to below 40% by February 5th, 2024. Soil moisture for well watered plants ranged between 69% to 74%. For drought stressed plants the soil moisture was maintained between 27 to 37% for the duration of the trial. 38 well watered plants, and 38 drought stressed plants received soil treatments of BioLiNE® Gold (BLG) Fulvic acid on Jan 16th, followed by two foliar applications on February 7th and February 14th. For the 38 plants under drought stress, both foliar applications occurred while the plants had soil moisture below 40% and were under stress conditions.

The temperatures inside the controlled environment chamber were maintained between average daily lows of 19.8 degrees Celsius and average daily highs of 24.4 degrees Celsius. The humidity was maintained between a low of 33.4% and high of 47.7% on average. **Table 8** below provides a summary of the results for the measurements made at the end of the trial for plant fresh weight, root fresh weight. The table compares the well watered control plants with the well water BLG treated plants and provides p-values for each data point. Similarly results of drought control are compared with drought treated.

Table 8: Report #3 - Summary of results with the associated p-values for this trial

	Well Watered Control	Well Watered BLG	% Diff.	p-value	Drought Control	Drought BLG	Percent Diff. (%)	p-value
Avg. Plant Fresh Wt. (g/plant)	11.896	14.732	23.8%	0.001	4.410	4.405	-0.1%	0.963
Avg. Root Fresh Wt. (g/plant)	4.399	6.881	56.4%	0.000	0.504	0.619	22.8%	0.085

Table 9: Descriptive Statistical Analysis for Plant Weight (g)

	Well Watered Control	Well Watered BLG	Drought Control	Drought BLG
Mean	11.896	14.732	4.410	4.405
Standard Error	0.621	0.563	0.124	0.105
Median	12.164	15.069	4.412	4.285
Standard Deviation	3.344	3.032	0.620	0.524
Sample Variance	11.179	9.195	0.384	0.275
Kurtosis	0.345	0.643	3.830	-0.465
Skewness	0.397	0.283	-0.955	0.073
Range	15.030	13.850	3.138	2.152
Minimum	5.719	9.002	2.398	3.332
Maximum	20.749	22.852	5.536	5.484
Sum	345	427	110	110
Count	29	29	25	25
IQR	4.854	4.292	0.662	0.804

Table 10: Descriptive Statistical Analysis for Root Weight (g)

	Well Watered Control	Well Watered BLG	Drought Control	Drought BLG
Mean	4.399	6.881	0.504	0.619
Standard Error	0.355	0.402	0.024	0.061
Median	4.213	6.812	0.496	0.537
Standard Deviation	1.912	2.165	0.118	0.305
Sample Variance	3.654	4.687	0.014	0.093
Kurtosis	-1.037	0.939	0.144	0.643
Skewness	0.137	0.876	0.263	1.097
Range	6.675	9.496	0.501	1.124
Minimum	1.086	3.558	0.281	0.291
Maximum	7.761	13.054	0.783	1.415
Sum	128	200	13	15
Count	29	29	25	25
IQR	3.173	2.871	0.172	0.487

Average fresh weight (FW) per plant:

Treatment with BLG resulted in almost a 24% increase in the average fresh weight of the green pea plants (p=0.00) under well watered conditions. This was a substantial increase that was unexpected given the favorable conditions under which the well-watered plants were grown in. Comparing the plant weight of the plants under drought condition the BLG treated, and control had effectively the same total above ground + below ground (root) weight. For the control plants the weight reduced from 11.9g to 4.4g due to the impact of drought. We would have expected to see an approximate 10% increase in plant weight for the drought treated plants as well, however this is not the first time that we have seen under severe and prolonged drought conditions the drought BLG treated plants having similar total plant weight as the drought control, however having a much larger portion of that weight be associated with the root weight. The reason for this is that under such dry soil conditions much of the nutrients needed to support metabolic activity can be locked-up, and the severity of the conditions are beyond what can be addressed by biostimulants. They are not magic.

Average Root fresh weight (FW) per plant:

The root weight increase for both well-watered and drought treated plants was significant due to treatment with BLG. Under well-watered conditions the average root weight of the green pea plants increased by more than 56% from 4.4g to 6.9g (p-value = 0.00). The drought conditions imposed severely reduced the root mass of the plants from 4.4g to 0.5g for the control (untreated check). For the BLG treated plants the root weight increased by about 23% to 0.62 (p-value = 0.08). Thus, BLG treatment supported the plants' ability to grow more roots in search of water and nutrients in severely dry soil conditions. By supporting the plants' ability to grow more roots, BLG provides crops experiencing drought an opportunity to access more water and survive longer until soil moisture is replenished.

Glutathione (GSH):

GSH levels were measured at three different timepoints. **Table 11,** below provides a summary of the GSH measurements for all three timepoints for both drought and well-watered plants. As the table indicates we did not obtain data for the well-watered control at time-point 1. The specimen collected had issues with their storage and were not usable for analyzing. However, as the specimen for the drought conditions show, even at timepoint #1 (Feb 9th), the measure values were significantly higher for the BLG treated versus drought control. There was about 31% increase (p-value=0) at timepoint #1, 36% increase at timepoint #2, and 13% increase in timepoint 3. It should be noticed that the BLG treated GSH levels increased from about 12.9 to 44.3 between timepoints #1 and #2 and reduced to 23.7 at timepoint #3.

Table 11: Report #3 - Results of Glutathione (GSH) measurements at three time-points throughout the trial

	Date	Well Watered Control	Well Watered BLG	% Diff.	p-value	Drought Control	Drought BLG	Percent Diff. (%)	p-value
Timepoint #1 GSH (µM/g FW)	Feb 9th		8.933	N/A	N/A	9.889	12.925	30.7%	0.000
Timepoint #2 GSH (µM/g FW)	Feb 12th	27.141	33.696	24.1%	0.008	32.632	44.310	35.8%	0.002
Timepoint #3 GSH (µM/g FW)	Feb 27th	11.332	17.353	53.1%	0.000	20.949	23.670	13.0%	0.083

The results of this study were in line with previous findings with Canola where we consistently found the foliar applications of fulvic acid further enhanced the production of GSH, a well-studied antioxidant. GSH can directly scavenge ROS, as well as regenerate other antioxidants like ascorbate, which in turn scavenge ROS.

Lipid Peroxidation as Measured by Malondialdehyde (MDA):

Treatment with BLG reduced the MDA concentration of drought stressed plants by almost 37% (p-value = 0.00). MDA values were lowered for both timepoints measured when compared with DC showing a strong treatment effect. Once again in this study we also show a reduction in MDA concentration when comparing well-watered conditions.

Table 12: Report #3 - Results of Lipid Peroxidation Reduction as Estimated by MDA Concentration

	Date	Well Watered Control	Well Watered BLG	% Diff.	p-value	Drought Control	Drought BLG	Percent Diff. (%)	p-value
Timepoint #1 MDA (µmol/g FW)	Feb 9th	3.11	3.04	-2.2%	0.155	3.36	3.02	-10.1%	0.02
Timepoint #2 MDA (µmol/g FW)	Feb 13th	2.15	1.34	-38.0%	0.003	4.66	2.94	-37.0%	0.000

Statistical Analysis:

The first step we took in analyzing the data was to obtain the descriptive statistics for each of the measurements. Due to the large number of biochemical assays at different timepoints, we have not included the descriptive statistics results in the main body of the report. The five descriptive analyses are included in dossier under **Appendix B**.

Single-Factor Anova test was run comparing each of the well-watered and drought FA treatments to control. The p-values for the results of the analysis are reported in the previous tables. We assigned an alpha-value of 0.15. The p-values calculate are provided in **table 8** for the plant and root weights, **table 11** for GSH and **table 12** for MDA. The plant weight under drought condition was the only parameter measured that had no treatment effect. Both plant and root weight increases were statistically significant under well-watered conditions, while only the root increase had statistically significant response to BLG treatment under drought condition. Treatment effect on concentrations of glutathione (GSH) and MDA was found to be statistically significant for both well-watered and drought conditions at all timepoints measured.

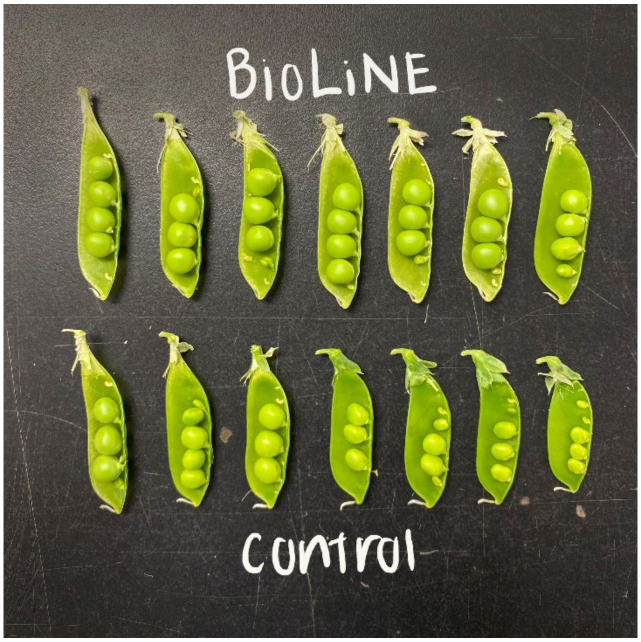
Conclusions:

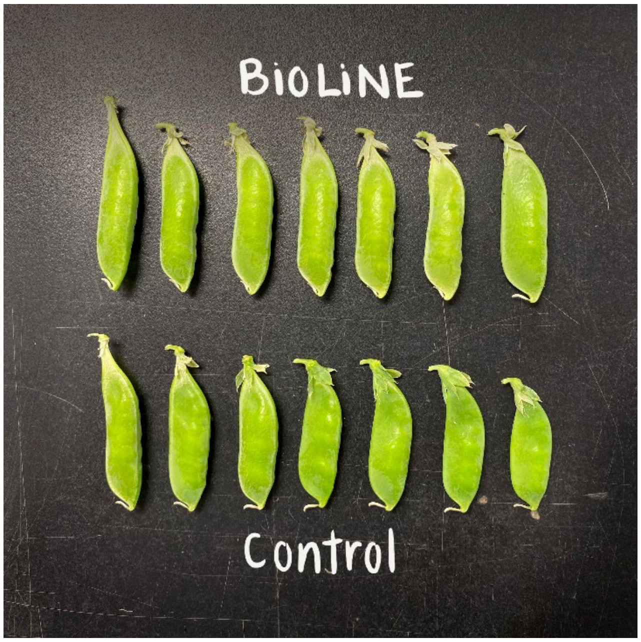
In conclusion, foliar applications of BLG fulvic acids significantly enhanced root growth in green pea plants for both well watered and drought stressed plants. The root mass increased by 56% for the well watered and 23% for the drought stressed plants. The total plant weight (above ground foliage + roots) the treatment with BLG only resulted in an increase in weight for the well-watered plants. The result is that for the control the ratio of root mass to above ground foliage was 11.4%. In comparison the BLG FA treated plants had a ratio of 14%. For both FA treated and control the root mass was substantially (>85%) lower under drought conditions. The severe drought conditions imposed by the trial had extremely negative impact on the physiological development of the green pea plants, however treatment with BLG FA helped ameliorate some of that impact by supporting increased root growth.

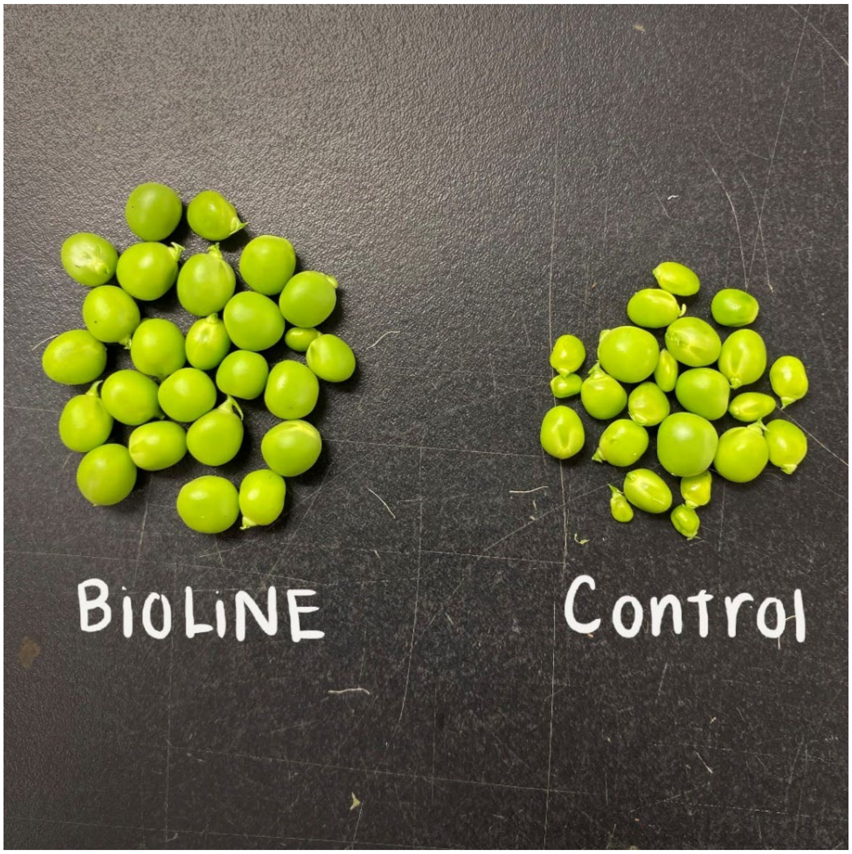
The glutathione (GSH) concentrations were measured at three different time-points corresponding with early, mid, and late-drought stress. Across all three time-points and for both well-watered and drought stressed green pea plants, the measure GSH levels were higher as a result of treatment with BLG FA, and the increases were all significant with p-values ranging between 0.00 and 0.08. As mentioned in other reports GSH is produced by plants as an adaptive response to the higher levels of ROS that are produced under stress conditions. GSH can directly scavenge ROS, as well as regenerate other antioxidants like ascorbate, which in turn scavenge ROS. Thus, the increase in GSH levels can help protect plants from oxidative damage caused by drought stress as found by several metabolic studies involving fulvic acid.

Under stress conditions, and resulting from accumulation of ROS in plants, lipid peroxidation levels increase. Measurements of MDA in plants as an estimation for lipid peroxidation is a well-established approach for its quantification. The findings in this study corroborated our previous findings with canola in measuring a significantly higher amount of MDA in the control plants as compared to the BLG FA treated. The overall trend of drought increasing MDA and FA application lowering MDA remained consistent.

Images:







Conclusions on Drought Stress Amelioration of BLG

In conclusion, this dossier provides a compilation of 3 trial results providing high quality data, using scientifically validated protocols. Using single-factor Anova test to analyze the probability of treatment effect, it can be concluded that:

1) Treatment with BLG improves growth of roots under both well-watered and drought conditions. Root growth data is provided for canola in report #1 where BLG treated plants had 31% increase in their root weight under well-watered conditions (p=0.03) and 45% increase under drought conditions (p=0.06). Similar results on root growth were observed with green peas, data for which is provided in report #3. The root weight increased by 56% for the well-watered plants (p=0.00) and by 22.8% (p=0.08) for plants under drought conditions due to treatment with BLG.
2) Treatment with BLG results in increased production and accumulation of antioxidants in plants under drought stress. The concentration of glutathione (GSH) for both well-watered and drought exposed plants increased in all three studies presented in this dossier in a statistically significant way. In the second and third reports GSH was measured at multiple time-points and across all time-points, the BLG treated levels were higher than non-treated with p-values approaching p=0.00. These results demonstrate very strong treatment effect. Ascorbic Acid (ASA) is another well-studied antioxidant. In report #1 ASA was also analyzed and there was a 17% increase (p=0.05) in ASA measured for canola plants under drought conditions due to treatment with BLG.
3) Treatment with BLG also resulted in the increased production and accumulation of antioxidant enzymes in plants under drought stress. Measurement of the catalase (CAT) activity of a plant is a well-established method for quantification of ROS control in plants under abiotic stress. In report #2, we found that foliar applications of BLG fulvic acids significantly enhanced activity of CAT in both well-watered and drought conditions. CAT is responsible for breaking down H_2O_2 into H_2O and O_2. There was a 58% increase in CAT activity (p=0.00) for well-watered plants treated with BLG and a 14% increase (p=0.02) for plants under drought conditions. It should be noted that the catalase activity more than doubled from 0.46 to 1.0 when comparing well-watered to drought control.
4) Treatment with BLG increased radical (ROS) scavenging activity as measured by quantifying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. Radical scavenging activity was quantified at two time points in report #2. There was a slight increase in radical scavenging between time point 2 and harvest across all treatments. The same trend observed in antioxidant and antioxidant enzyme activity was seen in the DPPH assay with drought stress causing increased radical scavenging activity, further enhanced by the application of fulvic acid for a consistent order of DFA > DC > WWFA > WC. Comparing the radical scavenging activity (DPPH) measurements for WWFA to WWC the increase at timepoint 2 was 3.8% with a p-value of 0.03 and for timepoint 3 was 5.4% with a p-value of 0.00. Comparing the DPPH for drought stressed samples the concentrations for DFA versus DC increased by 6.9% at timepoint 2 with a p-value of 0.00 and 4.0% for timepoint 3 but also with a p-value of near 0.00.
5) Treatment with BLG reduced lipid peroxidation as quantified by measuring the concentration of Malondialdehyde (MDA) in plants. Treatment with BLG reduced the MDA concentration of drought stressed plants by almost 50% (p=0.00) in canola plants as provided in report #2 and by as much as 37%

(p=0.00) in green peas (report #3). MDA reduction was observed in both well-watered and drought plants in a statistically significant way, showing a strong treatment effect. Once again in this study we also show a reduction in MDA concentration when comparing WWFA with WWC of about 8.5% with p-value = 0.02.

6) Treatment with BLG results in increased concentration of osmoprotectants such as proline and soluble proteins. In report #2 proline concentrations increased in canola plants from about 16 to 130 (µg/g FW) because of drought. Proline is a well-established osmoprotectant associated with abiotic stress tolerance and ROS control in plants. BLG treatment increase proline concentrations in both well-watered and drought conditions (p=0.00). Soluble protein content was also quantified and provided in report #2 at three different time-points. Across all three time-points there was an increase in soluble protein measured for both well-watered and drought condition plants resulting from treatment with BLG ($p<0.05$).

Thus, based on the data presented in this dossier, it can be concluded that BLG is an affordable, easy-to-use, versatile, and highly effective biostimulant for improving crop tolerance to abiotic stress. The biostimulant claims associated with the use of BLG are not specific to any genus, family, order, or class of plants (taxonomic grouping); growing media; or country. The modes of action involved in the abiotic stress amelioration caused by application of BLG are universal to all classes and most phylum of plants.

Appendix A – Descriptive Statistics for Report #2 Measured Parameters

Descriptive Statistics for Glutathione GSH (µM/g FW) Timepoint #1

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	33.25	36.34	38.88	42.27
Standard Error	0.45	0.41	0.86	1.57
Median	33.47	36.28	38.61	43.44
Standard Deviation	1.00	0.92	1.91	3.52
Sample Variance	1.00	0.84	3.66	12.38
Kurtosis	-1.43	-0.74	-1.97	4.10
Skewness	-0.63	0.34	-0.05	-1.94
Range	2.29	2.35	4.55	8.98
Minimum	31.85	35.26	36.47	36.14
Maximum	34.1	37.6	41.0	45.1
Sum	166	182	194	211
Count	5	5	5	5
Confidence Level(95.0%)	1.24	1.14	2.38	4.37

Descriptive Statistics for Glutathione GSH (µM/g FW) Timepoint #2

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	35.13	37.06	43.82	46.58
Standard Error	0.20	0.25	0.26	0.66
Median	35.14	36.82	43.52	46.60
Standard Deviation	0.45	0.55	0.59	1.48
Sample Variance	0.20	0.30	0.35	2.19
Kurtosis	1.71	-1.56	-2.13	-1.06
Skewness	1.25	0.69	0.63	-0.41
Range	1.14	1.31	1.37	3.66
Minimum	34.72	36.51	43.24	44.54
Maximum	35.9	37.8	44.6	48.2
Sum	176	185	219	233
Count	5	5	5	5
Confidence Level(95.0%)	0.56	0.68	0.73	1.84

Descriptive Statistics for Glutathione GSH (µM/g FW) Timepoint #3

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	36.72	39.76	44.97	49.51
Standard Error	0.09	0.59	0.65	0.60
Median	36.74	39.26	45.72	49.38
Standard Deviation	0.21	1.33	1.45	1.33
Sample Variance	0.04	1.76	2.10	1.78
Kurtosis	-0.61	-0.11	1.54	1.35
Skewness	-0.65	0.98	-1.45	1.19
Range	0.51	3.28	3.49	3.31
Minimum	36.42	38.49	42.61	48.33
Maximum	36.9	41.8	46.1	51.6
Sum	184	199	225	248
Count	5	5	5	5
Confidence Level(95.0%)	0.26	1.65	1.80	1.65

Descriptive Statistics for Catalase Activity (U) Timepoint #1

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	0.67	0.78	0.88	0.93
Standard Error	0.04	0.05	0.02	0.02
Median	0.70	0.81	0.90	0.92
Standard Deviation	0.09	0.11	0.04	0.04
Sample Variance	0.01	0.01	0.00	0.00
Kurtosis	-1.59	-0.12	1.16	-0.42
Skewness	-0.50	-0.46	-1.31	0.09
Range	0.23	0.29	0.09	0.10
Minimum	0.55	0.63	0.83	0.88
Maximum	0.8	0.9	0.9	1.0
Sum	3	4	4	5
Count	5	5	5	5
Confidence Level(95.0%)	0.12	0.14	0.05	0.05

Descriptive Statistics for Catalase Activity (U) Timepoint #2

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	0.78	0.88	0.93	1.06
Standard Error	0.02	0.02	0.02	0.05
Median	0.80	0.88	0.92	1.04
Standard Deviation	0.04	0.04	0.04	0.11
Sample Variance	0.00	0.00	0.00	0.01
Kurtosis	1.49	-1.78	2.89	2.80
Skewness	-1.33	0.17	1.69	1.23
Range	0.11	0.09	0.10	0.30
Minimum	0.71	0.84	0.90	0.94
Maximum	0.8	0.9	1.0	1.2
Sum	4	4	5	5
Count	5	5	5	5
Confidence Level(95.0%)	0.05	0.04	0.05	0.13

Descriptive Statistics for Catalase Activity (U) Timepoint #3

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	0.46	0.73	0.96	1.10
Standard Error	0.02	0.03	0.03	0.04
Median	0.46	0.77	0.99	1.13
Standard Deviation	0.03	0.08	0.07	0.08
Sample Variance	0.00	0.01	0.00	0.01
Kurtosis	1.56	-1.79	-3.04	2.84
Skewness	0.96	-0.79	-0.38	-1.72
Range	0.09	0.17	0.14	0.20
Minimum	0.43	0.63	0.89	0.95
Maximum	0.5	0.8	1.0	1.2
Sum	2	4	5	5
Count	5	5	5	5
Confidence Level(95.0%)	0.04	0.09	0.09	0.11

Descriptive Statistics for Radical Scavenging Activity DPPH (%) Timepoint #2

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	0.57	0.59	0.63	0.67
Standard Error	0.01	0.01	0.01	0.00
Median	0.57	0.58	0.63	0.67
Standard Deviation	0.01	0.01	0.01	0.01
Sample Variance	0.00	0.00	0.00	0.00
Kurtosis	2.07	-1.68	2.35	1.75
Skewness	-1.44	0.52	-1.50	1.28
Range	0.03	0.03	0.03	0.03
Minimum	0.55	0.58	0.61	0.66
Maximum	0.6	0.6	0.6	0.7
Sum	3	3	3	3
Count	5	5	5	5
Confidence Level(95.0%)	0.02	0.02	0.02	0.01

Descriptive Statistics for Radical Scavenging Activity DPPH (%) Timepoint #3

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	0.59	0.63	0.66	0.69
Standard Error	0.01	0.00	0.00	0.00
Median	0.59	0.62	0.67	0.69
Standard Deviation	0.01	0.01	0.01	0.01
Sample Variance	0.00	0.00	0.00	0.00
Kurtosis	-2.90	-0.35	2.33	1.45
Skewness	0.34	-0.59	-0.83	-1.36
Range	0.03	0.02	0.02	0.01
Minimum	0.58	0.62	0.65	0.68
Maximum	0.6	0.6	0.7	0.7
Sum	3	3	3	3
Count	5	5	5	5
Confidence Level(95.0%)	0.01	0.01	0.01	0.01

Descriptive Statistics for MDA (µmol/g FW) Timepoint #3

	WW Control	WW BLG	Drought Control	Drought BLG
Mean	1.86	1.70	3.04	1.53
Standard Error	0.04	0.02	0.24	0.03
Median	1.83	1.70	3.06	1.52
Standard Deviation	0.09	0.05	0.49	0.06
Sample Variance	0.01	0.00	0.24	0.00
Kurtosis	1.44	-2.89	-5.45	2.50
Skewness	1.34	-0.20	-0.04	1.44
Range	0.20	0.11	0.95	0.13
Minimum	1.78	1.64	2.56	1.48
Maximum	2.0	1.8	3.5	1.6
Sum	7	7	12	6
Count	4	4	4	4
Confidence Level(95.0%)	0.14	0.08	0.78	0.09

Appendix B – Descriptive Statistics for Report #3 (Green Peas) Measured Parameters

Descriptive Statistics for Glutathione GSH (µM/g FW) Timepoint #1

	WW BLG	Drought Control	Drought BLG
Mean	8.933	9.889	12.925
Standard Error	0.528	0.263	0.129
Median	8.386	9.637	12.878
Standard Deviation	1.294	0.643	0.317
Sample Variance	1.674	0.414	0.100
Kurtosis	-1.393	-1.620	2.044
Skewness	0.774	0.717	0.993
Range	3.230	1.564	0.949
Minimum	7.562	9.210	12.530
Maximum	10.792	10.775	13.480
Sum	54	59	78
Count	6	6	6
Confidence Level(95.0%)	1.358	0.675	0.333

Descriptive Statistics for Glutathione GSH (µM/g FW) Timepoint #2

	WW Control	WW BLG	D. Control	Drought BLG
Mean	27.141	33.696	32.632	44.310
Standard Error	0.872	1.805	0.566	2.794
Median	27.105	34.049	32.861	45.284
Standard Deviation	2.135	4.422	1.387	6.844
Sample Variance	4.560	19.554	1.924	46.838
Kurtosis	-2.420	-2.697	2.395	-2.833
Skewness	0.077	-0.094	-1.383	-0.166
Range	5.046	10.279	3.953	14.848
Minimum	24.743	28.321	30.106	36.143
Maximum	29.789	38.601	34.059	50.990
Sum	163	202	196	266
Count	6	6	6	6
Confidence Level(95.0%)	2.241	4.641	1.456	7.182

Descriptive Statistics for Glutathione GSH (µM/g FW) Timepoint #3

	WW Control	WW BLG	D. Control	Drought BLG
Mean	27.141	33.696	32.632	44.310
Standard Error	0.872	1.805	0.566	2.794
Median	27.105	34.049	32.861	45.284
Standard Deviation	2.135	4.422	1.387	6.844
Sample Variance	4.560	19.554	1.924	46.838
Kurtosis	-2.420	-2.697	2.395	-2.833
Skewness	0.077	-0.094	-1.383	-0.166
Range	5.046	10.279	3.953	14.848
Minimum	24.743	28.321	30.106	36.143
Maximum	29.789	38.601	34.059	50.990
Sum	163	202	196	266
Count	6	6	6	6
Confidence Level(95.0%)	2.241	4.641	1.456	7.182

Descriptive Statistics for MDA (µmol/g FW) Timepoint #2

	WW Control	WW BLG	D. Control	Drought BLG
Mean	3.113	3.045	3.357	3.019
Standard Error	0.034	0.028	0.091	0.083
Median	3.140	3.030	3.432	3.019
Standard Deviation	0.084	0.069	0.222	0.203
Sample Variance	0.007	0.005	0.049	0.041
Kurtosis	-0.964	-2.239	-1.669	2.271
Skewness	-0.613	0.390	-0.499	-1.104
Range	0.223	0.158	0.544	0.603
Minimum	2.988	2.979	3.073	2.657
Maximum	3.211	3.136	3.616	3.260
Sum	19	18	20	18
Count	6	6	6	6
Confidence Level(95.0%)	0.088	0.072	0.233	0.214

Descriptive Statistics for MDA (µmol/g FW) Timepoint #3

	WW Control	WW BLG	D. Control	Drought BLG
Mean	2.153	1.336	4.662	2.935
Standard Error	0.149	0.151	0.220	0.190
Median	2.024	1.217	4.769	2.968
Standard Deviation	0.365	0.369	0.540	0.464
Sample Variance	0.133	0.136	0.292	0.215
Kurtosis	1.255	0.553	-1.357	-0.314
Skewness	1.320	1.161	-0.524	0.386
Range	0.940	0.961	1.355	1.231
Minimum	1.859	1.003	3.892	2.422
Maximum	2.799	1.965	5.247	3.653
Sum	13	8	28	18
Count	6	6	6	6
Confidence Level(95.0%)	0.383	0.387	0.567	0.487

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Compilation of Original Research Efficacy Data Generated in Support of Nutrient Use Efficiency and Yield Claims for BioLiNE® Gold Fulvic Acids

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Product Introduction and Verification

1.1 Product Introduction

BioLiNE® Gold (BLG) is a purified fulvic acid that has been in the market in many U.S states and across Canada since 2018. Field trial work on the product began in 2015. The product has recently (2023) received CE certification under the PFC 6.B (Non-microbial Plant Biostimulants) based on the efficacy data dossier submitted.

1.2 Product Claims

The plant biostimulant product claims covered in this report are:

- Improved Nutrient Use Efficiency (NUE)
- Improved Yield

There are several agronomic indices that have been developed by scientists to evaluate the short-term impact of nutrient use efficiency. Agronomic efficiency (AE) measures the ability of a crop to utilize fertilizer nutrients applied to produce yield. Since yield is an important measure of biological benefit to the crop as well as economic benefit to the grower, establishing improved AE is perhaps the most powerful of all indices used in the assessment and quantification of nutrient use efficiency (NUE). Furthermore, AE is a crucial measure for sustainability of agricultural practices, and increasing the AE in agricultural system has definitive environmental benefits. The agronomic efficiency (AE) of supplied nutrients can be measured by comparing the crop yield of each treatment with the crop yield of the untreated check divided by the amount of nutrient applied in the trial.

In reporting the impact of BLG on nutrient use efficiency of crops we used the average yield values to calculate the agronomic efficiency (AE). The statistical uncertainty associated with the measured average yield will carry-over to the AE, given that AE is calculated from the measure yield and required no new or additional empirical data to be obtained. Thus, it should be noted that the p-values for the calculated AE are the same as the p-values for the average yield.

1.3 Target Crops and Crop Grouping

This dossier provides results from 2 field trials investigating nutrient use efficiency and yield improvements achieved from treatment with BioLiNE® Gold. Based on the data presented in this dossier, it can be concluded that BLG is a highly effective biostimulant for improving nutrient use efficiency in plants and supporting plants' ability to increase yield. The biostimulant claims associated with the use of BLG are not specific to any genus, family, order, or class of plants (taxonomic grouping); growing media; or country. The modes of action involved in the benefits of BLG are universal to all classes and most phyla of plants.

FA and HA have been studied extensively for their crop beneficial properties including their ability to improve nutrient use efficiency through the following modes of action:

- Carboxyl & phenolic groups complex/chelate ions increasing uptake & transport to metabolic targets.
- High concentration of electronegative donor atoms have higher ion affinity and increased cation exchange capacity.
- Increase bioavailability of phosphates, nitrates & sulfates in soils (sorption competition).

- Increase N fixation by stimulating root colonization or Rhizobium bacteria.
- Improve cell permeability of nutrients.
- Increase enzymatic activity of the proton pump (H+-ATPases).
- Improve enzyme activities / signaling involved in nitrogen assimilation and energy metabolism.
- Enzymatic activity associated with the glycolytic pathway and respiratory processes stimulate the activity of H+-ATPases in the root plasma membrane (root elongation through acid growth theory).

Table 1 below provides a summary of three trials carried out and included in this report. Details of protocols, data collection, analysis, and findings are included for each trial in the subsequent sections of this report. Each trial has its own separate report.

Table 1: Summary of Original Research Data on BLG impact on NUE

Trial #	Crop Group	Crop	Trial Type	Yield Inc. Full Fert.	Yield Inc. Red. Fert.	AE Inc. per kg of N	AE Inc. per kg of P_2O_5	AE Inc. per kg of K_2O	Plant / Root Wt. Inc.	Num. of Pods/ Fruit per Plant
1	1	Soybeans	RFP	**14.9% p=0.00**	**12.8% p=0.02**	N/A	**9.7**	**14.2**	**Pl - FF* 15.3% p=0.00 Pl - RF** 10.8% p=0.01**	**FF* 19.5% p=0.00 RF** 11.5% p=0.00**
2	1	Corn	RFP	**8.0% p=0.03**	N/A	**49.2**	N/A	N/A	N/A	N/A

* Pl-FF – Plant Weight Full Fertilizer

** PL-RF – Plant weight Reduced Fertilizer

1.4 Statistical Analysis Principals Applied

Statistical analysis used in this report are based on the recommendation made in the "US Biostimulant Industry Recommendations to Assess Efficacy, Composition, and Safety of Plant Biostimulant Products", published in the Journal of Regulatory Sciences, jointly by the Biological Products Industry Alliance (BPIA) and The Fertilizer Institute (TFI) Biostimulant Council (2022). According to the referenced document, "the main objective of the data analysis is to estimate the **magnitude of the difference** between the various treatments and provide a **measure of the variability** of those estimates. The decisions of acceptability or rejection of the treatment should not be based on p-value alone. P-values should be taken as a continuous measure of evidence against the null hypothesis, and p-values greater than 0.05 may be accepted depending on the study objectives."

In examining the significance of the efficacy data collected through field-trials, the principal criterion is that the product must produce **biological benefit to the crop or economic value to the grower.** The purpose of the statistical analysis is to establish that there was sufficient magnitude of difference resulting from treatment in relation to the variability within the dataset. This analysis is not arbitrary, and no single cut-off for p-value can be used across all efficacy trials. **One shoe will never fit all.** Researchers must take into consideration the experimental design, the type and number of datapoints collected in the determination of the acceptance or rejection of the null hypothesis. We hope that more researchers will adopt these principles and consult with statistician in the design of their experiments. This will increase the probability that they will employ the appropriate statistical analysis in their efficacy trials, rather than the use of an arbitrary cut-off p-value of 0.05 as the sole contributing factor to the acceptance or rejection of the null-hypothesis. Our preferred approach to statistical analysis of efficacy field-trials starts with the first step of obtaining the descriptive statistics for each of the measurements. Single-Factor Anova test were ran comparing treatments to control for key measurements in the trials including yield. The p-values for the results of the analysis are reported as calculated. We do not use any arbitrary p-value cut-off as the sole consideration for the acceptance and rejection of treatment effect.

Improving Nutrient Use Efficiency in Soybeans with BioLiNE® Gold Fulvic Acids (2023)

Report #1: Improving Nutrient Use Efficiency in Soybeans with BioLiNE® Gold Fulvic Acids (2023)

Introduction

BioLiNE® Gold (BLG) consists of purified fulvic acids that have very high levels of carboxylic acid and phenolic functional groups, and very small molecular size. Fulvic acids have been well documented in literature to contribute to improved crop yields due to their biostimulant impacts. Improving nutrient use efficiency is one of the primary biostimulant benefits of using BLG. The small molecular size and high concentration of oxygenated functional groups are the key factors that influence humic substances' capacity to complex nutrients, and improve uptake, transport, and assimilation of nutrients.

In this study, we look at reducing the application of monoammonium phosphate (MAP) and potash (K_2O) by 30% from the recommended usage rate and investigate the impact of using BioLiNE® Gold to improve nutrient use efficiency on soybeans. This study is similar in design to another replicated trial on corn.

Trial Objective:

- To determine the impact of BLG on soybean NUE, growth, and yield parameters in reduced nutrient conditions.
- The crop chosen for the study was **Soybeans** (Pioneer Xtend (Roundup Ready 2) variety P21A28X-HC21).
- Replicated field trial with 6 replicates per treatment. Each replicated plot is 10 m (32ft) x 4 rows with 2 rows of spacing between adjacent plots.
- The trial was located on a small section of farmland that has been under cultivation for many decades. Located on in Lambton County, ON, Canada (42.9^{o}N, 81.9^{o}W). The field was flat with very slight slope and imperfect drainage.

Trial Conditions:

Growing Season: 2023

Soil Type: Burford loam soil very gently sloping field with imperfect drainage.

Planting Date: May 19th, 2023

Soybean Seed Variety: Pioneer Xtend Soybeans variety P21A28X were used for the trial. This variety is Roundup Ready 2 and has been treated with LumiGEN® Seed Treatments, Lumisena® and Muniderm® which include fungicide, insecticide + Inoculant (*Bradyrhizobium japonicum*).

Planting Population Density: 300,000 plants per hectare (120,000 per acre) with 76cm (30") rows.

Crop Rotation: Corn-Soybean-Winter Wheat rotation. Prior to this trial there was corn grown on this field.

Experiment Design: Randomized complete block design with 6 replicates per treatment.

Soil Analysis:

Table 2: Report #1 – Soil Analysis results for a composite soil sample

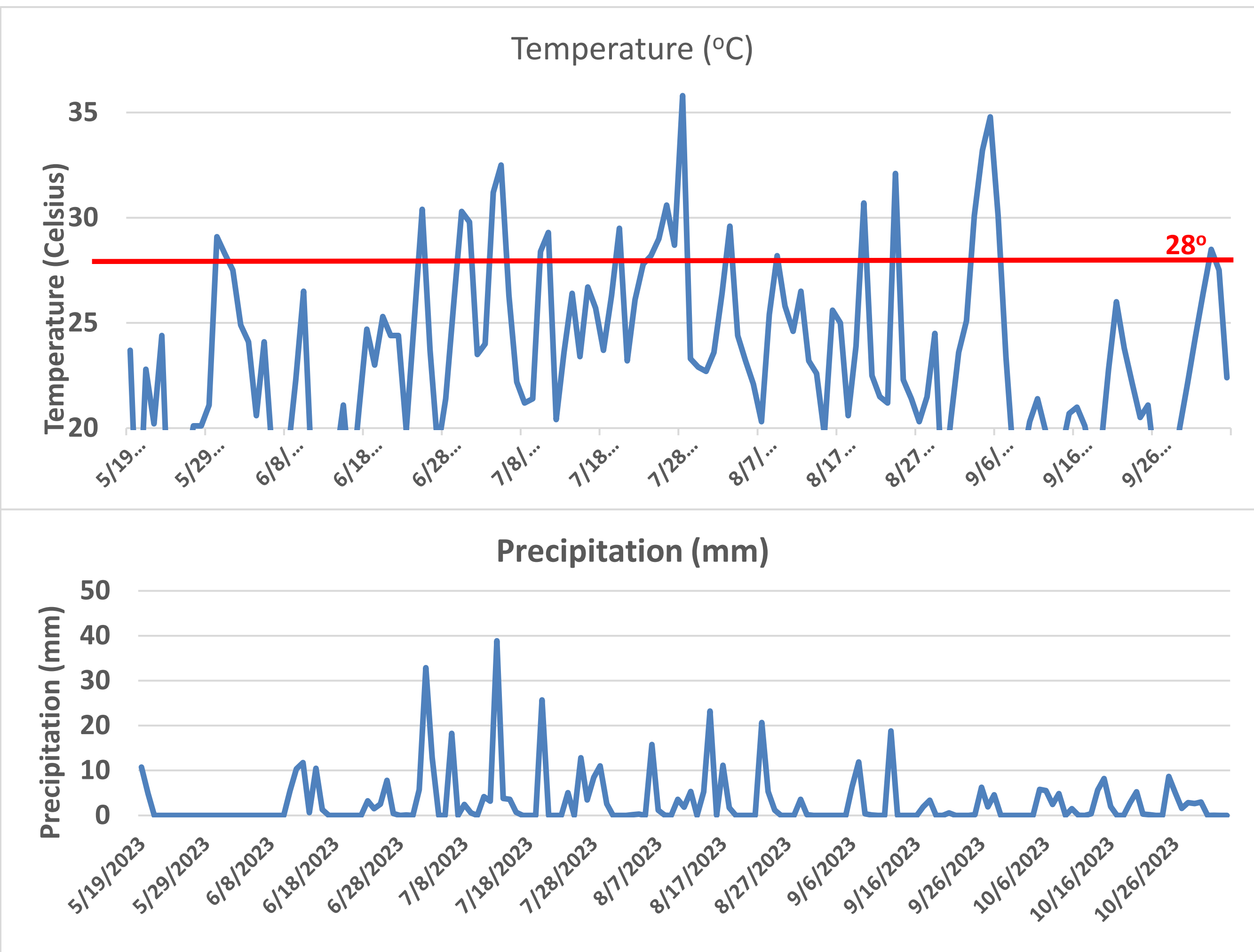
Sample	OM	Bicarb. P (ppm)	P (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	pH	CEC (meq/100g)
A2	3.9	34 good	89 high	286 very high	166 low	1930 med	17 low	6.4	14.2

% Base Saturation									
%K	%Mg	%Ca	%H	%Na	Sat. %P	Al (ppm)	Sat. %Al	K/Mg	ENR
5.2	9.7	67.9	67.9	0.5	15 high	771	0.3 good	0.54	51

Climatic Conditions:

The data on climate condition are measurements made by several weather stations near the location accessed through Visual Crossing (https://www.visualcrossing.com).

Figure 1: Temperature and precipitation profiles at trial location



Application of the Product:

Fertilizer Program: The trial had two fertilizer rates, full and reduced. The full rate of fertilizer was based on agronomist recommendations. The Reduced rates are 30% reduction of Urea and MAP applications.

Table 3: Report #1 – Full and reduced fertilizer application rates

Fertilizer	Composition (N-P-K)	Per acre (lbs)	Per hectare (kg)
Full (FPK)			
MAP	11-52-0	159	178.2
Potash	0-0-60	94	105.4
Reduced (RPK)			
MAP	11-52-0	111	124.4
Potash	0-0-60	71	79.6

Treatment Applications:

1. FPK Control – Control with water as an in-furrow/foliar and the full fertilizer rate
2. RPK Control – Control with water as an in-furrow/foliar and the reduced fertilizer rate
3. FPK + BLG – Full fertilizer rate with BLG as in-furrow/foliar
4. RPK + BLG – Reduced fertilizer rate with BLG as infurrow/foliar

Number of Replicates per treatment: There were 6 replicated plots per treatment.

Treatment Rates:

In-furrow BLG	In-furrow rate for all of 1 L/hectare (13.5 fl. oz/acre)
Foliar BLG	Foliar rate for all of 1,730 mL/hectare (23.7 fl. oz/acre) applied at V6 and VT

Treatment Dates:

- In-furrow at planting: May 19th, 2023
- First foliar applied on: July 5th, 2023
- Second foliar applied on: Aug 1st, 2023

Harvest Date: Soybeans were harvested on November 3rd, 2023

Data Measurements Collected:

- Number of pods/plant
- Seed weight
- Plant weight (at harvest)

Trial Results:

For each of the 24 plots (4 treatments x 6 replicates) exactly 30 plants were hand harvested for a total of 180 plants per treatment across the six replicates. The number of pods per plant were counted and recorded for each of the 180 plants per treatment at the time of harvest. This level of granular data is necessary to detect statistically significant differences as measured by p-value, in instances where the magnitude of difference between treatments is not very large. The weight of each of the 180 plants was also measured and recorded. For the yield data six soybean plants were combined into one sample and threshed to generate five sets of yield data per rep, and 30 yield data points per treatment. We didn't hand thresh the soybean plants individually, due to the time-consuming, labour-intensive nature of doing so. By threshing them in bundles we significantly reduced the amount of time it took to get the yield data for all 24 replicates, while still generating adequate granularity in yield data for statistical analysis. 30 datapoints per treatment is a good level of dataset from which to analyze the results. **Table 4** below provides a summary of all of the data collected from this trial.

Table 4: Report #1 – All data obtained from the trial

	FPK Control	RPK Control	FPK + BLG	RPK + BLG
Ave. Wt. of Plant (g)	31.6	29.4	36.5	32.6
Ave. Numb of Pods/Plant	41.9	41.5	50.1	46.3
Total Num. of Pods/Treatment	7,212	7,309	8,772	8,194
Average Moisture Content (%)	10.5%	8.4%	10.2%	8.8%
Avg. seed wt. per plant (g)	20.1	19.0	23.1	21.5
Total Wt. of Seeds/Treatment (g)	3623.2	3427.3	4062.7	3864.5
Yield (Bushels/Acre)	70.9	67.1	81.5	75.7

Average Weight of Plants at Harvest:

As the table indicates the average weight per plant reduced by about -7% from the FPK Control to RPK (p=0.06). Treatment with BLG resulted in plants that had increased weight of 15.3% (p=0.00) when comparing FPK + BLG with FPK Control, and an increase of 10.8% (p=0.01) for the RPK+BLG when compared to RPK Control.

Table 5: Report #1 – Comparison of Plant Size at Harvest for all-treatments

Treatment	Treatment Compared With	Percent Difference (%)	p-value
RPK Control	FPK Control	-7.0%	0.06
FPK + BLG	FPK Control	15.3%	0.00
RPK + BLG	RPK Control	10.8%	0.01

Table 5, above provides the comparison with percent difference calculations and the corresponding p-values. As the data indicates, even at reduced fertilizer application rate, the weight of the plant at harvest for BLG treated

plants was about 3% more than the full fertilizer plants FPK control. The extremely low p-values for the comparison are strong evidence of the probability of treatment effect for increase in plant size.

Increase in the Number of Pods/Plant:

The average number of pods per plants only decreased by 1.0% (p=0.78) when comparing the full fertilizer program (FPK) with the reduced fertilizer program (RPK). The relatively small magnitude of difference meant that the number of pods per plant did not decrease due to the reduction of nutrient applied in this trial. However, treatment with BLG did significantly increase the number of pods per plant for both FPK and RPK fertilizer programs. When comparing FPK + BLG with FPK control there was a 19.5% increase in the number of pods/plant from an average of 41.9 for control plants to an average of 50.1 for treated plants (p=0.00). Similarly, for the reduced fertilizer program (RPK), treatment with BLG resulted in 11.5% increase in the average number of pods per plant from 41.5 to 46.3 (p=0.00). The results once again show a strong statistical probability of BLG treatment causing an increase in the number of pods per plant for the soybean crop studied. **Table 6** provides the comparison data for the number of pods per plant and the p-values.

Table 6: Report #1 – Comparing the Average Number of Pods per Plant

Treatment	Treatment Compared With	Percent Difference (%)	p-value
RPK Control	FPK Control	-1.0%	0.78
FPK + BLG	FPK Control	19.5%	0.00
RPK + BLG	RPK Control	11.5%	0.00

Increase in Yield:

To calculate yield the soybean plants were randomly bundled in groups of 6 and threshed. Each replicate, thus, resulted in 5 measurements of seed weight. The total weight of the seeds for each group of 6 soybean plants was measured, providing 5 seed measurements per replicate and with 6 replicates per treatment, 30 measurements of seed weight per treatment. Three moisture measurements were taken from the seeds from each replicate. The average moisture content ranged between 8.4% for the RPK control to 10.5% for the FPK control. To remove the effect of moisture content variations on grain yield the average moisture content for each replicate was used to normalize the seed weights measured to a moisture content of 15.5%.

The normalized (15.5% moisture) average seed weights were divided by 6 to provide the per plant weights shown on **Table 4**. The total weight of seeds for the 180 plants per treatment is calculated by summing up the 30 weights taken per treatment. The total weight is used to calculate the yield in bushels per acre. The yield was calculated based on the 120,000 plants per acre (300,000 plants per hectare) planting rate. **Table 7** provides a comparison of the yield data across the four treatments. The yield decreased from 70.9 bu/ac for the FPK control to 67.1 bu/ac for the RPK control, equating to a decrease of 5.4% (p=0.27). From an economic perspective losing 5% of yield would be significant, but because only 30 data points contributed to these average values, the magnitude of the difference was not sufficient for these results to achieve our alpha target of 0.15. Thus it could be concluded that the 30% reduction in the applied fertilizer may have caused a reduction in the yield, but the difference in the yields observed is not large enough to make a conclusive claim of that.

Table 7: Report #1 – Comparison of Yield Data

Treatment	Treatment Compared With	Percent Difference (%)	p-value
RPK Control	FPK Control	-5.4%	0.27
FPK + BLG	FPK Control	14.9%	0.00
RPK + BLG	RPK Control	12.8%	0.02

When comparing the yield increase resulting for the application of BLG at full-fertilizer program (FPK) the yield increased by 10.6 bushels per acre from 70.9 to 81.5 equating to a 14.9% increase (p=0.00). At reduced fertilizer program use (RPK) treatment with BLG increased yield from 67.1 to 75.7 equating to a 12.8% increase (p=0.02). Both increases due to the treatment effect are both biologically and economically significant with a 10.5 bu/acre increase in yield under the full-fertilizer program and a 8.6 bu/acre increase in yield under the reduced fertilizer program. **Table 8** provides the descriptive statistics for the groups of 6 soybean plants seed weights from which the yield was derived.

Table 8: Report #1 – Descriptive Statistics of the Seed Weight Measurements (6 plants)

	FPK	RPK	FPK + BLG	RPK + BLG
Mean	120.8	114.2	138.7	128.8
Standard Error	3.7	4.5	4.1	4.1
Median	117.2	104.2	136.6	124.6
Standard Deviation	20.3	24.9	22.2	22.6
Sample Variance	413.4	619.8	494.8	509.0
Kurtosis	-0.9	0.5	6.0	1.5
Skewness	0.2	1.1	1.6	1.1
Range	73.1	97.9	121.0	98.0
Minimum	85.1	82.8	101.2	90.8
Maximum	158.2	180.7	222.3	188.8
Sum	3623.2	3427.3	4161.4	3864.5
Count	30	30	30	30
Confidence Level(95%)	7.6	9.3	8.3	8.4

Agronomic Efficiency:

For this trial we can calculate the AE based on the reduction of phosphorous and potassium fertilizer applications. **Table 9**, below, provides the AE calculations based on the total amount of phosphorous (P_2O_5) applied., while **Table 10** provides the AE calculations based on potassium (K_2O) for all four treatments. As the tables indicate, based on phosphorous the lowest AE is calculated as 65.2 for FPK control, while the highest AE is calculated for the RPK + BLG treatment at 99.6. Thus, by reducing the fertilizer application by 30% and using BLG, in this trial the AE of phosphorous application was increased by about 53%. The agronomic efficiency is improved with BLG treatment for both full fertilizer program and reduced fertilizer program.

The AE calculated on the basis of the difference in yield between BLG treated and control per unit of P_2O_5 applied for the full fertilizer program is calculated at 9.68. Thus, under FPK where 92.7 kg/ha of P_2O_5 was applied, BLG resulted in 9.68 kg/ha increase in soybean yield per kg/ha of P_2O_5 applied. The AE increases to 11.3 kg/ha of soybean yield increase per kg/ha of P_2O_5 applied under RPK conditions where the P_2O_5 applied was reduced by 30% to 64.7 kg/ha.

Table 9: Report #1 – Agronomic Efficiency (AE) calculation based on the amount of phosphorous applied

	Yield (kg/ha)	Yield (bu/ac)	Nutrient Applied (kg P2O5/ha)	AE
FPK	6,039	70.9	92.7	65.2
RPK	5,712	67.1	64.7	88.3
FPK + BLG	6,936	81.5	92.7	74.8
RPK + BLG	6,441	75.7	64.7	99.6

Similarly, when the AE is calculated based on potash application, the lowest obtained AE was for the FPK control at 95.5. Reducing the potash use by 30% from the recommended rate improved the AE to 119.6. Combining the reduction with BLG use increased the AE to 134.9. Overall, there was a 41% improvement in the AE comparing the FPK control with RPK + BLG. On the basis of K_2O application at 63.2 kg/ha usage rate (FPK), BLG application resulted in an AE of 14.2 kg/ha increase in soybean yield per kg/ha of K_2O applied. With reduced fertilizer program, there was only 47.7 kg/ha of K_2O applied, and the AE increased to 15.3 kg/ha of soybean yield increase per kg/ha of K_2O applied.

Table 10: Report #1 – Agronomic Efficiency (AE) calculation based on the amount of potash applied

	Yield (kg/ha)	Yield (bu/ac)	Nutrient Applied (kg K_2O/ha)	AE
FPK Control	6,039	70.9	63.2	95.5
RPK Control	5,712	67.1	47.7	119.6
FPK + BLG	6,936	81.5	63.2	109.7
RPK + BLG	6,441	75.7	47.7	134.9

Statistical Analysis:

The first step we took in analyzing the data was to obtain the descriptive statistics for each of the measurements. **Table 8** provided the descriptive statistics of the seed weight measurements in groups of 6 plants. This data was used to calculate the yield values in bushels per acre. **Table 11** below provides the descriptive statistics for the number of pods per plant. Single-Factor Anova test was ran comparing each of the treatments to control. The p-values for the results of the analysis are reported in the previous tables. We assigned an alpha-value of 0.15 to this efficacy trials and designed the experiment with the target of achieving a significance level lower than 0.15.

In examining the significance of our trial data, the principal criterion is that the product must produce biological benefit to the crop or economic value to the grower**.** Both of which were met in this trial with sufficient magnitude of difference resulting from treatment in relation to the variability within the dataset. Treatment with BLG resulted in p-values that were extremely low $p<0.02$ for average yield, plant weight and number of pods per plant.

Table 11: Descriptive Statistical Analysis of Pods per Plant

	FPK	RNPK	FPK + BLG	RPK + BLG
Mean	41.9	41.5	50.1	46.3
Standard Error	1.0	1.0	1.1	1.0
Median	41.0	41.0	48.0	46.0
Standard Deviation	13.2	13.5	14.4	13.5
Sample Variance	173.4	181.4	206.0	182.3
Kurtosis	-0.1	-0.6	0.1	-0.4
Skewness	0.2	0.1	0.4	0.2
Range	66.0	62.0	73.0	67.0
Minimum	12.0	11.0	15.0	14.0
Maximum	78.0	73.0	88.0	81.0
Sum	7212.0	7309.0	8772.0	8194.0
Count	172.0	176.0	175.0	177.0
Confidence Level(95%)	2.0	2.0	2.1	2.0

Conclusions:

In conclusion, BLG significantly improved the agronomic efficiency (AE) of MAP and potash applied to soybeans. In this study two different rates of fertilizers were used. The full fertilizer program was based on the recommended rate of use of MAP and potash by the growers' consulting agronomist. This rate was reduced by 30% to establish a reduced fertilizer program. The 30% reduction in MAP and potash use rate resulted in a **-5.4%** yield reduction, though due to the high calculated p-values (p=0.27) the magnitude of the difference wasn't large enough to reject the null-hypothesis. When BLG was applied, the nutrient use efficiency was improved and there was significant yield improvement for both the full (FPK) and reduced (RPK) fertilizer programs. Applying BLG under the FPK program resulted in 14.9% (p=0.00) yield increase over the control. Under the RPK fertilizer program, application of BLG resulted in 12.8% yield increase (p=0.02). As expected, the lowest yield results were seen in the RPK control with an average of 67.1 bu/ac (6,039 kg/ha) and the highest were seen when BLG treatment was used on the FPK program (FPK + BLG) with an average of 81.5 bu/ac (6,936 kg/ha). The agronomic efficiency was improved by 53% from 65.2 for the FPK to 99.6 for the RPK + BLG based on phosphorous application and by 41% from 95.5 to 134.9 based on potassium application.

Treatment with BLG also resulted in significant increase in the average plant weight at harvest of 15.3% (p=0.00) for FPK fertilizer program and 10.8% (p=0.01) for the RPK fertilizer programs. The number of pods per plant were also improved significantly by BLG treatment with an increase of 19.5% (p=0.00) for the FPK fertilizer program, and 11.5% (p=0.00) for the RPK program. Overall, the evidence generated through this study provides strong support for the biostimulant claim of improved nutrient use efficiency when using BLG fulvic acids on crops.

Improving Nutrient Use Efficiency in Corn with BioLiNE® Gold Fulvic Acids

Report #2: Improving NUE in Corn with BioLiNE® Gold Fulvic Acids – 2021

Introduction:

This trial was carried out by CNIRGY Agronomics. For these trials Dr. Kelly Morris (Technical Services Manager, CNI Ag) in collaboration with Cropsmith, Inc. carried-out the trials on corn (*Zea mays*). BioLiNE Gold was one of eight different products tested against the grower's standard in this replicated field trial.

Trial Objective:

- The intended claim evaluated by this trial was nutrient use efficiency.
- The crop chosen for the study was **corn** (Golden Harvest Hybrid GH 5122A)
- Replicated field trial with 5 replicates per treatment. Each replicated plot is 12m (40ft) x 3m (10ft)

Trial Conditions:

Growing Season: 2021

Trial Location: NE 80 Field, 26693 Prairie Chapel Rd, Farmer City, IL (40.2°N, 88.6°W)

Soil Type: Silty clay loam with 2 -10% slope

Number of Replicates: 5 reps per treatment, and eight products treated

Plot Size: Each replicated plot is 12m (40ft) x 3m (10ft)

Planting Date: June 1st, 2021

Corn Seed Variety: Golden Harvest Hybrid GH 5122A

Planting Population Density: 86,500 plants per hectare (35,000 per acre) with 76cm (30") row spacing.

Crop Rotation: Corn-Soybean rotation.

Experiment Design: Randomized complete block design with 6 replicates per treatment.

Soil Analysis (Lab Num 17818):

Table 12: Report #3 – Soil Analysis for the field where the trials were conducted

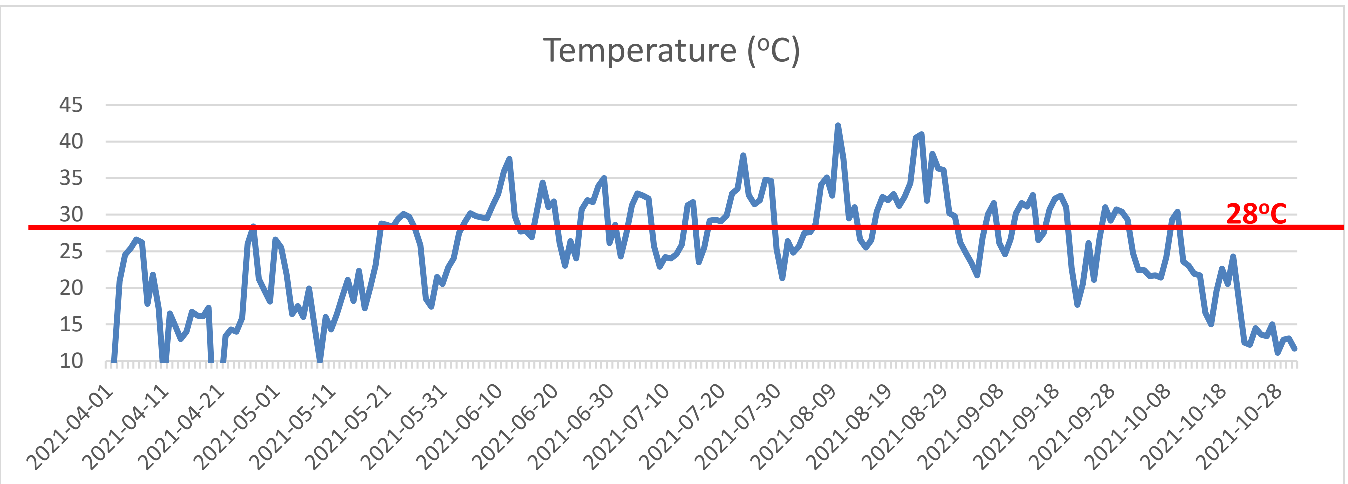
Column	Element	Value	Unit
OM_%t	OM	4.2	%
pH_1_1	pH	6.7	pH
CEC_meq_100g	CEC	16.8	meq 100g
P_Bray_1_ppm	P	11	PPM
K_ppm	K	105	PPM
Mg_ppm	Mg	400	PPM
Ca_ppm	Ca	2400	PPM

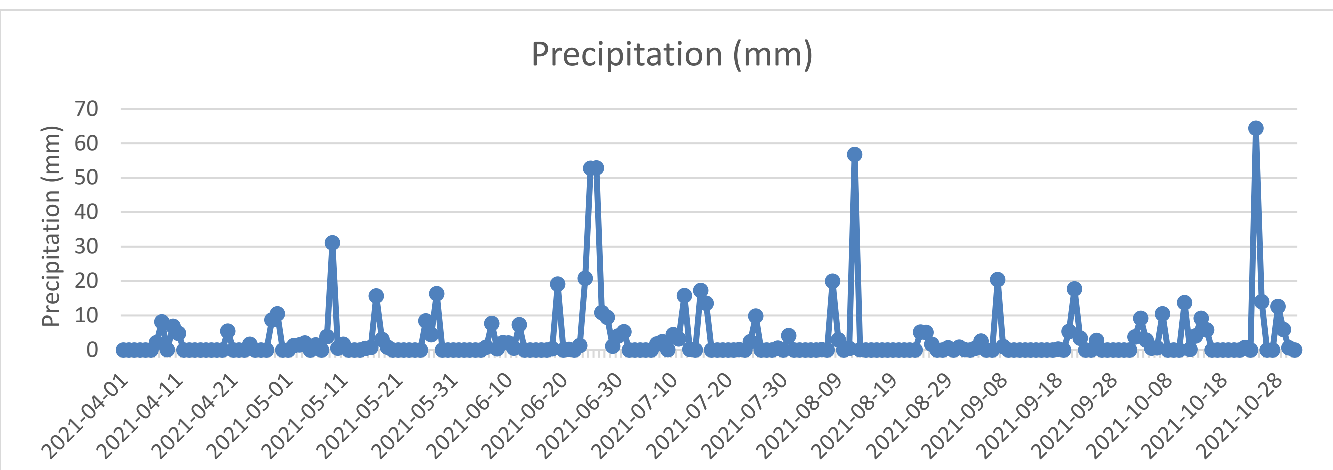
S_ppm	S	6	PPM
Zn_ppm	Zn	2.8	PPM
Mn_ppm	Mn	33	PPM
Fe_ppm	Fe	25	PPM
Cu_ppm	Cu	1.4	PPM
B_ppm	B	0.3	PPM

Climatic Conditions:

The data on climate condition are measurements made by several weather stations near the location accessed through Visual Crossing (https://www.visualcrossing.com).

Figure 2: Temperature and precipitation profiles at trial location





Application of the Product:

Growers Standard Dry Fertility program + Side-dress UAN:

- UAN (32-0-0) Side-dress 14.7 kg/ha (80 lbs/ac.)

Treatment Applications:

- Growers Standard
- BLG (In-Furrow) plus two foliar applications

Treatment Dates:

In-Furrow: June 1st, 2021 at 10 fl. oz/acre (740 mL/ha)

1st Foliar: July 2nd, 2021 with post emerge herbicide at 17 fl. oz/acre (1235 mL/ha)

2nd Foliar: At V4 stage at 17 fl. oz/acre (1235 mL/ha)

Data Measurements Collected:

For this trial the following data was collected at harvest:

- Stand count on June 16th, 2021
- Yield at Harvest

Trial Results:

The five replicated plots per treatment were randomly positioned for all nine treatments including the grower's standard. Number of plants per plot was assessed and converted to plants per hectare. **Table 13** provides a summary of the yield and stand count results for BLG treated plots compared to the grower's standard program. The table includes percent differences and the corresponding p-values.

Table 13: Report #3 – The average and standard deviation for ear girth (cm), ear length (cm), and kernel wt.

	Growers Standard	BLG Treatment	Percent Difference (%)	p-value
Stand Count (plants/ha)	82,478	82,209	-0.33%	0.44
Yield (kg/ha)	10,104	10,827	7.16%	0.10
Per Plant Yield (kg/plant)	0.131	0.142	7.98%	0.03

The stand count for the BLG treatment as compared to the grower standard did not change with only a 0.3% difference between the two and a high p-value of 0.44. There are many factors that can contribute to the emergence of the corn. We do not expect differences in stand count in well run small plot trials on good agricultural soils.

Yield and Yield Per Plant:

There was an economically meaningful and a statistically significant improvement in the corn grain yield measured for the BLG treated program in comparison with the grower standard program. The overall plot yield was converted from lbs/plot to kilograms per hectare resulting in a 723kg per hectare (7.2%) increase in yield. Using a single-factor Anova test to compare the results the p-value was measured as 0.10.

Table 14: Report #3 – Descriptive Statistics for the Yield Data

	Grower's Standard	BLG Treatment
Mean	10,111	10,890
Standard Error	356	232
Median	10,321	11,086
Standard Deviation	797	518
Sample Variance	634,584	268,589
Kurtosis	3.26	3.86
Skewness	-1.70	-1.90
Range	2,050	1,304
Minimum	8,757	9,991
Maximum	10,807	11,295
Sum	50,553	54,448
Count	5	5
Confidence Level (95.0%)	989	643

Table 14, provides the descriptive statistics for the yield data collected for the trial. We calculated the per plant yield for a given replicate by dividing the weight measured for the plot (yield) by the stand count for that respective plot. The per plant grain yield increased from 0.131 kg/plant to 0.142 kg/plant an increase of 8.0%. The p-value for the per plant yield increase was calculated as 0.03.

Agronomic Efficiency:

The agronomic efficiency (AE) of the UAN side-dress treatment can be calculated by dividing the yield by the weight of nitrogen applied from the UAN side-dress treatment. **Table 15**, below provides the AE calculations. There was 80 lbs/acre (14.7kg/ha) of N applied as side-dress to all plots. The calculated AE for the N applied in the untreated check is calculated at 687.3. Due to the increase in yield, the AE of the N applied increased to 736.6.

Table 15: Report #3 – Agronomic Efficiency of UAN based on kg of N applied

	Yield (kg/ha)	Yield (bu/ac)	Nutrient Applied (kg N/ha)	AE
Control (Growers Standard)	10,104	161	14.7	687.3
BLG Treatment	10,827	173	14.7	736.6

Statistical Analysis:

The first step we took in analyzing the data was to obtain the descriptive statistics. The descriptive statistics for the yield data is provided in **Table 14.** Single-Factor Anova test was run comparing the BLG treatments to control (growers standard). The p-values for the results of the analysis are provided in table 13.

Conclusions:

Corn (*Zea mays*) is an important staple cereal grain cultivated globally. In the U.S state of Illinois alone there were 4.5 million hectares (11 million acres) of corn planted in 2021. This trial was carried out by CNI Ag as part of their crop input evaluation program. The trial included eight different products that were compared to the growers standard and applied according to the manufacturers' timing and application rates. There was a total of 3.2 litres per hectare (44 fl. oz/acre) of BioLiNE® Gold (BLG) fulvic acids applied in the season. The application included 0.74 L/ha (10 fl. oz/acre) in-furrow at planting, plus two foliar applications of 1.2 L/ha (17 fl. oz/acre) applied. The first foliar application was tank-mixed with the post-emerge herbicide and the second was applied at V4 tank-mixed with a fungicide. The use of BLG fulvic acid improved the yield of corn. The average yield increase by 7.2% with a p-value of 0.10. When the average yield per plot was divided by the number of plants, as measured by the stand count, the resulting yield increase per plant was 8.0% with a p-value of 0.03. This was due to a slightly lower stand count for the BLG treated plots versus the grower standard. There was 14.7 kg per hectare of nitrogen applied in the form of UAN (32-0-0) side-dress resulting in 723kg/ha (12 bu/ac) increase in yield. This resulted in an increase in the AE of the nitrogen applied from 687.

Conclusions on the NUE and Yield Improvements Achieved by BLG Fulvic Acids

Reduction in fertilizer use can have significant positive impact on farm profitability as well as significant environmental benefits, including reduction in water pollution and GHG emissions. Fulvic acids (FA) are the smallest size fractions of humic substances that remain in solution at low pH. Higher concentrations of carboxylic acid and phenolic functional groups in FA have been associated with improved nutrient use efficiency in a variety of crops. The availability of electronegative donor atoms (oxygen) contributes to the much higher cation exchange capacity of FA and can chelate metals with a high affinity. The lower molecular size FA (< 3500 Da) have been demonstrated to reach the plasmalemma of higher plant cells, whereas larger size humic substances can only interact with the cell wall of plants.

FA and HA have been studied extensively for their crop beneficial properties including their ability to complex nutrients and improve nutrient uptake by plants. The application of fulvic acid in soil enhances the solubility and bioavailability of phosphates. Anionic sites in fulvic acids can compete with phosphate ions in soil for adsorption sites, reducing P fixation and requiring lower application of phosphate fertilizers.

Many researchers have studied the ability for fulvic acids to complex micronutrients, thereby improving the transport and assimilation of nutrients. The use of FA has been shown to improve cell permeability of numerous nutrients in plants. The high number of carboxylic, phenolic, hydroxyl, and amino functional groups in BLG allow for effective metal chelation.

Fulvic acid also increases the level of nitrogen fixation in the soil by aiding the colonization and activity of *Rhizobium* bacteria. Fulvic acids also aid in nitrogen retention in soil by binding to nitrogen compounds and increasing available nitrate-N. FA supports production of enzymes involved in inorganic nitrate assimilation and reduction in plants and have been shown to contribute to the direction of signaling pathways depending on the availability of nitrate. An increased degree of bioactivity of humic substances in nitrogen assimilation in plants is linked to a smaller molecular size and higher hydrophilic character, highlighting the advantages of fulvic acids over other humic products. These modes of action are universal to all plant taxonomic groups, and are not impacted by the country or growing media the plants are grown in. In this report we have complied three studies carried out using BLG to investigate the impact BLG FA has on improving nutrient use efficiency. The studies observed improved yield data when comparing BLG with control.

The agronomic efficiency of the nutrient applications has been calculated and in both reported trials there is significant improvement when BLG treatment is made. The statistical analysis of the data shows very strong evidence in support of treatment response. In the studies on soybean carried out in 2023, a 30% reduction in fertilizer use when combined with BLG application resulted in the highest agronomic efficiency, and increased profitability for growers. The economic benefits to growers are significant. Growers are encouraged to experiment with optimizing their nutrient use by incorporating BLG into their cropping program.