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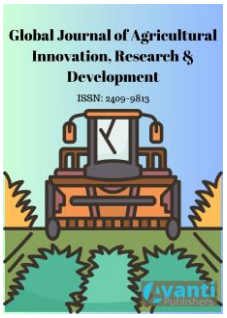

Priming Bean Seedlings to Boost Natural Plant Defenses Against Common Bacterial Wilt: Gas Exchange, and Fluorescence Results (Part 2)

Craig L. Ramsey[1,*], Vanessa M. Sandoval[2], Paul C. Freebury[3], Debra H. Newman[4], Greg Dooley[5], Leland J. Cseke[6] and Steven E. Newman[7]

[1]Retired – USDA, Fort Collins, CO 80526, USA; [2]Former Graduate Student, Colorado State University, Fort Collins, CO 80526, USA; [3]Colorado State University, Plant Growth Facilities, Fort Collins, CO 80526, USA; [4]Former Research Associate, Colorado State University, Fort Collins, CO 80526, USA; [5]Assoc. Prof. Dept of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80526, USA; [6]University of Missouri, Division of Plant Science and Technology, Interdisciplinary Plant Group, and CS Bond Life Sciences Center, Columbia, MO 65211-7310, USA; [7]Emeritus - Colorado State University, Fort Collins, CO 80526, USA

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ABSTRACT

This greenhouse study evaluated the effects of two chemical primers for kidney bean seedlings against a bacterial wilt (*Curtobacterium flaccumfaciens pv. Flaccumfaciens*) (CFF). The premise of this study was that the oxidant primers would mimic the signaling properties of radical oxygen species and initiate a cascade of molecular defenses. The factorial study included two levels for the foliar chlorine dioxide treatment, and two levels for the bacterial wilt inoculation treatment, plus two supplemental chemical treatments. The foliage response variables were gas exchange and fluorescence. There was a 36, 154, and 70% reduction in Pn, gs, and E, respectively, at 39 DAT when comparing the inoculated control to the non-inoculated control. The chlorine dioxide primers lowered leaf temperatures and leaf vapor pressure deficit in the CFF wilt inoculated plants. The chlorine dioxide primers improved gas exchange at 39 DAT when compared to the water treatments. Part 1 and 2 of this series conclude that the chlorine dioxide primers can activate a long-term, systemic acquired resistance (SAR) response in kidney bean plants infected with the CFF wilt. The Part 2 article also concludes that the EB treatments caused several inexplicable correlations among the gas exchange responses. A structured water premise was proposed as an explanation for the gas exchange anomalies due to the EB treatments. Intuitively, this study suggests that chlorine dioxide primers can initiate a series of ROS and salicylic acid signals that activate a suite of mechanisms that provide universal, multifaceted plant immunity that is sustained across a crop season.

*Corresponding Author
Email: clramsey37@gmail.com
Tel: +(1) 970-988-7949

1. Introduction

This article (Part 2) is the second of three articles that report on a two-year study involving the effects of chemical inducers on kidney bean seedlings inoculated with a vascular wilt disease. The first article (Part 1) [1] reported on the salicylic acid results while this article (Part 2) reports on the gas exchange, and fluorescence results. The third article (Part 3) will report on the leaf morphology, leaf area, and biomass results.

Development of chemical primers typically involves researching molecular signaling agents that activate a cascade of natural plant defenses for partial or complete protection against a broad spectrum of diseases, herbivores, and abiotic stresses [1-3]. Previous research has shown that several chemical primers have the potential to pre-program plant immunity systems in preparation for full activation against any pathogen attack [4-7]. Foliar application of chemical primers quasi-activate or prime plant defenses, much like vaccines boost immune systems to alleviate acute symptoms and speed recovery from viral attacks. Primed plants develop a series of innate defenses that allows a more rapid and robust response to pathogen attacks or abiotic stressors [8-10]. There is an ongoing debate, however, whether chemical primers can activate a Systemic Acquired Resistance (SAR) response that provides more long-term, systemic immunity [10-16].

Chlorine dioxide (ClO_2) has proven to be an effective chemical inducer for priming plant defenses [17-21]. Chlorine dioxide can be formulated with surfactants and still maintain their effective oxidant properties [17, 19-21]. The ClO_2 formulations evaluated in this study are patented, labeled for crop applications, and can be applied to plant foliage with minimal foliage injury. They also contain surfactants that plasticize the waxy leaf cuticle, allowing transport of the ClO_2 soluble gas into the vascular system. Once inside the phloem ClO_2 produces Radical Oxygen Species (ROS) signals that elicits a defense response [22-26]. Research has shown that single application of chlorine dioxide to plant foliage elevates gas exchange and fluorescence responses in plants [17, 18]. This same patented ClO_2 formulation was also evaluated as a foliage treatment to prime rhododendrons inoculated with *Phytophthora ramorum* in a field nursery study conducted by Ramsey *et al*. [17]. Another chemical inducer evaluated in this study is Actigard which is a commercial product that is an analog for salicylic acid.

Gas exchange and fluorescence measurements have been proven to be reliable monitoring methods to detect and determine the degree of disease severity and injury in infected crops [27-30]. Periodic monitoring of crop foliage provides insight into the temporal dynamics of crop indicators that help managers decide where and when to apply treatments to avoid crop losses to a pathogen outbreak. Photosynthesis (Pn) is a function of stomatal conductance (gs), internal CO_2 concentration (Ci) and transpiration (E), which in turn are correlated to the severity of wilt diseases [27-29]. In plants with a severe wilt infection, the microbes progressively clog the xylem which mimics water stressed plants with a concomitant reduction in gas exchange and fluorescence rates.

Numerous studies have investigated the relationships between diseased plants, gas exchange, and fluorescence responses. Bassanezi *et al*. [30] evaluated physiological responses of *Phaseolus vulgaris* to three diseases including monocycle of rust (Uromyces appendiculatus), angular leaf spot (Phaeoisariopsis griseola), and anthracnose (Colletotrichum lindemuthianum). They found an indirect correlation between disease severity and Pn, gs, and E rates. A study by Lorenzini *et al.* [27] evaluated Pn responses of tomato plants that were inoculated with two vascular wilt diseases. The wilt diseases were *Fusarium oxysporum* f. sp. *lycopersici* (Fol) and *Verticillium albo-atrum* (Vaa). Plants infected with Fol had reductions in Pn, gs and E at 7, 14 and, 21 days after inoculation (DAI) when compared to the control. Plants infected with Vaa also showed a reduction in Pn, gs, and E, at 21 DAI. They state that measuring asymptomatic leaves allowed detection of physiological injury due to the disease a week before visual observation of symptoms in the leaves.

Fluorescence measurements are a non-specific method of monitoring plant stress. A common fluorescence parameter used to monitor plant stress is maximum quantum efficiency (Fv/Fm). Several field studies have monitored seasonal, or long-term patterns in Fv/Fm measurements. A nursery study by Ramsey *et al*. [17] monitored the effects of the chemical inducers on rhododendrons inoculated with *Phytophthora ramorum* using three measurements of Fv/Fm over a five-month period. The temporal pattern of Fv/Fm over the three measurement dates showed a long delay of about 90 days before the chlorine dioxide inducers affected chlorophyll efficiency

levels. Also, at five months, inoculated plants treated with a single application of chlorine dioxide revealed a multi-month delay before there was an increase in chlorophyll efficiency levels. A citrus field study by Weng *et al*., [31] monitored citrus greening disease using Fv/Fm measurements in two orchards over two separate multi-month periods (6 and 12 months). They found that Fv/Fm had temporal patterns and accurately differentiated citrus greening positive leaves from citrus greening negative leaves with 92% accuracy [31]. This study didn't include any chemical treatments, but it did show that long term crop monitoring using Fv/Fm measurements is possible and offers high accuracy results. A study by Brouwer *et al*. [32] correlated the progression of pathogen biomass over time with Fv/Fm measurements in *Arabidopsis thaliana*. They found accurate correlations between the pathogen biomass, which is synonymous with disease severity, and reductions in chlorophyll efficiency. A study by Ierna found Fv/Fm differences in potato phenotypes when measured across two seasons [33].

This study evaluated the effects of chlorine dioxide and Actigard for priming the natural defenses of kidney bean (*Phaseolus vulgaris*) plants inoculated with a common bean bacterial wilt caused by *Curtobacterium flaccumfaciens* pv *flaccumfaciens* (CFF) [34]. The factorial study tested the interactions between five chemical treatments applied to the foliage and two CFF wilt levels (non-inoculated and inoculated treatments). The overall goal of this project was to evaluate chemical primers for their ability to induce a long-term, systemic plant immunity response and thereby reduce pesticide applications on row crops. This section of the study (Part 2) had two specific hypotheses. The first hypothesis was that the chemical primers would minimize the negative impacts of the CFF wilt on gas exchange responses when compared to the water control treatment. The second hypothesis was that the chemical primers would also minimize the negative impacts of the CFF wilt on chlorophyll efficiency (Fv/Fm) when compared to the water control treatment.

2. Materials and Methods

2.1. Study Design

The greenhouse study was conducted at the United States Department of Agriculture's Crop Research Laboratory greenhouses in Fort Collins, CO. The study had a factorial design with two factors, plus supplemental treatments using water and Actigard. The two study factors were chlorine dioxide applied at two rates, and plants inoculated or non-inoculated with the CFF wilt bacteria (Table **1**). Each treatment was replicated twelve times.

Table 1: Description of the chemical inducer treatments and CFF inoculation status.

Chemical Treatment	Concentration (mg/l)	CFF Inoculation Status
EB	200	Yes
EB	200	No
EB	400	Yes
EB	400	No
Actigard	60	Yes
Actigard	60	No
Water	0	Yes
Water	0	No

2.2. Description of Plant and Soil Material

Light red kidney beans (*Phaseolus vulgaris* L.) (Johnny Select Seeds, Winslow, Maine)were planted in fiber pots (6.8 L/pot) (Western Pulp, Corvallis, Oregon). The kidney beans were a bush variety with a determinate growth pattern. Also, kidney beans are annuals with a 60-to-80-day life span. The bean plants were fully mature and entering senescence at the end of this two-month study.

The pots were filled with Farfard 4-MP potting medium (Sun Gro Horticulture, Agawam, Massachusetts). This potting soil was a mix of Canadian sphagnum peat moss, processed pine bark, vermiculite, and perlite with an average field capacity range of about 75 to 85% soil moisture. Four bean seeds per pot were planted, and once the seeds germinated, they were culled to the two most vigorous seedlings. Two seedlings per pot were left to evaluate the different plant responses. One seedling was used for the first salicylic acid concentration measurements. The second seedling remained in the pot to take measurements on gas exchange, fluorescence, final SA measurement, leaf area, and biomass during the final harvest at 61-63 days after planting. Plants were watered on an "as needed" basis and kept midway to field capacity to avoid overwatering issues. Plants were fertilized with NPK (20-2.2-8.3) (Jack Peters Professional Lite 20-10-20 Allentown, Pennsylvania) soluble fertilizer through fertigation methods at 100 mg/l as irrigation was needed. After inoculation, plants were fertilized once per week. Greenhouse parameters were set for 27 C daytime temperatures and 17 C nighttime temperatures and 14:10 h light/dark schedule.

2.3. Description of Common Bean Bacterial Wilt

The bacterium used in this study was *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (CFF) which causes common bean bacterial wilt in *Phaseolus* species [34]. This disease was a problem for dry bean production in Colorado, Nebraska, and Wyoming during the 1960's to the early 1970's [34, 35]. The main dispersal of CFF wilt is through infected seed, but soil and infected debris can be a reservoir of inoculum [35, 36]. Symptoms of CFF wilt disease may include stunting and reduced yields for milder infections or even mortality for more severe cases [37]. The best management practice is to use wilt resistant cultivars and/or purchase clean seed since there is no pesticides that control CFF wilt in common beans [36, 37].

Common symptoms of CFF infection on *Phaseolus* are wilting of the leaves and necroticlesions with a yellow halo on the leaves [34, 37-39] . Vascular wilts are unique in that they thrive and multiply in the xylem where it is nutrient deficient [37, 39]. The bacterium clogs up the vascular system causing a drought-like stress that prevents water transport into the foliage [36, 37]. After the early 1970's CFF wilt was not a problem for bean production in the United States but has re-emerged as pathogen of concern for bean crops since 2003 [35, 40-42]. This disease has restricted international trade as CFF wilt disease is on the quarantine list for many countries [35, 37].

2.4. Common Bean Bacterial Wilt Inoculation Methods

The yellow race (B-528) of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (CFF) was used to inoculate the bean seedlings on four Days After Treatment (DAT). The CFF bacterium was cultured on nutrient broth yeast extract medium (NBY) and incubated at 22 C. Plates were re-cultured on new NBY plates to ensure a pure culture with a Colony Forming Unit (CFU) count of approximately 10^8. The NBY media ingredients were mixed in 1000 ml of distilled water and then autoclaved for 15 minutes. Then 6.16 g of $MgSO_4$ was dissolved into 25 ml of distilled water. Next 1 ml of the solution was added to the autoclaved agar mixture with a sterile syringe and Millex -GS 0.22 µm filter unit (Millipore Corporation Bedford, MA). The agar was added to petri dishes and allowed to continue to cool overnight under a flow hood.

To ensure optimal CFF inoculation conditions the greenhouse parameters were set for 32 ^{o}C and close to 100% relative humidity for inoculation one day prior to inoculation. After inoculation plants were conditioned for 48 hours under humid and hot conditions to ensure successful inoculation of CFF wilt in the kidney bean plants.

Plants were inoculated using the stem injection method when the seedlings were 20 days old, or 4 DAT and 16 Days After Planting (16 DAP). Plant inoculation took about two hours each morning. On the fifth day after chemical treatment (5 DAT) the plants were sampled for their first foliage harvest between 17 to 19 h after inoculation. Sterile 20-gauge BD Precision Glide (Becton Dickinson and Company, Franklin Lakes, NJ) needles were dipped in CFF pure cultures and then inserted at the cotyledonscar. A new sterile needle for inoculation was used for each treatment. Plants that were not inoculated were mock inoculated with sterile needles without CFF inoculum to ensure all plants were given the same mechanical injury from the needle and environmental treatment.

2.5. Description of Chlorine Dioxide and Actigard

The chlorine dioxide formulation used in this study was Electro-Biocide (EB) (SRO Inc., Denver, CO) which is a proprietary blend of chlorine dioxide, surfactant, and a pH buffer. The EB patents include a sarcosinate surfactant in the EB formulation that enhances droplet adherence and uniform spread on the foliage with minimal foliar injury. The surfactant also semi-plasticizes the epicuticle wax layer, which allows the ClO_2 to be absorbed and transported in the phloem. The EB formulations are available from SRO and Energis Solutions. Current EB labeling allows applications for row crops and hydroponic and aeroponic uses. Additional label enhancements are in process. The oxidant properties of EB mimic a surge of radical oxygen species (ROS) within the vascular system, which signals the initiation of a cascade of molecular processes that prime plant defenses [23-26].

Actigard (Syngenta, Basel,Switzerland) is formulated with the active ingredient acibenzolar-S-methyl (ASM), a functional analog to salicylic acid. Actigard has a unique mode of action that belongs to a category called "Host Plant Defense Induction; Group P1". Actigard induces host plant resistance by mimicking a SAR response found in most plant species, i.e., it has no direct activity against target pathogens. Actigard is a commercial chemical inducer, that primes plant defenses within four days after foliar application.

2.6. Chemical Application Methods

There were four different spray treatments. The EB formulations were prepared by Strategic Resource Optimization, (SRO, Denver, CO). Spray treatments were EB at 200 and 400 mg/l, Actigard at 60 mg/l, and tap water. Spray treatments were conducted on two consecutive days to allow enough time to measure each plant but maintain an equal number of measurement days after treatment.

Plants were sprayed by a low-volume, electrostatic sprayer (ESS Electrostatic Spraying, Watkinsville, GA) (Fig. **1**). The batteries were removed since the electrostatic charge from the sprayer would interfere with the inherent electrochemical charges in the EB formulations. Each plant had an 18 second spray application, with nine seconds for both the top and bottom side of the foliage. The spray application rate was 3.8 l/h or 1.055 ml/sec with an average droplet size of 40 microns. The liquid pressure was 103 mPa and the air pressure ranged from 207 to 276 mPa.

Figure 1: Chemical application to plant foliage using the ESS electrostatic sprayer.

2.7. Gas Exchange Methods

Gas exchange measurements of foliage physiology variables were measured 18, 32, and 39 DAT using a LICOR-6400 XT (LICOR Environmental, Lincoln, NE) (Fig. **2**). Plants were well watered each morning before taking the gas exchange measurements to ensure they had uniform soil moisture conditions. In addition, plants were placed under

LED plant grow lights for at least 15 minutes with a LED lamp light intensity of at about 1000 µmol photons/sq m/s PAR (Pro Max Grow, Tappan, NY). The measured light intensity at the leaf surface averaged 522 µmol photons/sq m/s PAR. The LED plant grow lights provided uniform light levels on plant foliage to avoid confounding measurement taken under widely varying sunlight levels over the multiple days of measurements.

Three leaves for each plant measurement date were selected from the upper foliage, using the youngest but fully expanded, healthy leaves. Leaves were selected with uniform phenological properties to avoid confounding measurement bias among immature and mature leaves, as well as healthy or partially healthy leaves. Leaf selection became essential in the inoculated plants as many of the leaves showed wilting or necrotic symptoms. In the inoculated plants the healthiest leaves were selected from the upper foliage, to avoid confounding measurements between leaves that were excessively wilted and average wilted leaves.

Figure 2: Measuring gas exchange of kidney bean foliage using the LICOR 6400 XT instrument and LED grow lamps to ensure uniform light conditions during measurements.

The LICOR 6400 XT was set at 1000 µmol/ sq m/s for photosynthetic active radiation (PAR). The air flow was set at 200 µmol/sq m/s, and relative humidity ranged between 50-70%. The chamber temperature was set at 20 ^{o}C, and the CO_2 mixer at 400 µmol/mol CO_2 concentration. Data collected included photosynthesis (Pn), stomatal conductance (gs), intercellular CO_2, (Ci), and transpiration (E).

Soil moisture and soil temperature were also collected during the foliage measurements using an ECHO-TM5 sensor (METER, Inc., Pullman, WA). This instrument measured volumetric water content (m^3/m^3) and temperature at 5 cm below the soil surface. The soil data was used as covariates in the gas exchange and fluorescence analysis.

2.8. Chlorophyll Fluorescence Methods

Chlorophyll fluorescence was measured using a LICOR-6400 XT (LICOR Environmental, Lincoln, NE) (Fig. **2**). Fluorescence data used the dark adapted, maximum quantum efficiency (Fv/Fm) variable that was measured at 2, 11 and 25 DAT. Plants were placed in complete darkness in a basement, so the plants were dark adapted for at least 15 hours to ensure baseline, or full rest condition before measuring Fv/Fm. An ultraviolet, blacklight was used to illuminate the foliage and instrument panel since ultraviolet wavelengths do not active chlorophyll. The LICOR 6400 XT was also used for the Fv/Fm measurements. Three leaves per plant were sampled for each of the measurement dates. At the time of chlorophyll fluorescence measurements, the plants were immature with only a few trifoliate leaves available for sampling. Leaf sampling included two leaflets on one trifoliate leaf, and another leaflet from another trifoliate was selected. At each measurement date the youngest fully expanded leaves were selected as the plants continued to grow. Soil moisture and temperature were also collected for each fluorescence measurement.

2.9. Time Series Photographs Observing Plant Phenology and Wilt Injury.

All 96 plants were photographed over four different time periods to observe any interactions between plant phenology and progression of symptoms of the CFF wilt disease. The first photos were taken during the foliar application of chemical treatments at 0 DAT and 16 DAP (Fig. **3**). On this date, the plants had generally grown to about 25 – 30 cm and had formed their second set of trifoliate leaves. A second set of photos were taken at 19 DAT and 34 DAP, and a third set of photos were taken on 42 DAT and 55 DAP (Fig. **4**). The plants grew to their full height of about 60 to 80 cm between 16 to 22 DAT. In other words, the plants doubled in height from 25 to 30 cm at 0 DAT to 60 to 80 cm at 16 to 22 DAT. The plants were harvested on 45 DAT and 60 DAP, which included all the foliage, i.e., both pre- and post-spray leaves were collected for final leaf area and biomass measurements.

Figure 3: Time series photos of CFF inoculated kidney bean plants for the water control treatment. Left photo on 6/7, (16 DAP or 0 DAT) and right photo on 6/7 (16 DAP or 0 DAT).

Figure 4: Time series photos of CFF inoculated kidney bean plants for the water control treatment. Left photo on 6/26, (34 DAP or 19 DAT) and right photo on 7/17 (55 DAP or 42 DAT).

2.10. Verification of CFF Wilt DNA in Inoculated Plants

The presence of CFF DNA in the CFF-inoculated plant tissue and absence of CFF DNA in the uninoculated plants was evaluated using plant tissue collected during the second plant harvest. To collect plant and CFF wilt DNA from plant tissue samples, a 5 cm section of stem tissue was collected from each plant during the second harvest, 62-63 days after planting (42-43 days post inoculation). Stem tissues were crushed with mortar and pestle to extract the sap that was then placed on DNA extraction cards (Whatman FTA Classic cards, GE Healthcare Life Sciences Pittsburg, PA) to extract DNA from both plant tissues and any CFF bacteria present within the stems [43]. The FTA cards also contained an indicator that would turn from pink to white when sample binding was sufficient for processing. The pestle with the crushed tissue was gently pressed against the FTA card to both extract and bind sample DNA from the sample liquid, while simultaneously devitalizing any living pathogens. All the extractor circles on the FTA cards were labelled for each plant along with its treatment and replication, and all mortars and pestles were washed, rinsed, and sanitized with alcohol and dried between each sample in preparation for additional extractions. FTA cards were allowed to dry completely to preserve the extracted DNA and then shipped to The University of Alabama in Huntsville, which was collaborating with this project, to analyze the cards for the presence of CFF DNA using qPCR reactions specific to CFF. Since the tissue sap collection process was not quantitative, the results were a qualitative test for the presence of CFF in infected plants. A very similar FTA procedure, which used different primers, was used to qualify *Phytophthora ramorum* in a study by Ramsey *et al*. [17].

Figure 5: Applying leaf tissue paste from a mortar pestle to a labelled FTA card.

2.11. Statistical Methods

JMP 11 (SAS Inc., Cary, NC) software was used to analyze the response data in this study. All fixed effects for all analyses were limited to two-way interactions. The JMP GLM Mixed model program was used to analyze the non-repeated measurements. Analyses of the gas exchange and fluorescence repeated measures over three measurement dates were conducted with the JMP REML model.

Multivariate tests were used to determine the most significant covariate, environmental terms to use in the gas exchange and fluorescence tests. All JMP Mixed and REML models were limited to two-way interactions to increase the value of hidden replication in the analysis. The JMP Profiler program integrated all interaction and covariate model terms to determine the interaction effects of the two study factors on the final response variables. The Student T test was used as the multiple range test to separate any differences among the Least Square Means estimates for the treatments ($\alpha \leq 0.05$). The regression and smoother functions within the JMP graph programs were used to create visual graphs to further explore several key plant responses to the study factors.

3. Results

3.1. Gas Exchange

Gas exchange measurements were taken at 18, 32, and 39 DAT. Due to a chemical treatment*date interaction term, the results for gas exchange parameters were reported in two tables. The first table reported on the non-inoculated plants by chemical treatment and by DAT. The second table reported on the CFF inoculated plants by chemical treatment and by DAT.

The initial data analysis included a multivariate correlation test. This test included the primary gas exchange parameters along with leaf temperature (Tleaf) and leaf vapor pressure deficit (vpdl) and soil moisture (Tables **2-3**). Table **2** lists the correlations across all three measurement dates, while Table **3** only lists the correlations occurring during the last measurement date (39 DAT). The two tables show that Pn, gs, and E increased in correlation strength with Tleaf and vpdl, and with soil moisture at the third measurement date.

Table 2: Multivariate correlation matrix among soil and gas exchange variables across all three gas exchange measurement dates. The correlations were analyzed across all chemical treatments and both non-inoculated and CFF inoculated treatments. All positive correlations are in blue text and all negative correlations are in red text.

	SM[a]	Pn	gs	Ci	E	VpdL	Tleaf
SM	1.0000	-0.0160	-0.0298	-0.0542	-0.0640	-0.0428	-0.0536
Pn	-0.0160	1.0000	0.6364	0.2861	0.6671	-0.4541	-0.3500
gs	-0.0298	0.6364	1.0000	0.7344	0.6775	-0.6885	-0.6376
Ci	-0.0542	0.2861	0.7344	1.0000	0.5579	-0.6584	-0.6498
E	-0.0640	0.6671	0.6775	0.5579	1.0000	-0.1967	-0.1936
VpdL	-0.0428	-0.4541	-0.6885	-0.6584	-0.1967	1.0000	0.9010
Tleaf	-0.0536	-0.3500	-0.6376	-0.6498	-0.1936	0.9010	1.0000

[a]SM = soil moisture, Pn = photosynthesis, gs = stomatal conductance, Ci = intercellular CO_2, E = transpiration, vpdl = vapor pressure deficit for leaf, Tleaf = leaf temperature.

Table 3: Multivariate correlation matrix among soil and gas exchange variables at 39 DAT. The correlations were analyzed across all chemical treatments and both non-inoculated and CFF inoculated treatments. All positive correlations are in blue text and all negative correlations are in red text.

	SM[a]	Pn	gs	Ci	E	VpdL	Tleaf
SM	1.0000	-0.0389	0.0307	0.0296	-0.0808	-0.1557	-0.2193
Pn	-0.0389	1.0000	0.7199	0.3380	0.7191	-0.6289	-0.5166
gs	0.0307	0.7199	1.0000	0.7597	0.8720	-0.7370	-0.6504
Ci	0.0296	0.3380	0.7597	1.0000	0.6551	-0.6725	-0.6596
E	-0.0808	0.7191	0.8720	0.6551	1.0000	-0.4920	-0.4661
VpdL	-0.1557	-0.6289	-0.7370	-0.6725	-0.4920	1.0000	0.9269
Tleaf	-0.2193	-0.5166	-0.6504	-0.6596	-0.4661	0.9269	1.0000

[a]SM = soil moisture, Pn = photosynthesis, gs = stomatal conductance, Ci = intercellular CO_2, E = transpiration, vpdl = vapor pressure deficit for leaf, Tleaf = leaf temperature.

Comparison of the two correlation matrices shows that most correlations are stronger among the variables at 39 DAT, indicating that data measurement date influenced the gas exchange responses. Soil moisture had a weak correlation with gas exchange, so it was not included as a covariate in the REML models. However, Tleaf and vpdl

were highly correlated with Pn, gs, and E variables, so they were included as covariates in the final models to avoid confounding of environmental conditions and gas exchange responses over two data collection dates.

3.2. Soil Moisture

All plants were watered together on an "as needed" basis and kept about midway to field capacity for potting soil. Soil moisture was measured during each of the three gas exchange measurement dates (Table **4**). Soil moisture was measured with 5 cm probes pushed into the soil; therefore, the moisture levels reflect soil moisture conditions between 5 to 8 cm from the soil surface. Soil moisture remained uniform across all the study factors and across the three measurement dates. The average soil moisture levels (ave. range 35-43%) show that the plants were not grown under water stress conditions over the three measurement dates. If soil moisture was not a limiting factor, then all gas exchange and fluorescence responses are only due to the progressive CFF wilt clogging of the xylem vessels. Analysis of soil moisture data shows that EB 200 and 400 mg/l and water control treatments had equivalent percent soil moisture levels at 39 DAT that ranged from 36 to 38% (Table **4**).

Table 4: Average soil moisture for each chemical treatment, for each CFF wilt status. The average percentage is listed for each of the three gas exchange measurement dates.

Chemical Treatment	Cff Inoculation Status	Ave. Soil Moisture at 18 DAT (%)	Ave. Soil Moisture at 32 DAT (%)	Ave. Soil Moisture at 39 DAT (%)
Actigard	no	37	34	35
Actigard	yes	34	41	32
EB 200	no	35	43	34
EB 200	yes	38	42	37
EB 400	no	37	39	39
EB 400	yes	36	43	36
Water	no	35	34	35
Water	yes	35	36	38

3.3. Photosynthesis

The Pn results were reported in two tables i.e., a table for CFF inoculated and a table for the non-inoculated plants. Each table included the Student T test letter and means by measurement days and chemical treatments. The non-inoculated plants generally had higher Pn rates than the non-inoculated plants (Table **5-6**). The only exception was EB 400 mg/l, which did not have a difference in Pn rates between inoculated and non-inoculated plants. At 32 DAT the non-inoculated EB 200 and EB 400 mg/l treatments were not different from each other. When compared to water control the EB 200 and EB 400 mg/l treatments had about 14% reduction in Pn levels at 32 DAT. At 39 DAT, Actigard, EB 200 mg/l, and water treatments for non-inoculated plants had equivalent Pn rates (Table **5**). Non-inoculated EB 400 mg/l had the greater Pn rate at 39 DAT than non-inoculated water control and EB 200 mg/l treatments. The Pn rates for Actigard and water declined from 32 DAT to 39 DAT. The decline to non-inoculated, water control treatment was probably due to the short life span of this bush type kidney beans, which spanned approximately two months. Previous research has shown that Pn declines as plants mature and enter senescence. In contrast, the EB 200 mg/l and EB 400 mg/l treatments did not decline from 32 to 39 DAT.

The Actigard and water treatment CFF inoculated plants decreased their Pn rates over time until they achieved an equivalent Pn rate at 39 DAT (Table **6**). In contrast, the CFF inoculated EB 200 and 400 mg/l plants had the highest Pn rate at 39 DAT. The EB 200 and 400 mg/l treatments had equivalent Pn rates which were 24-25% higher than the water control plants at 39 DAT (Table **6**). There was a general trend for a reduction in Pn levels for all the CFF inoculated chemical treatments from 32 to 39 DAT. The exception was EB 200 mg/l where Pn rate remained the same from 32 DAT to 39 DAT. There was a delayed response in the temporal dynamics of Pn for both inoculated and

non-inoculated plants. At 32 DAT, the EB 200 and 400 mg/l and water treatments had equivalent Pn rates for the inoculated plants. However, at 39 DAT the EB 200 and 400 mg/l treatment had higher Pn rates than the water control for the inoculated plants.

Table 5: Student T test for non-inoculated plant photosynthesis for days after treatment (DAT) and chemical treatment. All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (μmol CO_2 /sq m/s)
Actigard	18	CD	14.78
Actigard	32	AB	16.84
Actigard	39	E	12.58
EB 200	18	AB	17.22
EB 200	32	DE	13.72
EB 200	39	E	12.45
EB 400	18	A	17.57
EB 400	32	DE	13.63
EB 400	39	D	14.03
Water	18	AB	16.26
Water	32	BC	15.89
Water	39	E	12.46

Table 6: Student T test for inoculated plant photosynthesis for the two-way interaction model term between days after treatment (DAT) and chemical treatment. All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (μmol CO_2 /sq m/s)
Actigard	18	DEF	9.53
Actigard	32	BC	12.87
Actigard	39	F	8.03
EB 200	18	A	15.65
EB 200	32	CDE	11.13
EB 200	39	CD	11.30
EB 400	18	BC	12.86
EB 400	32	AB	13.99
EB 400	39	CD	11.38
Water	18	BC	12.87
Water	32	BC	12.28
Water	39	EF	9.10

3.4. Stomatal Conductance

The gs rates for non-inoculated plants was higher than the CFF inoculated plants for all chemical treatments (Table **7-8**). Also, the gs rates increased at 32 DAT but declined at 39 DAT for the non-inoculated, chemical treatments. At 39 DAT Actigard had the highest gs rate for non-inoculated plants (Table **7**).

Table 7: Student T test for non-inoculated plant stomatal conductance for chemical treatment and days after treatment (DAT). All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (mol /sq m/s)
Actigard	18	A	0.56
Actigard	32	A	0.61
Actigard	39	BC	0.37
EB 200	18	G	-0.20
EB 200	32	C	0.35
EB 200	39	E	0.19
EB 400	18	F	-0.06
EB 400	32	B	0.40
EB 400	39	D	0.27
Water	18	C	0.35
Water	32	B	0.42
Water	39	D	0.25

The CFF inoculated Actigard had the highest gs rate at 39 DAT. Also, at 39 DAT EB 200 and 400 mg/l and the water control at equivalent gs rates (Table **8**). The Actigard and EB 200 mg/l treatments had a high variation in gs rates over the 18 to 39 DAT time, but both treatments increased their gs rates over time. The water control increased its gs rate over the three measurement dates, while the gs rate for EB 400 mg/l maintained its gs rate over time (Table **8**).

Table 8: Student T test for inoculated plant stomatal conductance for chemical treatment and days after treatment (DAT). All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (mol/sq m/s)
Actigard	18	EF	0.10
Actigard	32	B	0.31
Actigard	39	A	0.46
EB 200	18	H	-0.18
EB 200	32	CD	0.15
EB 200	39	G	0.04
EB 400	18	CDEFG	0.14
EB 400	32	FG	0.08
EB 400	39	DEF	0.13
Water	18	DEF	0.13
Water	32	CDE	0.15
Water	39	C	0.18

3.5. Transpiration

The non-inoculated plants had a higher E rate than the CFF inoculated plants, for all chemical treatments across the three measurement dates (Table **9-10**). At 32 DAT, the non-inoculated Actigard plants had the highest E level while the EB 200 and 400 mg/l treatments had the second highest (Table **9**). At 39 DAT, the non-inoculated, water control and EB 400 mg/l treatments had equivalent E rates (Table **9**). For all non-inoculated chemical treatments, the

E rates decreased from 32 DAT to 39 DAT. The REML model predicted negative E rates for both EB formulations at 18 DAT (Table **9**).

Table 9: Student T test for non-inoculated plant transpiration for days after treatment (DAT) and chemical treatment. All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (mmol H_2O/sq m/s)
Actigard	18	B	7.69
Actigard	32	A	8.74
Actigard	39	CD	5.27
EB 200	18	G	-2.45
EB 200	32	D	4.81
EB 200	39	F	2.68
EB 400	18	G	-2.49
EB 400	32	D	4.70
EB 400	39	E	3.57
Water	18	D	4.80
Water	32	C	5.87
Water	39	E	3.40

At 39 DAT, Actigard had the highest E rate for the CFF inoculated plants, while EB 200 mg/l had the lowest E rate (Table **10**). There was a trend for the E rates to increase from 18 to 32 DAT, and then decrease from 32 to 39 DAT across the chemical treatments for the inoculated plants. The REML model predicted negative E rates for both EB formulations for the CFF inoculated plants at 18 DAT (Table **10**). It should be noted that Actigard had the highest E level at 39 DAT for the CFF inoculated plants, but it also had the highest Tleaf rates that ranged from 30 to 32 ^{o}C (Fig. **6A-B**). As E increases Tleaf should decrease as seen in (Fig. **6A**). for non-inoculated, water control plants. However, the REML model predicted that Actigard had the highest E rate for CFF inoculated plants at 39 DAT (Table **10**), but Actigard also had the highest Tleaf of 30 °C for CFF inoculate plants at 39 DAT (Fig. **6A-B**). Also, EB 400 mg/l and water treatments had the second highest E rates at 39 DAT, they also had higher soil moisture levels than Actigard at 39 DAT (Table **4**).

Table 10: Student T test for inoculated plant transpiration for the two-way interaction model term between days after treatment (DAT) and chemical treatment. All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (mmol H_2O/sq m/s)
Actigard	18	CD	2.22
Actigard	32	A	5.28
Actigard	39	AB	4.99
EB 200	18	F	-3.53
EB 200	32	CD	2.53
EB 200	39	E	0.95
EB 400	18	E	-0.69
EB 400	32	B	3.65
EB 400	39	D	2.28
Water	18	CD	2.44
Water	32	CD	2.64
Water	39	C	2.73

3.6. Intercellular Carbon Dioxide

The Ci rates were higher for the Actigard, EB 400 mg/l, and water control treatment for the non-inoculated plants when compared to the CFF inoculated plants (Table **11-12**). Also, the Ci levels were highest for Actigard and EB 400 mg/l at 39 DAT for the non-inoculated plants (Table **11**). However, if the Pn were compared with the Ci rates for Actigard and EB 400 mg/l at 39 DAT, there was a difference in CO_2 conversion between the two treatments. In other words, EB 400 mg/l had the highest Pn at 39 DAT, while Actigard had a low Pn rate that was equivalent to EB 200 and water treatments (Table **5**). This comparison shows that the high Ci for EB 400 mg/l was being converted into sugars due to the high Pn rates at 39 DAT. However, the high Ci levels for Actigard indicates a restriction in photosynthetic machinery with a subsequent buildup of CO_2 inside the intercellular space inside the leaves due to the low Pn rates at 39 DAT.

Table 11: Student T test for non-inoculated plant intercellular CO_2 for days after treatment (DAT) and chemical treatment. All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (µmol CO_2/mol air)
Actigard	18	A	324.98
Actigard	32	AB	332.18
Actigard	39	AB	315.61
EB 200	18	E	226.88
EB 200	32	B	311.57
EB 200	39	D	291.64
EB 400	18	E	243.48
EB 400	32	BC	305.23
EB 400	39	AB	315.95
Water	18	CD	296.84
Water	32	CD	298.64
Water	39	D	293.08

Table 12: Student T test for CFF wilt inoculated plant intercellular CO_2 for days after treatment (DAT) and chemical treatment. All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (µmol CO_2/mol air)
Actigard	18	BC	244.72
Actigard	32	A	324.95
Actigard	39	A	357.67
EB 200	18	E	133.21
EB 200	32	CD	231.98
EB 200	39	D	216.90
EB 400	18	BCDE	194.58
EB 400	32	BCD	242.49
EB 400	39	B	251.70
Water	18	BC	239.59
Water	32	CD	224.00
Water	39	BC	240.48

Actigard had the same pattern of high Ci levels for both non-inoculated and CFF inoculated plants at 32 and 39 DAT (Table **12**). This is consistent with the low Pn values for Actigard for the CFF wilt inoculated plants for these dates. Also, the EB 400 mg/l treatment had a high Ci value at 39 DAT, with a corresponding high Pn level at 32 DAT for CFF inoculated plants (Table **6**). This pattern of high Pn and Ci rates for the EB 400 mg/l treatment, but a low Pn rate and high Ci rate for the Actigard treatment is consistent for both non-inoculated and CFF inoculated plants. This pattern suggests that Actigard appears to restrict Pn rates in both non-inoculated and in CFF inoculated plants about a month after the spray application. Actigard had increased Ci, Tleaf, and vpdl rates for the CFF inoculated plants which is contrary to increased rates for gs, and E for Actigard (Tables **6**, **8**). As mentioned previously, Actigard had conflicting responses for the CFF inoculated plants which indicates that it may not be a useful primer for boosting natural plant immunity.

3.7. Leaf Vapor Pressure Deficit

The EB 200 and 400 mg/l treatments had the lowest vpdl levels for CFF inoculated plants at 39 DAT (Fig. **6A**). Further analysis of the E and gs data using the JMP profiler program shows that E and gs were optimized for both EB formulations when soil moisture was about 30%, Tleaf < 22.7 C and vpdl ranged from 0.75 to 1.25 kPa at 39 DAT for the CFF inoculated plants. The vpdl averaged 1.35 and 1.48 kPa for EB 200 and 400 mg/l for the CFF wilt inoculated plants at 39 DAT. Also, the vpdl averaged 0.8 and 0.9 kPa for EB 200 and 400 mg/l for the non-inoculated plants at 39 DAT. The vpdl rates for both EB formulations for the CFF inoculated plants were slightly higher than the optimized vpdl rates ranging from 0.75 to 1.25 kPa. The vpdl levels were close to optimal rates for both EB formulations which optimized the E and gs rates, while still maintaining high soil moisture levels at 39 DAT (Table **4**).

Due to the high correlations among gas exchange and leaf parameters they were also highly interconnected. In other words, all these parameters dynamically change when one parameter changes. The JMP profiler program is a quasi-simulator that interactively connects all the model terms so that they can be optimized together and avoid unnoticed negative interactions among physiological parameters. For example, there are threshold values for vpdl where any further decrease in vpdl rates doesn't reduce plant stress and improve plant health, but instead increases plant stress. For the EB formulations, as the vpdl levels drop below a range of 0.5 to 0.8 kPa, then E and gs rates also start decreasing followed by an increase in Tleaf leading to reduced Pn rates. Also, as E is reduced there is a reduction in nutrient transport to the leaves. Despite the dynamic correlations among the gas exchange variables, it is noteworthy that soil moisture is only slightly correlated with gs, E, Tleaf and vpdl (Table **2-3**).

At 39 DAT, both EB formulations had a lower vpdl for the CFF inoculated plants than Actigard or the water control treatment (Fig. **6B**). Along with a lower vpdl, both EB formulations had a higher gs at 39 DAT for the CFF inoculated plants (Table **8**, Fig. **6**). A regression plot also shows this sharp negative relationship between gs and vpdl for the EB formulations, for the CFF inoculated plants at 39 DAT (Fig. **6B**). The JMP REML model predicts that gs, E, and Pn

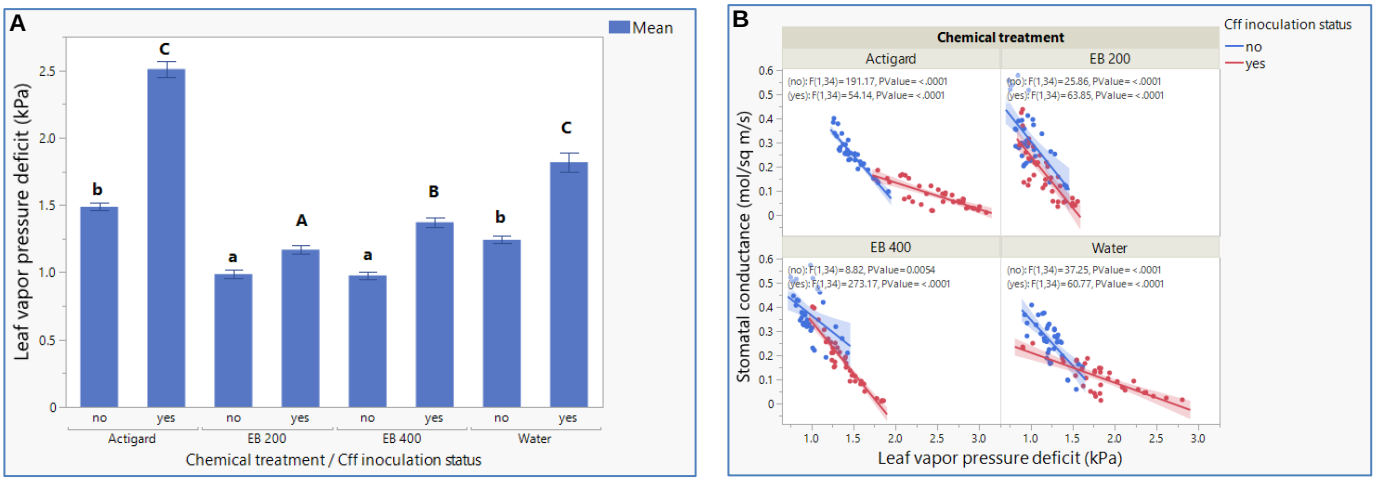

Figure 6: Student T test for leaf vapor pressure deficit (vpdl) at 39 DAT for chemical treatment and CFF inoculation status. Student T letters are upper case for CFF inoculated plants and lower-case letter for non-inoculated plants (**A**). Stomatal conductance over leaf vapor pressure deficit at 39 DAT for chemical treatment and CFF inoculation status (**B**).

increases by 75, 60, and 45%, respectively, as vpdl decreases from 1.5 to 1.0 kPa, for EB applied at 200 mg/l for CFF inoculated plants at 39 DAT.

When the twelve individual plants for each chemical treatment are plotted for gs and vpdl over DAT and by CFF inoculation status, a visual pattern is repeated for the EB formulations (Fig. **7**). The twelve plants were divided into five sets, with a smoother line depicting the patterns between gs and vpdl over the three measurement dates. The visual patterns in Fig. (**7**) show that gs and vpdl form "hockey stick" hooks for gs over DAT, and an inverted "u" pattern for vpdl over DAT for the CFF inoculated plants (Fig. **7**). Plotting the gs rates by individual plants shows that gs rates decrease at 32 DAT but start to recover at 39 DAT. Also, the vpdl rates start to increase at 32 DAT but then decrease again at 39 DAT. These patterns of recovery in EB formulations are generally in contrast to opposite patterns for the Actigard and water control treatments for the CFF inoculated plants. These smoother plots offer some evidence that the EB formulations were increasing gs and vpdl rates, or improving plant health status, at 39 DAT for the CFF inoculated plants, in comparison to the Actigard and water control treatments that showed no signs of improvement (Fig. **7**).

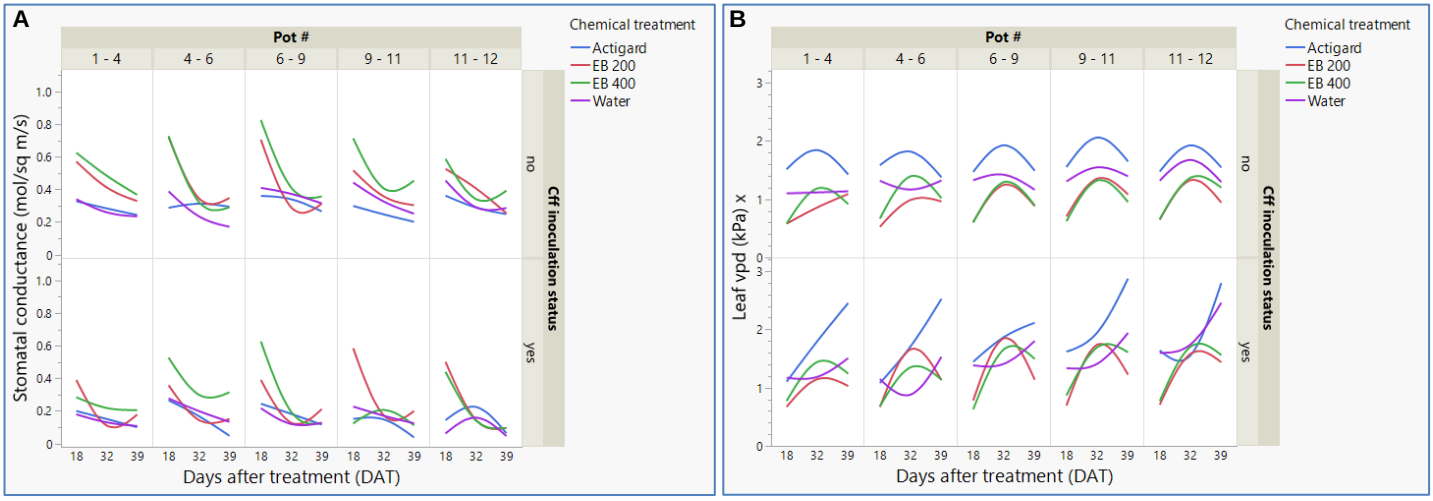

Figure 7: Stomatal conductance over DAT, for the twelve pots for CFF wilt status and chemical treatment (**A**). Leaf vpdl over DAT for the twelve pots for CFF wilt status and chemical treatment (**B**).

Literature involving common bean and soybeans states that unstressed plants have a gs range from 0.37 to 0.54 mol H_2O/sq m/s [38, 44]. In this study the average gs over the three dates, for non-inoculated plants was 0.32, 0.43, and 0.49 mol H_2O/sq m/s for the water, EB 200, and EB 400 mg/l treatments, respectively. The gs rates in this study are comparable to the gs rates in the literature, which suggests that the stomata were almost fully open for the EB formulations. Low vpdl values allows fully opened stomata which permits optimal gas exchange for CO_2 and water vapor with enhanced growth and yield in plants.

3.8. Leaf Temperature

The wilting symptoms for CFF disease are very similar to drought effects on plants. Excessive wilting is due to clogged xylem vessels, which in turn reduces water transport to the foliage. Inoculation with CFF wilt resulted in wilted leaves that also reduced E, which increases Tleaf. As CFF wilt inoculated plants increase in Tleaf there is a reduction in Pn and E (Fig. **8A**).

The EB formulations altered leaf properties such as leaf temperature and leaf vapor pressure deficit (vpdl). The plots show that Actigard and water treatments have a wide range in temperatures that extended up to 32 ^{o}C for the CFF wilt inoculated plants (Fig. **8A**). However, both the EB formulations have a lower temperature range for the CFF inoculated plants, ranging from 22 to 26 °C for CFF inoculated plants (Fig. **8A**). Also, both EB formulations show a concave pattern for Tleaf that increases by 32 DAT then decreases by 39 DAT for the CFF inoculated plants (Fig. **8B**). When comparing the Tleaf values with average soil moisture levels, both EB formulations had a lower Tleaf value, while still maintaining the highest, average soil moisture at 39 DAT (Table **4**) when compared to Actigard.

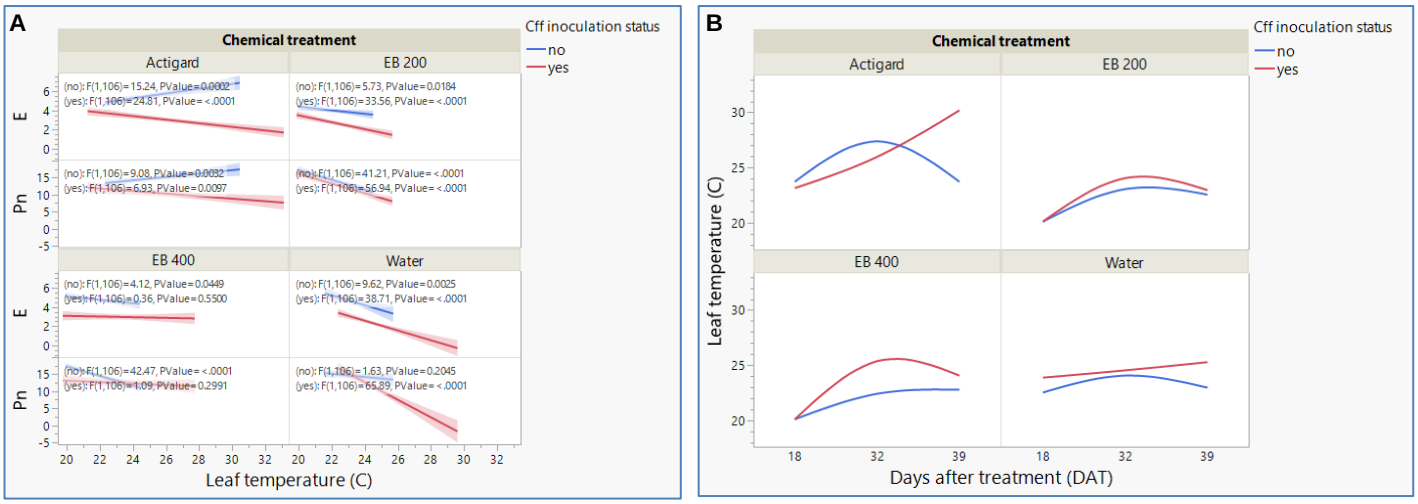

Figure 8: Photosynthesis and transpiration over leaf temperature, by chemical treatment and CFF wilt status (**A**). Leaf temperature over three collection dates by chemical treatment and CFF inoculation status (**B**).

3.9. Gas Exchange Anomalies

Several inexplicable anomalies were discovered when plotting gas exchange variables together (Fig. **9-11**). These anomalies suggest an unlinking of accepted and fundamental physiological relationships due to one or both EB formulations, due to their striking differences from the water treatments. One anomaly occurred for the EB 400 mg/l treatment when E and air temperature were plotted at 39 DAT (Fig. **9**). There is a sharp. positive relationship between E and air temperature for the non-inoculated, water control plants. In contrast, E maintained a relatively flat response for increasing air temperature for EB applied at 400 mg/l (Fig. **9**). In addition, EB applied at 200 mg/l showed a negative, or indirect relationship with increasing air temperatures. These flat, or negative relationships are contrary to widely recognized gas exchange patterns as air temperatures rise. These results suggest that E rates for the EB formulations were already at optimum levels at the lowest temperature, and thus could not increase with increasing air temperatures.

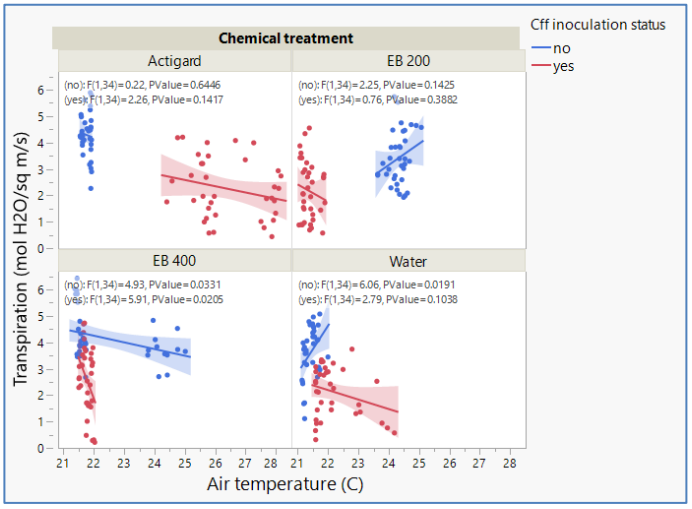

Figure 9: Leaf transpiration over air temperature by chemical treatment and CFF inoculation status at 39 DAT.

A related anomaly involves the relationship between leaf and air temperatures (Fig. **10**). It is expected that leaf temperature is positively related to air temperature as seen in the water treatment plots for the non-inoculated plants (Fig. **10**). The EB 200 mg/l treatment, however, had a negative relationship between leaf and air temperatures for the non-inoculated plants. Also, the 4EB 400 mg/l treatment had an almost flat relationship between leaf and air temperatures. Both EB formulation responses are contrary to the Actigard and water control treatment responses.

The contrary relationship among the treatments indicates that the measurements for the EB formulations were not due to instrument or operator errors.

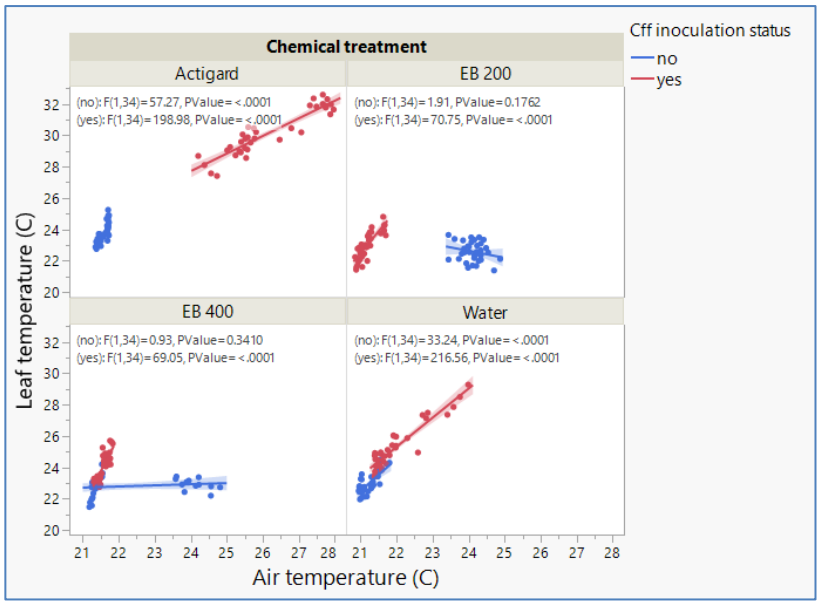

Figure 10: Leaf temperature over air temperature by chemical treatment for CFF inoculation status at 39 DAT.

A third anomaly is the relationship between Pn and Ci for the EB formulations (Fig. **11**). In the water treatment, Pn increases with increasing Ci in the non-inoculated plants. A study by Papathanasiou *et al*., [44] found that Pn and Ci levels were higher in well-watered plants, when compared to water stressed common bean plants, i.e., these relationships indicate a positive relationship between Pn and Ci in common beans. However, Pn decreases with increasing Ci, for the EB application at 400 mg/l for both the inoculated and non-inoculated plants. Also, Pn decreases for EB applied at 200 mg/l for the non-inoculated plants. These relationships are visually different for the water treatments for the CFF wilt inoculated plants (Fig. **11**).

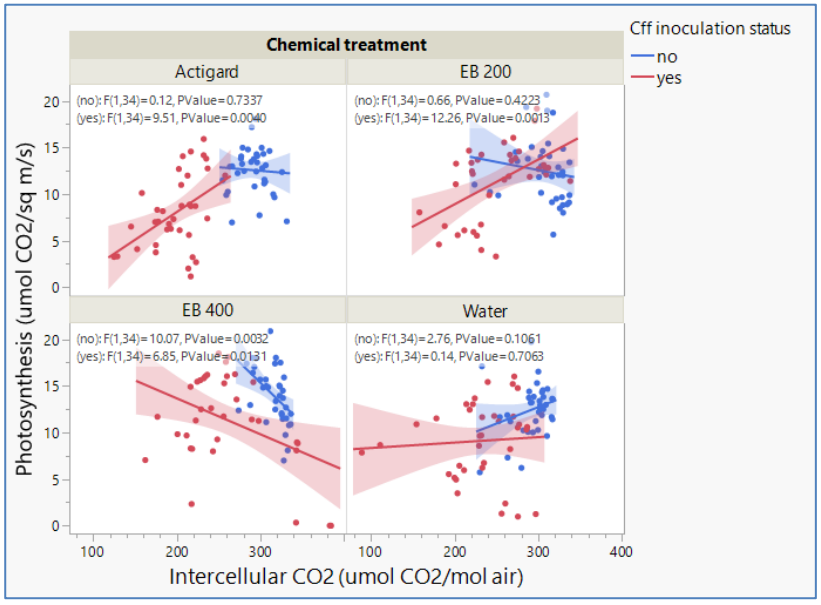

Figure 11: Leaf photosynthesis over intercellular CO_2 by chemical treatment and CFF inoculation status at 39 DAT.

A fourth anomaly involves the relationships between relative water content (RWC) and gs, E, vpdl and Tleaf for the EB 400 mg/l and water control treatment for the CFF inoculated plants (Table **13-14**). The EB 400 mg/l treatment

had a negative correlation between RWC and gs and E, but a positive correlation with vpdl and Tleaf (Table **13**). In contrast, RWC had a positive correlation between gs and E, but a negative correlation with vpdl and Tleaf for the water treatment (Table **14**).

Table 13: Multivariate correlation matrix among leaf and gas exchange variables at 39 DAT. The correlations were analyzed for the EB 400 mg/l treatment for CFF inoculated plants. All positive correlation is in blue text and all negative correlation are in red text. The stronger the correlation the brighter the color of the text.

	RWC[a]	WUE	Photo	Cond	Ci	Trmmol	VpdL	Tleaf
RWC	1.0000	0.0177	-0.0295	-0.4035	-0.0253	-0.3544	0.3233	0.2470
WUE	0.0177	1.0000	0.5779	-0.1302	-0.9716	-0.0012	-0.0956	-0.1565
Photo	-0.0295	0.5779	1.0000	0.5681	-0.4094	0.6933	-0.7041	-0.6226
Cond	-0.4035	-0.1302	0.5681	1.0000	0.2902	0.9787	-0.9430	-0.8193
Ci	-0.0253	-0.9716	-0.4094	0.2902	1.0000	0.1628	-0.1028	-0.0548
Trmmol	-0.3544	-0.0012	0.6933	0.9787	0.1628	1.0000	-0.9457	-0.7962
VpdL	0.3233	-0.0956	-0.7041	-0.9430	-0.1028	-0.9457	1.0000	0.9477
Tleaf	0.2470	-0.1565	-0.6226	-0.8193	-0.0548	-0.7962	0.9477	1.0000

[a]RWC = relative water content, Pn = photosynthesis, gs = stomatal conductance, Ci = intercellular CO_2, E = transpiration, vpdl = vapor pressure deficit for leaf, Tleaf = leaf temperature.

These correlations reveal a switch between negative and positive relationships, which indicates an unlinking or disassociation between relative water content and gas exchange and leaf conditions for the two chemical treatments applied to CFF inoculated plants. A decrease in gs and E with increased RWC and a increase in Tleaf with increased RWC for the EB 400 mg/l treatment was unexpected and contrary to general water stress physiological principles. The RWC results were included in this section on gas exchange anomalies to highlight the unlinking of fundamental physiological relationships. However, a full explanation of RWC methods and results will be fully detailed in Part 3 article.

Table 14: Multivariate correlation matrix among leaf and gas exchange variables at 39 DAT. The correlations were analyzed for the water control treatment for CFF inoculated plants. All positive correlation is in blue text and all negative correlation are in red text. The stronger the correlation the brighter the color of the text.

	RWC[a]	WUE	Photo	Cond	Ci	Trmmol	VpdL	Tleaf
RWC	1.0000	0.2873	0.3108	0.1840	-0.2242	0.2423	-0.2091	-0.2607
WUE	0.2873	1.0000	0.3432	0.0820	-0.7290	-0.0907	-0.4402	-0.4307
Photo	0.3108	0.3432	1.0000	0.8865	0.0650	0.8728	-0.7017	-0.6047
Cond	0.1840	0.0820	0.8865	1.0000	0.4581	0.8700	-0.8008	-0.6602
Ci	-0.2242	-0.7290	0.0650	0.4581	1.0000	0.3437	-0.2450	-0.1467
Trmmol	0.2423	-0.0907	0.8728	0.8700	0.3437	1.0000	-0.5262	-0.4946
VpdL	-0.2091	-0.4402	-0.7017	-0.8008	-0.2450	-0.5262	1.0000	0.9325
Tleaf	-0.2607	-0.4307	-0.6047	-0.6602	-0.1467	-0.4946	0.9325	1.0000

[a]RWC = relative water content, Pn = photosynthesis, gs = stomatal conductance, Ci = intercellular CO_2, E = transpiration, vpdl = vapor pressure deficit for leaf, Tleaf = leaf temperature.

These inexplicable anomalies are probably not due to faulty data collection, or instrument errors, because the water responses are considered the control treatment. If the water responses are valid, and mirror widely accepted physiological responses, then the other chemical treatment responses should also be valid. An alternative explanation is that the EB formulations altered or modified fundamental gas exchange relationships with environmental conditions or other gas exchange parameters. Further research is needed to verify if the EB formulations can alter fundamental gas exchange relationships, and/or relationships with environmental conditions.

3.10. Chlorophyll Fluorescence

Soil moisture, soil temperature, and vpdl were added as covariates in analysis of fluorescence data, thereby adjusting for environmental variations in these parameters over the three measurement dates. The 2 DAT data was analyzed separately because this data was collected two days before the CFF inoculation date while the data for the 11 and 25 DAT included both the chemical treatment and CFF wilt inoculation response. The 2 DAT results show that only Actigard reduced Fv/Fm. The water and both EB formulations had equivalent Fv/Fm values at 2 DAT. These results indicate that the EB formulations did not injury chlorophyll activities, but Actigard caused a slight reduction in maximum quantum efficiency.

Analysis of the 11 and 25 DAT data used the repeated measures model (JMP REML) to test the effects of the two data collection dates on chlorophyll efficiency (Table **15**). The final model showed a two-way interaction between chemical treatment x DAT. The CFF status term was dropped in the final model showing that the CFF wilt treatment had no effect on Fv/Fm for both 11 and 25 DAT. Therefore, the results were reported in a single table for the chemical treatment x DAT interaction results (Table **15**).

The range for Fv/Fm was narrow for all the chemical treatments across of the measurement dates, extending from 0.80 to 0.83. Chlorophyll efficiency can reach as high at 85 to 90% (Fv/Fm = 0.85 to 0.9) in healthy, non-stressed plants [41, 42]. The range of Fv/Fm values indicates that chlorophyll efficiency was operating near the upper ranges that is expected in healthy plants.

Table 15: Student T test for fluorescence (Fv/Fm) for days after treatment (DAT) and chemical treatment, as averaged across non-inoculated and CFF inoculated treatments. All levels that are not attached by the same letter are significantly different.

Chemical Treatment	DAT	Student T Test	Least Square Means (Fv/Fm)
Actigard	11	D	0.81
EB 200	11	D	0.81
EB 400	11	E	0.80
Water	11	CD	0.82
Actigard	25	A	0.83
EB 200	25	AB	0.82
EB 400	25	AB	0.83
Water	25	BC	0.82

At 11 DAT, the EB 400 mg/l had a Fv/Fm (0.80) lower than water control treatment (0.82). However, by 25 DAT the Fv/Fm values were 0.83 and 0.82 for the EB 400 mg/l and water control treatments, respectively (Table **15**). Also, at 25 DAT, the two EB formulations and the water control had equivalent Fv/Fm values. These results show that maximum quantum efficiency was not reduced over the long-term for this study.

3.11. Detection and Verification of CFF DNA in Plant Tissue

To verify the presence of CFF infection within the stem tissues of infected plants, we conducted a qualitative test using a qPCR approach, specific to CFF DNA. Analysis of stem tissue confirmed the expected presence of CFF wilt in the inoculated plants at 42-43 days post inoculation (Fig. **12**). The results also confirmed that non-inoculated foliar treatments were not infected with CFF wilt. Since the harvesting methods used were not quantitative when using FTA cards, the PCR methods used in this procedure were not designed for the enumeration of the CFF colony forming units for each treatment. Therefore, the relative ranking of the presence CFF DNA for each treatment was used to simply evaluate the success of the CFF inoculation methods.

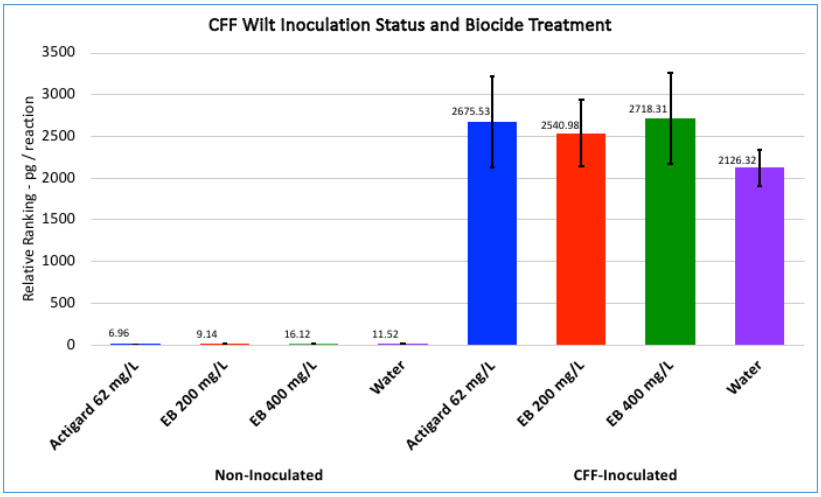

Figure 12: The mean CFF relative ranking score (y-axis), based on PCR analyses, is listed for each chemical treatment and CFF inoculation status.

3.12. Time Series Photographs

Four time series photos were taken for the water control treatment that was inoculated with the CFF wilt (See photo images in Part 1). The first two photos were taken on the spray application day (0 DAT and 16 DAP), when the plants were just developing their second and third set of trifoliate leaves. The photos of the mature plants show that the plants were about 30 to 40 cm taller than the seedlings on the spray date when the first two photos were taken. The leaves for the first sample date were recently chemically treated (5 DAT) and inoculated with the CFF wilt. However, the gas exchange measurements were taken on 18, 32, and 39 DAT on newly developed leaves. Also, fluorescence measurements were taken on 2, 11, and 25 DAT. Thus, gas exchange and fluorescence measured the physiological responses on the upper, newly matured leaves that formed between 11 to 39 DAT. These leaves were not present during the chemical treatments or the CFF wilt inoculation. Therefore, these gas exchange and fluorescence responses were due to systemic, phenological dynamics, and not due to direct effects of chemicals to the older foliage near the base of the plant.

4. Discussion

4.1. Gas Exchange

Gas exchange measurements were taken over an 21-day period at 18, 32 and 39 DAT where greenhouse temperatures were continuously increasing that caused bias in data collection across the three dates. This assumption was verified after running an initial multivariate analysis which revealed that Tleaf and vpdl were strongly correlated with the gas exchange responses and thus should be included as environmental covariates in the JMP REML models.

The water control treatment in the CFF inoculated plants shows a reduction in gas exchange rates due to partial clogging of the xylem which in turn restricts water flow and transpiration levels (Tables **3**, **5**, **7** and Fig. **6**). As the xylem becomes more restricted due to bacterial clogging, transpiration is reduced, leading to closing of the stomatal openings, and a reduction in photosynthesis. Also, leaf temperature increases with less transpiration. A study by Carmona *et al.,* evaluated the effects of a vascular wilt on tomato plants [38]. They found that Fv/Fm was reduced from about 0.72 to 0.52, and gs was reduced by 64% in the wilt inoculated plants. A study by Castillo-Arqaez *et al.,* evaluated the effect of laurel wilt on avocado trees. They found that the wilt disease reduced Pn, gs, and Fv/Fm by about 92, 82, and 75% in the laurel wilt inoculated plants at 32 days after inoculation [45]. In this study there was a 36, 154, and 70% reduction in Pn, gs, and E, respectively, at 39 DAT when comparing the inoculated control to the non-inoculated control. These two studies evaluated the effects of a wilt disease on plant gas exchange with somewhat similar results between the inoculated and non-inoculated controls. Wilt diseases cause a reduction in

gas exchange and fluorescence responses in non-primed, control plants. Therefore, chemical primers that can minimize these sharp reductions in these physiological responses may be cost effective.

Multiple gas exchange measurements show temporal interactions between the study factors over the first 40 days of the study. Gas exchange dynamics were reported for Pn (Tables **2-3**), gs (Table **5**, Fig. **6**), and E (Tables **6-7**). Monitoring temporal patterns in gas exchange rates reveals the progression of infection rates, the level of injury, and possibly any recovery or partial return to plant health. The dynamics in gas exchange responses are due to changes in environmental conditions and delays in plant responses to chemical treatments. Also, the interactions between the plant defenses and CFF inoculation shows a delayed response as the pathogen progressively grew and started clogging the xylem vessels. Finally, there appears to be a pattern of plant recovery for the EB 200 mg/l treatment with the CFF inoculated plants at 39 DAT (Fig. **7**, **11**). As expected, biological responses to foliage applications and CFF wilt inoculation exhibited delayed responses, however, by 39 DAT there is some evidence in plant health recovery for the EB treatments.

The gas exchange data for vpld and gs collected over the three measurement dates show a consistent pattern of a 'U-shaped and inverted U-shaped" recovery pattern for both EB formulations for CFF inoculated plants (Fig. **9**). This U-shaped, or inverted U-shaped, pattern over time implies that both vpdl and gs are on the path to recovery back to their initial parameter values. This pattern was not evident in the E and Pn levels over time, thus the recovery pattern was not consistent across all gas exchange responses for the EB treatments. Visual patterns are best recognized by graphing the gas exchange responses for each of the twelve pot replicates as seen in Fig. (**9**). Consistent patterns offer some clues on possible plant health recovery pathways over time even for annual crops with short life spans. Gas exchange is a non-destructive method for monitoring disease progression patterns that would otherwise remain unknown when relying solely on final biomass, leaf morphology and fruit yield results.

4.2. Vapor Pressure Deficit and Leaf Temperature

Leaf vapor pressure deficit is defined as the difference in vapor pressure inside the leaf intercellular airspace and the atmospheric vapor pressure (kPa) [46]. The intercellular airspace in a leaf is virtually the same as saturated air while the outside of the leaf has a much lower vapor pressure. This vapor pressure gradient between the inside and the outside of the leaf is one of the primary drivers for leaf transpiration. Research has shown that vpdl is a reliable leaf biomarker for abiotic stress levels, i.e., as vpdl increases there is a concomitant reduction in gas exchange rates [39, 47]. Due to the strong negative correlation between vpdl and stomatal conductance (Table **2-3**, Fig. **8B**) it is critical to fully understand the relationships between vpdl and stomatal conductance. Soil moisture levels averaged between 35 to 45% during the study period indicating that the plants were not water stressed (Table **2**). The relationships between gas exchange parameters and leaf parameters such as leaf temperature and vpdl are complex and interconnected (Fig. **8**) [48]. If the vapor pressure in the intercellular airspace can be reduced below saturation levels during low humidity, atmospheric conditions, then vpdl will be reduced and stomata will not start closing. Vapor pressure inside the leaf determines the rate that water transitions from a liquid to water vapor. The higher the vapor pressure at a given temperature, the higher the transition of water-to-water vapor. Structured water has a much lower vapor pressure that bulk water. Super cooled water at -40 ^{o}C contains 100% hexagonal ringed, structured water [49], and has a vapor pressure of 0.01 and 0.1255 kPa at -40 and -20 ^{o}C, respectively [50]. In contrast, bulk water has a vapor pressure of 3.17 kPa at 25 ^{o}C, or a 99% increase in vapor pressure over 100% structured water. In other words, as the structured water:free water ratio increases, the vapor pressure inside the leaf decreases, with a concomitant decrease in vpdl. In summary, as the structured water ratio increases inside the leaf, vpdl is reduced, and gs levels are maintained with a reduction in plant stress levels for water stressed plants. Two different premises will be offered in Part 2 and 3 of this series that provide possible explanations how the EB formulations may have increased the structured water:free water ratio inside the plant foliage. The first premise will be explained in the Gas Exchange Anomalies section (4.3) below.

The strong relationships between the gas exchange variables (Pn, g, and e) and leaf variables (vpdl and tleaf) are evident in the correlation tables across all chemical treatments and CFF inoculation status (Tables **2–3**). In general, the correlations become even stronger when analyzed just for the EB formulations. These positive correlations suggest that the EB formulations could improve gas exchange rates and ultimately increase plant and fruit biomass

for non-inoculated plants. However, the positive correlations for the EB formulations didn't translate into increased plant or fruit biomass in this study, for the non-inoculated plants. The mechanical injury to the vascular system in the plant stem due to the syringe needle in the non-inoculated plants resulted in a delayed recovery process that reduced the overall plant growth rate. However, another study that evaluated similar EB treatments on non-stem injected kidney beans did show increased plant biomass for the non-inoculated plants. This study will be reported in a future article.

4.3. Gas Exchange Anomalies

Several anomalies were discovered for the EB formulations involving the relationships between leaf temperature, air temperature, and gas exchange parameters for e, Pn, and Ci. These anomalies generally show a switch from a positive to a negative/neutral relationship among the chemical treatments. For example, the first anomaly shows that E is positively related to air temperatures for water and EB 200 mg/l treatments for non-inoculated plants at 39 DAT (Fig. **10**). However, Actigard and EB 400 mg/l were negatively affected by increasing air temperatures. This switch between positive and negative, linear relationships is an unlinking of accepted physiological relationships that are difficult to explain using the data collected in this study.

The second anomaly is that leaf temperature should be directly related to air temperature as seen in the water control plot in non-inoculated plants at 39 DAT (Fig. **11**). However, the EB formulations show an inverse or neutral relationship between leaf and air temperatures (Fig. **11**). The third anomaly is the relationships between Pn and Ci (Fig. **12**). The water treatment had a positive, linear relationship, while both EB formulations had a negative relationship between Pn and Ci for non-inoculated plants at 39 DAT. Also, Actigard had a neutral relationship between Pn and Ci. Both anomalies show a switch from a positive to a negative relationship which is unexplainable and contrary to widely accepted physiological responses. Such anomalies suggest that there is an unlinking of accepted physiological relationships that are fundamental, and widely acknowledged responses to abiotic stress events.

A previous study by Ramsey *et al*., investigated the effects of structured water on two legume species grown under water stressed condition to improve drought tolerance [46]. This study also had several anomalies among the gas exchange responses. The typical relationships between Pn, gs, and E with Tleaf were unlinked or had unexpected correlations with each other. Also, E was unlinked or had unexpected correlations with soil moisture. Both studies reveal that it is possible for chemical treatments, or structured water, to alter or result in unexpected relationships between plant physiological responses and environmental and/or leaf conditions. Such alterations are typically contrary to the widely held physiological principles involving abiotic and biotic plant stressors.

One possible explanation for the unlinking of accepted gas exchange relationships with leaf variables is that ClO_2 tends to temporarily reduce chlorophyll density [21, 51]. Chlorophyll A and B absorb light spectrum wavelengths from 400 – 500 nm (blue light) and from 600 – 700 nm (orange to red light). Given the assumption that ClO_2 reduces chlorophyll content in leaves [21, 51], then red light absorption by chloroplasts is also reduced due to lower density of chlorophyll A and B. Non-absorbed, red-light energy, however, is then free to be absorbed by biological cell water within the leaves [52-55]. The temporary reduction in Chlorophyll A and B levels due to EB treatments could be easily verified by monitoring chlorophyll density with a SPAD meter before and after foliar treatments with EB formulations over several weeks [56].

Any red-light energy that bypasses chlorophyll and is absorbed by unstructured, free water in cells can be converted to energized, structured water [54, 55]. A study by Chai *et al*., [55] found that Exclusion Zone thickness of structured water increased threefold after 5 min exposure to red light and infrared radiation. Biologically structured water molecules typically form a hexagonal ring, with strong H-bonds and delocalized electrons and protons [52-55, 57-60]. Biologically structured water is also referred to as bound water, non-freezing water, vicinal water, or interfacial water [60]. These water structures are energized due to the valence electrons absorbing photon energy and moving into higher orbits. Structured water forms sheets of hexagonal ringed water molecules, forming stacked layers of hydrogen bonded sheets, that hydrates all membrane surfaces [52-55, 57-60]. Cell hydration includes all organelles, proteins, and nucleic acids. Interfacial water has a myriad of cell functions including allowing correct 3-D folding of proteins and nucleic acids and increasing cell membrane potential. The delocalized electrons and

protons circling each hexagonal ring are exchanged in a host of redox reactions permitting redox homeostasis under very dynamic biological condition [52-55, 57-60].

Plants infected with a vascular wilt disease become water stressed, and progressively dehydrated. All cells have bound, or interfacial, water that cover all organelle and cell surfaces and hydrates all proteins and nucleic acid structures. Cell dehydration from wilt diseases may result in dehydration of proteins in chlorophyll such as the Photosystem I and II units that split water in the first stage of the photosynthesis cycle [61-64]. The PSII system splits water by taking in two water molecules and releasing four protons ($2H_2O$ - 4H+ + O_2 + 4e) [65]. Full hydration of PSII and PSI proteins with interfacial water ensures efficient proton conduction in the water channels and proton wires within the PSII and PSI structures [61-64]. Proton wires are nano-sized, with highly restricted water configurations that allows superconductivity of protons due to delocalization of energized protons [58]. The four electrons flow down the Electron Transfer Chain to eventually produce molecular oxygen (O_2). The four protons from splitting two water molecules flow at superconductivity rates along proton wires within PSII structures into the thylakoid lumen to recycle ADP to ATP as they cross the membrane into the stromata [58, 61-64, 66-67]. Sufficient hydration of chlorophyll and PSI and PSII proteins with vicinal, or bound water increases proton and electron flux rates in the light phase of photosynthesis and reduces the generation of ROS free radicals. Any treatments that may increase structured water content in leaves during water stress conditions, also increases chlorophyll efficiency and reduces photoinhibition.

Research at whole plant scale shows that increasing structured water, or bound water within plant foliage will reduce cell and plant injury to abiotic stressors, such as water stress. Rascio [65, 68-70] reviewed the relationships between bound water in plants and abiotic stresses. He reported that drought tolerant ferns and durum wheat had a high affinity for bound water on cell membranes [65]. He also postulated about the importance of bound water inducing drought resistance in plants by preventing cell dehydration under water stress conditions. Also, Kuroki *et al*., [71] found that water in resurrection plants (*Haberlea rhodopensis*) formed different molecular structures due to the number of H-bonds formed inside cells. The plant species could readily transition between the different water structures by reducing or increasing the H-bond numbers. They found that this plant species adjusted to extreme dehydration by increasing the number of H-bonds in water [71]. As the H-bonds increased, the structured or highly bound water increased in plant tissue thereby preventing cell damage. In summary, hydration at higher cellular levels with structured, bound water is as important as full hydration at subcellular levels that include membrane surfaces, organelles, proteins, and nucleic acids. Red light absorption by free water results in increased hydration levels of bound, interfacial water that reduces plant injury due to abiotic stressors.

A second advantage of increased levels of energized, structured biological water is the low energy inputs needed to split water into protons, electrons, and oxygen (O_2). An adequate flux rate of protons and electrons are needed for photosynthetic pathways to properly function. Structured water contains hexagonal ringed water with energized electrons in the valance π orbits. These energized water rings have an ionization potential of 12.06 eV, which is just under the ionizing threshold of 12.6 eV for water [57-60]. Red light has a wavelength around 680 nm, which has a quantum energy of 1.8 eV which has the energy potential to ionize structured, water molecules with an energized state of 12.06 eV. The energy input of about ~ 0.54 eV is enough to trigger the ionization or splitting of structured water at 12.6 eV [57-60]. Low energy requirements for water splitting ensures the first stages of photosynthesis are maintained near normal rates even in water stressed plants.

A third advantage of increased absorption of red-light energy and increased levels of structured water is the antioxidant properties of bound water. Plants grown under water stress conditions or infected with a vascular wilt disease are exposed to excess light, which results in the generation of radical oxygen species (ROS) [72-75]. Two articles by Hasanuzzaman *et al*., [76] and Dumanovic *et al*., [56] reviewed the effects of ROS and antioxidants on plant defenses and photosynthesis. They conclude that antioxidants are crucial in reducing photoinhibition in C3 plants that are exposed to abiotic stressors. Energized, structured water, also known as “biologically active water’, is an excellent antioxidant, due to the quasi-free, delocalized electrons in the two π orbitals around each hexagonal water ring [77]. The quasi-free, delocalized electrons can readily quench excess generation of free radicals produced along the electron transfer chain during photosynthesis in heat and/or water stressed plants [56, 74-78]. In summary, when red light is absorbed by water molecules inside cells there is a concomitant increase in structured water that

forms interfacial, bound water that enhances gas exchange rates, quenches free radical, or ROS species, which reduces the negative impacts of abiotic stressors on plants.

Research has shown that ozone, an oxidant like chlorine dioxide, also reduces chlorophyll density. An ozone injury study by Tenga *et al*., [79] found that ozone reduced chlorophyll density and SPAD readings. However, by 10 DAT the ozone treatment the SPAD reading recovered to pre-treatment levels. Another ozone injury study by Guidi *et al*., [80] found that bean plants exposed to ozone treatments had reduced Pn rates for 7 DAT before returning to pre-treatment Pn rates. These two studies show that ozone temporarily reduces chlorophyll density in ozone treated foliage, but chlorophyll recovers within a 7-to-10-day period. The Pn data for the EB 400 mg/l treatment shows that Pn decreased between 18 and 32 DAT but started to recover by 39 DAT for the non-inoculated plants (Table **5**). The EB rate appears to be high enough to cause a longer Pn recovery pattern.

At 39 DAT the EB 400 mg/l treatment had a gs, E, Fv/Fm, and RWC means of 0.212, 3.06, 0.82, and 73.5% for the CFF inoculated plants. In contrast, the water control treatment had a gs, E, Fv/Fm, and RWC means of 0.182, 2.87, 0.82, and 70.1% for the CFF inoculated plants. The RWC results will be explained in full detail in the Part 3 article. This is an unexpected, simultaneous increase in E rates and increased RWC for the EB 400 mg/l treatment. These results support the supposition that the gas exchange and leaf parameters had partially recovered to pre-CFF inoculation levels for EB 400 mg/l.

The anomalies mentioned above were unexpected but have noteworthy implications for altering fundamental plant responses to abiotic and biotic stressors. A possible explanation for the inexplicable correlations among of these relationships for the EB formulations was offered above, i.e., reduced chlorophyll density. A range of red-light wavelengths in the visible spectrum may have increased the structured, or bound water levels within the foliage due to reduced chlorophyll density that would otherwise absorb the same spectrum of light. Increased levels of bound water increase the efficiency of several physiological functions, thereby reducing plant stress levels when exposed to adverse, abiotic stressors. A second premise for increased structured water contents due to the EB treatments will be further explored in Part 3 of this series.

4.4. Direct Injection of CFF Wilt Culture into Plant Stems

Any reduction in Pn or g is a measure of the severity of the CFF attack on the plant vascular system. At 39 DAT there was a 36, 154, and 70% reduction in Pn, g, and e, respectively, when comparing the inoculated water control to the non-inoculated water control. The large reduction in gas exchange rates indicates that the CFF wilt severely infected the inoculated plants. The high level of infection was due to direct injection of the CFF wilt into the plant vascular system which bypassed the critical, front line defense systems in the plant foliage.

The inoculation method injected the CFF bacterium directly into the plant stem where it could enter the vascular system which ensured successful infection of the inoculated plants. However, direct injection also markedly increased severity of the injuries and symptoms of the CFF wilt, thereby obscuring or overshadowing any positive effects of the chemical treatments for boosting the immunity responses of the plants. If a foliage inoculation method was used instead, then the foliar plant defense proteins may have effectively slowly the spread of the bacteria into the vascular system. Also, the needles were dipped in pure cultures of CFF inoculum with a Colony Forming Unit (CFU) count of 10^8. Under real world conditions, leaf wound attacks would have much lower levels of inoculum entering the wound. A previous study by Ramsey evaluated the priming effects of EB on kidney bean plants inoculated with CFF using a floral frog instead of the direct stem injection. That study revealed that the non-inoculated EB treated plants had increased oven dry biomass when compared to the non-inoculated control plants. This study will soon be published in a future article.

4.5. Fluorescence

Chlorophyll fluorescence also measures biotic stress levels in plants [41, 42, 81]. As the disease progresses over time with increased wilting and elevated leaf temperatures, the maximum quantum efficiency (Fv/Fm) is reduced due to generation of free radicals and subsequent injury to chlorophyll [30-33, 82-84]. The temporal dynamics of fluorescence for both non-inoculated EB treatments at 2, 11 and 25 DAT the Fv/Fm values were the same or higher

than the non-inoculated water control plants. This indicates that EB did not degrade chlorophyll levels. The inoculated EB 200 mg/l plants had higher Fv/Fm values than the inoculated control at 2 DAT and at 25 DAT. The inoculated EB 400 mg/l treatment, however, had equivalent Fv/Fm values as the inoculated control plants.

Previous research has shown that plants infected with pathogens show a reduction in Fv/Fm levels [38]. However, recent studies with foliage treatments with salicylic acid have resulted in higher Fv/Fm values in infected plants [85-87]. This research suggests that maximum quantum efficiency is dependent on the timing and intensity of the salicylic acid signals in infected plants, or plants primed with salicylic acid. If infected plants are measured for Fv/Fm during the salicylic acid signal stages, the temporary increase in chlorophyll efficiency may be misleading and delay any decisions for foliage treatments. Repeated measurements for Fv/Fm may be necessary to detect any decrease in chlorophyll efficiency after the salicylic acid signals have subsided and plant defences are activated. The results from this study show that Fv/Fm increased between 11 and 25 DAT, which correlates with the salicylic acid results in the Part 1 article in this series. The complex interplay between maximum quantum efficiency, and activation of plant defences using salicylic acid signals may result in increased efficiency readings that could lead to faulty decision making about when and how to protect crops from disease outbreaks.

4.6. Time Series Photographs and Timing of Gas Exchange Measurements

The time series photos show the rate of growth and the growth stages for kidney bean seedlings (Fig. **3**, **4**). The time series photos were taken for the water control treatment that was inoculated with the CFF wilt (see Part 1 for images). The first two photos were taken on the spray application day (0 DAT and 16 DAP). The first leaf tissue collection date was on five DAT, or 21 DAP when the plants were just developing their second and third set of trifoliate leaves. The photos of the mature plants show that the plants grew an additional 30 to 40 cm between 5 (0 DAT) to 22 DAT. The growth rate of the plants suggests that the gas exchange and fluorescence measurements were taken on different, newly formed leaves over the course of the study. All foliage measurements were taken near the top of the plant, using the youngest, yet fully formed leaves. These measurements on newly formed, but mature leaves offer evidence that the chemical treatments and CFF wilt responses were systemic and affected the mature plant foliage. In other words, the gas exchange and fluorescence data from the newly formed leaves on the older plants mimicked the data taken from earlier measurements of the earlier, chemically treated leaves indicating activation of a SAR response.

4.7. Primers, SAR Activation, and Universal Immunity

The overall goal of this project was to evaluate chemical primers for their ability to induce a long-term, systemic plant immunity response and thereby reduce pesticide applications on row crops. Chemical priming of crops is an emerging research field that promises to alleviate or minimize negative impacts from biotic and abiotic stressors [88-90]. Chemical primers may activate an SAR response that provides a rapid and robust boost to plant immunity to minimize injury from most pathogen infections. Activation of such a response is systemic and has long-lasting, broad-spectrum properties [91-95]. SAR induced immunity is not disease specific, but a broad-spectrum response based on innate immunity. The systemic response includes mobile immune signals, pattern-recognition receptors, accumulation of dormant signaling enzymes, and alterations in chromatin state [91, 92, 95].

In Part 1 of this series, leaf tissue analysis revealed peaks in salicylic acid after the EB treatments [1]. Liquid chlorine dioxide is an oxidant. When ClO_2 is applied to foliage it produces a burst of ROS which in turn signals the biosynthesis of salicylic acid [22-26]. Both molecules are ubiquitous in plants, and they act as the primary activation signals for plant defenses Reactive oxygen intermediates mediate [22-26]. There are several literature reviews on the effects of salicylic acid signals for boosting plant immunity for both biotic and abiotic stresses [96-101]. A review by Lukan and Coll [96] evaluated the interplay between ROS signals and salicylic acid signals in activating and/or priming plant immunity. The results from this study suggest the chemical primers based on chlorine dioxide directly generate an ROS burst and a subsequent peak in salicylic acid in plant foliage. This combination and interplay of ROS signals and salicylic acid signals generated by chlorine dioxide primers activates long-term SAR response to protect plants against future pathogen attacks.

The findings from this study, however, also suggest that the interplay of ROS and salicylic acid signals can activate a full suite of defense mechanisms that is multifaceted, and non-specific. In other words, the combination of ROS and salicylic acid signals prime plant immunity for rapid and robust responses to both biotic and abiotic stresses and therefore acts as universal priming agents. The findings in this study are not compelling enough to assert that chlorine dioxide can activate a universal, non-specific plant immune system. However, the overall findings intuitively suggest that chlorine dioxide primers have the potential to boost plant immunity for both biotic and abiotic stresses. The question of the fitness cost tradeoffs between priming plants for non-specific, multifaceted immunity and maintaining plant growth and yield has been addressed in several reviews [102, 103]. The fitness cost for the chemical primers will be reported in Part 3 of this series.

The gas exchange results reveal that even a short, four-day head start in activating SAR for the EB formulations was enough time to reduce the negative impacts of the CFF wilt when compared to the water treatments. One of the first studies that investigated SAR responses in plants was conducted by Ross [102]. He found very quick SAR responses in tobacco plants inoculated with Tobacco Mosaic Virus (TMV). He reported increased plant defenses in leaves only two days after TMV inoculation, and maximum resistance to TMV at seven to ten days post-inoculation. The assumption that a SAR response takes approximately five to ten days, given the Ross results, effectively translates into a weeklong delay before plants develop an immune response to a pathogen attack. A multi-day delay before the innate immunity of plants is fully activated is also enough time for pathogens to severely injure crucial plant functions. Chemical primers may shorten this delay in plant defense activation by quasi-activation of the defenses and thereby reduce the risk of severe injury from a pathogen outbreak. This crude approximation for time savings due to the chemical primers is based partly on the SA accumulation time in Part 1 of this study [1].

This two-year study was divided into three parts (Parts 1-3) to highlight the stimulating yet challenging findings in each section. The first article [1] reported on the salicylic acid (SA) results, while Part 2 reported on the gas exchange and Fv/Fm results. The third article (Part 3) will report on the leaf morphology, leaf area, and plant biomass results. It is the hope that the three articles will build a convincing case of evidence that the chemical primers induced long-term plant immunity which may also include increased resistance to abiotic stressors. Chemical primers can enhance disease resistance and still be cost effective due to the low costs of a single foliar application. This study shows that chlorine dioxide primers have dual benefits by reducing overall pesticide costs, while simultaneously enhancing non-specific, general purpose plant defenses against both biotic and abiotic stressors such as low rainfall conditions. The findings of this study suggest that chlorine dioxide primers can initiate a series of ROS and salicylic acid signals that activate a suite of mechanisms that provide universal, multifaceted plant immunity that is sustained across a crop season. Agricultural forecasts predict a near-future with limited water resources, combined with global warming. Inexpensive, non-toxic universal primers have the potential to increase disease resistance, improve tolerance to heat and cold and enhance drought tolerance. Such primers should receive high priority research funding to improve crops that are grown under adverse weather conditions and limited water availability.

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