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Priming Bean Seedlings to Boost Natural Plant Defenses Against Common Bacterial Wilt: Leaf Architecture, Leaf area, Foliage Water Content, and Plant Biomass Results (Part 3)

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ABSTRACT

This greenhouse study evaluated the effects of two chemicals for priming kidney bean seedlings against bacterial wilt disease (*Curtobacterium flaccumfaciens* pv. *Flaccumfaciens*) (CFF). The premise of this study was that the oxidant properties of chlorine dioxide would mimic the signaling properties of radical oxygen species thereby initiating a cascade of molecular plant defenses. The factorial study included two levels for the foliar chlorine dioxide treatment, two levels for the bacterial wilt inoculation treatment, and two optional treatments. The biomass variables included oven dry total plant biomass, oven dry fruit biomass, and oven dry leaf biomass. Also, foliage and total plant water content data was collected, as well as total leaf area. Specific leaf area (SLA) was estimated from the leaf area and biomass data. The primers had equivalent leaf area, plant and fruit biomass as the water control for the CFF wilt inoculated plants. The EB 400 mg/l primer reduced SLA for the CFF inoculated plants. Both EB formulations increased aboveground water content in the CFF wilt inoculated plants. Multivariate tables revealed several significant correlations among leaf architecture, plant tissue water content, and biomass growth parameters for the EB primers and the water control treatment for the two CFF wilt treatments. Re-allocation of plant resources from plant growth to plant defenses due to chemical primers were estimated and discussed to determine the tradeoffs between plant yield and enhanced plant defenses. The three articles in this study show that chlorine dioxide primers can initiate a series of ROS and salicylic acid signals. This interplay of ROS signals and salicylic acid signals generated by the chlorine dioxide primers activates a long-term SAR response that protects plants against future pathogen attacks. In addition, interaction of the ROS and salicylic acid signals activates a suite of defense mechanisms that provide universal, multifaceted plant immunity that can be sustained across a crop season.

1. Introduction

This article (Part 3 is the last of three articles (Part 1 – 3) involving a greenhouse study that evaluated the effects of chemical inducers on kidney bean seedlings inoculated with a vascular wilt disease. The first article [1] reported on the salicylic acid results, while the second article (Part 2) [2] reported on the gas exchange and fluorescent results of this study. This two-year study was divided into three parts to keep each article readable and focused on specific findings. The third article (Part 3) describes the interactions of the chemical treatments and the CFF wilt with leaf area, leaf architecture, and plant or fruit biomass.

Priming plants with chemicals, heat treatments, magnetic fields, and light treatments for protection against biotic and abiotic stressors has evolved into a promising research field [3-5]. Over a decade of research has shown that several chemical primers have the potential to temporarily and quasi-activate natural plant defenses [6-8]. 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 [9-11]. However, there is an ongoing debate about whether chemical primers can activate a Systemic Acquired Resistance (SAR) response that provides more long-term systemic immunity [12-17]. An activated SAR response may translate into higher-scale enhanced defenses such as improved chlorophyll efficiency, enhanced gas exchange rates, or lower leaf temperatures.

Chlorine dioxide (ClO_2) has proven to be an effective chemical inducer for priming plant defenses [18-22]. Chlorine dioxide can be formulated with surfactants, maintaining their effective oxidant properties, and can be applied to plant foliage with minimal foliage injury [18, 20-22]. Chlorine dioxide formulations with surfactants are transported across the waxy cuticle of the leaves and transported as a soluble gas in the vascular system. Once inside the phloem, ClO_2 produces Radical Oxygen Species (ROS) signals that elicit a defense response [23-27]. Two studies have shown that a single application of chlorine dioxide to plant foliage elevates gas exchange and fluorescent responses in plants [18, 19]. This study evaluated the effects of a ClO_2 formulation applied as a single application to plant foliage to prime plants. The same ClO_2 formulation was also evaluated as a foliage application to prime rhododendrons inoculated with a fungal-like pathogen (*Phytophthora ramorum*) in a previous field nursery study by Ramsey *et al.* [18]. These studies evaluated the same ClO_2 formulation for priming plants to boost plant defenses against an inoculated disease.

Plants moderately or severely infected with vascular wilt diseases show wilt symptoms due to bacterial clogging of the xylem vessels. Wilt-infected plants suffer from water stress, or dehydration, that affects macro-tissues and systems like leaf morphology and foliage physiology [28, 29]. In addition, plant dehydration also occurs at the micro-scale by reducing water content within cells and negatively affecting cell functions [30, 31]. Cell water content can be classified as 1) tightly bound water attached to membrane and organelle surfaces and attached to micro-sites within proteins, enzymes, and nucleic acid structures, 2) loosely bound water that is not in contact with cell surfaces, and 3) free water [30-32]. The two types of biologically bound water consist primarily of structured water layers, referred to as interfacial, vicinal, or exclusion zone (EZ) water. Biologically free water is not structured with multiple water molecules bound together with stronger hydrogen bonds [30-32]. Also, free water has the same physical properties as tap water. Plant water (PWC) and foliage water content (FWC) were measured in this study to relate plant water content to gas exchange variables. Also, both water content variables were analyzed by both study factors. However, the water content in plant tissue was not further classified as bound or free water using foliage air-dry and oven-dry methods [31] or with a leaf pressure chamber that measured water potential [30]. Water content analysis provides further insight into the effects of chemical and vascular wilt disease treatments in plants.

Leaf architecture contributes to plant defenses by altering physical barriers such as lignin-fortified cell walls and thicker leaves. Specific leaf area (SLA) is the ratio of leaf area to the oven-dry leaf biomass (sq cm/g). Disease resistance research has found that leaf thickness and lower SLA values are positively correlated with disease resistance in plants [33-39]. A study by Toome *et al.* [33] investigated the effects of severity of willow leaf rust on

specific leaf areas of short-rotation coppice willows. They found that the severity of leaf rust increased with increasing SLA. In other words, as the SLA ratio decreases, plant defenses are activated, and plant disease resistance increases. Another study by Smith *et al.* [34] investigated leaf architecture traits associated with *Eucalyptus globulus* resistance to *Teratosphaeria* leaf disease. They found a common leaf trait known as Leaf Mass per Area (LMA), which is the inverse of SLA, was directly related to disease severity, i.e., as LMA increases, the disease severity is reduced. These two studies agree because LMA is the inverse of SLA. Thus as SLA decreases or LMA increases, plant defenses are enhanced, and disease resistance increases. A study by Vincent *et al.* [35] found that leaf thickness was negatively correlated with viral disease severity in Papaya. Research involving leaf physical defenses has conclusively shown that leaf thickness is directly associated with increased plant immunity or disease resistance. However, leaf economic spectrum research has proposed that as LMA increases or SLA decreases, there is a tradeoff in gas exchange rates, such as photosynthesis or transpiration rates, with a concomitant loss in plant growth.

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 bacterial wilt caused by *Curtobacterium flaccumfaciens* pv *flaccumfaciens* (CFF) [40]. The factorial study tested the interactions between five chemical treatments applied to the foliage and two CFF wilt levels (non-inoculated and inoculated treatments). Each of the three articles has its objectives specific to that article. There were two primary hypotheses for this third article. The first hypothesis was that the chemical primers would minimize the negative impacts of the CFF wilt on leaf properties. The second hypothesis was that the chemical primers would also minimize the negative impacts of the CFF wilt on plant growth rates and final harvest biomass.

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 the two-month study.

The pots were filled with Farfard 4-MP potting medium (Sun Gro Horticulture, Agawam, Massachusetts). The light red kidney beans were bush-type plants, which mature in six to eight weeks. Four bean seeds per pot were

planted, and once the seeds germinated, they were culled into 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 that required plant harvest 23 days after planting. The other seedling remained in the pot for further growth and evaluation for photosynthesis, fluorescence, final SA measurement, and biomass responses at the final harvest at 61-63 days after planting. 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. 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 [40]. This disease was a problem for dry bean production in Colorado, Nebraska, and Wyoming from the 1960s to the early 1970s [40, 41]. The primary dispersal of CFF is through infected seed, but soil and infected debris can be a reservoir of inoculum [41]. Symptoms of CFF may include stunting and reduced yields for milder infections or even mortality for more severe cases [42]. The best management practice is to use wilt-resistant cultivars and/or purchase clean seed since no pesticides control CFF in common beans [41, 42].

Common symptoms of CFF infection on *Phaseolus* are wilting of the leaves and necrotic lesions with a yellow halo on the leaves [40, 42]. Vascular wilts are unique because they thrive and multiply in the xylem, where it is nutrient deficient [43]. The bacterium clogs up the xylem, thereby preventing water transport into the foliage, foliage wilting, and reduced gas exchange in foliage [41-44]. After CFF re-emerged in 2003 in dry bean production fields in Nebraska, it has continued to be a minor bean crop pathogen in the USA. This disease has restricted international trade as CFF is on the quarantine list for many countries [43].

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. The CFF bacterium was cultured on nutrient broth yeast medium (NBY) and incubated at 22 °C. Plates were re-cultured on new NBY plates to ensure a pure culture. The NBY media ingredients were mixed in 1000 ml of distilled water and autoclaved for 15 minutes. Then 6.16 g of MgSO₄ was dissolved into 25 ml of distilled water. Then 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 cool overnight under a flow hood.

The greenhouse parameters were set at 32 °C and close to 100% relative humidity for inoculation one day before inoculation to ensure optimal CFF inoculation conditions. After inoculation, plants were conditioned for 48 hours under humid and hot conditions to ensure successful inoculation of CFF in the kidney bean plants.

Plants were inoculated using stem injection when the seedlings were 20 days old, four Days After Treatments (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 first set of plants were harvested for foliage samples 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 inserted at the cotyledon scar. 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), 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.

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 the category "Host Plant Defense Induction; Group P1". Actigard induces host plant resistance by mimicking the systemic activated resistance (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). 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 s spray application, i.e., nine s for the foliage's top and bottom sides. 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.

2.7. Time Series Photographs to Observe Plant Phenology

All 96 plants were photographed over four different periods to observe any interactions between plant phenology and the progression of symptoms of the CFF wilt disease. The first photos (Fig. 1) were taken during the foliar application of chemical treatments at 0 DAT and 16 DAP. 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 (Fig. 2) were taken at 19 DAT and 34 DAP, and a third set of photos were taken at 42 DAT and 55 DAP. The plants reached their full height of about 60 to 80 cm between 16 to 22 DAT. The plants were harvested on 45 DAT and 60 DAP, and all the pre and post-spray foliage were collected for final leaf area and biomass measurements.



Figure 1: 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 2: 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).

2.8. Plant Biomass and Leaf Area Methods

Plant biomass was harvested at the end of the study 61-63 DAP. Plants were harvested over three days due to the amount of plant material. Biomass measurements included leaf area, leaf fresh/dry biomass, stem fresh/dry biomass, and bean pod fresh/dry biomass. Total leaf area per plant and total oven dry leaf biomass per plant was used to calculate Specific Leaf Area (SLA) per plant, which was calculated as:

$$\text{SLA (sq cm/g)} = \frac{\text{total leaf area}}{\text{total oven dry leaf biomass}}$$

Aboveground fresh and oven-dry plant biomass measurements were collected for all aboveground biomasses, including stem, leaf, and fruit biomass. Fresh and oven-dry biomass data was used to determine foliage water content (FWC) for each treatment. FWC was calculated as follows:

$$\text{FWC (\%)} = \frac{\text{FFB} - \text{DFB}}{\text{FFB}} \times 100$$

Where FFB is fresh foliage biomass, and DFB is oven-dry foliage biomass. Also, the fresh and oven-dry biomass data was used to determine aboveground plant tissue water content (PWC) for each treatment. PWC includes all foliage, stem, and fruit plant tissue measured as fresh and oven-dry biomass. PWC was calculated as follows:

$$\text{PWC (\%)} = \frac{\text{FPB} - \text{DPB}}{\text{FPB}} \times 100$$

Where FPB is fresh aboveground plant biomass, and DPB is the oven-dry aboveground plant biomass. The daily growth rate (DGR) was estimated as the oven-dry aboveground plant biomass over the harvest date, based on Days After Planting (DAP). The daily growth rate is expressed as an average growth rate across the plant's life until the plant harvest date (g/day).

$$\text{DGR} = \frac{\text{Oven dry total plant biomass}}{\text{Days from seed planting to harvest}}$$

The Fitness Cost (FC) estimates the loss in oven-dry biomass due to the reallocation of plant resources for priming plants to enhance plant immunity. In other words, fitness cost is a quantitative estimate of the tradeoff

between maintaining growth rates or enhanced plant immunity based on biomass losses. Fitness costs were estimated using only the non-inoculated plant data, where FC is calculated as follows:

$$FC = \frac{(\text{Control biomass} - \text{Primer biomass})}{\text{Control biomass}} \times 100$$

Only aboveground plant components were harvested and separated into separate bags for detailed analyses of resource allocation among the plant components. The oven dryer was set at 67 C, and the bags were dried until they reached a constant weight when measured over two consecutive days for three randomly selected bags.

Leaf area was measured with a LICOR-3100C (LICOR Environmental, Lincoln, NE) area meter (Fig. 3). Only green and partially green leaves were measured for leaf area. The dead leaves were only weighed for oven-dry biomass.

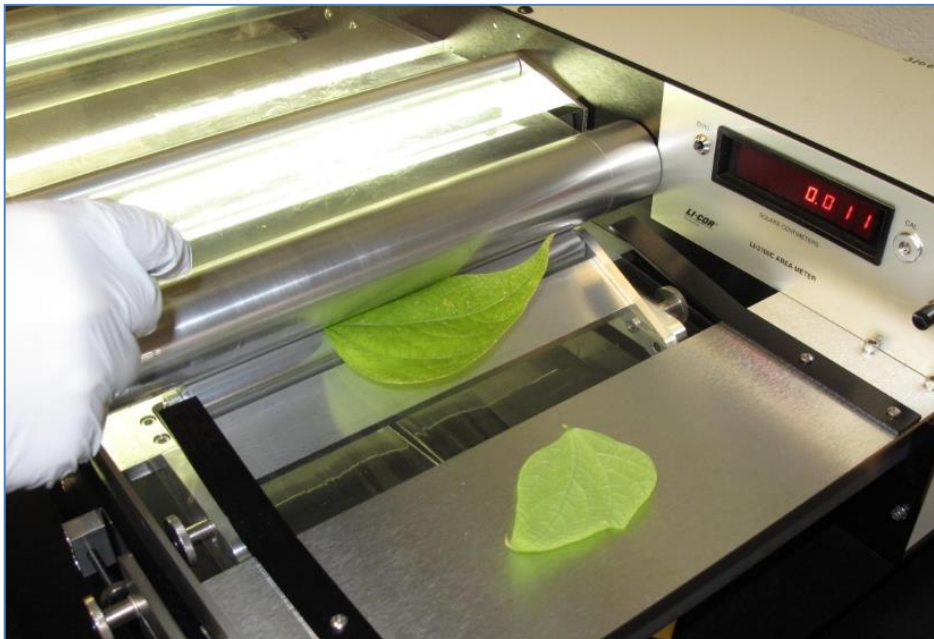


Figure 3: Measuring total leaf area using a LICOR-3100 instrument.

2.9. Verification of CFF Wilt DNA In Non-Inoculated and CFF Inoculated Plants

The presence of CFF wilt DNA in the CFF-inoculated plant tissue, and the absence of CFF wilt DNA in the uninoculated plants was determined during the final plant harvest. A 5 cm section of stem tissue was collected from each plant during the final harvest, 62-63 days after planting (42-43 days post inoculation) for DNA analysis of plant tissue. 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 [45]. The FTA cards also contained an indicator that would turn from pink to white when sample binding was sufficient for processing (Fig. 4). 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 labeled for each plant along with its treatment and replication, and all mortars and pestles were 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. A geneticist at this university was collaborating on this project and was asked 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 similar FTA procedure used different primers to qualify *Phytophthora ramorum* in a study by Ramsey *et al.* [18].

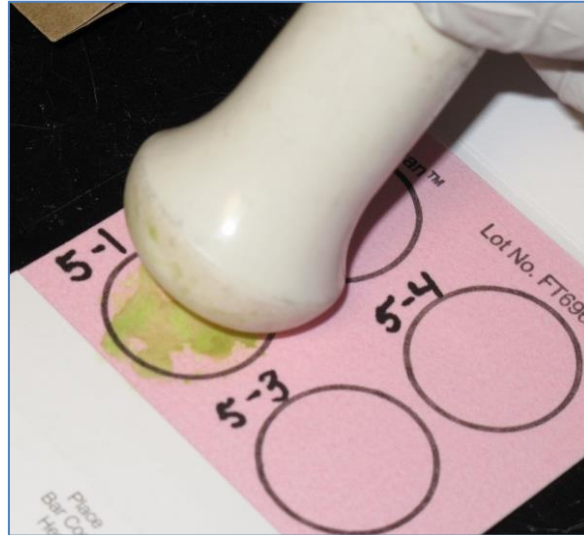


Figure 4: Applying Leaf Tissue Paste from a Mortar Pestle to a Labeled FTA Card.

2.10. 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. Analysis of Variance tests were used for non-repeated measurements. All non-significant terms in each final model were deleted but explained in the results section.

The leaf area and biomass analyses used the JMP fixed effects mixed model test. The 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 ($\alpha \leq 0.05$). The regression and smoother functions within the JMP graph programs were used to create visual graphs to explore further many aspects of all the plant responses to the study factors.

3. Results

3.1. Biomass Results

Analysis of the final plant harvest for leaf area and biomass showed that all the models had no two-way interaction terms. Therefore, the results could be reported by the significant terms in the model. All the leaf area and biomass models for total oven-dry biomass, oven-dry leaves, oven-dry pod, and oven-dry stems revealed equivalent levels among the chemical treatments. However, all the models showed that there was a large decrease in dry biomass when comparing the non-inoculated to the inoculated plants (Tables 2-4). The leaf area and biomass results show that the chemical treatments did not alleviate any negative or injurious responses for the CFF wilt inoculated plants. Plants were harvested 61-63 days after planting; thus, leaf morphology, leaf area, and plant biomass were single responses that integrated the physiological responses into a set of final plant harvest results.

Plants inoculated with the CFF wilt had a 62.6% decrease in total above-ground biomass when compared to non-inoculated plants (Table 2 and Fig. 5A). There was a 65.7% reduction in oven-dried fruit biomass when comparing non-inoculated plants to the CFF inoculated plants (Table 3 and Fig. 5B). Total leaf area was equivalent among the chemical treatments (Fig. 5C). However, there was an 88.8% reduction in leaf area per plant in the CFF inoculated plants compared to the non-inoculated plants (Table 4).

Table 2: Student T test for oven dry, aboveground plant biomass per plant by CFF wilt status. All levels that are not attached by the same letter are significantly different.

CFF Wilt Status	Student T-Test	Least Squares Means (g)
No	A	38.8
Yes	B	14.5

Table 3: Student T test for oven-dry fruit biomass per plant by CFF wilt status. All levels that are not attached by the same letter are significantly different.

CFF Wilt Status	Student T-Test	Least Square Means (g)
No	A	24.7
Yes	B	8.5

Table 4: Student T test for leaf area per plant by CFF wilt status. All levels that are not attached by the same letter are significantly different.

CFF Wilt Status	Student T-Test	Least Square Means (cm ²)
No	A	2,368.9
Yes	B	264.8

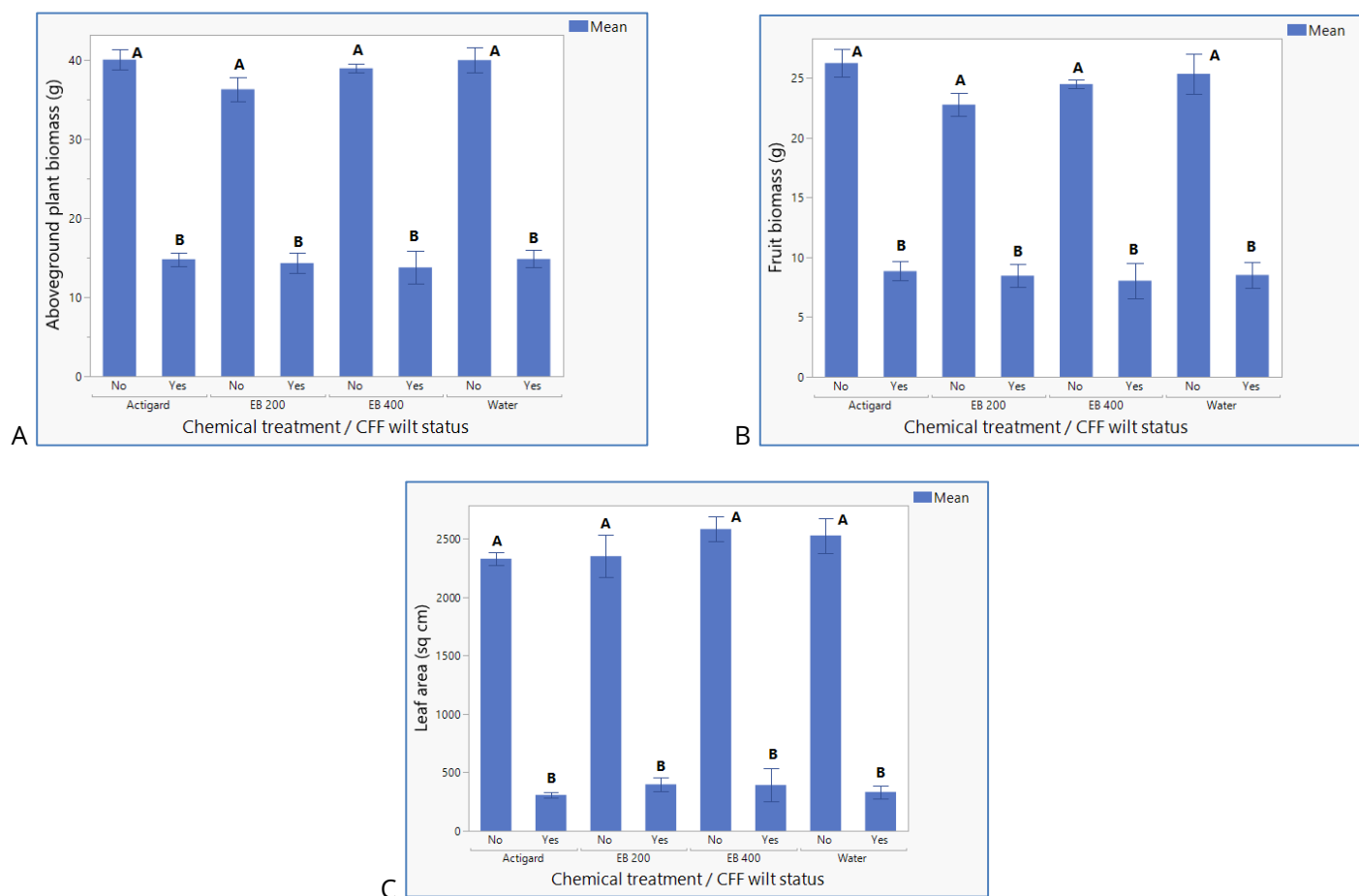


Figure 5: Mean Oven Dry, Aboveground Plant Biomass (A), Oven Dry Fruit Biomass (B), and Leaf Area (C), by Chemical Treatment and CFF Wilt Status.

The fitness cost tradeoff in biomass for the EB treatments for priming plants to boost plant immunity. The biomass tradeoff for EB at 200 mg/l ranged from 7 to 10% (Table 5). The fitness cost for EB at 400 mg/l had a smaller range from 3 to 5%, with one exception. The exception was an actual increase in leaf area of 2% for EB applied at 400 mg/l. The FC for Actigard was not estimated due to its combined poor performance in gas exchange and plant biomass results. From a fitness cost analysis, the optimal EB treatment would be EB applied at 400 mg/l, which had the lowest loss in plant biomass and also had increased leaf area.

Table 5: Fitness cost for two chemical primers for non-inoculated plants. fitness costs are estimated relative to water control treatment values.

Plant Variable	Water	EB 200 mg/g	EB 200 Fitness Cost (%)	EB 400 mg/g	EB 400 Fitness Cost (%)
DPB ^a (g)	40.0	36.3	9.2	38.8	2.6
DFB (g)	25.3	22.8	10.1	24.5	3.4
DLB (g)	7.4	6.7	10.0	7.0	5.4
LAP (sq cm)	2461	2283	7.2	2514	-2.1

^a DPB = oven-dry aboveground plant biomass, DFB = oven-dry fruit biomass, DLB = oven-dry foliage biomass, and LAP = leaf area per plant.

3.2. Foliage and Aboveground Plant Water Content Results

There was a two-way interaction term in the FWC model but not for the PWC final models (Table 6). Therefore, the FWC means were reported for both the chemical treatment and CFF wilt status (Fig. 6). The graph for the PWC means were reported for either the chemical treatment, averaged across the CFF wilt status (Fig. 7A), or reported by the CFF wilt status, and averaged across all the chemical treatments (Fig. 7B).

Table 6: Fixed effects models for foliage water content (FWC) p-values for chemical treatment and CFF inoculated plants. Aboveground plant tissue water content (PWC) p-values for chemical treatment and CFF wilt status.

Source	Foliage Water Content Model	Plant Water Content Model
	Prob > F	Prob > F
Chemical treatment	0.0002	<.0001
CFF wilt status	<.0001	0.0292
Chemical treatment*CFF wilt status	0.0005	0.4229

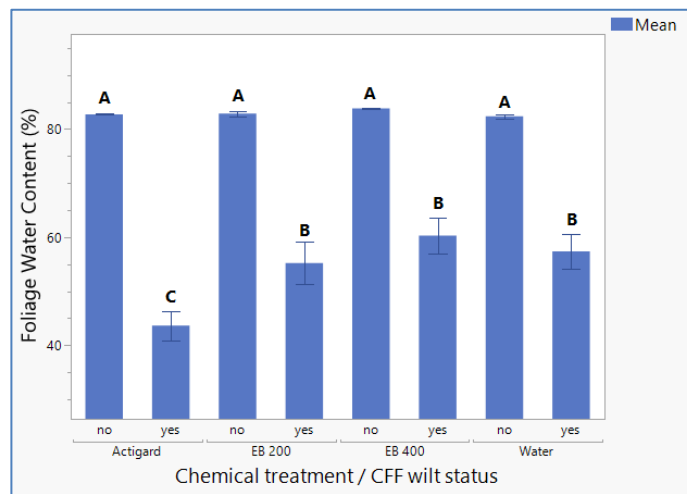


Figure 6: Foliage Water Content (FWC) for chemical treatments and CFF wilt status.

The FWC means were equivalent among the chemical treatments for the non-inoculated plants (Fig. 6). However, the EB 200 and 400 mg/l and water control treatment had higher FWC levels than the Actigard treatment

for the CFF inoculated plants. The CFF inoculated plants had FWC levels at or below 60% for all the chemical treatments, while the non-inoculated plants had FWC levels above 80%. The CFF wilt caused a loss in FWC of approximately 20% when measured at the end of the study.

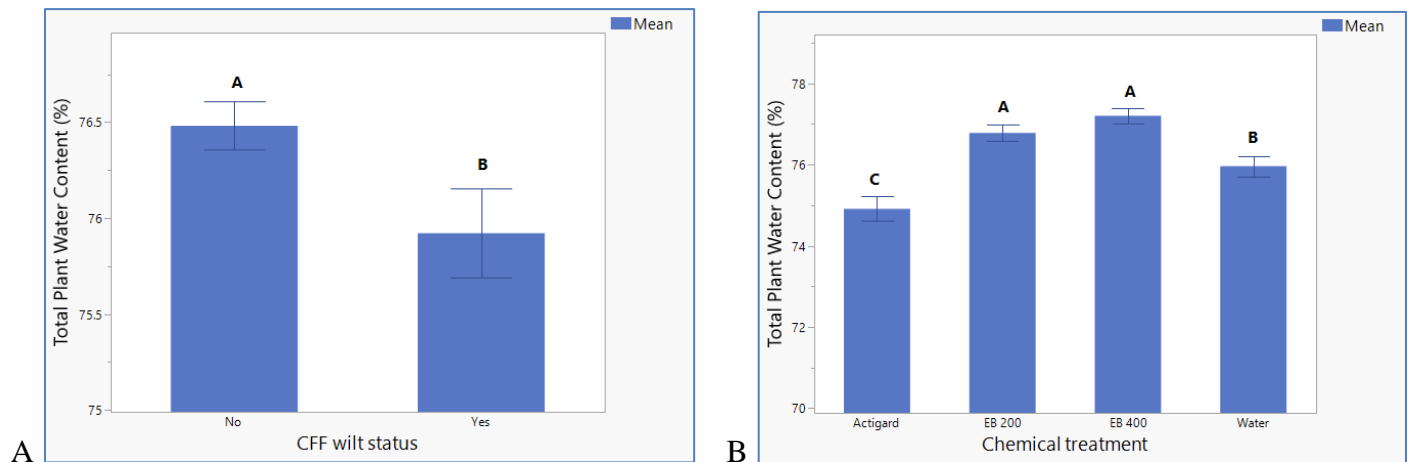


Figure 7: Plant Water Content (PWC) means for chemical treatments as averaged across both CFF wilt treatments (A). The PWC means for the CFF wilt treatments as averaged across all chemical treatments (B).

The EB 200 and 400 mg/l treatments had the highest PWC levels at the end of the study, as averaged across the CFF wilt factor (Fig. 7A). Also, the non-inoculated plants had a higher PWC level than the CFF wilt inoculated plants (Fig. 7B). In summary, the chemical treatments did not improve foliage water content at the end of the study. However, both EB formulations did enhance all the aboveground plant water content at the end of the study when compared to the Actigard and water treatments. Enhancing the water content in the main stem, branches, foliage, and fruit would be more important than just increasing the foliage water content.

The gas exchange and plant water content variables were analyzed with multivariate correlation tests to identify which variables were correlated with the internal carbon dioxide (C_i) to external carbon dioxide (C_a) ratio (C_i/C_a). Previous research has used the C_i/C_a ratio as a physiological indicator of photosynthesis efficiency in drought studies. As the C_i/C_a ratio increases, during water stress, photosynthetic efficiency also increases due to increased availability of CO_2 for the Calvin cycle [46-48]. Intracellular water can absorb CO_2 by using aquaporins [49]. Thus, internal carbon dioxide (C_i) is the concentration of CO_2 mixed in atmospheric gases inside the intercellular spaces in a leaf, plus the CO_2 absorbed into the bound and free intracellular water. As CO_2 is absorbed into intracellular water, the water acts as a temporary reservoir that readily absorbs and desorbs CO_2 as needed by chlorophyll consumption rates. A study by Talik [50] shows that fungi with high concentrations of non-freezing water (NFW) also had high levels of ionized minerals in NFW water. The research phrases for tightly bound water and non-freezing water are synonymous as they both describe structured, vicinal, or interfacial water that covers cell membranes. In other words, bound water has a high concentration of minerals which also has a higher capacity to absorb CO_2 [51, 52]. If the EB 400 mg/l treatment increased intracellular water content, then it could be assumed that the vicinal water also acted as a reversible reservoir by absorbing and releasing CO_2 on an 'as needed' basis, based on partial pressure dynamics.

Multivariate analysis was used to correlate the C_i/C_a ratio with plant water content and gas exchange variables for the EB 400 mg/l and water treatments, with and without the CFF wilt treatment at 39 DAT (Tables 7). Correlation analyses were conducted to provide evidence that the C_i/C_a ratio was related to water content for the EB 400 mg/l treatment but more related to gas exchange rates for the water treatment. For the non-inoculated plants, the C_i/C_a ratio was indirectly related to $vpdl$, $Tleaf$ and FWC for the water treatment, but for $Tleaf$ and Pn for the EB 400 mg/l treatment (Tables 7). Also, C_i/C_a was directly related to $vpdl$, gs , and E for the EB 400 mg/l treatment. Drought-tolerant studies have shown an indirect or inverse relationship between the C_i/C_a ratio and $vpdl$ [53], which is contrary to the effects of the EB 400 mg/l treatment on this relationship. Under typical

conditions, as vpdL increases, the plant becomes more stressed as they rely on stomatal conductance to regulate water vapor losses to drier atmospheric conditions, with a concomitant reduction in the Ci/Ca ratio. The EB 400 mg/l treatment shows that the Ci/Ca ratio increased with increasing vpdL rates, which may be due to the desorption or release of CO₂ from the intracellular water after the partial closure of stomata due to the increase in vpdL. In other words, increased vpdL rates induced stomata closure resulting in reduced gas flux rates, but also altered the internal Ci equilibrium, thereby releasing CO₂ from the intracellular water and raising the Ci/Ca ratio.

Table 7. Multivariate correlation tables for Ci/Ca relationships for water treatment with gas exchange, foliage and whole plant water content at 39 DAT for non-inoculated plants (A) and CFF wilt inoculated plants (B).

A) Water treatment with non-inoculated plants.

	FWC	PWC	Photo	Cond	Trmmol	VpdL	Tleaf	Ci/Ca
FWC	1.0000	-0.3427	-0.0051	0.0254	0.0329	0.0191	-0.0490	-0.0164
PWC	-0.3427	1.0000	0.1466	0.0068	-0.0655	-0.1203	0.0043	-0.0788
Photo	-0.0051	0.1466	1.0000	0.7987	0.7816	-0.4918	-0.4055	0.3565
Cond	0.0254	0.0068	0.7987	1.0000	0.9254	-0.7231	-0.5721	0.7995
Trmmol	0.0329	-0.0655	0.7816	0.9254	1.0000	-0.4420	-0.3315	0.7293
VpdL	0.0191	-0.1203	-0.4918	-0.7231	-0.4420	1.0000	0.7831	-0.7105
Tleaf	-0.0490	0.0043	-0.4055	-0.5721	-0.3315	0.7831	1.0000	-0.4953
Ci/Ca	-0.0164	-0.0788	0.3565	0.7995	0.7293	-0.7105	-0.4953	1.0000

^a FWC = foliage water content, PWC = aboveground plant water content, Pn = photosynthesis, gs = stomatal conductance, E = transpiration, vpdL, leaf vapor pressure deficit, Tleaf = leaf temperature, Ci/Ca = intercellular carbon dioxide concentration.

B) Water treatment with CFF wilt inoculated plants.

	FWC	PWC	Photo	Cond	Trmmol	VpdL	Tleaf	Ci/Ca
FWC	1.0000	0.8452	0.1567	0.0257	0.1503	-0.0570	-0.1791	-0.3066
PWC	0.8452	1.0000	0.1255	0.0081	0.0990	-0.0185	-0.1097	-0.2351
Photo	0.1567	0.1255	1.0000	0.8865	0.8728	-0.7017	-0.6047	0.1250
Cond	0.0257	0.0081	0.8865	1.0000	0.8700	-0.8008	-0.6602	0.5109
Trmmol	0.1503	0.0990	0.8728	0.8700	1.0000	-0.5262	-0.4946	0.3935
VpdL	-0.0570	-0.0185	-0.7017	-0.8008	-0.5262	1.0000	0.9325	-0.2912
Tleaf	-0.1791	-0.1097	-0.6047	-0.6602	-0.4946	0.9325	1.0000	-0.1875
Ci/Ca	-0.3066	-0.2351	0.1250	0.5109	0.3935	-0.2912	-0.1875	1.0000

^a FWC = foliage water content, PWC = aboveground plant water content, Pn = photosynthesis, gs = stomatal conductance, E = transpiration, vpdL, leaf vapor pressure deficit, Tleaf = leaf temperature, Ci/Ca = intercellular carbon dioxide concentration.

The correlation table for the CFF wilt inoculated plants shows a direct, positive relationship between the Ci/Ca ratio and Pn, gs, and E for the water treatment (Table 7). In contrast, the EB 400 mg/l treatment had a positive relationship with FWC, PWC, gs, and E but had a negative relationship with Pn (Table 8). These correlations show that as plant water content for both FWC and PWC increased, the Ci/Ca ratio also increased for the EB 400 mg/l treatment. In contrast, the Ci/Ca ratio decreased with increasing FWC and PWC but increased with Pn, gs, and E rates. These results provide additional evidence that as the water content increased, it absorbed CO₂ and thus increased the Ci/Ca ratio for the EB 400 mg/l treatment (Table 8). These correlations were further confirmed using the JMP fixed effect model with the profiler to test the interactions between the Ci/Ca ratio and the gas exchange and FWC data. The JMP profiler results show that the Ci/Ca ratio was significantly higher for the EB 400 mg/l treatment than the water treatment while adjusting for all the interactions among the Pn, gs, vpdL, and FWC variables for the CFF wilt treatment at 39 DAT (data not shown).

Table 8: Multivariate correlation tables for Ci/Ca relationships for EB 400 mg/l treatment with gas exchange, foliage and whole plant water content at 39 DAT for non-inoculated plants (A) and CFF wilt inoculated plants (B).**A) EB 400 mg/l treatment with non-inoculated plants.**

	FWC ^a	PWC	Pn	gs	E	VpdL	Tleaf	Ci/Ca
FWC	1.0000	0.7816	0.1301	-0.1412	-0.1620	0.0221	0.2449	-0.2646
PWC	0.7816	1.0000	0.1511	-0.0462	-0.1156	-0.0307	0.1653	-0.1515
Pn	0.1301	0.1511	1.0000	0.4934	0.1461	-0.6287	-0.0171	-0.3765
gs	-0.1412	-0.0462	0.4934	1.0000	0.8093	-0.4538	-0.7091	0.5839
E	-0.1620	-0.1156	0.1461	0.8093	1.0000	0.1285	-0.8375	0.6471
VpdL	0.0221	-0.0307	-0.6287	-0.4538	0.1285	1.0000	-0.0736	0.0205
Tleaf	0.2449	0.1653	-0.0171	-0.7091	-0.8375	-0.0736	1.0000	-0.6859
Ci/Ca	-0.2646	-0.1515	-0.3765	0.5839	0.6471	0.0205	-0.6859	1.0000

^a FWC = foliage water content, PWC = aboveground plant water content, Pn = photosynthesis, gs = stomatal conductance, E = transpiration, vpdL, leaf vapor pressure deficit, Tleaf = leaf temperature, Ci/Ca = intercellular carbon dioxide concentration.

B) EB 400 mg/l treatment with CFF wilt inoculated plants.

	FWC ^a	PWC	Pn	gs	E	VpdL	Tleaf	Ci/Ca
FWC	1.0000	0.9485	-0.0584	-0.2913	-0.2574	0.2262	0.1508	0.1538
PWC	0.9485	1.0000	0.0388	-0.1742	-0.1359	0.1126	0.0642	0.1849
Pn	-0.0584	0.0388	1.0000	0.6270	0.7423	-0.7390	-0.6388	-0.4130
gs	-0.2913	-0.1742	0.6270	1.0000	0.9793	-0.9386	-0.8061	0.2314
E	-0.2574	-0.1359	0.7423	0.9793	1.0000	-0.9377	-0.7789	0.0963
VpdL	0.2262	0.1126	-0.7390	-0.9386	-0.9377	1.0000	0.9462	-0.0412
Tleaf	0.1508	0.0642	-0.6388	-0.8061	-0.7789	0.9462	1.0000	-0.0087
Ci/Ca	0.1538	0.1849	-0.4130	0.2314	0.0963	-0.0412	-0.0087	1.0000

^a FWC = foliage water content, PWC = aboveground plant water content, Pn = photosynthesis, gs = stomatal conductance, E = transpiration, vpdL, leaf vapor pressure deficit, Tleaf = leaf temperature, Ci/Ca = intercellular carbon dioxide concentration.

3.3. Specific Leaf Area Results

As previously mentioned, specific leaf area (SLA) is the ratio of leaf area to the oven-dry leaf biomass (sq cm/g). Estimates of SLA were analyzed because SLA represents two primary components of leaf architecture, and it is an excellent bio-indicator for plant stress levels. Leaf area was 1,475, 1,370 and 1,488 sq cm/plant for the water control, EB 200, and EB 400 mg/l treatments, respectively. Oven dry foliage biomass was 27.9, 25.3, and 26.4 g/plant for the water control, EB 200, and EB 400 mg/l treatments, respectively. This study shows that EB applied at 400 mg/l decreased SLA (Fig. 8). As the SLA decreases, the ratio of leaf area to oven-dry leaf biomass decreases, and leaves become smaller, thicker, and denser. The tradeoff for enhanced leaf immunity is lower leaf area and lower sugar production. Healthy plants generally have an inverse relationship between SLA and AGPWC (Fig. 9).

The single application of EB at 400 mg/l appears to have initiated a strong enough oxidant signal after inoculation of the CFF wilt to activate a SAR response. The cascade of systemic SAR responses includes thicker leaves as evidenced by a significantly reduced SLA ratio estimated from the final leaf data (Fig. 8). A graph of SLA over PWC shows a positive, linear relationship for the non-inoculated, chemical treatments (Fig. 9). However, the water control treatment shows a negative relationship between SLA and PWC (Fig. 9 and Table 11).

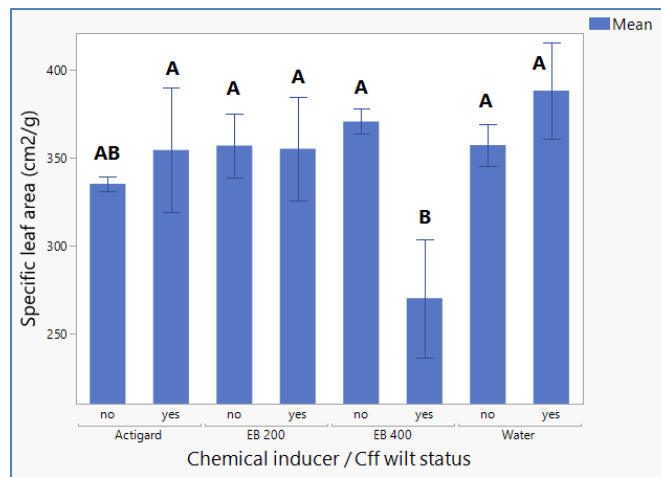


Figure 8: Specific leaf area means and student T-test for chemical treatments and CFF wilt inoculation status.

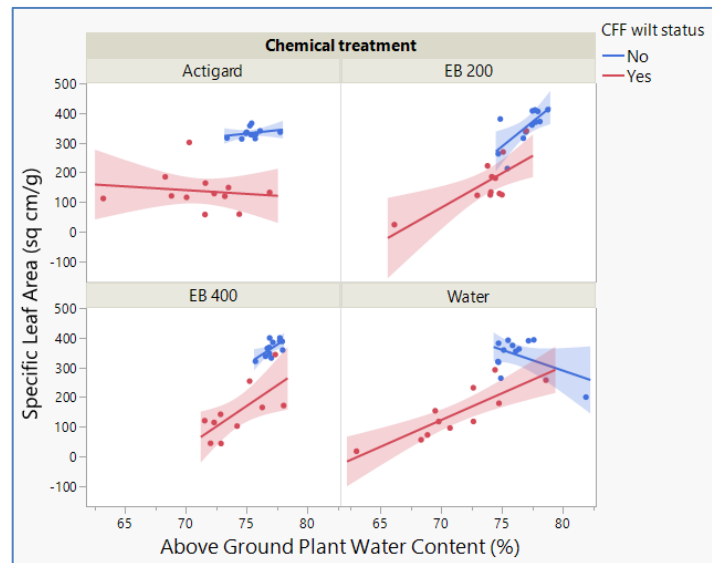


Figure 9: Specific leaf area over whole plant water content in plant tissue.

Table 9: Multivariate correlations among leaf properties, fruit biomass, water content, and daily growth rate for the EB 400 mg/l treatment and non-inoculated plants.

	Width	TLA	SLA	TGLC	FB	PWC	FSA	DGR
Width	1.0000	0.0659	-0.0292	-0.5479	0.4814	-0.1707	-0.0942	0.4346
TLA	0.0659	1.0000	0.8520	0.6744	0.6068	0.5717	-0.4446	0.7486
SLA	-0.0292	0.8520	1.0000	0.5761	0.3308	0.6481	-0.0515	0.4361
TGLC	-0.5479	0.6744	0.5761	1.0000	0.0966	0.3973	-0.1464	0.3305
FB	0.4814	0.6068	0.3308	0.0966	1.0000	0.0408	-0.4805	0.8903
PWC	-0.1707	0.5717	0.6481	0.3973	0.0408	1.0000	-0.1565	0.2107
FSA	-0.0942	-0.4446	-0.0515	-0.1464	-0.4805	-0.1565	1.0000	-0.5772
DGR	0.4346	0.7486	0.4361	0.3305	0.8903	0.2107	-0.5772	1.0000

^a Width = ave. leaf width, TLA = total leaf area per plant, SLA = specific leaf area, TGLC = total green leaf counts per plant, FB = oven-dried fruit biomass, PWC = aboveground plant water content, FSA = free salicylic acid, and DGR = daily growth rate.

Table 10: Multivariate correlations among leaf properties, fruit biomass, water content, and daily growth rate for the EB 400 mg/l treatment and CFF wilt inoculated plants.

	Width	TLA	SLA	TGLC	FB	PWC	FSA	DGR
Width	1.0000	0.7104	0.8758	0.6088	0.8517	0.4888	0.1142	0.7898
TLA	0.7104	1.0000	0.8862	0.9291	0.8717	0.5341	-0.0877	0.9194
SLA	0.8758	0.8862	1.0000	0.7831	0.9186	0.7047	0.1915	0.8985
TGLC	0.6088	0.9291	0.7831	1.0000	0.7988	0.5934	-0.1707	0.8530
FB	0.8517	0.8717	0.9186	0.7988	1.0000	0.5730	0.1928	0.9879
PWC	0.4888	0.5341	0.7047	0.5934	0.5730	1.0000	0.4106	0.5487
FSA	0.1142	-0.0877	0.1915	-0.1707	0.1928	0.4106	1.0000	0.1142
DGR	0.7898	0.9194	0.8985	0.8530	0.9879	0.5487	0.1142	1.0000

^aWidth = ave. leaf width, TLA = total leaf area per plant, SLA = specific leaf area, TGLC = total green leaf counts per plant, FB = oven-dried fruit biomass, PWC = aboveground plant water content, FSA = free salicylic acid, and DGR = daily growth rate.

Understanding leaf architecture using SLA estimates is essential to understanding the interplay between water content, water properties, and plant stress responses. Thinner leaves allow larger leaf size and total leaf area but also increase transpiration rates. Thicker leaves reduce leaf area and photosynthetic and transpiration rates but boost plant defenses.

The correlations in Tables 9-12 show that PWC plant water content is positively and highly related to SLA for the CFF wilt inoculated plants for both EB 400 mg/l and the water treatment (Table 10, 12). This correlation reverses to a negative relationship between SLA and PWC for the water treatment for the non-inoculated plants (Table 11). Analysis of SLA using FWC in the model also shows that both variables are closely correlated. As FWC increases, then SLA decreases, or as FWC decreases, the leaves become thinner and larger in size with a larger SLA value (data not shown). The multivariate tables show that leaf architecture depends on a complex interplay among many leaf and plant properties. As previously mentioned, SLA estimates are a bio-indicator for plant health and disease resistance. As SLA decreases, leaves become thicker and smaller, and disease resistance or plant defenses are increased. As a corollary, as SLA increases, then, plant resource allocation is switched from boosting plant immunity to plant growth and fruit production as leaves become thinner and larger in size. In this study, the EB 400 mg/l treatment reduced SLA to reallocate plant resources to boost immunity and defend against severe infection from the CFF wilt inoculation (Fig. 8).

The multivariate tables for EB 400 mg/l and water control treatments for the CFF wilt inoculated plants show a strong positive correlation among plant water content, daily growth rate, leaf area, and fruit biomass (Table 10, 12). These correlations show that plants allocate resources depending on plant defense or growth and reproduction needs, which generally follow accepted physiological principles. The EB formulations resulted in most of the exceptions or anomalies that were explained in Parts 2 and 3 of this series. For example, the EB 400 mg/l treatment had the smallest SLA or the thickest leaves to boost plant defenses against the CFF wilt inoculation (Fig. 8). However, the EB 400 mg/l treatment also had an equivalent oven-dry fruit biomass with all the other chemical treatments (Fig. 5). If the EB 400 mg/l treatment had the lowest SLA, thickest leaves to boost plant defense, how could it simultaneously allocate resources so that the oven-dry fruit biomass was equivalent to the other treatments that had much higher SLA with thinner leaves and fewer resources allocated to plant defenses? It appears that the EB 400 mg/l treatment boosted plant defenses without a tradeoff in fruit biomass production which is contrary to generally accepted plant defense principles and will be discussed in more detail in the Discussion section.

Table 11: Multivariate correlations among leaf properties, fruit biomass, water content, and daily growth rate for the water control treatment and non-inoculated plants.

	Width	TLA	SLA	TGLC	FB	PWC	FSA	DGR
Width	1.0000	0.1470	0.7072	-0.5293	0.5049	-0.3062	-0.3113	0.3045
TLA	0.1470	1.0000	0.6887	0.6948	0.6666	-0.3544	-0.4236	0.8353
SLA	0.7072	0.6887	1.0000	0.0665	0.8693	-0.4832	-0.4506	0.7617
TGLC	-0.5293	0.6948	0.0665	1.0000	0.2412	-0.2109	-0.0578	0.5299
FB	0.5049	0.6666	0.8693	0.2412	1.0000	-0.7957	-0.4123	0.9318
PWC	-0.3062	-0.3544	-0.4832	-0.2109	-0.7957	1.0000	0.2519	-0.7623
FSA	-0.3113	-0.4236	-0.4506	-0.0578	-0.4123	0.2519	1.0000	-0.3808
DGR	0.3045	0.8353	0.7617	0.5299	0.9318	-0.7623	-0.3808	1.0000

^a Width = ave. leaf width, TLA = total leaf area per plant, SLA = specific leaf area, TGLC = total green leaf counts per plant, FB = oven-dried fruit biomass, PWC = aboveground plant water content, FSA = free salicylic acid, and DGR = daily growth rate.

Table 12: Multivariate correlations among leaf properties, fruit biomass, water content, and daily growth rate for the water control treatment and off wilt inoculated plants.

	Width	TLA	SLA	TGLC	FB	PWC	FSA	DGR
Width	1.0000	0.6067	0.5887	0.4152	0.2418	0.6956	0.1524	0.2195
TLA	0.6067	1.0000	0.7870	0.9145	0.8319	0.6626	0.2064	0.8844
SLA	0.5887	0.7870	1.0000	0.6396	0.7748	0.8461	0.3626	0.6874
TGLC	0.4152	0.9145	0.6396	1.0000	0.8745	0.5471	0.1124	0.9168
FB	0.2418	0.8319	0.7748	0.8745	1.0000	0.6329	0.1768	0.9609
PWC	0.6956	0.6626	0.8461	0.5471	0.6329	1.0000	0.3918	0.5215
FSA	0.1524	0.2064	0.3626	0.1124	0.1768	0.3918	1.0000	0.2226
DGR	0.2195	0.8844	0.6874	0.9168	0.9609	0.5215	0.2226	1.0000

^a Width = ave. leaf width, TLA = total leaf area per plant, SLA = specific leaf area, TGLC = total green leaf counts per plant, FB = oven-dried fruit biomass, PWC = aboveground plant water content, FSA = free salicylic acid, and DGR = daily growth rate.

3.4. Detection and Verification of CFF DNA in Inoculated 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. 10). 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 of CFF DNA for each treatment was used to evaluate the success rate of the CFF inoculation methods.

4. Discussion

4.1. Biomass Results

At the end of the study, plants were harvested at 61-63 DAP. At this age, the plants were entering the senescence stage for bush-type kidney beans. Fresh and oven-dry biomass was measured for stems, leaves, and pods. The leaf area was taken on healthy green and partial green leaves only. Biomass analysis revealed that the

CFF-inoculated plants had much lower weight measurements for leaves, pods, stems, and leaf area when compared to the non-inoculated plants. Inoculated plants had a 64% decrease in above-ground total biomass when compared to non-inoculated plants. Neither non-inoculated nor CFF-inoculated plants had any biomass differences due to the chemical treatments.

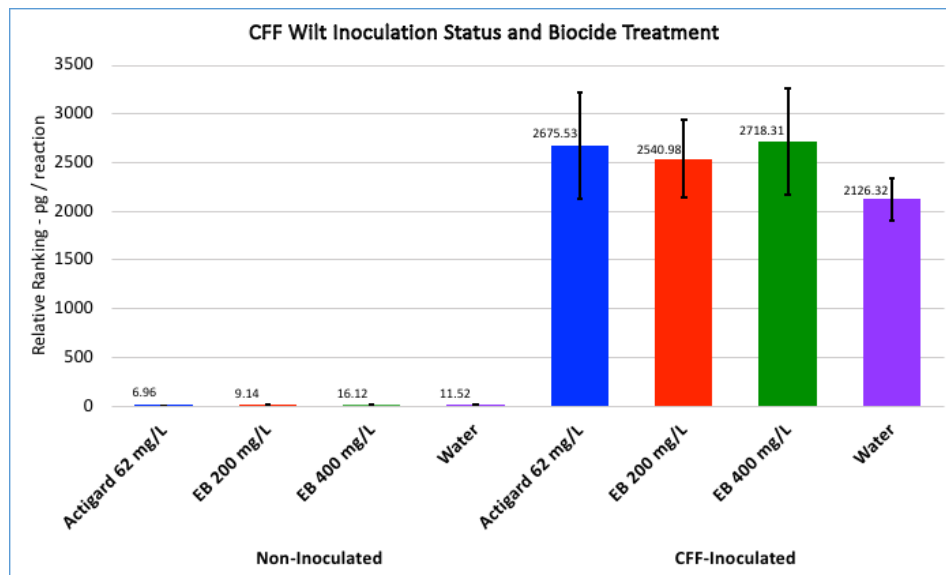


Figure 10: Mean CFF relative ranking score (y-axis). The score was based on PCR analyses. Scores are listed by each chemical treatment, and CFF wilt inoculation status.

Estimation of fitness cost for the chemical primers quantifies the reallocation of resources from plant growth and crop yield to boosting plant immunity. There is an ongoing debate, however, whether primers can induce all-out immunity, or just prime plant defenses, based on primer properties, concentrations, and repeat applications. Of these three priming factors, it seems intuitive that plants would have the ability to respond less robustly to a single primer application versus repeated primer applications. In Part 1 of this series, the single primer treatments generated a double-peaked curve in free salicylic acid (SA) concentration in leaf tissue [1]. These peak SA concentrations subsided back to baseline levels within 24 hours after the CFF injection date and five days after the primer treatments for both the CFF inoculated and non-inoculated plants. In contrast, the CFF wilt inoculated plants had 15-fold higher free SA concentrations in leaf tissue at 61 days after planting, as averaged across all primer treatments. These higher levels of free SA in the leaf tissue represent an all-out activation of plant defenses due to the continuous presence of the CFF wilt in the plants. This 15-fold difference in free SA between a single primer application and the continuous presence of the CFF pathogen within the plants is a bio-indicator whether plant defenses were just primed or were fully operational.

It could be argued that the water control treatment was the most cost-effective treatment because it had the same plant and fruit biomass and leaf area as the chemical primers for the CFF wilt inoculated plants without the additional primer and application cost. This can be refuted to some extent by two arguments. The first argument involves the direct injection of CFF wilts into the plant stem, which is the site of abundant xylem vessels. This injection site completely bypassed all the plant defenses located in the foliage. The CFF culture was injected into the xylem vessels where the bacterium thrives, and there are virtually no plant defenses. Thus, the primers were useless for defending against the pathogen in this study. An exploratory study by Ramsey was conducted a year earlier than this study which involved similar EB treatments with kidney seedlings inoculated with the CFF wilt. However, this exploratory study used a multi-pin device (a floral frog with 50 pins) to inoculate the CFF culture into a section of a leaf on each plant. The daily growth rate (DGR) for the seedlings in this study was 0.37 and 0.25 g/day for the EB and water treatment, respectively, for the CFF inoculated plants. The EB treatment had a 47% higher daily growth rate than the water control due to using the floral frog to inject the CFF wilt culture in the leaves instead of direct injection into the stem of the plants. This study will soon be published to show the differences between the two different CFF injection sites on plants.

The second argument is that the EB primers did reduce plant stress levels in the non-inoculated and CFF inoculated plants. As evidenced in Part 2 of this series, the EB primers reduced leaf temperature, pdf, and other gas exchange responses. Also, in Part 3, the EB primers reduced SLA and increased FWC. The combination of these responses reduced the overall plant stress levels, allowing more plant resources to be allocated to plant growth instead of plant defenses.

4.2. Foliage and Plant Water Content Results

Foliage and total plant water content (FWC and PWC) levels were measured at 61 – 63 DAP after the plant harvest. The FWC data includes water classified as free or intracellular, bound water in the foliage. The free water is generally associated with apoplastic and symplastic water pathways, while the intracellular water content is typically associated with the bound water in plant tissue [54]. Other research terms synonymous with structured bound water include vicinal water, interfacial water, exclusion zone (EZ) water, non-freezing water, and gel-like water. Bound water consists of stacked sheets of hexagonal ringed water molecules that are layered together with hydrogen-bonded sheets that hydrate all membrane surfaces [54-62]. The tightly bound and loosely bound water contents in plant tissue range from 85 to 94% of the total water content, while free water ranges from 6-15% [57]. As the ratio of free water to bound water increases, gas exchange rates improve in non-water-stressed plants [63-65]. However, as intracellular, bound water contents increase in water-stressed plants, the plants become more adaptive and express more drought-tolerant attributes [66-68]. Drought-tolerant plants develop modulated responses to swings in soil moisture levels and show less variation in plant tissue water content [66-68].

Both EB formulations increased PWC levels for the CFF wilt inoculated plants at 39 DAT (Fig. **7A**). The design of this study did not include any measurements to quantify the percent of free or bound water in the foliage or plant tissue. However, there is some evidence that the intracellular water levels were increased, which in turn suggests that the levels of bound water also increased. In Part 2 of this article series, one explanation was offered for the assumed increase in intracellular water contents [2]. This explanation suggested that the EB formulations temporarily reduced the chlorophyll density, thereby allowing red light to be absorbed by water molecules which results in higher levels of bound water inside the cells.

There is another possible explanation that could also explain why the EB formulations may have increased the bound water in the CFF wilt plants. This second explanation correlates the increased free salicylic acid (SA) concentrations for both EB formulations with a possible increase in intracellular bound water for both treatments. As mentioned in Part 1 of this series, both EB formulations increased free SA levels when measured at 5 DAT for the chemical treatments [1]. Please review Part 1 for a full explanation of how the EB formulation generated an oxidative burst that resulted in the free SA peaks in the foliage tissue at about 5 DAT. A structured water study by Sharma *et al.* [69] found that salicylic acid increased the exclusion zone of water by approximately 20 to 40% when water was exposed to a SA concentration of approximately 0.718 mg SA/l [69]. As mentioned previously, exclusion zone water and bound water are synonymous research terms. From these results, it can be deduced that both EB formulations increased free salicylic acid levels in the foliage, which in turn increased the exclusion zone or bound water levels in the intracellular water of the foliage. The free SA concentration tested in the Sharma *et al.* study [68] was quite high compared to the free SA concentrations reported in Part 1 of this series which ranged in units of ng/g. Although the concentration of free SA affected the size of the EZ zone in the study by Sharma *et al.* [69], it is very plausible that SA can increase bound water levels under average physiological conditions. Future studies should be designed to measure oxidant burst effects on free SA concentrations while simultaneously measuring the percentage of free and bound water in the foliage.

Most abiotic stressors, such as water stress, induce the generation of free SA in plants to activate the defense systems. Several drought tolerance studies have shown that foliar applications of salicylic acid do increase the relative water content in water-stressed crops [70-74]. A maize drought tolerance study by Rao *et al.*; [70] shows that a foliar application of salicylic acid plus L-tryptophan increased RWC to 79% when compared to 52.3% RWC in the check plants. A rice drought tolerance study by Farooq *et al.* [71] shows that RWC was 60 % in water-stressed rice plants treated with a SA foliage application when compared to an RWC of 50% in the control plants. Another rice drought study by Sohag *et al.* [72] reveals that RWC was 55 and 85% in the water-stressed control and SA-treated plants, respectively. These studies conclude that SA improves several bio-indicators related to drought

tolerance which includes enhancing water content in water-stressed plants. Currently, it is widely accepted that foliage treatments with SA formulations can substantially improve drought [73-76] and cold tolerance [77, 78] in field crops.

As leaf tissue dehydrates during drought or cold temperatures, drought and cold-tolerant plants can alter the structural properties of interfacial and vicinal water and thereby increase the structured water content in their foliage. Three researchers, including Ignatov *et al.*, [32], Morita *et al.*, [79], and Kuroki *et al.*, [80], investigated the ability of an extreme drought-tolerant plant species, *Haberlea rhodopensis*, to alter its interfacial, or vicinal water properties by increasing the structured water content when plants were water stressed. They conclude that extreme drought-tolerant plants can create structured water through the formation of five- and six-point water molecule rings in the vicinal, bound water zone. Also, as the structured water content increases in the foliage, there is a concomitant increase in drought tolerance [81, 82]. A water study by Jhon [83] found that supercooled water at -40 °C remains liquid and is 100% structured water. A review of structured water by Pollack [84] states that structured water will remain liquid in confined spaces as temperatures drop to -80 °C. A water study by Gallo states that supercooled water has structured water properties [85]. These studies show that structured water has non-freezing properties and remains liquid well below 0 °C. Intuitively, vicinal or bound water is also non-freezing water, and therefore, as vicinal or bound water increases, cold tolerance also increases.

Vicinal, structured water also increases heat tolerance which was explained in more detail in Part 2 of this series [2]. Structured water has lower vapor pressure properties, as explained in Part 2, which means it changes from a liquid to a gas phase more slowly than free water. Thus, vicinal water remains in the liquid phase under higher temperatures and thereby reduces cold. A lower vpdl occurs when there is a smaller gradient between the intercellular space in the leaves and the atmospheric vapor pressure. As previously mentioned, a lower vpdl reduces the potential for stomatal closure, thereby maintaining adequate flux rates for CO₂. The collective research findings for the interplay between structured water and heat and cold tolerance are aligned with the Sharma *et al.* [69] findings. Together all the findings suggest that as SA increases structured water content in plant foliage, there is a correlated increase in heat and cold tolerance.

In summary, salicylic acid foliage treatments enhance both drought tolerance and cold or heat tolerance by acting as a signaling agent for activating plant defenses and by altering interfacial, bound water properties. There is ample evidence that SA treatments enhance plant defenses against water, heat, and cold stress. However, there is a paucity of research on the interactions between SA treatments, increased structured intracellular water levels, and improved plant defenses against abiotic stressors. If SA foliage treatments can be linked to increased structured water levels in plants, then it can be rationally concluded that EB treatments can also increase structured water in plant tissue, based on the finding in Part 1 of this series [1]. EB formulations can activate SA signals with sufficient oxidative bursts, then EB formulations can be substituted for expensive SA foliage treatments.

4.3. Development of Two Proposed Hypotheses for Plant Responses to EB Primers.

Results from Part 2 of this series also show that the two EB formulations had the lowest E (Table 10 Part 2), gs (Table 8 Part 2) and vpdl (Fig. 7 Part 2) for the CFF wilt inoculated plants at 39 DAT. The two EB formulations also had high Ci/Ca means (Table 12 Part 2) and high FWC (Fig. 6 Part 3), and equivalent total and fruit biomass and leaf area with the other chemical primers (Fig. 5 Part 3) for the CFF wilt inoculated plants. In general, the two EB formulations had low gas exchange rates and yet still maintained plant growth rates, leaf area, and fruit production that were equivalent to the other treatments.

These incongruous results suggest that the EB formulations altered basic physiological processes and thereby altering and reducing the dependence of foliage biological functions on water volume or quantity. Instead, the efficiency of foliage physiological activities became more dependent on water quality and structure when the CFF wilt progressed into excessive water stress on the foliage. This combination of observing specific foliage responses with prior knowledge of structured water on plant biological functions persuaded the primary author to suggest two hypotheses proposals that were stated in the Part 2 and 3 Discussion sections. The two proposed hypotheses were: 1) the chlorine dioxide primers reduced chlorophyll density in leaves, which resulted in increased absorption

of red-light energy by cell water that increased its structured water properties, and 2) the chlorine dioxide primers resulted in a burst of ROS and salicylic acid signals in the foliage which increased the structured water levels within foliage cells. The first hypothesis could be tested using a SPAD meter to test chlorophyll density after foliage treatments. The second hypothesis could be tested using any method used to estimate bound water in food processing or food drying articles. These hypotheses are based on the putative theory that biotic and abiotic stresses in plants can be minimized by maintaining sufficient levels of biologically structured water at the cell level.

This putative theory was directly evaluated and tested in a greenhouse study using simulated irrigation with structured water for water-stressed legumes that was conducted and reported by Ramsey [86]. The findings from this study show a 25% reduction in Pn and a 34% reduction in stomatal conductance in velvet beans when grown under a deficit irrigation schedule. Despite the reduction in gas exchange rates, there was only a 6.8% reduction in oven-dry foliage biomass when grown under moderate to high water stress conditions (40 to 50% less water) when irrigated with structured water [86]. These study findings were the basis for the two proposed hypotheses in Part 2 and 3 articles in this series.

The putative theory that structured water can enhance plant defenses against abiotic stresses such as heat or water stress is further confirmed by a myriad of magnetized water studies. A study by Shalatonin *et al.* [87] found that magnetic fields expanded water exclusion zones. They define water exclusion zone (EZ water) as structured or vicinal water, and they consider the two terms synonymous. From their study, it can be deduced that magnetized irrigation water has a higher ratio of structured water to free water than conventional irrigation water. A review of magnetized irrigation water by Alattar *et al.* [88] and a study by Abd El-Basir [89] also state that magnetic fields alter several water properties, including altering H-bonding configurations and increasing the ratio of structured water to free water. Current literature included numerous magnetized irrigation water studies conducted on crops grown under deficit irrigation schedules or water stress conditions [90-100]. The reviews and studies substantiate and support the putative theory that structured water, when applied as irrigation water, minimizes and alleviates abiotic stresses in plants or crops. The findings from these studies also underpin the two proposed hypotheses concerning the EB formulations in Parts 2 and 3 of this article series.

4.4. Specific Leaf Area Findings

Leaf morphology dynamics in this study focused on SLA interactions between the two study factors. Research has shown that SLA is a good indicator of foliage responses to abiotic and biotic stressors [33]. As the ratio of leaf area to oven-dry leaf biomass (SLA) decreases, leaves become thicker, which increases the physical barriers to disease entry into leaves [33, 34]. Thicker leaves also have less surface area, thicker cuticles, and reduced water losses [33, 34]. Disease resistance literature shows that leaf thickness is positively related with increased plant immunity. The literature also states that resistant crop varieties generally have lower SLA ratios to increase the physical barriers against pathogen infections [35-38].

The SLA was 270.19 and 387.98 sq cm/g for EB 400 mg/l and the water treatment, respectively, for the CFF inoculated plants. Thicker leaves for EB applied at 400 mg/l increased the physical barriers for re-entry of any CFF wilt impinging on the leaves. The second leaf harvest was not based on sampling but measured all the green and partially green leaves for each plant. Thus, the SLA data included all the new leaves that developed over 57 to 58 days between the chemical application and the final harvest when the plants grew about 30 to 40 cm in height. The SLA results show thicker leaves developed after EB was applied at 400 mg/l on the CFF inoculated plants.

The single application of EB at 400 mg/l appears to have initiated a strong enough oxidant signal after inoculation of the CFF wilt to activate a SAR response. The cascade of systemic SAR responses includes thicker leaves, as evidenced by a significantly reduced SLA estimated from the final harvest data (Fig. 8-9). Also, the SLA for EB applied at 400 mg/l was significantly lower than all the other chemical treatments for the CFF inoculated plants (Fig. 8). The increased leaf thickness for EB applied at 400 mg/l is significant evidence of a SAR response that resulted in increased leaf properties indicative of enhance plant defenses in the mature plants.

The salicylic acid (SA) results reported in the Part 1 article show a peak in free SA at 1300 h for EB applied at 400 mg/l. This sudden oxidant peak putatively activated an SAR response which resulted in a systemic response

with a lower SLA and thicker leaves in the two months following the chemical treatments. In conclusion, the EB formulation developed thicker leaves in post-spray foliage, which is indicative of an activated, systemic SAR response to develop physical barriers in new foliage to prevent water loss and/or minimize reinfection from neighboring plants.

4.5. Time Series Photographs and Plant Growth

The time series photos show the rate of growth and the growth stages for kidney bean seedlings. The photos reveal that the seedlings were very young during the first leaf sampling and very mature during the second leaf collection date. Four-time series photos were taken for the water control treatment that was inoculated with the CFF wilt (Fig. 1-2). The first two photos (Fig. 1) 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 trifoliolate leaves (Fig. 1, right photo). The photos of the mature plants (Fig. 2) show that the plants were about 30 to 40 cm taller than the seedlings. The time series photos conclusively show that the two leaf collection dates occurred when the plants were seedlings and again when the plants were fully mature. The leaves for the first sample date were recently chemically treated (5 DAT) and inoculated with the CFF wilt. However, the leaves on the second harvest date included both pre-spray and post-spray leaves that were never chemically treated or CFF inoculated.

4.6. Oxidant Primers and Redox Biology

Several reviews were published for chemical primers [5-11]. Oxidant primers such as hydrogen peroxide or ozone generate radical oxygen species (ROS) that act as signals for activation of plant defenses [101-103]. Both oxidants and ROS species are signaling agents that can activate plant defenses against biotic and abiotic stressors. Herrera-Vasquez *et al.* [104] state that ROS and salicylic acid interplay both downstream and upstream of salicylic acid signaling for plants grown under abiotic stress conditions. Voeikov [105-109] investigated the functionality of oxygen-free radicals for signaling, energy generation, and immunity for structured water. He concludes that living cells are constantly converting a high percentage of inhaled oxygen into free radical oxygen species and immediately converting the free radicals into less injurious compounds [104-109]. In other words, if there is an abundance of biologically available oxygen, then free radicals can act as signaling agents and/or activate immune responses and then be quenched into benign intermediate species. In other words, Voeikov is suggesting that in healthy cells with abundant oxygen and/or structured water, radical oxygen species perform a myriad of signaling functions and are rapidly quenched or decomposed into benign molecules without causing excessive injury within cells.

A literature search shows that oxidant-based primers can enhance plant defenses. A hydroponic study by Graham *et al.* [110] evaluated the effects of ozonated water on sanitizing recycled water that was used to grow tomatoes. They found that oxidant water properties increased leaf area, shoot dry matter, and stem thickness in the hydroponically grown tomatoes but not in Pn, g, or Ci when measured after 9, 20, 26, 34, 41, and 48 DAT. A primer study by Sohag *et al.*; [72] found hydrogen peroxide increased drought tolerance in rice plants. Another primer study by Habib *et al.*; [111] also found that hydrogen peroxide reduced water stress in wheat plants. A chemical primer and water stress study by Rahman *et al.*; [112] found that RWC, Pn, g and e significantly increased after a hydrogen peroxide primer was applied to mildly stressed soybeans when measured at 3, 6, and 8 DAT. Finally, a maize study by Araújo *et al.*; [113] found that a hydrogen peroxide primer increased Pn, g, and e in maize plants that were salt stressed for twelve days. In this study, the EB primers increased Pn and g in the CFF-inoculated plants (Tables 3, 5). In addition, the EB primer at 400 mg/l increased PWC but decreased SLA in inoculated plants (Fig. 7B, 8). Finally, the EB primer at 400 mg/l increased the Pn/Ci ratio (Fig. 11 in Part 2 article [2]) and reduced leaf temperatures and vpdl in inoculated plants (Fig. 7, 8). In Part 1 of this series, when EB was applied at 400 mg/l, there was a 15-fold increase in free salicylic acid as averaged across CFF inoculated and non-inoculated plants at 5 DAT, and 23 to 25 h after CFF inoculation. Oxidant primers can boost plant immunity for both biotic and abiotic stressors with minimal injury to plant growth and yield.

Along with boosting plant immunity, oxidant primers can enhance or improve overall plant or cellular health. A basic research study by Martino and Castello [114] investigated the modulation of peroxide species in cells (H₂O₂

and O_2^-) for maintaining cell health. A review by Sherin *et al.*; [115] states that primers can enhance photosynthesis in plants. A study by Ramsey *et al.*; [18] found that the EB formulation used in this study also improved the maximum quantum efficiency of chlorophyll in the chemical control rhododendrons after a single foliage spray application. Also, a thesis by Hammack [116] found that the EB formulations tested in this study also improved chlorophyll efficiency in camellia plants. Together these studies offer preliminary evidence that the EB formulations can improve plant growth and chlorophyll efficiency in the woody ornamentals and legume species that are not under any stressor conditions. Further research may show that the EB formulations with the proprietary surfactants have a dual benefit for improving crop health: 1) improving crop health and growth when grown under normal, non-stressor conditions, and 2) boosting plant immunity for plants or crops grown under biotic and abiotic stressor conditions.

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 [117, 118]. Chemical primers may activate a 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 [119-123]. 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 [119-121].

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 the foliage, it produces a burst of ROS, which in turn signals the biosynthesis of salicylic acid [23-27]. Both molecules are ubiquitous in plants, and they act as the primary activation signals for plant defenses [23-27]. There are several literature reviews on the effects of salicylic acid signals for boosting plant immunity for both biotic and abiotic stresses [124-128]. A review by Lukan and Coll [103] 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 a 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 are 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 to 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 [129, 130].

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 [131]. 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 challenging findings in each section. The first article [1] reported on the salicylic acid (SA) results, while Part 2 [2] reported on the gas exchange and Fv/Fm results. 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. 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. 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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