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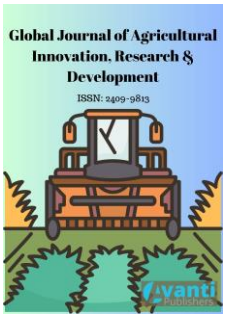

Priming Bean Seedlings to Boost Natural Plant Defenses Against Common Bacterial Wilt: Salicylic Acid Responses to Chemical Primers (Part 1)

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ABSTRACT

This greenhouse study evaluated the effects of two chemical inducers for priming kidney bean seedlings against a bacterial wilt disease. This study's central premise was that chlorine dioxide's oxidant properties would mimic the signaling properties of radical oxygen species, thereby initiating a cascade of molecular plant defenses, including the synthesis of salicylic acid (SA). This signaling agent then initiates a cascade of pre-defense activities to provide a more rapid and robust natural defense against pathogen attacks. This factorial study included two levels for a foliar chlorine dioxide treatment and two for a bacterial wilt inoculation treatment. The two plant response variables were free and conjugated salicylic acid levels sampled in leaf tissue over two collection dates. Half of the 96 plants were inoculated with a bacterial culture that causes common bean wilt disease. Leaf tissue was harvested 17 to 32 h and 960 h after the wilt inoculation to determine the temporal dynamics of SA due to chemical treatments. Also, PCR tests were used to verify wilt presence in the inoculated plants. Inoculation of the wilt disease did not affect free SA when leaf tissue was sampled from 17 to 32 h. after wilt inoculation. However, chlorine dioxide applied at 400 mg/l and sampled at 20 h after inoculation resulted in a 15-fold increase in free SA over the control. Also, chlorine dioxide applied at 400 mg/l with leaf tissue sampled at 26 h after inoculation resulted in a 33-fold increase in conjugate SA levels compared to the control plants.

Leaf tissue sampled at 960 h after the inoculation showed no free SA differences among the chemical treatments. However, the inoculated plant had a 15.9-fold increase in free SA compared to the non-inoculated plants. The priming effect on kidney bean seedlings using a single chlorine dioxide foliage application temporarily increased free and conjugate SA. The free and conjugate SA levels for the non-inoculated plants returned to baseline levels when sampled at 960 h. These results indicate that primed plants elevate SA up to several weeks with a slow decline back to baseline levels. Stem injection of the bacterial wilt bypassed the immunity mechanisms present in leaves, which significantly increased the wilt injury levels. Stem injection negated much of the foliar defenses, which overshadowed the priming effects of the chemical treatments on plant immunity and foliar defenses. The second leaf sampling on newly formed leaves reveals elevated SA levels in the inoculated plants but not in the non-inoculated plants.

1. Introduction

Molecular signals activate a cascade of natural plant defenses in vascular plants for partial or complete protection against a broad spectrum of diseases, herbivores, and abiotic stresses. Over a decade of research has also shown that several chemicals can act as inducers, temporarily and partially activating natural plant defenses. Foliar application of chemical inducers activates or primes plant defenses, much like vaccines boost immune systems to alleviate acute symptoms and speed recovery from viral attacks. Primed plants develop a series of temporary, quasi-defense mechanisms that allow a more rapid and robust response to pathogen attacks or abiotic stressors [1-9].

A primary signaling agent for activating plant defense responses is salicylic acid (SA) [10-16]. Once SA accumulates to an activation threshold, a signal activates a series of defense responses, including activation of the Systemic Acquired Resistance (SAR) response [12, 13, 17]. Two forms of SA are produced during a plant defense response, free and conjugated SA [17]. Free SA is the active form of SA that is responsible for activating a defense response. When free SA is conjugated with small organic molecules such as glucose, an inactive form called conjugated SA is generated. Conjugated SA is translocated and stored in the plant vacuoles. Conjugate SA is comprised mainly of *O*-β-*D*-glucoside within plants and is an important reserve needed to rapidly convert into free SA for a quick accumulation response to a pathogen attack [17].

Recent reviews have succinctly detailed the myriad pathways and mechanisms related to priming plants to boost their immunity. In addition to conventional priming constructs, these reviews also included information on the accumulation of SA to initiate long-term SAR defense responses [18-22]. The reviews also mentioned potential plant resource tradeoffs between maintaining the primed status or diverting resources back into plant growth and reproduction needs [18-22]. Finally, the reviews covered priming effects on immunity "memory" and the transformation of immunity systems to provide long-term protection against delayed or late-in-the-season pathogen attacks.

A chemical inducer with an established record for priming plant defenses is acibenzolar S-methyl (ASM), a functional analog to salicylic acid [23-26]. Actigard (Syngenta, Basel, Switzerland) contains ASM and is commercially available in the USA. Acibenzolar S-methyl is linked to reducing disease severity by priming plant defenses without exhibiting any inherent antimicrobial properties [23-26]. In a study by Neerja et al. [25], they evaluated rice plants treated with ASM to determine the plant defense responses against *Rhizoctonia solani*. The results showed a lower disease severity with inoculated ASM-treated plants compared to the inoculated control. In addition to reducing disease severity, ASM applied to the non-inoculated plants resulted in a higher yield than the control non-inoculated plants. The metabolic costs of priming have yet to be fully understood. However, it may be possible that there is a minimal tradeoff in priming metabolic costs and plant resources allocated to growth and yield.

Chlorine dioxide has been proven to be an effective chemical inducer for priming plant defenses [27-33]. Chlorine dioxide liquid formulations have oxidant properties and can be applied to plant foliage using non-toxic concentrations [27, 29-33]. Chlorine dioxide formulations with surfactants are easily transported across the waxy cuticle of the leaves and transported as a soluble gas in the vascular system. Once inside, the ClO_2 mimics the oxidant signals of other radical oxygen species, eliciting a defense response. One such response is the synthesis of salicylic acid [11, 34-36].

This study evaluated the effects of chlorine dioxide and Actigard for priming the natural defenses of kidney bean (*Phaseolus vulgaris*) plants inoculated with common bacterial wilt caused by *Curtobacterium flaccumfaciens* pv *flaccumfaciens* (CFF) [37]. 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 primary hypothesis of this study was that the chemical treatments would prime the kidney bean plants, thereby increasing the SA responses in both the CFF-inoculated and possibly the non-inoculated plants. The second hypothesis was that the free and conjugate salicylic acid levels would reveal different temporal patterns when leaf tissue was collected over two sampling dates. The third hypothesis was that the SA responses would be higher in the CFF-inoculated plants when compared to the non-inoculated plants. The fourth and third hypothesis was that the free and conjugate levels of salicylic acid in the leaf tissues would show different temporal patterns over the sample dates.

This two-year study will be divided into three articles (Part 1 – 3). This article is Part 1 that describes the interactions of priming plants with chemical inducers and inoculation with a vascular wilt disease on free and conjugate forms of SA in kidney bean seedlings. The second article (Part 2) will describe the interactions of the chemical treatments and the CFF wilt with gas exchange and fluorescent responses. The third article (Part 3) will report on the effects of chemical treatments and CFF wilt on leaf morphology, leaf area, and biomass for the kidney bean plants.

2. Materials and Methods

2.1. Study Design

The greenhouse study was conducted at the United States Department of Agriculture's Crop Research Laboratory (USDA CRL) greenhouses in Fort Collins, CO. The study had a factorial design with two factors, plus additional 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. Each treatment was replicated twelve times. The study factors are listed in Table **1**.

Table 1: Description of chemical treatments and *Curtobacterium flaccumfaciens* pv *flaccumfaciens* (CFF) inoculation status.

Biocide Type	Concentration (mg/l)	CFF Inoculation Status
ElectroBiocide	200	Yes
ElectroBiocide	200	No
ElectroBiocide	400	Yes
ElectroBiocide	400	No
Actigard	60	Yes
Actigard	60	No
Water	0	Yes
Water	0	No

2.2. Description of Plant 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 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 seedlings remained in the pot for further growth and evaluation for photosynthesis, fluorescence, final SA measurement, and biomass responses at the final harvest 61-63 days after planting. Plants were fertilized with 20N-2.2P-8.3K (Jack Peters Professional Lite 20-10-20 Allentown, Pennsylvania) soluble fertilizer through fertigation methods at 100 mg $N{\cdot}L^{-1}$ 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 a 14:10 h light/dark schedule.

2.3. Description of Common Bean Bacterial Wilt

Curtobacterium flaccumfaciens pv. *flaccumfaciens* (CFF) is a bacterium that causes common bean bacterial wilt disease in *Phaseolus* species [37]. This disease was a problem for dry bean production in Colorado, Nebraska, and Wyoming from the 1960's to the early 1970s [36, 37]. The main dispersal of CFF is through infected seed, but soil

and infected debris can be a reservoir of inoculum [37, 38]. Symptoms of CFF may include stunting and reduced yields for milder infections or even mortality for more severe cases [39]. The best line of defense is to plant resistant cultivars and purchase clean seeds since there are no pesticides that control CFF in common beans [39, 40].

Common symptoms of CFF infection on *Phaseolus* are wilting of the leaves and necrotic lesions with a yellow halo on the leaves [37, 39]. Vascular wilts are unique because they thrive and multiply in the xylem, which is nutrient deficient [40]. The bacterium clogs up the vascular system causing a drought-like stress that prevents water transport into the foliage [38, 39]. After the early 1970's CFF was a problem for bean production in the United States in 2003. CFF remerged on dry bean production fields in Nebraska [37, 41]. This disease has restricted international trade as CFF is on the quarantine list for many countries [37, 39].

2.4. Common Bean Bacterial Wilt Inoculation Methods

The yellow race (B-528) of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (CFF) was used to inoculate the light red kidney bean plants. 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. 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. 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 cooled overnight under a laminar flow hood.

To ensure optimal CFF inoculation conditions, the greenhouse parameters were set for 32 °C and close to 100% relative humidity one day before inoculation. The greenhouse shade cloths were closed to achieve high humidity conditions, and the exhaust fans were turned off. After inoculation, plants were exposed to humid and hot conditions for 48 h to enhance optimal inoculation conditions.

Plants were inoculated using the stem stab method 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 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 cotyledon scar at a downward angle. A new sterile needle for inoculation was used for each treatment. Plants 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, and additional label enhancements are currently 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 plants and boost plant immunity.

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 "Host Plant Defense Induction; Group P1" category. 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, Bailey, 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 and 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 both the top and bottom sides 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.

2.7. Time Series Photographs to Observe Plant and CFF Wilt Interactions

All 96 plants were photographed over five different periods to observe any interactions between plant phenology and the 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. On this date, the plants generally grew to about 25-30 cm and 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 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.

2.8. Plant Tissue SA Sample Collection and Extraction

The first salicylic acid leaf tissue analysis harvest started at 17 to 19 h after inoculating CFF in the plants and five DAT. Foliage harvest time started at 0700 in the morning and ended at 2200 (military time) on the same day. One leaf from each plant was collected every three hours. The selection of leaves was the large leaves for SA measurement while avoiding cotyledon leaves (unless no other leaves are available). Six leaves were collected per plant over the 22 h sampling period. SA's second and final leaf harvest was collected 61 days after planting (44 DAT). This leaf harvest evaluated any differences in SA concentrations among the chemical treatments at the end of a typical growing season for these beans. At each collection time, one leaf sampled per plant was inserted into a sterile 50 ml centrifuge tube and immediately submerged into liquid nitrogen. Leaf samples were then placed into a -80 °C freezer until ready for analysis. Plant samples were then removed from the -80 °C freezer, lyophilized for 24 hours, and ground to a fine powder with mortar and pestle.

2.9. Leaf Tissue Preparation for Salicylic Acid Analysis

Leaf samples were analyzed at Colorado State University, Department of Environmental and Radiological Health Sciences. The director of the analytical laboratory developed a custom method for free SA and total SA measurement using a liquid chromatography and tandem mass spectrometry (LC-MS/MS) instrument. Each leaf sample was measured for free SA and total SA. Conjugated SA was calculated by subtracting free SA from total SA concentrations.

Leaf samples were prepped for LC-MS/MS by measuring 15 mg (± 0.5 mg) into a 13 ml test tube. Free SA was extracted by adding 1 ml of 10% acetic acid and 20 µl of an internal standard of 15 µg/ml D4-salicylic acid. Samples were then vortexed for about 30 seconds until well mixed and placed in a sonicating bath for 10 minutes. Samples were then vortexed for a second time for 30 seconds and placed into the sonicating bath for an additional 10 min. The samples were then centrifuged for 10 minutes at 3400 revolutions per minute. An 0.5 ml aliquot of the liquid was then transferred to 1.5 ml vials for LC-MS/MS analysis of free SA.

Total SA was extracted by acid hydrolysis. From the same sample used for free SA, 500 µl of supernatant was transferred to a clean 13 ml glass tube and diluted with 400 µl of 10% acetonitrile/0.1% acetic acid. Then 50 µl of 10 % hydrochloric acid solution was added and vortexed. The pH was then verified to be between 2-3 pH. Samples were then placed into an 80 C sand bath for 30 minutes. Samples were then allowed to cool for about 10 minutes,

and then 50 µl of 15% ammonium hydroxide was added. After being vortexed, samples were verified for a pH of 4 to 5. Samples were centrifuged for 3 minutes at 14000 revolutions per minute, and 0.5 ml aliquot was transferred to a 1.5 ml vial for LC-MS/MS analysis of total SA.

2.10. Salicylic Acid Liquid Chromatography (LC-MS/MS) Analysis

The liquid chromatography (LC) instrument (Agilent 1290 UPLC) was coupled to a quadruple mass spectrometer (Agilent 6460). The mass spectrometer was equipped with an electrospray ionization source using Agilent Jet Stream Technology (Agilent, Santa Clara, CA). SA was separated on a Zorbax Eclipse Plus C18 column (2.1mm x 100mm, 3.5 µm particle size) at 40 °C. A sample volume of 10 µL was injected, and a binary mixture of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B) at a flow rate of 0.4 mL/min. The gradient was 20% B increasing to 100% B at 4 min and held for 1 min. The ionization source conditions used were as follows: negative polarity, nebulizer gas flow of 5 L/min at 30 °C and 45 psi, sheath gas flow of 11 L/min at 250 °C; nozzle voltage of 500V, and the capillary voltage at 3500 V. Salicylic acid was identified by comparison of retention times with analytical standards, individual multiple reaction monitoring (MRM) mass transitions, and with MS/MS ion ratios. In this method, SA had a retention time of 2.95 minutes; the MRM transitions were 137 > 93.1 and 65.1 m/z, and an ion ratio of 9.4. Peaks matching retention within 5% with ion ratios with 20% of the standard ratio were considered acceptable for SA. D4-salicylic acid was confirmed by retention time (2.95 mins) and the MRM transition 141.1 > 69.1 m/z. The data collection and processing were performed using Agilent Mass Hunter Quantitative software.

2.11. Leaf Tissue CFF Wilt DNA Extraction and Qualification

To test for the presence or absence of CFF infection within the CFF-inoculated and uninoculated plants, 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 [42]. 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 extract and bind sample DNA from the sample liquid while devitalizing any living pathogens. All the extractor circles on the FTA cards were labeled for each plant, along with its treatment and replication. 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, which was collaborating with this project, to analyze the cards for the presence of CFF DNA using qPCR reactions specific to CFF bacteria. Since the stem tissue sap collection process was not quantitative, the results were a qualitative test for the presence of CFF in infected plants.

2.12. Statistical Analyses

JMP 11 (SAS 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 was used for non-repeated measurements. All non-significant terms in each final model were deleted but explained in the results section. The SA samples were analyzed as a repeated measures study, with sample time as a random variable nested within the primary model for the first leaf tissue harvest. Analysis of the two leaf tissue harvests was reported as two separate tests due to a date interaction term in the model when the two dates were tested for significance. In other words, the SA results differed between the two tissue harvests, necessitating reporting the two harvest results separately.

The Student T-test was used as the multiple range test to separate any differences among the Least Square Means for SA levels among the treatments ($\alpha \leq 0.05$). 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

Analysis of the compiled SA data was divided into two tissue sampling dates as there were differences in SA levels between the two sampling dates. In addition, the SA data was divided into free and conjugate SA variables due to concentration differences within each sampling date. The first sampling date was analyzed using a repeated measures model due to the six sampling times within the sample date. The second sampling date involved one time and was not tested using repeated measures. The first leaf tissue sampling determined free and conjugate SA from leaves collected 17 h after the CFF inoculation starting at 0700 to 2200 (military time) at five DAT. The second leaf tissue sampling determined free and conjugate SA from leaves collected 61 days after planting, 44 DAT, and 960 h after CFF inoculation.

The study factor interactions for each set of analyses will be reported separately, based on the four subsections listed below, including 1) free SA for a first date, 2) conjugate SA for a first date, 3) free SA for a second date, and 4) conjugate SA for a second date.

3.1. Free Salicylic Acid for First Leaf Tissue Sample Date

The repeated measures model showed that CFF status did not affect free SA levels. The final model dropped the CFF status term but included the chemical treatments term. Also, there was a two-way interaction between leaf tissue sample time and chemical treatment. These results indicate that taking SA samples only 17 to 32 hours after inoculation was too early for any plant responses to the inoculation of the pathogen. The control plants had equivalent free SA concentrations overall sample collection times (Fig. **1** and Table **2**). Actigard also did not show a difference in concentration of free SA from 0700 to 2200 hours. Actigard and water were not different from each other over all sample times.

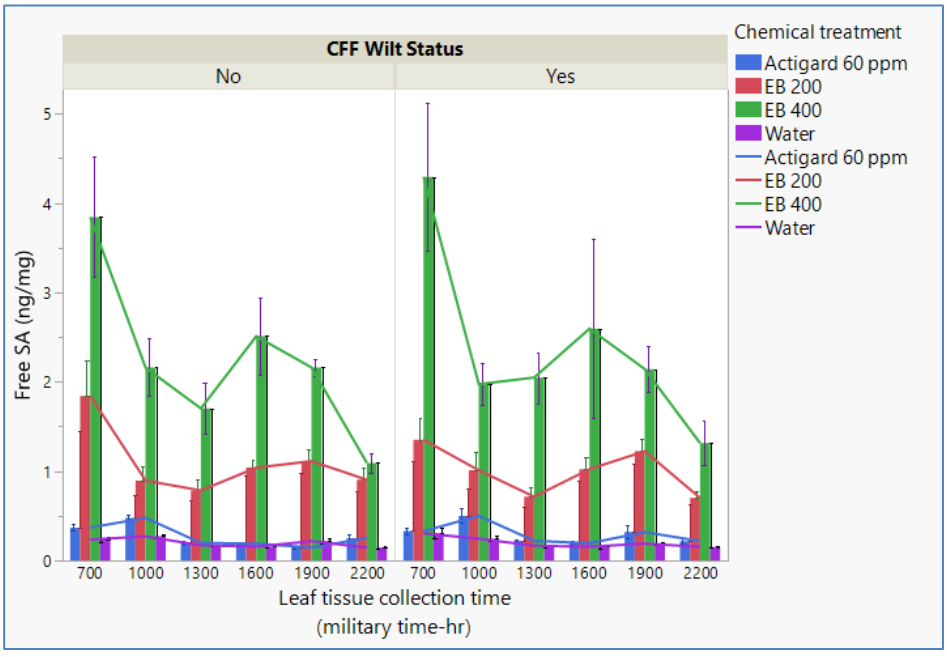

Figure 1: The least-square means for free SA (y-axis) are plotted over tissue sample time (x-axis), and the chemical treatments are labeled in the legend. The standard error is represented by black error bars (n=12).

Leaves treated with EB 400 mg/l had a higher free SA concentration than all other spray treatments (Fig. **1** and Table **2**). Leaves treated with both concentrations of EB had increased levels of free SA. The free SA for EB 400 mg/l at 0700 h was 15-fold greater than the water control. EB at 400 mg/l was consistently higher than Actigard, and water-treated leaves were collected from 0700 to 2200 h. Leaves treated with EB 200 mg/l had the second greatest concentration of free SA. Leaf tissue treated with EB 200 mg/l and sampled at 0700 h was 2.5-fold lower in free SA than tissue treated with EB 400 mg/l but also had a 5.9-fold increase in free SA levels than the control plants.

Table 2: Least square means, and Student T-test results for free SA are listed by chemical treatment and tissue sample time for the first foliage harvest due to the two-way interaction term in the analysis*.

Chemical and Concentration	Leaf Tissue Sample time (Military Time)	Student T Test (α≤0.05)	Least Squares Mean (ng/mg)
Actigard	700	HIJ	0.35
EB 200	700	DE	1.60
EB 400	700	A	4.06
Water	700	IJ	0.27
Actigard	1,000	GHI	0.49
EB 200	1,000	FG	0.95
EB 400	1,000	BCD	2.07
Water	1,000	J	0.26
Actigard	1,300	J	0.21
EB 200	1,300	FGHI	0.75
EB 400	1,300	CD	1.88
Water	1,600	J	0.17
Actigard	1,600	J	0.19
EB 200	1,600	F	1.03
EB 400	1,600	B	2.55
Water	1,900	J	0.16
Actigard	1,900	J	0.23
EB 200	1,900	EF	1.17
EB 400	1,900	BC	2.15
Water	1,900	J	0.20
Actigard	2,200	J	0.24
EB 200	2,200	FGH	0.80
EB 400	2,200	EF	1.20
Water	2,200	J	0.15

*The CFF wilt term was not in the final model, so it was not included as a factor in this table. All SA means not assigned to the same letter are significantly different.

Although there is no effect of the CFF inoculation study factor on free SA leaf tissue levels across all chemical treatments in the factorial model, there were two peaks in free SA levels for the EB formulations (Fig. **2**). There is a pronounced peak in free SA at 1300 h (Fig. **2**) for EB at 400 mg/l, which is five DAT, and 23 to 25 h (1300 to 1500) after the CFF inoculation into the seedling stem. Also, there is a smaller but still noticeable peak in free SA for EB at 200 mg/l at 1300 h. In contrast, the conjugate SA levels remain relatively level across all sampling times. The free SA for Actigard and water remained near baseline levels across all sampling times. In summary, the EB formulations primed the kidney bean seedlings to synthesize free SA within 23 to 25 h after CFF inoculation of the plants.

These two peaks in free SA levels for the EB formulations need to be revised. The peaks in free SA were 5 DAT for the chemical treatment, and the CFF wilt inoculation had no effect in the final model. In other words, what caused those two peaks in free SA for the EB formulations? If the two peaks were due to the EB treatments, then the free SA burst was delayed by five days, which seems unlikely. If we assume that the free SA burst was due to stem injury from the needle injection 23 to 25 h prior to the peak free SA levels. In that case, the control treatment should also show a similar peak in free SA. These possible explanations fail to fully explain why there were two peaks in free SA for both inoculated and non-inoculated plants for the EB formulations at five DAT and 23 to 25 h after the CFF inoculation. One possible explanation that seems most logical is that both EB formulations primed

the plants for future stress events. When the EB-primed plants were injured by the needle injection for both the inoculated and non-inoculated plants, the plants responded with a peak in free SA levels at 23 to 25 h after the needle injury. As mentioned in the methods section, the non-inoculated plants also received a blank needle injection so that all the treatments had the same needle injury responses when evaluating the temporal dynamics of the SA levels in the foliage tissues. This explanation has limitations but offers a possible cause for an otherwise mysterious peak in free SA for the EB formulations at five DAT. That peak is absent from the other two treatments.

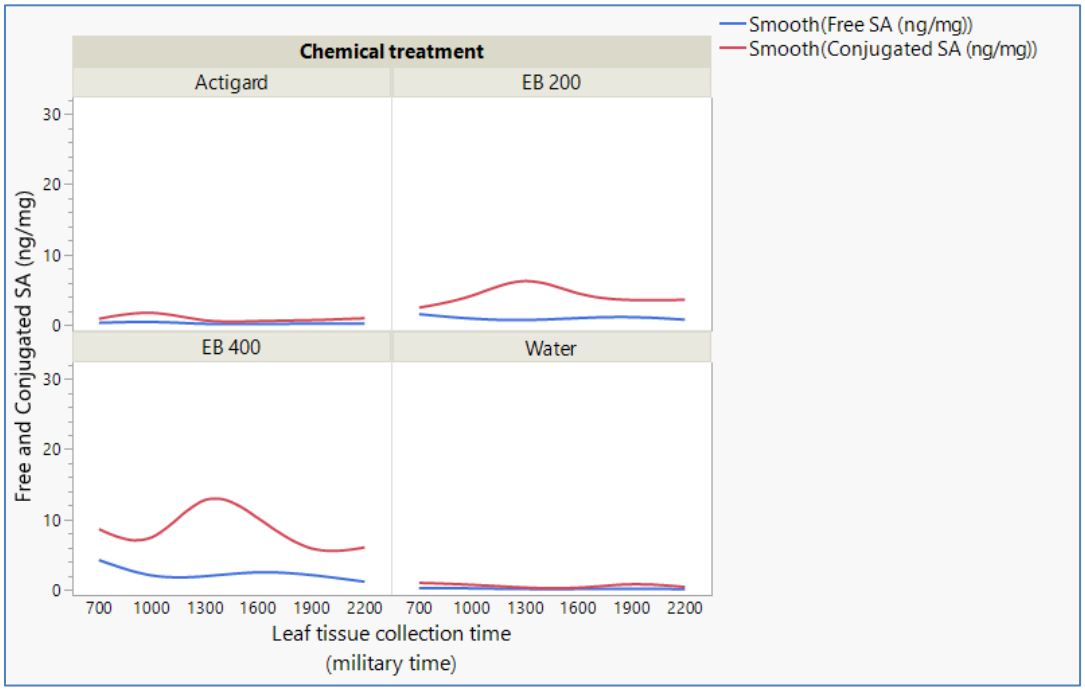

Figure 2: Statistical smoother graphs for free and conjugate SA (y-axis) are plotted over leaf tissue collection time (x-axis), and the free and conjugate SA labels are in the legend. The free and conjugate SA data included both inoculated and non-inoculated plants, as there was no CFF term in the final models for both free and conjugate SA analyses.

The underlying premise for using the two EB formulations was that chlorine dioxide at 200 and 400 mg/l was not injurious to the kidney bean physiology or growth. In addition, the two formulations showed the potential to boost plant immunity against pathogen infections, which should translate into less wilting, less foliar injury, and less reduction in biomass and fruit yield compared to the control. One method of testing these two hypotheses is to visually compare the relationships between free SA during the first harvest with above-ground, oven-dry plant biomass, total leaf area, and oven-dry fruit biomass for each chemical treatment and CFF inoculation status (Fig. **3**-**4**). If the first premise is valid, there should be a direct or neutral relationship between free SA and plant/fruit biomass and leaf area. There was a direct relationship between free SA when sampled at 1300 h and plant/fruit biomass and leaf area for EB at 200 mg/l for the non-inoculated plants (Fig. **3**). However, there was an indirect relationship between plant biomass and leaf area for EB at 400 mg/l which indicates that this formulation was injurious to overall plant growth. Both EB formulations generally had a positive, direct relationship between plant/fruit biomass and leaf area for the CFF inoculated plants (Fig. **3**). These direct relationships reveal that the EB formulations primed the plants, boosting their immunity against the CFF wilt disease over the 62-day study.

In contrast, the water and Actigard treatments generally showed a negative relationship between plant/fruit biomass and leaf area for the non-inoculated plants (Fig. **4**). These relationships show that increasing rates of free SA at 1300 h was injurious to plant growth. The relationships between free SA and plant/fruit biomass and leaf area were uniform for the CFF-inoculated plants (Fig. **4**). In other words, increasing free SA rates at 1300 h had no effect on the seedling biomass, leaf area, or fruit yield for either the water or Actigard treatments. The visual relationships are a generalized validation of the two study hypotheses for the EB formulations. A full description of the study factors on plant growth and leaf area will be published in the future, second article installment.

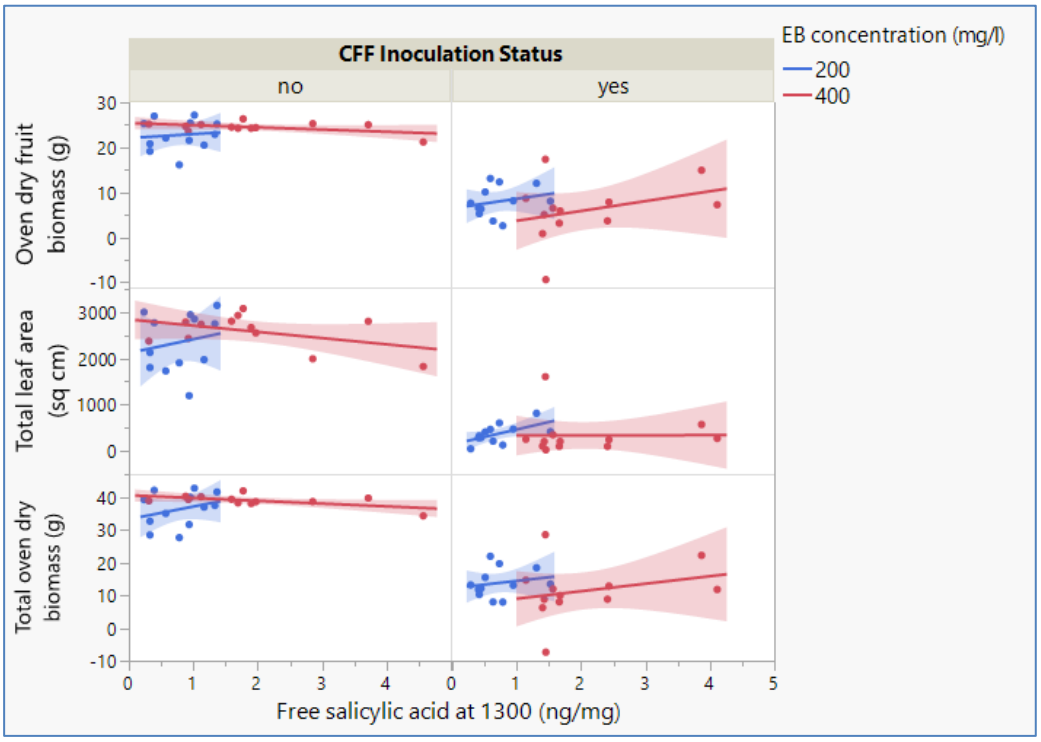

Figure 3: Total oven-dry plant biomass, total leaf area, and total oven-dry fruit biomass (y-axis) over free salicylic acid (x-axis) leaf tissue concentrations measured at 1300, or 23 to 25 h after CFF wilt inoculation. The CFF inoculation status (upper x-axis) and the EB concentrations (legend) further delineate the linear relationships over the whole time of the study.

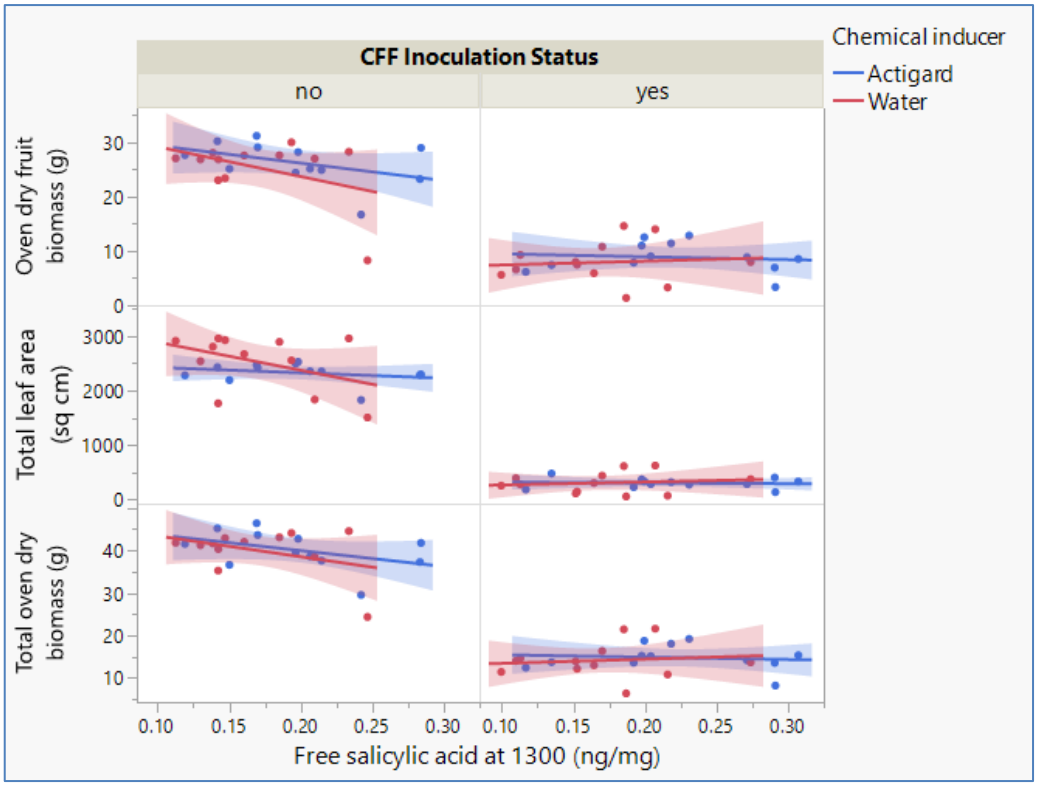

Figure 4: Total oven-dry plant biomass, total leaf area, and total oven-dry fruit biomass (y-axis) over free salicylic acid (x-axis) leaf tissue concentrations measured at 1300, or 23 to 25 h after CFF wilt inoculation. The CFF inoculation status (upper x-axis) and the Actigard and water treatments (legend) further delineate the linear relationships over the whole time of the study.

3.2. Conjugate Salicylic Acid for First Leaf Tissue Sample Date

The repeated measures model for the conjugate SA data included two two-way interactions. The two interaction terms were: 1) leaf tissue collection time * CFF wilt status and 2) leaf tissue collection time * chemical treatment. The leaf collection time*CFF wilt interaction shows a peak in conjugate SA at 1300 h (Fig. **5**). The leaf collection time*chemical treatment interaction was reported in a Student T-test table (Table **3**). When averaged across chemical treatments, the inoculated leaves had reduced conjugate SA levels compared to the non-inoculated leaves at 0700 and 1600 h.

Actigard and water were not different for all leaf tissue collection times, whether inoculated or not (Fig. **5** and Table **3**). The lack of free and conjugate SA concentrations for Actigard was likely due to ASM being a functional analog of SA. It does not necessarily induce a SA response without recognizing a pathogen. EB, on the other hand, does induce an SA response due to the ROS interaction within the plant. Since the first harvest for SA was too early for interaction with the CFF, there was no response with water control or Actigard-treated leaves. If the interaction of CFF had been present, there might have been some differences with the water control.

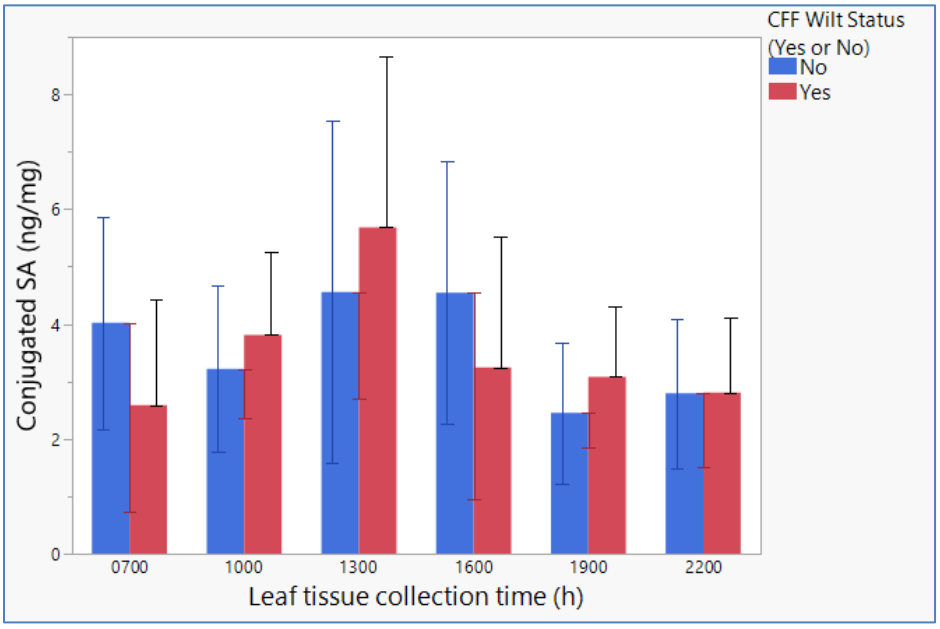

Figure 5: Least square means for conjugate SA (y-axis) over tissue sample collection time (x-axis) as averaged over the chemical treatments, based on the sample time*CFF wilt interaction term. The standard error is represented by black error bars (n=12).

Both EB treatments had the greatest concentration of conjugate SA (Table **3**). Conjugate SA showed EB 400 mg/l having the highest conjugate SA and EB 200 mg having the second highest. EB 400 mg/l had its highest peak of conjugate SA at 1300 h. The difference in water and EB 400 mg/l at this peak was a 33-fold increase for EB 400 mg/l. EB 200 mg/l had the second highest concentration and peaked at 1300 h. This was a 15.6-fold increase for EB 200 mg/l at 1300 h compared to the water control.

The conjugate SA was much higher in the EB treatments compared to the water treatment. This shows that the leaves were still actively producing SA five DAT, and the free SA was not converted from the conjugate SA reservoir. Previous studies have shown that SA accumulation is necessary to prime plants for disease resistance [10, 14, 38]. The accumulation of SA in the foliage of the inoculated leaves confirms that the leaves were successfully primed with a single application of EB to the foliage. A study by Sharma [40] evaluated the effects of ozone treatments on Arabidopsis leaves for inducing SA concentrations in foliage. They found that free SA levels increased between 0.8 to 0.9 ng/mg at 6 h after exposure to the ozone treatments. Our study results show that free SA increased to 1.0 and 2.6 ng/mg for the EB 200 and 400 mg/l treatments at 6 h after foliar applications. These two studies generally agree that free SA levels rapidly increase in the foliage (within 6 h) upon application of either gaseous or liquid oxidants.

Table 3: Least square means and Student T-test results for conjugated SA are listed for chemical treatment and tissue sample time for the first foliage harvest*.

Chemical and Concentration	Leaf Tissue Sample Time (Military Time)	Student T Test (α≤0.05)	Least Squares Mean (ng/mg)
Actigard	700	H	0.91
EB 200	700	FG	2.52
EB 400	700	B	8.96
Water	700	GH	1.05
Actigard	1,000	GH	1.82
EB 200	1,000	E	4.17
EB 400	1,000	C	7.30
Water	1,000	H	0.78
Actigard	1,300	H	0.67
EB 200	1,300	C	6.39
EB 400	1,300	A	13.57
Water	1,300	H	0.41
Actigard	1,600	H	0.62
EB 200	1,600	DE	4.46
EB 400	1,600	B	10.15
Water	1,600	H	0.32
Actigard	1,900	H	0.74
EB 200	1,900	EF	3.60
EB 400	1,900	C	5.86
Water	1,900	H	0.87
Actigard	2,200	H	1.01
EB 200	2,200	EF	3.63
EB 400	2,200	C	6.11
Water	2,200	H	0.46

*The interaction of the two study factors requires that means should be listed by both factors. All SA means not assigned to the same letter are significantly different.

3.3. Salicylic Acid Results for Second Leaf Tissue Sample Date

Analysis for the free SA only included the two study factors, and only the CFF wilt status was included in the final model (Table **4**). Also, there were no two-way interaction terms in the final model. Non-inoculated leaves treated with EB 200 and 400 mg/l had a SA response at the first foliage harvest, but the second harvest showed that SA levels returned and were equivalent to the control.

Table 4: Least square means and Student T test for free salicylic acid during the second foliage harvest*.

CFF Inoculation Status	Student T-Test (α≤0.05)	Least Squares Means
Yes	A	6.56
No	B	0.41

*The only significant term in the final model was the CFF inoculation status. All levels that are not attached by the same letter are significantly different.

The interactions between sample time*chemical treatment and sample time*CFF wilt for free and conjugate SA are illustrated in Fig. (**6**-**7**). All non-inoculated Actigard, EB 200 mg/l, EB 400 mg/l, and water treatments had equivalent free and conjugate SA levels for the second harvest Fig. (**6**-**7**). These results indicate that dicot leaves may require multiple spray treatments to maintain the priming levels needed for the long term.

The SA response for inoculated leaves continued to increase for all the chemical treatments compared to the SA levels in the first harvest. During the first foliage harvest, the inoculated Actigard and control-treated leaves showed no increased SA responses (Fig. **3**-**4**). Actigard and control inoculated leaves increased the free and conjugate SA levels in the second harvest (Fig. (**6**-**7**).

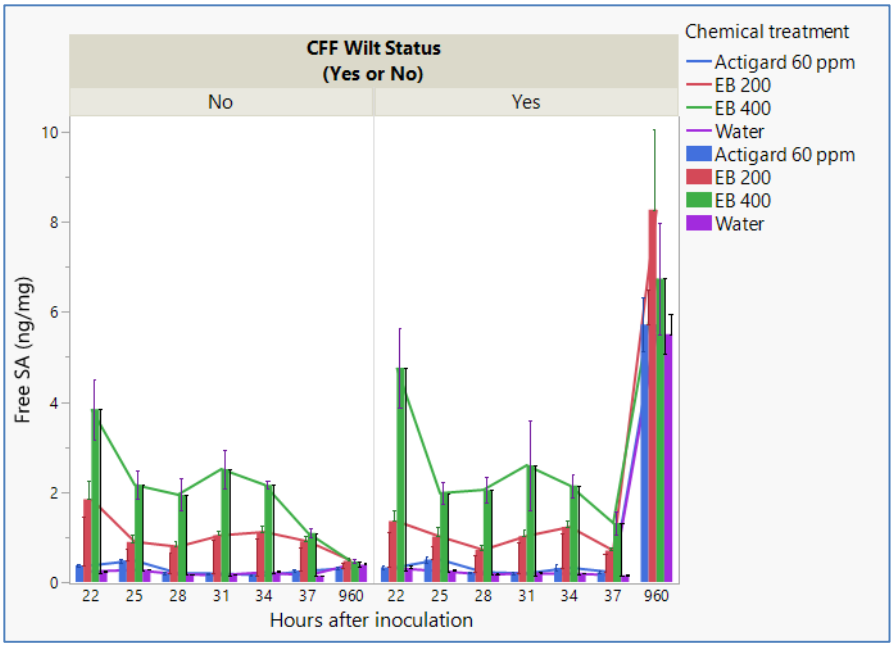

Figure 6: Free salicylic acid (y-axis) was plotted over hours after CFF inoculation time (x-axis). The first leaf collection occurred between 22 and 37 h after CFF inoculation into each stem. The second leaf collection was at the end of the study at 61 days after seed planting or 960 h after CFF inoculation. The standard error is represented by black error bars (n=12).

The model for the second sample date for conjugated SA included a two-way interaction between chemical treatment and CFF wilt status (Table **5**). Inoculated EB 200 mg/l and water control leaves had the highest mean concentrations for conjugate SA and were not different from each other (Table **5**). The second highest in conjugate SA was with EB 400 mg/l treated leaves. Inoculated Actigard had the lowest of all spray treatments. The high concentrations indicate that the leaves struggled to defend against CFF.

Table 5: Least square means for the conjugate salicylic acid for both study factors are listed for the second foliage harvest*.

Chemical and Concentration	CFF Inoculation Status	Student T-Test (α≤0.05)	Least Square Mean (ng/mg)
Actigard	Yes	A	38.75
EB 200	Yes	A	37.51
EB 400	Yes	B	27.49
Water	Yes	C	11.86
Actigard	No	D	2.92
EB 200	No	D	1.40
EB 400	No	D	1.37
Water	No	D	1.06

*The Student T-test lists any differences among the study factors. All conjugate SA levels not attached by the same letter are significantly different.

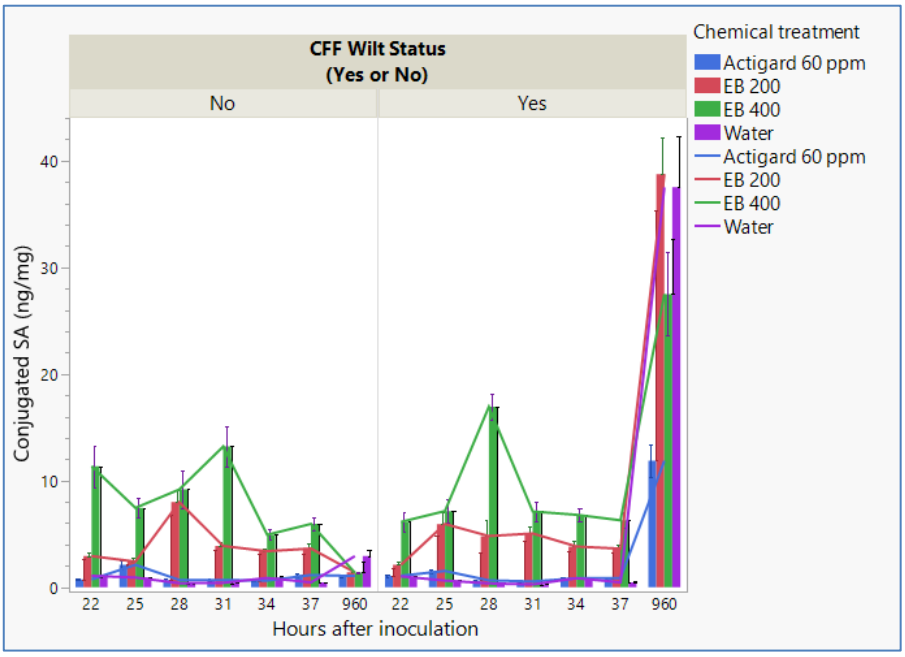

Figure 7: Conjugate salicylic acid (y-axis) was plotted over hours after CFF inoculation time (x-axis). The first leaf collection occurred between 22 and 37 h after CFF inoculation into each stem. The second leaf collection was at the end of the study at 61 days after seed planting or 960 h after CFF inoculation. The standard error isrepresented by black error bars (n=12).

3.4. Qualification of CFF DNA using PCR

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. **8**). 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 to enumerate 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.

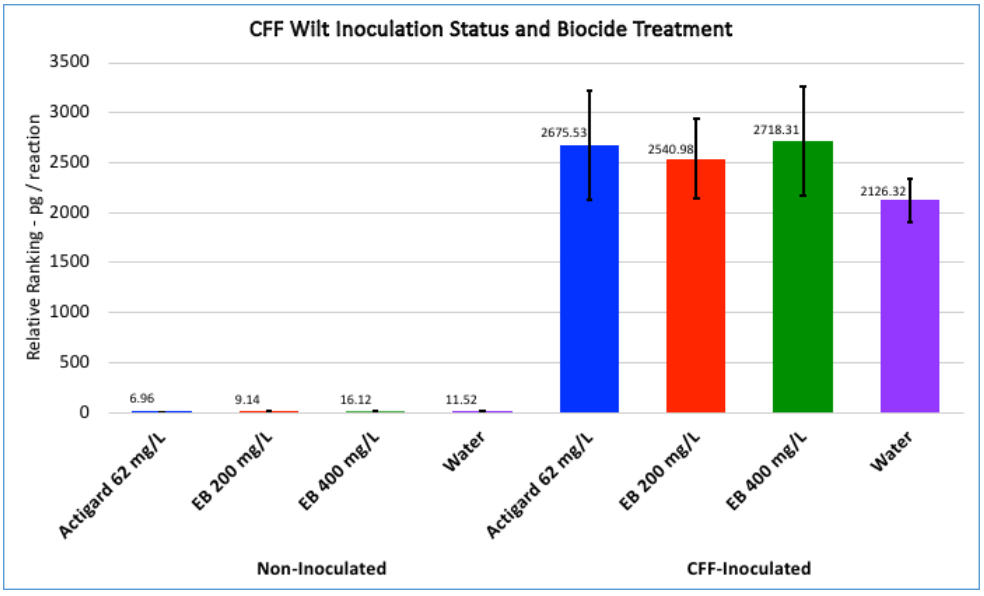

Figure 8: The mean CFF relative ranking score (y-axis), based on PCR analyses, is listed for each chemical treatment and CFF inoculation status (x-axis) (n=12).

4. Discussion

The foliar application of EB has promise due to its proposed dual functionality in boosting natural plant defenses [27]. The first function of EB was its disinfectant properties that semi-sanitized leaf surfaces. The second function of EB acts as an oxidant signal in the vascular system that initiates the synthesis of SA. For the first leaf tissue harvest, EB 200 and 400 mg/l treatments showed increased free and conjugate SA at five DAT. There was a 5.9 and 15.4-fold increase in free SA for EB applied at 200 and 400 mg/l, respectively, at their peak tissue concentration compared to water (Table **2**). Conjugate SA for EB 200 and 400 mg/l was higher than water treatments. Conjugate SA concentrations remained at baseline concentrations during the first tissue harvest for the water treatment, which indicates that EB 200 and 400 mg/l were actively producing SA due to priming five DAT. The overall increase in SA concentration for free SA and conjugate SA showed that EB successfully primed the plants' defenses for protection against pathogens. It indicates that EB could be taken into the plants to interact and elicit an SA response. The free or conjugate SA tissue concentrations for Actigard were equivalent to water when compared to water SA responses. The lack of SA activation by Actigard is due to its functional analog SA properties, i.e., Actigard replaces or supplements SA in leaf tissue. Therefore it does not elicit the synthesis of additional SA.

At the last harvest at 44 DAT (61 days after planting), there were differences in free and conjugate SA in inoculated versus non-inoculated. For non-inoculated leaves, EB treatments free and conjugate SA concentrations returned to baseline levels and were equivalent to the water control. Inoculated leaves for free SA continued to rise for all chemical treatments. Free SA for inoculated EB 200 and 400 mg/l had equivalent foliage concentrations at the end of the study. Inoculated EB 200 mg/l had a greater free SA concentration than Actigard and the water control. The conjugate SA EB 200 mg/l and water had the highest concentrations, and EB 400 mg/l had the second highest.

The first leaf tissue harvest shows that both EB treatments increased free and conjugate SA tissue concentrations (Table **2**, **3**). These findings confirm the study hypothesis that the oxidant properties of the EB formulations would activate a SA response. These results also reveal that when EB was sprayed onto foliage, it was stable enough not to be degraded during exposure to light on the leaf surface. Stability continued as EB was transported across the cuticle, entered the phloem, and maintained a strong enough signal to elicit a SA response at five DAT. The first harvest was only one day after CFF inoculation. However, the free SA peaks in the leaf tissue for the EB formulations (Fig. **2**) suggest that even as short as 23 to 25 h after CFF inoculation, there was a synergistic response between the two study factors that activated a burst in SA concentrations. Also, both EB formulations had much smaller conjugate SA peaks.

The overall results reveal that EB applied at 200 mg/l resulted in lower free SA activation rates during the first tissue harvest. However, by the second tissue harvest, EB at 200 and 400 mg/l had equivalent free SA concentrations. Also, EB at 200 mg/l had a higher conjugate SA concentration (Table **5**). At first glance, these results suggest that EB at 200 mg/l was the optimal EB formulation. However, due to bypassing the natural foliar plant defenses by injecting the CFF wilt into the plant vascular system, the plants were exposed to high disease severity conditions that compromised many physiological functions. Preliminary analysis of the other plant responses, including oven-dry plant biomass, total leaf area, and fruit biomass, reveals that EB applied at 400 mg/l resulted in equivalent or higher biomass and leaf area responses when compared to EB at 200 mg/l. In other words, the two EB formulations should be compared only after analyzing all the gas exchange, fluorescence, biomass, and leaf area responses due to the severity of the CFF wilt attack. The second and third part articles will compare all the plant responses to determine the optimal treatment given the severity of the CFF wilt attack.

The first leaf tissue sampling evaluated the temporal responses of free and conjugate SA due to the chemical treatments and CFF inoculation. The chemicals were applied five days before the first leaf harvest to allow the foliage time to synthesize salicylic acid in response to the oxidant stimulus. Leaf tissue sampling from 0700 to 2100 h the day after the CFF inoculation does reveal temporal patterns for at least the EB formulations. Smoother curves reveal the temporal patterns for the chemical treatments and CFF inoculation, which show an early (23 to 25 h) synergistic response for the EB formulations. The temporal patterns suggest that both EB treatments primed plant defenses, which rapidly responded within 23 hours after the CFF inoculation event with peaks in free SA

tissue concentrations (Fig. **2**). As previously mentioned it is unclear why the free SA peaks occurred for the EB treatments, either due to the needle injury, or to a unexplainable, synergistic response to both the chemical treatment and the CFF injection the day before the first leaf sampling date. The control leaves confirmed the dynamics of SA synthesis because the free and conjugate SA concentrations remained at baseline levels across all sampling times (Fig. **2**).

In summary, these SA results show that EB has a dual role in natural plant defenses against pathogens. The first role of EB was the oxidant property of chlorine dioxide to kill pathogens on the surface of plant foliage. The second role was to prime the natural defenses by eliciting a SA response to allow a more rapid and robust defense against future infections.

The second leaf collection was completed at the end of the study, or 44 DAT (62 days after planting). The results show that all the non-inoculated leaves had nominal baseline SA concentrations for both free and conjugate SA. Since the temporal dynamics of SA concentrations in the non-inoculated treatments return to normal levels at 44 DAT, multiple applications of EB may be needed to ensure long-term protection against possible pathogen attacks. The CFF-inoculated plants, however, revealed a temporal increase in both free and conjugated SA for the second leaf tissue harvest at 960 h after inoculation (Fig **6**, **7**). These sharp and robust SA patterns in the inoculated leaves suggest that oxidant chemical inducers can act as a signaling agent for synthesizing free SA, thereby providing a more robust and rapid boost in plant defenses against the CFF pathogen attack.

Accumulation of free and conjugate SA over two months for the CFF inoculated plants, due to priming with EB formulations, may have also initiated a SAR immunity response with concomitant maintenance of primed plant protection over the entire crop growing season. A study by Ramsey et al. [27] found that rhododendrons treated with a single chlorine dioxide (EB) foliar application increased maximum quantum efficiency (Fv/Fm) over a five-month period which suggests a long-term effect of a single application on foliar physiological responses. The rhododendron field study also reveals that EB treatments have positive, multi-month effects on gas exchange responses. These long-term temporal patterns in foliar physiology may also underlie plant priming responses. The additional plant responses in this study included gas exchange parameters, fluorescence, and plant biomass parameters. At least two of these responses suggest that a long-term SAR response was activated in the leaves treated with chlorine dioxide, which will be reported in the second and third part articles for this study.

The chlorine dioxide concentration used in this study has been evaluated for plant foliar injury in several field and greenhouse studies [27, 29-33, 43, 44]. This study found a dose-response with increasing free SA levels as chlorine dioxide increased. Oxidant properties of

Figure 9: 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 the Right photo on 6/7 (16 DAP or 0 DAT).

Figure 10: 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 the Right photo on 7/17 (55 DAP or 42 DAT).

Chlorine dioxide mimics a sudden burst of radical oxygen species, which activates the synthesis of free SA. There was a 15- and 33-fold increase in free and conjugated SA for EB applied at 400 mg/l within five days after chemical application. This oxidant burst likely stimulated resistance mechanisms within treated tissues and may have initiated a SAR response that offers long-term protection against pathogen attacks.

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 matured during the second leaf collection date. Four-time series of photos were taken for the inoculated water control treatment with the CFF wilt (Fig. **9**-**10**). The first two photos (Fig. **9**) 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 developing their second and third set of trifoliate leaves (Fig. **9**, right photo). The photos of the mature plants (Fig. **10**) 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 on two different sample leaf conditions. 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 sample date were newly formed and had never been chemically treated or recently inoculated. In other words, any evidence of increased free or conjugated SA levels in the second leaf samples would be due to the activation of systemic mechanisms that are long-term and are present in newly formed plant foliage. When SAR functionality is activated, the process is systemic across all plant components. Results from the second leaf collection revealed significant increases in free and conjugated SA levels in the newly formed leaves in the inoculated plants at 960 h after CFF inoculation (Tables **4**-**5** and Fig. **6**-**7**).

In summary, the time series photos show that the newly formed leaves sampled during the second leaf collection had significantly elevated free and conjugated SA levels for the EB and control treatments. Also, EB applied at 200 mg/l had an increased level of free SA than the control treatment at 960 h for the inoculated plants. However, the total and conjugate SA levels were equivalent among the EB and water treatments at 960 h after CFF inoculation, indicating that inoculation with the CFF wilt was the primary mechanism that activated the SAR response. The difference is that the EB formulations had a more rapid and robust activation of the SAR response that resulted in less negative gas exchange and biomass responses to the CFF inoculation over the course of the study.

A SAR response to a pathogen attack develops from a localized infection or oxidant burst into a systemic, whole-plant response that is translocated via the vascular system. The systemic response includes mobile immune

signals, pattern-recognition receptors, dormant signaling enzyme accumulation, and chromatin state alterations [45, 46]. The systemic SAR response confers a long-lasting memory of primary pathogen attack that is less specific than adaptive immune memory in animals [45]. Consequently, SAR provides long-term protection that is rapid and robust due to a heightened state of defense against a broad range of pathogens. The results of this study indicate that a SAR response was initiated due to the inoculation of CFF into plants for the EB and water treatments. However, the elevated levels of free and conjugated SA levels within five days of the foliage application of chlorine dioxide did not translate into increased SA levels during the second leaf collection date for the inoculated plants, except for EB applied at 200 mg/l for free SA levels.

Direct injection of the CFF bacteria into the plant stems ensured successful infection of the inoculated plants. However, direct injection also markedly increased the 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. Another study with similar study factors also evaluated the effects of EB formulations on kidney bean seedlings inoculated with the CFF wilt using a foliar frog that did not bypass the plant defenses in the foliage. This study will be reported in the future, the third article installment of this plant priming project.

The effects of the EB formulations on plant physiology and growth will be in a second article installment. The interactions between free SA and plant/fruit biomass and leaf area were discussed in this article (Fig **3-4**). The physiological and growth responses will provide insight into the overall temporal plant responses to the EB treatments.

The initial premise for testing the chemical primers was to induce a long-term, systemic plant immunity response and thereby reduce pesticide applications on row crops. This two-year study will be reported in three articles (Part 1–3) that will reference each other. Each of the three articles focus on specific chemical, physiological and biomass responses to the two study factors. The conclusions from the three articles will offer proof that the chemical primers induced long-term plant immunity. If the chemical primers can boost disease resistance from a single foliar application, the potential for reducing overall pesticide costs and applications is substantial.

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