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Use of Foliar Chemical Treatments to Induce Disease Resistance in Rhododendrons Inoculated with *Phytophthora ramorum*

Craig L. Ramsey[1,*], Paul C. Freebury[2], Debra H. Newman[3], Wolfgang Schweigkofler[4], Leland J. Cseke[5] and Steven E. Newman[6]

[1]*Retired– USDA Fort Collins, CO, 80526, USA*
[2]*Colorado State University, Plant Growth Facilities, Fort Collins, CO, 80526, USA*
[3]*Colorado State University, Horticulture and Landscape Architecture, Fort Collins, CO, 80526, USA*
[4]*Dominican University of California, Department of Natural Sciences and Mathematics, San Rafael, CA, 94901, USA*
[5]*Christopher S. Bond Life Sciences Center and Interdisciplinary Plant Group, University of Missouri, Columbia, MO 65211, USA*
[6]*Colorado State University, Horticulture and Landscape Architecture, Fort Collins, CO, 80526, USA*

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*Corresponding Author
Email: clramsey37@gmail.com
Tel: +1 (970) 416-6326

ABSTRACT

A field study was conducted at the National Ornamental Research Site at Dominican University California (NORS-DUC). The study goal was to evaluate three chemical inducers applied as foliar treatments for controlling *Phytophthora ramorum*, on *Rhododendron x 'Cunningham's White'* nursery plants. The inducers were chlorine dioxide (ElectroBiocide), hydrogen peroxide (OxiDate 2.0), and acibenzolar-s methyl (Actigard). Water samples from the electrostatic sprayer were measured for three physicochemical water properties. Visual assessment of plant foliage, based on the Horsfall- Barratt scale, was conducted at three and five months after chemical treatments. Foliar fluorescence (Fv/Fm) was measured over three dates. The success of *P. ramorum* inoculations were determined using qPCR methods. Visual assessment across both months showed no signs of *P. ramorum* infection or chemical injury symptoms. However, *P. ramorum* infection vis-à-vis qPCR analysis was confirmed. The September Fv/Fm results revealed that all the chemical inducer treatments were equivalent to the water treatment, except for Actigard. The qPCR results were in general agreement with the Fv/Fm results indicating that the rhododendrons were successfully inoculated with *P. ramorum* but were non-symptomatic. The electrostatic sprayer ionized the water droplets, resulting in increased Fv/Fm values for the water treatments 90 days after application. There was a three-month delay in fluorescence responses to the most effective chemical applications, indicating that woody plants may need to be monitored over the long term to determine accurate responses to foliar treatments.

1. Introduction

Phytopthora ramorum, the causal agent of Sudden Oak Death and ramorum blight, is an oomycete pathogen that infects many native forest plants and ornamental nursery species in the western U.S. The genus *Phytophthora* is one of the most destructive plant pathogens in agriculture and nursery production today [1]. With a host range of over 135 species, identifying, containing, treating, and eradicating *P. ramorum* has proven to be a difficult task since its discovery in the United States in the 1990s [2 - 3].

Over the past two decades, there has been a growing research interest in priming plants for disease resistance and evaluating chemical inducers for stimulating plant defenses [4 - 8]. Numerous studies have tested inducers to chemically prime, plant immune systems to minimize disease severity in crops [9 - 11]. Oxidant disinfectants can act as chemical inducers because they are semi-stable free radicals, which are the primary biomolecules used to signal and elicit plant responses when exposed to biotic and abiotic stressors. Liquid formulations of chlorine dioxide and hydrogen peroxide were evaluated as oxidant inducers that act as semi-stable, free radical signaling agents that prime innate plant defenses against pathogen infections [12 - 17]. Two commercial formulations of chlorine dioxide and hydrogen peroxide that were evaluated in this study are: 1) ElectroBiocide (Strategic Resource Optimization, Bailey CO, USA) a proprietary blend of chlorine dioxide, pH buffer, and a sarcosinate surfactant, and 2) OxiDate 2.0 (BioSafe Systems, Hartford, CT, USA) is an EPA registered biocide. OxiDate contains hydrogen peroxide (27.1%) and peroxyacetic acid (2%), and is an EPA labeled disinfectant for 40+ row crop species for foliar applications for disease control.

A commercial inducer that is specifically designed to induce disease resistance in crops is Actigard (Syngenta Crop Protection, LLC, Greensboro, NC USA). Actigard contains the active ingredient acibenzolar-S-methyl (ASM), which is a functional analog to salicylic acid [8, 11]. Actigard was evaluated in several field studies for its ability to prime plants and reduce disease severity [6,10,18]. Soylu *et al*. [6] found that Actigard increased superoxide dismutase (SOD) and glutathione S-transferase in tomato plants resulting in a 75% reduction in disease severity against a bacterial canker, combined with 62% suppression in bacterial growth. Hong *et al*. [19] found that Actigard mixed with thymol decreased bacterial wilt incidence from 80 to 93% in a tomato crop. They also found a 57 to 94% increase in tomato yields in a two-year study.

The degree of plant-pathogen infection rate or plant disease severity can be indirectly measured using foliar fluorescence [20 - 24]. Fluorescence measures the degree of any biotic and abiotic stress on a chlorophyll protein sub-unit called Photosystem II and is, therefore, a non-specific, biomarker for plant stress [25 – 28]. The maximum quantum efficiency of Photosystem II (Fv/Fm) is estimated from the formula ((Fm-Fo)/Fm =Fv/Fm) where Fm is the maximum fluorescence and is measured when plants are dark-adapted and the chlorophyll reaction centers are closed [26 - 27]. Maximum quantum efficiency can be used to estimate the severity of pathogen infection by reducing the confounding effects of environmental conditions on fluorescence measurements. Minimizing the effects of environmental conditions can be accomplished by holding the environmental variables as constant as possible during measurements or measuring the environmental variables and adding that data as covariates in fluorescence analysis of treatment effects. Research has shown that healthy plants generally can reach a maximum quantum efficiency (Fv/Fm) of approximately 0.83, i.e., about 83% of the photons received by the chlorophyll are converted into plant sugars [28].

The physicochemical properties of water for two water treatment technologies have been evaluated for effects on redox potential dynamics and redox biology responses in plant growth studies [29 - 32]. These studies show that water properties such as oxidation reduction potential (ORP), electrical conductivity, and pH can decrease or increase plant growth, depending on the range of each water property and the plant watering methods. Husson *et al.* [33] reviewed the effects of redox potential and pH on the interactions between soil, plants, and microbes. The literature generally shows that plant chlorophyll efficiency is improved as the water redox potential becomes more negative.

A field study was conducted to evaluate the effects of chemical inducers, mentioned above, on priming plant defenses for rhododendrons that were foliar inoculated with *P. ramorum*. The rhododendron responses to the three *P. ramorum* inoculation treatments and six chemical inducer treatments were evaluated using two methods (fluorescence and qPCR) to discern whether these evaluation methods could be used to validate each other's results.

2. Materials and Methods

2.1. Study Design

The field study was conducted at the National Ornamentals Research Site at Dominican University of California (NORS-DUC) research nursery located in San Rafael, CA, USA. Since *P. ramorum* is endemic to California, and NORS-DUC was established to study quarantined pathogens in an open field setting, this site was selected to conduct the field study. The goal of the study was to evaluate the effects of inducer/chemical treatments on Rhododendron plants inoculated with *P. ramorum* zoospores.

The objectives of the study were: 1) Determine the effects of three inducer/chemical treatments using three plant responses, 2) Determine the effects of three *P. ramorum* inoculation treatments using three responses, and 3) Determine the effects of leaf puncturing with a floral frog on fluorescence responses. The study was replicated by using either five or six rhododendrons for each specific treatment. Three plant responses were measured in this study including: 1) fluorescence parameter, maximum quantum efficiency (Fv/Fm), 2) visual assessment of plant foliage for *P. ramorum* symptoms, and 3) qPCR analysis of plant foliage to quantify the level of *P. ramorum* DNA. Foliar fluorescence was measured at three dates (35, 94, and 160 days after treatments (DAT)) and visual assessments were collected on two dates (94 and 160 DAT).

The study was a factorial design with two study factors including three chemical treatments and three *P. ramorum* inoculation treatments. Rhododendrons were randomly assigned to treatment groups using the random assortment generator using SAS-JMP software (SAS Institute Inc., Clary, NC, USA).

2.2. Plant and Chemical Treatments

The rhododendron hybrid selected for this study was *Rhododendron* "Cunningham's White". The woody ornamental is a semi-dwarf, evergreen shrub with waxy leaf cuticles, and is a primary host for *P. ramorum*. Plant size ranged from 40 to 65 cm in height with total leaf counts ranging from 20 to 60 leaves per plant. The leaf size ranged from 5 cm to 10 cm. One hundred, one-year old plants were purchased in November 2014 in number one trade pots which were transferred to number two trade pots in March 2015. The rhododendrons were randomized based on size, labeled, and sorted by their *P. ramorum* inoculation status. The three inoculation treatments were: 1) 33 plants inoculated nine days before chemical treatments (IB), 2) 33 plants inoculated nine days after chemical treatments (IA), and 3) 34 plants that were non-inoculated, but received chemical treatments (NI).

The chemical treatments included the two oxidant disinfectants: ElectroBiocide and OxiDate 2.0. ElectroBiocide was applied at 0, 200, 400, and 600 mg/L, and OxiDate 2.0 were applied at 10,000 mg/L. The third chemical was Actigard (Syngenta Crop Protection, LLC, Greensboro, NC USA) that was applied at 62 mg/L. In the first two weeks of April the chemical inducers and *P. ramorum* inoculation treatments were completed.

2.3. Validation Control Rhododendrons

Three rhododendron plants, not included in the study, were inoculated earlier in the spring by the NORS-DUC scientists and used to validate fluorescence measurements for the positive control plants in the study. These rhododendrons were successfully inoculated and showed visual symptoms by mid-summer. These symptomatic plants were used as "validation controls" (VC), which were positively identified with *P. ramorum* symptoms. Three leaf samples were selected from each plant for two foliage classes including leaves with and without *P. ramorum* symptoms during the September plant measurements.

2.4. Greenhouse and Plant Irrigation Description

A completely enclosed, white polyethylene film covered hoop house was built at the field nursery to contain the rhododendrons and increase relative humidity as shown in Figure **1**. The hoop house was placed in a larger "containment plot" sealed with a thick pond liner. The 100 pots were placed on pallets to prevent spore contaminated water from being taken up by the plant roots. The non-inoculated plants were used as the negative controls and they were separated from the inoculated rhododendrons with a plastic partition to prevent accidental inoculation from windblown spores or water uptake by the roots.

An overhead, automated irrigation system was used to uniformly water all the plants, and the irrigation schedule was nine min/day. The irrigation schedule was designed to mimic springtime rainfall conditions for coastal California and provide optimal conditions for inoculation and "infectivity success" for *P. ramorum* zoospore. Immediately after the *P. ramorum* inoculation, the irrigation was turned on to raise the relative humidity to increase disease infection rates for the inoculated plants. The hoop house temperature was monitored to prevent overheating in the summer months.

Figure 1: Photo of hoop house (left photo) and rhododendron plants inside hoop house (right photo).

2.5. P. Ramorum Zoospore Inoculation Methods

A *P. ramorum* isolate (Pr-1418886) was grown for three weeks on V8-juice agar (100 mL filtered V8 juice, 0.1 g CaCO3 and 900 mL distilled water [34]). Sporangia production and zoospore release was conducted as described in Widmer [35]. The *P. ramorum* zoospore suspensions were prepared in the morning of the plant inoculations in order that the zoospore were fresh and actively moving. The zoospore concentration was counted using a hematocytometer and adjusted to 1 x 104 spores/ml. The *P. ramorum* zoospores are motile and require wet surfaces for the spores to move to stomatal openings, enter the leaves, and initiate the infection process.

A portable fabric shelter was used to inoculate the rhododendrons individually and to prevent cross-contamination among the plants. A hand bottle (Double Mist Trigger Sprayer, Kwazar, UK) was used to apply the *P. ramorum* zoospore suspension onto the axial and abaxial sides of the foliage. A total of 30 – 40 ml of zoospore suspension was applied to each plant, with 15 - 20 ml to the axial and 15 - 20 ml applied to the abaxial side of the foliage as shown in Figure **2**.

All the IA and IB plants were leaf punctured with a floral frog to improve the inoculation success rate as shown in Figure **3**. A floral frog was used to puncture four leaves per plant before applying the *P. ramorum* suspension for the IA and IB plants. The floral frog had 66 steel pins that created a 4 cm circle of holes in each leaf. Two immature leaves were selected from the top of the plant, and two mature leaves were selected from the middle of the plant. The NI plants were not inoculated and did not require leaf puncturing to improve infectivity success.

However, half of the NI plants (17 plants) were leaf punctured to evaluate the effects of leaf injury and chemical injury from punctured leaves on fluorescence measurements.

The two methods used to improve the success of the inoculation process were: 1) puncture four leaves per plant before applying the inoculant, and 2) raise the relative humidity inside the enclosed hoop house for 72 hours after applying the inoculant to ensure wet leaves for motile zoospore to enter leaf cuticles. All inoculated plants were placed inside the hoop house with the automated irrigation scheduled to maintain wet leaf surfaces for 72 hours after leaf inoculation. The end flaps for the hoop house were opened after the week of chemical applications to prevent overheating in the hoop house.

Figure 2: Photo of floral frog used to puncture selected rhododendron leaves (left photo) and leaf with puncture holes (right photo).

Figure 3: Photo of spray droplets from foliar application of *P. ramorum* zoospore on adaxial (left photo) and abaxial surface of leaves (right photo).

2.6. Electrostatic Sprayer

An air-assisted, electrostatic sprayer (Model SC-EB, Electrostatic Spraying Systems Inc. (ESS), Watkinsville, GA, USA) was used to apply the chemical treatments to the plant foliage as shown in Figure **4**. The spray application rate was 3.8 l/hr or 1 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. The negative electrostatic charge on the spray droplets ranged from -5 to -10 µamps. The applied voltage at the nozzle electrode ranged from 1,200 to 1,300 V. Each chemical treatment was individually applied to each plant. A digital timer was used to set the total spray time for each plant at 10 s/plant. Spray time was set at 5 s/plant for the upper foliage and 5 s/plant for the underneath foliage. The estimated chemical spray volume was 10. 6 ml/plant (1.055 ml/sec x 10 sec = 10.6 ml). Each plant was timed and sprayed on

a rotating platform to ensure complete coverage. The spray lines were purged between each chemical treatment, but not between replicates.

The physicochemical water properties were measured using charged water droplets collected after spraying the air-assisted, electrostatic sprayer. Approximately 40 ml of charged spray droplets were collected in pre-sterilized vials. Filtered tap water and the charged water samples were measured with an Oakton ORP/EC/pH meter (PC 650 meter, Oakton Instruments, Vernon Hills, IL, USA). The oxidation reduction potential (ORP), electrical conductivity (EC) and pH were measured immediately after collecting the charged water samples and at 24 and 75 hours after the time of sample collection.

Figure 4: Photo of applying chemical inducer treatments with air-assisted, electrostatic sprayer (left photo) and timing each foliar treatment with a second person with a stopwatch (right photo).

2.7. Foliage Assessment

Plant foliage was visually assessed for *P. ramorum* infection rates using the Horsfall- Barratt scale [36] which is based on a scale of 1-10 (Image 5). The two foliage assessments occurred in July and September, or three and five months, respectively, after the plant treatments. A third plant mortality assessment occurred in Mach 2016, or 340 days after the chemical treatments.

2.8. Fluorescence Methods

Fluorescence was measured with a portable gas exchange/fluorometer (LICOR-6400 XT, LI-COR Environmental, Lincoln, NE, USA). Foliar fluorescence was measured on dark-adapted leaves for maximum quantum efficiency, or Fv/Fm, in May, July, and September, or one, three and five months after chemical treatments, respectively. The IA, IB, and NI plants were separately measured over a three-day period. Rhododendrons were dark-adapted approximately 16 to 18 h inside two on-site, enclosed, light blocking, fabric shelters before collecting fluorescence measurements as shown in Figure **5**. Fluorescence measurements were collected between 6 and 10 am to reduce erroneous measurements due to increased mid-day temperatures inside the shelters. Also, fluorescence is influenced by diurnal plant activities, so measurements were restricted to a 4 h window to reduce diurnal measurement errors. Black lights were used to illuminate the shelter and maintain plants in dark-adapted mode.

Three leaves per plant were selected for fluorescence measurements. Each leaf was identified with colored fluorescence zip ties to ensure that the same leaves were measured over the three study dates. The uppermost, youngest, but fully expanded leaves were selected for fluorescence measurements. Fluorescence, soil moisture, and soil temperature data collected for all three study dates were compiled into a single dataset.

Volumetric soil moisture (m^3/m^3) and soil temperature were measured with an ECH_2O data logger (METER Environment, Pullman, WA, USA) and a soil sensor (5-TM) for each fluorescence measurement. Plants were irrigated to maintain soil moisture between 0.15 to 0.28 m3/m3, or about 15 to 28% soil moisture.

Figure 5: Photo of *P. ramorum* symptoms on rhododendron foliage (left photo), enclosed shelters for fluorescence measurements (center photo) and collecting fluorescence measurements inside the shelter (right photo).

2.9. qPCR Analysis of Plant Foliage

Quantitative polymerase chain reaction (qPCR) tests were conducted to verify the presence of *P. ramorum* located inside the leaf tissues of inoculated plants. The NORS-DUC lab cultured *P. ramorum* zoospore to develop standard curves for the qPCR leaf tissue analysis. The initial *P. ramorum* density was approximately 1.0 x 10^4 CFU/ml, which was serially diluted to 10^1, 10^2, 10^3 and 10^4 CFU/ml to develop the standard curve. The initial *P. ramorum* culture was verified to be 100% pure *P. ramorum* zoospores to eliminate cross contamination issues with any other *Phytophthora* species. The standard curve was used to quantify *P. ramorum* in the leaf tissue collected at 160 days after foliar treatments. The pure *P. ramorum* culture was also used to accurately identify the *P. ramorum* DNA extracted from the leaf samples.

Three rhododendron leaves, located just below the most recent flush of new growth, were selected at random from each plant. The upper and lower leaf surfaces were thoroughly cleaned to prevent leaf sample contamination from zoospore or other fungal segments residing on the surface. This was accomplished using a facial scrub pad containing diluted Tween-20 to wash the leaf surfaces. Leaf surfaces were then completely rinsed with ddH_2O and dried with sterile paper towels. The three leaves were then individually flash frozen in liquid nitrogen for DNA extraction. A total of 15 leaf samples (5 plants x 3 leaves/plant) per treatment were collected and examined separately for the qPCR analysis.

Leaf tissue (200mg/leaf) was ground in liquid nitrogen, diluted with 300 ul of ddH_2O, centrifuged at 14,000 rpm, and 40 ul of the supernatant from each collection was pipetted onto separate FTA Elute cards (Whatman Inc., Clifton, NJ, USA) for DNA storage. The Flinders Technology Associates filter papers (FTA card) are cotton-based cellulose membranes impregnated with a chaotropic agent that inactivates infectious micro-organisms, lyses cellular material, and fixes DNA and/or RNA within the fiber matrix [37]. Thus, such samples are no longer infectious and do not pose a biohazard [38 – 40]. For elution of DNA from the cards, 3mm disks were made using a 3 mm Harris punch. These disks were transferred to microcentrifuge tubes and washed with 500 µl of sterile DNA/DNase free water. After discarding the wash solutions, disks were eluted with 30 µl sterile DNA/DNase free water heated to 98°C for 30 minutes.

Quantitative PCR reactions were performed using the Mastercycler ep realplexReal-time PCR System (Eppendorf, Enfield, CT, USA) with the Maxima SYBR Green/ROX qPCR Master Mix kit (Thermo Scientific, Waltham, MA, USA). Several published *P. ramorum* primer sets for the 18S rRNA subunit internal transgenic spacer (ITS) region was tested and found to have non-specific amplification, at least on the available equipment using SYBR Green methods. Thus, we designed and optimized new ITS primers as part of ongoing barcoding studies, and these were determined to be specific in this study. The *Rhododendron caucasicum x ponticum* primers, used to assess the amount of plant DNA, were specific to a portion of the Ribulose-1,5-bisphosphate carboxylase

oxygenase subunit (rbcL). PCR parameters were as follows: 2 min at 95°C, followed by 45 cycles of 30 s at 95°C, 20 s at 61°C, and 30 s at 72°C. The optimized ITS and rbcL primer sequences are listed in Table **1**. Each reaction was performed using 4-6 biological replicates using 2 µl of each DNA eluted from the FTA® Elute cards. Specificity of the amplification was verified using dissociation curve analysis at the end of each run using the MxPro software (Stratagene, Santa Clara, CA). The software calculated threshold level (fluorescence 850) represented the approximate midway point of the exponential phase of all amplifications, and this was applied consistently across all amplifications to obtain the threshold cycle (C_T) values. Relative *P. ramorum* target DNA levels were normalized to the plant rbcL DNA levels as a means of normalizing for plant tissue delivered to each FTA card following established ΔC_T methods [41]. Along with positive and negative controls, *P. ramorum* zoospore DNA was also serially diluted and replicated in triplicate on each *P. ramorum* assay plate to produce standard curves that allowed both the testing of variation between experiments and the estimation of the absolute quantity of *P. ramorum* as shown in Figure **6**.

Table 1: Primers used for qPCR reactions.

Primer Description	Primers
Pram ITS F	GCT GCG GCG TTT AAT GGA GGA G
Pram ITS R	GTT TCC CAA ATG GAT CGA CCC TCG
Rhod rbcL F	CCA CAT CGA GCC TGT TGC TGG
Rhod rbcL R	CCT TGG AAC GTT TTA GCA TAC GCT GC

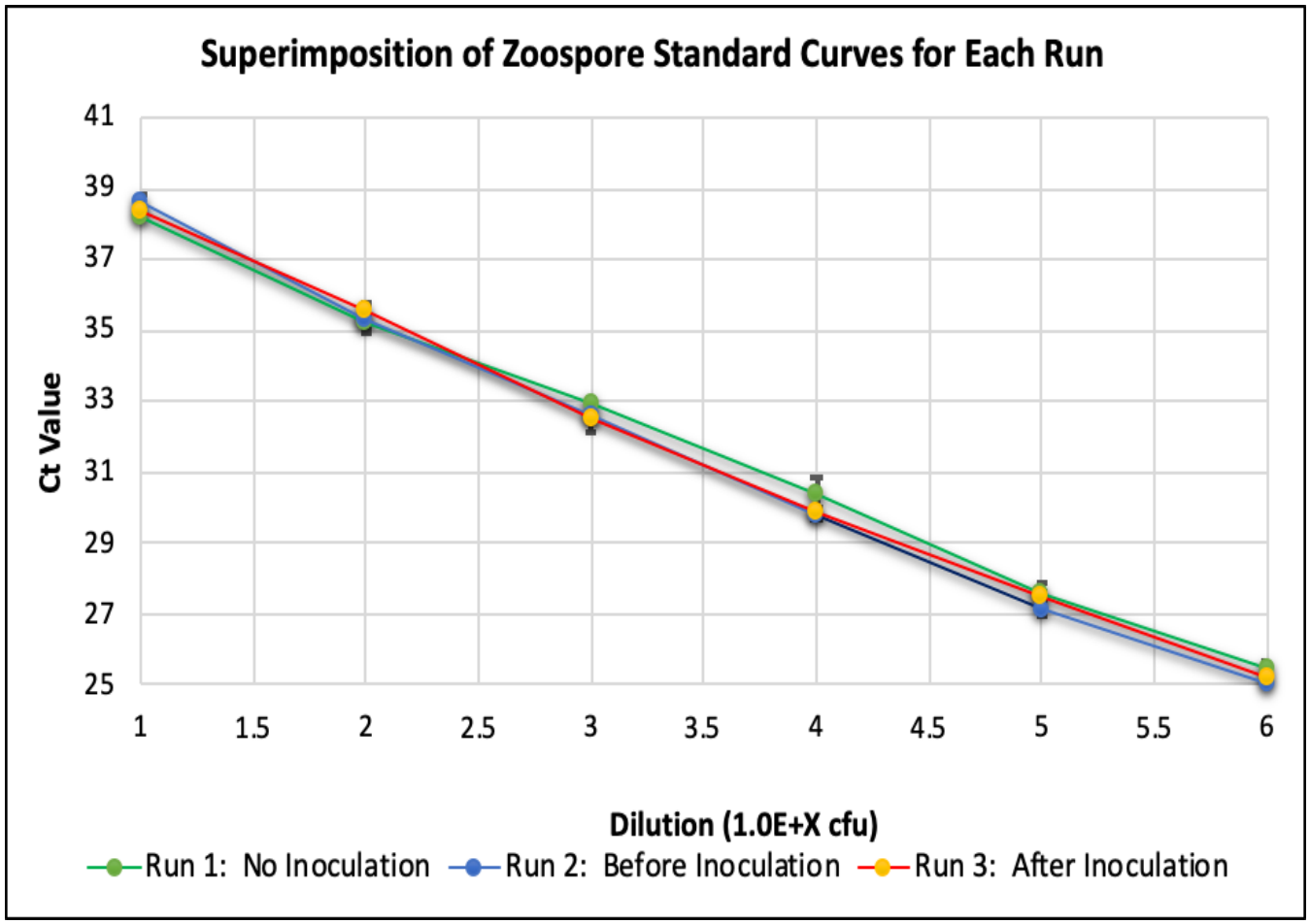

Figure 6: *P. ramorum* zoospore DNA was assayed for Ct values for the IA, IB, and NI runs to produce standard curves to test the variation between experiments and estimate the absolute quantity of *P. ramorum*. Error bars represent standard error."

2.10. Statistical Analysis

The study design was developed with JMP software (SAS Institute Inc., Clary, NC, USA), using the Design of Experiment (DOE) program to reduce the number of samples. The DOE design used hidden replication by limiting interaction terms to two-way interactions. Restricting the final model to two-way interactions achieved 49 statistical replications for each treatment. Results were significant at α = 0.05. The JMP Least Squares Fit model was used for the *P. ramorum* visual symptom analyses. Fluorescence was measured over three dates, so the JMP repeated measure program titled Restricted Maximum Likelihood Method (GLM- REML) was used to analyze the data. In addition, fluorescence measurements were collected from three leaves per plant. The leaf number was converted into a nested, random variable in the GLM- REML model to account for the inherent variability among leaves. The charged water properties (ORP, EC and pH) were analyzed with the Fv/Fm responses to determine any water redox effects on chlorophyll efficiency.

3. Results

The maximum quantum efficiency (Fv/Fm) data were analyzed for the compiled data across the three study dates, and for the September data only. Multivariate analysis showed that soil moisture, soil temperature, and leaf vapor pressure deficit (VPDL) were the three environmental covariates that affected fluorescence the most as shown in Table **2**. Leaf vapor pressure deficit is physiologically correlated to soil moisture and including two environmental covariates that were correlated into the GLM model would introduce unnecessary errors into the model. Therefore, only soil moisture and soil temperature were included as covariates in the final model.

Maximum quantum efficiency (Fv/Fm) was affected by soil moisture, soil temperature, and *P. ramorum* inoculation when analyzed across all three study dates as shown in Table **3**. Statistically smoothed graphs show the predicted Fv/Fm values, based on the GLM model, over the three measurement dates, the *P. ramorum* inoculation status, and five chemical treatments as shown in Figure **7** to **9**. The Fv/Fm patterns start to diverge after the second collection date, or three months after initiating the study for all three *P. ramorum* inoculation treatments (IA, IB, and NI). Therefore, the September data (five months after initiating the study) were analyzed separately to determine the long-term effects of the treatments on Fv/Fm.

Table 2: Multivariate analysis of covariates for maximum quantum efficiency.

	Fv/Fm	**Soil Moisture**	**Soil Temp**	**VpdL**	**Tleaf**
Fv/Fm	1.0000	0.0397	0.0912	0.0487	0.0630
Soil Moisture	0.0397	1.0000	-0.2596	-0.1569	-0.1023
Soil Temperature	0.0912	-0.2596	1.0000	0.2868	0.5111
VpdL	0.0487	-0.1569	0.2868	1.0000	0.8809
Temperature of Leaf	0.0630	-0.1023	0.5111	0.8809	1.0000

Table 3: Generalized Linear Model (GLM) results for maximum quantum efficiency (Fv/Fm) for three data collection dates. Note that measurement date is not a model term, so the effects of time were not tested in the model.

Source	**F Ratio**	**Prob>F**
Soil Moisture	4.4620	0.0354*
Soil Temperature	18.2009	<.0001*
Chemical Treatment	1.0518	0.3878
Inoculation Status/Timing	6.7449	0.0014*

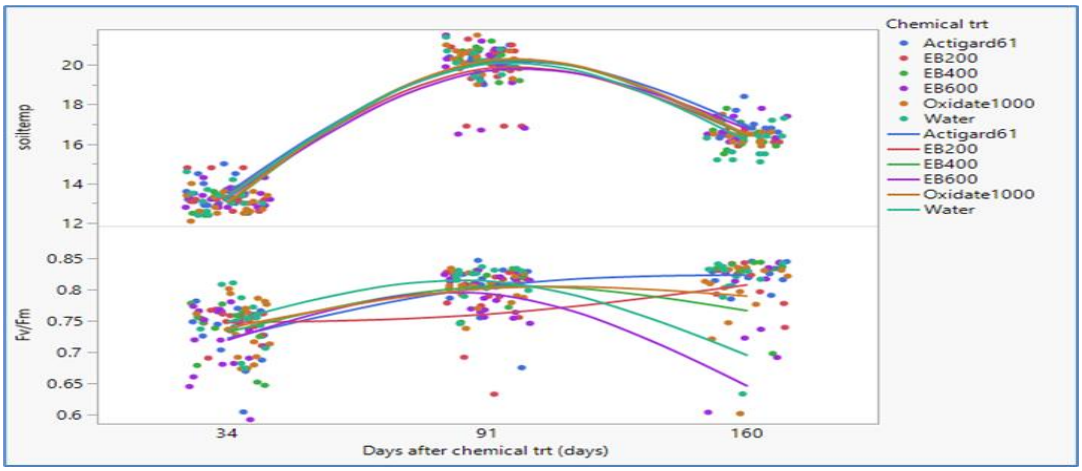

Figure 7: Statistical smoother curves illustrating the temporal pattern of maximum quantum efficiency (Fv/Fm) and soil temperature (y-axis) over measurement dates (x-axis) for non-inoculated plants (NI treatment). Upper graph is soil temperature and lower graph is maximum quantum efficiency for five chemical treatments.

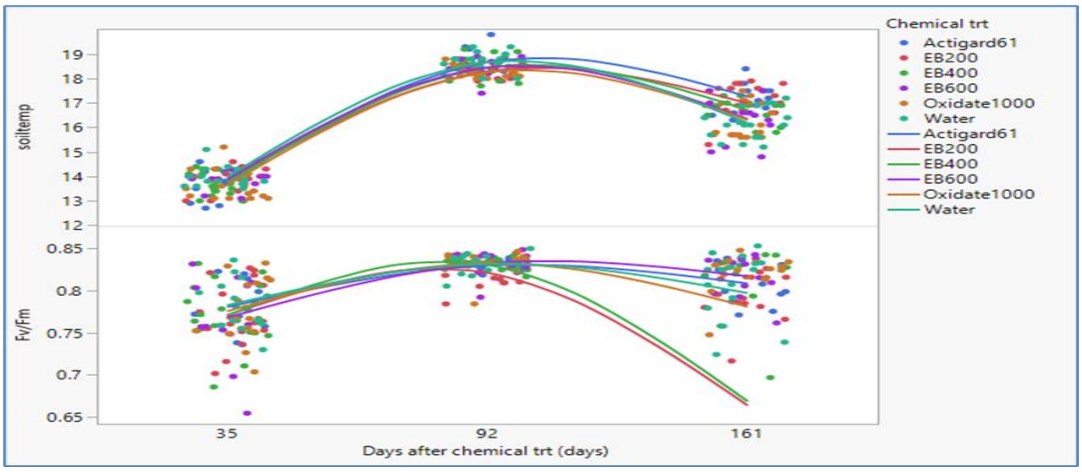

Figure 8: Statistical smoother curves illustrating the temporal pattern of maximum quantum efficiency (Fv/Fm) and soil temperature (y-axis) over measurement dates (x-axis) for plants inoculated after foliar treatments (IA treatment). Upper graph is soil temperature and lower graph is maximum quantum efficiency for chemical treatments.

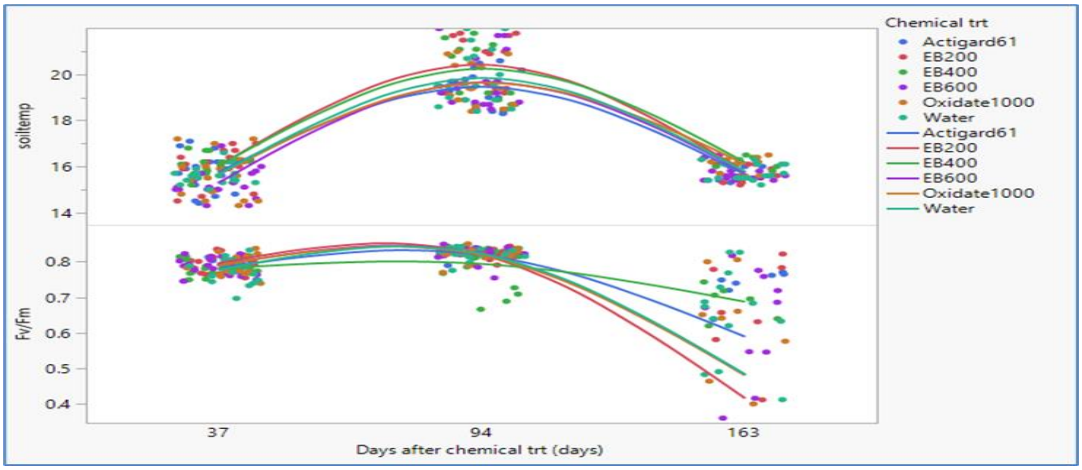

Figure 9: Statistical smoother curves illustrating the temporal pattern of maximum quantum efficiency (Fv/Fm) and soil temperature (y-axis) over measurement dates (x-axis) for plants inoculated before foliar treatments (IB treatment). Upper graph is soil temperature and lower graph is maximum quantum efficiency for five chemical treatments (legend). Fv/Fm for 3 dates for Green line is EB400 is highest Fv/Fm at 163 days after treatment. OxiDate had the second highest Fv/Fm at 163 days after treatment.

Two covariates (soil moisture and temperature), chemical treatments, and three interaction terms were included in the final GLM model for the September Fv/Fm data as shown in Table **4**. The predicted Fv/Fm estimates for the IB treatments were lower than the IA treatment at five months measurement date as shown in Figure **10**. The percent chlorophyll efficiency (Fv/Fm x 100), based on the GLM model for the water alone treatment, was 80, 47, and 71% for the IA, IB, and NI *P. ramorum* inoculation treatments, respectively, at 160 days after the treatments. Due to a mechanical failure in the automated irrigation of the IB plants during the weekend before the September fluorescence measurements, it appears that the Fv/Fm measurements were affected by the recovery of the water stressed plants. Rhododendrons treated with Actigard nine days after the plants were inoculated with *P. ramorum* had the highest Fv/Fm, i.e., 86% of the photons received by the chlorophyll were converted into plant sugars at 160 days after treatment.

Table 4: Generalized Linear Model (GLM) results for maximum quantum efficiency (Fv/Fm) for September collection dates.

Source	F Ratio	Prob>F
Soil Moisture	0.0876	0.7675
Chemical Treatment	3.2331	0.0081*
Soil Temperature	5.7061	0.0177*
Inoculation Status/Timing	23.5984	<.0001*
Soil Mstr*Trt	3.0719	0.0107*
Chemical Trt*Soil Temp	3.1052	0.0098*

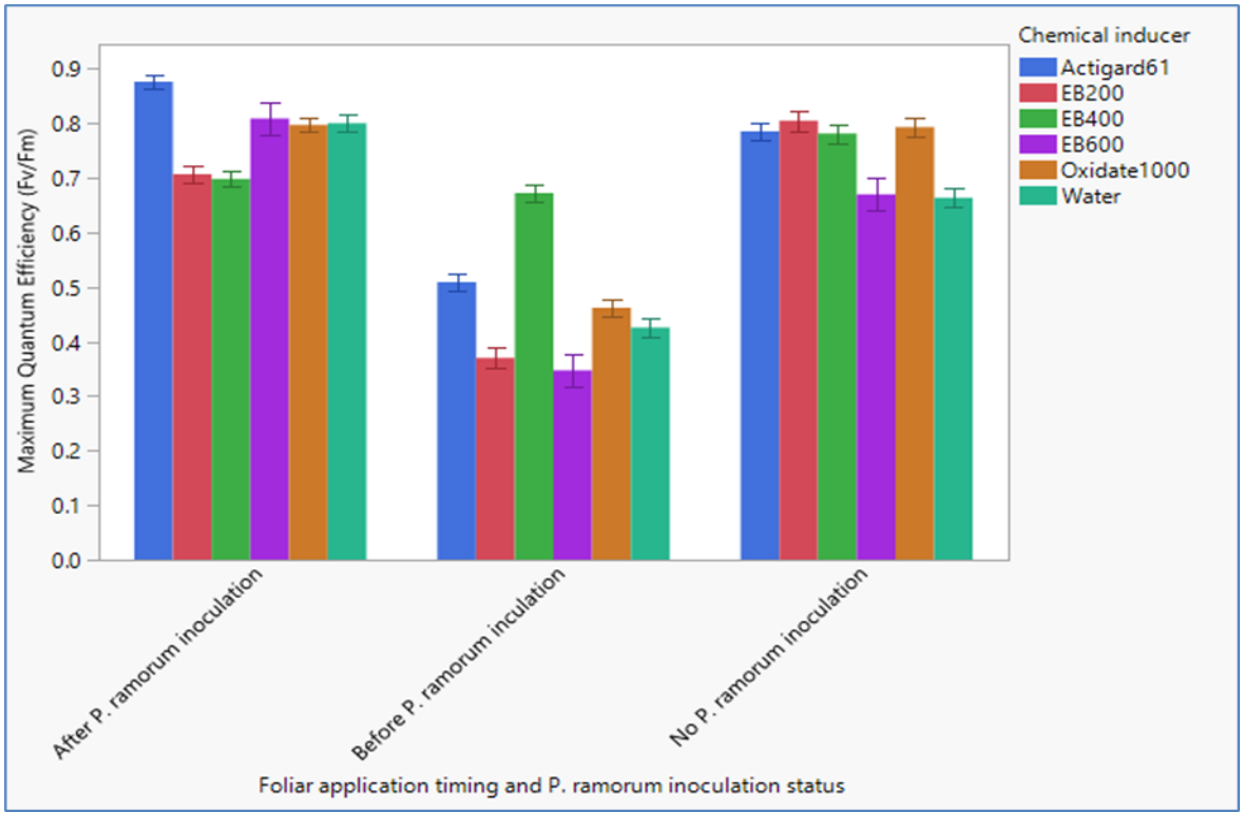

Figure 10: Predicted maximum quantum efficiency (Fv/Fm), based on the GLM model, at 160 - 163 days after treatment for three *P. ramorum* inoculation treatments (x-axis) and six chemical treatments (legend). Rhododendrons sprayed with chemical inducers after *P. ramorum* inoculation had the highest Fv/Fm 160 days after treatments.

The three-validation control (VC) plants that were infected with *P. ramorum* were measured for Fv/Fm in September for both leaf sample types (with and without *P. ramorum* symptoms). Both leaf types had equivalent Fv/Fm estimates (*p*-value = 0.9766). These fluorescence results were compared to the positive controls that were inoculated with *P. ramorum* in the main study. The IA, IB water treatment leaves, and the VC leaves had equivalent Fv/Fm estimates as shown in Figure **11**.

The Fv/Fm values for the positive controls, or the inoculated (IA and IB) and non-inoculated (NI) rhododendrons given the water treatments were also compared as shown in Figure **12**. The Fv/Fm estimates were higher for the non-inoculated (NI) water treatments when compared to the inoculated rhododendrons (IA and IB), across the July and September measurement dates. Percent efficiency (Fv/Fm x 100) was 79, 74, and 83%, based on the GLM model with two covariates, for the IA, IB, and NI treatments, respectively, for the water only treatments across both measurement dates.

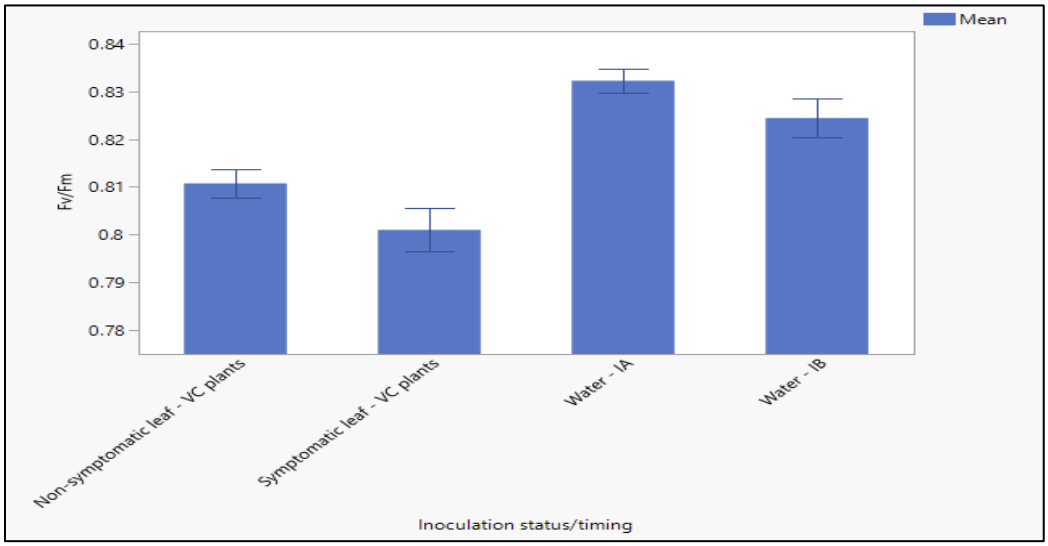

Figure 11: Predicted maximum quantum efficiency (Fv/Fm) for foliage samples collected from four sets of *P. ramorum* infected plants. The GLM model used soil moisture and soil temperature as covariates and showed no differences among the four groups of foliar samples labeled in the graph (p-value = 0.2760).

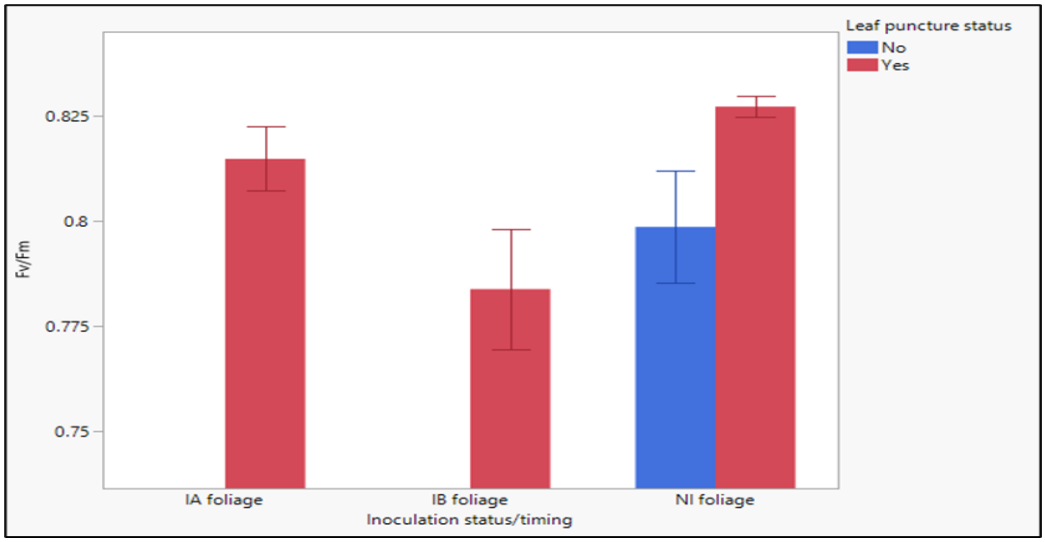

Figure 12: Predicted Fv/Fm, based on the GLM model, for rhododendrons inoculated with *P. ramorum* (IA and IB) and non-inoculated (NI) plants. Fv/Fm estimates were from water treatments only, and Fv/Fm estimates were averaged over the 94 and 160 day measurement dates. The non-inoculated (NI) estimates included both punctured and non-punctured leaves (legend). The non-inoculated rhododendrons had significantly higher Fv/Fm estimates than the IA or IB plants if soil moisture was 20% and soil temperature was 16.6 C.

The NI water treatments were divided into a subset of plants that had either punctured or non-punctured leaves. This dataset was analyzed for leaf injury effects in tandem with chemical injury effects on Fv/Fm estimates. Analysis for leaf injury effects shows that puncturing four leaves per plant, with a floral frog (approximately 66 pins), had no effect on maximum quantum efficiency across all two study dates. Analysis of the September dataset also showed no leaf injury effect on Fv/Fm estimates. Although there was no leaf injury effect from puncturing four leaves, the arc patterns over five months for the Fv/Fm data for each of the chemical treatments for punctured leaves had different divergence patterns. The non-punctured plants had parallel Fv/Fm arcs over time while the punctured plants had a convergence of Fv/Fm arcs at 94 days after treatment, followed by a wide divergence in arc patterns due to chemical treatments. These divergent patterns suggest that puncturing four leaves per plant allowed some chemical treatments to directly enter the leaf wounds, generating a free radical signal and eliciting a positive response in Fv/Fm for EB-200 and water treatments as shown in Figure **13**.

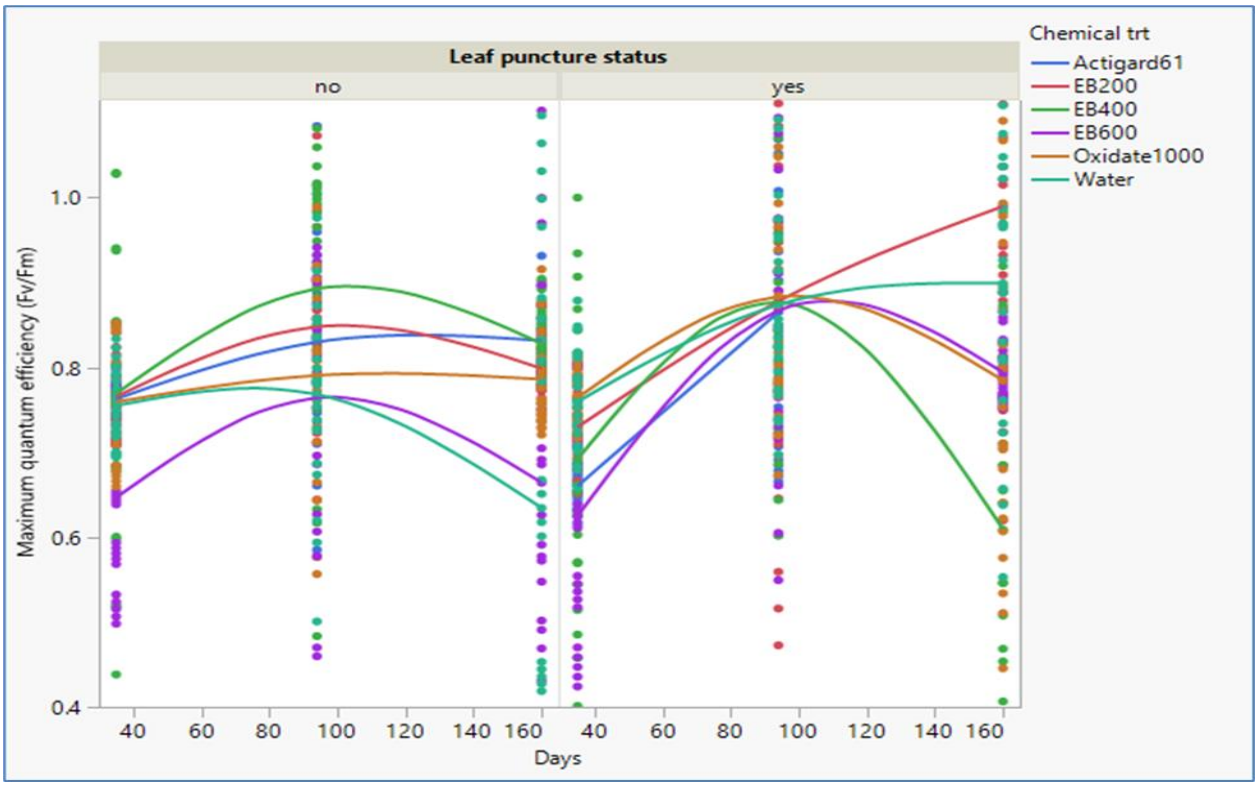

Figure 13: Statistical smoother curves for the predicted maximum quantum efficiency (Fv/Fm) for non-inoculated rhododendrons that had leaf punctured and non-punctured leaves. Fv/Fm was graphed over three study dates (x-axis), leaf status (upper x-axis), and five chemical inducer treatments.

Results from the Fv/Fm and visual *P. ramorum* symptom analyses did not correlate well across the data collection dates. Visual evaluation of plant foliage for symptoms of *P. ramorum* infection revealed no inoculation treatment effects across all two study dates as shown in Table **5**. Virtually all the inoculated plants remained non-symptomatic during the five-month study.

The presence of *P. ramorum* in the leaf tissue of each treatment was confirmed using qPCR analysis specific to the 18S rRNA subunit internal transgenic spacer (ITS) region as shown in Figure **6**. Here, the water treatment samples provided both negative (NI samples) and positive (IB and IA samples without chemical treatment) controls, demonstrating detection of *P. ramorum* within new leaf material in the absence of chemical treatments. Significant levels of *P. ramorum* were also detected in IB samples treated with EB (200 ppm), EB (600 ppm), and Actigard, whereas IA samples for EB (400 ppm) showed only very low levels of *P. ramorum*. Zoospore standard curves were developed and used to quantify *P. ramorum* levels in leaves, which also demonstrated good consistency of amplification between experiments as shown in Figure **14**.

These qPCR tests show that *P. ramorum* inoculation successfully infected many rhododendrons. The qPCR tests also show that the non-inoculated plants remained healthy and non-infected at 160 days after the inoculation treatments. In addition, the qPCR tests show that three out of the five chemical inducer treatments (EB (200 ppm), EB (600 ppm) and Actigard) had *P. ramorum* in the leaf tissue for the rhododendrons inoculated before they were applied with chemical inducers (IB treatments). Both the fluorescence (Fv/Fm) and qPCR tests show that the rhododendrons inoculated before they were applied with chemical inducers (IB treatments) had a lower Fv/Fm and higher qPCR value when compared to the other treatments as shown in Figure **10** and **14**. Thus, both Fv/Fm and qPCR substantially validate each other, i.e., that the foliage was successfully inoculated with *P. ramorum* and that the three of the chemical inducers inactivate *P. ramorum*, from partial to virtually complete inactivation, in the sampled leaf tissue as shown in Figure **14**.

The mishap with the irrigation system for the IB plants in September, however, confounded the fluorescence results for this treatment. Fluorescence can be reduced by both abiotic stress (water stress) and biotic stress (*P. ramorum* inoculation). The IB inoculation treatments had lower Fv/Fm values across all five chemical inducer treatments, which reflects fluorescence responses to both abiotic and biotic stressors. However, the qPCR results show that only two of the five chemical inducer treatments had elevated *P. ramorum* infection rates. The differences between the Fv/Fm and qPCR results could be explained by the irrigation mishap, i.e., Fv/Fm was lower across all five chemical inducer treatments due to lack of water which overshadowed the fluorescence responses to the *P. ramorum* inoculation success rate.

Table 5: Visual *P. ramorum* foliage symptoms based on Horsfall - Barratt scale for chemical treatments. The plants were monitored on two dates.

Chemical Treatment	Inoculation Status/Timing	Ave. Score, (05/14/2015)	Ave. Score, (07/06/2015)
Actigard	Inoc After	1	1
Actigard	Inoc Before	1.3	1
Actigard	No Inoc	1	1
EB200	Inoc After	1.3	1
EB200	Inoc Before	1	1
EB200	No Inoc	1	1
EB400	Inoc After	1	1
EB400	Inoc Before	1.2	1
EB400	No Inoc	1	1
EB600	Inoc After	1	1
EB600	Inoc Before	1	1.3
EB600	No Inoc	1	1
OxiDate	Inoc After	1	1.2
OxiDate	Inoc Before	1.3	1
OxiDate	No Inoc	1	1
Water	Inoc After	1	1
Water	Inoc Before	1	1
Water	No Inoc	1	1

Actigard had the highest Fv/Fm (86%), followed by EB (600 ppm) with the second highest Fv/Fm of 82% when compared to the other four IA treatments at 160 days after treatment as shown in Figure **10**. Also, both Actigard and EB (600 ppm) had low *P. ramorum* counts for the IA treatments as shown in Figure **14**.

The air-assisted, electrostatic sprayer changed the three physicochemical water properties when compared to tap water properties as shown in Table **6**. The charged water samples had a higher electrical conductivity, a more negative ORP, and a higher pH. The stability of the three water properties were estimated by remeasuring the same water samples at 24 and 75 hours after sample collection. The three physicochemical water properties degraded slightly after 24 hours, with a sharper decrease in water properties after 75 hours.

Table 6: Physicochemical water properties for filtered tap water and the air-assisted, electrostatic sprayer charged water samples. Average electrical conductivity, oxidation reduction potential, and pH for water samples at 0, 24 and 75 hours after sample collection time.

Water description	Electrical conductivity (uS/cm)	Oxidation reduction potential (mV)	pH
Filtered tap water	140	-46	7.8
ESS at 0 hour	332	-67	8.1
ESS at 24 hours	335	-56	7.9
ESS at 75 hours	341	-36	7.6

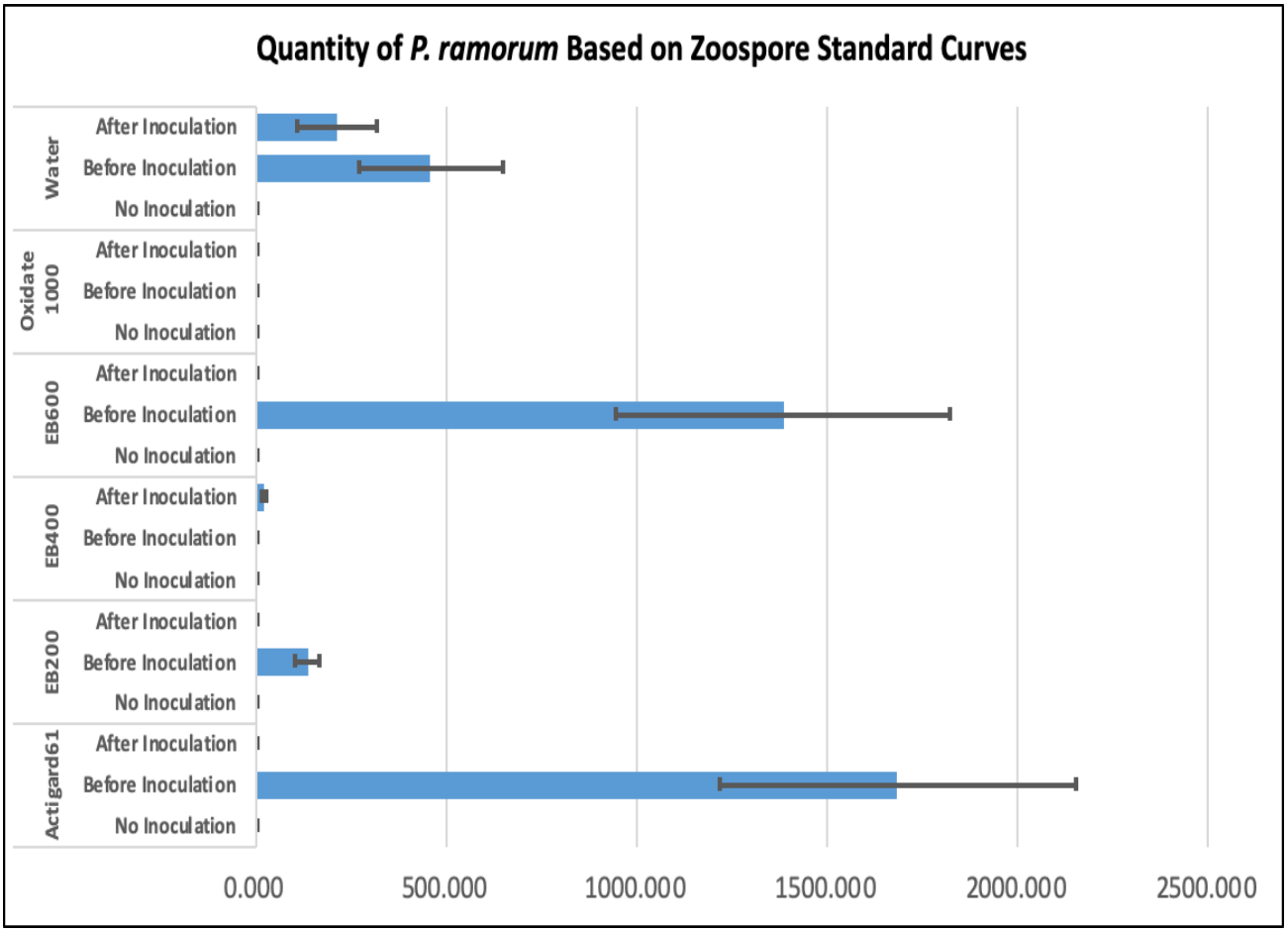

Figure 14: Quantitative polymerase chain reaction (qPCR) verification of *P. ramorum* infection of Rhododendron x 'Cunningham's White' leaves averaged for five plants inoculated with *P. ramorum* before and after the chemical inducer treatments were applied and compared to plants that were not inoculated with *P. ramorum* (no inoculation - NI).

4. Discussion

4.1. Maximum Quantum Efficiency (Fv/Fm) Dynamics Over Time

The arc patterns of the smoother graphs for Fv/Fm show that the chemical inducer treatments diverge over the three measurement dates. The predicted Fv/Fm estimates for each chemical inducer treatment remain close together at 35 days, but then start diverging after the July measurements. The arc pattern for Fv/Fm parallels the arc trajectory for soil temperature as shown in Figure **7** to **9**, i.e., Fv/Fm increased with summer temperatures at the 97-day data collection but decreased for data collected 160 days after initiating the study.

Fluorescence measurements include estimates of instantaneous maximum quantum efficiency (Fv/Fm), which is a measure of the fraction or proportion of absorbed photons that are engaged in photochemistry, or the production of plant sugars. Chlorophyll efficiency (Fv/Fm) acts as a physiological biomarker for many abiotic and biotic stressors [24]. Because Fv/Fm is a non-specific biomarker and measures foliar responses to many stressors, data analysis should always include the most important environmental covariates such as soil temperature and moisture, as determined by multivariate testing. The GLM model in this study included two environmental covariates to minimize the confounding effects of environmental conditions on fluorescence that occurred over the five-month study as shown in Tables **2** and **3**. Also, if the plants were grown under uniform cultural and environmental conditions during the study time, then any cultural differences in watering schedules and lighting conditions would be minimized. In this study, the watering schedule was uniform across all the plants, and temperature and light conditions were kept as uniform as possible. The final GLM model accounts for the most important environmental variables that results in higher overall accuracy for plant responses that are specific to the study treatments.

The significance of collecting Fv/Fm data over a five-month time span is illustrated in the smoother arc patterns for each chemical inducer treatment as shown in Figures **7** and **9**. The arc patterns show that the effects of each chemical treatment on Fv/Fm were only expressed after the 97-day (July) measurements. Analysis of Fv/Fm data, for the three measurement dates, shows that all three study factors were not significant due to the long delay in foliar responses to the chemical inducers and *P. ramorum* inoculation treatments, except for the two environmental covariates and *P. ramorum* inoculation factor as shown in Table **1**. The GLM model for September data shows that the chemical inducer treatments, *P.* ramorum inoculation, soil temperature, and two interaction terms affected fluorescence and ultimately chlorophyll physiology as shown in Table 2. The Fv/Fm arc patterns show that rhododendron foliar responses can be delayed from three to five months after the chemical inducer applications and *P. ramorum* inoculation treatments. The authors do not know of any other studies with such long-term longitudinal data collection for fluorescence for woody or ornamental plant species. It is apparent from this study that woody ornamentals such as rhododendrons may exhibit delayed foliar responses to chemical treatments that may only be measured several months after treatment applications.

Measurement of maximum quantum efficiency over five months facilitates longitudinal data analysis to determine if there is a long lag phase in *P. ramorum* infection dynamics, or if the plants remain visually non-symptomatic after *P. ramorum* inoculation, or if they were not successfully inoculated. The Fv/Fm estimates at 160 days indicate that there is a long lag period before rhododendron responses to chemical treatments and *P. ramorum* inoculation treatments were able to be detected. Combining the Fv/Fm and visual injury results together indicates that the inoculated plants (IA and IB) appear to be infected with *P. ramorum*, however, infections are non-symptomatic as shown in Table **5**. In summary, there is a three-month delay before chemical treatments appear to reduce chlorophyll injury resulting from *P. ramorum* inoculation.

4.2. Visual Assessment of Foliar Symptoms

Visual assessment across both months found no signs for *P. ramorum* symptoms nor chemical injury symptoms on plant foliage. In other words, at 94 days after the chemical applications, the average foliar injury was

3 – 5% for inoculated and chemically treated plants, which was the same injury level as the control plants. Theplants were visually assessed again, at 340 days after chemical application, in March 2016. This data revealed that 33 out of 100 plants were either cut or dead, which was probably the long-term mortality result of an irrigation failure in September 2015 (unpublished data). However, the 77 plants that remained alive still did not show any foliar symptoms for *P. ramorum* infection, or for chemical injury at 11 months after treatment.

4.3. qPCR *Test Confirmation*

The quantitative polymerase chain reaction (qPCR) results were averaged across 15 leaf samples collected from five plants per treatment and indicate that *P. ramorum* was present in leaf tissue in some of the inoculated plants as shown in Figure **6**. Leaf samples for qPCR analysis were collected randomly just below the last flush of immature leaf growth and the plants themselves were inoculated with *P. ramorum* in leaves centrally located on the plant, therefore it is unlikely that the collected qPCR analysis tissues were the same tissues that were initially inoculated. Moreover, positive identification of *P. ramorum* in these tissues, combined with the rigorous leaf surface washing prior to tissue disruption, suggests that the pathogen moved through the plant tissues systemically to infect newer leaves. This is especially observable in the EB (200 ppm), EB (600 ppm), Actigard, and water control IB tissues, only the water control treatment and to a lesser extent EB (400 ppm) were found positive for *P. ramorum* for IA samples as shown in Figure **6**. These qPCR results also suggest that treatment with OxiDate 2.0 at 10,000 ppm can prevent such systemic spread of *P. ramorum*. Other research has found that rhododendrons planted in potting soil infected with *P. ramorum* were able to translocate the pathogen via the vascular system, especially from wounded or cut roots [42 - 43].

P. ramorum was not detected in the non-inoculated treatments showing that the qPCR methods generated no false positive results as shown in Figure **6**. Also, the IA treatments generally had low qPCR counts indicating that applying the chemical inducers after the plants were inoculated resulted in higher P. *ramorum* inactivation levels across all these foliar applications.

The qPCR test was designed to specifically confirm that the *P. ramorum* inoculation treatments successfully infected the rhododendron plants. However, the qPCR data could not be compiled with the Fv/Fm data to analyze the combined responses for treatment effects. Both the qPCR and Fv/Fm tests show that some of the inoculated plants were successfully infected with *P. ramorum*. During the experiment, the plants were semi-contained in a plastic hoop house to prevent infection from *P. ramorum* zoospore spreading by wind or rain from nearby infected plants. However, infections may have occurred from water droplets from the overhead irrigation system that bounced from an infected leaf to a non-infected leaf. Although this is possible, it is much more likely that the *P. ramorum* inoculations were successful and that the zoospores were transported to non-inoculated leaves via the vascular system. Lewis [42] and Parke and Lewis [43] found that *P. ramorum* could be translocated in the vascular system of rhododendrons when planted in potting soil inoculated with the pathogen. Natural infections with *Phytophthora* species on sentinel plants at NORS-DUC were observed only during the rainy season with a spike from January to March, with no symptom development during the dry summer months [44]. Non-symptomatic growth of *P. ramorum* in the foliage of woody plants has been reported by Denman *et al*. [45] and McCartney *et al*. [46].

4.4. Validation Control Plant Confirmation

Maximum quantum efficiency measurements of the three validation control plants tested fluorescence responses for both symptomatic and non-symptomatic leaves on the same plant. In addition, these plants acted as a validation control (VC) for the positive control plants (IA and IB) within the study. The Fv/FM estimates for the symptomatic and non-symptomatic VC leaves were equivalent (p -value = 0. 2760). In addition, the VC fluorescence results were compared to the IA and IB plants sprayed with the water treatment as shown Figure **11**. The predicted

Fv/Fm results, adjusted for soil moisture and soil temperature, were equivalent across all four groups of leaf samples, providing indirect evidence of the presence of *P. ramorum* in all the foliage samples.

The qPCR tests for the IA and IB inoculated plants shows that the water applications to these plants had detectable DNA levels in the foliage, five months after the inoculation treatments. Combining the results from the qPCR tests and the Fv/Fm tests for the VC, IA and IB inoculated plants correlates the successful inoculation of the IA and IB plants with the finding that the VC, IA, and IB foliage had equivalent Fv/Fm estimates. Therefore, it can be implied that the VC leaf samples for both symptomatic and non-symptomatic foliage were also infected with *P. ramorum*.

Comparison of Fv/Fm estimates for the non-inoculated (NI) and inoculated plants (IA and IB) shows that fluorescence can detect responses to *P. ramorum* infections even when the foliage is non-symptomatic. The random selection of measurement leaves increases the probability that all the leaves on the plant are uniformly infected with *P. ramorum*. The GLM model used to predict the Fv/Fm estimates included soil moisture and temperature as covariates which allowed standardization of the environmental variables across the five-month study. The model shows that the NI water treatment had a higher Fv/Fm estimate than the IA or IB water treatments if soil moisture and temperature was held at 20% (v/v) and 16.6 C, respectively as shown in Figure **12**.

4.5. Electrostatic Sprayer and Water Property Effects on Fv/Fm Responses

The air-assisted, electrostatic sprayer generated charged water droplets with altered the physicochemical water properties. The redox potential of water depends on the dissolved oxygen, dissolved hydrogen, and hydrogen ion concentrations (pH). The oxidation-reduction status of a solution is based on the collective electron activity within the solution, including all oxygen ion species, and becomes negative as pH becomes alkaline.

The literature indicates mixed plant responses as ORP changes in energized or treated water. Azad and Ishikawa [47] studied tap water treated with either quartz porphyry or quartz porphyry ceramic beads. They found that a 30-minute ceramic water treatment reduced ORP by 155 mV and increased pH by 0.95 compared to the untreated water. They also found that the ceramic filtered water increased *Brassica rapa* growth by 17%. In contrast, Thirumdas *et al* [48] reviewed the effects of plasma activated water (PAW) on water properties. Their review concluded that PAW activated water increased both ORP and electrical conductivity, and may increase seed germination and plant growth. Jiafeng *et al* [49] found that *Triticum* spp. seedlings had increased growth when irrigated with PAW activated water. Achiwa *et al*. [50] evaluated the effects of electrolyzed water on leek growth. The electrolyzed acidic water had a pH of 2.5 - 2.7, and EC of 500 - 3, 000 µS/cm., while the electrolyzed alkaline water had a pH of 11.8 - 12.0 and EC of 2,000 - 2, 500 µS/cm. They found that watering leek seedling with acidized and alkaline water on alternate weeks resulted in the maximum growth (39%) based on oven dry biomass compared to the control treatment. The literature shows that the physicochemical water properties can improve plant growth, but the conflicting responses to the three water properties shows that more research is needed to better understand the interactions between the water properties and plant growth.

The September water treatments for non-inoculated and non-punctured plants had a much lower Fv/Fm estimate than the punctured and non-inoculated plants. The smoother are patterns for Fv/Fm over the three data for the water treatment datasets shows different patterns for the punctured and non-punctured plants as shown Figure **13**. This graph shows that the punctured leaves had a delayed response, but chlorophyll efficiency increased between 90 and 160 days after treatment.

The applied voltage of the electrostatic sprayer nozzle electrode averaged 1,200 to 1,300 V. Also, the electrostatic charge on the water droplets ranged from -5 to -10 µamps. This electrical charge may have energized water molecules above the ionization energy needed to split water which is 12.60 eV. Assuming the electrical charge was sufficient to ionize the water molecules, then the H^+ and OH^- ion concentrations would have increased thereby temporarily, or permanently, changing the physicochemical water properties.

The electrostatic sprayer increased electrical conductivity and pH and produced a more negative oxidation reductional potential when compared to filtered tap water. The electrostatic sprayer produced charged water droplets with biologically favorable redox properties that were relatively stable over 24 h storage time as shown in Table **6**. Assuming an average leaf deposition coverage of 80% for the electrostatic sprayer, then the total foliar coverage was approximately 8.5 ml. The water treatment results suggest that the reduced redox potential combined with punctured leaves allowed the charged water to enter the leaf tissue that resulted in long-term effects on chlorophyll efficiency. The putative ionized water only treatments from the electrostatic sprayer increased Fv/Fm by approximately 11.6%, for the punctured foliage on the non-inoculated plants between 90 and 160 days after foliage treatments were applied, when compared to the non-punctured leaves. In other words, the percentage of intercepted photons increased from approximately 71 to 83% that were then converted into plant sugars for punctured leaves treated with the charged water. Continued research is needed for foliage treatments with charged, or ionized water applications, to improve chlorophyll efficiency in ornamental and crop plants.

Recent research has shown that all three of these water properties are linked to favorable redox biology responses in plants. The effects of the electrostatic sprayer on the water properties were unexpected and were not designed into the study variables. However, probing the data for possible changes in water properties and associated plant responses has posed important questions relating to energized or structured water and possible interactions with plant growth and physiology. Research involving structured water that is generated from custom generators has shown promise for enhanced crop growth, increased drought tolerance, and increased disease resistance [51 - 55].

4.6. Oxidants as Chemical Inducers to Prime Plant Defenses

Oxidants such as chlorine dioxide and hydrogen peroxide are commonly formulated as EPA registered disinfectants used for decontamination of surfaces. However, these oxidants were also evaluated as free radical molecular agents used to prime or induce disease resistance in plants [15 – 17, 50]. A two-year study by Sandoval [56] found that chlorine dioxide induced partial disease resistance in kidney bean seedlings that were inoculated with *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (CFF), or common bean wilt. Lui *et al*. [57] found that foliar application of hydrogen peroxide protected cucumbers from osmotic stress. Van Wyk *et al*. [58] evaluated the effects of hydrogen peroxide on *Fusarium* infections in South African forest nurseries. The results from this study show that chlorine dioxide has some potential for priming plants for increased disease resistance.

4.7. Structured Water and Redox Biology Interactions with Plant Defenses

A working hypothesis can be derived from integrating the results from this study with other studies that investigated the interactions between energized water properties, redox biology, and plant defenses [59 – 67, 4]. The overall findings of current research suggest that physicochemical water properties could be manipulated to enhance plant immune systems and/or protect crops from biotic and abiotic stressors. Research involving structured water treatments and interactions with plant physiology or crop growth is still in the early stages and is incomplete concerning the many complex interrelationships. For example, in this study, the charged water foliar applications appear to alter the long-term redox biology of woody plants. Chlorophyll efficiency in the punctured leaves in the water treatments only increased between the 90 and 160 measurement dates. Rhododendrons have waxy cuticles thus leaf puncturing may have allowed the charged water to enter the leaf and contact the inner leaf tissue. In this study, the charged water foliar applications had a delayed response in chlorophyll efficiency that extended over three months.

The electrostatic sprayer results for the foliar applications with charged water poses further questions whether crops without waxy cuticles need leaf puncturing to improve chlorophyll efficiency after foliar water applications with an electrostatic sprayer. Also, research is needed to determine if there are any delays in improved chlorophyll efficiency in field crops, and how long does the improved response last.

Additional research is needed to evaluate the long-term effects of commercial and novel chemical inducers on plant defense systems. Also, long term studies are needed to determine whether treatments to stimulate plant innate immunity systems could return infected plants to complete health and the plants be considered non-infected. Studies are also needed to determine the interactions between water properties, redox biology, innate plant defenses, and potential treatments.

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