THESIS

INITIATING A PLANT HERBIVORY RESPONSE INCREASES IMPACT OF FUNGAL PATHOGENS ON A CLONAL THISTLE

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ABSTRACT

INITIATING A PLANT HERBIVORY RESPONSE INCREASES IMPACT OF FUNGAL PATHOGENS ON A CLONAL THISTLE

Cirsium arvense, or Canada thistle, is one of the most detrimental weeds for agricultural production and rangeland health. The autecious fungus, *Puccinia punctiformis*, or CT-rust, shows potential as a control agent but rarely reaches epidemic proportions in natural populations. Manipulating plant defense hormones could alter host susceptibility and allow CT-rust to have more widespread impact. To determine if applying hormones increases the infection by the fungal pathogen, Canada thistle plants were inoculated and sprayed with jasmonic acid (JA) and salicylic acid (SA). Results show that the addition of JA at the time of inoculation increases the infection rates, both the incidence and severity, and impact of CT-rust which could make it a more effective control agent for Canada thistle. In the first greenhouse experiment, I found that JA increased infection rates by nearly 20 %. Infection consistently reduced root biomass and this reduction was 45 % greater with the addition of JA compared to water. We also found that, while JA does have a slight effect on plant growth, applying herbivory defense hormones does augment CT-rust's action as a biological control.

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INTRODUCTION

Cirisum arevense, also known as Canada thistle or creeping thistle, is native to Eurasia. This species first spread to North America in the 1600's and is now on more state noxious weed lists than any other weed in the United States (Guggisberg *et al*., 2012, USDA Plants Database). As a problematic weed in temperate zones across the globe, Canada thistle has invaded millions of acres of agricultural and natural land, impacting both crop plants and native vegetation through direct competition and allelopathy (Stachon & Zimdahl, 1980, Schroder *et al.*, 1993). Due to its clonal nature and underground nutrient stores, Canada thistle is resilient to many environmental stresses and control efforts (Skinner *et al*., 2000). The expansive root system makes control difficult, often requiring multiple herbicide applications and mechanical treatments to control the weed (Beck & Sebastian, 2000; Demers *et al*., 2006). To reduce the reliance on herbicides, several classical insect biological control agents have been introduced to combat Canada thistle. However, the last half a century of efforts has not produced a reliably successful agent. This is due in part because insects such as the Canada thistle stem-mining weevil (*Hadroplontus litura*), Canada thistle gall-fly (*Urophora cardui*) and the tortoise beetle (*Cassida rubiginosa*) do not attack the regenerative below ground biomass of the thistle (Cripps *et al*. 2009, Cripps *et al.* 2011a; Winston *et al*., 2008). Further, it has proven difficult to find insect biocontrol agents that are specific enough given the large numbers of native *Cirsium* species in North America (Winston *et al*., 2008).

The use of plant pathogens as biological control agents has been proposed as an economical and sustainable management method for weedy plants (Charudattan, 2001). Numerous plant pathogens, such as rust fungi, have proven to be more specific than insect

biological control agents with no reports of unexpected infection of nontarget plants (Barreto *et al.* 2012, Barton, 2004, Scott *et al.* 2012, Woods *et al.* 2010) and show potential as biocontrol agents for invasive, clonal weeds. Canada thistle is systemically infected by the autoecious rust *Puccinia puntiformis*, or CT-rust. The rust was first proposed as a biocontrol agent against Canada thistle over 100 years ago (Wilson, 1969). CT-rust completes all five life stages solely on Canada thistle beginning with wind borne aeciospores that infect leaves in late spring. These give rise to uredinia and then teliospores by late summer (Baka & Losel, 1992). A combination of temperature, humidity, and plant-produced semiochemicals from the thistle cue teliospore germination, which gives rise to basidiospores that systemically infect the host (Berner *et al*., 2013). Symptomatic ramets die early or are stunted and covered in spores (Thomas *et al.*, 1994), yet it is possible that many infected thistles never exhibit this symptomatic response (Berner *et al*., 2015). Natural infection levels of CT-rust vary within populations from year to year and among populations but usually reduce cover of Canada thistle over time (Frantzen & Vanderzweerde, 1994, Kluth *et al*., 2005, Turner *et al*., 1981) yet infection frequency is rarely high enough to provide consistent control. Despite the history of previous research, there is still uncertainty on the life stage of the rust and the time of year that result in systemic infections. Previous studies have focused on inoculum pressure as the limiting step (Berner *et al*., 2013, Berner *et al*., 2015b, Frantzen, 1994) in depleting Canada thistle populations with varying results. These studies focus on timing of inoculation of the plant with overwintering teliospores and found that fall inoculations of rosettes provided higher frequency of infection than spring inoculations. Other researchers noted higher infection levels in plants that also experienced herbivory by *Ceratapion onopordi*, leading them to conclude that successful infection requires an insect vector (Muller *et al*., 2011, Wandeler *et al*., 2008, Wandeler & Bacher, 2006.) –

presumably one that transmits spring and summer aeciospores or urediniospores. However, global patterns of CT-rust infection suggest that insect vectors are not required and may not increase infection rates (Cripps *et al.* 2009, Cripps *et al*., 2010a, Berner *et al*., 2015).

Plants induce plant defenses to increase resistance to harmful pathogens and herbivores (Heil & Bostock, 2002). The responses are mediated by two main defense hormones: salicylic acid (SA) and jasmonic acid (JA). SA is upregulated in response to pathogen attack and JA is upregulated in response to herbivory. There is crosstalk between these pathways which work antagonistically; an increase in one is usually mirrored by a decrease in the other (as reviewed in Pieterse *et al*., 2012, Walling, 2000). Thus, in several systems, increasing herbivory defense response by spiking levels of JA leads to higher susceptibility to plant pathogen infection through secondary effects on the SA pathway (Brooks, *et al*., 2005, Nakano & Mukaihara, 2018, Nomura, *et al*., 2005). While some studies have looked at how upregulating JA increases susceptibility to pathogen attack, these have focused mainly on understanding processes with model organisms or protecting crop species rather than mitigating pests (Bostock, 2005, Heil & Baldwin, 2002, Heil & Bostock, 2002, Walters & Heil, 2007).

In addition to optimal timing or insect transmission, it is possible that SA and JA mediated defense systems alter the susceptibility of Canada thistle to CT-rust, and that interactions between these pathways plays a role in infection frequency in natural populations. We hypothesized SA mediated host resistance is a limiting factor to the development of systemic infections by CT-rust and manipulating plant defense hormones would alter host susceptibility to pathogens. Specifically, that infection would increase with the addition of JA (simulating insect herbivory pressure) and decrease with the addition of SA treatments compared to control.

MATERIALS AND METHODS

Two main greenhouse experiments were conducted to test the hypothesis. Experiment 1 was designed to test the effects of plant defense hormones on CT-rust infection rate and the combined effect of hormone treatment and rust inoculation on plant biomass. Experiment 2 was then performed to verify results and the interaction between defense hormones and inoculum.

Plant materials and growth conditions

To ensure that experimental plants were initially infection free, Canada thistle was grown from seed in the greenhouse. All seeds were collected from multiple individuals at one location in Fort Collins (40°35'14.5"N 105°01'49.7"W) and sanitized with 75 % ethanol and 2.5 % hypochlorite solutions. Experiment 1 began in March 2016 when seeds were germinated in petri dishes on Whatman 1 filter paper in the lab (~21 °C) for one week. After one week, radicles were approximately 1 cm and sufficiently long for healthy seedlings to be transferred into potting soil in 3.8 cm diameter containers. Seedlings were grown for an additional week and then 48 uniformly sized seedlings were separated and planted into 2.5 L pots in a mixture of 5:1 potting soil (PRO-MIX) and quartz sand (Quikrete play sand).

The above methods were modified slightly for Experiment 2 (started in November 2017). After germinating on filter paper, seeds were transferred to quilt batting for one week and then to potting soil in a seedling tray. Seedlings were grown in the green house for an additional two weeks and, of the surviving seedlings, 124 were potted in 9 cm square pots for application of treatments. Following the incubation period and when plants were 8 weeks post germination, they were transferred to 2.5 L pots with the same soil mixture and grown in the greenhouse. Greenhouse temperatures were between 18 - 24 °C with 10-30 % humidity. Supplemental

lighting maintained a 16 light:8 dark photoperiod with mean daytime PAR of 323 ± 58 μmol $m^{-2}s^{-1}$.

Spore pre-germination and plant inoculation

Spore germination cue was produced from a hexane extraction of volatile compounds from the roots of ~0.5 g sanitized and crushed 7 day old Canada thistle seedlings (modified from French *et al.*, 1988 and French & Lightfield, 1990). The interaction between spores and cue solution was observed under a microscope as sub-samples counted 3 times over a grid system at 10x magnification after 1 week to verify viability of the spores. At this time, an average of approximately 5 % of teliospores had germinated and produced basidiospores.

Thistles were grown for 2 weeks until they were at a basal rosette stage. The rosettes were inoculated with a solution of CT-rust spores (20 urediniospores: 1 teliospores) isolated from infected thistle leaves through filtration. The spores were 2 years old and had been stored at room temperature prior to filtration. Spores were rehydrated and suspended in with 100 mg per 200 ml of DI water and 2.5 μl of germination cue was added to stimulate germination 3 days prior to inoculation. For better distribution of spores, 50 μl of Tween 80 (Polyoxyethylene sorbitan monooleate) was added as a surfactant. Spore concentration was determined with a haemacytometer to be 2 x 10^5 spores ml^{-1}.

Adjustments were made to improve teliospore germination rates in Experiment 2. Inoculum solution consisted predominantly *P. punctiformis* teliospores. The teliospores had been collected in the field in the summer of 2017 (6 months prior to inoculation) and isolated from infected thistle leaves. Spores were removed from leaves by vigorously shaking them in DI water with Tween 80 and then filtered first through cotton straining cloth to remove plant tissue and

then through coffee filters to remove spores from solution. Spores were stored at 4 °C until use when 100 mg of teliospores were rehydrated in 500 ml of DI water (1 x 10^5 spores ml^{-1}) with 50 µl of germination cue to stimulate germination along with 50 µl of Tween 80 1 day prior to inoculation. The germination cue was made, again, from a hexane extraction of volatile compounds from 7 day old Canada thistle seedling but just from roots with green tissue removed. With this cue the teliospore germination rate was >10 %. Inoculum was applied as a foliar spray to runoff or ~2 ml per plant as done previously.

Experimental design

To determine how altering plant defense hormones effects CT-rust infection rate, hormone solutions were applied as foliar treatments. The experimental design for Experiment 1 included 4 treatments with 12 replicates for each. The treatments were 1) water and no inoculation (control), 2) 0.2 mM methyl jasmonate and inoculation (JA), 3) 0.2 mM salicylic acid and inoculation (SA), or 4) water and inoculation (H_2O). After being transferred to soil, thistles were grown for an additional 2 weeks until they were at a basal rosette stage. Plants in the three inoculated treatments received a foliar spray of the pre-germinated teliospore solution as described above for Experiment 1; 2 ml of the solution was applied to each plant. Plants were separated by treatment and placed in four incubators (Precision 20 cf Refrigerated Incubator 815, #3721, Thermo Scientific, Mariette, OH, USA) with 12 pots in each. Temperature was set at 18 °C and soil was kept moist to simulate optimum germination environment for teliospores (Berner *et al*, 2013, Thomas *et al.*, 1994). No artificial light was added during the incubation period and, while moisture was maintained, humidity was not controlled. Plants were sprayed to runoff with a foliar spray consisting of ~2 ml methyl jasmonate (0.2 mM meJA dissolved in DI water and 0.1

% ethanol with 0.1 % Tween 80 added for even distribution), salicylic acid (0.2 mM of SA dissolved in DI water and 0.1 % ethanol with 0.1 % Tween 80), or water (with 0.1 % ethanol and 0.1 % Tween 80) (Crampton *et al*, 2009; Sathiyabama & Balasubramanian, 1999; Shahzad *et al*, 2015). A fourth treatment also received the water foliar spray and was left non-inoculated to act as a control. In order to capture the probable timing of teliospore germination and subsequent infection, the spray was applied immediately prior to inoculation and every other day during the 2 week incubation period and the following week after they were returned to the greenhouse.

Similar methods were applied for Experiment 2 except that we also included non-inoculated hormone controls for all treatments. This created a full factorial with 6 treatments; JA with spores, JA without spores, SA with spores, SA without spores, water with spores, water without spores. Each treatment had 20 replicates (n = 20) and the foliar application for each treatment was applied every other day during incubation and for the following week as in the previous experiment. All 120 pots were moved to incubators and half were inoculated with the other half receiving the DI water and Tween 80 control solution (January of 2018). After incubation, plants were transplanted to 2.5 L pots, as described above, and monitored for symptoms.

Symptom monitoring and harvest

Plants were monitored for infection in the greenhouse every other day until the plants were harvested. Infection was determined with the appearance of bright yellow aeciospores that usually emerged first on new ramets on the underside of their leaves. The number of ramets per pot and presence of infection were quantified weekly. In Experiment 1, plants did not show signs of infection until 12 weeks after inoculation. Plants were harvested after four months of growth

in the greenhouse. At this point, the primary stems had already flowered and were senescing; for this reason, heights were inconsistent and not measured. Most plants had begun to produce tertiary ramets, or ramets that were produced after the secondary ramets had started to mature. The number of primary, secondary, tertiary, and all infected ramets were quantified prior to harvest. Above ground stems and leaves were removed, dried at 25 °C for a minimum of 5 days and weighed for biomass. Below ground roots and rhizomes were removed from soil, washed, and weighed for a wet biomass.

Plants in Experiment 2 first exhibited signs of infection 5 weeks after inoculation and were harvested ten weeks post incubation. At this point primary ramets had begun to bolt or send up flowering stems and infected tissues were starting to senesce. Prior to harvest, plants were photographed and the height of each ramet was measured. Leaf surface area was measured using a LICOR LI-3100C leaf area meter (LI-COR, Inc., Lincoln, NE, USA). Above ground biomass was determined with added green leaf and stem tissue weights, separated by infected and non-infected material. For below ground biomass, roots were washed, sampled for diagnosis, and weighed (wet).

Molecular detection of infection

During harvest of experiment 1, 2 cm sections were sampled from the stems of the primary ramet (S1), two uniformly sized, adjacent secondary ramets (S2), and a tertiary ramet. The rhizomes connecting these stems were also sampled in 2 cm sections, two sections attached to the primary stem (R1), sections leading away from the two secondary stems (R2) and connected to the tertiary ramet (R3) (Fig. 1). The samples were randomly collected without regard to signs of infection, but presence of CT-rust spores was noted. The segments were

surface sanitized in 75 % ethanol for 1 min, rinsed with DI water, submersed in a 2.5 % hypochlorite solution, agitated on a shaker for 5 min and rinsed with DI water three times. They were weighed, cut to a maximum weight of 100 mg, and ground using liquid nitrogen in a sterile mortar and pestle. DNA was extracted using a QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). Extracted DNA samples were stored -80 °C. No non-inoculated plants exhibited symptoms of rust infection. DNA from these plants was pooled and tested for the presence of CT-rust DNA. Individual samples from inoculated plants were tested to verify presence or absence of CT-rust DNA and whether it was present in both symptomatic and asymptomatic ramets.

To verify the presence of CT-rust and test for latent infections, DNA was amplified using fungus-specific primers from transcribed spacer region 2 (ITS2-F3: 5'-TGC TGC TAT ATA GCT CAC TT-3' and ITS2-B3: 5'-GCT TAT TGA TAT GCT TAA GTT CAG-3'). The PCR reaction mixture was made up of 10 µM deoxynucleotide triphosphate (dNTP Mix from Thermo Scientific), 10 µM primers, 10x Standard Taq Reaction Buffer and Taq DNA polymerase (New England BioLabs). Conventional PCR was performed in 20 µl volumes with 1 µl of DNA at a 0.01 – 0.005 dilution with an approximate concentration of 10 ng/µl. Amplification was achieved with an initial denaturation step of 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 2 min at 58 °C, 1 min 30 s at 72 °C, and a final extension of 7 min at 72 °C.

Amplified DNA was separated by gel electrophoresis on 1 % agarose gel with red nucleic acid gel stain (Gold Biotechnology) with a 1 kb GeneRuler ladder (Thermo Scientific). All gels produced either no detectable band or a single band of ~250 bp long, the same size as predicted and that was produced by DNA extracted from CT-rust spores. Presence of bands at the appropriate length was recorded and imaged using GeneSys. Amplified DNA that showed

positive for rust DNA was purified using a QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and sequenced by Quintara Biosciences (Hayward, CA). Sequences were compared against known sequences through BLAST searches of the NCBI database.

Statistical Analysis

Plant characteristics and harvest data was analyzed in JMP Pro 13 (SAS Institute, Oct. 2018). Proportion infected plants (incidence) and proportion infected ramets (severity) were fit using a generalized linear model with logit link and binomial distribution. Above and below ground biomass were fit using an identity link and gaussian distribution and were log transformed as needed to meet the assumptions for analysis of variance. Treatments (control vs. inoculated and foliar treatments) and their interaction were included as fixed effects. A Type 1 error rate of $\alpha = 0.05$ was used for assigning statistical significance. Pairwise comparisons of means were analyzed for significance with a Tukey's HSD post hoc test, and we used custom contrasts to compare the relative effects of inoculation on plant performance between foliar treatments when there were significant hormone by inoculation interaction terms.

RESULTS

Incidence and severity of CT-rust disparately affected by hormone application

First symptoms of infection in Experiment 1 were expressed nearly 4 months after inoculation. Proportion of pots infected, or pots with at least one infected ramet, was 0 % for the control, 58.3 % for JA, 8.3 % for SA, and 16.7 % for H_2O (Fig. 2). The infected ramets were

almost entirely tertiary ramets. Hormone treatments significantly affected the severity of infection, or proportion of total ramets infected, with plants that received JA applications having a significantly greater proportion of ramets infected than plants in any of the other treatments (See Table 1 for main effect test statistics). There were no other significant differences in infection severity between any of the other treatments.

Effects of treatments on above- and below ground biomass

In Experiment 1, dry above ground biomass was not significantly different between treatments (Fig 3a). Below ground wet biomass had greater differences (Fig. 3b) and there was a significant effect of treatment. Inoculated plants treated with JA had reduced biomass by 29.546 % ($p = 0.0013$) and H_2O was reduced by 23.590 % ($p = 0.0100$) had significant lower below ground biomass than non-inoculated controls. Below ground biomass for inoculated, SA treated plants was also lower, but not significantly so ($p = 0.1163$). Although infection rates were greater in JA vs. water or SA treated plants (see above), there were no significant differences in below ground biomass between plants in any of the inoculation treatment.

Effects of hormone application on the onset and severity of infection

In the second experiment we used a full factorial design so that we could separate the direct effects of hormone treatments on plant performance from the indirect effects of hormones on infection levels and their impacts on performance. Following inoculation, the first visual sign of infection appeared within one week, during the incubation period. Most inoculated plants began to show signs within 5 weeks of inoculation. By the end of week 10 (at harvest), every inoculated plant had some sign of infection (Fig. 4). The non-inoculated plants had no visible

signs of infection. For the inoculated treatments, the proportion of ramets that were visibly infected were 65.7 % JA, 41.1 % SA, and 47.5 % water. Again, the JA treatment showed significantly greater rates of infection than water ($\chi^2_{(1, 47)} = 9.450$, $p = 0.002$) and SA ($\chi^2_{(1, 47)} = 20.109$, $p < 0.0001$). There were no significant effects of hormone treatments ($F_{(2, 113)} = 0.5711$, $p = 0.5666$) or inoculation ($F_{(1, 113)} = 1.2687$, $p = 0.1767$) on final height at harvest (See Table 2 for main effect test statistics).

Effects of hormone application on root: shoot ratio

Inoculation ($F_{(1, 113)} = 70.141$, $p < 0.0001$) and its interaction with hormone treatments ($F_{(2,113)}$, $p = 0.003$) significantly affected above ground biomass. For this variable, inoculation reduced above ground biomass for plants treated with H_2O by 59.770 % and SA by 46.834 % but did not significantly reduce above ground biomass for JA. Further, JA by itself reduced above ground biomass relative to non-inoculated, water treated plants ($F_{(2, 114)} = 34.8899$, $p < 0.0001$), but not relative to SA treated plants (Fig. 4a). Similarly, inoculation significantly reduced leaf surface areas ($F_{(1, 113)} = 159.7304$, $p < 0.0001$) but there was no difference between hormone treatments.

The impact of hormones in the presence of inoculum had a different result for below ground biomass. For this variable, hormone treatment ($F_{(2, 113)} = 9.3931$, $p = 0.0002$), inoculation ($F_{(1, 113)} = 339.1268$, $p < 0.0001$) and their interaction ($F_{(2,113)}$, $p = 0.024$) were all significant (Fig. 5b). In the absence of fungus, there were no significant differences between hormone treatments. Inoculated plants always had lower biomass than the control plants across all treatments, and there was a 44.690 % greater effect of the fungus on plants in the presence of JA than H_2O ($F_{(1,}$

$_{114}$) = 141.0084, $p < 0.0001$). In contrast, the effect of CT-rust on SA plants was 21.988 % less than with H_2O ($F_{(2, 114)} = 97.8074$, $p < 0.0001$).

The difference between the final above and below ground biomass in Experiment 2 is emphasized when comparing the root to shoot ratios (Fig. 5). All treatments had similar ratios except for the inoculated JA treatment whose ratio is reduced by 37.778 % compared to the JA control.

Molecular identification of infection status

The DNA extracted from stem and rhizome segments from Experiment 1 was molecularly diagnosed for the presence of CT-rust. The 38 asymptomatic pots had 9 samples each that were pooled by pot and none of these 38 pooled samples, including the controls, produced bands with the rust-specific primers. All 10 of the 10 pots with symptoms of CT-rust produced bands with rust specific primers. Of the 90 rhizome and stem samples cut from the primary, secondary and tertiary stems of symptomatic pots, 53 were positively diagnosed with the rust specific primers even though fewer than half of the sampled ramets were visually symptomatic for infection. 70 % of the primary stems (S1), 60 % of the primary rhizomes (R1), 60 % of the secondary stems (S2), 55 % of the secondary rhizomes (R2), 60 % of the tertiary stems (S3) and 50 % of the tertiary rhizomes (R3) were positively diagnosed for the rust (Table 3). Samples producing positive bands were sent for sequencing and compared to other sequences available through NCBI. In all cases the top returned sequences were between 87.37 % and 98.92 % matches with *Puccinia carduorum* or a 100 % match with *Puccinia calcitrapae*. It was verified that no fungus was present in the control treatment. None of the control treatment samples tested positive for the fungus.

DISCUSSION

The results support the hypothesis that systemic infection rates and severity in Canada thistle would increase with the addition of JA. Experiment 1 showed a 41.6 % increase in the incidence of infection with the application of JA compared to the water control. There was also a 7.5-fold increase in the severity of the infection due to the JA treatment. In the second experiment we did not see an effect of hormone treatments on incidence, as all inoculated thistles became infected. However, JA treatments increased the severity and timing of the infections.

The addition of JA also increases the impact of CT-rust on Canada thistle. Below ground biomass of the JA treatment in Experiment 1, although not significantly different from the other inoculated treatments, was 6.51 % lower than H2O and 14.59 % lower than SA. However, in Experiment 2 the effect of fungal inoculation on root biomass was significantly greater for JA treated plants than SA or H_2O treated plants.

The impact of JA and inoculation is more dramatic when examining the ratio of root to shoot biomass. The application of JA increased the impact of the pathogen on root biomass but had no effects on above ground biomass. Leathwick & Bourdot, (2012) suggest that above and below ground biomass are strongly correlated in Canada thistle. Here, our data shows that CT-rust differentially impacts roots. Thus, the impact of the pathogen on natural populations may be less obvious than has been assumed. As most carbon stores and regenerative power lie in the roots of this plant (Nunes & Kotanen, 2017), the low root biomass with JA could signify that the fungus shifts plant resources away from root production and towards the production of above ground biomass and spores. There was no strong evidence that SA reduces the rate, severity or

impact of infection. Thistles treated with SA had lower rates of infection and greater belowground biomass than water treatments, but never to a significant degree.

To avoid altering plant growth, yet ensure hormonal cross-talk, hormone concentrations in foliar sprays were kept at 0.2 mM (Kazan & Manners, 2008, Mur *et al.*, 2006). At higher concentrations, JA has been shown to increase lateral root growth and inhibit primary root growth in different plants, (Huang et al. 2017, Tung et al. 1996, Staswick, Su, & Howell, 1992) especially in early development. There was no significant difference in below ground biomass for non-inoculated treatments; thus, the reduction in biomass for inoculated, JA thistles was likely due to the interaction of the hormone and fungal pathogen infection.

Several researchers have detected asymptomatic rust infections in other plant species (Kropp *et al.*, 1996, Zhao *et al.*, 2007, Baskarathevan *et al.,* 2016) and Berner *et al.* (2015) suggested that asymptomatic infections of CT-rust could be as high as 60 % within an infected population of Canada thistle (Berner *et al*., 2015). However, our molecular diagnostic did not detect any evidence of infection in thistle plants that were asymptomatic. Although we found no evidence for latent infections, we did detect the presence of CT-rust DNA within asymptomatic ramets that were produced by and attached to symptomatic plants. Whether these ramets and or shoots would have eventually become symptomatic is unclear – however, in our trials ramets begin expressing symptoms at the time of emergence from the soil. Primary stems in Experiment 1 had the greatest proportion of samples that were molecularly positive for rust fungus even though none of these stems were symptomatic. This suggests that the fungus can spread within the plant and between ramets without expressing symptoms in established stems. It is an open question whether roots that appeared healthy at the time of harvest would have survived to produce additional shoots, and if these shoots would have expressed disease symptoms.

The greater incidence, severity and impact of CT-rust with the application of JA indicate that insect herbivory could play a role in the epidemiology and impact of the pathogen. Insect feeding upregulates the production of JA in many plant species (Howe & Jander, 2008) and has been shown to interfere with the SA defense pathway in several species (Doherty et al, 1988, Felton et al 1999, Thaler et al. 2002, van Wees et al., 1999). Kluth *et al.* (2001) found that simulating herbivory by perforating Canada thistle leaves resulted in significantly higher levels of infection after 2 weeks with either natural or artificial CT-rust inoculation. If herbivory does stimulate JA production in Canada thistle, as it does in many other plant species, then it may have a synergistic impact with the fungus. It has been hypothesized that insect herbivores or pollinators could act as vectors for CT-rust because early spring infections emit a fragrant odor similar to that of the thistle's blooms co-incident with the production of pycnidia which attract many insects (Connick & French, 1991, Theis, 2006). Many studies have attempted to test this hypothesis by using or simulating an insect vector to establish infection (Muller et al. 2011; Sciegienka et al. 2011; Wandeler et al., 2008). Muller *et al.* (2011) applied infected leaf material to Canada thistle and observed an infection rate of 30 %. This increased to approximately 50 % when they injected pure urediniospores in solution into stems, mimicking the stem mining weevil *Ceratapion onopordi*. Other studies have shown that *C. onopordi* not only promotes infection with CT-rust but also has a mutualistic relationship with the fungus and preferentially feeds and oviposits on infected thistle (Friedli, & Bacher, 2001a, Freidli & Bacher, 2001b). However, few other herbivores exhibit this preference and are undeterred in their feeding on infected plants (Kluth *et al.,* 2001, Kruess, 2002), and systemic infections occur independent of weevil feeding (Berner *et al.* 2013, Cripps, 2009). This makes it difficult to conclude that insect vectors play an important role in CT-rust epidemiology; but these studies along with the data here do offer

evidence that insect feeding could indirectly contribute to higher rates of fungal infection via hormonal cross-talk. It is likely that synergistic effects have a more substantial impact below ground, even when not as obvious above ground (Cripps *et al.*, 2011b).

It is possible that CT-rust infection rates in the field could be increased by applying JA or JA-precursors to natural populations of Canada thistle, either with or without artificial inoculations. This could increase the efficacy of current control efforts that are spreading the spore inoculum in an attempt to start new (or increase old) systemic infections. Similar methods could be tested with other fungal pathogens on Canada thistle that show limited or short-term reductions in thistle population size (Bourdot *et al.*, 2006).

Altering the susceptibility of Canada thistle may be key to effective control by this fungal pathogen. The interactions between hosts and their pathogens can be complex as there is strong selective pressure on each. A change in one can cause slight resistance or susceptibility in the other (Burdon & Thrall 2008). We see that the combination of pathogens and herbivores, the order in which they attack the host, the abiotic conditions at the time and interactions with other hormones all change plant defense and resistant or susceptibility (Ponzio *et al.* 2013, Scala *et al.* 2013). The cross talk between JA and SA results in the plant's inability to apply all defense pathways simultaneously (Stam *et al.,* 2013), and we can take advantage of this inability and induce one response, such as the JA defense, to make plants more susceptible to the detrimental effects of pathogen infection.

TABLES AND FIGURES

Table 1. Harvest data from experiment 1. Data are means ± SE. *Infection rate is the number of infected shoots divided by new shoots.

Means ±SE of harvest data, Experiment 1

	Control	**JA**	**SA**	**H_2O**	**F Ratio**	**Prob > f**
Number of secondary shoots	14.917 ± 2.696	12.500 ± 2.696	27.167 ± 2.696	18.583 ± 2.696	5.679	0.0022
Number of tertiary shoots	13.917 ± 1.742	15.500 ± 1.742	19.917 ± 1.742	19.667 ± 1.742	2.981	0.0415
Number of total shoots	28.833 ± 3.971	28.000 ± 3.971	47.083 ± 3.971	38.250 ± 3.971	5.124	0.004
Infected shoots	0 ± 0.291	1.583 ± 0.291	0.167 ± 0.291	0.250 ± 0.291	5.507	0.0027
Infection rate*	0 ± 0.045	0.199 ± 0.045	0.013 ± 0.045	0.026 ± 0.045	4.268	<0.0001
Aboveground biomass – dry (g)	34.409 ± 1.674	31.903 ± 1.674	35.711 ± 1.674	35.003 ± 1.674	0.979	0.4113
Belowground Biomass - dry (g)	203.725 ± 10.300	145.533 ± 10.300	170.392 ± 10.300	155.667 ± 10.300	6.083	0.0015

Table 2. Harvest data from experiment 2. Data are means ± SE.

	H_2O Control	H_2O Inoculated	JA Control	JA Inoculated	SA Control	SA Inoculated	F Ratio*	Pro > F*
Number of Total shoots	13.15 ± 0.904	9.35 ± 0.904	8.72 ± 0.953	7.85 ± 0.904	15 ± 0.980	8.89 ± 0.927	3.89	0.023
Proportion Infected	0	0.4747 ± 0.0385	0	0.6566 ± 0.0385	0	0.4114 ± 0.0395	10.7298	0.0001
Height (cm)	26.25 ± 2.813	23.6 ± 2.813	23.944 ± 2.965	32.0 ± 2.813	25.059 ± 3.051	27.632 ± 2.886	1.2025	0.3129
Leaf Weight (g)	52.055 ± 2.332	18.69 ± 2.332	41.811 ± 2.458	22.745 ± 2.332	51.124 ± 2.529	21.947 ± 2.393	4.7888	0.0102
Leaf Area (cm^2)	14.97.205 ± 73.577	562.098 ± 73. 577	1138.917 ± 77.557	600.265 ± 73.577	1466.065 ± 79.806	598.342 ± 75.489	3.9778	0.0215
Stem Weight (g)	16.925 ± 1.198	9.065 ± 1.198	12.8 ± 1.263	11.535 ± 1.198	17.341 ± 1.3	10.621 ± 1.229	4.143	0.0185
Aboveground Biomass – dry (g)	68.99 ± 4.184	27.755 ± 4.184	49.15 ± 4.184	34.28 ± 4.184	58.195 ± 4.184	30.94 ± 4.184	4.969	0.0085
Belowground Biomass – wet (g)	73.91 ± 2.871	28.105 ± 2.871	67.428 ± 3.026	15.545 ± 2.871	71.453 ± 3.114	36.026 ± 2.945	3.8586	0.0241
Root: Shoot	0.984 ± 0.073	1.057 ± 0.073	1.268 ± 0.077	0.479 ± 0.073	1.07 ± 0.079	1.171 ± 0.075	22.8447	<0.0001

Table 3. Fraction of primary stems (S1), primary rhizomes (R1), secondary ramets (S2), secondary rhizomes (R2), tertiary ramets (S3) and teritary rhizomes (R3) that tested positive for presence of rust DNA. These individual samples were taken from symptomatic pots from Experiment 1. All non-symptomatic pots were also pooled and tested but did not produce bands matching *Puccinia* DNA.

Symptomatic Plants	S1	R1	S2	R2	S3	R3
JA 15	1/1	0/2	1/2	0/2	0/1	0/1
JA 17	1/1	2/2	2/2	0/2	1/1	0/1
JA 18	1/1	1/2	2/2	2/2	1/1	1/1
JA 20	1/1	2/2	2/2	2/2	1/1	1/1
JA 21	1/1	1/2	0/2	0/2	0/1	0/1
JA 22	1/1	1/2	1/2	2/2	0/1	1/1
JA 24	0/1	1/2	0/1	1/2	0/1	0/1
SA 31	0/1	0/2	2/2	0/2	1/1	0/1
H2O 37	1/1	2/2	1/2	2/2	1/1	1/1
H2O 46	0/1	2/2	1/2	2/2	1/1	1/1

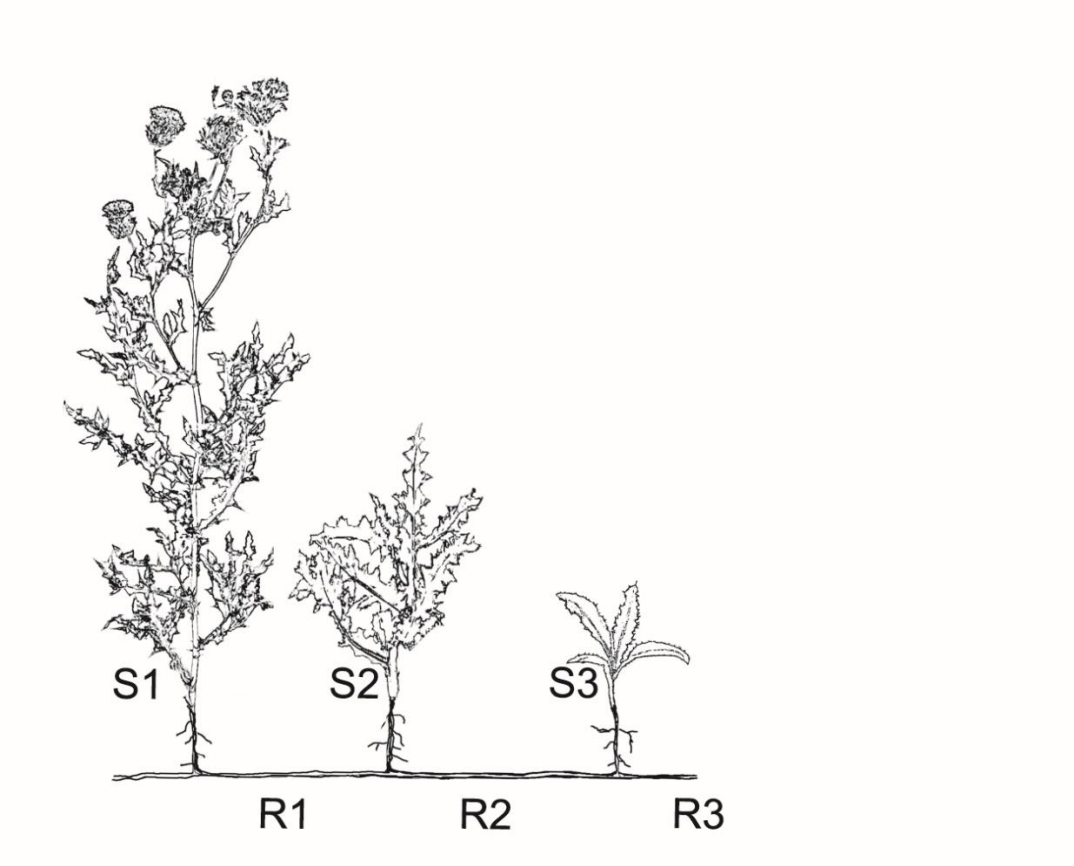

Figure 1. Sampling scheme for molecular identification of CT-rust infections. S1- primary shoot; R1 rhizome between primary and secondary shoots; R2 secondary shoot; R2 – rhizome between secondary and tertiary shoot; S3 tertiary shoot; R3 rhizome between tertiary and quaternary shoots. S1 was inoculated, visible infections were only found on S2 and S3.

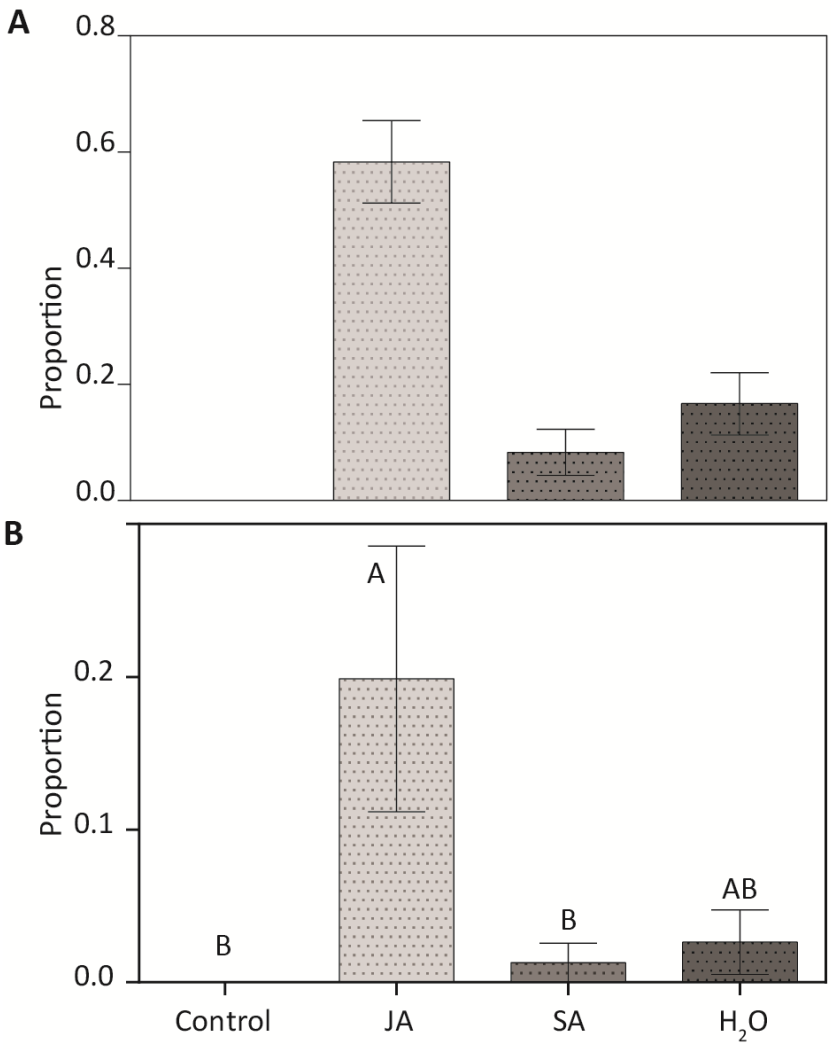

Figure 2. Panel A shows incidence of infection or proportion of pots with symptoms of infection. Panel B shows the overall proportion of new ramets that were infected or the mean number of infected ramets per pot divided by the total number of tertiary ramets per pot, by treatment. Letters show mean difference based off a Tukey's HSD test, and error bars show standard error of the mean.

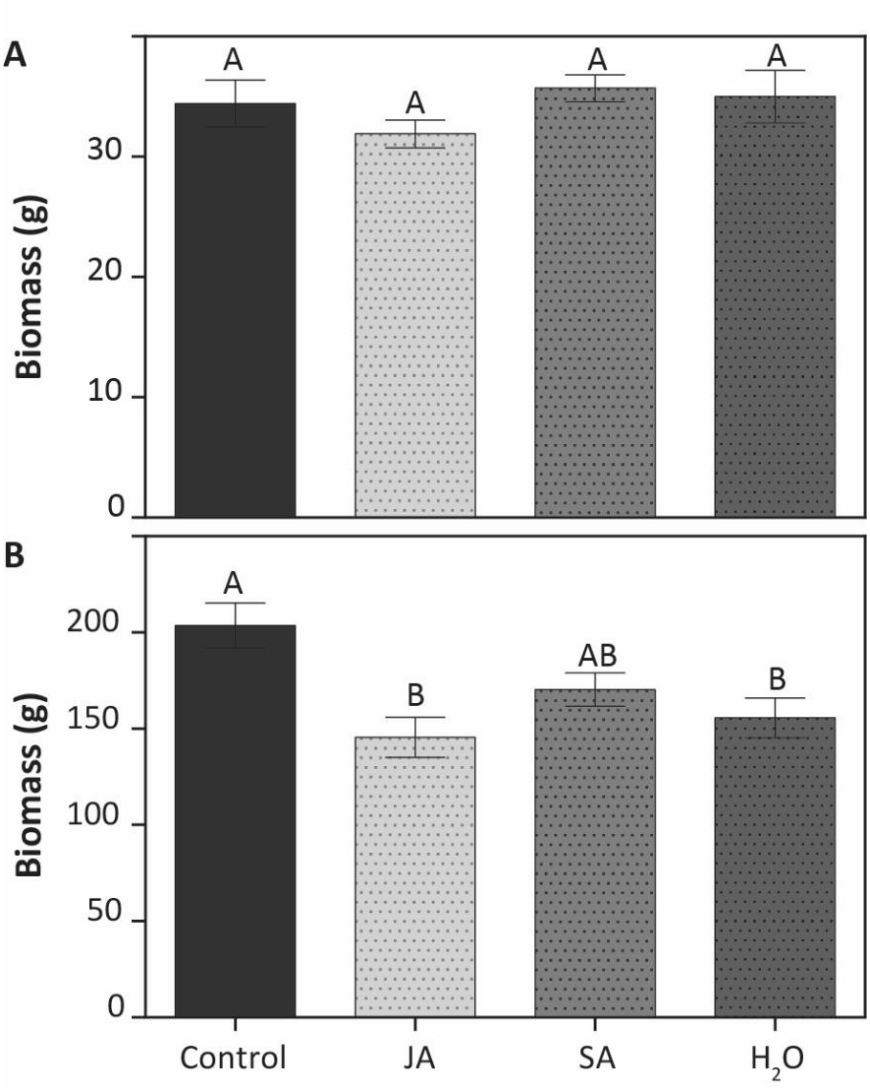

Figure 3. Panel A shows the mean above ground biomass (g) for each treatment in Experiment 1. There was no significant difference between treatments. Panel B shows the mean below ground biomass (g) by treatment for experiment 1. Treatment effect is significant (p-value = 0.0015), letters show mean difference based off a Tukey's HSD test, and error bars show standard error of the mean.

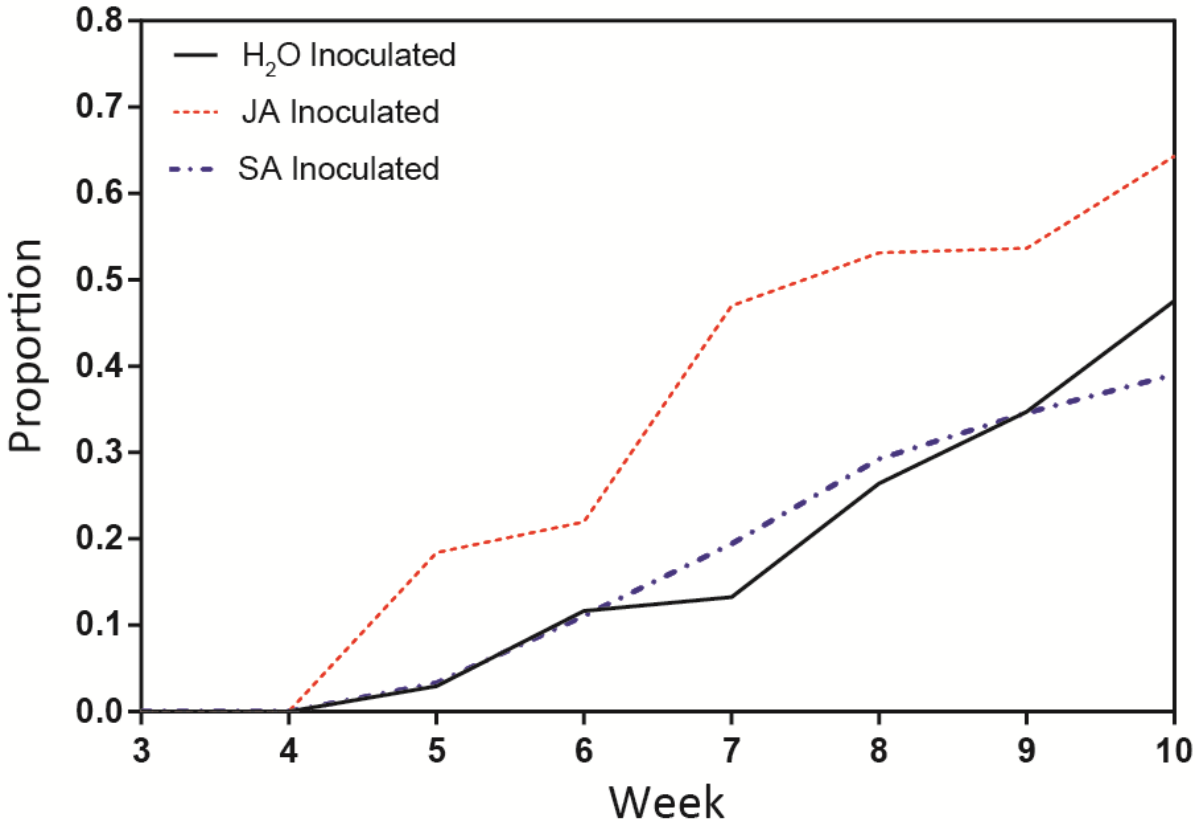

Figure 4. The mean total infected ramets in experiment 2 divided by the mean total ramets each week per pot, by treatment. Control treatments were not inoculated, and no pots exhibited symptoms. One pot in the JA Inoculated pot produced a symptomatic ramet during incubation which died and is not included in the data.

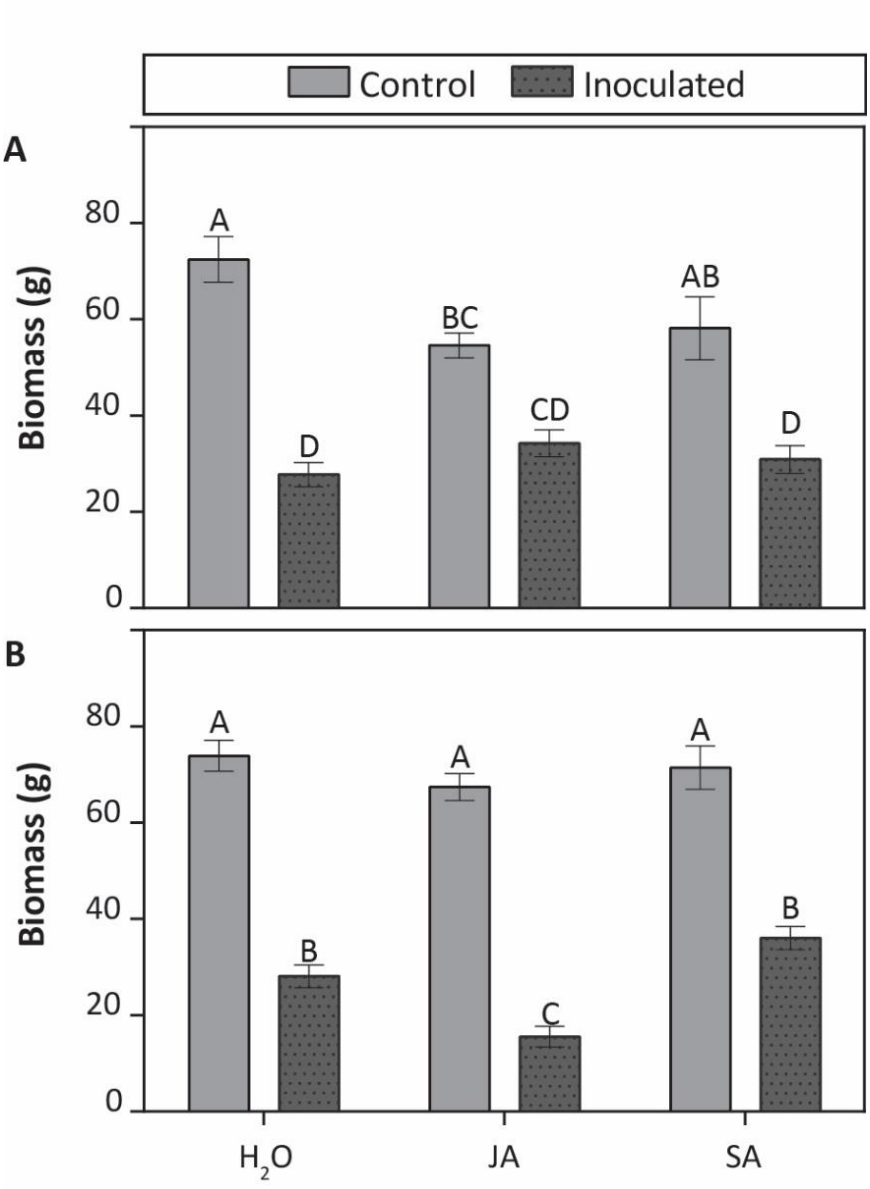

Figure 5. Final biomass measurements for experiment 2. Panel A shows the mean above ground biomass (g) which differed for inoculation (p-value < 0.0001) and interaction with foliar treatments (p-value = 0.0026). Panel B shows mean below ground biomass (g) for each treatment. Effects of foliar treatment, inoculation, and the interaction were significant (p-value = 0.0002, < 0.0001, 0.0241). Letters show mean difference based off a Tukey's HSD test. Error bars are standard error of the mean.

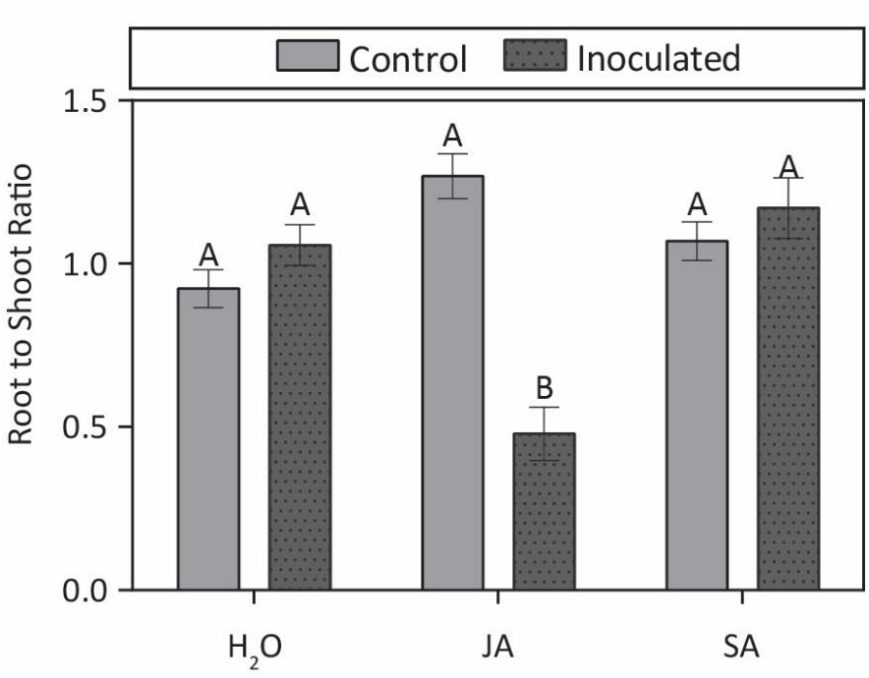

Figure 6. Mean root to shoot ratio per pot by treatment. Effects of the foliar treatments ($F_{(2, 113)}$ = 5.3689, p = 0.006), inoculum ($F_{(1, 113)}$ = 11.239, p = 0.0011) and the interaction ($F_{(2, 113)}$ = 22.8447, p < 0.0001) are all significant. Letters show mean difference based off a Tukey's HSD. Error bars show standard error of the mean.

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