The Effects of Salinity and Activated Charcoal on the Herbivory of *Arabidopsis thaliana* by *Myzus persicae*

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SUMMARY

Road salt is commonly applied in the winter and inevitably percolates into surrounding areas where it is absorbed by plants. The detrimental effects of salinity on plants have been studied extensively, with recent literature investigating potential mitigative methods, including the application of biochar. This study explores the use of activated carbon, a modified version of charcoal with increased adsorptive ability, as a remediation technique for salt stress. Using model organisms *Arabidopsis thaliana* (thale cress) and *Myzus persicae* (green peach aphids), the aim was to determine the individual and combined effects of salinity and activated charcoal on plant performance and aphid populations using a factorial design. Overall findings presented a statistically significant effect ($p=0.0201$) on *M. persicae* herbivory between 25 mM salt and activated charcoal treatment groups. Although changes in plant biomass were not observed, there were a greater number of aphids occupying the plants without activated charcoal than on plants with activated charcoal for 25 mM salt treatments. Therefore, activated charcoal presents the opportunity for an accessible method of treatment for salt-stressed plants.

Received: 03/05/2017 **Accepted**: 03/30/2017 **Published:** 07/31/17
URL: https://journals.mcmaster.ca/iScientist/article/view/1454

Keywords: *Arabidopsis thaliana, Myzus persicae,* phloem-feeding insects, activated charcoal, road salt, plant-herbivore interactions, plant stress

INTRODUCTION

Road salt is universally applied to delay the formation of ice by lowering the freezing temperature of water. This application significantly reduces vehicular accidents in the winter (Turunen, 1997). However, its excessive use also entails severe, and possibly irreversible, environmental consequences for surrounding ecosystems (Moghaieb, Saneoka and Fujita, 2004; Parida and Das, 2005). Some countermeasures must be implemented to protect environmental integrity.

Without protective measures, road salt can have severe environmental consequences. Approximately 90% of road salt percolates as far as 6 metres from the road's edge as the snow melts, increasing the salinity of surrounding soil and groundwater (D. Williams, N. Williams and Cao, 2000; Norrström and Bergstedt, 2001). Road salt also interferes with various soil properties, such as cation exchange capacity, by leaching vital minerals involved in photosynthesis and cell structure maintenance out of the soil (Demarty, Morvan and Thellier, 1984; Berkowitz and Wu, 1993; Akselsson, et al., 2013). This leaching effect substantially decreases overall plant performance, which in turn affects the entire ecosystem (Maron and Crone, 2006).

Plants are the basis of most terrestrial ecosystems as they provide energy and nutrients for herbivores (Bowdish and Stiling, 1998). When plant performance decreases, the resulting effects reverberate throughout the entire ecosystem and cause changes in both herbivory patterns and herbivore populations (Hairston, Smith and Slobodkin, 1960; Bowdish and Stiling, 1998). The application of various countermeasures to areas at risk of road salt exposure helps to avoid these detrimental effects. Studies have investigated the efficacy of protective methods, including silicon and proline application (Zhu, et al., 2004; Hoque, et al., 2007). A more recent study by Thomas, et al. (2013) also found that biochar can mitigate the detrimental effects of road salt. However, the impacts of activated charcoal, a substance that closely resembles biochar, on reducing salt toxicity are not entirely understood (Thomas, et al., 2013). Activated charcoal, otherwise referred to as activated carbon, is prepared through the pyrolysis of charcoal at temperatures between 950°C and 1000°C. This process stacks carbon atoms randomly, leaving interstices, or pores. This structural arrangement increases the overall surface area and thus increases the adsorptive ability of activated charcoal. This heightened adsorption is often exploited to remove unwanted tastes and smells as well as undesired organic and inorganic matter (Bansal and Goyal, 2005).

This study, conducted in September 2016, aims to determine how activated charcoal and salt water solutions influence aphid herbivory. To address whether activated charcoal reduces the impact of varying levels of soil salinity, we experimentally simulated a ditrophic ecosystem. *Arabidopsis thaliana* plants were inoculated with *Myzus persicae*, and treated with combinations of salt and activated charcoal. *A. thaliana* was selected as a model organism due to its relatively fast growth rate and *M. persicae* was selected due to its high fecundity (Baugh and Phillips, 1991; Somerville and Koornneef, 2002). In stressful conditions, *M. persicae* gives birth to winged alates, which can be interpreted as an indication of treatment-induced plant stress (Baugh and Phillips, 1991). Our experiment aims to answer three research questions:

1. Does varying salt concentration have an effect on the herbivory of *A. thaliana* by *M. persicae*?
2. Does activated charcoal have an effect on the herbivory of *A. thaliana* by *M. persicae*?
3. Does the interaction between activated charcoal and varying salt concentration affect the herbivory of *A. thaliana* by *M. persicae*?

MATERIALS & METHODS

To determine the effect of activated charcoal and salt concentration on aphid herbivory, a factorial design was generated involving 24 randomly selected wild type *A. thaliana* in the rosette stage of development. The McMaster Biology Greenhouse in Hamilton, Ontario, supplied all plants used in the study. *A. thaliana* plants were then partitioned into six groups of four, and each division was assigned an identifying letter corresponding to treatment received, as indicated in Table 1.

Table 1: Experimental Groups of *Arabidopsis thaliana*

	No Salt	Low Salt	High Salt
No Activated Charcoal	A	C	E
With Activated Charcoal	B	D	F

Indicators of plant performance such as number of leaves, rosette diameter, and stem length, were measured on day 1 prior to aphid inoculation and treatment administration. The number of leaves included all leaves visibly attached to the plant by a petiole, and excluded new buds. Rosette diameter was considered to be the longest distance from one leaf tip of the plant to the other, without extending any curled leaves. Stem length was measured as the length of the entire bolting stem extending from the top of the rosette, and was recorded as zero for non-bolting plants. Since all *A. thaliana* were initially selected to be in the rosette stage, each plant's day 1 stem length measurement was zero. These quantities were measured again on days 5, 8, and 12.

Activated charcoal was supplied by the McMaster Biology Greenhouse. Once all initial plant measurements and observations were recorded, activated charcoal was added to plants in groups B, D, and F (Table 1). A thin layer of activated charcoal, approximately 1-2 mm thick, was applied to the soil surrounding the plant on day 1 only.

Next, saline solutions were prepared by dissolving 0.3652 g and 0.7305 g of NaCl in 250 mL of distilled water to obtain the desired concentrations of 25 mM and 50 mM, respectively. These concentrations were chosen on the basis that *A. thaliana* can tolerate up to 100 mM of NaCl (Zhu, 2000). Plants that were not receiving saline treatments were watered with distilled water. Each plant was given a 20mL solution of their designated treatment on days 1, 5, 8, and 12.

M. persicae were raised on either *A. thaliana* or tobacco plants in the McMaster Biology Greenhouse. The aphids we used were in varying life stages, excluding winged alates. After watering, each individual plant was inoculated with three aphids. They were placed on the plant towards the middle of the rosette with a metal inoculating rod. On days 5, 8, and 12, the number of living aphids was recorded.

Plants were housed in two plastic trays throughout the duration of the experiment. To limit aphid transfer between plants to only winged-alates, paper dividers were positioned between plants, as seen in Figure 1.

Figure 1: Experimental design of *A. thaliana* trays. There were two trays with 12 plants each, separated by dividers to limit *M. persicae* movement to winged alates.

After watering and inoculation, plants were evenly distributed amongst the trays using a block-randomized method to minimize the effects of confounding variables. The two plant trays were divided in half for a total of four blocks (Figure 2). One plant of each experimental group (A-F) was included in each block. Additionally, the placement of plants within each block was randomized (Table 2). The trays were covered with a plastic lid containing two mesh-covered ventilation openings, and placed by a north-facing window for the duration of the study.

Block 1		**Block 2**	
D16	E20	D15	C12
A4	F21	E17	B5
B6	C11	F22	A1
Block 3		**Block 4**	
A3	D14	B8	C9
C10	B7	D13	A2
F24	E19	F23	E18

Figure 2: Summary of placement of each plant after block randomization. Numbers 1-24 refer to the specific plant, and letters A-F identify the treatments administered.

STATISTICAL ANALYSIS

The effect of salt concentration and activated charcoal along with their interaction on aphid herbivory was evaluated using a two-way analysis of variance (ANOVA) with interaction using statistical analysis software (Urbanek, Bibiko and Iacus, 2016). The data for number of aphids were log-transformed to improve homoscedasticity and normality, as aphids reproduce exponentially (Helms and Hunter, 2005). This also generated a better F critical value, indicating that the model was a better fit. The block effect due to the block randomization distribution of plants was accounted for in the ANOVA. Finally, Tukey's Honestly Significant Difference test was used to determine which interactions had significant variance in their means.

After the data was collected, one *A. thaliana* plant treated with low salt and no activated charcoal was removed due to an infestation of fungus gnat larvae. Any leaves touching the soil were wilted and irreparably damaged. This plant was an outlier within the data set and its poor performance was attributed to the fungus gnat larvae, leading us to exclude it in the analysis of variance.

RESULTS

Table 2: Analysis of variance results for effects of salt concentration, charcoal presence, and their interaction on the log-transformed population size of *M. persicae* on plants

Sources of Variation	Salt	Activated Charcoal	Salt & Activated Charcoal	Error
Degrees of Freedom	2	1	2	17
Mean Square	0.193	0.065	2.068	0.416
F Critical Value	0.4636	0.1556	4.9616	
p-value	0.6368	0.6981	0.0201	

As seen in Table 2, there was no significant effect of salt concentration on aphid population (p=0.6368) or activated charcoal on aphid population (p=0.6981). However, a significant effect was seen in the interaction between salt concentration and activated charcoal on aphid population (p=0.0201). This supports the hypothesis that the interaction between salt concentration and activated charcoal affects aphid herbivory. Thus, the effect of salt concentration on aphid herbivory is dependent on the presence or absence of activated charcoal and vice versa.

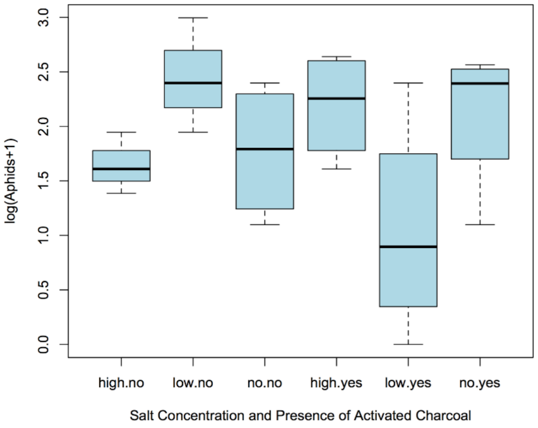

Figure 3: Mean value of ($\log_{10}$+1)-transformed number of aphids on treated plants. There is a significant difference between means for low salt with activated charcoal and low salt without activated charcoal ($p<0.05$).

Figure 3 illustrates the mean value of log-transformed number of aphids on day 12 with their standard deviation for each treatment group. The difference in mean aphid populations is greatest for plants treated with a low salt concentration.

Figure 4 illustrates the mean value of log-transformed number of aphids on plants treated with no, low concentration, and high concentration salt water in either the presence or absence of activated charcoal. Plants treated with no salt or high salt had approximately the same number of aphids regardless of whether or not charcoal was present. Only groups of *A. thaliana* treated with low salt showed significant differences in number of aphids depending on the presence or absence of activated carbon.

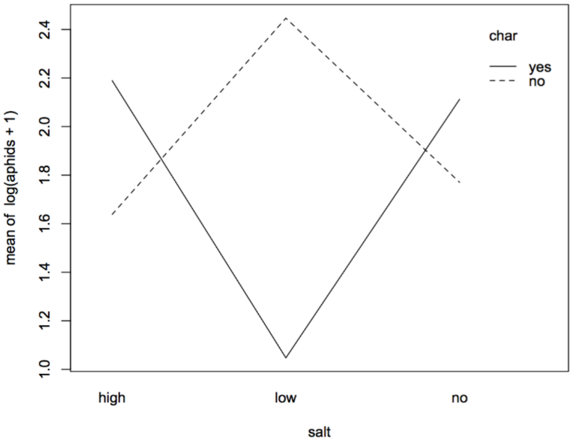

Figure 4: Interaction plot showing mean value of ($\log_{10}$+1)-transformed number of aphids for each of the treatment groups. There is a significant difference in means of *M. persicae* on *A. thaliana* treated with low salt with and without activated carbon ($p<0.05$).

Salt concentration and activated charcoal did not have an effect on any measured plant biomass variables, such as number of leaves, stem length, or rosette diameter. The experiment was organized with a block random design to reduce the probability of confounding variables biasing the results. An analysis of variance showed the blocks had no significant effect on aphid herbivory.

DISCUSSION

Road salt is necessary for safe winter driving, but can abiotically stress plants and negatively impact plant performance. Biochar has been researched as a countermeasure for salt-induced plant stress (Thomas, et al., 2013). However, the effects of activated charcoal on plant-herbivore relationships are not yet well understood. A further understanding of activated charcoal's effects provides the possibility for a protective countermeasure against salt damage on sensitive plants. This study examined how the relationship between *M. persicae* and *A. thaliana* was affected by the introduction of varying concentrations of salt water and activated charcoal. A two-way ANOVA with interaction showed that the effect of plants treated with 25 mM salt concentration on aphid herbivory significantly depended on the presence or absence of activated charcoal. However, aphid herbivory for plants treated with no salt or 50 mM salt concentration did not depend on the presence or absence of activated charcoal. Additionally, salt concentration and activated charcoal did not independently affect aphid herbivory.

There were more aphids on plants treated with low salt in the absence of charcoal than on plants treated with low salt in the presence of charcoal. These results indicate that aphid herbivory was higher in the absence of activated charcoal (Figure 4), which supports the plant stress hypothesis. This hypothesis states that herbivores preferentially feed on plants that are under biotic and abiotic stressors over unstressed plants (Joern and Mole, 2005). Piercing-sucking insects, including *M. persicae*, perform better and have higher reproductive potential on stressed plants compared to non-stressed plants (Koricheva, Larsson and Haukioja, 1998). This pattern could occur for several reasons: the alteration of biochemical source-sink relationship allocations, a change in foliar chemistry, and decreased defense levels (Joern and Mole, 2005). The changes in biochemical source-sink relationship allocations affect internal resource partitioning within stressed plants (Joern and Mole, 2005). A study performed by Lincoln (1993) found that, on leaves with elevated carbon content, there was increased consumption by herbivorous insects. This was due in part to the newly enhanced leaf digestibility and reduced efficiency of nitrogen use (Lincoln, 1993). The introduction of stressors to plants also induces a change in foliar chemistry by affecting the levels of nitrogen-containing compounds available within the leaves. These compounds include amino acids, amides, and imino acids (Mansour, 2000). Herbivores preferentially feed on stressed plants with higher nitrogen concentrations, and tend to survive longer as a result (Mattson, 1980).

There was no significant effect on the amount of aphid herbivory for plants that were treated with distilled water, independent of whether or not charcoal was present. This indicates that activated charcoal alone does not affect herbivory. Regardless of activated charcoal's ability to mitigate the negative effects of toxic chemicals, we found no evidence that it improved plant performance. Similarly, there was no relationship between the plants belonging to the two groups that were treated with a 50 mM salt solution. It may be that the charcoal's adsorptive ability was not high enough to mitigate the effect produced by a greater salt concentration. Independent of charcoal's presence or absence, when the salt concentration is 50 mM aphids are equally successful at reproducing on the salt-stressed plants. This suggests that the effect of activated charcoal on aphid herbivory depends on the level of salt concentration.

Aphids on *A. thaliana* stressed with 25 mM salt water without charcoal performed better than aphids on plants stressed with high concentration salt water without charcoal (Figure 4). This agrees with the pulsed stress hypothesis, proposed by Huberty and Denno (2004). This hypothesis states that herbivores, especially phloem feeders, perform better on plants that cycle through periods of stress and recovery (Huberty and Denno, 2004). Elongated periods of stress negatively affect the amount of nutrients that phloem-feeding insects can extract from a plant, while short cyclic periods maintain nutrient availability while decreasing plant performance (Huberty and Denno, 2004). Since the experiment had a relatively short time span (12 days), *A. thaliana* treated with low salt without charcoal experienced a stress period that allowed for increased performance of *M. persicae*. Plants given high salt without charcoal had a lower number of aphids compared to those exposed to low salt without charcoal due to the increased stress of *A. thaliana.* This reduced the nutrients available to *M. persicae* therefore causing a decrease in population. If the experiment were to be conducted over a longer period of time, plants treated using low salt without charcoal would be under an elongated period of stress, likely producing conditions less favourable for aphid herbivory.

The study results indicate that the presence or absence of activated charcoal alone did not have an effect on aphid herbivory. This is due to the fact that activated charcoal works to adsorb harmful toxins rather than to provide the plants with nutrients (Bansal and Goyal, 2005). It would appear that activated charcoal does not help nor hinder plant performance or independently affect aphid herbivory.

There are, however, innate limitations to this study's design. Within each of the 6 treatment groups, there were only 4 individual *A. thaliana*. This sample size is extremely small, due to resource restrictions. In small sample sizes, the presence of one outlier has more influence on prevalent trends within the data than in larger sample sizes. Since there were only 4 plants within each treatment group, having one outlier meant that a quarter of the data collected from that treatment group must be disregarded. Also, this experiment was performed over the course of 12 days, which is a very short time frame relative to *A. thaliana*'s life span of 2.5 months (Tocquin, et al., 2003). With a longer experimental time frame, the results would better represent changes in rosette diameter, number of leaves, and stem length. There was not enough time to allow for plant biomass to significantly accumulate or diminish over the course of the experiment, which likely contributed to the insignificance of plant performance results.

The interaction between activated charcoal and salt water should be examined in greater detail. Activated charcoal could be considered as a method for protecting sensitive environments to alleviate plant stress and decrease insect herbivory. This study did not analyze the effects of charcoal on organisms occupying other trophic levels. Future research could investigate the possible detrimental effects of activated carbon on different species within the food chain, a factor that could not be tested due to the ditrophic nature of our experiment.

The results of this experiment have several future implications. This gives insight into the efficacy of applying activated charcoal in tandem with road salt to minimize anthropogenic impacts and maintain road safety. Since our study only focused on the interaction between *A. thaliana* and *M. persicae*, the impact on a more complicated multitrophic ecosystem can be extrapolated from our simple design.

CONCLUSION

The interaction of 25 mM salt solutions with activated charcoal has a significant effect on the herbivory of *M. persicae* with an increased number of aphids on plants growing in the absence of activated charcoal. These results support the plant stress hypothesis and provide insight for a new method of protection for salt-stressed plants. With further research, activated charcoal could be widely applied to decrease insect herbivory and mitigate salinity effects on plants in heavily salted areas.

ACKNOWLEDGEMENTS

We would like to thank Dr. Susan Dudley for her guidance throughout the study and suggestions on the paper. We also thank Sebastian Irazuzta, Geneviève van Wersch, and Russ Ellis for their supervision during data collection days. Materials for the study were provided by the McMaster Biology Greenhouse in Hamilton, Ontario.

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