Can We Save the Common Ash?

"An Investigation Into the Jasmonic and Salicylic Acid Interplay's Effect on the Plant – Pathogen Interaction of *Fraxinus Excelsior* and *H.fraxineus*"

Introduction

The Common Ash Tree (*Fraxinus Excelsior)* is a vital part of our biodiversity (Ireland's tallest most common native tree) and culture (primary source of wood for hurley manufacturing). Both our culture and diversity are being significantly impacted by *H.fraxineus,* more commonly known as Ash Dieback. Ash Dieback is a chronic fungal disease that causes leaf loss, crown dieback, and bark lesions in affected trees. This consequently succumbs the wood, therefore making it useless for commercial production.

Upon reading a study on the effect of two plant hormones Salicylic and Jasmonic Acid on boosting the defence of Poplars against pathogenic fungi, it was found that these defence hormones do not always suppress one another in regulating plant chemical defences against pests and pathogens. Instead it was found that the positive interaction of the two hormones not only increases the defence of the poplar tree but also has no negative effects on the growth of the tree. This inspired us to investigate the potential effect that these two hormones had in relation to the plant pathogen interaction of *H.fraxineus* and *Fraxinus Excelsior,* to determine if we could reduce or prevent the effects of the fungus.

We conducted our experiment using three genotypes (genetic make up of the tree) of Common Ash trees. Genotype 1 trees were the worst affected by *H. fraxineus* showing severe signs of the disease Genotype 2 trees were moderately affected with some evidence of ash dieback and Genotype 3 were the least affected trees with little to no symptoms of the disease and appeared healthy. We did this to see the effects which our three treatments had on the growth of the fungus with different genotypes of trees, to see if we could find a treatment which preformed consistently across genotypes.

Experimental Methods and Data Collection

We planned to conduct our experiment in vitro, meaning we conducted our experiment on petri dishes of ah agar with a sample size of 180 petri dishes. The first steps of our experiment was to collect the leaf material for this as well as the specific leaf collection of different genotypes from which we planed to harvest our leaf extract.
The leaf extract was made by grinding 1.2g of the set genotype into 10ml of water. Once a fine pasted formed this mixture was then strained and purified in order to obtain to leaf extract for that genotype. This was repeated a total of 6 times, two trees for each genotype.

The ash agar was made by blending leaf material into 1L of water. The agar agar powder was added and then the mixture was heated to 121 *c for decontamination. after cooling it was then poured into the petri dishes.

Treatments and their concentrations

treatment	Concentration	Ratio
1	High, 0.04756 M:0.02378 M	JA:SA 2:1
2	Low, 0.0002378 M:0.0004756 M	JA:SA 1:2
3	Low, 0.0004756 M:0.0002378 M	JA:SA 2:1

The solutions of the two hormones were made by taking them in their concentrated form and diluting them to a set molar concentration of 0.04756 M. This was then diluted acordingly depending on treatment. The treatments can be seen in the table to the right.

Once all components were asembled the dishes were then brought to Teagasc research centre in order to inoculate the plates with the fungus within a setrile environment. After all dishes were assembled, the fungal plug in the center and the different leaf extracts and/or treatments depending on genotype applied on filter paper disks surrounding the edge of the plate, the dishes were then sealed and brought back to our lab for data collection.

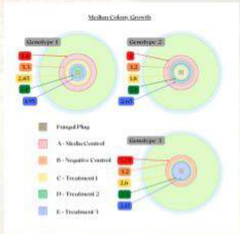
After two weeks we collected overfirst set of data, we gathered the colony diameter of every plate. To keep this consistent this was done by placing a 1mm grid over the petri dish which was placed on top of a light box, this allowed us to acuratly measure the conlony diameter. A picture was then taken and the result was added to the filing system of data we created.

On our 5th week of data collection we noticed some fungal growth (which was not *H.fraxineus)* on the plates which could have effected our experiment. These plates were discarded bringing our original population of 180 down to 150. We repeated the result collection every two weeks for seven weeks and in the end we used our week seven data to showcase our results. As it was the most accurate and reliable, as we had observed the results becoming more consistent with fewer outliers as the weeks went on.

Our statistics were conducted using the percent growth inhibition . This is the percentage at which the growth was inhibited when compared to the media control. The control for our experiment then became the media control compared to the negative control (just the leaf extract and fungus) and our treatments 1, 2, and 3 also being compared separately to the control. Our sample size decreased to 120 due to the comparison with the media control.

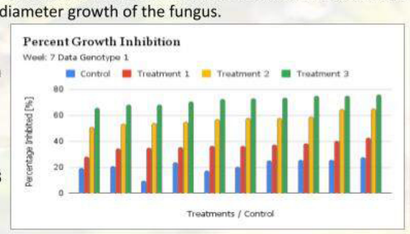
Once our results were applied to the percent growth inhibition we moved on to statistical analysis of our data.

Results and Analysis

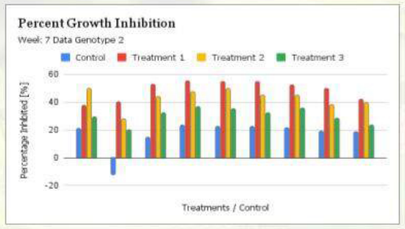


We graphed the median colony diameter of each treatment and control group within the genotypes separately for our week 7 data. These show visually that for genotype 1, treatment 2 worked best and for genotype 3, treatment 2 and 3 were the most effective. It also shows that for genotype 2 (which is the tree that was only moderately affected by ash dieback) treatment 1 was the most effective. Due to our data being non-normally distributed (as the result for the Shapiro wilk test show that at an alpha level of 0.05 there is a significance of <0.000001 which shows our data is severely skewed.), we used the Kruskal Wallis H-Test for further analysis. If we found statistical significance in a test we could then complete a Mann Whitney U test for each pair within the samples. Each week, seven Kruskal Wallis' were carried out and four were compared across each treatment. This indicated the effect the genotype had on how well the treatment performed. The other three tests were completed across each of the genotypes, indicating (for each genotype) which treatment had the most effect on the colony diameter growth of the fungus.

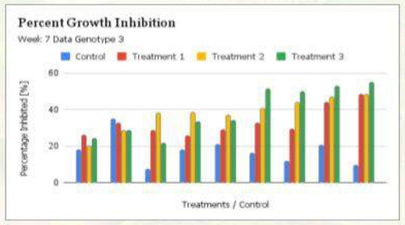
<u>Genotype 1 Analysis</u>
After comparing our data from genotype 1 for week 7 we obtained a statistically significant result from our Kruskal Wallis with a p-value of <0.00001. When comparing our treatments to the control, we found each were significant, meaning that each treatment inhibited growth of *H.fraxineus* significantly when compared with the control. When comparing treatments we found that both treatments 2 and 3 worked significantly better than treatment 1 but there was no significance between them.



<u>Genotype 2 Analysis</u>
Analysis of genotype 2 data for week 7 showed that there was a significant difference when comparing all three pairings to the control with p-values of 0.00018, 0.000132, and 0.00736. Therefore, all treatments significantly inhibited the growth of *H.fraxineus.* We found a significant difference when comparing treatment 1 and 3 (p-value of 0.00024), and treatment 2 and 3 (p-value of 0.00114). Therefore we can state that treatment 1 and treatment 2 worked best for genotype 2 with no significance between them.



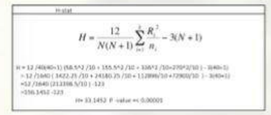
<u>Genotype 3 Analysis</u>
Analysis of genotype 3 data for week 7 showed that there was a significant difference when comparing each of the treatments to the control. Therefore all treatments significantly inhibited the growth of *H.fraxineus* with the p-values of 0.00132, 0.00058 and 0.00078. We found no statistical significance when comparing treatments to each other, therefore although the treatments inhibited growth they all inhibited it to the same degree.



<u>Analysis of Treatments and Controls</u>
When comparing data across treatments/control groups we found that there was no significance between the control groups. When comparing the effectiveness of treatment 1 we found there to be a significant difference between genotypes 1 and 2 as well as genotypes 2 and 3, with the p values of 0.00331 and 0.00512 respectively. We can therefore state that treatment 1 worked significantly better on genotype 2. When comparing the effectiveness of treatment 2 we found there to be a significant difference between genotypes 1 and 2 as well as genotypes 1 and 3, with the p values of 0.00018 and 0.00906 respectively. From this analysis we can state that treatment 2 worked significantly better on genotype 1 than it did on the other genotypes. When comparing the effectiveness of treatment 3 we found a significant difference when comparing genotype 1 and 2, as well as genotype 1 and 3 with p-values of 0.00018 and 0.00906 respectively. From this analysis we can state that treatment 3 worked significantly better on genotype 1 than it did on the other two genotypes.

<u>Example of analysis across Genotype for week 7</u>

Genotype 1 comparison



Control	58.5	Treatment 1	155.5	Treatment 2	336	Treatment 3	270
19.23077	3	25	7.5	53.84615	10	42.30769	10
20.40816	5	37.2544	17	64.70588	29	50.98039	21
9.0909	1	34.69388	13	65.30612	31	53.06122	22
23.40425	6	36.17021	15	68.08511	32.5	57.44681	26.5
17.0731	2	38.29787	18	68.08511	32.5	57.44681	26.5
20	4	36.36364	16	70.45455	34	56.81818	25
25	7.5	34.14634	12	73.17073	37	58.53658	28
25.53191	9.5	35	14	75	38.5	65	30
25.53191	9.5	40	19	75	38.5	72.5	35
27.27273	11	54.54545	24	75.75758	40	72.72727	36

The H-stat we received for the Kruskal Wallis comparing the genotype 1 values for control / treatment groups is 33.1452. The chi square critical it must fall above for our test to make it lower than the alpha value of 0.05 is 7.8147, therefore we obtained a statistically significant result with a p-value of <0.00001. We then compared the pairs individually using a Mann- Whitney U Test to find out if the treatments had a statistical difference in regard to percent growth when compared to the control, and if so which treatment performed best.

P- value = 0.00005

control	R1	Treatment 1	R2
9.0909	1	25	7.5
17.0731	2	34.14634	12
19.23077	3	34.69388	13
20	4	35	14
20.40816	5	36.17021	15
23.40425	6	36.36364	16
25	7.5	37.2544	17
25.53191	9.5	38.29787	18
25.53191	9.5	40	19
27.27273	11	54.54545	20

control

$U_1 = n_1 \times n_2 + \frac{1}{2} n_1 (n_1 + 1) - 2R_1$
$U_1 = 10 \times 10 + \frac{1}{2} 10(10+1) - 58.5$
$U_1 = 96.5$

Treatment 1

$U_2 = n_1 \times n_2 + \frac{1}{2} n_2 (n_2 + 1) - 2R_2$
$U_2 = 10 \times 10 + \frac{1}{2} 10(10+1) - 151.5$
$U_2 = 3.5$

P- value = 0.00018

Control	R1	Treatment 2	R2
9.0909	1	53.84615	11
17.0731	2	64.70588	12
19.23077	3	65.30612	13
20	4	68.08511	14.5
20.40816	5	68.08511	14.5
23.40425	6	70.45455	16
25	7	73.17073	17
25.53191	8.5	75	18.5
25.53191	8.5	75	18.5
27.27273	10	75.75758	20

Control

$U_1 = n_1 \times n_2 + \frac{1}{2} n_1 (n_1 + 1) - 2R_1$
$U_1 = 10 \times 10 + \frac{1}{2} 10(10+1) - 55$
$U_1 = 100$

Treatment 2

$U_2 = n_1 \times n_2 + \frac{1}{2} n_2 (n_2 + 1) - 2R_2$
$U_2 = 10 \times 10 + \frac{1}{2} 10(10+1) - 155$
$U_2 = 0$

After completing the Mann Whitney U tests for genotype 1 which compared the control to the three treatments, there was a significant statistical difference when comparing all three pairings. With p-values of 0.00018, 0.00018 and 0.00005 respectively, all comparisons fall under the new alpha level added after Bonferroni correction of 0.0125. After analysing these results, we can state that all three treatments had a significant effect in regards to inhibiting the growth of the fungus when compared to the control.

P- value = 0.00018

Control	R1	Treatment 3	R2
9.0909	1	42.30769	11
17.0731	2	50.98039	12
19.23077	3	53.06122	13
20	4	56.81818	14
20.40816	5	57.44681	15.5
23.40425	6	57.44681	15.5
25	7	58.53658	17
25.53191	8.5	65	18
25.53191	8.5	72.5	19
27.27273	10	72.72727	20

Control

$U_1 = n_1 \times n_2 + \frac{1}{2} n_1 (n_1 + 1) - 2R_1$
$U_1 = 10 \times 10 + \frac{1}{2} 10(10+1) - 55$
$U_1 = 100$

Treatment 3

$U_2 = n_1 \times n_2 + \frac{1}{2} n_2 (n_2 + 1) - 2R_2$
$U_2 = 10 \times 10 + \frac{1}{2} 10(10+1) - 155$
$U_2 = 0$

P- value = 0.00024

Treatment 1	R1	Treatment 2	R2
25	1	53.84615	10
34.14634	2	64.70588	12
34.69388	3	65.30612	13
35	4	68.08511	14.5
36.17021	5	68.08511	14.5
36.36364	6	70.45455	16
37.2544	7	73.17073	17
38.29787	8	75	18.5
40	9	75	18.5
54.54545	11	75.75758	20

Treatment 1

$U_1 = n_1 \times n_2 + \frac{1}{2} n_1 (n_1 + 1) - 2R_1$
$U_1 = 10 \times 10 + \frac{1}{2} 10(10+1) - 56$
$U_1 = 99$

treatment 2

$U_2 = n_1 \times n_2 + \frac{1}{2} n_2 (n_2 + 1) - 2R_2$
$U_2 = 10 \times 10 + \frac{1}{2} 10(10+1) - 154$
$U_2 = 1$

P- value = 0.00044

Treatment 2	R1	Treatment 3	R2
53.84615	4	42.30769	1
64.70588	9	50.98039	2
65.30612	11	53.06122	3
68.08511	12.5	56.81818	5
68.08511	12.5	57.44681	6.5
70.45455	14	57.44681	6.5
73.17073	17	58.53658	8
75	18.5	65	10
75	18.5	72.5	15
75.75758	20	72.72727	16

Treatment 2

$U_1 = n_1 \times n_2 + \frac{1}{2} n_1 (n_1 + 1) - 2R_1$
$U_1 = 10 \times 10 + \frac{1}{2} 10(10+1) - 137$
$U_1 = 18$

Treatment 3

$U_2 = n_1 \times n_2 + \frac{1}{2} n_2 (n_2 + 1) - 2R_2$
$U_2 = 10 \times 10 + \frac{1}{2} 10(10+1) - 73$
$U_2 = 82$

P- value = 0.00044

Treatment 1	R1	Treatment 3	R2
25	1	42.30769	10
34.14634	2	50.98039	11
34.69388	3	53.06122	12
35	4	56.81818	14
36.17021	5	57.44681	15.5
36.36364	6	57.44681	15.5
37.2544	7	58.53658	17
38.29787	8	65	18
40	9	72.5	19
54.54545	13	72.72727	20

Treatment 1

$U_1 = n_1 \times n_2 + \frac{1}{2} n_1 (n_1 + 1) - 2R_1$
$U_1 = 10 \times 10 + \frac{1}{2} 10(10+1) - 58$
$U_1 = 97$

Treatment 3

$U_2 = n_1 \times n_2 + \frac{1}{2} n_2 (n_2 + 1) - 2R_2$
$U_2 = 10 \times 10 + \frac{1}{2} 10(10+1) - 152$
$U_2 = 3$

After completing the Mann Whitney U tests for genotype 1 which compared the effectiveness of the treatments to each other, we found there was a significant statistical difference when comparing treatment 1 to treatment 2 and 3. With p-values of 0.00024 and 0.00044 respectively, both comparisons fall under the new alpha level added after Bonferroni correction of 0.0125. After analysing these results, we can state that treatment 1 performed the worst out of the three treatments and that there was no statistical difference to the effectiveness of treatment 2 and 3.

Conclusion

After the analysis of Week 7 results, we found that when we compared the effect of the treatments against each other for genotype 1, treatment 2 and 3 worked significantly better than treatment 1. For genotype 2 we found that treatments 1 and 2 worked significantly better than treatment 3 and we found for genotype 3, there was no significance amongst the percentage at which the treatments could inhibit the growth of H.fraxineus. Treatment 1 performed best on genotype 2 (the tree which was moderately affected by the disease). Treatment 2 performed best on genotype 1 (the tree which was most affected by the disease) while treatment 3 also performed best on genotype 1. Based on the analysis of our data we would recommend the use of treatment 2, JA:SA, at 1:2 of a lower concentration. This treatment had a significant effect on inhibiting the growth of H.fraxineus, regardless of how affected the tree was initially, while also supporting the ash tree population from further decline by inhibiting the most growth on trees which were most severely affected by the disease. Although we can state from the results of our statistical analysis that treatment 2 was the most effective treatment at reducing the growth of H. fraxineus, we can conclude that this is a pilot study on reducing the growth of H. fraxineus. There is still a lot more research to be carried out on the reduction and prevention of Ash Dieback over a much longer period of time. To continue our research we would like to investigate if we would achieve the same results if the experiment were to be carried out using trees themselves over a period of months and years as opposed to weeks with a higher sample size to ensure that the result is consistent. With this approach we would be able to examine any physical changes that may develop in the trees over a longer period of time. We would also be able to investigate if the JA and SA solution negatively impacted the tree using this method. In the study on the effects of these two hormones on Poplar trees it was found that the solutions did not negatively affect the growth of the tree. However, this study did not examine trees at different stages of infection of the disease. In the future, cheaper and more sustainable ways of sourcing Jasmonic acid need to be established initially for continued research purposes, but as well for commercial use if this becomes a successful treatment for Ash Dieback.