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THE EFFECTS OF PHOSPHOROUS AND SELENIUM TREATMENTS ON ARSENIC UPTAKE AND PLANT GROWTH IN RICE (*Oryza sativa* L.)

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Abstract

Phosphorus (P) and Selenium (Se) supplementation to rice plants grown in Arsenic (As) contaminated conditions as be found by many studies to reduce As uptake and benefit growth in such conditions, however there are some inconsistencies as to how effective these treatments are. This study investigates the effect of 0, 0.5, 1, 1.5 and double the recommended concentration of P and Se on the growth of rice seedlings both with and without the presence of As over a maximum 20 day period. Analysis of the growth data collected indicated that there is no significant difference in the leaf, maximum and minimum root lengths, leaf and root numbers or the As content of the plant material. This study finds that different concentrations of P and Se do not affect growth at early stages and do not affect As uptake.

Keywords: *phosphorus, selenium, arsenic, contaminated, plant material.*

INTRODUCTION

Rice (*Oryza sativa L.*) is a staple food source in many countries (Lee, *et al*., 2015) providing 35-60% of total calorie intake in Asian countries (Liang, *et al*., 2016) where 90% of rice is produced (FAOSTAT, 2017). Rice requires a large water input with its average water productivity being half that of wheat, at 0.4kg grain m^{-3} in Asia (Bouman, *et al*., 2007). Borin, *et al* (2016) states that the amount of water utilised by the rice plant in a traditional flood irrigation system is between 8000 and $10000m^3$ ha^{-1} in Brazil, which given the average yield of 5.2 tonnes (FAOSTAT, 2017) gives a similar water productivity to that of Asia.

The Production of rice in Asia uses around 80% of the total fresh water resources (Yang, *et al*., 2016), but this resource is becoming increasingly scarce due to the demand from industries, urban areas and through heavy metal contamination (Liang, *et* al., 2016). One such heavy metal is arsenic (As), which is a greater problem in rice than other cereal crops as a greater volume of water is used. The flooded conditions also provide lower redox potentials, enabling arsenic to be more bioavailable (Bouman, *et al*., 2007). The World Health Organisation (2016) state that arsenic is one of their 10 chemicals of major public health concern, and have a recommended limit of 10µg/L in drinking water. Arsenic contaminated ground water affects many areas in which rice is grown (Dixit, 2016; Srivastava, *et al.*, 2015) and is believed to be one of the major sources of entry into the human body (Lee, *et al.*, 2015).

In these areas arsenic contamination can exceed 1000µg/L (Srivastava, *et al.*, 2015), with Dixit, *et al* (2016) stating that in some areas in south east Asia As levels in drinking water are as high as 3200µg/L.

Reasoning for study. As contamination of agricultural land, more specifically which used for rice, is a major issue for food security in less economically developed Asian countries. It reduces yields, plant health and is harmful to humans. P and Se applications are potential treatments for this, but not all research has indicated that this is true. There is also the issue that the research done has paid little attention to the effect of the treatments on the plant health, which has been the main focus of this research.

The Null Hypothesis for this research is that there is no difference in the arsenic content, germination rate or growth of the plant, between the different concentrations of the treatments, between the treatments and their controls, or between each treatment

MATERIALS AND METHOD

Phosphorus. It has been the assumption for many years that phosphorus (P) can be used to inhibit uptake of As, as it shares the same uptake channel as phosphate. This is however only true for the inorganic species As(V) which is the least prevalent of the two inorganic species in rice paddies. This is due to anaerobic conditions which are more suitable to As(III), whose uptake channel is the same as silicon (Dixit, *et al.,* 2016). Therefore, it should be assumed that increasing the P concentration in the soil would have little effect on the arsenic uptake by the plant, as it is not affecting the major species, this however may or may not be the case.

Studies have ended with opposing views as to the effects of P treatments. In an experiment conducted by Lee, *et al* (2015) concerning the effects of P application on As toxicity in rice, concluded that there was the possibility that the addition of phosphate increased the concentration of As in the soil water, but this depended greatly on soil properties. Lee, *et al* (2015) also concluded that competitive uptake of As and P by rice seedlings did not occur even at high P concentrations, nor did it decrease the bioavailability of As to the rice. Despite the conclusion of the experiment Lee, *et al* (2015) also stated that the assumption that P application reduces As uptake has been supported by several studies. For example, Lu, *et al* (2010) found that there was a correlation between the molar ratio of P/As and uptake. Talukder, *et al* (2012) found that competitive uptake of P and As occurs though this was under aerobic conditions. An experiment by Bolan, *et al* (2013) supports the conclusion of Lee *et al* (2015) that the addition of phosphorus increases the concentration of As in the soil water, but then states that this increases its bioavailability, contradicting the findings of Lee, *et al* (2015).

Selenium. As and selenium (Se) have similar chemical properties but the interactions between them depend on their chemical forms (Pandey and Gupta, 2015; Kumar, *et al.*, 2013). Se is an essential element to animals and acts as a cofactor for several enzymes (Kumar, *et al.,* 2013), the presence of Se also supports the expression of selenoprotein, which acts as an antioxidant, and is supported by reports that the addition of Se reduces the oxidative damage caused by As (Pandey and Gupta, 2015).

The studies into the effects of selenium on arsenic accumulation in plants are also similar to those of phosphorus, in that the conclusions drawn from the experiments where varied and had opposing outcomes. Feng, *et al* (2013) compared the results of different papers focusing on the effects of selenium on heavy metal toxicity in plants. Although similar quantities of As and Se were used on different plants, the effects of the Se were not consistent with each experiment. Two different species of Se were used (Se IV and Se VI) but there was no connection between the Se species and the effect exerted on arsenic (Feng, *et al.,* 2013).

Malik *et al* (2012) and some of the papers highlighted by Feng, *et al* (2013) indicate that low levels of Se may be beneficial to the plants growth, and also promote resistance of some abiotic stresses such as drought and other metal toxicity (Kumar, *et al.*, 2013). However, Feng, *et al* (2013) stated that in a previous experiment involving rice, they found that concentrations of 0.8 mg L^{-1} of Se were toxic to the plant.

Preliminary Trials. A preliminary trial was carried out to determine the germination rate of the rice seed. In this preliminary trial 50 rice seeds were placed in filter paper in a petri dish and water added to dampen, this was replicated 4 times. The petri dishes were placed in a germination chamber with environmental conditions of 20^{o}C and 16 hours of light. After 3 days the seeds were removed from the chamber and germinated seeds counted. Seeds were counted as germinated if they had a stem/root which was greater than 2mm. Some seeds had started to grow mould, so it was decided that the seeds for the experimental trials would be sterilised prior to germination. This trial was also carried out using each treatment solution in place of the water to see if there was any difference in the germination rate.

Trial 1 – Envelopes

Rice seeds were sterilised in 10% bleach for 5 minutes to help prevent mould growth. The Seeds were then germinated in water for 5 days in environmental conditions of 20^{o}c and 16 hours of daylight. After this period, seedlings of equal length were transferred into envelopes for growth, as can be seen in Figure 1.

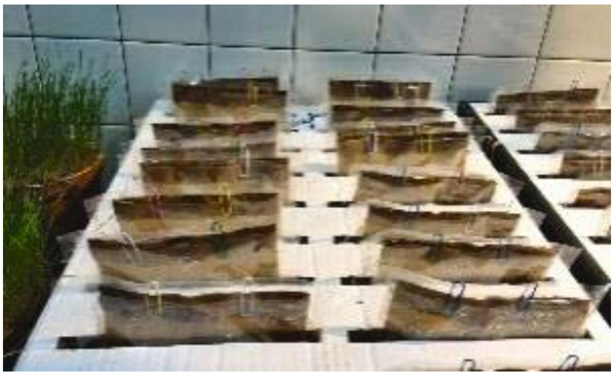

Figure 1. Envelopes containing rice seedlings

The envelopes were chosen as a growth medium as they contained no nutrients which could be utilised by the plant and interfere with the results, but also to ensure that the seeds were never submerged in the solutions. Three seeds were placed in each envelope and each treatment was replicated 4 times, totalling 12 seeds per treatment. The seeds were separated with 50mm intervals, as recommended for mature plants. The placement of the envelopes was decided through use of a random number generator. Each envelope was given 50 ml of full strength Kimura B solution, Composition - 0.36 mM (NH4)2SO4, 0.36 mM KNO3, 0.54 mM MgSO4·7H2O, 366 µM Ca(NO3)2·4H20, 25.1 µM H3BO3, 2.01 µM MnSO4·4H2O, 2.02 µM ZnSO4·7H2O, 1.19 µM CuSO4·5H2O and 0.49 µM MoO3 (Syu, *et al.,* 2017). 30.6 µM ferric sodium was used instead of Fe-citrate due to issues with dilution. Previous studies have used a half strength solution for the first week or two and then increased it to full strength, but as different concentrations of treatments were being tested in this study, it was decided that the use of the full-strength solution throughout the study would be more appropriate than switching after a period of time, as the change in nutrient balances may have affected the plants.

The solution excluding phosphorus was one of the treatments being tested, added in the form of 0.18 mM $NaH_2PO_4{\cdot}2H_2O$ (Syu, *et al.,* 2017), changing the volume added to the solutions. Se was added in the form of 7.7 µM Na_2SeO_3 (Feng, *et al.,* 2013). The treatment groups contained 0, 0.5, 1, 1.5 and double the recommended values for growth of phosphorus and selenium. In the P test groups, no Se was not included as it is not applied as in a field situation. In the Se test groups a concentration of P1 was included as P is essential to growth. The treatments were tested with and without the presence of arsenic, at 1mg/L as these levels can be present in the natural environment (Srivastava, *et al.*, 2015). The treatment groups were tested without the presence of arsenic to determine if the concentration of arsenic influenced seedling growth and if the treatments would alleviate its effect, or if any effects observed were solely due to the concentration of the treatment.

The envelopes were placed under a multispectral light and kept at a constant temperature of 19 ^{o}C. The rice was left to grow, but after a period of 15 days it became apparent that there would be insufficient growth to measure the arsenic level in the plant material. At this point the root and leaf numbers were recorded and leaf length, maximum and minimum root length were measured. The liquid in the envelopes was collected for analysis and any excess was disposed of. The arsenic content of the water was analysed using a Thermo scientific ICE 3000 series AA spectrometer.

Trial 2 – Perlite

A secondary test was performed using only the P1, Se0.5, Se1 and Se1.5 treatment groups and their As contaminated equivalents and was carried out using perlite as the growth medium, as can be seen in Figure 2.

Figure 2. Rice Seedlings grown in perlite

The seeds were sterilised in 10% bleach for 6 minutes after observing mould in the first trial.

The seeds were germinated in the same conditions as the first trail but only left for 3 days, as a sufficient number of seeds had germinated. In this trail the separation between seeds was reduced from 50mm to 20 mm as the growth during the first trial period was insufficient to require the space. A total of 32 pots were used, 4 repeats for each test group, with 10 seedlings per repeat. The treatment solutions were added to the pot, each given 80ml of solution which half-filled the pots. The seeds were planted about 1cm below the surface of the perlite, so they were never submerged in the solution. The pots were placed under the same environmental conditions as the envelopes, and after 7 days growth was sufficient to begin measurements. The leaf length above the surface of the perlite was measured every 3 to 4 days after this point, as measurements were taken solution was also added to keep the pots half full of liquid. After 17 days the seedlings were removed from the perlite, root and leaf numbers were recorded and root maximum length, root minimum length and leaf length were measured. The leaves were separated from the seed and root system so that As content could be analysed in both the root and the leaves, to determine the transportation and storage of As in the plant at early growth stages. Due to lack of total plant material, the replicates of each treatment were put together to be analysed.

Arsenic Analysis of Plant Material

The samples were dried for 4 hours at 100^{o}c and the dry weights of the leaves and roots measured. The dry material was then digested in an acid solution – containing 5 parts 70% perchloric acid and 2 parts 70% nitric acid. The material was left to digest overnight and then heated until all the solution had evaporated. 10 ml of HCl was then added and brought to a simmer for 5 minutes. The remaining solution was then cooled, filtered and made up to 50ml with distilled water. This solution was then analysed using a Thermo scientific ICE 3000 series AA spectrometer.

RESULTS AND DISCUSSIONS

Germination and Establishment.

The preliminary trial in which rice seeds were germinated in water, found that the germination rate was 72.5%, this was compared to the preliminary test solutions using a chi squared test which found that there was no significant difference in the germination rate between the treatment solutions or the control groups (P=0.986).

The establishment rates of the experimental trials were analysed using a chi-squared goodness of fit test, which found that in both trials, none of the treatments had a significantly different establishment rate when compared to the expected value. Trial 1 used envelopes to grow the seedlings and contained treatment solutions of P or Se at 0, 0.5, 1, 1.5 or double the recommended value and were grown both with and without As. The expected establishment for trial 1 was 6.3 seedlings of 12 and the P-Value was 0.980. The second trial which used perlite, testing solutions of P1, Se0.5, Se1 and Se1.5, with and without As, had an expected establishment of 20.25 seedling out of 40 and a P-Value of 0.540.

Trial 1 – Envelopes

Normality tests were conducted on the data collected from the first trial and all data was found to be significant ($P<0.05$) indicating that the data did not follow a normal distribution. All data sets were then analysed using a Kruskal-Wallis Test, the null hypothesis being that there is no difference between the treatment groups. The P-Values calculated through the Kruskal-Wallis test indicate that there is no significant difference in the growth of the leaves and the roots of the rice plant in the presence of arsenic and in the presence of different treatment concentrations, which can be seen in Figures 3-7. Regardless of the measurements taken, for example stem length, the range of the values for each treatment group overlap with most other groups in that data set. For root min length and stem length the median values for the treatment groups are all present in a 1 cm range (either side of 1.0cm for min root length and around 3.0cm for stem length). In comparison, the medians for root number and max root length are more spread across the range of data.

The Arsenic content of the water (also analysed with a Kruskal-Wallis test) found that there was no significant difference in the arsenic levels in the water between different treatments (see Figures 8 and 9). However, the difference in arsenic content in the As contaminated groups was very close to being significant, with a P-Value of 0.054. The treatment group P0 has a visibly lower arsenic content (see figure 9) than the other treatment groups with no overlapping data.

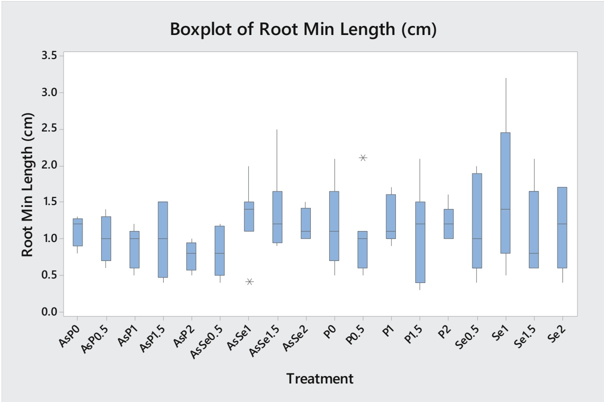

Figure 3. Boxplot of minimum root length showing the variation of values and the overlapping of data

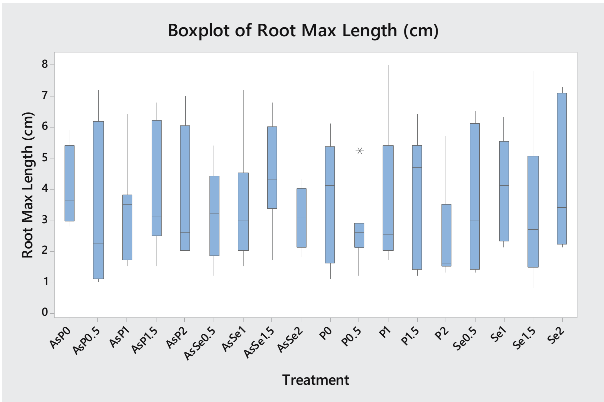

Figure 4. Boxplot of maximum root length. Overlapping data indicates that there is little difference between the treatment groups

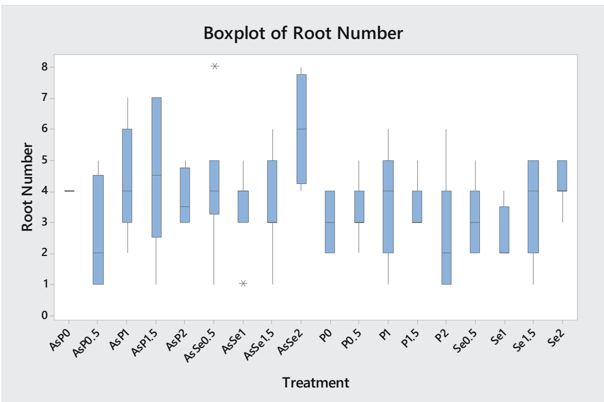

Figure 5. Boxplot of root number. Overlapping values indicate that there is no significant difference between the treatment groups

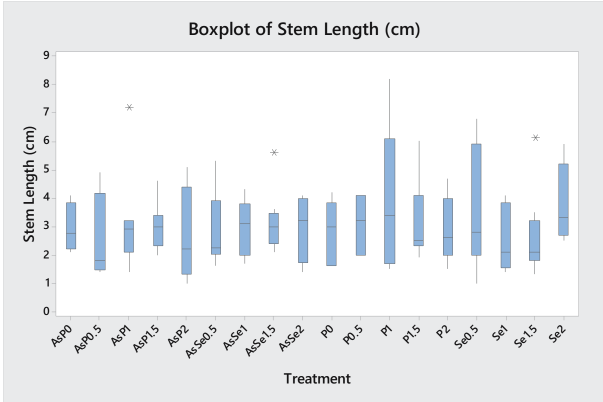

Figure 6. Boxplot of stem length. Values overlap between treatments, indicating no significant difference between them

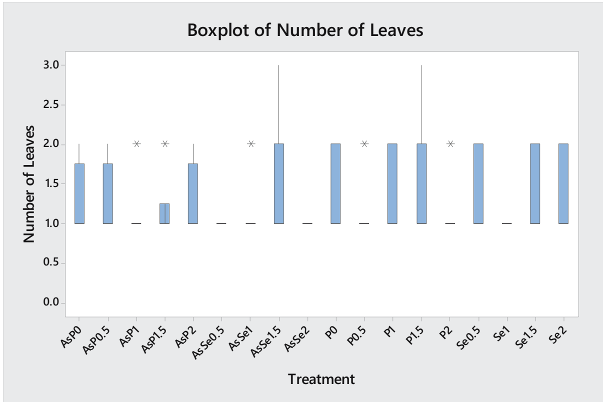

Figure 7. Boxplot of number of leaves. Little variation in the data so no significant difference between treatment groups

Figure 8. Boxplot of the As reading for the non-As contaminated groups. All median values range between 0.4 and 0.6 mg/l As with no visible trend. The boxes overlap indicating no significant difference in the values between the treatments

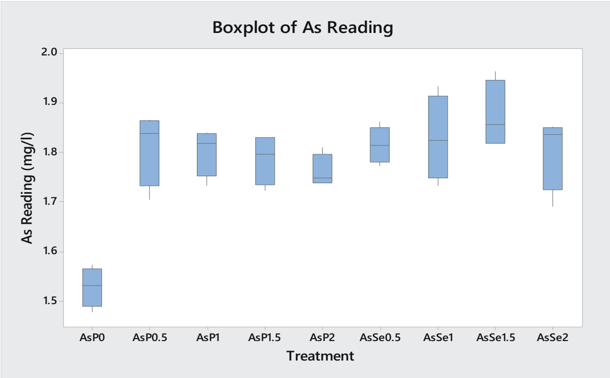

Figure 9. Boxplot of As reading for the As contaminated groups. The median values for all treatment groups except AsP0 lie around 1.8 mg/l. The range of values for these treatments overlap, indicating no significant difference.

Trial 2 – Perlite

Normality tests were conducted on the data collected from the second trial and all of the data was found to be significant ($P<0.05$) indicating that the data did not follow a normal distribution. All data sets were analysed using a Kruskal-Wallis test, with all P-Values being >0.05 indicating no significant difference in the growth of the seedlings between the treatment groups or in the presence of increased arsenic. Figures 10 to 13 show boxplots of the leaf growth in each treatment growth over a period of 10 days. In all figures the median values are relatively close together, though the difference between the smallest and largest median grows over time. In Figure 10 for example the greatest difference between the medians is about 0.75 cm where as in figure 13 the difference is 1.9 cm. However, in all figures the boxes overlap indicating that there is no significant difference in the leaf growth between treatments.

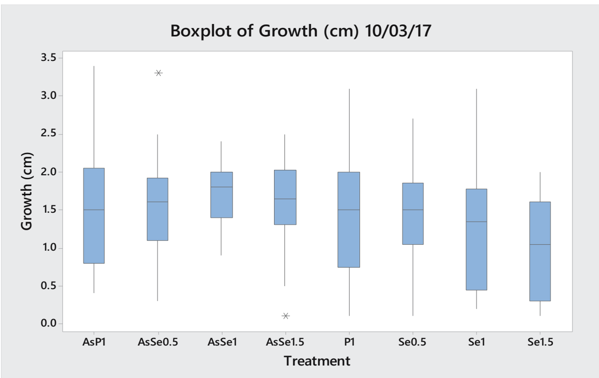

Figure 10. Boxplot of leaf growth above the perlite surface on 10/03/17. All medians are around 1.5 cm growth and all boxes overlap, indicating no significant difference in growth between the treatments

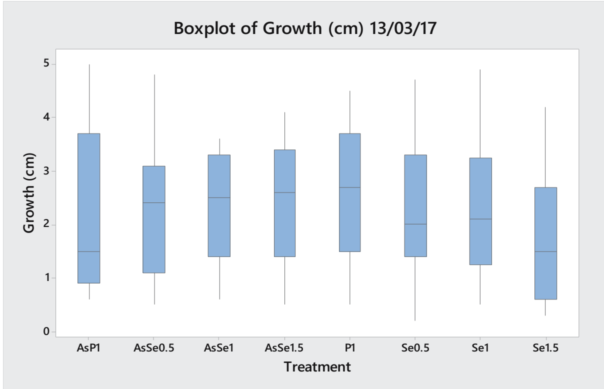

Figure 11. Boxplot of leaf growth above the perlite surface on 13/03/17. Medians more sped out than previous measurements, median values centred around 2.0 cm and all boxes overlap indicating no significant difference in growth between treatments

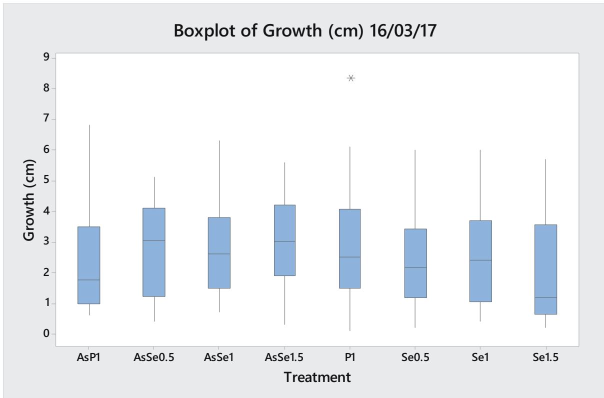

Figure 12. Boxplot of leaf growth above the perlite surface on 16/03/17. Medians are all similar and lie around 2.5cm growth, the boxes overlap indicating no significant difference in growth between the treatments

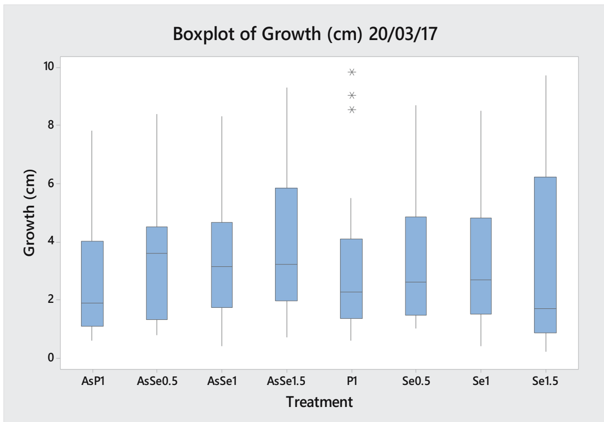

Figure 13. Boxplot of leaf growth above the perlite surface on 20/03/17. The medians are similar to those of the previous data set and lie around 2.5 cm growth, all boxes overlap indicating no significant growth between treatments

Figures 14 and 15 show the change in median growth of each treatment over the growth period. In groups with additional arsenic (Figure 14), the addition of Se (at all test values) had a more beneficial effect on the median growth than the P1 group without the additional Se. The measurements at 20/03/17 indicate a difference in the medians of 1.25 cm between the AsP1 and the selenium treatment groups. Though this may indicate a trend in the data, there was no significant difference in the leaf lengths at this stage in growth. In the test groups which were not given additional arsenic (Figure 15), the Se1.5 group had the lowest median growth of the test groups, though the difference is less pronounced compared the As groups, only having a difference in medians of 0.55 cm at 20/03/17. The greatest difference was observed at 16/03/17 at 0.95 cm.

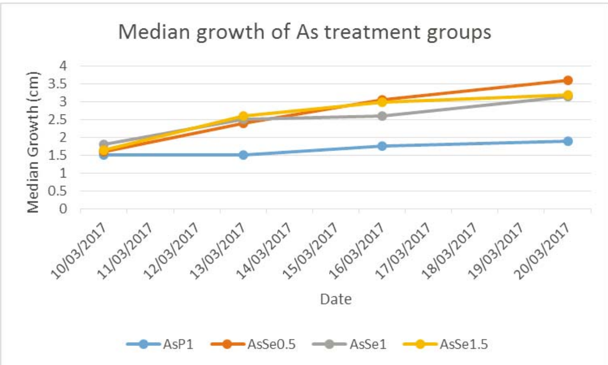

Figure 14. The median growth of As treatment groups over a 10 day period

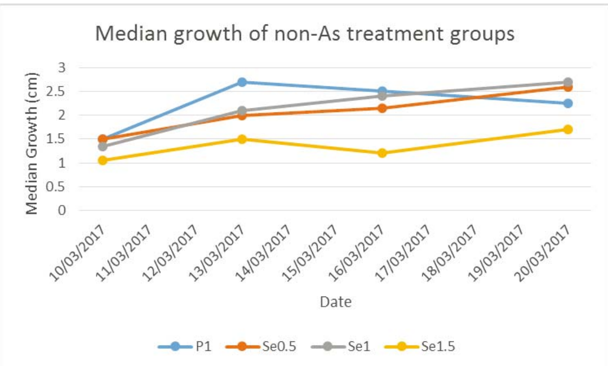

Figure 15. The median growth of non-As treatment groups over a 10 day period

Final Growth and As Analysis

The normality test performed on the final growth data concluded that none of the data followed a normal distribution. The Kruskal-Wallis tests indicated that there was no significant difference in the maximum root length, minimum root length, leaf length, root number or leaf number between the different treatment groups.

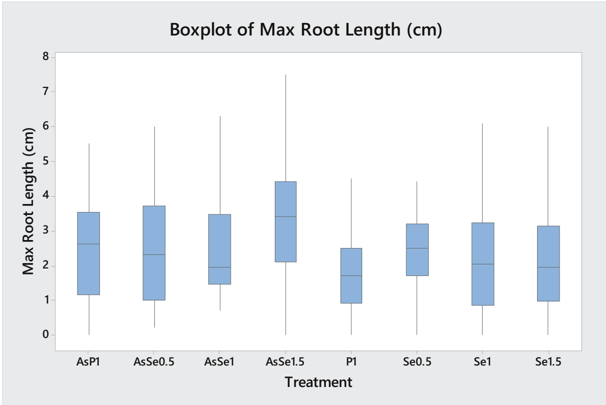

Figure 16. Boxplot of maximum root length for each treatment. The range of each treatments values overlap, indicating no significant difference between the maximum root length and the treatment group. The is no visible trend

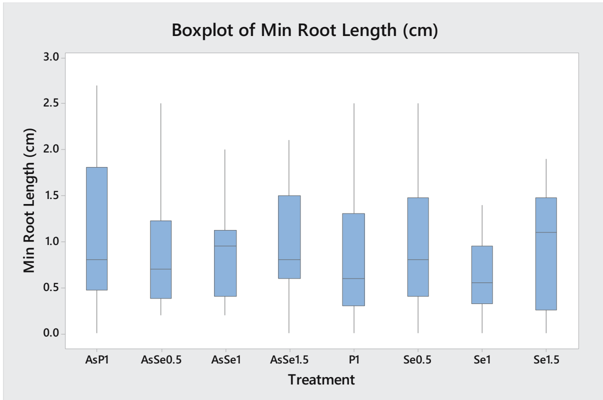

Figure 17. Boxplot of minimum root lengths for each treatment. The range of values for each treatment overlap indicating that there is no significant difference in the minimum root length between the treatment groups. There is no visible trend

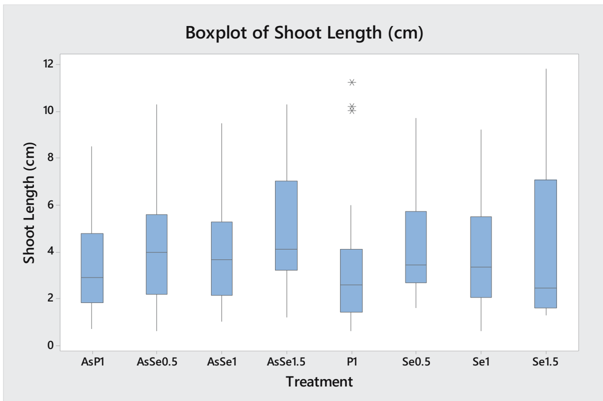

Figure 18. Boxplot of the shoot/leaf length of each treatment. The range of values for each treatment overlap, indicating that there is no significant difference in the leaf length between different test groups. There is no visible trend

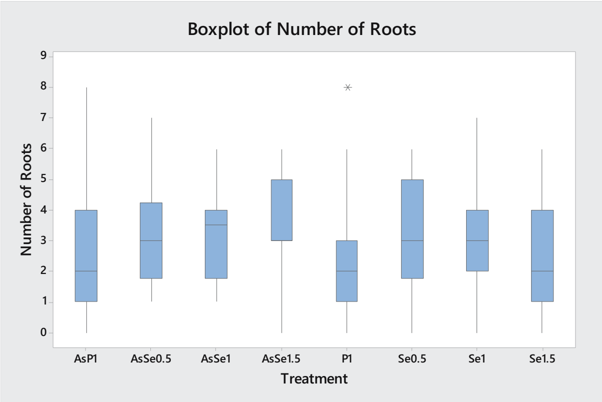

Figure 19. Boxplot of the number of roots the seedlings had in each test group. The boxes of AsSe1.5 and P1 do not overlap but their range of values do, there is therefore no significant difference in the root numbers observed in each treatment group

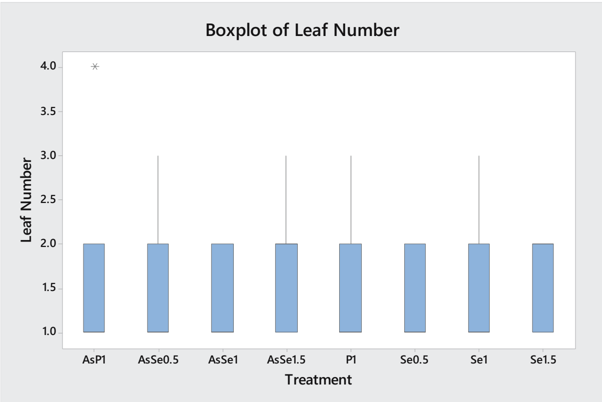

Figure 20. Boxplot of leaf numbers observed on seedling of each treatment group. The boxes overlap with very little variation, indicating that there is no significant difference in the leaf numbers observed in each test group

The relationship between the mass of plant material and the arsenic content of the material were analysed using a Pearson correlation, which indicated that there was no correlation between the root or the leaf dry mass and the arsenic content in the plant material, with P values of 0.273 and 0.924 respectively.

Figures 21 and 22 show the correlation between the dry mass of the plant material and the As content. The roots (Figure 21) may have a slight positive correlation between the dry matter and the As but the correlation between the data is not significant.

There is also no correlation between the treatment group and the As content.

There is no visible correlation between the leaf dry mass and the As content of the plant material, figure 22 shows that every group apart from AsSe1.5 had a similar arsenic concentration (between 0.025 and 0.035 mg/l) regardless of the dry mass.

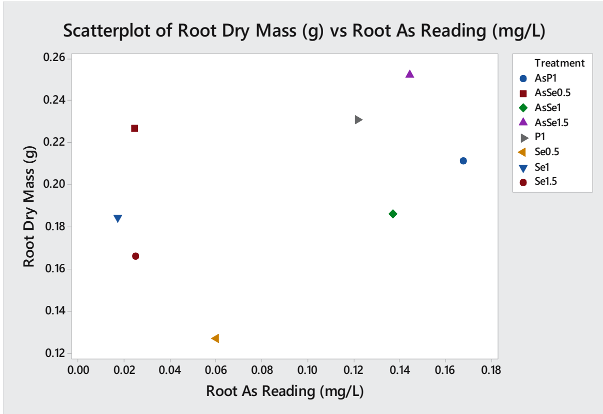

Figure 21. A scatterplot of the correlation between the root dry mass of each treatment and their respective arsenic concentration. There is no visible correlation

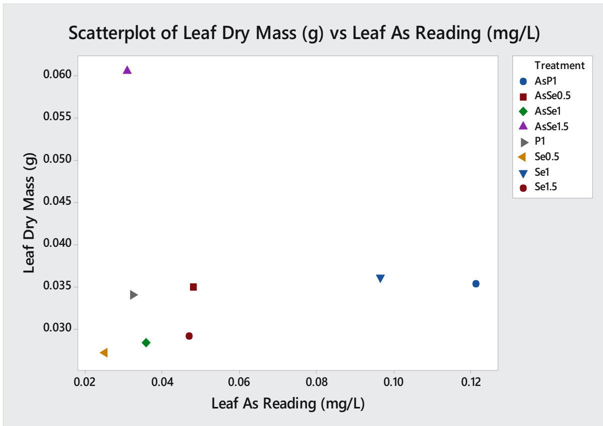

Figure 22. A scatterplot of the correlation between the leaf dry mass of each treatment and the As content. There is no visible correlation

Discussions

Establishment. The preliminary trial in which rice was germinated in water, had a germination rate of 72.5%, with there being no difference statistically between the germination rate of the treatment solutions and the controls.

In the trials, previously germinated seeds were placed in different growth mediums, the establishment rates in both mediums were just over 50%, which is greater than establishment rates which occur in a field situation, where establishment is between 20 and 40% (NSW, 2016).

This increased establishment rate is present for all test populations, indicating that this is not likely to be due to the solutions but due to the consistent environmental conditions.

Trial 1 – Envelopes. The trials which utilised envelopes as a growth medium found that there was no significant difference in the growth of the seedlings between the treatment groups.

This may be due to the fact that the trials were only carried out over a short period of time where the rice seedlings do not have a large nutrient requirement, due to low maintenance costs (as the seedlings are small) and stored nutrients in the seed being utilised.

What may have been expected, if grown over a longer period, is that treatment groups with lower P concentrations than the recommended value would be stunted compared to other treatment groups. A study carried out by Bolan, et al (2013) showed that with rice grown in a nutrient solution, increasing P had a positive impact on the dry matter of both roots and shoots (so show increased growth), whereas increasing As concentration decreases the dry matter.

The study carried out by Bolan, et al (2013) had the rice growing in solution for a longer period of 8 weeks, which may account for lack of variation in this study, as the rice seedlings were only grown in the solution for 2 weeks. Previous studies have found that Se is toxic to rice at 8mg/l (Se2) (Feng, et al., 2013) and yet the Se2 treatment groups were unaffected and had similar growth to other treatment groups. As the rice was only grown for a short period it will have required very little nutrients for growth and therefore may not have exhausted the background P in the solution. Similarly, as the root systems were small, and the trial taken place over a short period of time the plants may not have taken up and stored sufficient Se for a toxic response to occur.

The arsenic concentrations of the water samples obtained from the envelopes were not statistically significant. However, the readings for the As contaminated treatments were very close to being significant, as the treatment AsP0 had a much lower arsenic content than the other treatment groups. This may be showing that the complete lack of P enables As to be taken up by the plant more readily, several study support the fact that increasing P concentrations reduces As uptake (Lu, *et al*., 2010 and Talukder, *et al*., 2012), however no other P treatment group resulted in an As reading which even slightly varied from that of the other treatments. From this there are two possible conclusions, either at this level of As contamination, 0.08 mM of P is sufficient to produce the maximum restriction to As uptake, or that as the difference in the arsenic contents are not significant statistically, the concentration of P has no effect on the As uptake of rice.

The study carried out by Bolan, *et al* (2013) looked at the As concentration in the root and shoot material at different concentrations of As and P. The study found that increasing the P concentration reduced the As concentration in both the root and shoot material when As is present. Where no P was present root As concentration was significantly greater compared to treatments containing P. Trial 1 of this study shows a near significant value at AsP0 which would support the findings of Bolan, et al (2013) however the concentration of As in this study (13µM) is closer to the zero concentration of As in the study by Bolan, et al (2013) which showed no significant difference in the As concentration in both shoot and root at All P concentrations.

Trial 2 – Perlite. In the trial which used perlite as a growth medium, no significant difference was found in the growth of the plant (leaves and roots) between the different treatment groups. However, a potential trend can be observed in the As contaminated treatments when the growth data is taken into account over a period of time. Figure 14 shows that the treatment group AsP1 had a constantly lower median growth value compared to the other As treatments, which could indicate that Se application can increase growth under conditions of As contamination. This trend is not observed in the non-As treatment groups and is not statistically significant, so the conclusion of this study is that Se has no effect on the growth of rice at early stages.

There also appears to be no correlation between the arsenic content of the plant material and the dry mass of the material or the treatment type. However, the sensitivity of the atomic absorption spectrometer had a minimum sensitivity to arsenic of 0.4mg/l, which all the measured values are below, it is therefore likely that the data is unreliable.

In a similar study by Chauhan, *et al* (2017), who looked at the effects of different concentrations of Se (at similar levels to this study) on As toxicity (As at double the concentration in this study). They observed a significant difference in root and shoot lengths and biomass as well as reductions in As accumulation in both root and shoot material with increasing Se concentrations. In the study, the rice seedlings were grown in a Hewitt nutrient medium (which has greater concentrations of nutrients compared to the Kimura B solution) and was grown in temperatures of around 26°C for around a month. As this study is similar to that done by Chauhan, *et al* (2017), it may be an indication that given a longer time period or stronger environmental conditions, sufficient growth would have occurred for more reliable analysis of the As content of the plant material. However, it cannot be said that the trends in the data would be similar to that of Chauhan, *et al* (2017) as there is no significant trend present in this data.

CONCLUSIONS

Although a different growth medium was used between trials 1 and 2, the environmental conditions and solution concentrations remained the same. Both trials indicate that Se concentrations do not affect plant growth or As uptake in rice seedlings. The P concentrations in trial 1 were also shown to have no significant effect on the seedling growth or As uptake.

The growth period for both trials was the main limiting factor in this investigation, as the lack of seedling growth limited the plant material available for analysis and did not allow for much variation in growth. It would therefore be beneficial for future studies to increase the growth period or the number of replicates for each treatment so that reliable analysis can be performed. As none of the data collected has proven to be statistically significant, the null hypothesis is accepted, indicating that there is no difference in the arsenic content, germination rate or growth of the plant between the different treatment concentrations and between each treatment.

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