



Development of LA-ICP-MS and Associated Techniques Towards Analysis of Crop Enhancement Products

READY, Joseph David

Available from the Sheffield Hallam University Research Archive (SHURA) at:

<https://shura.shu.ac.uk/34331/>

A Sheffield Hallam University thesis

This thesis is protected by copyright which belongs to the author.

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.

Please visit <https://shura.shu.ac.uk/34331/> and <http://shura.shu.ac.uk/information.html> for further details about copyright and re-use permissions.

Development of LA-ICP-MS and Associated Techniques Towards Analysis of Crop Enhancement Products

Joseph David Ready

This thesis is submitted in partial fulfilment of the
requirements of Sheffield Hallam University for the degree
of Doctor of Philosophy

April 2024

Candidate Declaration

I hereby declare that:

1. I have not been enrolled for another award of the University, or other academic or professional organisation, whilst undertaking my degree.
2. None of the material contained in this thesis has been used in any other submission for an academic award.
3. I am aware of and understand the University's policy on plagiarism and certify that this thesis is my own work. The use of all published or other sources of material consulted have been properly and fully acknowledged.
4. The work undertaken towards the thesis has been conducted in accordance with SHU Principles of Integrity in Research and the SHU Research Ethics Policy.
5. The word count of the thesis is 47,090

Name	Joseph David Ready
Date	30/04/2024
Award	PhD
Research Institute	Industry and Innovation Research Centre
Director of Studies	Professor Neil Bricklebank
Signature	

Abstract

Wheat is the most significant crop grown globally for human consumption, contributing around 20 % of the total dietary calories and protein consumed worldwide. It is also the most widely grown crop in the world, cultivated on 217 million hectares of agricultural land. Zinc is an essential micronutrient which plays a vital role in healthy plant growth and development. Zinc is the only metal to be present in all six classes of enzymes found in plants and is a cofactor in more than 300 proteins. It has been shown that zinc is the most important micronutrient when it comes to crop yields and a deficiency of zinc has the greatest impact on yield of any micronutrient deficiency. Zinc deficiency is the most common micronutrient deficiency globally, with over 50 % of agricultural land classified as zinc deficient.

Seed applied treatments are commonplace in agriculture, with some being used as a method to deliver micronutrients to plants during the early stages of development. Seed treatment is an environmentally approach to nutrient delivery to plants due to the targeted nature of the application and the smaller amounts of active ingredients used compared to crop spraying.

Adjuvants are materials added to formulations, designed to increase the efficacy of active ingredients. This thesis outlines novel methods and results investigating the effect of adjuvant inclusion in a ZnO-containing seed coating on the uptake and effects of Zn, using winter wheat as a model crop.

Analysis methods include ICP-MS, and LA-ICP-MS, both of which are widely established in life sciences, but have never been applied to study the effects of seed-applied adjuvant on nutrient uptake. The thesis also includes supporting data using micro-CT and SEM to image seed-applied Zn for the first time.

The aim of this research was to investigate the effect of adjuvants on the uptake of zinc from seed film coatings, to determine the effect of adjuvant on zinc uptake, localisation and concentration in winter wheat, and to determine the extent to which adjuvant inclusion may negate the drawbacks of using ZnO as the Zn source for the film coatings.

Acknowledgements

“Science is magic that works” Kurt Vonnegut.

I would first like to thank my Director of Studies Professor Neil Bricklebank for his ongoing support, guidance, and wisdom throughout this project, not to mention his patience with my thesis writing around full-time work.

Thank you to Dr Catherine Duckett for her technical guidance with the analytical work, as well as always being willing to listen to me moaning about one thing and another. I wish her the best of luck in her new career.

Thank you to Professor Malcolm Clench for his analytical support as well as encouraging and enabling my conference attendance throughout the project. I wish him all the best in his well-earned retirement.

I am incredibly grateful to my industrial supervisor Dr Kathryn Knight, as well as advisors Martin Shaw, Laura Weatherhead, Laura Bishop, and Andy Fowles. Each one of them were supportive throughout, as well as providing me with opportunities to gain experience within the Croda business. Special mention to Jahmel Neblett and Rob Kempster for all their support with the growth trials included in this work.

Special thanks go to Dr Rob Hutchinson and Dr David Price for their support with the development of my skills with the Laser Ablation kit and the ICP-MS, respectively, for keeping me on track with the thesis writing and for their unwavering support with my career since leaving the lab.

I would like to thank the BMRC technical staff, especially Mick Cox for his endless support when the instruments were not playing ball. Countless days would have been lost without his help.

I would like to thank my family and friends for their unwavering support throughout my journey so far. Thanks to my housemate throughout my PhD, Arron Bird, having someone who was facing the same challenges to share the journey with was invaluable. Thank you to my partner Kara, who has been subjected to my thesis-induced moods and offered unconditional support.

Finally, I would like to thank the BBSRC and Croda for providing the funding for my PhD studies.

List of figures & tables

<u>Figure 1:</u> World map depicting locations of agricultural land which have been found to be zinc deficient.	31
<u>Figure 2:</u> Cross-section of a root, showing the structures involved in transport within the plant.	34
<u>Table 1:</u> Summary of Croda adjuvants which were included in the work undertaken throughout this thesis.	39
<u>Table 2:</u> The formulation of the flowable suspension used as the base formulation for the treatment of wheat seeds throughout the work contained within this thesis.	48
<u>Figure 3:</u> Schematic of the internal components of the PerkinElmer NexION ICP-MS to exemplify the workings of a typical ICP-MS instrument.	52
<u>Figure 4:</u> Schematic of the basic principle of a quadrupole when filtering charged ions according to mass to charge ratio (m/z).	53
<u>Figure 5:</u> Images taken from the epoxy embedding of wheat seeds for analysis by LA-ICP-MS. The hardened epoxy block is removed from the sample mould, sanded down using P400 and P800 sandpaper, before being returned to the mould for mounting into the sample chamber.	62
<u>Equation 1:</u> Calculation of best scan speed for LA-ICP-MS imaging	64
<u>Figure 6:</u> Schematic to illustrate the importance of selecting the correct scan rate for LA-ICP-MS imaging experiments.....	65

<u>Figure 7:</u> Examples of LA-ICP-MS images of gelatin embedded wheat seed generated using the UP213 laser ablation system from New Wave Research, now Elemental Scientific Lasers (Bozeman, MT, USA).	66
<u>Figure 8:</u> Examples of the interaction of the laser beam with agar sample matrix, showing how water-based matrices can negatively affect ablation, and what a good ablation of such a matrix would look like.	67
<u>Figure 9:</u> Examples of LA-ICP-MS images of epoxy embedded wheat seed sections generated using the imageBio 266 from Elemental Scientific Lasers (Bozeman, MT, USA).	68
<u>Equation 2:</u> Equation used to convert mg/L values from ICP-MS/ICP-OES analysis of digested material, to elemental content in mg/Kg.	76
Figure 10: A representation of the Zn concentration in mg/L (y-axis) of a range of solvents (x-axis), analysed after they had been used to 'wash' ZnO-treated wheat seeds carried out by ICP-MS in solution mode.	82
<u>Figure 11:</u> Zn content of ground and acid digested material from seeds treated with the ZnO formulation (reference) and the same formulation with added adjuvant (A-D) after they had been washed for 24 hours in nitric acid. As well as digested unwashed reference seed as a positive control. The analysis was carried out by ICP-OES. Each data point corresponds to one repeat, the height of the bar corresponds to the median value.	84
<u>Figure 12:</u> Zn content of ground and acid digested material from seeds treated with the ZnO formulation (reference) and the same formulation with added adjuvant (A-D) after they had been washed for 24 hours in nitric acid. The	

analysis was carried out by ICP-OES. Each data point corresponds to one repeat, the height of the bar corresponds to the median value. 85

Figure 13: A plot to show the median Zn concentration in acid-washed samples of each of the seed treatments, at each of the timepoints, compared to the unwashed reference control. The height of each bar corresponds to the median value and the error bars depict the standard deviation between the repeats. The analyses were carried out by solution mode ICP-MS.....87

Figure 14: Zn content of ground and acid digested material from seeds treated with the ZnO formulation (reference) and the same formulation with added adjuvant (A-E) after they had been washed for 24 hours in nitric acid. Each chart represents a different period of dry storage between treatment and acid washing. Ref control refers to a treated seed of the same age which has not been washed. The analysis was carried out by ICP-MS. The height of the bar corresponds to the median value and the bar represents the 5 % confidence interval.....89

Figure 15: Zn content of ground and acid digested material from seeds treated with the ZnO formulation (reference) and the same formulation with added adjuvant (A-E) after they had been washed for 24 hours in nitric acid. Each chart represents a different period of dry storage between treatment and acid washing. The analysis was carried out by ICP-MS. The height of the bar corresponds to the median value and the bar represents the 95 % confidence interval.....90

Figure 16: LA-ICP-MS generated Zn distribution images of epoxy-embedded sections of washed wheat seeds 12 months after they were treated with the reference coating and the reference coating with a range of adjuvants. There is

also an image of the unwashed ref control seed as a positive control. Each image is generated on the same intensity scale (0-1000000 CPS). 92

Figure 17: LA-ICP-MS generated Zn distribution images of epoxy-embedded sections of washed wheat seeds 12 months after they were treated with the reference coating and the reference coating with a range of adjuvants. There is also an image of the unwashed ref control seed as a positive control. Each image is generated on the same intensity scale (0-1000000 CPS). Each column contains three repeat analyses. 95

Figure 18: LA-ICP-MS images of freshly coated unwashed seeds. Left to right on the top row are ZnO (reference coating), ZnO + A (reference coating including adjuvant A), ZnO + B (reference coating including adjuvant B). The bottom row left to right are ZnO + C (reference coating with adjuvant C), ZnO + D (reference coating with adjuvant D) and ZnO + E (reference coating with adjuvant E). The images are semi-quantitative, spatially resolved plots of ^{66}Zn signal intensity in counts per second, normalised to the baseline signal. Each image is produced on the same scale (0-1,000,000 CPS). 97

Figure 19: LA-ICP-MS semi quantitative spatially resolved plot of the ^{66}Zn signal intensity of an untreated wheat seed section. The signal intensity is measured in counts per second (CPS) and normalised to the baseline. The scale was 0-1,000,000 CPS.....98

Figure 20: LA-ICP-MS generated distribution images of epoxy-embedded sections of 3 treated wheat seeds immediately after they were treated with the reference coating and the reference coating with a range of adjuvants, alongside an untreated seed for comparison. Cu images were generated on a 0-100,000

CPS scale, Mn on a 0-1,000,000 CPS scale and Mg on a 0-2,000,000 CPS scale.	100
<u>Figure 21:</u> A side-by-side comparison of two 3D micro-CT images of an untreated (left) and a Reference ZnO-treated (right) seed. Both images were generated on the same relative density scale (0-1).	102
<u>Figure 22:</u> A side-by-side comparison of two micro-CT threshold images of an untreated (left) and a Reference ZnO-treated (right) seed. Both images were generated on the same relative density scale (0-1). The threshold was set such that any density common to both images (corresponding to the seed) was removed, leaving only the areas of density which were present in the treated seed and not the untreated seed visible in the image.	104
<u>Figure 23:</u> micro-CT 3D images of a treated seed after washing in nitric acid, showing a separation of the seed coating from the endosperm. These are supported by threshold analysed images of the same data on the right-hand side showing changes to the topography of the seed surface after washing.	107
<u>Figure 24:</u> SEM images of winter wheat seeds. Untreated is a winter wheat seed with no treatment applied. Ref is a winter wheat seed with the base ZnO flowable suspension seed coating applied, Ref + A-E refers to the base ZnO seed coating with the addition of adjuvant A-E.	109
<u>Figure 25:</u> SEM EDX elemental analysis for zinc on the surface winter wheat seeds. Untreated is a winter wheat seed with no treatment applied. Ref is a winter wheat seed with the base ZnO flowable suspension seed coating applied, Ref + A-E refers to the base ZnO seed coating with the addition of adjuvant A-E. L α 1,2	

denotes the relaxation of electrons from the M to the L energy shell, the size of this shift is characteristic of the element, in this case Zn.	112
<u>Figure 26:</u> SEM EDX analysis of Cl on an untreated compared to a reference treated winter wheat seed. K α 1 denotes the relaxation of electrons from the L to K energy shells, the size of this shift is characteristic of the element, in this case Cl.	114
<u>Figure 27:</u> Schematic representation of the randomised placement of the different treatments in the glass house for the proof-of-concept growth trial carried out at Plant Impact.	123
<u>Table 3:</u> Breakdown of the masses of constituent parts of Part A of the Hoagland's solution as made up in 3 L deionised water.	125
<u>Table 4:</u> Breakdown of the masses of the constituent parts of Part C of the Hoagland's solution including zinc as made up in 1 L deionised water.	126
<u>Table 5:</u> Breakdown of the masses of the constituent parts of Part C of the zinc-free Hoagland's solution as made up in 1 L deionised water.	127
<u>Figure 28:</u> Photograph of the Zn-free (orange) and full Hoagland's (red) treated wheat material after four weeks of growth, showing yellowing of leaves in the plants starved of zinc.	128
<u>Figure 29:</u> Summary of the fresh shoot and root mass of the plant material from the proof-of-concept growth trial. The plants grown in compost are shown in blue, the plants grown with full Hoagland's solution (with Zn) are shown in red, the plants grown with Zn-free Hoagland's are shown in orange and the plants treated only with water are shown in green.	129

<u>Table 6:</u> Summary of the fresh shoot and root masses from the proof-of-concept growth trial.	130
<u>Table 7:</u> A summary of the results of the pairwise statistical analysis of the fresh shoot mass data from the proof-of-concept trial.....	131
<u>Table 8:</u> A summary of the results of the pairwise statistical analysis of the fresh root mass data from the proof-of-concept trial.	132
<u>Figure 30:</u> Results of the ICP-MS analysis of Zn content in digested plant material from the proof-of-concept growth trial. The height of the column represents the median value, whilst the error bars depict the 95 % confidence interval. Each data point represents one replicate.....	134
<u>Table 9:</u> A summary of the results of the pairwise statistical analysis of the Zn content of plant material from the proof-of-concept growth trial.	135
<u>Figure 31:</u> A comparison of the recorded plant height for the untreated compared to the treated plants on the day of harvest from the development assay.	138
<u>Figure 32:</u> A comparison of the recorded fresh mass for the untreated compared to the treated plants on the day of harvest from the development assay.	139
<u>Figure 33:</u> A comparison of the recorded dry mass for the untreated compared to the treated plants after drying in a 60 °C oven overnight.	141
<u>Figure 34:</u> A comparison of the Zn concentration by ICP-MS of the digested plant material from the development assay growth trial. Each data point represents one separately digested repeat.....	142

<u>Figure 35:</u> A representation of the number of plants emerged in each pot by treatment at time points throughout the growth trial. The total number of seeds sown in each pot was three.	147
<u>Figure 36:</u> A representation of the number of plants emerged in each pot by treatment at time points throughout the growth trial. The total number of seeds sown in each pot was three.	149
<u>Table 10:</u> Fresh masses recorded following the third growth trial.	150
<u>Figure 37:</u> A comparison of the recorded dry mass of the plant material for each of the treatments harvested at the end of the full assay growth trial after drying overnight in a 60 °C oven.....	151
<u>Table 11:</u> Mean dry mass results from the third growth trial.	152
<u>Figure 38:</u> A comparison of the Zn concentration by ICP-MS of the digested plant material from the full assay growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.	153
<u>Table 12:</u> A summary of the results of the pairwise Conover-Inman statistical analysis of the Zn content of plant material from the full assay growth trial. ...	154
<u>Figure 39:</u> A comparison of the Cu concentration by ICP-MS of the digested plant material from the full assay growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.	156
<u>Figure 40:</u> A comparison of the Mn concentration by ICP-MS of the digested plant material from the full assay growth trial. Each data point represents one	

separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.	158
<u>Table 13:</u> Tabulated concentrations of Zn, Cu and Mn by ICP-MS data of the digested plant material from the full assay growth trial. Values stated are median mg/Kg values	159
<u>Table 14:</u> Summary of mean dry masses (g) of plant shoot material from the fourth growth trial	162
<u>Figure 42:</u> A comparison of the Zn concentration by ICP-MS of the digested plant material from the full-scale growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.	163
<u>Figure 43:</u> A comparison of the Cu concentration by ICP-MS of the digested plant material from the full-scale growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.	164
<u>Figure 44:</u> A comparison of the Mn concentration by ICP-MS of the digested plant material from the full-scale growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.	165
<u>Table 15:</u> Tabulated concentrations of Zn, Cu and Mn by ICP-MS data of the digested plant material from the fourth growth trial. Values stated are median mg/Kg values.	166

<u>Figure 45:</u> Photographs of the trays containing silver sand and Chinese mustard seeds at the first irrigation point, on day 3 after sewing.	176
<u>Figure 46:</u> Collation of photographs showing the growth of each of the treated Chinese mustard trays, shown on days 5, 7 and 10 after sewing.	179
<u>Figure 47:</u> Graphical representation of the dry masses recorded after harvesting and drying the plant material from the proof-of-concept trial.	180
<u>Table 16:</u> Table of dry masses of the Chinese mustard plants in grams once harvested, separated by Zn treatment applied to each tray.	180
<u>Figure 48:</u> Zn concentration in digested Chinese mustard plant material, grown during the proof-of-concept trial, analysed by ICP-MS.	182
<u>Table 17:</u> Zn concentration in digested Chinese mustard plant material, grown during the proof-of-concept trial, analysed by ICP-MS.	182
<u>Figure 49:</u> Uptake of Zn normalised to plant mass in digested Chinese mustard plant material, grown during the proof-of-concept trial, analysed by ICP-MS.	184
<u>Table 18:</u> Uptake of Zn normalised to plant mass in digested Chinese mustard plant material, grown during the proof-of-concept trial, analysed by ICP-MS.	184
<u>Figure 50:</u> Graphical representation of the dry masses of Chinese Mustard plant material generated as part of the second growth trial. Dry mass was recorded using a 4 figure balance, following drying in the freeze dryer to constant mass.	186
<u>Table 19:</u> Dry masses (g) of the plant material grown as part of the second growth trial.	187

<u>Figure 51:</u> Zn concentration in digested Chinese mustard plant material, grown during the second growth trial, following microwave digestion and analysis by ICP-MS.	188
<u>Table 20:</u> Zn concentration in digested Chinese mustard plant material, grown during the second growth trial, analysed by ICP-MS.	189
<u>Figure 52:</u> Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the second growth trial, analysed by ICP-MS.....	190
<u>Table 21:</u> Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the second growth trial, analysed by ICP-MS.....	190
<u>Figure 53:</u> Graphical representation of the dry masses of Chinese Mustard plant material generated as part of the third growth trial following harvest and freeze drying of the resultant plant material to constant mass.	194
<u>Table 22:</u> Dry masses (g) of the plant material grown as part of the third growth trial.	195
<u>Figure 54:</u> Zn concentration in digested Chinese mustard plant material, grown during the third growth trial, analysed by ICP-MS.	196
<u>Table 23:</u> Zn concentration in digested Chinese mustard plant material, grown during the third growth trial, analysed by ICP-MS.	196
<u>Figure 55:</u> Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the third growth trial, analysed by ICP-MS.	197
<u>Table 24:</u> Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the third growth trial, analysed by ICP-MS.	198

<u>Figure 56:</u> Semi-quantitative LA-ICP-MS images of Chinese Mustard Leaves treated with a range of Zn sources. All images were generated on the same 0-1,000,000 counts per second (CPS) scale. From top left to bottom right the treatments are: Control, ZnO, ZnO + E, ZnSO ₄ and Zn(NO ₃) ₂	200
<u>Figure 57:</u> Graphical representation of the dry masses of Chinese Mustard plant material generated as part of the fourth small-scale growth trial, featuring all of the adjuvants as treatments.	205
<u>Table 25:</u> Dry masses (g) of the plant material grown as part of the fourth small-scale growth trial which encompassed all 5 adjuvants as treatments.	206
<u>Figure 58:</u> Zn content of Chinese Mustard plant material (mg/Kg), grown during the fourth growth trial, analysed by ICP-MS.....	208
<u>Table 26:</u> Zn Content (mg/Kg) of digested Chinese mustard plant material, grown during the fourth growth trial, analysed by ICP-MS.....	209
<u>Table 27:</u> Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the fourth growth trial, analysed by ICP-MS.	211
<u>Figure 60:</u> Semi-quantitative LA-ICP-MS images of Chinese Mustard Leaves treated with a range of Zn sources. All images were generated on the same 0-1,000,000 counts per second (CPS) scale. From top left to bottom right the treatments are: Control, ZnSO ₄ , Zn(NO ₃) ₂ , ZnO and ZnO+A-E.	215
<u>Figure 61:</u> Fresh mass of winter wheat plant material recorded during the harvest of plants from the sources of Zn glasshouse growth trial, The horizontal line represents the median value whilst the individual points denote individual plants.	220

<u>Figure 62:</u> Dried mass of winter wheat plant material recorded after drying the plants from the sources of Zn glasshouse growth trial, The horizontal line represents the median value whilst the individual points denote individual plants.	221
<u>Figure 63:</u> Graphical representation of the number of plants which emerged per pot, out of a total of 6 by treatment. The horizontal top of the bar gives the median value per treatment, whilst each point represents one pot.	222
<u>Figure 64:</u> Graphical representation of the percentage of plants which emerged by treatment, during the sources of zinc glasshouse growth trial.....	223
<u>Figure 65:</u> Graphical representation of the mean plant mass per number of plants harvested by each treatment, in the sources of Zn glasshouse growth trial....	225
<u>Figure 66:</u> Zn content (mg/Kg) of digested winter wheat plant material harvested following the sources of Zn glasshouse growth trial, analysed by ICP-MS.	226
<u>Figure 67:</u> Zn uptake (μg) normalised to dry mass of Winter Wheat plant material, grown during the sources of Zn glasshouse growth trial, analysed by ICP-MS.	227
<u>Table 28:</u> Results by treatment for each of the measurements carried out during the sources of Zn full scale glasshouse trial.	229
<u>Figure 68:</u> Calibration graph for Zn standards by ICP-MS – standards used to quantify the Zn content of digested seed material throughout Chapter 4 of this thesis, R ² value is shown, and the equation of the straight line is used to calculate the limit of detection for the analysis (LOD) below.	273

Table of Contents

Candidate Declaration	2
Abstract	3
Acknowledgements	4
List of figures & tables	6
Table of Contents	19
List of Abbreviations	26
1.0. Thesis Introduction	28
1.1. Crop Nutrition	28
1.1.1. The role of zinc in plants	28
1.1.2. The importance of zinc in crops	29
1.1.3. Zinc deficiency	29
1.1.4. Zinc uptake in plants	31
1.1.5. Transport of zinc within plants.....	32
1.1.6. Accumulation of zinc within plants.....	35
1.1.7. Similarities between zinc and other cations	36
1.1.8. Wheat as a crop of global importance.....	37
1.2. Introduction to adjuvants.....	37
1.2.1. Adjuvants in agriculture	37
1.2.2. Adjuvants included in the study.....	38

1.3.	Introduction to seed treatments	39
1.3.1.	Types of seed coatings	41
1.3.2.	Seed coating active Ingredients	42
1.3.3.	Challenges and Future Development of seed coatings.....	44
1.4.	Seed coating formulation studied throughout this thesis.....	47
1.5.	Research project aims and objectives	49
2.0.	Analytical Techniques and Method Development.....	50
2.1.	Analytical Techniques.....	50
2.1.1.	Inductively Coupled Plasma Mass Spectrometry	50
2.1.2.	Mass Spectrometry	52
2.1.3.	Quadrupole mass spectrometry	53
2.1.4.	Polyatomic interferences in ICP-MS.....	54
2.1.5.	Mitigation strategies for polyatomic interferences in ICP-MS	54
2.2.	Inductively Coupled Plasma Mass Spectrometry Sample Introduction Methods	55
2.3.	Introduction to Laser Ablation	55
2.4.	Coupling of Laser Ablation to Inductively Coupled Plasma Mass Spectrometers	56
2.5.	Fundamentals of LA-ICP-MS sampling for imaging	56
2.6.	Laser Ablation Hardware	57

2.7.	Inductively coupled plasma optical emission spectroscopy (ICP-OES) ..	59
2.8.	Comparison of ICP-MS and ICP-OES	59
2.9.	Scanning Electron Microscopy	60
2.10.	Energy Dispersive X-ray Spectroscopy	60
3.0.	Method Development.....	61
3.1.	Seed material sample preparation	61
3.2.	Laser Ablation Method Considerations	63
3.3.	Development of LA-ICP-MS image quality throughout the project.....	65
4.0.	Seed Treatment Mechanism of action	71
4.1.	Introduction.....	71
4.2.	Aims of the chapter.....	73
4.3.	Methods.....	74
4.3.1.	Seed variety selection	74
4.3.2.	Development of Washing Methodology.....	74
4.3.3.	Washing of Treated Seeds.....	75
4.3.4.	12-month washing experiment design.....	75
4.3.5.	Sampling of Seed.....	76
4.3.6.	Equation for the calculation of mg/Kg values	76
4.3.7.	Preparation of Seed for LA-ICP-MS Analysis.....	76

4.3.8.	Laser Ablation Parameters.....	77
4.3.9.	ICP-MS settings for Imaging	77
4.3.10.	Generation of spatially resolved LA-ICP-MS Images	78
4.3.11.	Development of LA-ICP-MS Capabilities	78
4.3.12.	Micro-CT Analysis of Seeds.....	80
4.3.13.	Thresholding of micro-CT data.....	81
4.3.14.	Scanning Electron Microscopy Imaging of Treated Wheat Seeds ..	81
4.3.15.	Energy Dispersive X-ray Spectroscopy imaging of treated wheat seeds	81
4.3.16.	Development of Washing Methodology.....	82
4.4.	Results.....	84
4.4.1.	Washing of Treated Seeds.....	84
4.4.2.	Washing and Analysis of Treated Seeds Over 12 Months	86
4.4.3.	LA-ICP-MS Images of Zn in freshly treated wheat seeds.....	96
4.4.4.	LA-ICP-MS images of other metals in freshly treated wheat seeds	99
4.4.5.	Micro-CT images of treated and untreated wheat seeds.....	102
4.4.6.	Thresholding of micro-CT images for highly dense particles.....	104
4.4.7.	Micro-CT imaging of treated-washed Wheat seed.	106
4.4.8.	SEM Analysis of Whole Wheat Seeds	108
4.4.9.	EDX Analysis of Zn on Seed Surface.....	111

4.5.	Conclusion	115
5.0.	Glass House Wheat Growth Trials	119
5.1.	Introduction	119
5.2.	Aims of this chapter	121
5.3.	Greenhouse Facilities	122
5.4.	Growth trial 1	122
5.4.1.	Growth trial 1 design	122
5.4.2.	Preparation of Hoagland's solution	124
5.4.3.	Growth trial 1 results	128
5.5.	Growth Trial 2 – Development Assay	137
5.5.1.	Experimental Design	137
5.6.	Results of the Development assay	138
5.7.	Growth Trial 3 – Full Assay	145
5.7.1.	Growth trial 3 study design	145
5.7.2.	Results Growth Trial 3 – Full Assay	146
5.8.	Growth Trial 4	160
5.8.1.	Growth Trial 4 – study design	160
5.9.	Conclusion	168
6.0.	Studies on the effects of the source of Zn	171
6.1.	Introduction	171

6.2.	Aims of the chapter.....	173
6.3.	Development of small-scale uptake experiments	173
6.3.1.	Development of small-scale uptake experiments Introduction	173
6.3.2.	Methods – development of small-scale uptake experiments.....	174
6.3.3.	Proof of concept trial methods	175
6.3.4.	Second Growth Trial Methods.....	177
6.4.	Results of Development of small-scale uptake experiments.....	178
6.5.	Proof-of-concept results.....	178
6.6.	Second growth trial results – addition of DI water control	186
6.7.	Expansion of small-scale growth experiments to include adjuvant ...	192
6.7.1.	Methods for small-scale growth trial including adjuvant.	192
6.7.2.	Results for small-scale growth trial including adjuvant	194
6.8.	Expansion of small-scale growth method to include all adjuvants	203
6.8.1.	Methods for expansion of small-scale growth method to include all five adjuvants	204
6.8.2.	Results for expansion of small-scale growth method to include all five adjuvants	205
6.9.	LA-ICP-MS Analysis of plants from the small-scale growth trials	212
6.9.1.	Method for semi-quantitative analysis of Chinese mustard shoots	213
6.9.2.	Mounting of shoots for LA-ICP-MS analysis.....	213
6.9.3.	LA-ICP-MS parameters for imaging of Chinese mustard shoots	213

6.9.4.	LA-ICP-MS image generation	214
6.9.5.	Results for semi-quantitative analysis of Chinese mustard shoots	214
6.10.	Large-scale greenhouse sources of Zn trial	217
6.10.1.	Sources of Zn glasshouse trial method	217
6.10.2.	Preparation of plant material for analysis by ICP-MS.....	218
6.10.3.	Results of sources of Zn glasshouse trial.....	220
6.11.	Sources of Zn study conclusion	231
7.0.	Conclusions	234
8.0.	Recommendations for future work	237
9.0.	References	241
10.0.	Other research contributions whilst undertaking this research.....	269
11.0.	Appendix	273
11.1.	ICP-MS seed washing zinc analysis method validation.	273

List of Abbreviations

LA-ICP-MS	Laser Ablation Inductively Coupled Plasma Mass Spectrometry
LA	Laser Ablation
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
AAS	Atomic Absorption Spectroscopy
mg/Kg	milligram per kilogram
CPS	Counts Per Second
μ-CT	micro computed tomography
micro-CT	micro computed tomography
SEM	Scanning Electron Microscopy
EDX	Energy-dispersive X-ray spectroscopy
XANES	X-ray absorption near edge structure analysis
ATP	Adenosine triphosphate
NA	Nicotianamine
M	molar (moles per litre)
NP	Nanoparticle
nAChRs	Nicotinic acetylcholine receptor
K	Kelvin
AC	Alternating Current
DC	Direct Current
KED	Kinetic Energy Discrimination
nm	nanometre
DCI	Dual Concentric Injector
TwoVol3	Two Volume 3 (laser ablation sample chamber)
CMC	Carboxy methyl cellulose
PVC	Polyvinyl chloride
ESL	Elemental Scientific Lasers
SR	Scan Rate
DCM	Dichloromethane
Ref	Reference (base ZnO-containing seed treatment formulation)
ZnAL-LDH	Zinc aluminium layered double hydroxides
AMF	Arbuscular Mycorrhizal Fungi
PEG	Polyethylene glycol
RO	Reverse osmosis
m/z	Mass to charge ratio

Page left intentionally blank

1.0. Thesis Introduction

1.1. Crop Nutrition

1.1.1. The role of zinc in plants

Zinc (Zn) is an essential micronutrient. It is only required in small quantities but is vital to the healthy growth of the plant. Optimal zinc nutrition in plants is considered to be in the range of 20-60 mg/Kg dry mass.¹ Zinc plays a vital role in plant growth and development.² Moreover, Zn is essential for the survival of other organisms, including humans.³

The role of zinc in plant biology is multifaceted. It is the only metal to be present in all six classes of enzymes (oxidoreductase, transferase, hydrolases, lyases, isomerases and ligases). It also acts as a cofactor in more than 300 proteins including zinc finger proteins, RNA polymerases and DNA polymerases.⁴ Zinc also plays a role in a range of physiological processes, it is important for membrane integrity and stabilization, the alleviation of oxidative stress, hormone regulation and the maintenance of the CO₂ concentration in the mesophyll.^{5,6} Most notably, zinc is vital for the creation of auxin, the plant growth hormone, and for the production of chlorophyll and cytochrome pigments.⁷

1.1.2. The importance of zinc in crops

Zinc has been shown to be the most important micronutrient in terms of increasing crop yield, as discussed by Sillanpaa who conducted field trials of the growth of commercially important crops, including wheat and barley, across 15 different countries. Locally important crops were grown with fertilizer treatments containing macronutrients and 6 micronutrients (boron, copper, iron, manganese, molybdenum, and zinc) using the 'all-minus-one' principle, whereby one micronutrient was excluded from the treatment each time. The removal of zinc had a greater negative impact on the crop yield than the removal of any other micronutrient. The order of yield reduction by individual micronutrient deficiency was as follows: Zinc 49% > boron 31% > molybdenum 15% > copper 14% > manganese 10% > iron 3%.⁸ These results were further supported by the findings from a similar study carried out by Zou *et al.* in China who also reported zinc to be the most important micronutrient in terms of its negative effect on crop yield when deficient.⁹

1.1.3. Zinc deficiency

Zinc deficiency is the most common micronutrient deficiency in crops. It is estimated that more than 50% of the world's agricultural land is considered to be deficient in zinc.¹⁰ Zinc deficiency can result in a significant reduction in crop yields and quality. Crop yield can be reduced by as much as 20% before any visual symptoms of the deficiency occur.¹¹ This is due to variance in the mobility of zinc within a plant, depending on its availability in the soil or growing medium. When zinc availability is adequate, it is readily translocated from older to younger

leaves. However, when zinc is deficient, movement of zinc from older leaves to younger ones is delayed.¹² Therefore, zinc deficiency is first visually identifiable in middle leaves. The symptoms of zinc deficiency can include the following: stunting (reduced height), interveinal chlorosis (yellowing of leaves), brown spots on upper leaves, and distorted leaves. Since there is a delay in the visibility of symptoms in zinc deficient plants, visual symptoms are usually only seen in severely affected plants.¹³

There are a number of factors which control the amount of plant available zinc in the soil. These include: the total zinc content of the soil, pH, and redox conditions, CaCO_3 and organic matter contents, the concentration of ligands which are capable of forming organo-zinc complexes, microbial activity in the rhizosphere, concentrations of other micronutrients, concentrations of macro-nutrients (especially Phosphorous) and the soil moisture status.¹⁴ The soil types most commonly associated with zinc deficiency in plants are generally soils with one or more of the following: "Low total zinc concentration, high CaCO_3 contents, high organic matter contents (>3%), neutral or alkaline pH, high available P concentration, high bicarbonate and/or magnesium concentration, prolonged water logging, and high sodium, calcium, ,magnesium and bicarbonate concentrations."¹⁵ The soil pH plays a key role since the activity of Zn^{2+} in soils is directly proportional to the square of the proton activity. Therefore, the solubility of zinc will decrease with increasing soil pH.¹⁶ Moreover, at a pH range of 5.5-7, the soil concentration of soluble zinc decreases significantly by between 30-fold and 45-fold for each unit increase in pH.¹⁷ Alloway *et al.* reported an atlas which shows areas of widespread and medium deficiency and their geographical

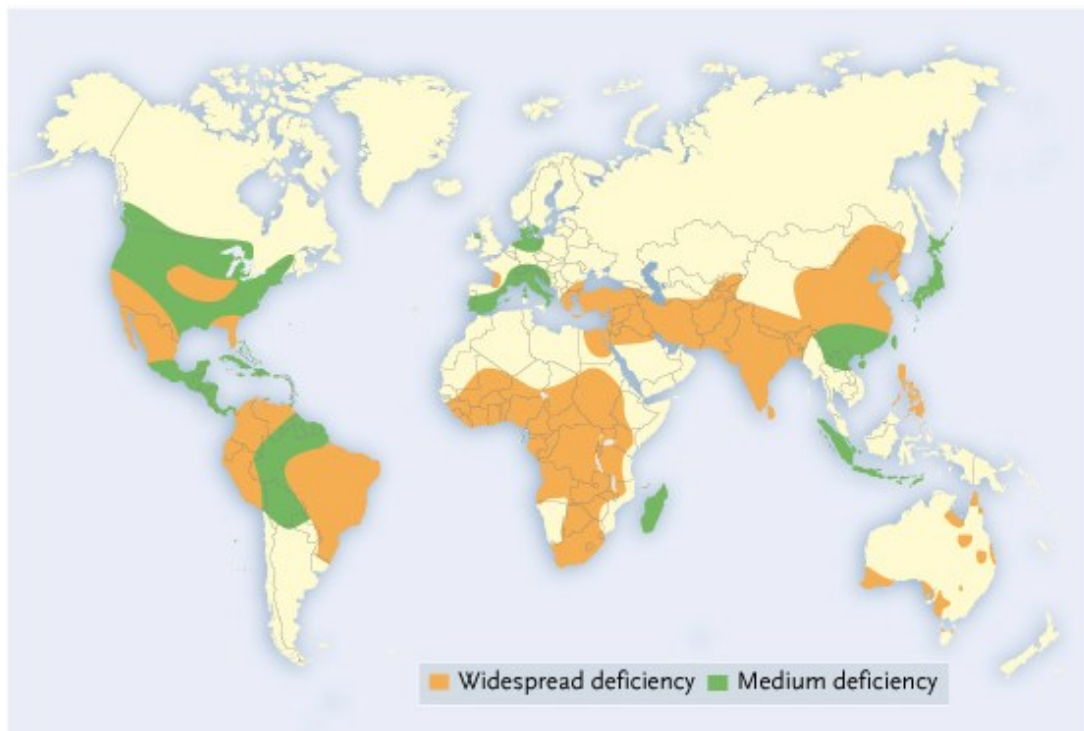


Figure 1: World map depicting locations of agricultural land which have been found to be zinc deficient.

location, this is shown in figure 1.¹⁸ The image is generated from data reported in the international literature where cases of zinc deficiency in crops have been reported. This not only outlines how widespread the problem of soil zinc deficiency is, demonstrating that zinc deficiency really is a global issue.

1.1.4. Zinc uptake in plants

The most common mechanism of the uptake of zinc in plants is by the uptake of the divalent zinc cation (Zn^{2+}) by the roots. In some cases, organic ligand-Zn complexes are also taken up by plant roots. Plants employ two main physiological strategies for the uptake of divalent cations such as zinc. The first involves the efflux of reductants, organic acids, and H^+ ions, which increase the solubility of

Zn-complexes and release Zn^{2+} ions for absorption by root epidermal cells. The second strategy involves the release of phytosiderophores which form stable complexes with zinc and their subsequent influx into root epidermal cells. This absorption mechanism is restricted to cereal roots.¹⁹ Phytosiderophores are low molecular weight organic compounds which have a high affinity for metals, so chelate to and acquire them.²⁰ They are biosynthesised by the condensation of S-adenosylmethionine to form nicotianamine, which is a phytosiderophores precursor.²¹

The main driving force in Zn^{2+} uptake is the hyperpolarisation of the root cell-plasma membrane. This is mediated by the active transport of H^+ ions out of the root cells. This reduces the soil pH, thus increasing the uptake of cations such as Zn^{2+} . Unlike water, cations are not able to cross cell membranes freely.²² Therefore, the cations are transported across the membrane by specific transporter proteins, known as ZIP proteins.²³ The uptake of cations is known to be passive rather than active since the ZIP proteins have no association with the breakdown of ATP. (ATP breakdown is indicative of an active process).²⁴ In addition to the selective ZIP proteins, Zn^{2+} uptake also takes place by non-selective cation channels which enable the passive diffusion of a range of cations.²⁵

1.1.5. Transport of zinc within plants

Once zinc has crossed the membrane of the root epidermal cell, it must pass through several tissues before reaching the xylem, in which it is transported to the shoot. The tissues which must be passed through are the epidermis, the

cortex, the endodermis and the pericycle, which ultimately loads the xylem. This transport of minerals and water towards the stele (central part of the root or stem containing the vascular tissues) is known as radial transport.²⁶ Metal cations accumulate in the pericycle before being loaded into the xylem.²⁷

There are two main routes involved in the transport of mineral ions to xylem. These are the symplastic route and the apoplastic route. The cytoplasm of adjacent cells in root tissues are connected by cytoplasmic bridges in the cell walls. This forms a symplastic continuum without membrane barriers. The symplast of a plant is the inner side of the plasma membrane, in which water and low molecular weight solutes can diffuse. In the symplastic pathway diffusion of ions occurs through the cytoplasm. In addition to this, water and mineral ions can move through cell walls and intercellular spaces, the apoplast. The apoplast is the non-protoplasmic parts of the tissue such as the cell wall and intracellular space. In the apoplastic pathway, water and mineral ions diffuse across cell membranes by passive diffusion. Both mechanisms contribute significantly to the transport of zinc within roots.²⁸ However, transport via the apoplastic route is interrupted in some regions of the endodermis due to the deposition of suberin lamellae, formed from lipophilic biopolymer that acts as a barrier to the movement of water and mineral ions, in the casparian strip. Suberin is very impermeable to water and charged ions. Therefore, water and ions must pass through the membrane before the casparian strip so that they can be transported further via the symplastic pathway.²⁹

Once the zinc has reached the pericycle and xylem parenchyma (cells bordering the xylem), it is transferred into the xylem through a process known as xylem

loading. This is the key determining step in the export of zinc from root tissues. The constant activity of H⁺-ATPase in the xylem parenchyma causes membrane hyperpolarisation which causes the Zn²⁺ ions to be loaded into the apoplastic xylem (an active process).³⁰

Within the xylem, metal ions are transported mainly as metal complexes with organic acids and nicotianamine (NA). NA is chelator of several metal cations in plants, including zinc.³¹

Wheat and many other crops are monocotyledon plants (monocots). Meaning they have only one seed leaf. They also tend to have fibrous roots and are amphistomatous, meaning they have stomata on both the upper and lower surface of their leaves.³² A diagram of the structure of a monocot root is shown in figure 2.

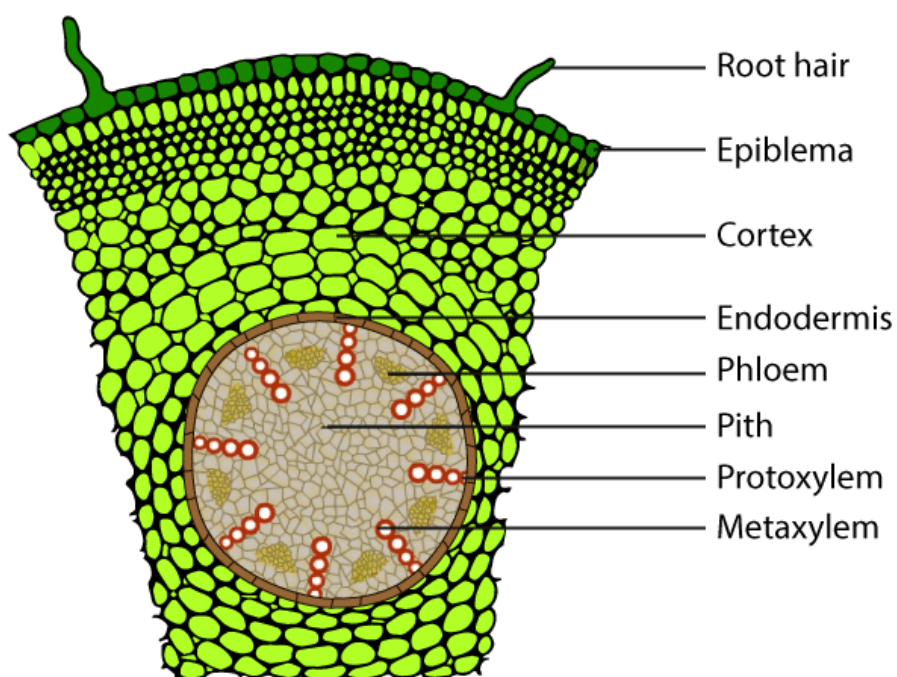


Figure 2: Cross-section of a root, showing the structures involved in transport within the plant.

1.1.6. Accumulation of zinc within plants

The accumulation of zinc within plants is affected by the level of zinc available to the plant. When plants have an adequate supply of zinc, the concentration of zinc tends to be higher in growing tissue than in more mature tissue. Examples of growing tissues include roots, shoots, and reproductive tissues. The transport of zinc in xylem does not necessarily coincide with the transport of water. Zinc has variable mobility depending on its supply. Zinc can be translocated to a greater extent when it is in adequate supply. The movement of zinc out of older leaves coincides with their senescence (loss of a cell's power of division and growth as it deteriorates with age) and can be delayed by zinc deficiency.^{33,34}

It is thought that a wide range of membrane proteins and chelators could be involved in zinc redistribution and homeostasis. However, relatively little is known about the mechanism by which they are expressed and how zinc redistribution is controlled. To do this it would be necessary to determine the sub-cellular localisation of the proteins, which would enable the significance of their expression under various conditions to be understood.³⁵

Zinc deficiency seems to be first detected by plants in the shoots, signals are then thought to be sent to the roots where the cation transporters are functioning.³⁶ Assuncao et al. have proposed a model for the mechanism by which plants sense and respond to zinc deficiency. They proposed that bZIP19 and bZIP23 (proteins involved in signalling) are in some way activated by zinc deficiency, which in turn leads to the expression of ZIP genes, thus increasing zinc uptake and redistribution.³⁷ They do, however admit that this is a proposal based on what is

currently known, and that the model only provides the first step in truly understanding the mechanism by which plants can respond to zinc deficiency.

1.1.7. Similarities between zinc and other cations

Plant essential elements exist in the soil as a mixture of cations and anions. Cations (including zinc) tend to be less mobile in the soil as they are more readily bound to the soil. Although this depends on the soil conditions in question. The essential elements which exist as cations are Nitrogen (ammonium form), potassium, calcium, magnesium, iron, manganese, and zinc.³⁸ However, other essential elements exist in soil as anions: nitrogen (nitrate form), phosphorous, sulfur, chlorine, boron, and molybdenum.³⁹

Since the uptake of zinc is due, at least in part, to zinc-specific ZIP transporter proteins, it cannot be assumed that a treatment which enhances the uptake of zinc would increase the uptake of other cations in the same way. However, there are many reports in the literature of zinc and cadmium interactions in plants.

Since both zinc and cadmium are divalent cations, they can both be taken up by the roots by the same mechanism. Cadmium is toxic to plants and is known to cause oxidative stress. Tkalec et al. reported an antagonist interaction between cadmium and zinc, whereby the oxidative stress of tobacco plants grown in cadmium containing soil were to some degree alleviated by the application of zinc. This, they suggested, was due to the two divalent cations sharing the same uptake mechanisms.⁴⁰

1.1.8. Wheat as a crop of global importance

Winter wheat was selected as the model crop for this body of work, in part due to the interest of the project's industrial sponsors, but also because wheat is the most significant crop grown globally for human consumption.⁴¹ Wheat contributes around 20 % of the total dietary calories and proteins consumed worldwide⁴² Wheat is cultivated on an estimated 217 million hectares of land globally, which makes it the most widely grown crop in the world, in terms of land use.⁴³

1.2. Introduction to adjuvants

1.2.1. Adjuvants in agriculture

The term 'adjuvant' in the context of agrochemicals refers to a material added to formulations which is designed to increase the efficacy of the active ingredient, thus improving the performance of the product formulation.⁴⁴

Croda International Plc. are a UK-based global speciality chemical company, one of their business areas is the supply of adjuvants to agrochemical companies for a variety of applications in products such as fertilisers and crop spray treatments.⁴⁵ Croda's adjuvants cover a range of chemistries and mechanisms of action. The overarching aim of the project was to explore the effect of adjuvant inclusion on the uptake seed-applied zinc using winter wheat as a model crop.

1.2.2. Adjuvants included in the study

Five adjuvants were selected for the study, to be applied on top of a ZnO containing seed film coating formulation at 15 % of the level of active ingredient. They were selected to cover a broad range of chemistry and activity. The adjuvants included were: Atplus UEP100 an alkoxyated trimethylpropane trioleate (A), Tween L1010 a polyoxyethylene polyoxypropylene sorbitan monolaurate (B), Tween 24 a polyoxyethylene sorbitan monolaurate (C), Atplus PFA a polypropylglycol cetheth (D) and Hydravance 200 a surfactant blend of ethoxylated sorbitan ester, polyoxyethylene polyoxypropylene block co-polymer and alcohol ethoxylate (E).⁴⁶ The adjuvants are proprietary to Croda, as such specific information about their structure and chemistry is confidential. Table 1 gives a summary of their existing uses as advertised by Croda.

Adjuvant	Code letter	General usage
Atplus UEP100	A	Designed to enhance the uptake of systemic or translaminar active ingredients with additional spray retention properties.
Tween L1010	B	Designed to increase spray retention and spreading for contact active ingredients
Tween 24	C	Designed to enhance surface activity to provide better wetting, lower equilibrium surface tension and lower contact angle
Atplus PFA	D	A pourable liquid adjuvant developed to improve the performance of fungicidal applications
Hydravance 200	E	enhances the penetration of water into hydrophobic soil, improving the wetting and percolation of water throughout the soil profile.

Table 1: Summary of Croda adjuvants which were included in the work undertaken throughout this thesis.

1.3. Introduction to seed treatments

Seed treatment is a process which involves the application of materials of interest to the surface of a seed. It is often done to change the physical properties of a seed. The size, shape and weight of a seed can be manipulated to improve the flowability and sowing success rate of a particularly troublesome seed variety. Seed treatments can also be used as an application route for active ingredients such as nutrients, protectants and soil adjuvants.⁴⁷ They are often considered to be a useful method for homogeneously distributing low rates of fertiliser.⁴⁸ Seed

treatment is also commonly used as an application route for antimicrobial or fungicidal agents.⁴⁹

Seed treatments are considered to be a more environmentally friendly method of application of pesticides, due to the small amounts used relative to crop spraying, and the targeted nature of the application.⁵⁰ However, they are not without compromise, seed treatments containing imidacloprid, a neonicotinoid have been associated with a reduction in bee populations. This is due to the dust released during the drilling of crops. Maize is of particular concern since it is drilled during the main honey flows.^{51,52} Schaafsma et al investigated neonicotinoid residues in subsurface drainage and open ditch water around maize fields where seed treatments including neonicotinoid insecticides had been used. They found that there is a 40- to 50- fold dilution of neonicotinoid insecticide residues in surface waters found in streams, compared to what is found in ditches draining fields where seed treatments have been applied. This suggests that the residues in ditch water most likely come from contamination from dust during planting, or via settled dust which is redistributed by wind or rain. This indicates that the contamination is not due to leeching of active ingredients from planted seed.⁵³ The implication of this would be that adaptation of the planting machinery may be a more effective method to mitigate the off-target effects of seed treatments, rather than changes to the formulation of the seed treatments themselves.

Seed treatment is a large and growing industry. In the United States, between 2012 and 2014, 90% of corn, 76% of soybean, 62% cotton and 56% of winter wheat acres were planted with treated seed.⁵⁴ In 2021 the global seed coating

market was valued at \$1.9 bn dollars, this is forecast to grow at a rate of 8.5 % a year, reaching \$3.6 bn by the year 2028.^{55,56}

1.3.1. Types of seed coatings

There are three main mechanisms of seed coating which are in commercial use: Pelleting, encrusting and film coating.⁵⁷ Pelleting involves the coating of small, difficult to handle seeds with ingredients such as clay and soil. Pelleting ensures uniformity and smoothness of the seed giving greater efficiency in the planting process. By ensuring that the pellets are as close to spherical as possible, automated planting devices can work much more effectively. The pellets can also include protection additives to maximise the germination rate of the planted crop.⁵⁸

Encrusting involves the coating of seeds with a mixture of liquids and solid particulates, such that the seed surface is completely covered but the seed's original shape is retained. Encrusted seeds have been shown to have significantly improved germination rates, though the seed coating thickness can have an impact on germination rate, with encrusted seeds taking longer to germinate than untreated or film treated seeds.⁵⁹

Film coating refers to the practice of encapsulation of applied protectant is needed but pelleting is not required to allow for precision planting. Protectants such as antifungals and insecticides are fixed to the seed surface along with bio stimulants and other active ingredients. Film coatings have the advantage that the act to reduce dust-off during the mechanical planting process which is

especially important when using insecticides. Film coatings are coloured which enables differentiation between seed varieties as well as between applied treatments.⁶⁰

1.3.2. Seed coating active Ingredients

Seed coatings are often used as a vehicle for the application of micronutrients to seeds. Micronutrient deficiency in soil is a widespread problem and can be caused by excessive irrigation and the excess application of chemical fertilisers.⁶¹

It has been shown that the treatment of Corn seeds with molybdenum and potassium has a positive increase on the number of seedlings which germinate. Gomes et al treated corn with increasingly concentrated Mo and K treatments, using germination, first count, seedling length, seedling dry matter and the cold test to assess the influence of the dosage of Mo and K in a seed treatment on the physiological potential of Corn. They found that whilst the number of seedlings germinating increased with increasing dose, smaller doses of molybdenum resulted in a higher percentage of normal seedlings. This suggests that the physiological quality of corn seeds is affected by increasing Mo doses.⁶²

As outlined in section 1.1.2 above, it is well established that Zn is one of the most vital nutrients required for the growth and development of plants. A study which examined the use of a range of concentrations of Zn solutions as a primer prior to sowing mungbean seeds showed that seed priming with 0.01 M Zn solution increased the grain yield by 35 % and the grain Zn concentrations by 50 %.⁶³

Rizwan et al primed wheat seeds by continuous aeration for 24 hours with a range of concentrations of zinc oxide (ZnO) and iron oxide (FeO) nanoparticles (NPs).

The seeds were sown in soil which was contaminated with cadmium (Cd) due to long-term application of sewage water. They observed that both the plants treated with ZnO and FeO showed increased growth under the Cd stress. Both NPs increased chlorophyll content and gas exchange in the Cd-stressed plants. Both NPs reduced oxidative stress in the plants and decreased the wheat Cd concentration while enhancing Fe and Zn concentrations in the wheat.⁶⁴

Naturally then, there is interest in the application of Zn to crop seeds as a seed treatment. Where Zn deficiency and drought stress take place concurrently wheat grain yields can be reduced by as much as 15 %. In a study on the impact of different Zn application strategies on yield, it has been shown that the application of Zn containing seed treatment improved wheat yield under drought stress by 14 %. When seed treatment was combined with the application of a Zn-containing foliar spray, the yield was increased by 17 % compared with the untreated control. It was also shown that the application of the seed coating combined with the foliar spray improved the chlorophyll content compared to the untreated control.⁶⁵

However, the regulations on the use of nanoparticles in agriculture are restrictive in many parts of the world, including some of the largest seed coating markets. As a result, despite the growing evidence of the potential benefits of the use of nanomaterials in agriculture, very few NP-containing treatments have made it to market.⁶⁶ The majority of work on micronutrient seed coatings has avoided the use of NPs for this reason.

1.3.3.Challenges and Future Development of seed coatings

Over the past 10 years, regulations around the use of Neonicotinoids have been tightening due to growing evidence of their harmful effects on the population of pollinators, especially bees.⁶⁷ Neonicotinoid is the umbrella term for a family of insecticides which are broadly based on the structure of nicotine and contain the nitromethylene functional group. They work by targeting nicotinic acetylcholine receptors (nAChRs).⁶⁸ They exhibit highly selective toxicity to insects over mammals.⁶⁷ In 2013, the use of three neonicotinoids (imidacloprid, thiamethoxam and clothianidin) on flowering crops and cereals was banned by the European Commission. In 2020 this ban was extended to all uses of the three neonicotinoids.⁶⁹ Their use in seed coatings is of particular concern due to the exposure of pollinators caused by the distribution of the insecticide throughout the plant such as pollen and nectar. There is also concern about exposure due to the release of dust created by coated seeds through processing using certain kinds of mechanical planters.⁷⁰

The ban is not unanimously supported. The primary concerns for beekeepers in respect to bee population losses are Varroa mites (which act as vectors for a number of viral diseases), nutrition and disease.⁷⁰ It has been shown using a causal analysis approach that the major factor in reduced survival of bee colonies is Varroa mites; with neonicotinoid pesticides unlikely to be the sole cause of reduced survival, though they could not be ruled out as a contributing factor.⁷¹

The tightening of regulations in the pesticide and fertilizer markets, there is an increase in demand to replace synthetic crop enhancement products with organic

alternatives.⁷² Chitin, a carbohydrate polymer made up of repeating 2-(acetylamino)-2-deoxy-D-glucose units, is a promising alternative. Chitin is most commonly obtained from the exoskeleton of shellfish, insects, or fungal cell walls.⁷³ Chitin has been shown to be an effective fertilizer, soil conditioning agent and plant disease control agent. Chitin and its derivatives are known to enhance natural plant defence mechanisms so they can also be considered to be plant growth regulators, growth stimulants, anti-stress agents, and induce the production of secondary metabolites.⁷⁴ Chitin has also been shown to be effective as a seed treatment. It has a beneficial effect on seed germination as well as increasing the production of carbohydrates and improving frost tolerance.⁷⁵

It is widely established that plastic pollution presents one of the greatest human-caused threats to the ecosystem. Microplastics, commonly defined as particles smaller than 5 mm in size, have been widely studied in aquatic ecosystems where consumption of plastic pollutants has been shown to not only adversely affect the organism which ingests them but also provides a route for hazardous substances to be absorbed into the aquatic food chain.^{76,77}

Whilst seed treatment is a standard practice for many crop seeds, there is increasing interest in the benefits of seed treatments for improving the effectiveness of ecological restoration projects.⁷⁸ Seed treatment would help to accelerate and synchronise germination whilst improving seed vigour and seedling establishment. Seed treatments could also be employed to give different varieties of seed similar size and shape properties, enabling simultaneous and

automated planting which would open the possibility of planting highly biodiverse ecosystems quickly and easily.^{58,79}

Traditionally, restoration or 're-wilding' projects were carried out by the planting of seedlings due to the fact that fewer than 10 % of seeds deployed to such projects establish to produce a mature plant.⁸⁰ Such a low success rate limits the feasibility of seed based restoration making it an unsustainable strategy to pursue; without the inclusion of seed treatments.⁸¹ The most striking advantage of seed coating for restorative applications is the ability to tailor the applied treatment to the conditions and barriers to seedling growth observed in the target environment. Examples include the inclusion of a soil surfactant in the coating of wheatgrass which improved seedling emergence and plant survival in water repellent soil following a fire.⁸² Seed coatings incorporating salicylic acid improved plant survival and growth of native species in Australia, whilst coatings including predator repellents have been shown to reduce seed consumption rates by rodents and therefore improve plant establishment.^{83,84,85}

Whilst the development of seed treatments tailored to the seed type and the conditions in which they will be planted presents an enormous opportunity in this space, the volume of research which would be required to optimise a seed coat for every seed type for every challenging environment is enormous.

1.4. Seed coating formulation studied throughout this thesis

The studies undertaken throughout this thesis are based upon a ZnO flowable suspension designed for application to winter wheat seeds prior to sowing. The treatment formulation, ingredients, preparation, and application methods were provided by Croda. The formulation is broken down in table 2.

Substance	Function	g/100 mL	Equivalent %
Zinc Oxide	Micronutrient	85	49.70
Atlox PN100	Wetting agent and dispersant	10.3	6.02
Pricerine 9091	Anti-freeze	8.55	5.00
Silcolapse 5020	Anti-foam	0.52	0.30
Proxel GXL	Antibacterial	0.05	0.03
Kelzan RD	Structuring agent	0.52	0.3
Unisperse RED 3RS-E2	Pigment	20.50	11.99
Deionised water	Continuous phase	45.58	26.65

Table 2: The formulation of the flowable suspension used as the base formulation for the treatment of wheat seeds throughout the work contained within this thesis.

The formulation in table 2 is used throughout this thesis and is referred to as the Reference, or Ref formulation. Where the adjuvants outlined in 3.3.2 are included on top of the Reference formulation they are referred to as Ref + (A-E) where A-E refers to the adjuvant which has been added.

1.5. Research project aims and objectives

The aims of the research project were to investigate the effect of adjuvants on the uptake of zinc from seed film coatings, to determine the effect of adjuvant on zinc uptake, localisation and concentration in winter wheat, and to determine the extent to which adjuvant inclusion may negate the drawbacks of using ZnO as the Zn source for the film coatings.

The research also aimed to develop methods using LA-ICP-MS and related techniques for the analysis of crop enhancement products more widely.

2.0. Analytical Techniques and Method Development

2.1. Analytical Techniques

2.1.1. Inductively Coupled Plasma Mass Spectrometry

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is a widely established and sensitive analytical technique, a type of mass spectrometry which uses a plasma to completely ionise the sample material. Almost all of the ions produced are positive ions, balanced by the loss of an electron. The sample is atomised creating ions, which are then detected. It is used to detect metals and can work at very low concentrations. It is also able to detect different isotopes of the same element, meaning it can be used for isotope ratio tracking or for detection of isotopic labels in samples.

The plasma is formed by inductively heating argon gas with an electromagnetic coil. Argon ICP temperatures are typically in the range of 5,500 to 6,500 K which is approximately equivalent to that of the surface of the sun. The plasma is sustained inside a quartz torch consisting of two concentric tubes, with a quartz injector inserted through the centre. The end of the torch is placed within the induction coil and supplied with a radio-frequency magnetic field. Argon flows between the two tubes of the torch at a flow rate of between 13 and 18 Lmin⁻¹. A brief electric current is applied to introduce free electrons into the argon stream. In the induction coil an alternating high frequency magnetic field (in excess of 27 million cycles per second) accelerates the electrons in one direction and then the other. The accelerated electrons collide with atoms of argon, causing them to lose an electron which is in turn accelerated and collides with another argon atom.

The plasma is sustained when the rate of release of new electrons caused by electron-argon collisions is balanced with the rate of recombination of free electrons with the generated argon ions. The resulting plasma is made up mainly of argon atoms, with a comparatively small proportion of electrons and argon ions. Alongside the high temperatures, the plasma also intensely emits ultraviolet light, so it is most commonly viewed via a mirror rather than directly.

The plasma is retained within the torch as the flow of argon gas through the outermost tubes of the torch keeps the plasma away from the walls of the torch. A second flow of around 1 Lmin^{-1} is introduced between the inner tube and the injector to keep the plasma away from the end of the injector. A third flow of argon travels through the injector, keeping a cooler channel down the centre of the plasma. Samples to be analysed are introduced through the injector, most commonly in the form of a mist of liquid.

Figure 3 gives a schematic of the principal components of the PerkinElmer NexION series ICP-MS instrument. The other commercially available ICP-MS systems have similar components, though they often use an ion mirror to deflect the ions instead of the quadrupole ion deflector.

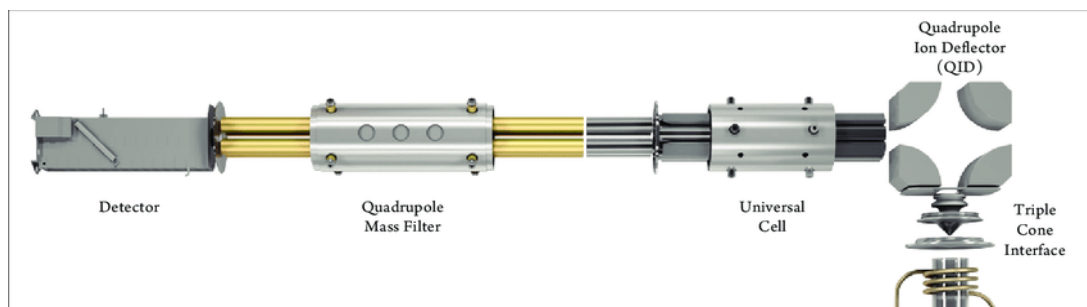


Figure 3: Schematic of the internal components of the PerkinElmer NexION ICP-MS to exemplify the workings of a typical ICP-MS instrument.

2.1.2. Mass Spectrometry

The generated ions pass into the mass spectrometer via a series of cones. The cones are most commonly made of nickel (although platinum is also used for some applications) and decrease in aperture size progressively which acts to focus the ion beam. The mass spectrometry is carried out by a quadrupole where the ions are separated on the basis of their mass-to-charge ratio (m/z) and the multiplier detector receives an ion signal proportional to the analyte concentration. As an alternative to quadrupole-based systems, time of flight ICP-MS systems are also available, in which ions are separated on the basis of the time of flight through a flight tube of fixed distance with and electric field of known strength.

2.1.3. Quadrupole mass spectrometry

The quadrupole mass filter consists of four parallel cylindrical rods and separated the mass-to-charge ratio of ions based on the stability of their trajectories in an oscillating magnetic field. Each opposing pair of rods are connected to each other electronically, an AC current is passed along them with a DC offset applied between one pair of rods and the other. The fluctuating current causes the ions to travel between the rods, only ions of a certain mass-to-charge ratio will reach the detector. Other ions have unstable trajectories and would collide with the rods. This mode of action allows for certain mass-to-charge ratios to be selected. When the instrument scans for that m/z ratio, only those ions will reach the detector. Figure 4 gives a schematic of the workings of a quadrupole.

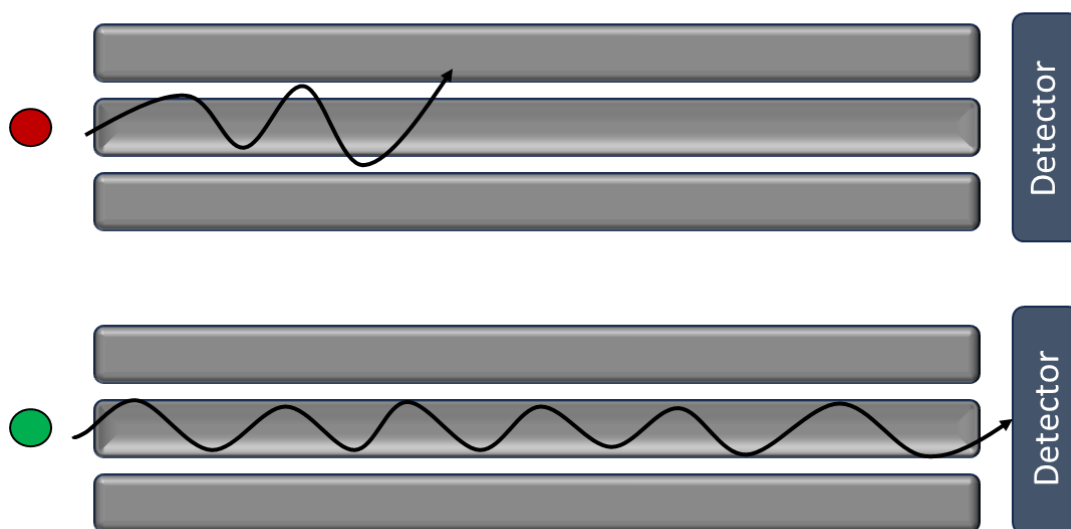


Figure 4: Schematic of the basic principle of a quadrupole when filtering charged ions according to mass to charge ratio (m/z).

2.1.4. Polyatomic interferences in ICP-MS

ICP-MS does have its challenges which must be considered and minimised where possible. Most critical is the possibility of polyatomic interferences forming in the plasma. These species which can form in the plasma have the same m/z ratio of an analyte of interest and can lead to a falsely high reading for that analyte. For example, the most abundant isotope of iron is ^{56}Fe . However, this has an interference with $^{40}\text{Ar}^{16}\text{O}$ since the plasma is an argon plasma this presents a serious challenge since it is not possible to determine whether the signal is being generated by ions of iron, or by ArO .⁸⁶

2.1.5. Mitigation strategies for polyatomic interferences in ICP-MS

One option would be to monitor for one of the less abundant isotopes of the analyte of interest, this comes with two risks, either the sensitivity may not be sufficient, or the alternative isotope may have interferences of its own. The second option is to introduce an inert collision gas, such as helium into the reaction cell (between the quadrupole and the detector). This is known as Kinetic Energy Discrimination (KED).⁸⁷ The helium gas flows in the opposite direction to the flow of ions and reduces the incidence of polyatomic interferences based on collision theory. Polyatomic species will be larger than individual ions, as such the He is more likely to collide with the polyatomic species, thus reducing their effect on the analysis. This does reduce the sensitivity of the analysis to the analyte of interest as their concentration will also be reduced by the He flow,

whether this is an issue will depend on the abundance of the analyte of interest in the sample.⁸⁸

Some ICP-MS systems have the capability to introduce a reactive gas into the reaction cell, the most commonly used being hydrogen, oxygen, and ammonia. In the case of ^{56}Fe , oxygen reaction can be carried out giving rise to a mass shift to $^{56}\text{Fe}^{16}\text{O}$, by monitoring at m/z 72 the polyatomic interference of $^{40}\text{Ar}^{16}\text{O}$ can be avoided.

2.2. Inductively Coupled Plasma Mass Spectrometry

Sample Introduction Methods

There are two main sample introduction methods for ICP-MS, namely solution mode and laser ablation mode. In solution mode, the analyte will be digested in acid, often nitric acid, before being introduced into the plasma via tubing and powered by a peristaltic pump. The second, less common sample introduction method is laser ablation, the sampling of solid material using a laser to generate particles which are introduced into the plasma.

2.3. Introduction to Laser Ablation

Lasers were first reported in the early 1960s, with early examples of the effects of laser energy on solid samples being reported shortly thereafter, notably by Ready et al. in 1965.^{89–92} It was around this time that laser-induced emission of ions and atoms from solid surfaces were first analysed using a mass spectrometer.⁹³

Sampling by laser ablation (LA) involves a laser beam being focussed on the surface of a sample to produce a plume of fine particles of ablated material. These particles are then transported using a carrier gas to the plasma of an ICP-MS.^{94–96} Most current generation LA systems will use helium (He) as a carrier gas, though some older systems may use Argon (Ar).⁹⁷ Once in the plasma the particles go through a number of processes, including ionisation before travelling through the mass spectrometer to the detector.⁹⁸

2.4. Coupling of Laser Ablation to Inductively Coupled Plasma Mass Spectrometers

By far the most common analysis method for laser ablation today is coupled with an ICP-MS. The laser is coupled to the injector of the ICP-MS, often via PEEK tubing. The ablated material from the solid sample is transported by a carrier gas direct to the injector of the ICP-MS and into the plasma, from here on the material is ionised and analysed in the ICP-MS as described in 1.1.⁹⁹

2.5. Fundamentals of LA-ICP-MS sampling for imaging

To generate LA-ICP-MS images, the laser scans along the tissue at a given scan rate. The scan rate is set according to the duty cycle of the ICP-MS. As the laser scans, continuously firing, the plumes of material are transported into the ICP-MS as they are generated, and the signals recorded. Once the timings of the signal of the ICP-MS have been mapped to the timings of the laser firing and location relative to the tissue have been aligned, elemental images can be generated using processing software.

2.6. *Laser Ablation Hardware*

At the outset of the project, the Laser Ablation unit installed in the lab was an aging New Wave Research 213 nm Laser Ablation system. The system was manufactured in 2004, and the world of laser ablation has come a long way between then and now. In the final year of the project, the laser system was replaced with a brand new imageBio266 with a wavelength of 266 nm (Elemental Scientific Lasers, Bozeman, MT, USA). The new system had a number of important improvements over the original system. The 213nm system had used argon as the carrier gas, whilst the 266 nm system used helium as the carrier gas. Helium is a much more efficient carrier gas than argon, it has been shown that the maximum sensitivity at the ICP-MS can be increased by up to a factor of three when using helium as a carrier gas as opposed to argon. This increase in sensitivity allowed elements of lower concentration to be detected, whilst also allowing smaller spot sizes to be used for analysis, thus increasing the resolution of the images generated. The 266nm system also had advantages in terms of the range of spot sizes available, enabling a minimum spot size of 1 μm as opposed to 4 μm . In reality the 213nm did not offer sufficient sensitivity to reach spot sizes as low as 4 μm , the lowest spot size used with sufficient sensitivity to generate images was 6 μm . Due to the improved design of the sample chamber, the 266 nm system had a much larger sample chamber, whilst also having a much faster sample washout time than the 213 nm system. This sped up analysis in two ways, firstly the sample washed out more quickly, so the laser could scan faster, and the ICP-MS could acquire data more quickly. Secondly, multiple samples could be analysed consecutively without the need to open and re-purge the chamber,

meaning less downtime between samples. The speed of washout had a compounding effect on the overall analysis. Due to the longer washout time of the 213 nm system, the image had to be generated from a series of individual lines, with a 30 second warm up time at the beginning of each line and a 40 s washout time at the end of each line. The fast washout of the newer chamber design enabled the whole sample to be analysed in one continuous acquisition, with just one warm up at the beginning of the raster. The washout time and transport efficiency of the 266 nm system was especially strong as it was fitted with an optional TwoVol 3 ablation chamber which was capable of washout times of less than 1 ms. As it was set up in the lab the washout time was around 30 ms. The chamber fitted to the original 213 nm system was around 30 s. The system was also fitted with a Dual Concentric Injector (DCI). This reduces the number of connections in the tubing and delivers the plume of ablated material direct to the end of the injector at the base of the plasma, using an additional flow of argon to improve transport efficiency. It has the additional benefit of keeping ions 'on axis' so they are more efficiently transmitted through the ICP-MS giving an improvement of signal intensity. The laser wavelength itself offered benefits on samples which were mounted on glass slides, 266 nm laser energies are much less likely to ablate the glass on which the samples is mounted than 213 nm lasers, enabling complete ablation of the sample, without the risk of the metals in the glass causing interference when ablated.

2.7. Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Inductively coupled plasma optical emission spectroscopy (ICP-OES) is an alternative argon plasma-based technique for the analysis of chemical elements. Ions are generated by aspirating the sample into the plasma in much the same way as in ICP-MS. The detection differs in that identification is based on the wavelength of light emitted by excited ions as they return to their ground state, quantification is based upon the intensity of that light.

2.8. Comparison of ICP-MS and ICP-OES

ICP-MS has a number of advantages over ICP-OES. ICP-MS is highly specific due to the identification of ions by mass to charge ratio. In ICP-OES the wavelength range of similar ions can overlap slightly, making absolute identification challenging.¹⁰⁰ Secondly, ICP-MS is capable of greater absolute sensitivity than ICP-OES.¹⁰¹ It is generally considered to be the case that where the concentration of analyte in the sample is consistently below 10 ppb, the best option would be to use ICP-MS. Take for example a comparison of the Agilent 5110 ICP-OES and the equivalent Agilent 7900 single quadrupole ICP-MS system. The dynamic range of the OES system is given as 10ppb-10000 ppm, whereas the dynamic range of the ICP-MS system is given as <1ppt-1000ppm. Another benefit of ICP-OES is that the instruments are much cheaper to buy and have lower ongoing running costs than and ICP-MS system as they can run on lower purity argon than the 99.99% pure argon required by the ICP-MS system.¹⁰²

2.9. Scanning Electron Microscopy

The Scanning Electron Microscope (SEM) is designed for the analysis of microstructure morphology of a sample. SEM works by replacing the light energy which would be used in optical microscopy with a high-energy electron beam.¹⁰³ The surface of a sample is imaged by scanning it with a focussed beam of electrons. The electrons interact with the atoms of the sample, producing signals which inform on the topography and composition of the sample. Most commonly, secondary electrons which are emitted by the sample atoms following excitation by the focussed electron beam are detected using a secondary electron detector. Some SEMs can achieve a resolution of less than one nanometer.¹⁰⁴

2.10. Energy Dispersive X-ray Spectroscopy

Energy dispersive x-ray spectroscopy (EDX) is an analytical technique used to determine the composition of a solid material. The excitation of electrons close to the nucleus caused more distant electrons to drop energy levels to replace them, as the vacant energy states are filled x-ray radiation is emitted. Each element emits x-rays of differing frequencies and so these emission enable the elemental identification of the sample.¹⁰⁵

3.0. Method Development

3.1. Seed material sample preparation

Significant advances in the sample preparation of seed material for laser ablation analysis made throughout the project. Due to the small amount of research which had been carried out previously on seed analysis by LA-ICP-MS, there were very few established protocols on which the methodology could be based. Prior work within the group had prepared samples by embedding seed in gelatin or CMC. Seeds were snap frozen and embedded in the gelatin or CMC before being cut into 20 µm thick sections using a Cryostat. This has been shown to be successful, however the sectioning is not a straightforward process. Due to the difference in density between gelatin and seed tissue, during sectioning separation can occur between the gelatine and the seed, causing the sample to be lost. Furthermore, where sections are successfully generated, due to gelatine being water-based, there can be interference on the images generated, showing up as a fuzziness or hazing around the periphery of the seed. To counter this, a number of alternative options were investigated. The first was embedding in paraffin wax and microtome sectioning. However, this led to the same incompatibility meaning no sections were successfully generated by this method. The final method was to embed the seed within a fast-setting two-part epoxy resin. Once set, the resin block was ground down using sandpaper (P400 followed by P800) to reveal the seed sections. This method not only alleviated the difficulties associated with microtome or cryostat sectioning, but it also generated images which were much sharper as there was no water interference around the edge of the seed. Examples are given in section 3.3. The secondary advantage of this method was

that multiple seeds could be mounted within one resin block, meaning it would be possible to carry out multiple repeats without having to open, and subsequently purge the ablation chamber on the laser ablation system.

The resin blocks were mounted into a plastic mould of 25 mm internal diameter. This was necessary to enable the resin block to be mounted into the sample holder supplied with the ImageBio 266 laser ablation system. These were custom fabricated by cutting 25 mm PVC conduit into 13 mm lengths using a lathe to match the diameters of the commercially available ESL sample moulds. Resin embedded seed and the sample holder are shown in figure 5.

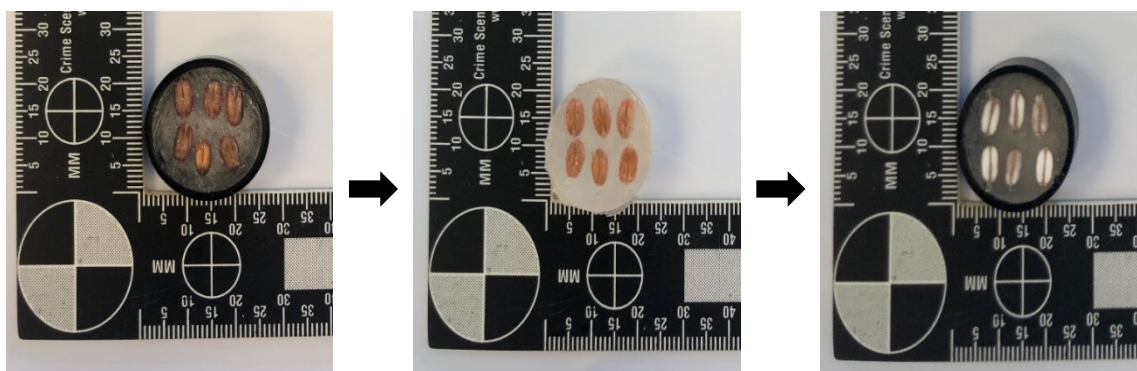


Figure 5: Images taken from the epoxy embedding of wheat seeds for analysis by LA-ICP-MS. The hardened epoxy block is removed from the sample mould, sanded down using P400 and P800 sandpaper, before being returned to the mould for mounting into the sample chamber.

3.2. *Laser Ablation Method Considerations*

For reliable LA-ICP-MS data it is imperative that the laser ablation parameters to be used are optimised. First amongst which is the laser spot size. The spot size must be selected based on the size of the tissue and the features you wish to resolve. A smaller spot size will increase the resolution of the image but will reduce the signal intensity and increase the analysis time. The user can often adjust the shape of the ablation spot. Where possible it is preferable to use a square ablation spot. This ensures the tissues receives the same dosage across the ablation line and ensures that sampling is always taking place from the same X position. It is vitally important that the laser power (fluence) is selected appropriately. The laser fluence being too low will lead to incomplete sample ablation and low signal intensities. The laser fluence being too high can introduce noise to the signal which will negatively affect the signal to noise ratio. Excessive fluence can also lead to ablation of the glass slide on which the sample is mounted. The laser fluence will be optimum at the point at which the signal from your elements of interest is high but there is no ablation of the glass slide. The laser wavelength of the LA system can have a noticeable impact on the propensity of the laser to ablate glass. The longer the laser wavelength, the less likely the laser is to ablate glass at a given fluence level. This is to say that at a higher laser wavelength, you could use a higher fluence rate without ablating glass than you could with an equivalent system with a shorter laser wavelength. Another consideration is the laser repetition rate. This refers to the number of laser shots per second i.e., the frequency of laser shots. The higher the repetition rate (measured in Hz) the more laser shots per second. The optimal repetition

rate is selected based on the hardness of the tissue; harder samples will often require a higher laser repetition rate. The repetition rate will also affect the amount of ablation at a given laser power, so some degree of optimisation between the laser power and repetition rate is likely to be necessary. For the highest quality LA-ICP-MS images, it is imperative that the scan rate (SR) is calculated correctly. The aim is for the laser to travel the distance of one laser spot diameter within a single duty cycle (DC) of the ICP-MS. This will generate square pixels, which improves the quality of the final image.

To calculate the scan rate, the following equation is used:¹⁰⁶

$$\text{scan rate } (\mu\text{m/s}) = \text{spot size } (\mu\text{m}) / \text{ICP acquisition time (s)}$$

Equation 1: Calculation of best scan speed for LA-ICP-MS imaging

If the scan rate is too slow, the ablations will overlap, causing the same area to be analysed more than once. The pixels will be narrow, making the image difficult to view. Conversely, where the scan rate is too fast, there will be undersampling of the tissue. The pixels will be stretched and will not represent the laser sampling accurately.¹⁰⁷ A schematic to illustrate the importance of scan rate is given in figure 6.

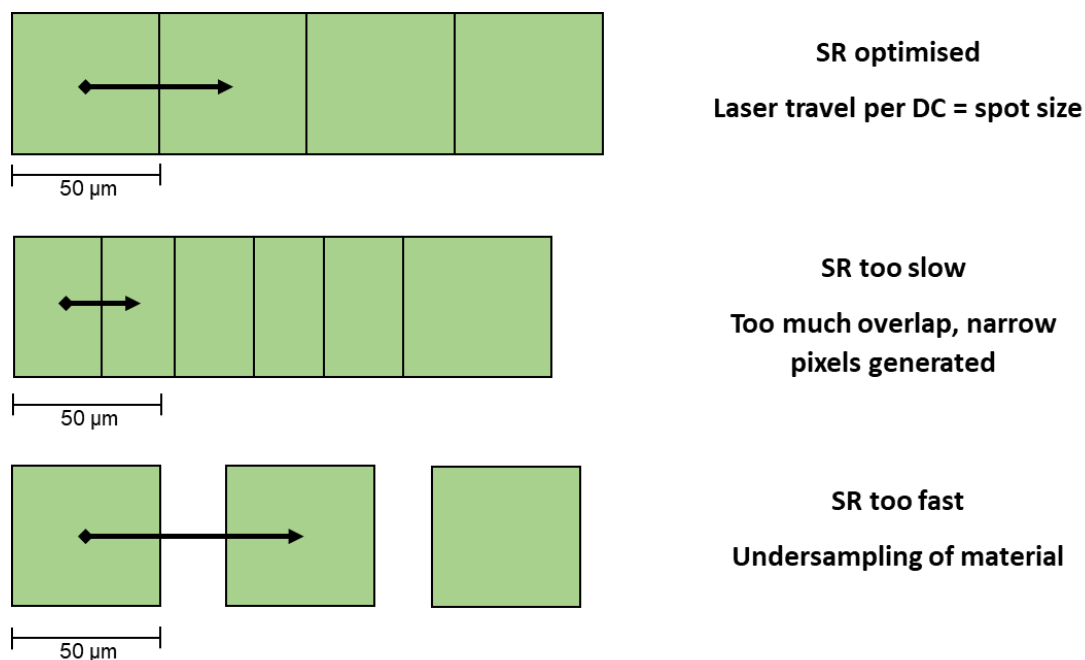


Figure 6: Schematic to illustrate the importance of selecting the correct scan rate for LA-ICP-MS imaging experiments.

3.3. Development of LA-ICP-MS image quality throughout the project

Throughout the project, a large amount of progress was made to improve the quality of the data generated and by extension the quality of the images generated. These improvements were brought about by a combination of factors. There were improvements to the sample preparation methods, and improvements in the understanding of laser method optimisation as outlined in 2.5, as well as the move to the new laser ablation unit. The faster sample chamber washout time of the ImageBio 266 laser ablation system compared with the

previous New Wave Research 213 nm unit, enabled much faster imaging meaning a smaller spot size could be used to give higher resolution images. The final improvement came from the upgrade of the ICP-MS software. The original software was limited to 25,000 data points per run. This limitation meant that images had to be acquired line by line, once updated the maximum number of data points per run was increased to 250,000 which meant that images could be acquired in one run using a raster technique. This meant there was no need to wait for the ablation chamber to wash out between lines, vastly decreasing total sampling time.

Figure 7 gives an example of the images generated at the outset of the project. These were generated using a 100 μm laser spot and took an average of 2 hours to collect the raw data.

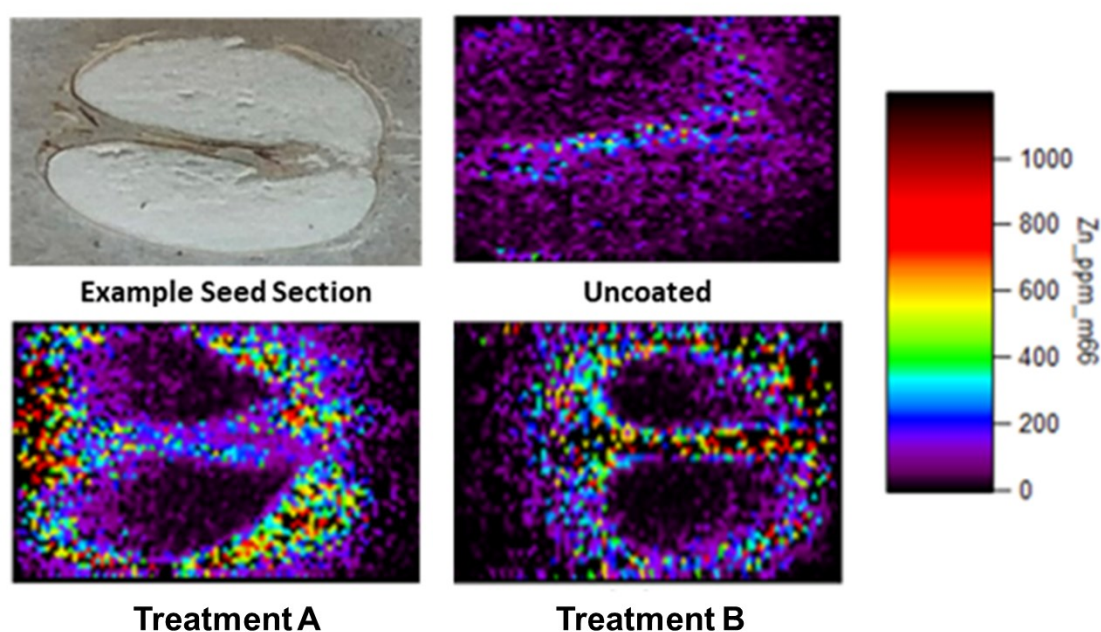


Figure 7: Examples of LA-ICP-MS images of gelatin embedded wheat seed generated using the UP213 laser ablation system from New Wave Research, now Elemental Scientific Lasers (Bozeman, MT, USA).

The images presented in figure 7 appear to have a fuzziness around the seed periphery. We hypothesised that this may be because the seeds were embedded in gelatin and sectioned. Gelatin is made up with 95 % water, this may interfere in two ways, the first would be that the water, if not completely dried would interfere with the ablation of the laser – leading to surface boiling as opposed to ablation of particles. Secondly, embedding the seed in gelatin could cause diffusion of the seed coating active ingredients away from the seed surface and into the surrounding gelatin.

Figure 8 gives an example of the issues which can be faced when ablating a material with a high water content, this time Agar.

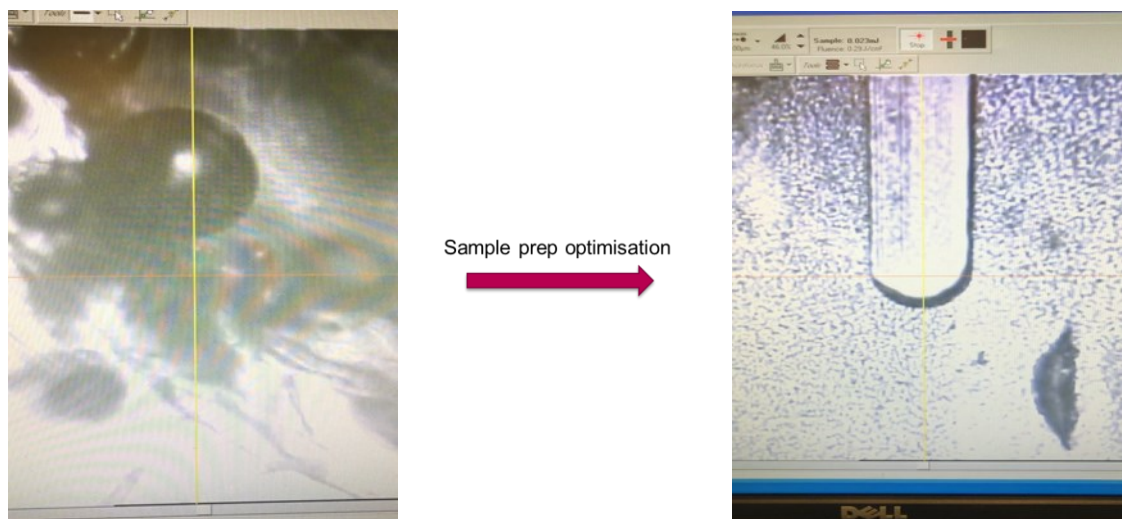


Figure 8: Examples of the interaction of the laser beam with agar sample matrix, showing how water-based matrices can negatively affect ablation, and what a good ablation of such a matrix would look like.

The two examples demonstrate the issues which can be faced when analysing high water matrices, as well as what a good ablation should look like when the sample preparation has been optimised. On the left we see large bubbles being formed as the water boils off on contact with the laser, with no visible ablation crater. On the right, when the sample preparation had been optimised we see a clean ablation crater with sharp edges, showing no signs of the laser interfering with the surrounding matrix. In this case the ablation characteristics were optimised by ensuring that the sample was flash frozen in liquid nitrogen before being sectioned and prepared for analysis.

Although the issue of water interfering with the ablation could be worked around, there was still a concern embedding treated seed in a water-based matrix could lead to interference of the matrix with the seed treatment. This was mitigated by embedding seeds in epoxy resin, as shown in figure 5. Examples of the image quality generated at the end of the project, after the hardware and method improvements mentioned in this chapter, are given in figure 9.

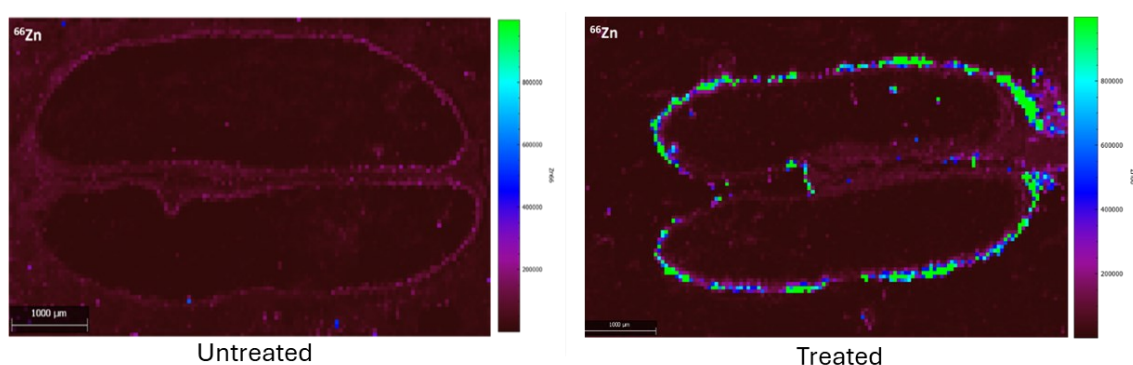


Figure 9: Examples of LA-ICP-MS images of epoxy embedded wheat seed sections generated using the imageBio 266 from Elemental Scientific Lasers (Bozeman, MT, USA).

The faster sample washout and laser repetition rate meant that imaging could be carried out at a smaller spot size, in a shorter time, giving greater image resolution. These images were generated using a 50 μm spot as opposed to the 100 μm spot used for the images in figure 7. Furthermore, due to the images being prepared in epoxy, there was no water interference around the seed periphery, with the images sharply portraying the edge of the seed section.

Page left intentionally blank

4.0. Seed Treatment Mechanism of action

4.1. Introduction

Seed coatings are chemical solutions applied to the surface of seeds to provide protection, promote seedling establishment, and facilitate nutrient uptake.¹⁰⁸

There are several forms of seed coating in the market, this work focussed on the use of a water-based Zn-containing film seed coating. Film coating was originally developed in the pharmaceutical industry, before being adapted for seed coatings. The aim is to uniformly apply a thin layer of active ingredient, with as little modification of the size and shape of the seed as possible in the process.⁵⁹

The intention of the seed coating in this case was to increase the Zn content of the seedling as it germinated and established itself. The hypothesis was that the Zn would be released into the root zone surrounding the seed before being taken up via the roots in the form of Zn^{2+} . It has been reported that the delivery of nano-zinc oxide particles through a seed coating leads to increased germination percentage, improved root growth and similar crop growth with nano-zinc oxide as with zinc sulphate application.¹⁰⁹

Adjuvants in agriculture are surfactants which, when included in agrochemical products, increase the effectiveness of the active ingredients through a variety of mechanisms.¹¹⁰ The inclusion of adjuvants in the seed coating formulation sought to increase the efficacy of the Zn uptake, either by retaining the Zn in an available form in the root zone to enable uptake, or by increasing the Zn uptake itself. The adjuvants selected were a range of Croda proprietary adjuvants which have

previously been shown to have a beneficial effect on the uptake of active ingredients in other crop care applications.

In practice, seeds are most often provided to farmers in a pre-treated form. Therefore, there is often a period of storage of the treated seeds prior to sowing. Little is known about the effect this period of storage would have on a coated seed and whether there would be any reduction in the efficacy of the seed coating over time, for example by movement of the Zn into the body of the seed itself, rather than remaining on the seed surface to be available for root uptake after sowing. It was also not known whether the inclusion of adjuvant within the seed coating formulation would influence the localisation of the Zn, when combined with a period of storage.

A seed washing method has been developed to remove the Zn from the seed surface after a period of storage following treatment. Any Zn remaining on the seed following the complete removal of Zn from the surface to which it was applied would be an indication that the Zn had, by some mechanism, been transported into the endosperm of the seed rather than remaining on the seed surface. Or that it was not completely removed from the seed surface during the washing process.

To investigate this, following storage and washing at selected time points, washed seed was digested and prepared for bulk elemental analysis. This allowed the Zn concentration within the seed tissue to be analysed and compared to the untreated and treated controls. To provide insight as to the localisation of any latent Zn following the washing process, samples of the seeds were prepared as longitudinal sections for bio-imaging by LA-ICP-MS. To our knowledge, there

are no previously published studies on the washing of treated seeds to remove or study the effect on the active ingredients contained within the seed coating formulation.

4.2. *Aims of the chapter*

The aim of the experiments presented in this chapter was to determine the extent of Zn movement into the body of the seed following the application of ZnO in the form of a Flowable suspension seed coating formulation to the seed surface. This chapter also aimed to investigate the extent to which a period of storage following seed treatment would affect the distribution, and by extension the efficacy and mechanism of action of the Zn-containing seed treatment.

4.3. Methods

4.3.1. Seed variety selection

Winter wheat was chosen as the model crop for the work presented within this thesis. As discussed in 3.1.5, wheat is a crop of global importance, as well as a crop which is widely treated with seed treatments in modern agriculture. KWS Zyatt (KWS, Cambridge, UK) was selected due to being one of the most popular grade 1 wheat varieties used in the UK. It also has very consistent growth characteristics from season to season.¹¹¹

4.3.2. Development of Washing Methodology

A methodology has been developed to determine the localisation and mechanism of uptake of Zn in wheat seeds treated seed treatment formulations containing Zn and a range of adjuvants which involved washing the treated seed to see if all of the coating could be removed. A number of solvents were evaluated to develop the seed washing methodology. The tested solvents were: 10 % nitric acid (derived from AnalaR NORMAPUR analytical grade 69 % Nitric acid, VWR, UK), deionised water, acetone ($\geq 99\%$, VWR, UK), ethanol ($\geq 99.8\%$, VWR, UK), and DCM (VWR, UK). To determine which would remove the Zn from the seed coat, seeds were washed in each solvent under agitation for 24 hours. The seeds were then removed, and the solvents were retained for analysis by ICP-MS. To prepare the samples for analysis by ICP-MS, the solvents were evaporated using a rotary evaporator set to $60\text{ }^{\circ}\text{C}$. The resulting solution was then diluted in 5% Nitric acid and aspirated to the ICP-MS. The Zn content of the residue of each

solvent was then determined to measure the effectiveness, or zinc removing power' of each solvent.

4.3.3. Washing of Treated Seeds

Treated seeds were washed for 24 hours in 10 % nitric acid with agitation on a rotary mixer at 60 rpm. The seeds were then removed from the nitric acid and allowed to air dry in a fume hood for a further 24 hours. Once dried, the seed material was then ground using an electric coffee grinder (Morphy Richards, Swinton UK) for 60 seconds to a homogenous powder. The seed material was then digested in triplicate by microwave digestion in 69 % nitric acid (AnalaR NORMAPUR analytical grade 69 % Nitric acid, VWR, UK) and 30 % hydrogen peroxide (Merck, UK) before being diluted in ultrapure deionised water (18.2 MΩ) for analysis by ICP-OES.

4.3.4. 12-month washing experiment design

To investigate the mechanism of uptake of seed-applied zinc into wheat, a 12-month washing experiment was designed. A large batch of winter wheat seed were treated using the formulation outlined in 1.4. Samples were then taken at time points to be digested and analysed by ICP-MS. The time points selected were: 24 hours, 6 weeks, 3 months, 6 months, 9 months, and 12 months. Bulk ICP-MS data gathered at each time point was supported by LA-ICP-MS images of seed sections, both freshly coated and after 12-months. As well as LA-ICP-MS images of aged, washed seeds. The experiment included seeds treated with the base ZnO formulation (referred to as 'Ref' or 'Reference') alongside seeds treated with the base ZnO formulation with added adjuvant (adjuvants A-

E).

4.3.5. Sampling of Seed

At each time point 5 g of seed were randomly sampled to be washed from the stock of treated seed. The seed was stored for the 12-month duration of the experiment in large Ziplock plastic bags to prevent the ingress of moisture. The stock of seed consisted of 200 g of seed with each treatment initially, with 5 g sampled at each timepoint. Once the 5 g had been washed, it was all ground. Of the ground seed material, three 0.2 g (± 0.05 g) samples were weighed out using a 5-figure balance to be microwave digested and analysed.

4.3.6. Equation for the calculation of mg/Kg values

The following equation was used to calculate the elemental content within the digestion material:

$$mg/Kg \text{ analyte} = \left(\frac{(sample \text{ conc. (mg/L)} - Digestion \text{ blank (mg/L)}) \times Dilution \text{ factor}}{(1000 \div Digestion \text{ volume (mL)} \times sample \text{ mass (g)})} \right) \times 1000$$

Equation 2: Equation used to convert mg/L values from ICP-MS/ICP-OES analysis of digested material, to elemental content in mg/Kg.

4.3.7. Preparation of Seed for LA-ICP-MS Analysis

Seeds were embedded in a two-part, fast setting epoxy resin (Diall, UK) in a custom-fabricated mould of 25 mm internal diameter such that the epoxy disk, once set, could be securely fitted into the sample holder of the laser ablation unit. Six seeds were added to each of the moulds. The moulds were produced by precision cutting 25 mm PVC tubing into disks of 13 mm tall, figure 5 shows the custom moulds in use. Once set the epoxy block was ground down using P400

sandpaper to reveal the seed sections. Once the sections were revealed, the blocks were further sanded using P800 sandpaper to prepare the surface for ablation. Once sanded, the blocks were cleaned using acetone followed by deionised water.

4.3.8. Laser Ablation Parameters

Laser ablation was carried out using an ImageBio 266 laser ablation unit (Elemental Scientific Lasers, Bozemann, MT, USA) with a 266 nm diode-pumped solid-state laser and a TwoVol 3 fast washout chamber set-up in analytical mode. Helium was used as the carrier gas for optimised sample transport efficiency at a flow rate of 600 mLmin⁻¹. The imaging was carried out in 'image mode' using a raster to select the area to be ablated using left to right scanning. Ablation was carried out using a 50 x 50 µm square laser spot, with a raster space of 50 µm and a laser repetition rate of 200 Hz. The laser power was set to 40 % which equated on this set-up to ~ 3 Jcm⁻¹ laser fluence. The scan rate was 240 µms⁻¹.

4.3.9. ICP-MS settings for Imaging

The LA unit was coupled to a NexION 350x single quadrupole ICP-MS (PerkinElmer, Waltham, MA, USA) via an Elemental Scientific Dual Concentric Injector (DCI). The analysis was carried out in 'standard' mode, meaning no collision or reaction gases were used. The isotopes acquired were: ⁶³Cu, ⁵⁷Fe, ⁵⁵Mn, ⁶⁶Zn and ²⁵Mg. The dwell time for each isotope was set to 5 ms with 8 sweeps per reading giving a total duty cycle of 0.208 s. The number of readings

per analysis varied with the overall length of the raster pattern of the laser ablation.

4.3.10. *Generation of spatially resolved LA-ICP-MS Images*

Images were generated using iolite v4 software (Elemental Scientific, Bozeman, MT, USA). Iolite combines the laser log information from the laser ablation unit with the mass spectral data from the ICP-MS in the form of an NC file. The laser log file contains information on the location of the laser spot at a given point in time, the mass spectral data for the same time point can then be integrated against the baseline to calculate the signal intensity for the isotope of interest at that given time-point. Using this data, a colour-coded signal intensity map is generated. Separate images were generated for each of the isotopes selected in the ICP-MS method.

4.3.11. *Development of LA-ICP-MS Capabilities*

At the outset of the project, the Laser Ablation unit installed in the lab was an aging New Wave Research 213 nm Laser Ablation system. The system was manufactured in 2004, and the world of laser ablation has come a long way between then and now. In the final year of the project, the laser system was replaced with a brand new imageBio266 with a wavelength of 266 nm (Elemental Scientific Lasers, Bozeman, MT, USA). The new system had a number of important improvements over the original system. The 213nm system had used argon as the carrier gas, whilst the 266 nm system used helium as the carrier gas. Helium is a much more efficient carrier gas than argon, it has been shown

that the maximum sensitivity at the ICP-MS can be increased by up to a factor of 3 when using helium as a carrier gas as opposed to argon. This increase in sensitivity allowed elements of lower concentration to be detected, whilst also allowing smaller spot sizes to be used for analysis, thus increasing the resolution of the images generated. The 266nm system also had advantages in terms of the range of spot sizes available, enabling a minimum spot size of 1 μm as opposed to 4 μm . In reality, the 213nm did not offer sufficient sensitivity to reach spot sizes as low as 4 μm , the lowest spot size used with sufficient sensitivity to generate images was 6 μm . Due to the improved design of the sample chamber, the 266 nm system had a much larger sample chamber, whilst also having a much faster sample washout time than the 213 nm system. This sped up analysis in two ways, firstly the sample washed out more quickly, so the laser could scan faster, and the ICP-MS could acquire data more quickly. Secondly, multiple samples could be analysed consecutively without the need to open and re-purge the chamber, meaning less downtime between samples. The speed of washout had a compounding effect on the overall analysis. Due to the longer washout time of the 213 nm system, the image had to be generated from a series of individual lines, with a 30 second warm up time at the beginning of each line and a 40 s washout time at the end of each line. The fast washout of the newer chamber design enabled the whole sample to be analysed in one continuous acquisition, with just one warm up at the beginning of the raster. The washout time and transport efficiency of the 266 nm system was especially strong as it was fitted with an optional TwoVol 3 ablation chamber which was capable of washout times of less than 1 ms. As it was set up in the lab the washout time was around 30 ms. The chamber fitted to the original 213 nm system was around 30 s. The system

was also fitted with a Dual Concentric Injector (DCI). This reduces the number of connections in the tubing and delivers the plume of ablated material direct to the end of the injector at the base of the plasma, using an additional flow of argon to improve transport efficiency. It has the additional benefit of keeping ions 'on axis' so they are more efficiently transmitted through the ICP-MS giving an improvement of signal intensity. The laser wavelength itself offered benefits on samples which were mounted on glass slides, 266 nm laser energies are much less likely to ablate the glass on which the samples is mounted than 213 nm lasers, enabling complete ablation of the sample, without the risk of the metals in the glass causing interference when ablated.

4.3.12. *Micro-CT Analysis of Seeds*

Micro Computed Tomography (micro-CT) is a high resolution, non-destructive imaging technique based on x-rays. It can be used to generate a 3D image of a sample, without destroying it. The technique is sensitive to differences in relative density, which meant it may be useful for imaging the localisation of applied ZnO particles on the surface of the seed. Micro-CT was performed using a SkyScan 1272 (Bruker, MT, USA). Seeds were partially embedded in a malleable dental wax before being mounted securely onto the stage. No filter was applied, and the spot size was set to 5.19 μm . The source voltage was set to 35 kV and the source current was 200 μA . The rotation step was set to 0.250 ° and scanning was carried out through a full 360 ° cycle.

4.3.13. *Thresholding of micro-CT data*

Thresholding on the acquired whole seed micro-CT images was carried out using Batch Analyser software (Bruker, DE). The threshold was adjusted for each image such that the density which was common to both was removed, leaving only the areas of density which were present in the treated seeds but not in the untreated seeds. The threshold used was a minimum of 190 AU for both the treated and untreated seed.

4.3.14. *Scanning Electron Microscopy Imaging of Treated Wheat Seeds*

Scanning Electron Microscopy (SEM) is a surface technique which scans the topography and composition of a sample using a beam of electrons. The electrons interact with the atoms in the sample producing signals which can be interpreted to create 2D images of the sample surface. SEM analysis was carried out by Francis Sweeney of the Materials Engineering Research Institute at Sheffield Hallam University. Treated seeds were prepared and supplied by the author, SEM analysis and generation of images presented within this chapter was carried out by Francis Sweeney using a Philips XL-30 ESEM-EDX (Philips, Amsterdam, NL).

4.3.15. *Energy Dispersive X-ray Spectroscopy imaging of treated wheat seeds*

Energy Dispersive X-ray Spectroscopy (EDX) is carried out alongside SEM. Upon interaction with X-rays each unique atomic structure on the sample gives a unique

set of peaks on an electromagnetic emission spectrum. EDX was used to confirm whether the particles detected by the micro-CT were, indeed, Zn based. EDX imaging of wheat seeds was carried out by Francis Sweeney of the Materials Engineering Research Institute at Sheffield Hallam University. Treated seeds were prepared and supplied by the author, EDX analysis and generation of images presented within this chapter was carried out by Francis Sweeney.

4.3.16. Development of Washing Methodology

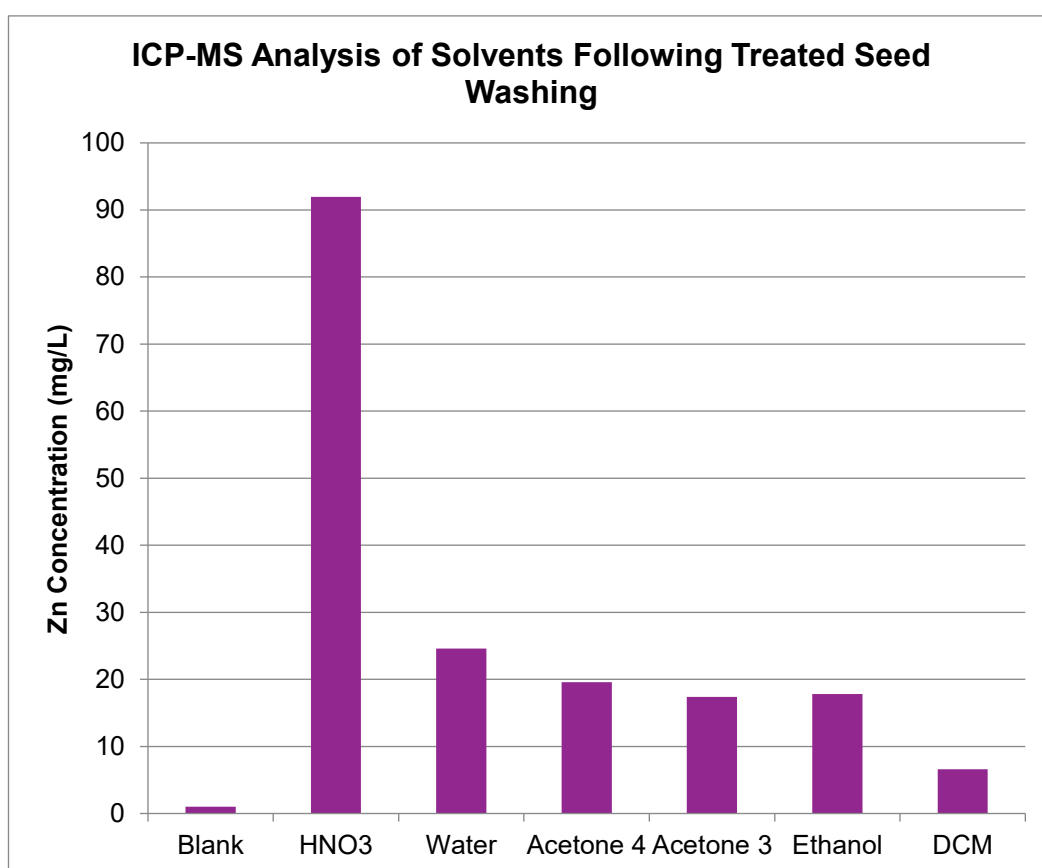


Figure 10: A representation of the Zn concentration in mg/L (y-axis) of a range of solvents (x-axis), analysed after they had been used to ‘wash’ ZnO-treated wheat seeds carried out by ICP-MS in solution mode.

Seeds were washed using a wide range of solvents, the wash solutions were then retained and analysed by ICP-MS to determine the Zn content of the wash solutions with results given in figure 10. The more Zn present in the wash solution, the more Zn had been removed/washed from the seed surface. The results show that water, acetone, and ethanol contained similar levels of Zn. The DCM wash solution contained a noticeably lower concentration of Zn compared with the other solvents tested. Nitric acid, however, contained a much higher concentration of Zn compared with the other solvents tested, suggesting it had been much more effective in removing Zn from the seed surface upon washing than any of the other solvents.

This result is consistent with the literature since it has been widely documented that ZnO solubility increases with decreasing pH.¹¹²

Although washing seeds in acid is not directly representative of the soil environment, the majority of agricultural soils around the world are at least slightly acidic.^{113,114} A collation of European soil data, carried out on behalf of the European commission showed that the vast majority of soils in western Europe had an average pH value of less than 6.5, with the exception of some areas in south east Spain and southern Italy.¹¹⁵ It is proposed that whilst the Zn may not be stripped from the seed surface as efficiently in a slightly acidic soil as it is in aqueous Nitric acid, the process would be chemically similar and the method is therefore representative.

4.4. Results

4.4.1. Washing of Treated Seeds

The results of the analysis of washed seed material by ICP-OES are given in figure 11.

Zn Content of Treated Seeds After 24 Hr Wash in HNO₃ - Analysed Using ICP-OES

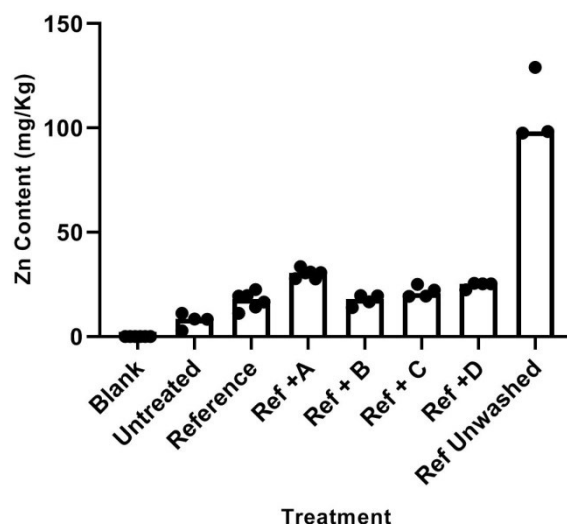


Figure 11: Zn content of ground and acid digested material from seeds treated with the ZnO formulation (reference) and the same formulation with added adjuvant (A-D) after they had been washed for 24 hours in nitric acid. As well as digested unwashed reference seed as a positive control. The analysis was carried out by ICP-OES. Each data point corresponds to one repeat, the height of the bar corresponds to the median value.

The analysis by ICP-OES of washed seed material data showed that treated seeds which were unwashed were significantly higher in Zn than the treated washed seed and the untreated washed seed. This suggested that the washing methodology had significantly reduced the level of Zn contained within the treated seed. However, there were differences between the untreated seed and the

treated washed seed. This suggested that there was some zinc retained by the seed after the washing had been carried out.

Zn Content of Treated Seeds After 24 Hr Wash in HNO₃ - Analysed Using ICP-OES

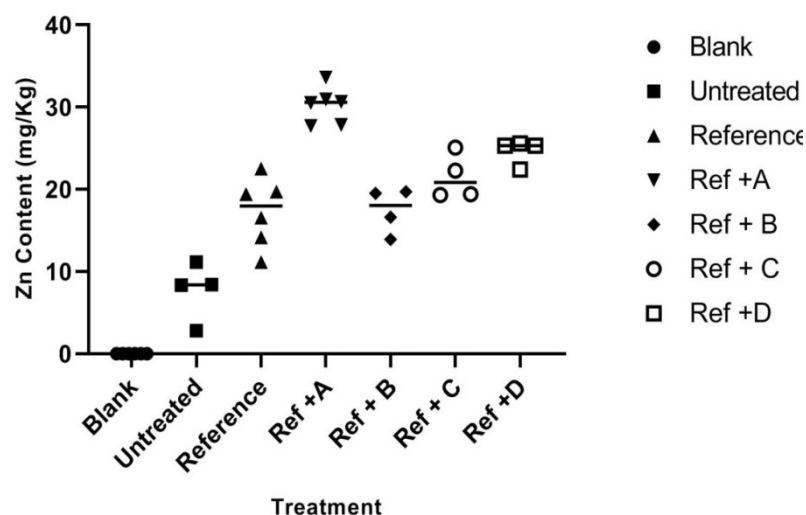


Figure 12: Zn content of ground and acid digested material from seeds treated with the ZnO formulation (reference) and the same formulation with added adjuvant (A-D) after they had been washed for 24 hours in nitric acid. The analysis was carried out by ICP-OES. Each data point corresponds to one repeat, the height of the bar corresponds to the median value.

The plot from figure 11 is shown in figure 12 with an adjusted scale to examine the differences in the Zn content of the different treated seeds compared with the untreated control. Each treatment retained more Zn than the untreated control. There appeared to be some differences in the Zn retained by the seeds with some treatments compared to others. Ref + A and Ref + D had retained more Zn than the Ref, Ref + B and Ref + C. This suggested either there had been movement of Zn into the endosperm of the seed where it would not be removed during the washing process, or the Zn was not fully stripped from the seed surface by the washing process. The differences between treatments containing different

adjuvants suggested that the presence of adjuvant may influence either the movement of Zn into the seed or the reduced efficiency of the removal of the seed treatment from the surface.

4.4.2. Washing and Analysis of Treated Seeds Over 12 Months

The calculated mg/Kg value of Zn was determined for the washed seed of each treatment and compared to an untreated control and a treated, unwashed control (Ref control). The collated data for each timepoint is given in figure 13.

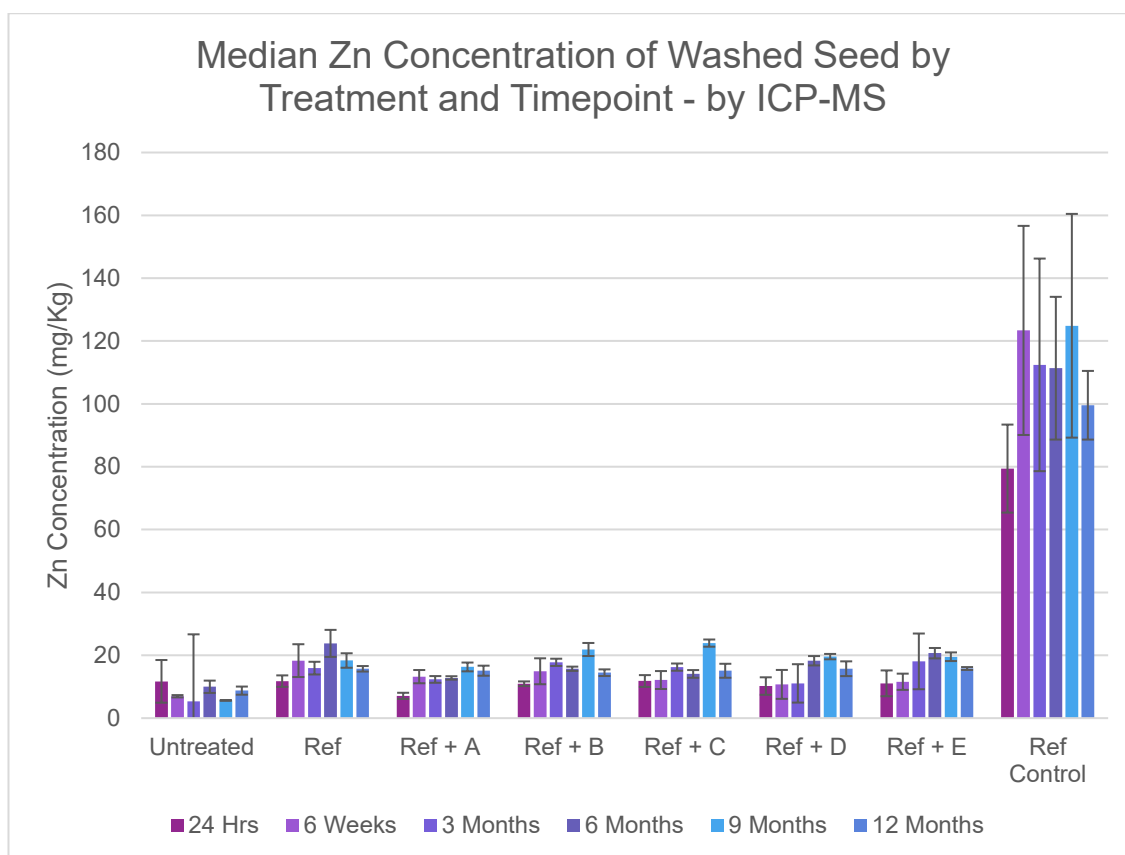


Figure 13: A plot to show the median Zn concentration in acid-washed samples of each of the seed treatments, at each of the timepoints, compared to the unwashed reference control. The height of each bar corresponds to the median value and the error bars depict the standard deviation between the repeats. The analyses were carried out by solution mode ICP-MS.

The Zn concentration which was measured in the untreated seed at each timepoint was largely consistent. Whilst there was some slight variation in the measured Zn concentration there was no significant difference in the Zn concentration at each of the measurement points. As you would not expect the Zn content of wheat seeds to change over time, this result is reassuring in terms of the repeatability of the analytical workflow. The results for each of the washed treated seeds were similar. There was some slight variation in the measured Zn concentration at the different timepoints, but these differences were not

statistically significant. They were most likely due to heterogeneity in the treatment of seeds or variation due to sampling. Ref control gave similar results, with no significant differences in the Zn content of unwashed seed being detected. Though the median value for the 24-hour analysis was slightly lower than the values from the subsequent analyses. Again, this could be a sampling or uneven coating effect.

There were some differences detected between the treated washed seeds and the untreated seeds across a number of the time points. This again suggested that there was some retention of Zn within the seed and that it was not being completely removed from the seed surface during the acid washing. If it were, it would have expected to see the Zn content return to the untreated Zn level as this is analogous with the endogenous Zn content of this batch of wheat seeds.

Individual plots for the analysis at each of the timepoints are given in figures 14 and 15.

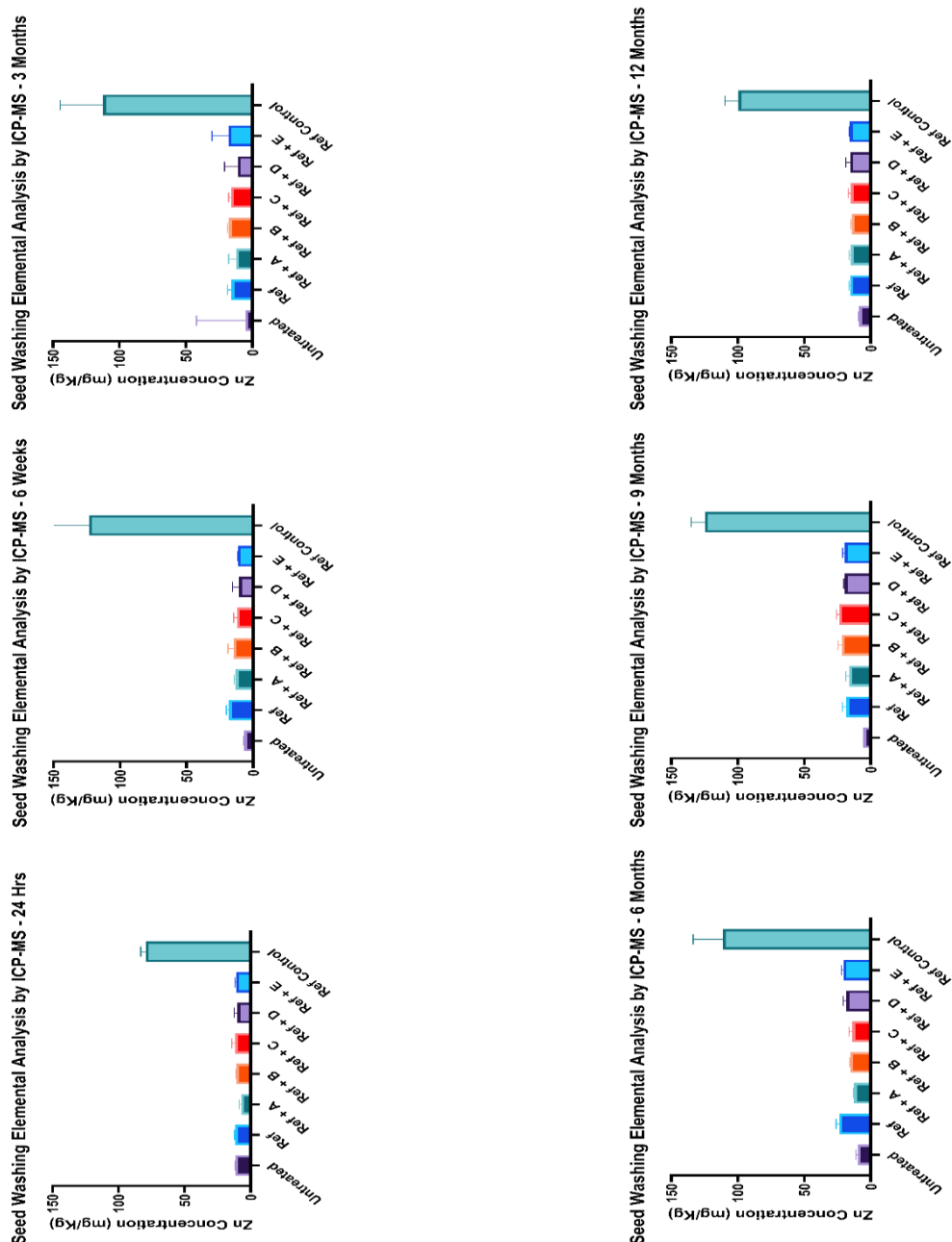


Figure 14: Zn content of ground and acid digested material from seeds treated with the ZnO formulation (reference) and the same formulation with added adjuvant (A-E) after they had been washed for 24 hours in nitric acid. Each chart represents a different period of dry storage between treatment and acid washing. Ref control refers to a treated seed of the same age which has not been washed. The analysis was carried out by ICP-MS. The height of the bar corresponds to the median value and the error bars represent the 5% confidence interval.

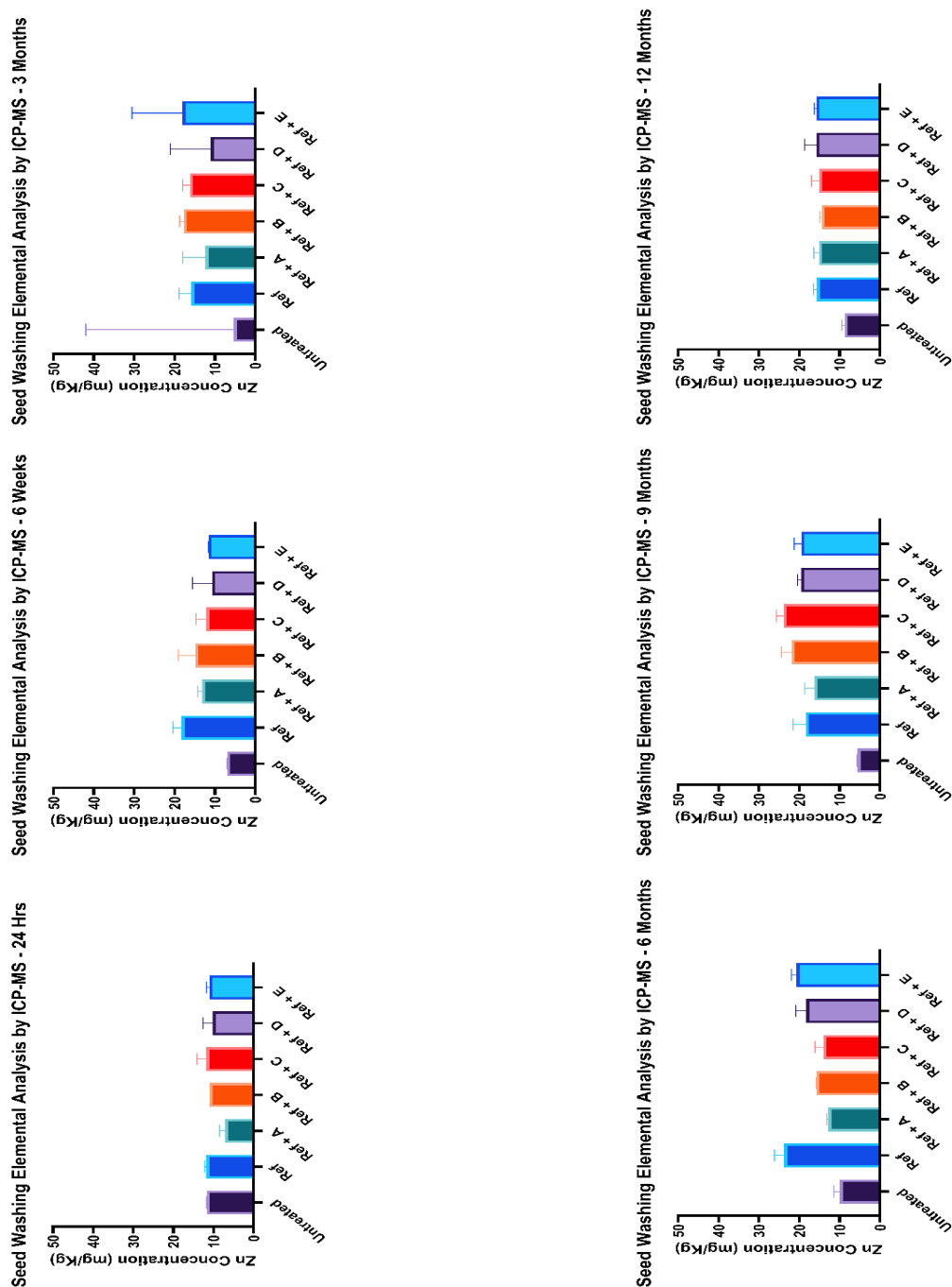


Figure 15: Zn content of ground and acid digested material from seeds treated with the ZnO formulation (reference) and the same formulation with added adjuvant (A-E) after they had been washed for 24 hours in nitric acid. Each chart represents a different period of dry storage between treatment and acid washing. The analysis was carried out by ICP-MS. The height of the bar corresponds to the median value and the bar represents the 95 % confidence interval.

The data shows that when the seeds were washed and analysed 24 hours after treatment there was no significant difference between the untreated and the treated washed seeds ($P=0.068$). At each subsequent time point there was a difference between the Zn content of the untreated seed and the treated washed seeds ($P<0.05$).

Figure 16 is a collated image giving the LA-ICP-MS images of epoxy-embedded wheat seed sections. Examples of all the treated seeds were analysed alongside an untreated control.

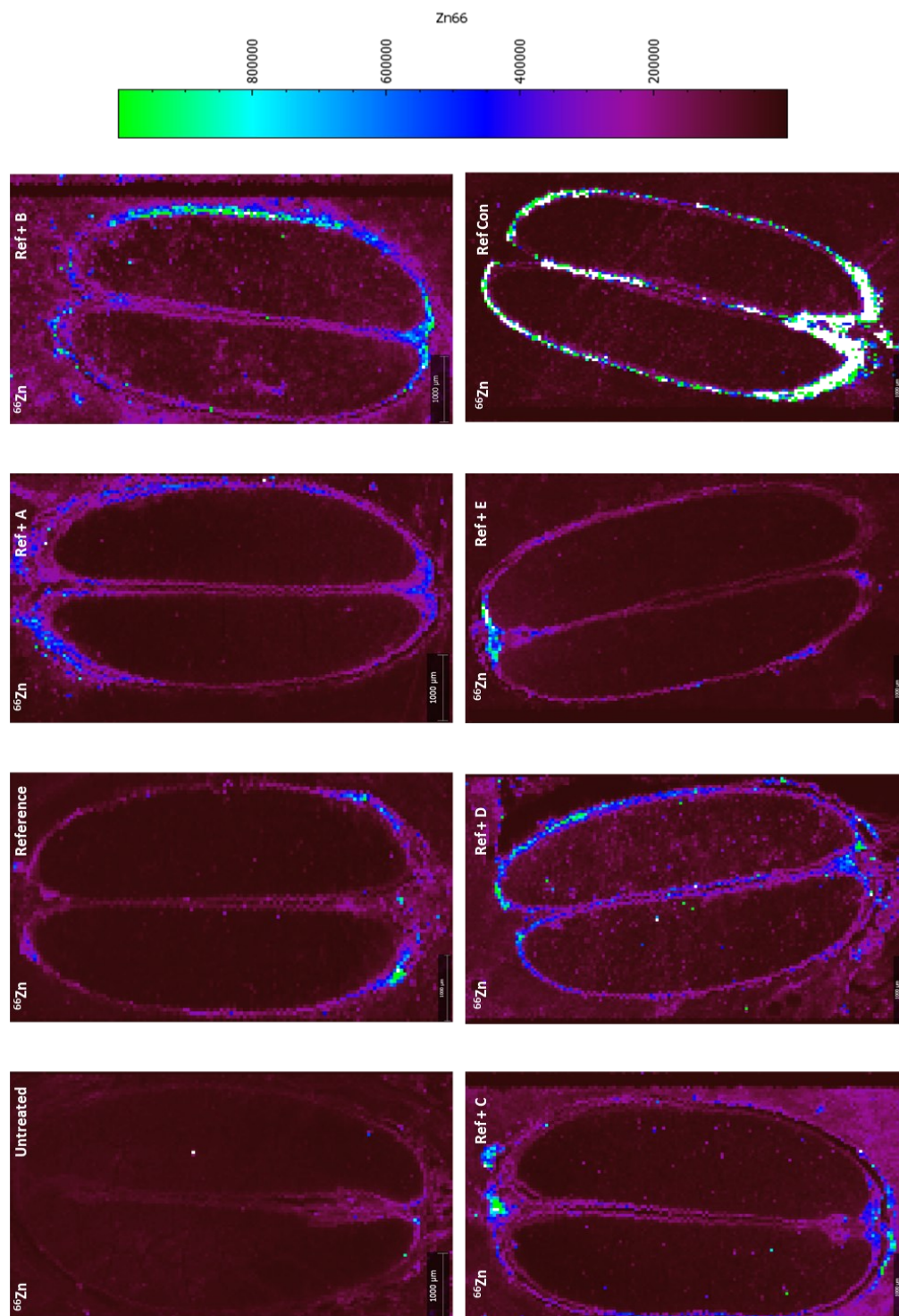


Figure 16: LA-ICP-MS generated Zn distribution images of epoxy-embedded sections of washed wheat seeds 12 months after they were treated with the reference coating and the reference coating with a range of adjuvants. There is also an image of the unwashed ref control seed as a positive control. Each image is generated on the same intensity scale (0-1000000 CPS).

There is a clear difference in Zn signal intensity between the Reference control (unwashed) seed image and the treated washed seed images. The Zn can be seen to be localised around the periphery of the seed section, which corresponds to the seed coat. This is consistent with the bulk analysis experiments which reported a significant difference in Zn concentration between the treated seed and the treated, washed seed. A difference in Zn signal intensity can also be observed between the untreated seed and the treated, washed seeds. The retained Zn can be seen to be localised around the seed coat with areas of greater signal intensity around the seed section periphery which were not observed on the untreated seed.

The reference control seed can be seen to have strong signal intensity across the entire seed periphery. This suggests the seed coating has coated the seed evenly.

There appears to be a slight increase in the Zn signal intensity in the endosperm of the Ref + D and Ref + B treated seed, suggesting that there may have been some proliferation of Zn into the endosperm. This could either be due to the inclusion of the adjuvant having a direct effect when applied to the surface of the seed, or due to the adjuvant having an effect during the acid washing process and causing Zn to be up taken into the endosperm. These images were only semi-quantitative and were intended to investigate the localisation, rather than the absolute concentration of Zn within the seed. As such it would be difficult to draw firm conclusions on the significance of the slight increase in signal intensity in the endosperm of the seeds treated with adjuvant's B & D.

The results given in figure 16 constitute a selection of one image from each treatment for comparison purposes. The analysis was carried out in triplicate. The full results are given in figure 17.

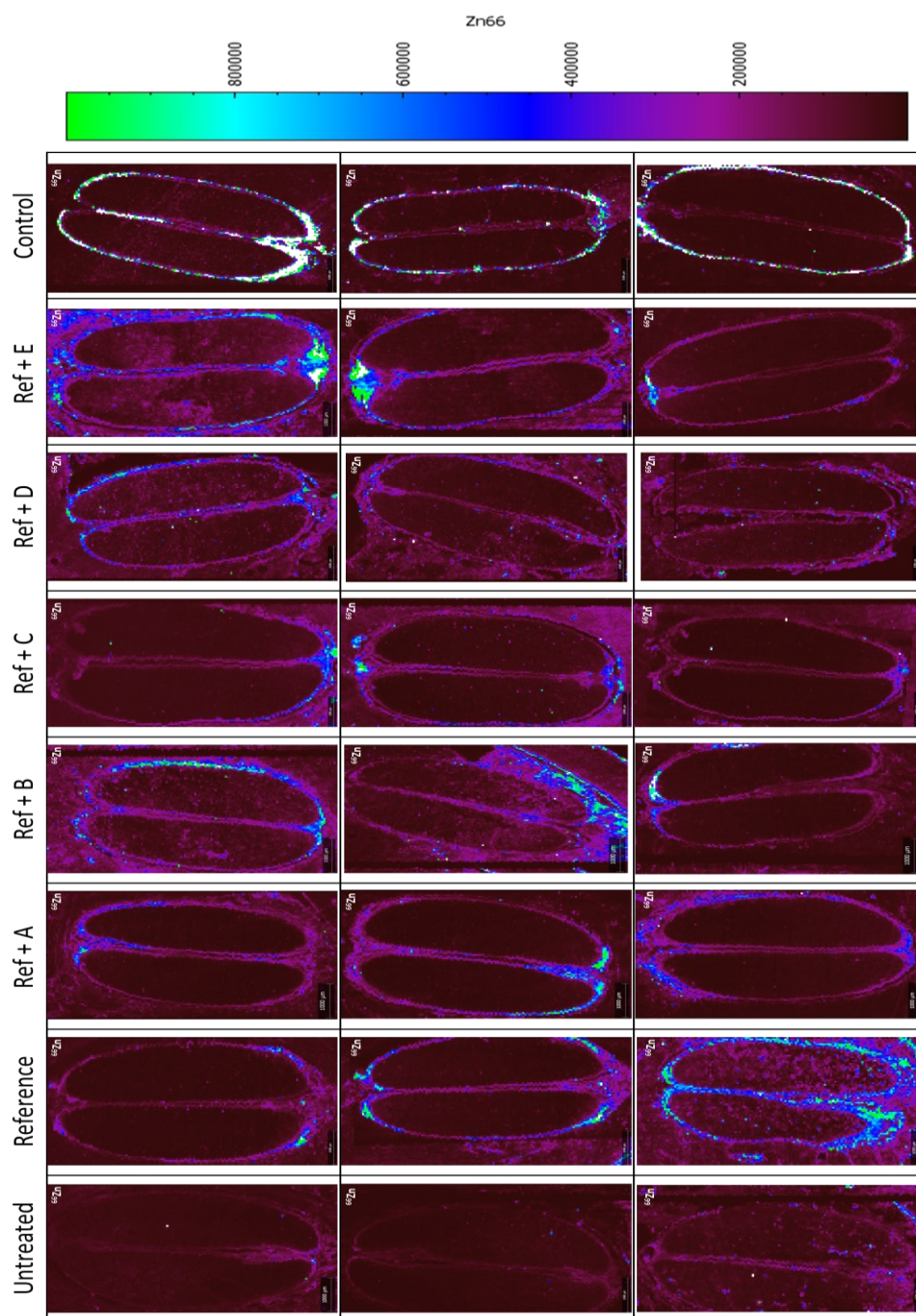


Figure 17: LA-ICP-MS generated Zn distribution images of epoxy-embedded sections of washed wheat seeds 12 months after they were treated with the reference coating and the reference coating with a range of adjuvants. There is also an image of the unwashed ref control seed as a positive control. Each image is generated on the same intensity scale (0-1000000 CPS). Each column contains three repeat analyses.

The results were consistent across each of the three repeats, with each repeat of each treatment giving similar Zn signal intensities and Zn signal localisations.

4.4.3. LA-ICP-MS Images of Zn in freshly treated wheat seeds

LA-ICP-MS analysis was carried out on seeds treated with the ZnO-containing reference coating and the reference coating plus the 5 adjuvants of interest. The images were compared side-by-side to investigate any effect of the inclusion of adjuvant withing the seed coating formulation on the localisation of the active ingredient, in this case ZnO on the finished treated seed. The images are given in figure 18. LA-ICP-MS analysis was also carried out on an untreated control seed to allow for comparison with the treated seeds. These results are given in figure 19.

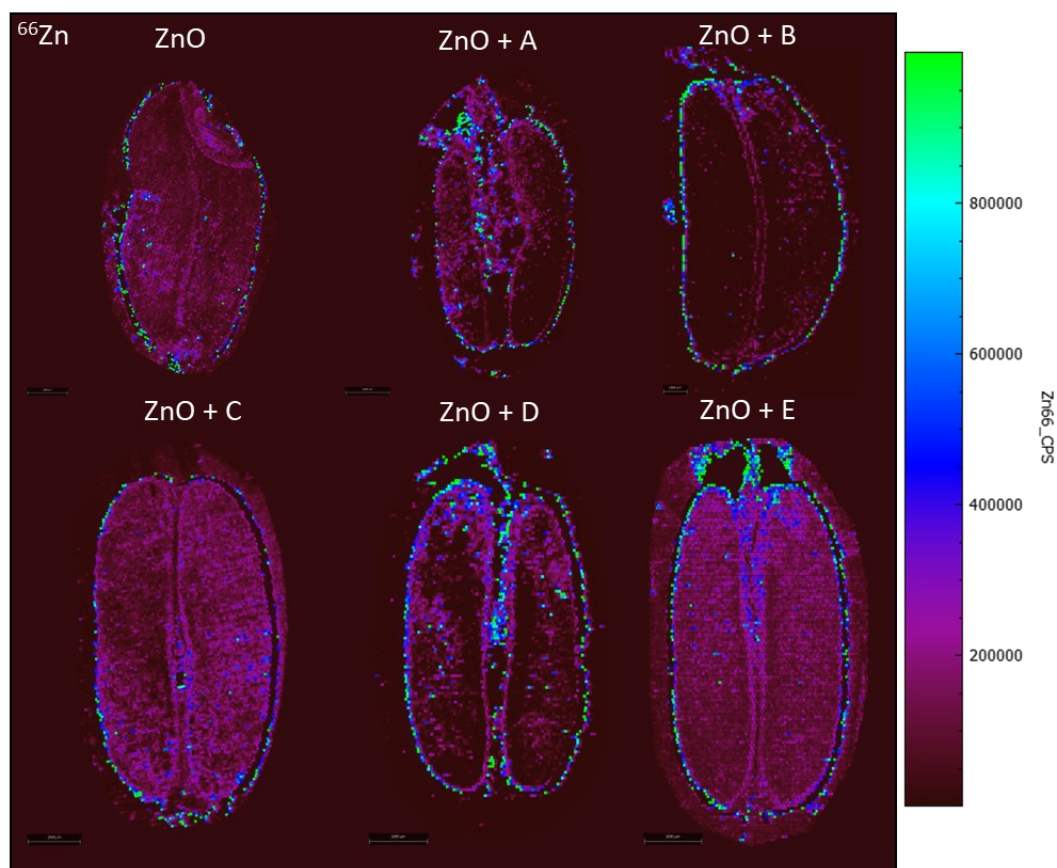


Figure 18: LA-ICP-MS images of freshly coated unwashed seeds. Left to right on the top row are ZnO (reference coating), ZnO + A (reference coating including adjuvant A), ZnO + B (reference coating including adjuvant B). The bottom row left to right are ZnO + C (r reference coating with adjuvant C), ZnO + D (reference coating with adjuvant D) and ZnO + E (reference coating with adjuvant E). The images are semi-quantitative, spatially resolved plots of ^{66}Zn signal intensity in counts per second, normalised to the baseline signal. Each image is produced on the same scale (0-1,000,000 CPS).

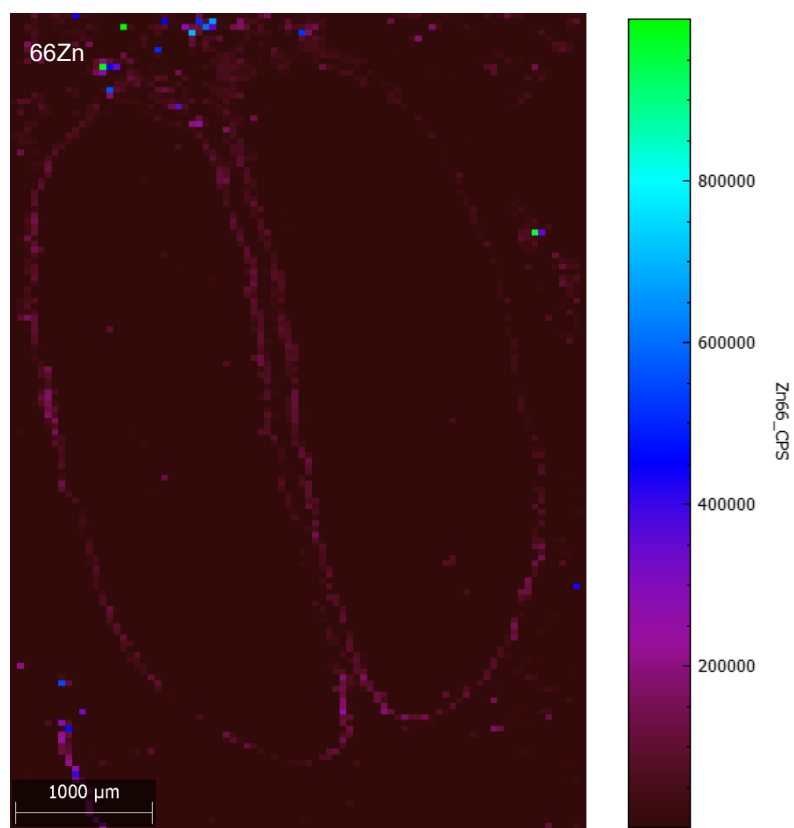


Figure 19: LA-ICP-MS semi quantitative spatially resolved plot of the ^{66}Zn signal intensity of an untreated wheat seed section. The signal intensity is measured in counts per second (CPS) and normalised to the baseline. The scale was 0-1,000,000 CPS.

The LA-ICP-MS imaging shows no influence of adjuvant inclusion on the localisation of Zn signal in the semi-quantitative analysis of a transverse section of treated wheat seeds. The signal intensity in all cases is localised around the periphery of the seed section, corresponding with the seed coat, as would be expected. There is no evidence of any increased penetration of Zn into the endosperm with the inclusion of adjuvant, nor any evidence of an effect of adjuvant on the distribution of Zn around the surface of the seed coat.

4.4.4.LA-ICP-MS images of other metals in freshly treated wheat seeds

Semi-quantitative distribution maps were also generated for a range of metals which are endogenous to the seed. The results are presented in figure 20.

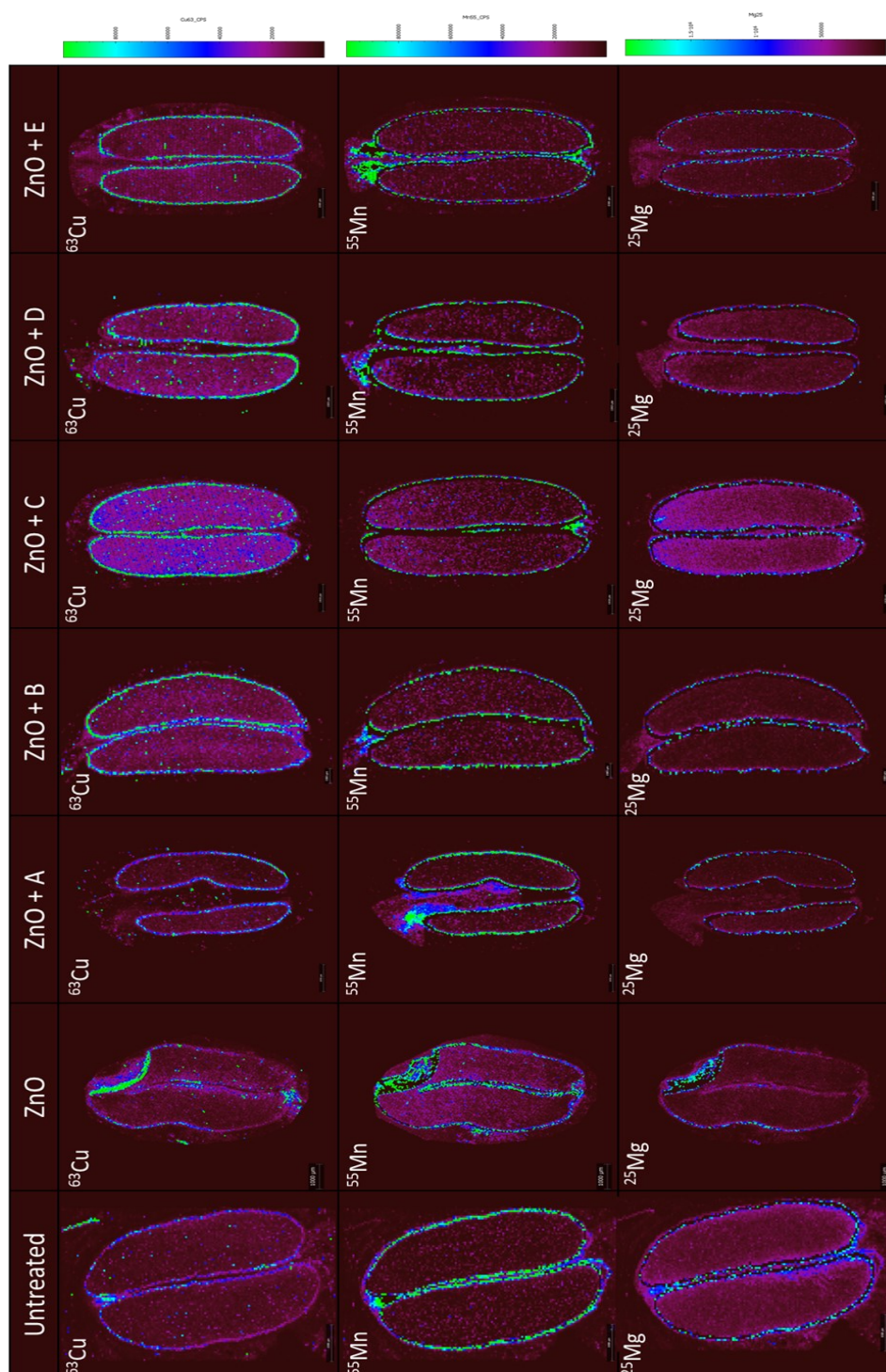


Figure 20: LA-ICP-MS generated distribution images of epoxy-embedded sections of 3 treated wheat seeds immediately after they were treated with the reference coating and the reference coating with a range of adjuvants, alongside an untreated seed for comparison. Cu images were generated on a 0-100,000 CPS scale, Mn on a 0-1,000,000 CPS scale and Mg on a 0-2,000,000 CPS scale.

The results indicate that there is no adverse effect of the application of reference coating on the distribution of endogenous metals (Cu, Mn & Mg). The localisation of these endogenous metals is unchanged after treatment, irrespective of the inclusion of adjuvant in the seed treatment formulation.

4.4.5. Micro-CT images of treated and untreated wheat seeds

The results of the micro-CT analysis of untreated and reference (ZnO-treated) seeds are given in figure 21.

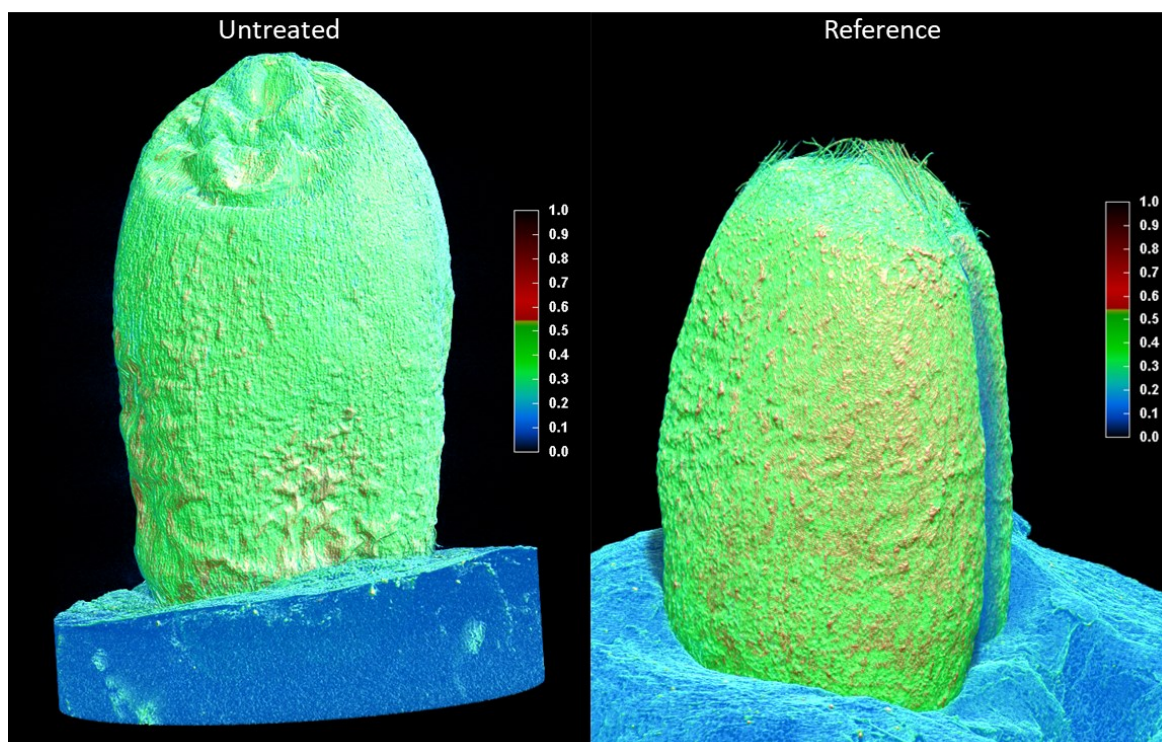


Figure 21: A side-by-side comparison of two 3D micro-CT images of an untreated (left) and a Reference ZnO-treated (right) seed. Both images were generated on the same relative density scale (0-1).

The micro-CT analysis showed areas of higher density material deposited on the surface of the treated seed, highlighted in red on the images, which were not present on the untreated seed. Although little work has been carried out on ZnO analysis on seeds using micro-CT, there is published work on the detection of

ZnO nanoparticles on the retinas of rats. The images in this study show particle-shaped areas of increased density on the surface of the retina.¹¹⁶ Much like the particle-shaped areas of increased density detected on the surface of the coated seeds in this work. Micro-CT has been used as a means to map the 3D morphology of tomato seeds, with a view to predicting germination outcomes. This work was mainly focussed on the physical characteristics of the seed, both internal and external and how they related to germination outcomes.¹¹⁷ A similar method has employed micro-CT in Barley and Thale Cress to examine the internal 3D phenotypic information to study flower development stages. Previously, this would have been done using a time-consuming and destructive technique involving fixation, sectioning, and microscopy.¹¹⁸ Other work has used micro-CT as a means to study the localisation of calcium oxalate crystals within the cotyledon of legume seeds. The study was able to show that the calcium oxalate crystals formed during stage two of germination (imbibition), but were not used up, suggesting that they are not used as a source of calcium for the growing plant.^{119,120}

Micro-CT had been used to study wheat grains, Le et al. used micro-CT to analyse the morphology of wheat grains through the development cycle, by capturing 3D images at various development stages. The stages imaged varied from 60 to 310 days after flowering. The work was able to identify changes in the volumes of the various compartments within the grain by development stage.¹²¹

4.4.6. Thresholding of micro-CT images for highly dense particles

When the thresholding methodology was applied to the micro-CT dataset, it was possible to remove the areas of density which were attributed to the seed tissue in both images, leaving behind only the dense particles, thought to be ZnO. The resulting images are given in figure 22.

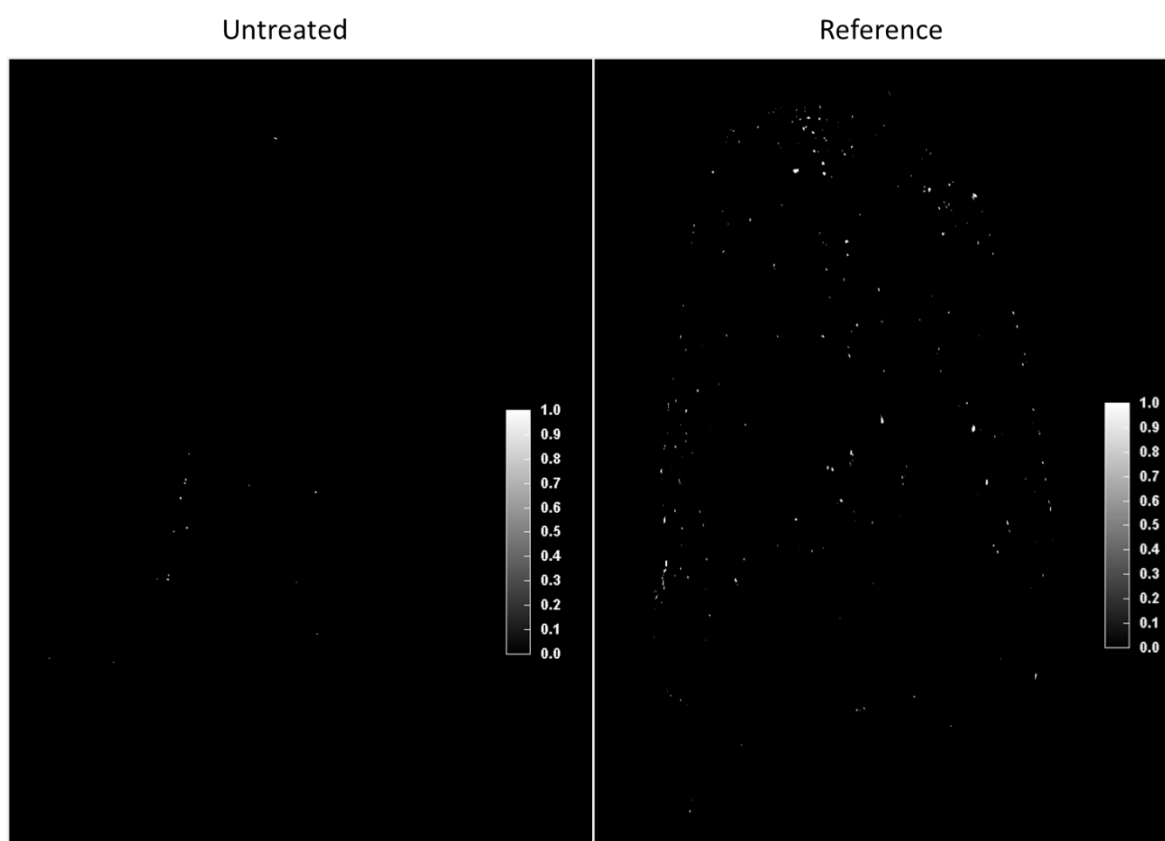


Figure 22: A side-by-side comparison of two micro-CT threshold images of an untreated (left) and a Reference ZnO-treated (right) seed. Both images were generated on the same relative density scale (0-1). The threshold was set such that any density common to both images (corresponding to the seed) was removed, leaving only the areas of density which were present in the treated seed and not the untreated seed visible in the image.

The thresholding method was developed by adjusting the minimum density which would be displayed in the method. This was adjusted until the tissues present in both the untreated and reference treated seeds were excluded from the image. This left behind only the dense particles which were present on the surface of the reference treated seed, but not detected on the surface of the untreated seed. Since both seeds were the same type of seed, from the same batch and approximately matched for size, the only difference between the two is the application of the ZnO-containing seed coatings. Although it is a possibility that the areas of density detected may be caused by other constituents of the seed coating formulation, the only one likely to have high enough density to be detected in this way by micro-CT would be ZnO. In order to be completely sure, it was necessary to characterise the particles on the seed surface using an alternative technique. Samples were provided for Scanning Electron Microscopy (SEM) analysis to enable in-situ elemental analysis to be carried out on the particles detected on the seed surface.¹²² There are numerous publications which have characterised ZnO using SEM, including in plant samples.^{123,124}

The results of this SEM analysis are given in sections 4.4.8 and 4.4.9 of this chapter.

4.4.7. Micro-CT imaging of treated-washed Wheat seed.

Micro-CT analysis was carried out on a treated-washed wheat seed. There is no evidence of the particle-like areas of high density on the image. However, there is evidence of changes in the topography of the seed surface. It appears that there has been an expansion of the seed coat and that it has separated from the inner seed coat and the endosperm. This is particularly evident on the cross-sectional images. This tells us that the swelling of the seed begins as soon as it is exposed to sufficient moisture, and this is the first stage of germination.

Since the nitric acid wash solution is aqueous, the most likely explanation for this is that during the washing process the seed goes through, at least partially the imbibition process. Imbibition is an early stage of germination in which the seed absorbs moisture, causing the endosperm to swell and forcing the seed coat to expand.¹²⁵ Water is absorbed by cell walls and protoplasmic macromolecules such as proteins and polysaccharides.¹²⁶ The movement of water involves water moving down a diffusion gradient from a region of higher to lower water potential.¹²⁷ When the seed is dried, the endosperm would then shrink, leading to the separation between the seed coat and the endosperm which we observe in these images.¹²⁸ This would also explain why there is a difference between the untreated seed Zn levels and the treated washed Zn levels. The acid strips the ZnO from the seed surface. The ZnO is then suspended in the nitric acid. The seed undergoes imbibition, absorbing the nitric acid and along with it some of the zinc. The results are given in figure 23.

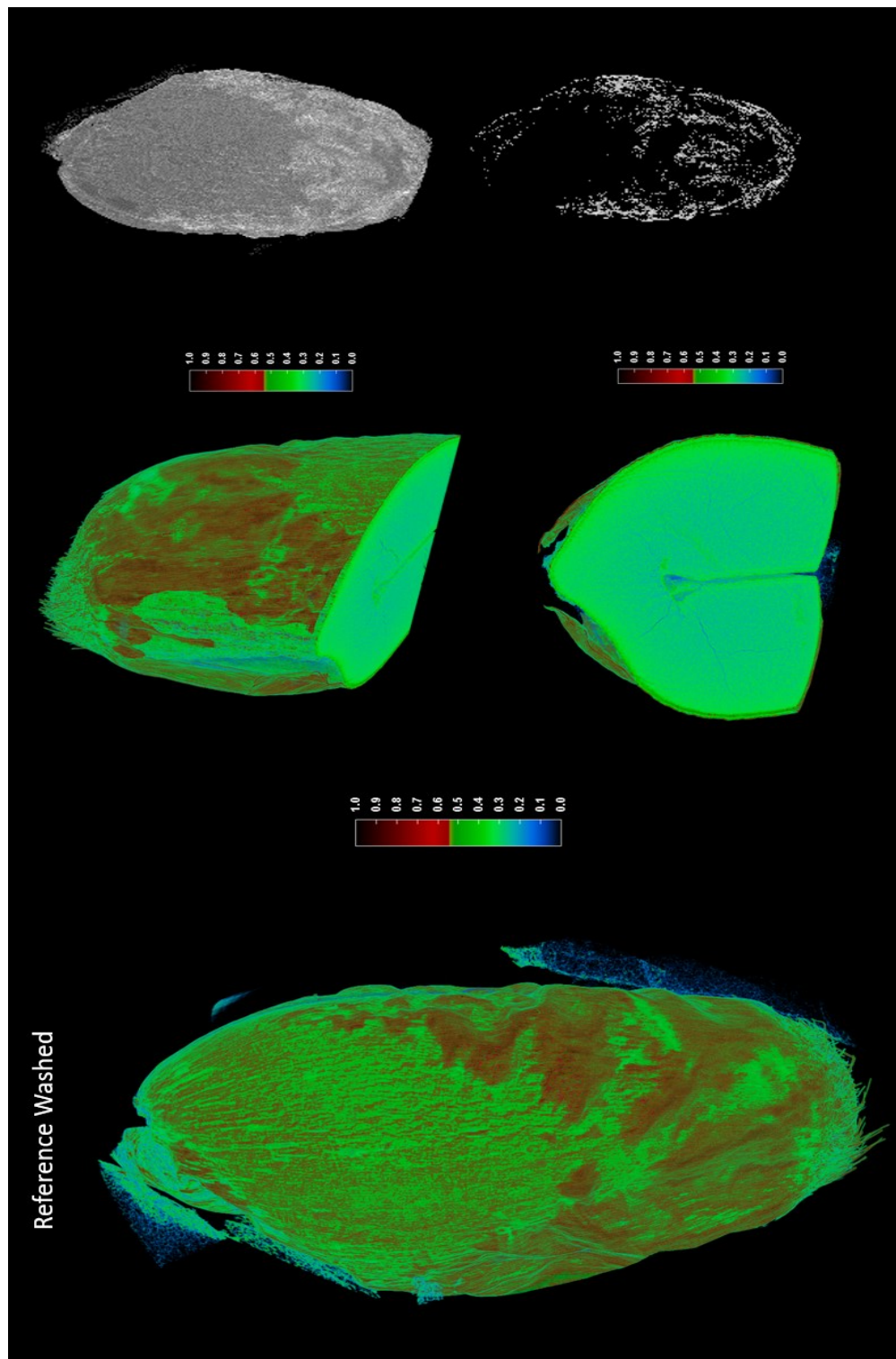


Figure 23: micro-CT 3D images of a treated seed after washing in nitric acid, showing a separation of the seed coating from the endosperm. These are supported by threshold analysed images of the same data on the right-hand side showing changes to the topography of the seed surface after washing.

4.4.8. SEM Analysis of Whole Wheat Seeds

The results of the SEM analysis of the surface of whole wheat seeds are given in figure 24 in the form of 2 dimensional black and white images depicting seeds including the untreated control, along with the Ref (ZnO) coating and Ref A-E which was base ZnO coating with the inclusion of adjuvant.

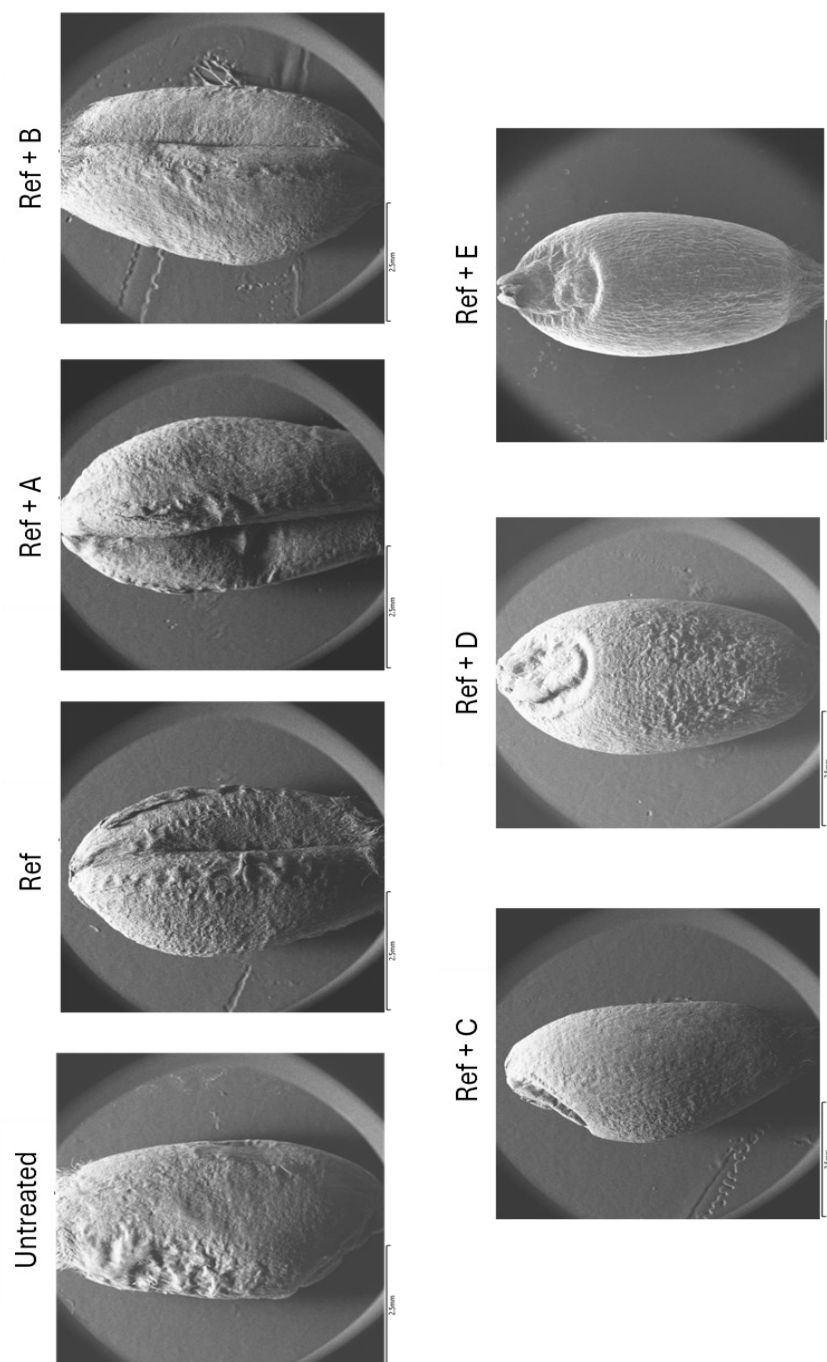


Figure 24: SEM images of winter wheat seeds. Untreated is a winter wheat seed with no treatment applied. Ref is a winter wheat seed with the base ZnO flowable suspension seed coating applied, Ref + A-E refers to the base ZnO seed coating with the addition of adjuvant A-E.

The SEM results show that SEM. can clearly view the surface of the seed This is no great surprise since there have been several published reports of SEM being used for the analysis of seeds, including wheat seeds.^{129–131} The SEM images show very similar seed surface topography to that which was observed using the micro-CT in section 4.4.5. This suggests that the micro-CT images do indeed represent the seed surface and by extension suggests that the density thresholding parameters used for the micro-CT work were appropriate for the seed material. Although there has been no previous published work utilising both micro-CT and SEM on wheat seeds, work has been carried out by Yamauchi et al who investigated the distribution of calcium oxalate crystals in quinoa seeds using a combination of the two techniques.¹³² They also observed the complementary nature of SEM and micro-CT for seed analysis applications such as the one reported in this chapter.

In order to determine whether the particles observed by micro-CT in 4.4.6 were, indeed, Zn particles from the application of ZnO-containing seed coatings, the seeds were also analysed by EDX, as detailed in 4.4.9

4.4.9.EDX Analysis of Zn on Seed Surface

The results of the EDX analysis of the surface of whole wheat seeds are given in figure 25 in the form of 2 dimensional images depicting seeds including the untreated control, along with the Ref (ZnO) coating and Ref A-E which was base ZnO coating with the inclusion of adjuvant. The EDX analysis also looked at other elements, none of which appeared to show any differences, apart from Cl being present in the treated seeds and not present on the surface of the untreated seeds. These images are given in figure 26.

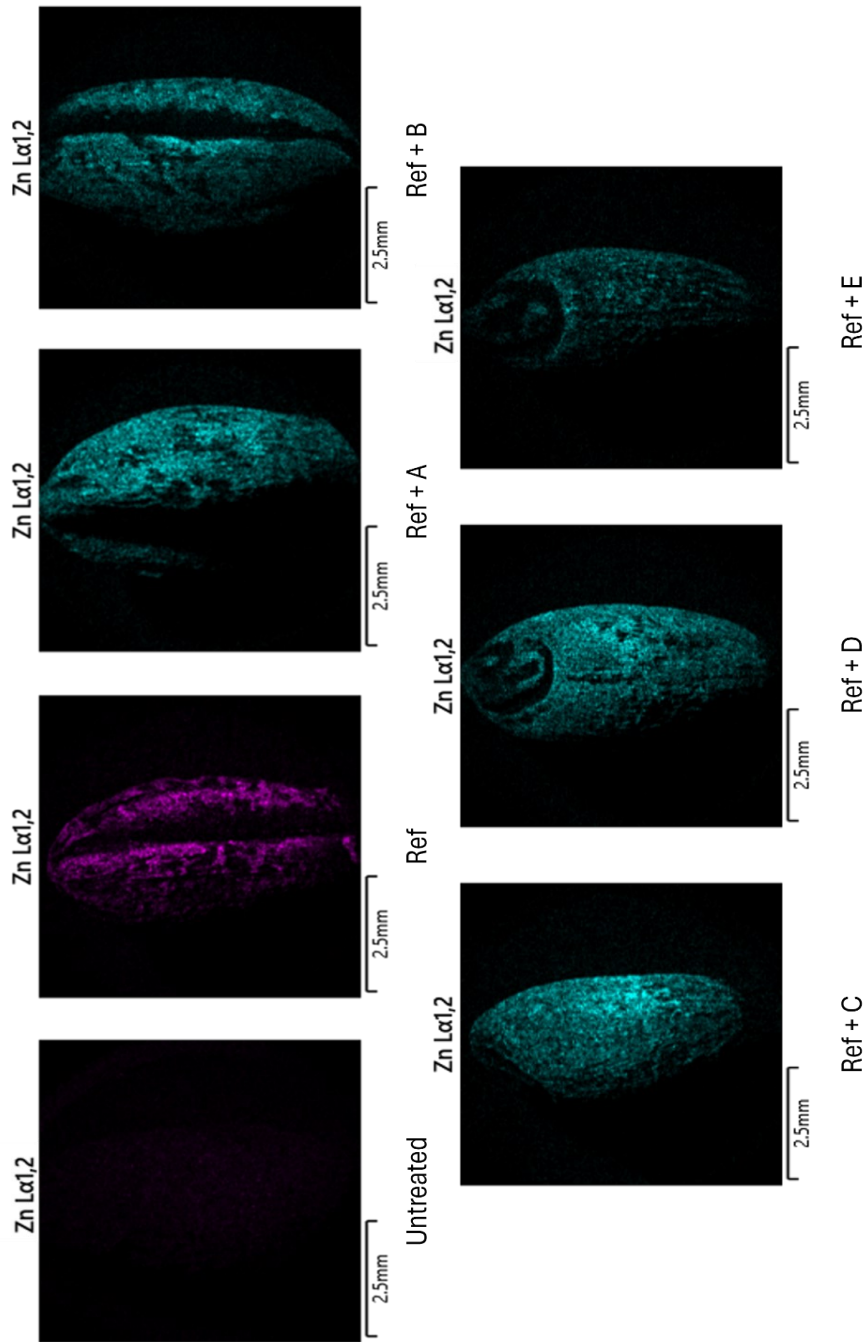


Figure 25: SEM EDX elemental analysis for zinc on the surface winter wheat seeds. Untreated is a winter wheat seed with no treatment applied. Ref is a winter wheat seed with the base ZnO flowable suspension seed coating applied, Ref + A-E refers to the base ZnO seed coating with the addition of adjuvant A-E. $L\alpha_{1,2}$ denotes the relaxation of electrons from the M to the L energy shell, the size of this shift is characteristic of the element, in this case Zn.

To the best of our knowledge, there have been no published examples of EDX to analyse ZnO microparticles on the surface of seed coating treated wheat seeds, nor have there been any reported applications of EDX to study the effect of adjuvant addition to nutrient-containing seed treatment.

Nonetheless, there are examples of published work to which we can turn to contextualise the results reported in this chapter. The most pertinent of which is work published by Montanha et al. They used EDX to characterise ZnO nanoparticles which were applied by way of a seed coating to soybean seeds. When the seeds were coated with ZnO nanoparticles with a particle size of 40 nm, at very high resolution they were able to detect clear particles on the surface of the seed which were not present in the untreated control seeds.¹³³

The results of the EDX analysis support the previous findings of this chapter, which indicate seed applied Zn remains on the seed surface, in the form of particles even after a period of storage. This supports the hypothesis that the effect of seed applied Zn on the plant relies on removal of the Zn from the seed surface into the root zone and uptake via the roots. This also suggests that the adjuvant most likely to influence Zn uptake may be adjuvant E, due to its known soil moisture retention properties. It is likely that adjuvant E would assist with Zn uptake by holding moisture, and by extension Zn in the soil around the seed and around the roots from which it can be up taken into the plant.

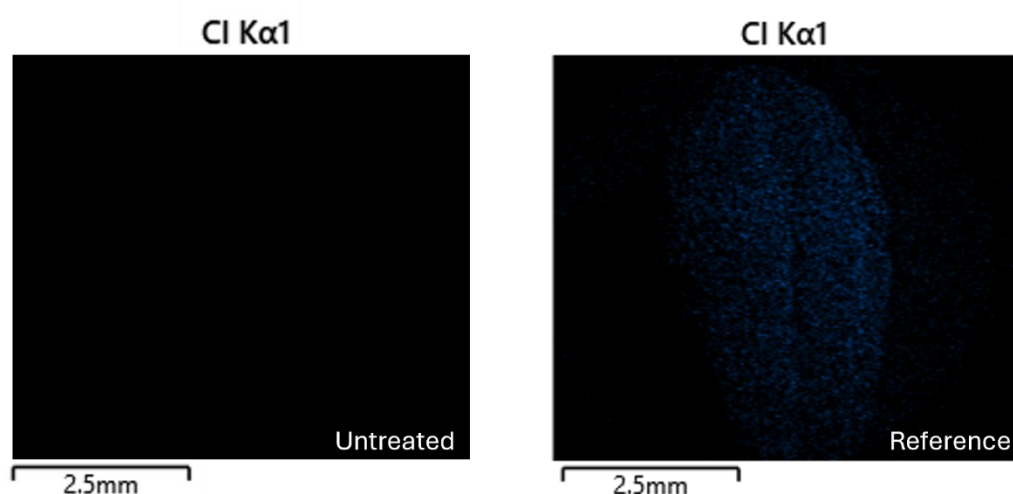


Figure 26: SEM EDX analysis of Cl on an untreated compared to a reference treated winter wheat seed. K α 1 denotes the relaxation of electrons from the L to K energy shells, the size of this shift is characteristic of the element, in this case Cl.

The presence of Cl in the seed treatment can be explained by the presence of the red dye included in the formulation. The dye included in the seed treatment formulation is Unisperse Red (BASF, Ludwigshafen, Germany), molecular formula $C_{24}H_{16}Cl_3N_3O_2$.¹³⁴ This observation provides further evidence that the Zn seen in the LA-ICP-MS, micro-CT and the EDX images presented within this chapter, does indeed correspond to the seed coating applied to the seeds being analysed.

4.5. Conclusion

In this chapter, a method has been presented for the washing of seed coatings from the seed surface. A method has been developed for the bulk analysis of seed material by ICP-MS and ICP-OES. A method has been developed for the LA-ICP-MS imaging of treated and untreated wheat seed sections. A method has been developed which enables the imaging of whole wheat seeds by micro-CT. It is worth noting that the washing of seeds in Nitric acid, whilst it may simulate the removal of seed treatment in a slightly acidic aqueous environment, it does not mirror exactly what would happen in the natural environment. In soil there would be no agitation of the seed to aid in the complete removal of Zn from the seed coat. Further work of interest would be to investigate a method which would be more directly representative of the natural environment and assess any effect of the nitric acid and the agitation which is not replicated in the natural environment.

The results show that there is some retention of Zn observed in treated seeds after washing, though there was no evidence of any diffusion of Zn into the endosperm of treated seeds over time, even in seeds which were treated 12 months prior to analysis. The micro-CT data presents what are thought to be ZnO particles deposited on the surface of the treated seeds. The particles are present on the treated seeds and can be isolated from the seed tissue by density thresholding. The micro-CT data also shows swelling of the seed following washing in Nitric acid. It is known that seeds expand during the imbibition process as they take up moisture to begin germination. It is proposed that the swelling is evidence of imbibition taking place, since this would be taking place in the nitric

acid which has stripped the Zn from the seed coating, This Zn entering the space between the endosperm and the expanded seed coat could explain the latent Zn levels in seeds which have been washed. This correlates with the bulk Zn content data, as well as the localisation of Zn in the LA-ICP-MS images of washed seed sections. Hotspots were observed, particularly in the ZnO + E treated seeds following washing, which appeared to show an accumulation of Zn in the germ of these seeds. This suggests that adjuvant E may have an effect of encouraging Zn uptake into the germ during the imbibition process.

Nitric acid washing is not directly representative of in-soil conditions, but it does give an indication of the mechanism of the release of ZnO from the seed surface and the subsequent uptake of Zn from the root zone. The development of this method presents a step towards a representative method to study the mechanism of action of seed applied nutrients, and any impact of changes in the seed treatment formulation on the mechanism. This is crucial as hitherto there were no reported methods which would enable such a study to take place.

The SEM and EDX data presented within this chapter strongly supports the results of the LA-ICP-MS analysis and the micro-CT analysis, all of which showed that prior to exposure to moisture, the applied ZnO-containing seed coating remained on the surface of the seed in crystalline form, irrespective of the period of storage prior to analysis.

Micro-CT could further be deployed in a time-course experiment to measure the swelling of seeds at time points through the washing process. This can be

achieved using the analysis functions of the micro-CT software which enable in-situ measurements to be taken and compared to other samples.

Page left intentionally blank

5.0. Glass House Wheat Growth Trials

5.1. Introduction

As noted previously, zinc is an essential micronutrient in plants and has been shown to be the most impactful micronutrient in terms of increasing crop yields.^{135,136} It is also known to be the most important micronutrient in terms of its negative effect on crop yields when deficient.^{137,138}

Zinc deficiency is the most common micronutrient deficiency in crops worldwide, with more than 50 % of the world's agricultural land considered to be zinc deficient.¹³⁹ Zinc deficiency can result in significant reductions in crop yields and quality.¹⁸

Over the years glasshouse trials on wheat crops have been carried on a wide range of research areas from phosphorous sensitivity to tolerance to soil acidity, crown rot resistance, and resistance to aphids.^{140,141,142,143} Trials have also been carried out studying the uptake of nutrients in wheat. Growth trials have been reported which assessed the effect of plant growth-promoting rhizobacteria (PGPR) as a biostimulant in combination with nitrogen-based fertilisation. The study found that across a range of N concentrations, plant biomass was significantly increased with inoculation with PGPR. The study also found that whilst the tissue concentration of N, P, Mn and Cu were reduced, due to the increase in biomass the overall uptake of these nutrients was increased.¹⁴⁴ Work has also been reported which focussed on the potential phytotoxic effect of the environmental release TiO₂ nanoparticles (NPs) on wheat crops. Since TiO₂ NPs are the most widely produced NPs in the world, their release into the environment

is inevitable. Whilst there was no significant effect of TiO_2 on plant growth, when the wheat was treated hydroponically with 40 mg/mL TiO_2 there was a significant increase in plant N, P, Cu and Zn concentrations.¹⁴⁵

There have been several reports of growth trials involving zinc in a range of crops. Notably a meta-analysis study was carried out in 2014 which assessed the results of 104 articles covering 263 growth trials on the impact of arbuscular mycorrhizal fungi (AMF) on improving plant tissue zinc concentration in a range of crops over a range of soil conditions. The meta-analysis showed that AMF had a positive effect on Zn concentration of all plant tissue types and that the positive effect was modulated mainly by soil texture. They also reported that soil pH and soil Zn concentration affected AMF-mediated shoot Zn concentration, whilst soil P concentration influenced fruit Zn concentration.¹⁴⁶ Work has also been reported on the use of Zn-aluminium layered double hydroxides (ZnAl-LDH) with borate association as a controlled-release mechanism for Zn and B to be used as a micronutrient delivery method for crop nutrition in tomato plants. The results showed that the Zn and B improved plant growth and assisted with macronutrient uptake when combined with NPK fertiliser.¹⁴⁷

Several studies have been carried out on Zn nutrition in wheat crops specifically. One study compared the effect of treatment with ZnO NPs with ZnSO_4 treatment in wheat using a combination of petri dish and glasshouse pot experiments. The wheat concentration increased significantly in both the plants treated with ZnO NPs and the plants treated with ZnSO_4 . ZnO NPs were found to be more effective at increasing grain Zn content whilst ZnSO_4 was more effective at increasing leaf

Zn content. Both caused moderate increases in biomass and grain yield at moderate doses. At high doses ZnSO₄ was shown to be more toxic than the ZnO NPs.¹⁴⁸ Another study carried out in field trials over two consecutive years in winter wheat found that the application of ZnSO₄ at 7 kg ha⁻¹ in combination with boron at 0.25 kg ha⁻¹ was found to significantly increase in plant height, dry weight and protein content in the grains of wheat crops.¹⁴⁹

Comparisons between glasshouse data and field trials have shown that screening in a glasshouse environment is a good predictor of field agronomic performance.¹⁴⁰

5.2. *Aims of this chapter*

The aim of this work was to determine whether a glass house experiment could be developed which enabled a Zn deficiency to be induced and detected by elemental analysis. Secondly, the work aimed to determine whether the glass house method could be used to detect the effect of a Zn-containing seed coating on early plant growth and plant Zn content. Finally, the work aimed to determine the effect of adjuvant inclusion within the ZnO seed coating formulation on the uptake of Zn in winter wheat plants grown under controlled glass house conditions.

5.3. Greenhouse Facilities

Plant Impact Plc is a Croda subsidiary based at Rothamsted Research (Harpenden, UK) with access to extensive greenhouse facilities which enable growth trials to be carried out under controlled conditions.

Four trials were carried out at Plant Impact in the form of three development trials followed by a final trial which built on the findings of the first three and incorporated the 5 adjuvants included in the project throughout.

5.4. Growth trial 1

5.4.1. Growth trial 1 design

The first trial to be carried out was a proof-of-concept trial set-up in the greenhouse at Plant Impact. In this trial untreated seed were sown in a sand and perlite mix alongside a positive control of untreated seed sown in nutrient-rich compost, Advance Pot & Bedding M3 Compost was selected (Levington Horticulture Ltd, Suffolk, UK). Of the sand and perlite sown seeds, 1/3 were treated with water as a control, 1/3 were treated with Zn-free Hoagland's solution and the final 1/3 were treated with complete Hoagland's, including zinc.

Forty 4" pots were prepared in total, ten per treatment and placed in a randomised order in the greenhouse. The greenhouse temperature was set to 21 °C daytime temperature and 19 °C nighttime temperature, with 14 hours of supplementary lighting. The randomised order is given in figure 27.

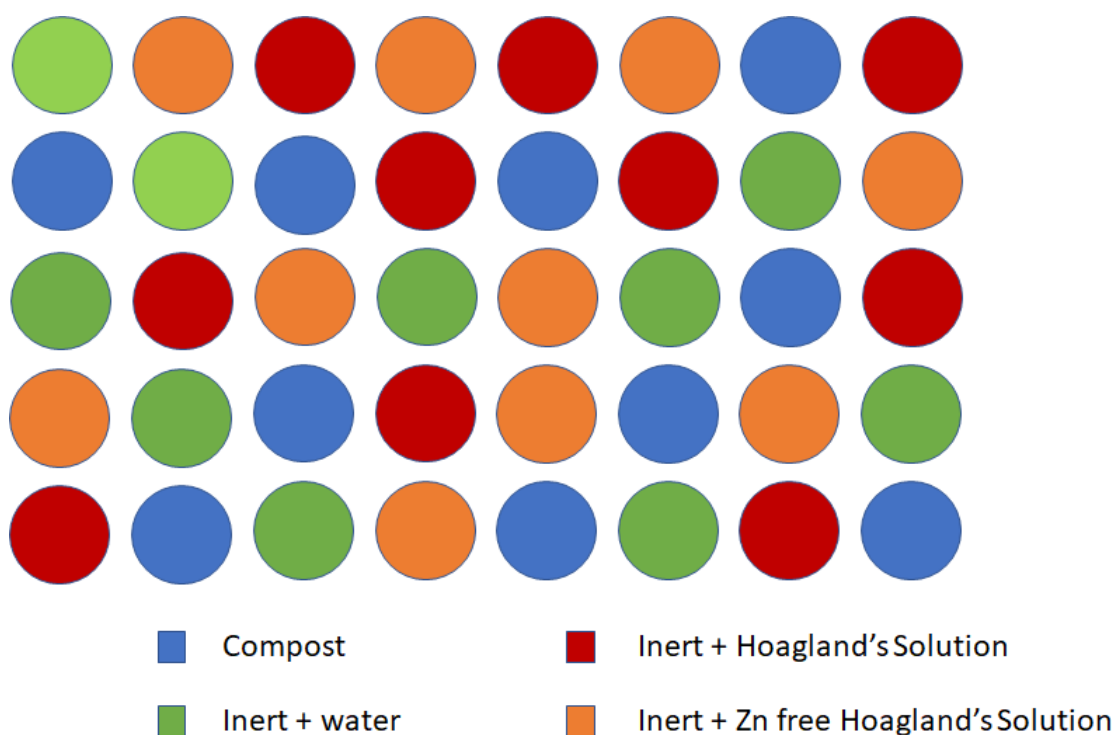


Figure 27: Schematic representation of the randomised placement of the different treatments in the glass house for the proof-of-concept growth trial carried out at Plant Impact.

The plants were irrigated three times per week for a total of four weeks until Zn deficiency was visibly identifiable in the control and Zn-free Hoagland's treated plants.

Pots in each treatment group were removed from the randomised design and irrigated. Plants were watered to excess with the same volume of solution to try and reduce the differences in water availability across treatment groups. Treatments were applied in order to reduce contamination, with water only, then Zn-free Hoagland's solution followed by Zn-containing Hoagland's solution.

5.4.2. Preparation of Hoagland's solution

Hoagland's solution is a hydroponic nutrient solution first developed in 1933. which contains a range of macro and micronutrients including N, K, Ca, P, S, Mg, B, Fe, Mn, Zn, Cu and Mo.¹⁵⁰ As such, Hoagland's solution provides every nutrient necessary for plant growth, making it one of the most used nutrient solutions for growing plants, particularly in a scientific context.¹⁵¹

Due to incompatibilities, Hoagland's solutions are made up in 3 parts.

Part A = 100 x feedstock solutions

Part B = 500 x $\text{Ca}(\text{NO}_3)_2$ solution

Part C = 4000 x micronutrient stock solution

Part A was made up in 3 L of deionised water as shown in table 3.

Nutrient	Mass (g)
KNO ₃	45.8
MgSO ₄	37.8
NH ₄ H ₂ PO ₄	8.6
Fe EDTA (6% v/v)	4.5

Table 3: Breakdown of the masses of constituent parts of Part A of the Hoagland's solution as made up in 3 L deionised water.

Part B was made up with 47.2 g Ca(NO₃)₂ in 1 L deionised water.

Part C was made up in 1 L deionised water in two batches. The first batch was the micronutrient solution with Zn and is given in table 4.

Nutrient	Mass (g)
H_3BO_3	2.86
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.54
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.08
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.22
MoNa_2O_4 dihydrate	0.13

Table 4: Breakdown of the masses of the constituent parts of Part C of the Hoagland's solution including zinc as made up in 1 L deionised water.

The second batch was the Zn-free micronutrient solution which was made up in 1 L of deionised water. The nutrients included are given in table 5.

Nutrient	Mass (g)
H_3BO_3	2.86
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.54
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.08
MoNa_2O_4 dihydrate	0.13

Table 5: Breakdown of the masses of the constituent parts of Part C of the zinc-free Hoagland's solution as made up in 1 L deionised water.

5.4.3. Growth trial 1 results

After four weeks of growth, Zn deficiency was visible in the plants starved of Zn nutrition in the form of yellowing of the leaves. Figure 28 shows the difference in colour between the Zn-free and the full Hoagland's treated wheat plants two weeks after sowing.



Figure 28: Photograph of the Zn-free (orange) and full Hoagland's (red) treated wheat material after four weeks of growth, showing yellowing of leaves in the plants starved of zinc.

Chlorosis of leaves as a symptom of zinc deficiency has long been established, including in the zinc deficiency chapter of the book *Advances in Agronomy* from 1957.¹⁵² Yellowing of leaves has been identified as an indicator of leaf

chlorosis.¹⁵³ Because of this, yellowing of leaves has widely been attributed to zinc deficiency in the literature, examples include Xing et al, who sampled naval orange leaves with varying yellowing of leaves to measure their nutrient contents. They reported a decrease in chlorophyll content with increasing yellowness of leaves which was also correlated to reduced zinc content.¹⁵⁴ The yellowing of leaves in this trial is no great surprise, but it did give an initial indication that the methodology was eliciting a zinc deficiency and giving the expected physiological effect.

Fresh root and shoot mass were also recorded and the results are given in figure 29,

Fresh Mass Data from POC Growth Trial

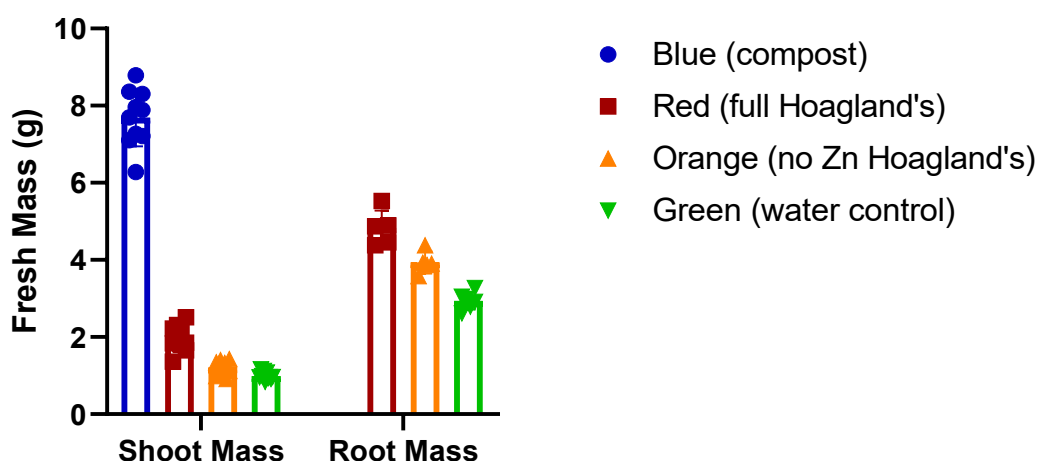


Figure 29: Summary of the fresh shoot and root mass of the plant material from the proof-of-concept growth trial. The plants grown in compost are shown in blue, the plants grown with full Hoagland's solution (with Zn) are shown in red, the plants grown with Zn-free Hoagland's are shown in orange and the plants treated only with water are shown in green.

The results presented in figure 29 are tabulated in table 6.

None of the plants grown in inert soil, the water control, were as high in shoot mass as the plants grown in nutrient rich compost. Due the advanced stage of root growth, the roots could not be separated from the compost to record an accurate mass. The plants grown in compost were significantly higher in fresh mass than all the different inert soil grown plants. There was a clear difference in fresh plant and root mass between the inert soil grown plants treated with and without Zn in the Hoagland's solution applied to them, will the Zn-treated plants higher in mean shoot mass and mean root mass.

Treatment	Mean Fresh Mass (g)	Mean Root Mass (g)
Compost	7.69	N/A
Full Hoagland's	1.97	4.83
No Zn Hoagland's	1.22	3.93
Water Control	0.99	2.94

Table 6: Summary of the fresh shoot and root masses from the proof-of-concept growth trial.

The fresh masses were statistically analysed using a one-way ANOVA followed by Tukey pairwise comparisons. For the shoot masses, all of the shoot masses were found to be significantly different to each other, with the exception of no Zn Hoagland's vs water control. The p values for this analysis are given in table 7.

Treatments	Significance	P value
Compost v Full Hoagland's	Significant	P < 0.0001
Compost v No Zn Hoagland's	Significant	P < 0.0001
Compost v Water control	Significant	P < 0.0001
Full Hoagland's v No Zn Hoagland's	Significant	P = 0.0015
Full Hoagland's v Water control	Significant	P < 0.0001
No Zn Hoagland's v Water control	Not significant	P = 0.633

Table 7: A summary of the results of the pairwise statistical analysis of the fresh shoot mass data from the proof-of-concept trial.

The fresh root masses were also statistically analysed using a one-way ANOVA followed by Tukey pairwise comparisons. The compost grown plants were not included in the analysis as the roots could not be removed from the soil effectively. All the root masses analysed were found to be statistically different to each other. The p values for the analysis are given in table 8.

Treatments	Significance	P value
Full Hoagland's v No Zn Hoagland's	Significant	P = 0.0025
Full Hoagland's v Water control	Significant	P < 0.0001
No Zn Hoagland's v Water control	Significant	P = 0.0007

Table 8: A summary of the results of the pairwise statistical analysis of the fresh root mass data from the proof-of-concept trial.

Compost was included as a growth medium to act as a positive control. Compost is nutrient rich and as such it was included in the experiment to demonstrate how the plants would grow where there was no deficiency of any nutrient, including zinc.¹⁵⁵ In fact, compost has been shown to supply considerable amounts of Zn to plants in pot growth trials.¹⁵⁶ Compost also retains moisture far more effectively than the sand and perlite mix would be expected to. This is largely because the level of moisture retention in soil can be directly linked to the organic matter content of the soil.¹⁵⁷ Therefore, it is no surprise that the plants grown in the compost outperformed those grown in the sand and perlite mix, irrespective of the nutrient solution applied to them. The difference between the plants grown in sand and perlite mix with and without Zn included in the Hoagland's solution represents a direct effect of the absence of Zn on the shoot and the root growth

of the plants. Whilst it is well known that Zn has an effect on plant growth, this result provided an indication that the glasshouse assay which had been designed for this experiment was capable of delivering a zinc deficiency, and that this deficiency could be observed in terms of plant shoot and root growth differences. Similar work was carried out in a glasshouse trial with maize which also showed reduced plant growth and ultimately crop yield with zinc deficiency.¹⁵⁸

Once the plants were harvested, the shoots were collected, dried, digested and analysed by ICP-MS. The results from this analysis are given in figure 30.

Zn Content of Wheat Shoot Material from POC Growth Trial - by ICP-MS

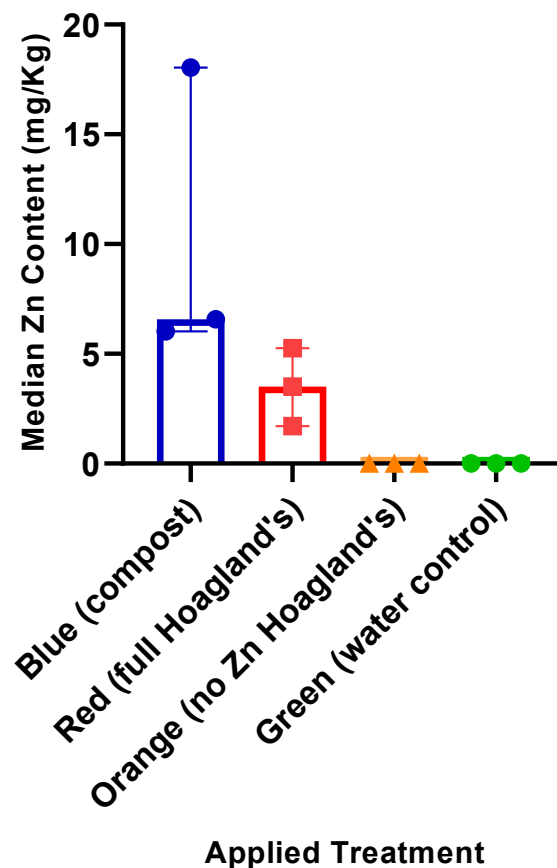


Figure 30: Results of the ICP-MS analysis of Zn content in digested plant material from the proof-of-concept growth trial. The height of the column represents the median value, whilst the error bars depict the 95 % confidence interval. Each data point represents one replicate.

Analysis by ICP-MS detected Zn in both the compost grown plants at 6.58 mg/Kg and the full Hoagland's treated plant material at 3.51 mg/Kg. No Zn was detected in the plants treated only with water or in the plants treated with Zn-free Hoagland's solution.

The results were statistically analysed using a one-way ANOVA followed by Tukey pairwise comparisons, the results are given in table 9. There was no significant difference in Zn content found between the plants grown in compost

and the plants grown in inert soil and treated with a full Hoagland's solution. The level of Zn in the full Hoagland's treated plants was significantly higher than the level found in the Zn-free Hoagland's treated plants as well as the water control plants.

Treatments	Significance	P value
Compost v Full Hoagland's	Not significant	P = 0.1657
Compost v No Zn Hoagland's	Significant	P = 0.03
Compost v Water control	Significant	P = 0.03
Full Hoagland's v No Zn Hoagland's	Significant	P = 0.0099
Full Hoagland's v Water control	Significant	P = 0.0099
No Zn Hoagland's v Water control	Not significant	P > 0.9999

Table 9: A summary of the results of the pairwise statistical analysis of the Zn content of plant material from the proof-of-concept growth trial.

The results of the proof-of-concept growth trial showed that the assay developed was able to generate and show a Zn deficiency. The yellowing of leaves associated with a Zn deficiency could be visibly detected. The yellowing is known as chlorosis and is a symptom of plants producing insufficient chlorophyll, since

chlorophyll is responsible for the green colour of leaves.¹⁵⁹ A benefit of plant Zn availability was detected in terms of fresh root and shoot mass, whilst the level of Zn within the plants was significantly higher in the plants exposed to Zn than those which were starved of Zn.

5.5. Growth Trial 2 – Development Assay

5.5.1. Experimental Design

A secondary growth trial was carried out to develop a method which would be able to detect the effect of a Zn-containing seed coating on the Zn uptake of wheat crops in the glasshouse, based upon the methodology used in growth trial one.

This time all seeds were sown in the sand and perlite mix used in growth trial one, all pots were treated exclusively with Zn-free Hoagland's solution. There were two treatments included in the trial: Untreated seed and seed with a ZnO-containing seed treatment.

Winter wheat seed (KWS Zyatt) were treated with a ZnO-containing flowable suspension. The formulation of the flowable suspension is outlined in section 1.4 of this thesis. The treatment rate for wheat was 0.6 g of active ingredient per Kg of wheat seed.

The seeds were again sown in 4" pots and randomly arranged in the greenhouse to account for any variation brought about by the pot's location within the glasshouse.

The aim of growth trial 2 was to determine whether the effect of Zn-containing seed coating application could be detected in plants grown under glasshouse conditions.

Plant height was recorded immediately prior to harvest, the fresh mass of plant material was recorded. Once dried the dry mass of plant material was recorded. Elemental analysis was then carried out on the dried plant material by microwave digestion followed by analysis by ICP-MS.

5.6. Results of the Development assay

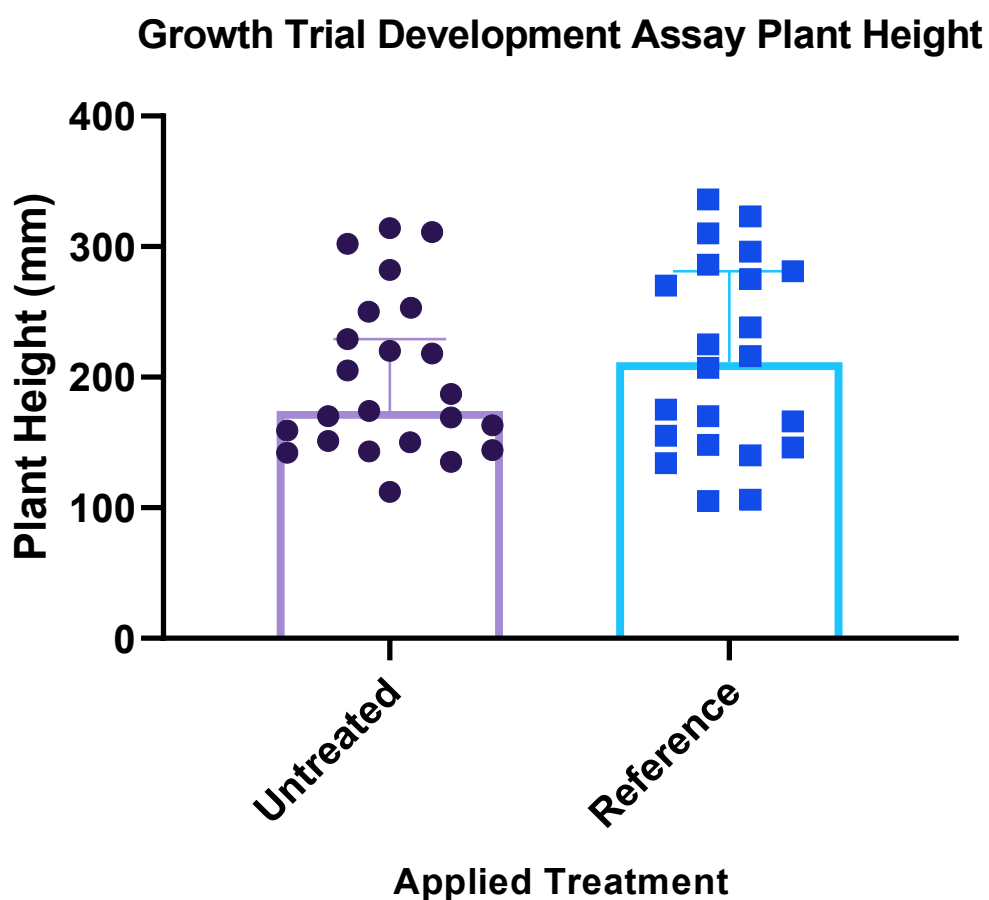


Figure 31: A comparison of the recorded plant height for the untreated compared to the treated plants on the day of harvest from the development assay.

Immediately prior to plant harvest, the height of each plant was measured with the results given in figure 31. Although the mean plant height value of the treated plants was higher, at 214 mm, than the mean height value of the untreated plants at 199 mm, using a one-way ANOVA there was no significant difference ($p=0.467$) in plant height between the treated and untreated plants.

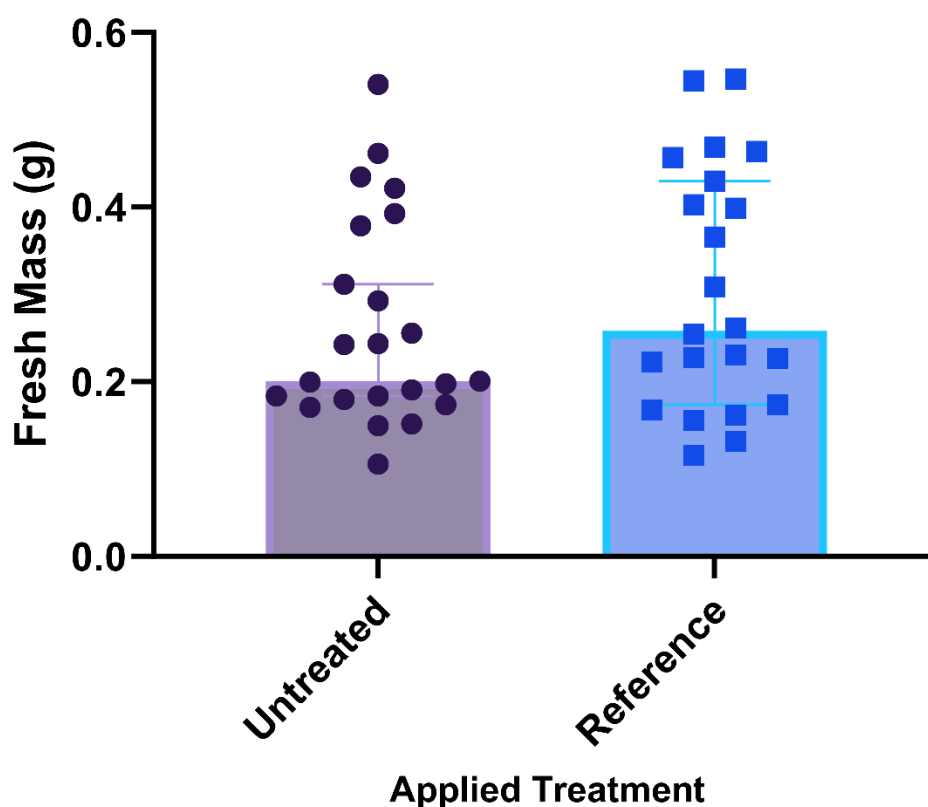


Figure 32: A comparison of the recorded fresh mass for the untreated compared to the treated plants on the day of harvest from the development assay.

The fresh mass of each plant was measured immediately after harvest and is given in figure 32. Whilst the mean fresh mass of the treated plants was higher than the mean fresh mass of the untreated plants, the data was statistically analysed using a one-way ANOVA, the results showed no significant difference ($P=0.2547$) in fresh mass between the plant material from the ZnO treated seed and the plant material from the untreated seed.

Following drying of the plant material, the dry mass data was recorded and statistically analysed using a one-way ANOVA. The results are given in figure 33.

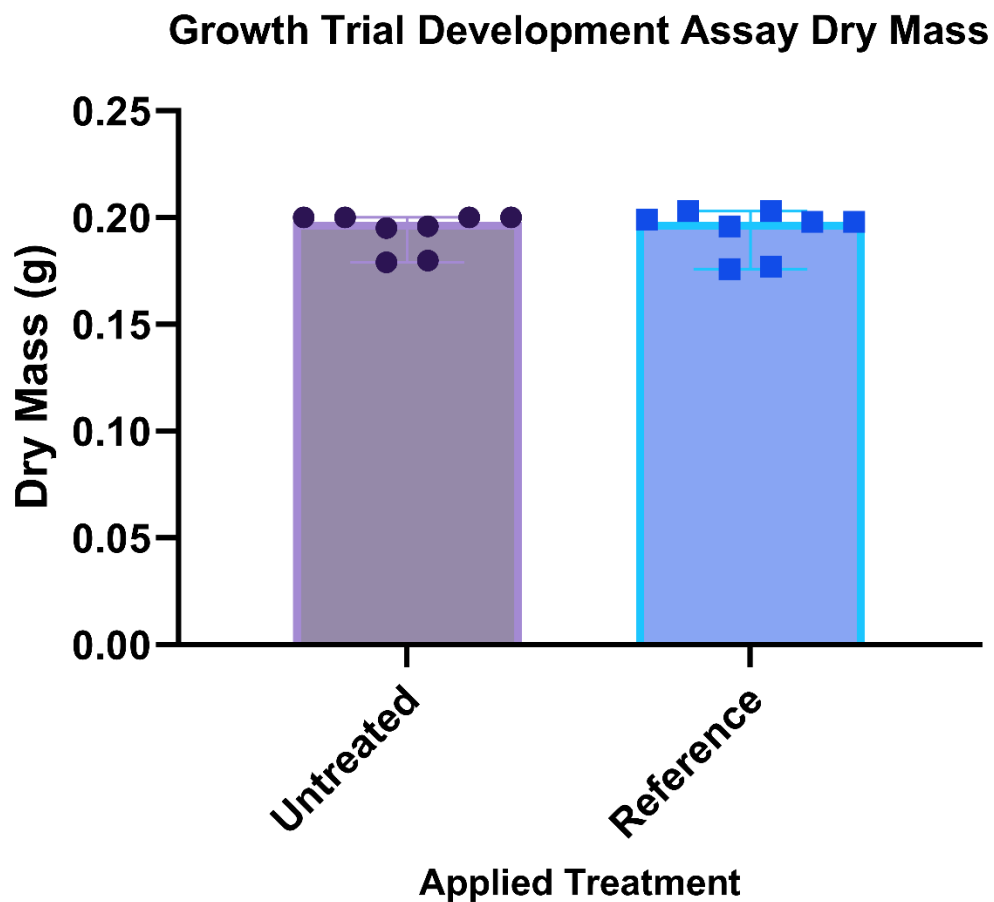


Figure 33: A comparison of the recorded dry mass for the untreated compared to the treated plants after drying in a 60 °C oven overnight.

The results showed no significant difference ($P=0.6714$) in dry mean mass between the plant material from the ZnO treated seed (0.194 g) and the plant material from the untreated seed (0.195 g).

Following microwave digestion, the plant material was analysed by ICP-MS in solution mode to calculate the Zn concentration within the plant material. The data is given in figure 34.

Development Assay Plant Material Zn Content - by ICP-MS

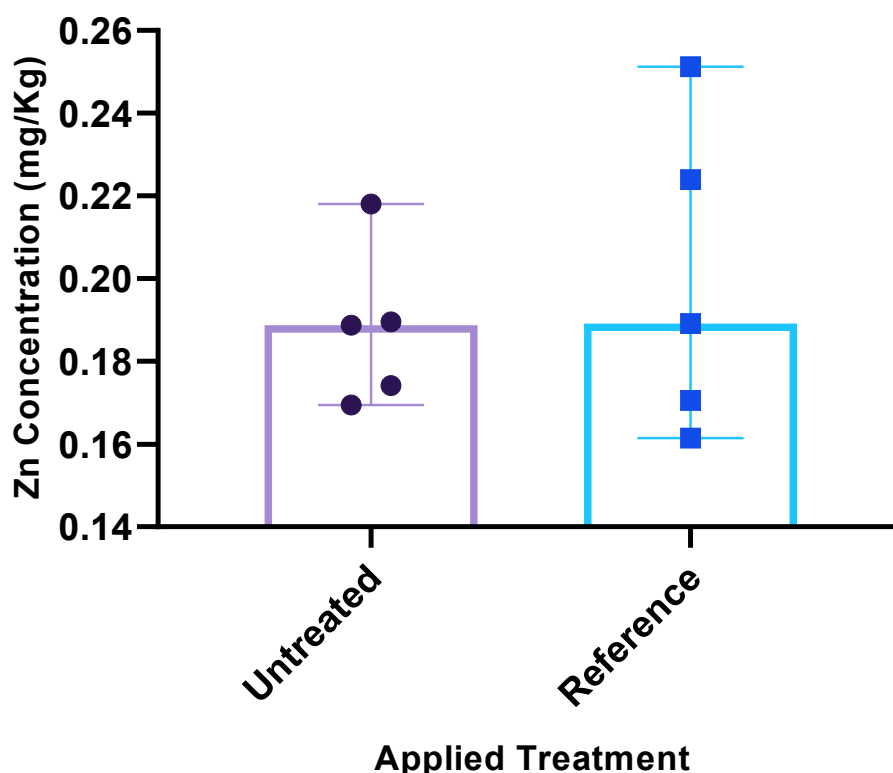


Figure 34: A comparison of the Zn concentration by ICP-MS of the digested plant material from the development assay growth trial. Each data point represents one separately digested repeat.

Elemental analysis of the plant material by ICP-MS gave a slightly higher median Zn content for the treated plant material compared to the untreated material. The median value for treated plant material was 0.206 mg/Kg compared to 0.189 mg/Kg for the untreated plant material. However, one-way ANOVA, this difference was not found to be statistically significant ($P=0.581$). There has been very little published work on Zn content of wheat plant material at such an early growth stage and, to the best of our knowledge, there has been no published

work which reported the Zn content of early-stage wheat plants grown in inert soil or under glasshouse conditions.

Given the only source of Zn available to the plant in this experiment (aside from the endogenous Zn stored in the seed) was the Zn applied to the treated seed in the form of the ZnO-containing seed coating, it is not surprising that such low values for plant Zn content were recorded.

In each of the parameters observed in this experiment, the treated plant material performed marginally better than the untreated plant material. However, none of these differences were found to be significant.

Comparison of these results is difficult due to the lack of comparable published literature. The Zn content of wheat is heavily dependent on the Zn content of the soil, or other growth medium, in which it has been grown. A study of 599 wheat crops and corresponding soil samples found a positive correlation between soil Zn content and final wheat grain Zn content.¹⁶⁰ Studies of the zinc content of different types of soil and from different geographical locations have shown that the zinc content can vary wildly, from as low as 10 mg/kg to as high as 300 mg/kg.¹⁶¹ The most vulnerable soils to zinc deficiency are sandy, calcareous, saline and wetland soils.^{162,163} Given the sand and perlite mix was used as the basis for the growth trials, it is no surprise that low levels of Zn were reported in the plant material as sandy soils would be expected to have a low level of retention of the applied zinc-containing solution.

Where plant zinc content has been reported in the literature, it is usually the zinc content of the grain of mature wheat plants. This is because it is often investigated to establish the effect of different treatments and soil conditions on the nutritional

content of the grain as a human food source. There is a significant difference between the Zn contents which have been reported in mature wheat grain and the Zn concentration reported in the young plants in this work. The Zn content varied widely between regions but was in the range of 14-51 mg/kg in the work reported by Huang et al based on a large-scale study of zinc in wheat cropping regions of China.¹⁶⁰

A wider study by Wang et al. reported Zn content in wheat grain from all over the Globe. The worldwide average was 31.84 mg/kg, although there was some variation across the continents. In Europe the average was 25.10 mg/kg, in Africa it was 29 mg/kg, Asia was 33.63 mg/kg and North America was 33.91 mg/kg. They did report that Zn fortification during breeding of the crops was effective in increasing the final Zn content of the grain, and by extension of the flour, which was produced from it, reporting an average value of 40.45 mg/kg with the combined treatment of soil Zn application and foliar spraying. The combination of soil treatment and seed priming also gave rise to a slight increase in grain zinc content, with an average of 34.30 mg/kg.¹⁶⁴

The focus of this study was not to determine the effect of seed treatment on the final grain, rather to determine any effects on the early stages of plant growth. As such, whilst these studies serve as an indicator that treatment with Zn can be an effective means of boosting the level of Zn within the plants, comparison with the absolute values reported in such studies would be misleading.

5.7. Growth Trial 3 – Full Assay

5.7.1. Growth trial 3 study design

The methodology used in growth trial 2 (3.1) was expanded to include a range of Zn-containing treatments. The treatments included the ZnO seed coating (reference) outlined in section 1.4, as well as five versions the same ZnO treatment containing one of 5 different adjuvants. The adjuvants were included at 15% (w/v) of the active ingredient (ZnO). The adjuvants were selected to cover a wide range of adjuvant chemistries, as described in section 1.2.2.

Each treatment was planted in the same sand and perlite mix used in the previous trials, the pots were randomised in the glasshouse and all treatments were treated with Zn-free Hoagland's. A positive control was included, this was an uncoated seed treated with full Hoagland's solution throughout. This was included to enable the effect of the localisation of Zn on the seed coating to be investigated. The trial was carried out for a total of 6 weeks.

The plant emergence rate was recorded the fresh mass was recorded immediately after harvest. Once the plant material was dried, the dry mass was recorded, and the plants were microwave digested and analysed by ICP-MS.

The aim of this trial was to determine whether the effect of adjuvant inclusion in a Zn-containing seed treatment could be determined in a glasshouse trial.

5.7.2.Results Growth Trial 3 – Full Assay

As the trial was carried out, the pots were monitored daily to record the emergence of shoots. Emergence data by day and by treatment is given in figure 35.

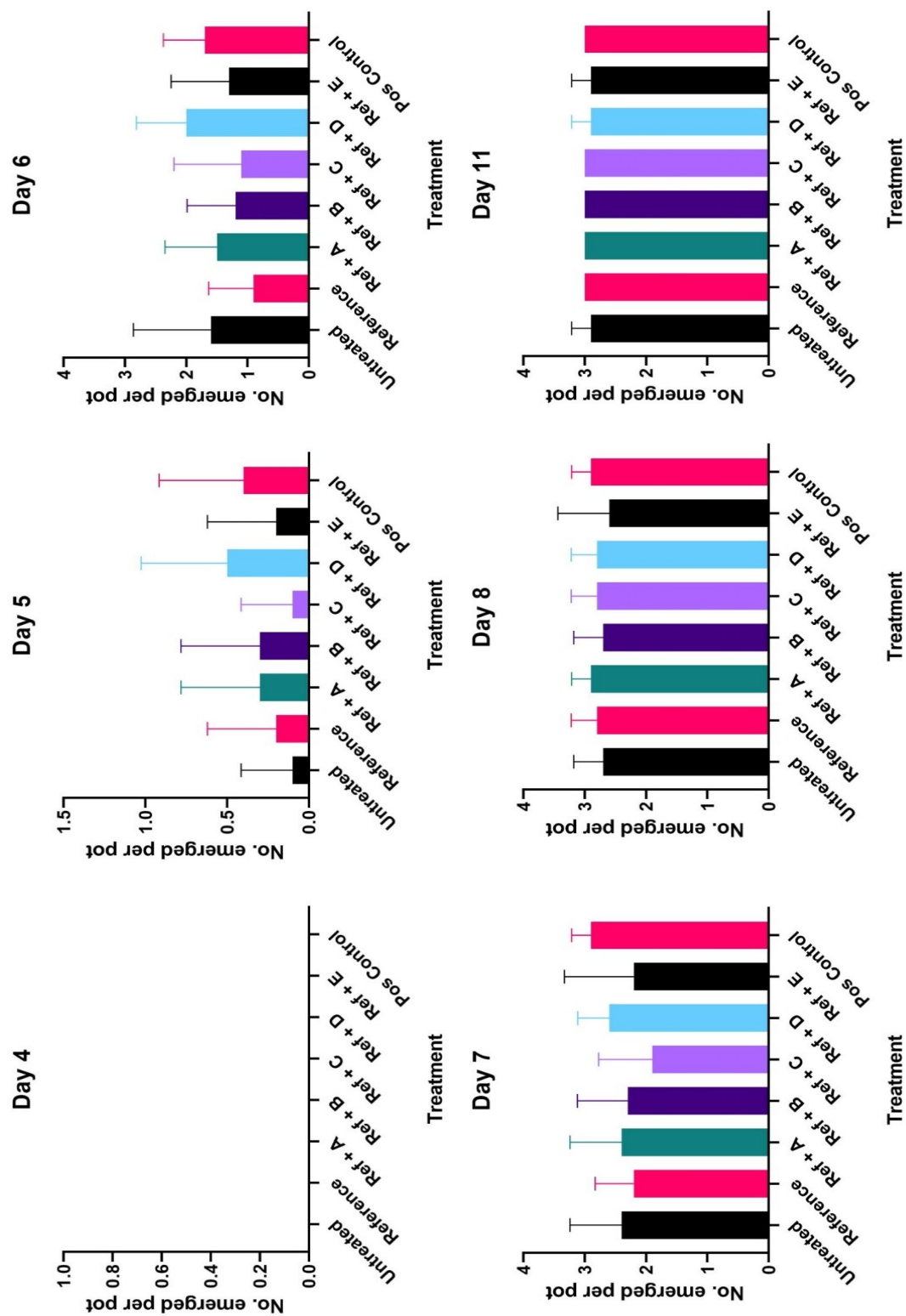


Figure 35: A representation of the number of plants emerged in each pot by treatment at time points throughout the growth trial. The total number of seeds sown in each pot was three.

The emergence data given in figure 35 showed no significant difference in the rate of emergence of shoots, regardless of the treatment applied. None of the plants had begun to emerge by day 4, whilst all of them showed signs of emergence by day 5. Although there were differences in the mean number of plants emerged per pot between some of the treatments, these were not statistically significant. Waseem Abass *et al* reported similar findings from a laboratory experiment which compared the germination and emergence rate for wheat seedlings treated with a range of commercially available seed film coatings, compared with primed seeds and untreated controls. No significant difference was reported in the emergence rate between untreated seedlings and seedlings treated with any of the commercial seed film coatings.¹⁶⁵ The seed treatments used in the experiment reported by Abass *et al* included a range of Incotec products, although none of them contained zinc, they did contain a range of biostimulants including PEG 6000 and CaCl_2 .²³

The recorded fresh mass data is represented in figure 36 and tabulated in table 10.

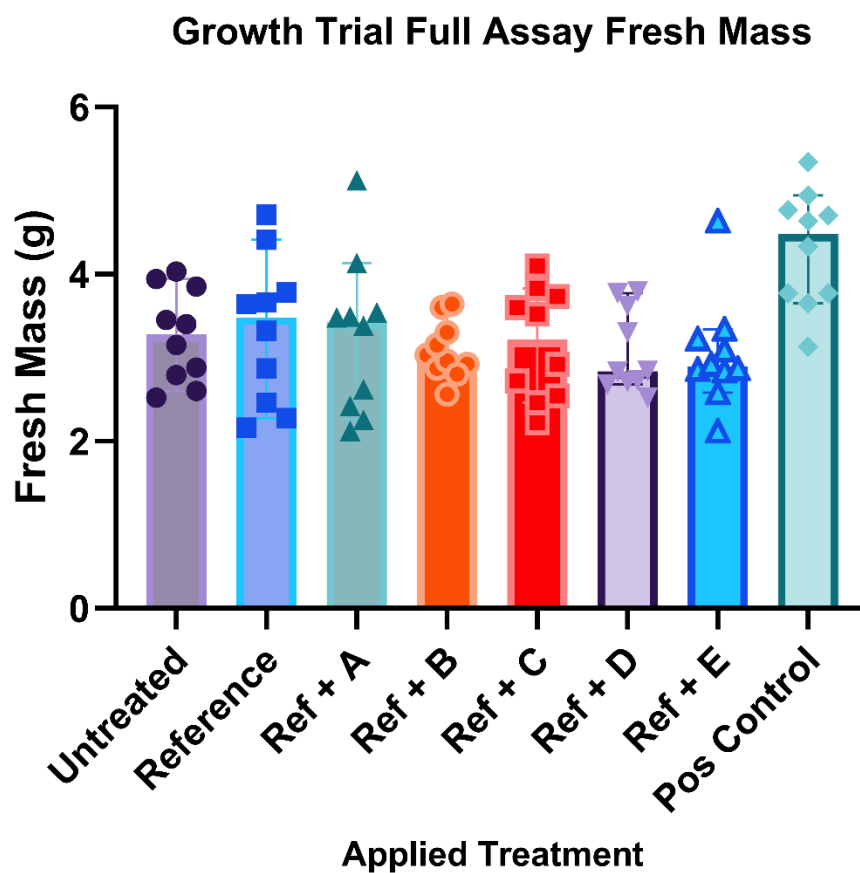


Figure 36: A representation of the number of plants emerged in each pot by treatment at time points throughout the growth trial. The total number of seeds sown in each pot was three.

Treatment	Mean Fresh Mass (g)
Untreated	3.26
Reference	3.33
Ref + A	3.26
Ref + B	3.08
Ref + C	3.16
Ref + D	3.08
Ref + E	3.05
Pos Control	4.30

Table 10: Fresh masses recorded following the third growth trial.

None of the seed treatments resulted in a significant increase in fresh mass when compared to the untreated plants. The fresh masses of the positive control plants (untreated seeds treated with full Zn Hoagland's) were significantly higher than the fresh masses of the untreated seeds and of all of the seed treatments. Therefore, there was no observable benefit of seed treatment in terms of fresh mass detected through this trial.

The masses of plant material once dried also indicated no significant benefit of seed treatment when compared to the untreated plant material, the results are given in figure 37 and table 11.

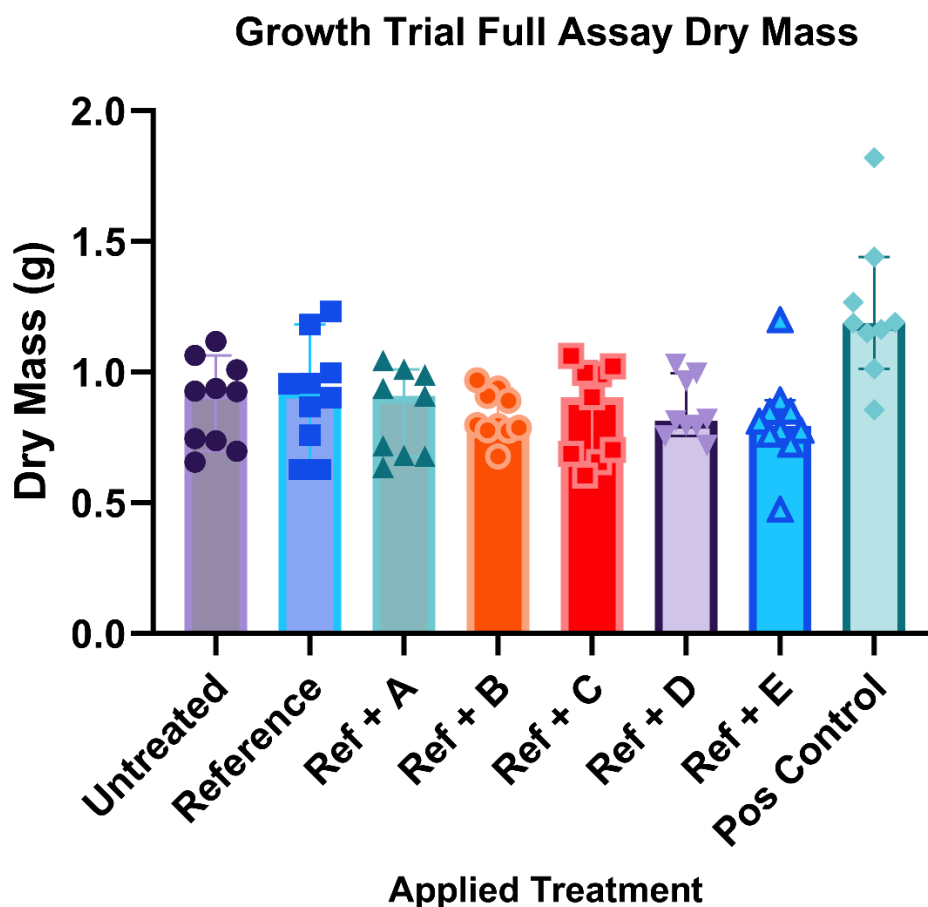


Figure 37: A comparison of the recorded dry mass of the plant material for each of the treatments harvested at the end of the full assay growth trial after drying overnight in a 60 °C oven.

Treatment	Mean Dry Mass (g)
Untreated	0.882
Reference	0.911
Ref + A	1.00
Ref + B	0.832
Ref + C	0.914
Ref + D	1.053
Ref + E	0.811
Pos Control	1.205

Table 11: Mean dry mass results from the third growth trial.

Like the fresh mass data, the dry mass of the positive control plants was significantly greater than the dry masses of any of the plant material from treated seed.

The results of the elemental analysis of the plant material from the full assay growth trial is given in figure 38 and table 13.

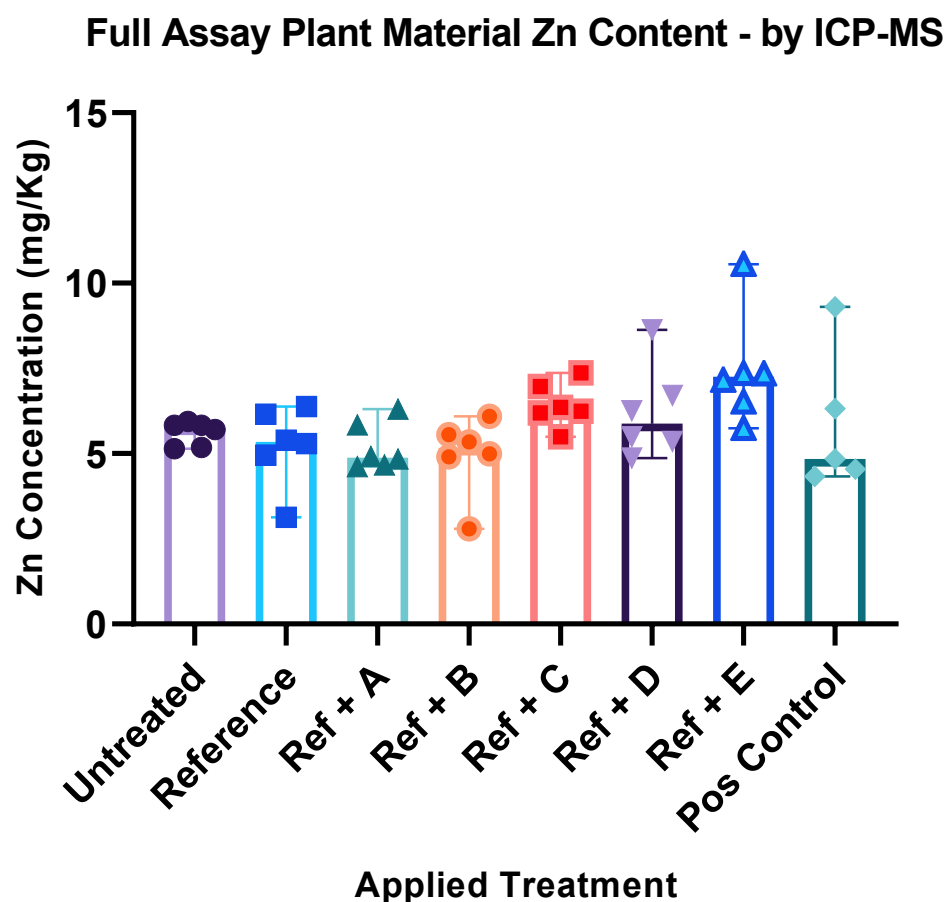


Figure 38: A comparison of the Zn concentration by ICP-MS of the digested plant material from the full assay growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.

The Zn content by ICP-MS data was analysed using the Kruskal Wallis test followed by Conover-Inman pairwise comparisons, the significant results are given in table 12.

Treatments	Significance	P value
Untreated v Ref + E	significant	P = 0.0147
Reference v Ref + C	significant	P = 0.0369
Reference v Ref + E	significant	P = 0.0058
Ref + A v Ref + E	significant	P = 0.001
Ref + A v Ref + C	significant	P = 0.0079
Ref + B v Ref + C	significant	P = 0.009
Ref + B v Ref + E	significant	P = 0.0011

Table 12: A summary of the results of the pairwise Conover-Inman statistical analysis of the Zn content of plant material from the full assay growth trial.

The inclusion of adjuvant E into the seed treatment formulation significantly increased Zn uptake compared to the plant material from the untreated seed, the ZnO treated seed (no adjuvant), the ZnO with adjuvant A treatment and the ZnO with adjuvant B treatment.

Whilst the inclusion of adjuvant C in the ZnO seed treatment was significantly beneficial compared to the ZnO treatment, ZnO with adjuvant A and ZnO with adjuvant B, the plant material from the Ref + C seed treatment was not significantly higher than the Zn content of the untreated plants.

The Cu and Mn content of the plant material was also analysed by ICP-MS as these elements were included in the Hoagland's solution but would not be present in the seed coatings. The results are given in figures 39 and 40 respectively, as well as in table 13.

Adjuvant E is an adjuvant which is made up of a blend of surfactants. Internal Croda testing has shown that E has strong 'soil wetting' characteristics.¹⁶⁶ Adjuvant E is known to retain moisture in the soil significantly more effectively than untreated soils. It is possible that the increased Zn content in the plants treated with the seed treatment containing adjuvant E is due to the retention of moisture around the seed zone. The localisation of Zn due to being applied as a seed coating combined with the moisture retention properties of adjuvant E, may create a more favourable environment for Zn-uptake by the roots of the growing plant.

Full Assay Plant Material Cu Content - by ICP-MS

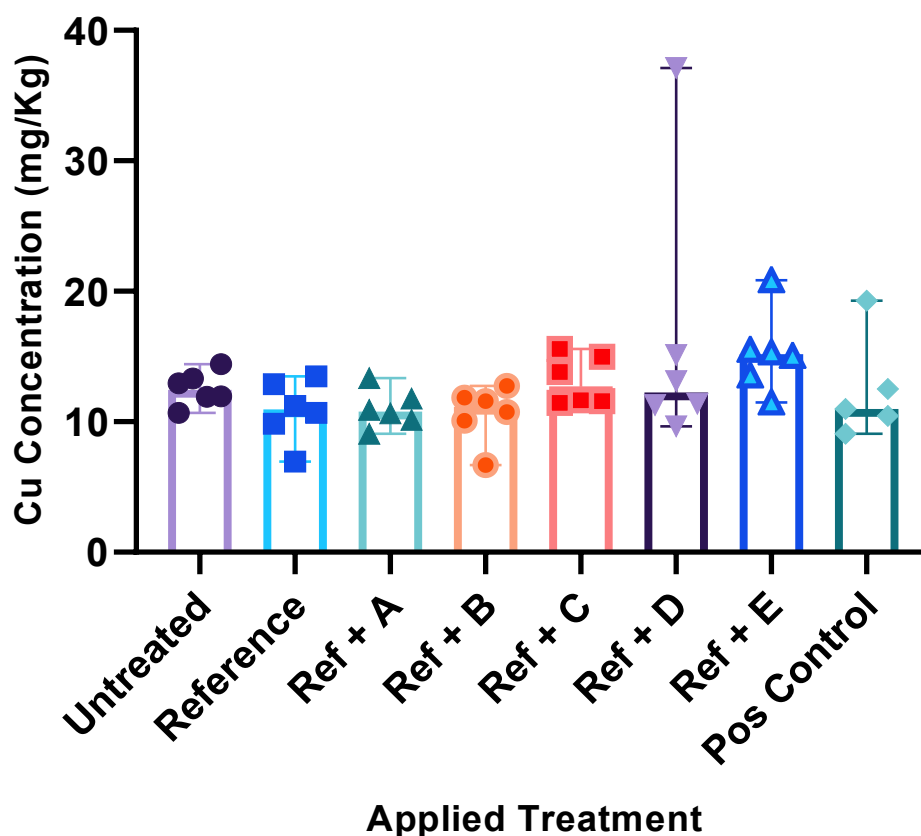


Figure 39: A comparison of the Cu concentration by ICP-MS of the digested plant material from the full assay growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.

The Cu content by ICP-MS data was analysed using the Kruskal Wallis test, followed by Conover-Inman pairwise comparisons. In this case, none of the treated plants were significantly higher in Cu content than the untreated plants. Since the source of Cu would be the Zn-free Hoagland's solution and this was applied to all of the samples (apart from the positive control), it can be concluded that the experiment showed no benefit of adjuvant inclusion in the seed coating

formulation on the uptake of nutrients from the wider growing medium. This would suggest that the co-localisation of the nutrient and the adjuvant may be an important factor in any increased uptake observed.

Full Assay Plant Material Mn Content - by ICP-MS

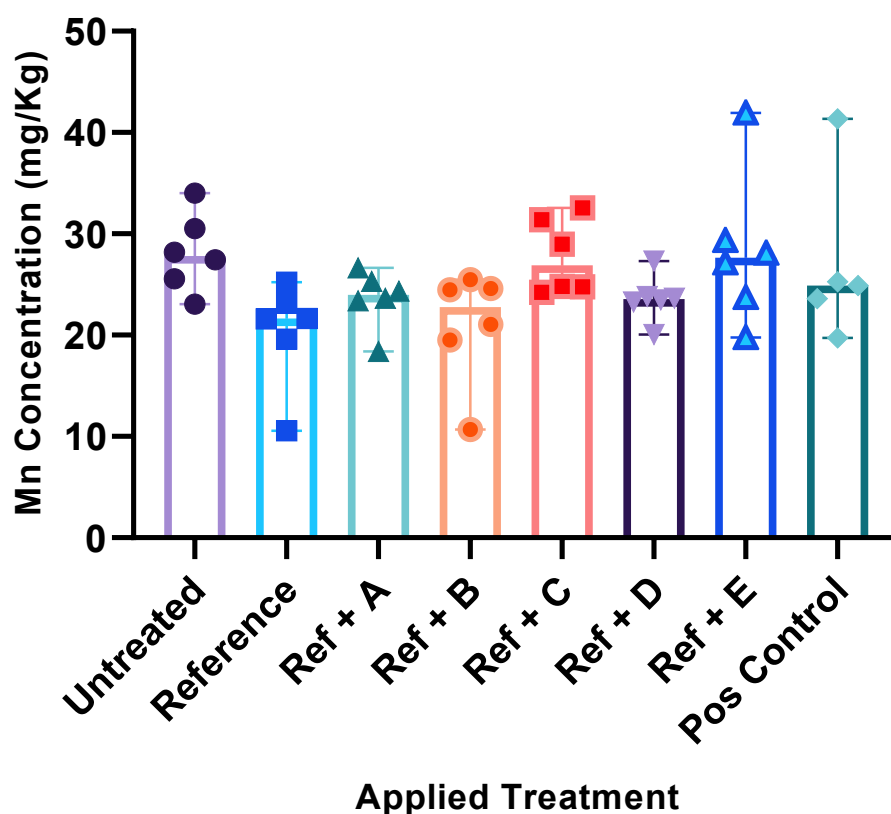


Figure 40: A comparison of the Mn concentration by ICP-MS of the digested plant material from the full assay growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.

Similar to the Cu uptake data, there was no evidence that the presence of seed coating, whether it be with or without adjuvant in the formulation had no beneficial impact on the uptake of Mn. Like Cu, Mn would only have been introduced to the plants via the Hoagland's solution. In fact, the untreated plants were significantly higher in Mn than the Reference ($P=0.0006$), Reference with adjuvant B ($P=0.0233$) and Reference with adjuvant D treatments ($P=0.0087$). This suggests

that the seed treatment may have hindered the uptake of nutrients other than the Zn applied to the seed, rather than have an additional benefit to the intended increase in Zn nutrition.

Treatment	Median Zn Concentration (mg/Kg)	Median Cu Concentration (mg/Kg)	Median Mn Concentration (mg/Kg)
Untreated	5.71	13.3	30.5
Reference	4.95	9.8	19.6
Ref + A	4.84	10.6	24.3
Ref + B	5.00	11.6	24.6
Ref + C	6.19	11.6	24.8
Ref + D	5.36	11.3	23.3
Ref + E	7.35	15.3	29.4
Positive Control	4.55	10.5	23.6

Table 13: Tabulated concentrations of Zn, Cu and Mn by ICP-MS data of the digested plant material from the full assay growth trial. Values stated are median mg/Kg values

5.8. Growth Trial 4

5.8.1. Growth Trial 4 – study design

Following the results of growth trial 3, it was posseted that growing the plants for 6 weeks was too long to be able to detect the effect of a seed applied micronutrient solution. As such, another growth trial was carried out. This time with more plants grown for 4 weeks to give a similar overall mass of plant material, but from earlier stage plants. The effect of a micronutrient-containing seed coating on plant growth was expected to be most apparent during the early stages of plant development and so the reduction in growth time with this trial was designed to increase the likelihood of detecting such an effect.

The dry mass of the plant material was recorded after the material was dried overnight in an oven at 60 °C. The results are given in figure 41.

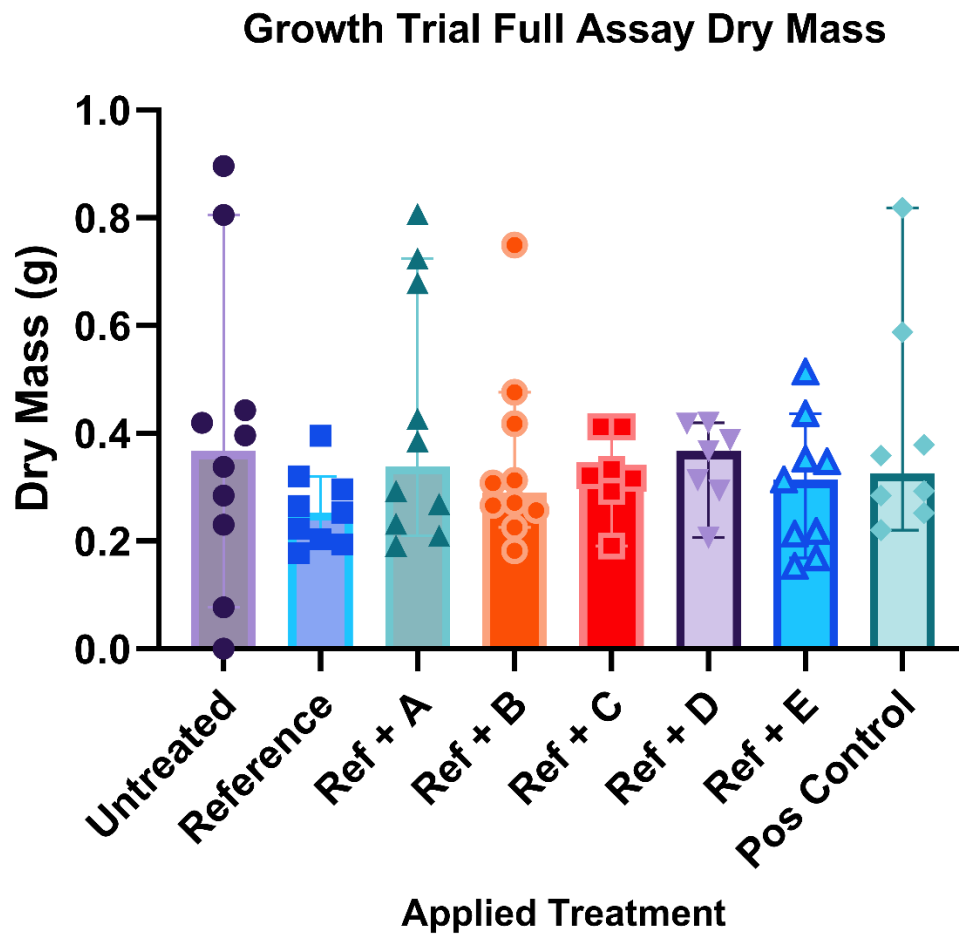


Figure 41: A comparison of the recorded dry mass of the plant material for each of the treatments harvested at the end of the fourth growth trial after drying overnight in a 60 °C oven.

Treatment	Mean Dry Mass (g)
Untreated	0.39
Reference	0.32
Ref + A	0.42
Ref + B	0.35
Ref + C	0.24
Ref + D	0.27
Ref + E	0.39
Pos Control	0.45

Table 14: Summary of mean dry masses (g) of plant shoot material from the fourth growth trial

Whilst there was no significant difference observed between the mean dry masses of the plant material ($P=0.0601$), there did appear to be some trend differences in the results, with the mean value for Ref appearing to be lower than the rest of the treatments, since $P>0.05$ these differences cannot be considered to be significant. This was consistent with the shorter growth period of this trial,

generally differences in plant mass are not detected in growth trials until much later in the growth cycle.

Once the plants were harvested, the shoots were collected, dried, digested and analysed by ICP-MS. The results from this analysis are given in figure 42 and summarised in table 15.

Growth Trial 4 Plant Material Zn Content - by ICP-MS

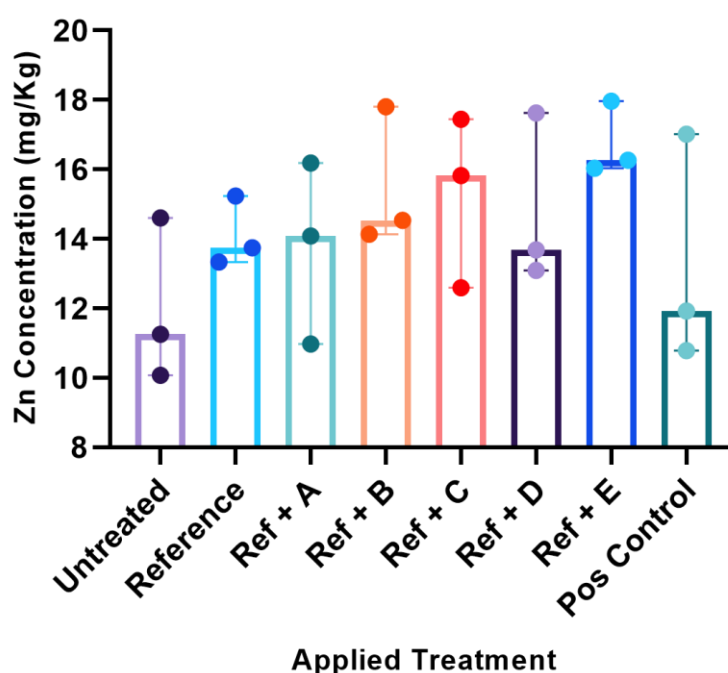


Figure 42: A comparison of the Zn concentration by ICP-MS of the digested plant material from the full-scale growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.

The elemental analysis of the plant material by ICP-MS showed a significantly higher level of Zn in the plant tissue of the seeds treated with the Ref + E seed coating compared with the untreated seed ($P = 0.0279$). Although the other

treatments showed a trend of increased Zn compared to the untreated seed, none of these differences were statistically significant.

The results of the Cu and Mn analysis are given in figures 43 and 44, as well as in table 15.

Growth Trial 4 Plant Material Cu Content - by ICP-MS

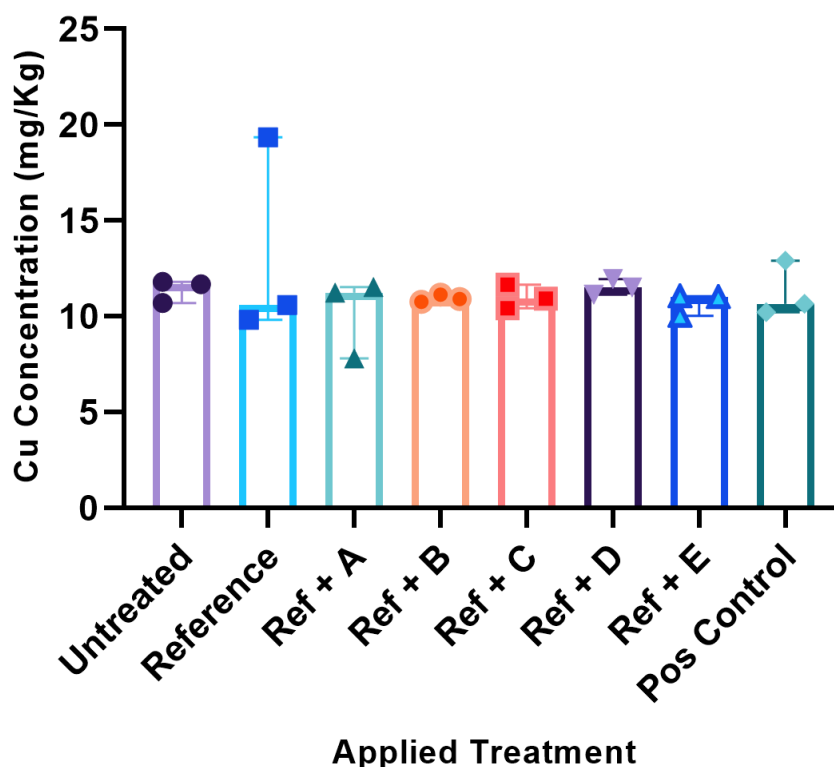


Figure 43: A comparison of the Cu concentration by ICP-MS of the digested plant material from the full-scale growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.

Growth Trial 4 Plant Material Mn Content - by ICP-MS

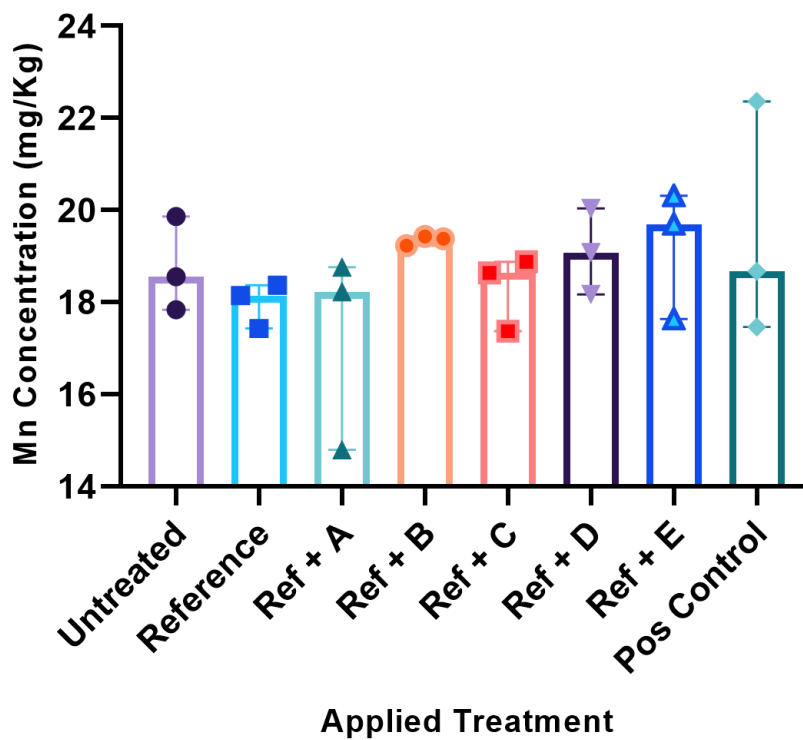


Figure 44: A comparison of the Mn concentration by ICP-MS of the digested plant material from the full-scale growth trial. Each data point represents one separately digested repeat, the height of the column represents the median and the error bars represent the 95% confidence interval.

Treatment	Median Zn Concentration (mg/Kg)	Median Cu Concentration (mg/Kg)	Median Mn Concentration (mg/Kg)
Untreated	11.25	11.68	18.55
Reference	13.74	10.60	18.15
Ref + A	14.08	11.24	18.23
Ref + B	14.54	10.90	19.38
Ref + C	15.82	10.92	18.64
Ref + D	13.68	11.51	19.07
Ref + E	16.27	11.01	19.70
Positive Control	11.92	10.66	18.67

Table 15: Tabulated concentrations of Zn, Cu and Mn by ICP-MS data of the digested plant material from the fourth growth trial. Values stated are median mg/Kg values.

The elemental analysis showed no significant difference between any of the seed treatments in terms of the Cu content of the analysed plant material. The Cu was included in the analysis as it was not present in the seed treatment but would be introduced via the Hoagland's solution applied to all of the samples at the same rate. The fact that there was no significant difference suggests that the presence of adjuvant in a seed treatment does not increase the uptake of metals which are introduced via irrigation of the growing medium. This is supported by the Mn results from the elemental analysis. There was no significant difference in the

uptake of Mn with any of the treatments. The fact that the uptake of nutrients only present in the Hoagland's solution is not affected by the inclusion of adjuvant, while the uptake of seed applied nutrient combined with seed applied adjuvant does show a significant effect suggests that the combination of nutrient-containing seed coating and adjuvant is required to see the beneficial effects. The inclusion of adjuvant in a seed coating does not seem to have further effects on the uptake of nutrients contained within the soil. The benefit is limited to increased uptake of nutrient contained within the seed treatment formulation.

5.9. Conclusion

In conclusion, this work presents the development, and subsequent results of a controlled glasshouse growth trial methodology coupled with elemental analysis by ICP-MS which was able to identify a Zn deficiency in young wheat plants, investigate the effects of Zn-containing seed treatment of young wheat plants, and to investigate the effects of adjuvant inclusion on Zn-containing seed treatments. The method was able to identify a zinc deficiency based on the difference in plant Zn content when grown in inert soil treated with Zn-containing Hoagland's solution, compared to plants grown in inert soil treated with Zn-free Hoagland's solution. The second iteration of the growth trial study examined the effect of Zn-containing seed coated seeds compared to untreated seed. There were small increases in the plant height, fresh mass and the plant Zn content of the treated seeds compared to the untreated seeds. There was no difference in the recorded dry masses from the treated and untreated seeds. Once the trial was expanded to include a range of adjuvants included in the Zn-containing seed coating formulation, adjuvant effects were identified. The inclusion of adjuvant E gave rise to an increase in the Zn content of the young wheat plants compared to the other treatments tested. The increase was detected for Zn, but it was not detected for Cu or Mn. These were included in the elemental analysis as they were applied as part of the Hoagland's solution, so any increase detected would be due to the effect of the adjuvant on the nutrients in the surrounding growth medium. This was not observed in these studies, which means that the increase was only seen for Zn as this was applied in the same seed coating formulation as the adjuvant. The co-localisation of the Zn and the adjuvant both being applied

to the seed was required to see the beneficial effects of the adjuvant application. Adjuvant E has strong 'soil wetting' capabilities. We propose that the mechanism of action may be that the adjuvant holds Zn and moisture close to the seed to which they are applied as it grows, increasing the concentration of Zn in the root zone, thus leading to the increased uptake of Zn during the early stages of plant growth.

Page left intentionally blank

6.0. Studies on the effects of the source of Zn

6.1. Introduction

As outlined previously in 1.1.2, zinc is an essential micronutrient for plant health and was the key active ingredient in the reference ZnO-containing seed coating which is being investigated throughout this work. It is involved in various cellular and physiological functions in plants, as well as playing a role in growth, development, and crop yield. Zn also plays crucial structural, catalytic, and regulatory roles in large numbers of proteins and enzymes.^{167,168}

Not all of the zinc in the soil is available to plants all of the time, its availability depends on the physico-chemical properties of the soil, the activity of plant roots and microflora in rhizosphere as well as other non-edaphic factors. Some Zn exists as insoluble complexes or is adsorbed and exchangeable. Water soluble forms of zinc are the most freely available to plants. Root activity, such as ion exchange and the release of organic acids make exchangeable forms of zinc available to some extent.¹⁶⁹ Therefore, to maximise the Zn uptake potential from soil, it would have to be present in the root zone in Zn^{2+} form.¹⁷⁰

Hyperaccumulators are plants which are capable of accumulating large concentrations of heavy metals in their above ground tissues, far in excess of the levels found in most species, without suffering from phytotoxic effects. The three key distinctions between hyperaccumulators and non-hyperaccumulators is that they have a strongly enhanced rate of metal uptake, faster root to shoot translocation, and a greater ability to detoxify and sequester heavy metals in leaves.¹⁷¹ *Brassica juncea*, commonly known as Chinese or Indian Mustard, is a

species of mustard plant. *Brassica juncea* grows quickly and is known to be a hyperaccumulator of heavy metals, including zinc.^{172,173}

The use of hyperaccumulators, including *Brassica juncea* as a means to study the uptake of heavy metals such as zinc has been widely reported in the literature. Previous work by Singh et al has studied the effect of zinc application rate as well as zinc source on uptake in *Brassica juncea*, reporting significant differences between plants treated with ZnO compared to plants treated with zinc sulphate reported.^{174 175}

The formulation of the seed treatment studied in this work is a flowable suspension, in which ZnO micro particles are suspended in a water-based solution by a blend of surfactants and other formulation aids. As such, the Zn source used must be a particle and thus not soluble in water. It has been widely established that the form of Zn applied to soils has a noticeable impact on the Zn nutrition of plant material.^{176,177} The literature also suggests that a more soluble Zn source would be more available for uptake by plants.¹⁷⁸ However, in this case a soluble Zn source would not be compatible with the flowable suspension seed coating which is being investigated.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is a sensitive technique for elemental analysis. The sample is ionised in the plasma before being analysed on the basis of its mass to charge ratio.¹⁷⁹ ICP-MS has been widely used for the study of the elemental composition of plant material.¹⁸⁰

6.2. Aims of the chapter

The aim of this work was to determine whether a small-scale Chinese mustard method could be developed to quickly differentiate Zn content of plants grown under different Zn treatment conditions, with a range of Zn sources and to determine the effect of the inclusion of adjuvant on the Zn uptake. The full-scale growth trial was carried out to translate the small-scale Chinese Mustard results into wheat and into a larger scale in the glasshouse.

6.3. Development of small-scale uptake experiments

6.3.1. Development of small-scale uptake experiments

Introduction

A methodology was developed to assess the uptake of Zn into plant material on a small, lab scale, to enable the effect of various sources of Zn on plant uptake to be assessed quickly and cheaply in the laboratory. Chinese Mustard (*Brassica juncea* L.) was chosen for these experiments as it is fast and easy to grow, as well as being a known hyperaccumulator of metals including zinc.^{181,182}

6.3.2. Methods – development of small-scale uptake experiments

Plastic trays were prepared for use in the growing experiments by drilling four 2 mm holes in the base of the tray, one in each corner to allow for drainage and to prevent the trays from becoming waterlogged during the experiment. The base of the tray was lined with two overlapping 150 mm filter papers to prevent the growing medium from passing through the drainage holes. The growing medium used was pure silver sand (Fisher Scientific, Loughborough, UK). Chinese Mustard Green seed (*Brassica juncea* L.) was purchased from Seed Co-operative (Spalding, UK).

A separate tray was prepared for each treatment. Each tray was placed on a separate impermeable plate to enable re-uptake of drain-off and to prevent contamination of each tray from the drain-off of another.

Each tray contained 350 g silver sand, with 5 g of Chinese mustard seed spread evenly across the sand. The tray was dampened on day 0 using 100 mL of the solution to be tested. At each irrigation point (days 3, 5 and 7) a further 100 mL of solution was added to each of the trays.

All growth experiments were carried out in a temperature and humidity-controlled growth cabinet (manufacturer) which was set to 22°C and 90 % humidity with the light set to level 3 24 Hrs a day.

6.3.3. *Proof of concept trial methods*

To determine whether the methodology could be used to observe differences in Zn uptake between different sources of Zn, a proof-of-concept trial was carried out. Three trays were prepared as described in 2.1 to examine three treatments. The three treatments to be included were zinc oxide (ZnO), zinc sulphate (ZnSO₄) and zinc nitrate (ZnNO₃)₂. Each Zn source was prepared to equate to 50 ppm Zn per solution. Each tray was prepared before being placed in the growth cabinet under the conditions described in 6.3.2. As ZnO is not completely soluble in water, each treatment was shaken vigorously for 30 seconds prior to application, to ensure each was as homogenous as possible. Figure 45 is a photograph of the trays at the first irrigation point after sewing (day 3) showing how the seeds were arranged in the sand and the set-up of the trays.

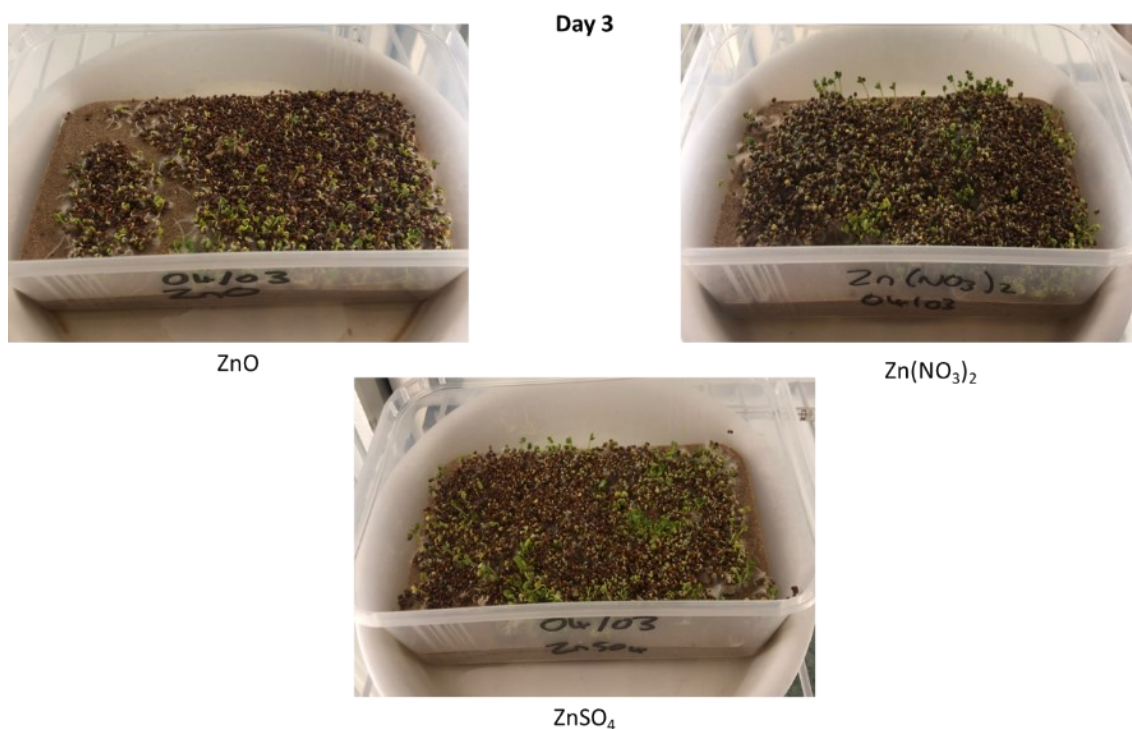


Figure 45: Photographs of the trays containing silver sand and Chinese mustard seeds at the first irrigation point, on day 3 after sewing.

On day 10, the plant material was harvested. Whole shoots, including root material were separated from the silver sand matrix by gently removing them by hand, before rinsing the root zone in deionised water to remove any remaining sand residue.

The plant material was then dried in a freeze drier to constant mass and the total dry mass of the plant material from each treatment was recorded.

The material was ground using an electric coffee grinder for 120 seconds, until a homogenous powder was formed. This powder was then randomly sampled in triplicate for digestion and analysis by ICP-MS.

6.3.4. Second Growth Trial Methods

The second growth trial was a repeat of the proof-of-concept trial, with the addition of a control treatment. The control was treated only with deionised water. The trays were prepared and treated as per the methodology outlined in section 6.3.2. Again, Zn treatments, ZnO, ZnSO₄ and Zn(NO₃)₂ were prepared to 50 ppm. On day 10, the plant material was harvested. Whole shoots, including root material were separated from the silver sand matrix by gently removing them by hand, before rinsing the root zone in deionised water to remove any remaining sand residue.

The plant material was then dried in a freeze drier to constant mass and the total dry mass of the plant material from each treatment was recorded.

The material was ground using an electric coffee grinder for 120 seconds, until a homogenous powder was formed. This powder was then randomly sampled in triplicate for digestion and analysis by ICP-MS.

6.4. Results of Development of small-scale uptake experiments

6.5. Proof-of-concept results

By day 3 the seeds from each of the treatments had shown signs of emergence, with green shoots being seen emerging from a number of the sewn seeds. By day 5 all of the treatments had continued to emerge and were starting to grow and form leaf structures, by this point the tray was completely covered in growing plants with few gaps in between visible. By day 7, the treated mustard had continued to grow and were growing upwards towards the top of the tray. The plants treated with ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$ appeared to be marginally taller at this point than the mustard treated with ZnO , though this was not measured or quantified so is only a visual interpretation. By day 10 there was very little visual difference between the mustard from any of the treatments. They had all grown evenly with the majority of the plant heights just below the rim of the container. A compilation of photographs from each irrigation point are given in figure 46.

Day 5	ZnO	ZnSO ₄	Zn(NO ₃) ₂
Front			
Top			
Day 7	ZnO	ZnSO ₄	Zn(NO ₃) ₂
Front			
Top			
Day 10	ZnO	ZnSO ₄	Zn(NO ₃) ₂
Front			
Top			

Figure 46: Collation of photographs showing the growth of each of the treated Chinese mustard trays, shown on days 5, 7 and 10 after sewing.

The dry mass of plant material was recorded and are shown in table 16 and are displayed in figure 47.

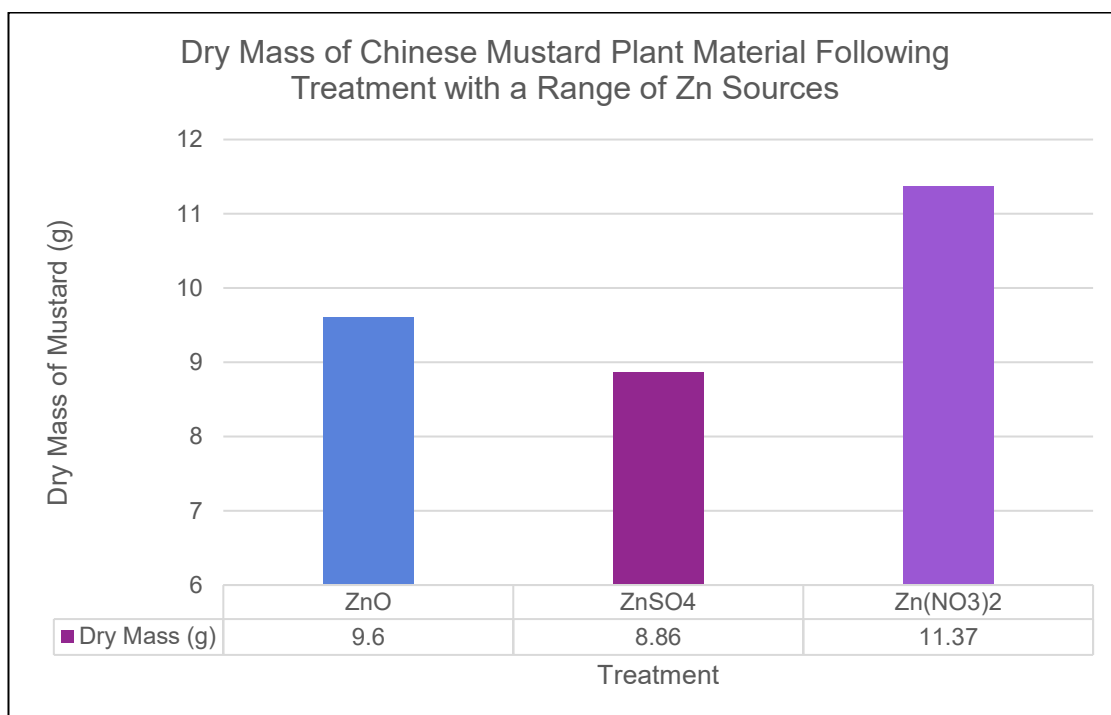


Figure 47: Graphical representation of the dry masses recorded after harvesting and drying the plant material from the proof-of-concept trial.

Treatment	Median Dry Mass (g)
ZnO	9.6
ZnSO ₄	8.9
Zn(NO ₃) ₂	11.4

Table 16: Table of dry masses of the Chinese mustard plants in grams once harvested, separated by Zn treatment applied to each tray.

The dry mass of the plant material treated with zinc nitrate was 11.4 g, the ZnO treated plant material was 9.6 g whilst the zinc sulfate treated plant material was 8.9 g. The increased dry mass of the zinc nitrate treated plant material suggests that the presence of nitrate may have a beneficial effect on plant growth.

This is not surprising since it is well established that nitrogen fertilisation increases plant growth, as such nitrogen is the basis of a large number of fertilisers in use around the world.¹⁸³ Furthermore, it has been shown that within the Brassica genus of plants (broadly defined as cruciferous vegetables, cabbages or mustard plants), treatment with nitrate has a significantly positive impact on the yield of plant material, both in terms of overall mass and the mass of shoots.¹⁸⁴

The plant material was then sampled, microwave digested in triplicate and analysed using ICP-MS. The total Zn content was measured for the plant material from each of the treatments to give a bulk measure of the amount of Zn contained within the plant tissue in mg/Kg. The results are given in figure 48 and table 17.

Uptake of Zn from a Range of Sources in Chinese Mustard - by ICP-MS

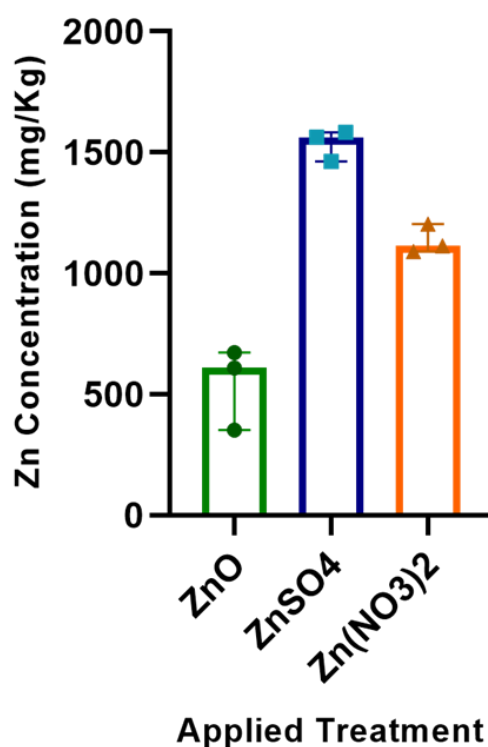


Figure 48: Zn concentration in digested Chinese mustard plant material, grown during the proof-of-concept trial, analysed by ICP-MS.

Treatment	Median Zn Content (mg/Kg)
ZnO	609
ZnSO ₄	1562
Zn(NO ₃) ₂	1114

Table 17: Zn concentration in digested Chinese mustard plant material, grown during the proof-of-concept trial, analysed by ICP-MS.

Both the zinc sulfate and zinc nitrate treated plant materials were significantly higher in Zn content than the zinc oxide treated plant material. Zinc sulfate was significantly higher in Zn than Zn nitrate.

Due to the variation in plant dry mass, it was theorised that the tissue Zn concentration could be adversely affected by the extra plant material of a treatment which gave rise to a greater dry mass. More plant material could mean that the tissue Zn concentration would be lower, even though the total amount of zinc which had been up taken may have been greater. Given the aim of this assay was to determine the effect of the source of Zn on the total uptake of Zn by the plant, it was decided that the Zn content should be normalised to the plant's dry mass to give a measure of the amount of Zn uptake by the plants. Furthermore, it is expected that Zn would be localised at the growing tip of the plant. Extra biomass was likely to be in the shoot, rather than the leaves and so the extra biomass could reduce the tissue concentration, even if the Zn level localised in the growing parts of the plant was higher. The normalisation accounted for the plant growth benefit of nitrate fertilisation and isolated the availability of Zn from the various sources as the only variable. This was a similar approach to data analysis as taken by Watts-Williams et al. They investigated the effect of mycorrhizal root zone colonisation in combination with soil amended with a range of Zn sources in tomato plants. As part of their data analysis, they normalised the plant Zn concentration to the plant mass to give a Zn uptake per plant value.¹⁷⁷

The normalised data is given in figure 49 and table 18.

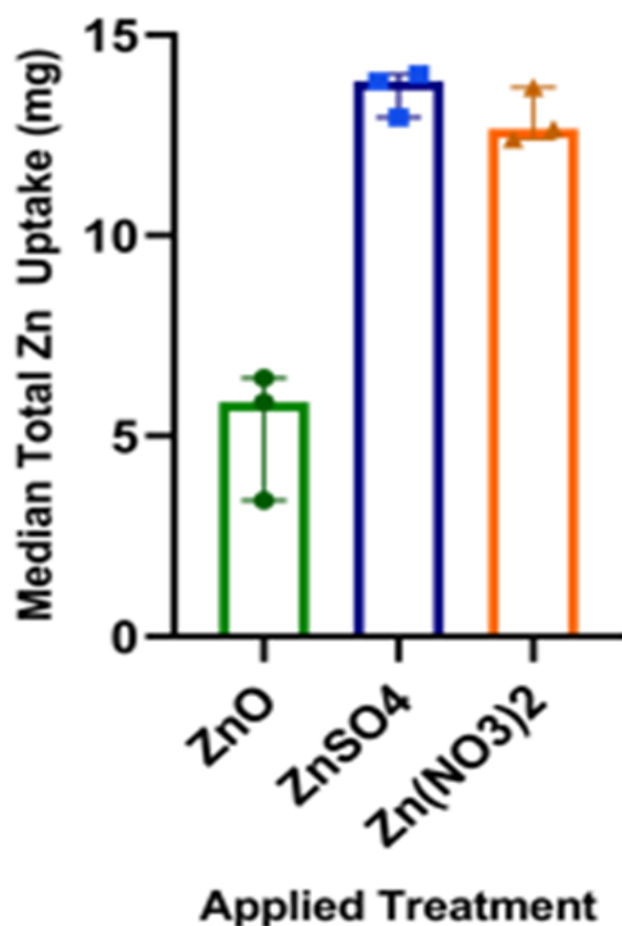


Figure 49: Uptake of Zn normalised to plant mass in digested Chinese mustard plant material, grown during the proof-of-concept trial, analysed by ICP-MS.

Treatment	Median Zn Uptake (mg)
ZnO	5.9
ZnSO ₄	13.9
Zn(NO ₃) ₂	12.7

Table 18: Uptake of Zn normalised to plant mass in digested Chinese mustard plant material, grown during the proof-of-concept trial, analysed by ICP-MS.

After normalisation, the zinc nitrate and zinc sulfate treated plant material had still taken-up significantly larger amounts of zinc compared to the zinc oxide treated plant material. This time, however, there was no significant difference between the sulfate and nitrate treated plant material.

This study was designed as a proof of concept, firstly to investigate whether Chinese Mustard was a suitable plant species to assess the uptake of Zn in different forms, and whether the method for treatment, digestion and analysis of plant material was suitable for this study. The results show uptake of Zn by Chinese Mustard when applied at the 50 ppm Zn concentration as used in this study and analysed by ICP-MS. Secondly the results suggest that differences in uptake with differing zinc treatments may be observable using this assay design. As such, and as detailed throughout the rest of this chapter, this proof of concept was used as the basis for increasingly robust and wider encompassing experiments.

6.6. Second growth trial results – addition of DI water

control

Plants were grown according to the protocol and conditions laid out in section 6.3.4 Once the plants were harvested the dry masses of each were recorded. The results are given in figure 50 and table 19.

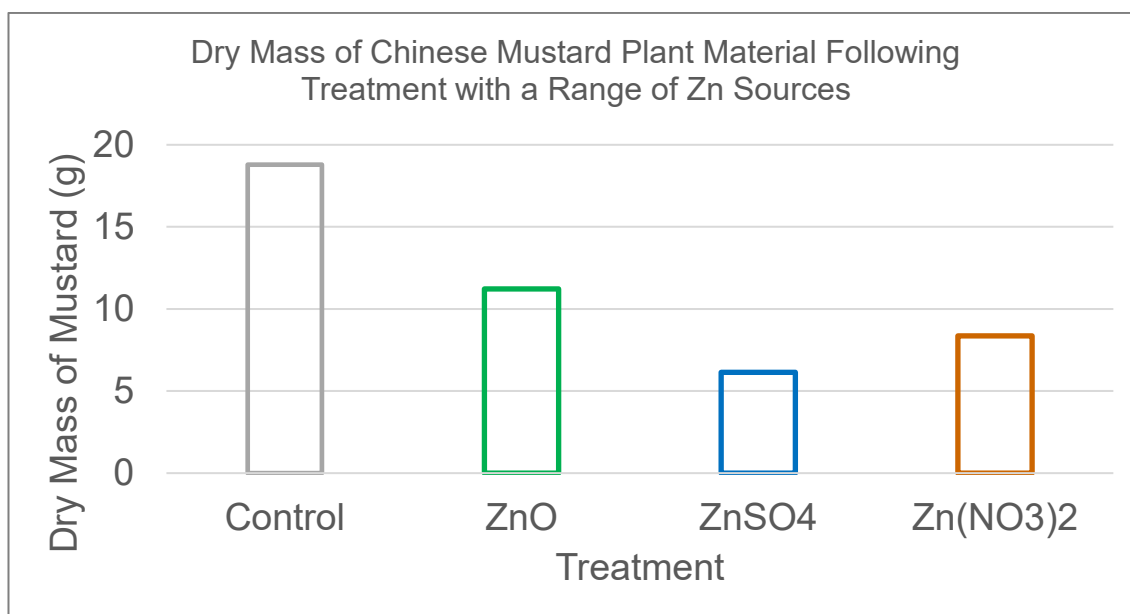


Figure 50: Graphical representation of the dry masses of Chinese Mustard plant material generated as part of the second growth trial. Dry mass was recorded using a 4 figure balance, following drying in the freeze dryer to constant mass.

Treatment	Dry Mass (g)
ZnO	11.22

ZnSO₄	6.14
Zn(NO₃)₂	8.36
Control	18.79

Table 19: Dry masses (g) of the plant material grown as part of the second growth trial.

The results show that the dry mass of plant material from each of the zinc treatments was lower than the water treated control, with water control having a mass of 18.79 g, compared to 11.22, 6.14 and 8.36 g for ZnO, ZnSO₄ and Zn(NO₃)₂, respectively. This would suggest that, at the 50 ppm concentration of Zn used for these experiments, irrigation with Zn-containing solutions may have a negative effect of mustard growth.

The plant material was digested and analysed by ICP-MS. The results are shown in figure 51 and table 20.

Uptake of Zn from a Range of Sources in Chinese Mustard - by ICP-MS

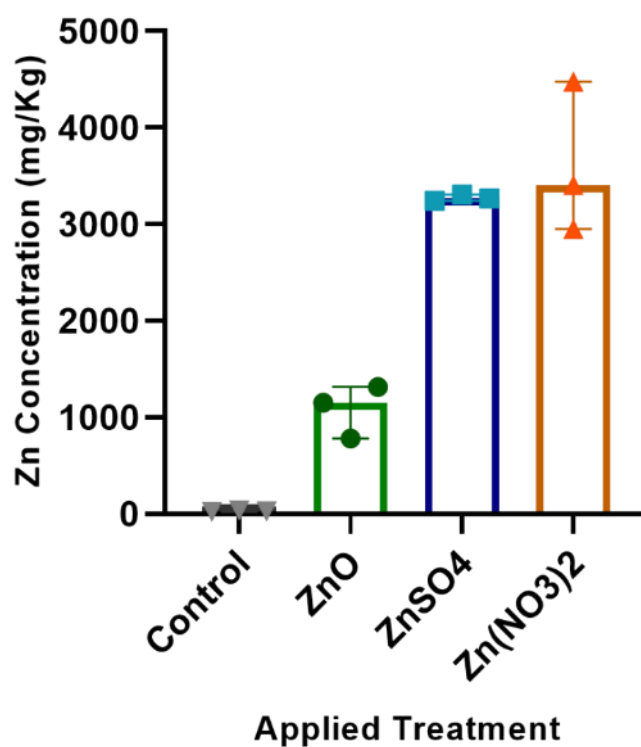


Figure 51: Zn concentration in digested Chinese mustard plant material, grown during the second growth trial, following microwave digestion and analysis by ICP-MS.

Treatment	Median Zn Content (mg/Kg)
ZnO	1154.0
ZnSO ₄	3266.8
Zn(NO ₃) ₂	3404.6
Control	26.6

Table 20: Zn concentration in digested Chinese mustard plant material, grown during the second growth trial, analysed by ICP-MS.

The results show a difference between all of the zinc treatments and the water-treated control. This indicates that there was an increase in the Zn content of the plant tissue when treated with Zn, regardless of the form of Zn which was applied. The results also show a significant difference between ZnSO_4 and ZnO as well as between $\text{Zn}(\text{NO}_3)_2$ and ZnO . There appears to be no significant difference in plant zinc content between those treated with ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$.

To account for the differences in plant mass by treatment, the Zn content was normalised to the dry mass harvested by multiplying the value in mg/Kg by the dry mass to give the total mass of Zn taken-up by the plant for each treatment in mg. Similar data processing methodologies have been reported in the literature. For example, Shaver et al. reported calculating the Zn uptake in mg per pot from the tissue Zn concentration whilst studying the effect of fertilizer solubility on Zn uptake in corn.¹⁸⁵ The normalised data is given in figure 52 and table 21.

Total Zn Uptaken by Chinese Mustard Following Treatment with Zinc Solutions Prepared with a Range of Sources of Zn - by ICP-MS

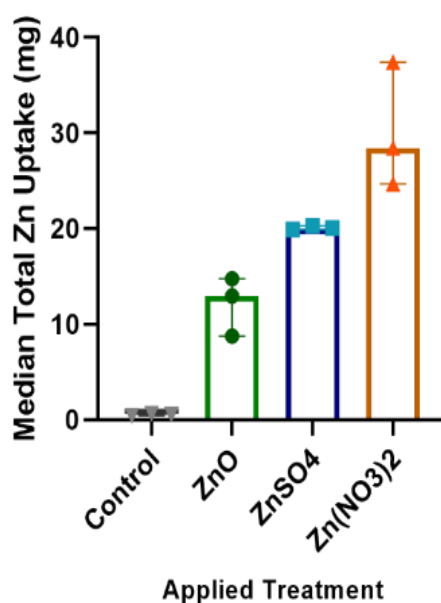


Figure 52: Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the second growth trial, analysed by ICP-MS.

Treatment	Median Zn Uptake (mg)
ZnO	12.9
ZnSO ₄	20.1
Zn(NO ₃) ₂	28.5
Control	0.5

Table 21: Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the second growth trial, analysed by ICP-MS.

Again, all treatments are significantly higher in total Zn uptake than the control treatment. Both ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$ are significantly higher in total Zn uptake than the sulfate and nitrate treated plants. Whilst there was no difference between the sulfate and nitrate in terms of tissue Zn concentration, there was more total uptake of Zn in the plants treated with $\text{Zn}(\text{NO}_3)_2$ compared with those treated with ZnSO_4 once the differences in plant mass were accounted for, though this difference was not statistically significant.

Both ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$ are salts and so they are considered to be soluble in water, ZnO in microparticle form as used here is not so soluble in water.^{186,187} The order of solubility in water is $\text{Zn}(\text{NO}_3)_2 > \text{ZnSO}_4 > \text{ZnO}$. Goodwin et al report the solubility of ZnO in water at 18 °C as 0.00042 g/100 cm³ water, compared with 53.8 g/100 cm³ water for ZnSO_4 and 211.5 g/100 cm³ water for $\text{Zn}(\text{NO}_3)_2$.^{188–190} The results broadly correspond to the relative solubility of each Zn source and appear to suggest that the more soluble sources of Zn result in an increased uptake of Zn by the plant. This seems to be reasonable since it has been widely shown that the solubility of a metal cations under soil conditions has a large impact on its bioavailability to a plant, so by extension has a large impact on the uptake of the metal by the plant. Whilst water soluble zinc would be freely available to plants, uptake of insoluble zinc would take place via the roots which efflux reductants and organic acids which enhance the solubility of Zn-complexes, enabling their uptake.¹⁹

6.7. Expansion of small-scale growth experiments to include adjuvant

The small-scale Zn uptake method described in 2.2 was adapted to include the use of adjuvant alongside ZnO to assess any effect of adjuvant inclusion on the uptake of ZnO from non-soluble sources, such as ZnO. The hypothesis was that the inclusion of adjuvant may increase ZnO uptake to be similar to that of the soluble Zn sources (ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$ in this case). This was important in relation to seed coatings as the seed coatings in question are formulated as a flowable suspension, with ZnO particles suspended in an aqueous medium using surfactant chemistry.

6.7.1. Methods for small-scale growth trial including adjuvant.

The third growth trial was a repeat of experiment reported in section 6.3.4 above, with the addition of adjuvant. The control was treated only with deionised water. The trays were prepared and treated as per the methodology outlined in section 6.3.2 Zn treatments, ZnO, ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$ were prepared to 50 ppm concentration of zinc. Adjuvant was included at 15% of the Zn content, in accordance with the way in which seed treatments were formulated. For this initial experiment adjuvant E was selected to act as a proof-of-concept. Given our understanding of the mechanism of Zn uptake, namely uptake via the roots, and the known activity of adjuvant E as a soil wetting adjuvant, it was selected as the adjuvant most likely to show a difference. The hypothesis was that the inclusion

of adjuvant E would retain moisture in the root zone, which would retain Zn in the root zone by extension, and therefore increase the uptake of Zn.

On day 10, the plant material was harvested. Whole shoots, including root material were separated from the silver sand matrix by gently removing them by hand, before rinsing the root zone in deionised water to remove any remaining sand residue.

The plant material was then dried in a freeze drier to constant mass and the total dry mass of the plant material from each treatment was recorded.

The material was ground using an electric coffee grinder for 120 seconds, until a homogenous powder was formed. This powder was then randomly sampled in triplicate for digestion and analysis by ICP-MS.

6.7.2. Results for small-scale growth trial including adjuvant

The dry mass results for the third growth trial are given in figure 53 and tabulated in table 22.

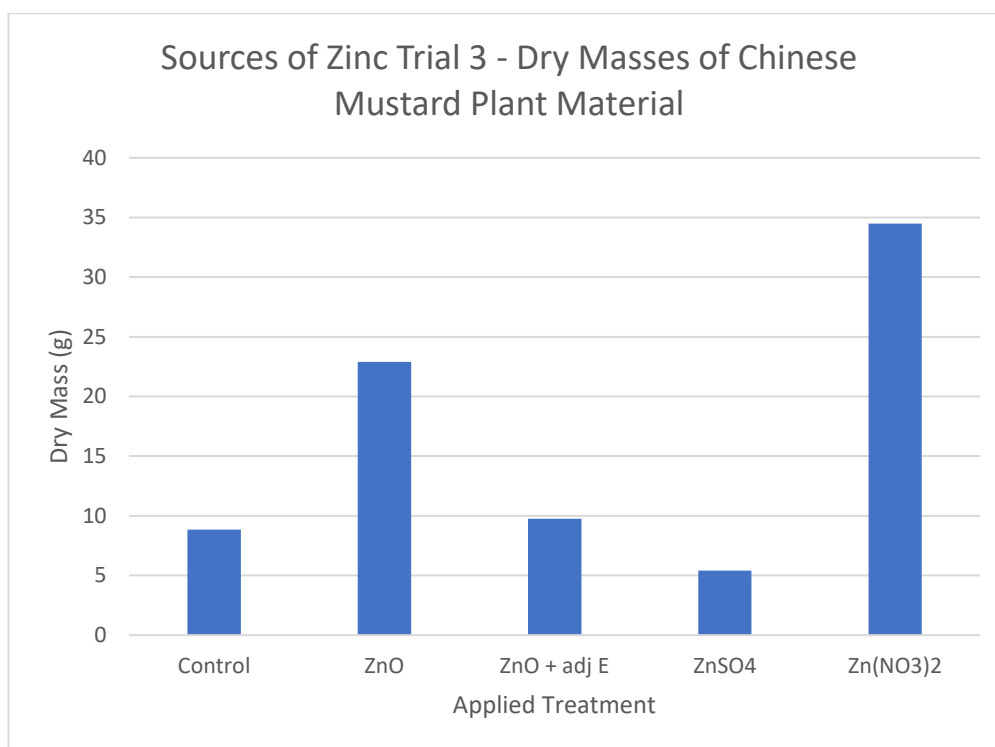


Figure 53: Graphical representation of the dry masses of Chinese Mustard plant material generated as part of the third growth trial following harvest and freeze drying of the resultant plant material to constant mass.

Treatment	Dry Mass (g)
ZnO	22.9
ZnO + E	9.73
ZnSO ₄	5.4
Zn(NO ₃) ₂	34.5
Control	8.85

Table 22: Dry masses (g) of the plant material grown as part of the third growth trial.

In this trial, the dry mass for Zn(NO₃)₂ was substantially higher at 34.5 g than those of the other Zn treatments and the water control. The ZnO treatment also appeared to result in an increase of dry mass, with a result of 22.9 g

As before, the plant material was digested, and the Zn content was analysed by ICP-MS. The elemental analysis results are given in figure 54 and table 23.

Zn Content in Chinese Mustard Treated with a Range of Zn Sources - by ICP-MS

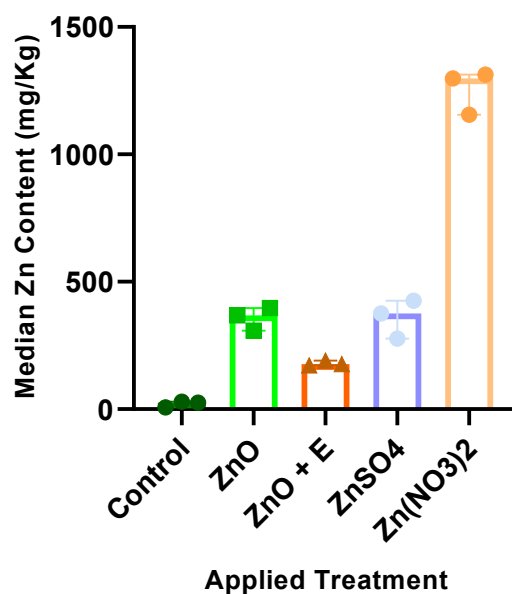


Figure 54: Zn concentration in digested Chinese mustard plant material, grown during the third growth trial, analysed by ICP-MS.

Treatment	Median Zn Content (mg/Kg)
ZnO	367.7
ZnO + E	176.7
ZnSO ₄	375.2
Zn(NO ₃) ₂	1298.7
Control	25.9

Table 23: Zn concentration in digested Chinese mustard plant material, grown during the third growth trial, analysed by ICP-MS.

As in previous trials, the results show a significant benefit in terms of plant Zn content when treated with $\text{Zn}(\text{NO}_3)_2$. However, this time there was no difference found between the ZnO and the ZnSO_4 treated plant material, which contradicts the findings of the previous trials. Furthermore, the inclusion of adjuvant E appears to have a negative effect on the plant Zn content in this trial.

Due to the large variation in dry mass yields, it was again necessary to account for those differences by normalising the plant Zn concentration to the dry mass to give a measure of the total mass of Zn up taken during the experiment.

These results are given in figure 55, and table 24.

Uptake of Zn from a Range of Sources in Chinese Mustard - by ICP-MS

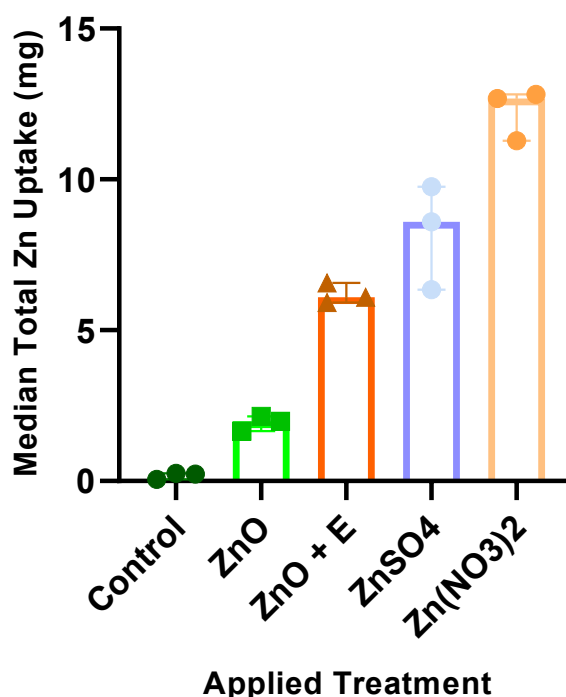


Figure 55: Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the third growth trial, analysed by ICP-MS.

Treatment	Median Zn Uptake (mg)
ZnO	2.0
ZnO + E	6.1
ZnSO ₄	8.6
Zn(NO ₃) ₂	12.7
Control	0.2

Table 24: Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the third growth trial, analysed by ICP-MS.

The ZnO and Zn(NO₃)₂ results appear to give a similar trend to the previous trials, where the more soluble Zn source leads to a higher Zn content in the plant tissue. The inclusion of adjuvant E in the ZnO treatment appeared to have a significantly beneficial effect on the Zn concentration recorded within the plant material. The ZnO + E treatment resulted in a significantly higher plant Zn concentration than ZnO alone.

To the best of my knowledge, there have been no published studies on the effect of adjuvant on Zn uptake in Chinese mustard, or similar crops, when applied in this way. The adjuvant profile of adjuvant E though; is of a soil-wetting adjuvant. That is to say, that adjuvant E was selected for this study because it increases the retention of water within the soil. Knowing that the majority of plant Zn uptake

is in the Zn^{2+} form and from the soil via the roots, it follows logically that the addition of adjuvant which would retain the aqueous solution, in this case containing ZnO in the soil and therefore in the root zone may increase the uptake of Zn. If only by increasing the length of time for which the soil is wet with ZnO solution which could be transformed to Zn^{2+} , taken-up by the roots and translocated within the plant.

These results were complimented by semi-quantitative LA-ICP-MS images which are given in figure 56.

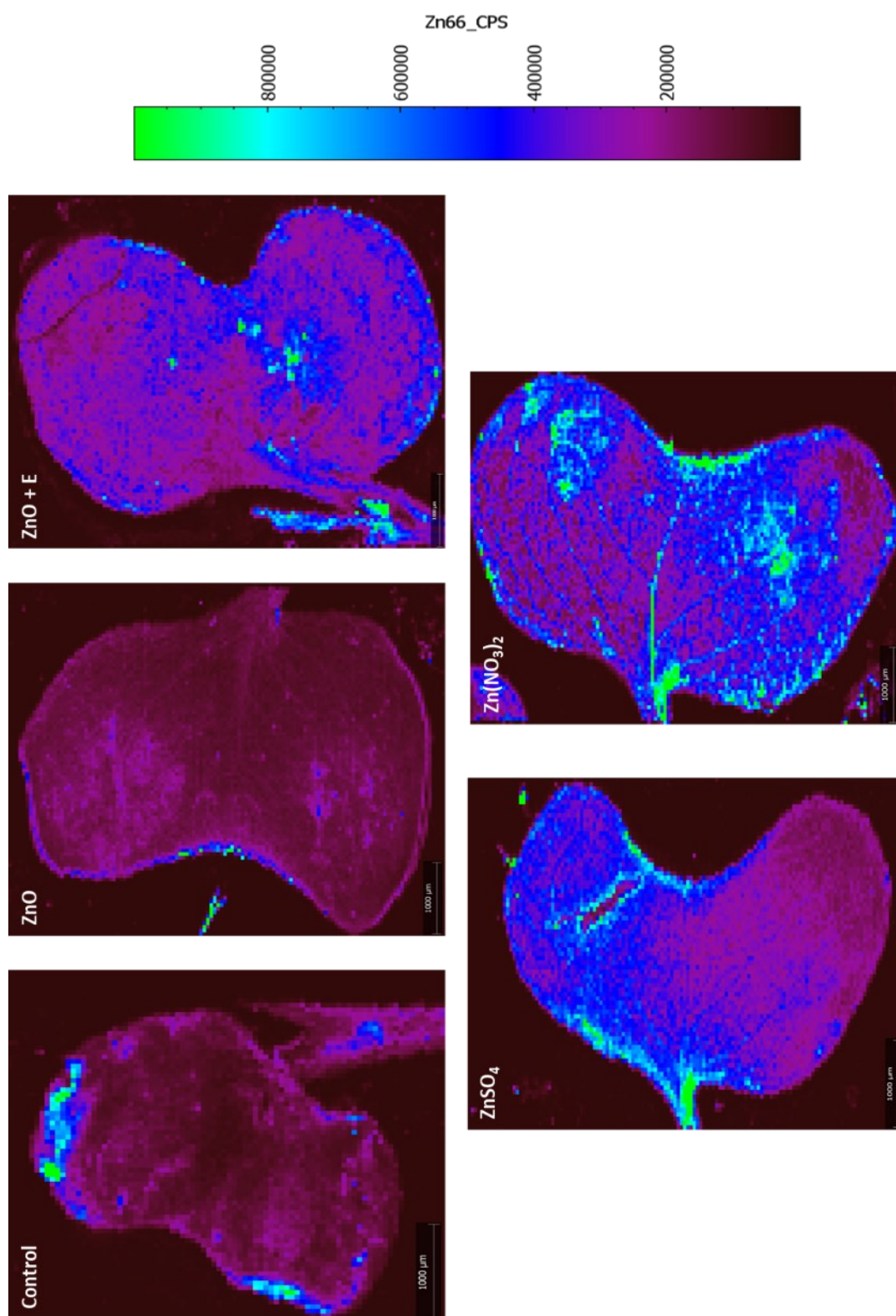


Figure 56: Semi-quantitative LA-ICP-MS images of Chinese Mustard Leaves treated with a range of Zn sources. All images were generated on the same 0-1,000,000 counts per second (CPS) scale. From top left to bottom right the treatments are: Control, ZnO, ZnO + E, ZnSO₄ and Zn(NO₃)₂.

The five images are all generated on the same 0-1,000,000 cps scale to allow for comparison. The images clearly show much greater intensity of signal across the leaf tissue for the ZnSO_4 , and the $\text{Zn}(\text{NO}_3)_2$ treated leaves, with some evidence of localisation in the vascular structures of the leaves. The ZnO treated leaf appeared to have no increase in Zn intensity and showed only minor localisation at the growing edge of the leaf. The inclusion of adjuvant E appeared to generate an increase of the intensity of Zn signal across the leaf image, with some evidence of localisation in central parts of the leaf. Interestingly, this $\text{ZnO} + \text{E}$ treatment did not appear to give rise to localisation of Zn in the leaf vasculature in the same way that the two soluble Zn sources. This could be due to the fact that ZnO is largely insoluble in water, as such it may still be present in the form of ZnO particles, as opposed to in the form of the Zn^{2+} ion.

The water treated leaf does appear to show two small regions of Zn localisation at the edges of the leaf. The difference in localisation may suggest a difference in the plant's utilisation of Zn when it is deficient in the plant's growth medium.

LA-ICP-MS is a technique of growing interest for the analysis of plant tissues, enabling the study of the spatial distribution of a wide range macro and micro elements.

Hong-Xia et al have previously studied Indian mustard using LA-ICP-MS to image the distribution of a range of elements including Cd and Zn. Much like in this work they selected the mustard species as it is a known metal hyperaccumulator. They concluded that LA-ICP-MS was a useful technique for the study of metal distribution in plant samples, specifically mustard varieties. Although their focus was to image cross sections of the shoot to investigate the localisation of metals

within the vasculature of the plant structure. They reported a localisation of Zn in the Xylem, suggesting that this indicates that Zn is largely transported via the Xylem.¹⁹¹ This both supports the idea that water soluble Zn would be more readily transportable within the plant, but also correlates with the localisation of Zn in the vasculature, especially with $\text{Zn}(\text{NO}_3)_2$ and ZnSO_4 – the more water-soluble Zn sources reported in figure 56.

There was some variation in the dry masses between this trial and the two previous trials. Whilst the plants were grown in a growth chamber, it was heated but it was not temperature controlled, so there was room for the temperature to increase above the desired 18 °C on particularly warm days. This could have had an effect on the outcomes of the two trials as the temperature throughout each trial could not be guaranteed. This provided a note of caution that comparing results between trials may give rise to error which could not be quantified. Therefore, the most robust way to interpret this data would be to compare results between treatments within each individual trial, where each treatment was grown within the same growth chamber. As such, even though the temperature could not be guaranteed to be 18 °C, each of the treatments were grown in the same cabinet and would have experienced the same conditions. Another potential source of variation in the dry masses was the method by which the plant material was separated from the sand. It was possible that washing the plants with deionised water until the sand was removed may have affected the mass of the plants, and if different volumes of water were used to clean different treatments, this could give rise to variation. Furthermore, where root material was particularly developed it was extremely difficult to completely remove the sand from the roots.

Any residual sand would have been counted in the dry mass and would have affected the result. The method for the following trials was adjusted, such that plants would be harvested by cutting at the base of the stem, to avoid any issues with separating the root material from the sand. This also ensured that this small-scale growth experiments replicated the large-scale glasshouse trials, as outlined in 6.10 where plants are harvested by cutting them at the base of the stem, as close to the growth medium as possible.

6.8. Expansion of small-scale growth method to include all adjuvants

The small-scale Zn uptake method described in 6.3.2 was adapted to include the use of five different adjuvants alongside ZnO to assess any effect of adjuvant inclusion on the uptake of ZnO from non-soluble sources, such as ZnO whilst also assessing differences in the efficacy of the different adjuvants included in the trial. The hypothesis was that the inclusion of adjuvant may increase ZnO uptake to be similar to that of the soluble Zn sources (ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$) in this case. This was important in relation to seed coatings as the seed coatings in question are formulated as a flowable suspension, with ZnO particles suspended in an aqueous medium using surfactant chemistry. The adjuvants included in this trial, as well as throughout this work were adjuvants A-E, chosen to cover a wide range of adjuvant chemistries and activities such that any differences detected may give information about the mechanism of their action when related to the differences in adjuvant chemistry with any adjuvant which did not show a significant difference.

6.8.1. Methods for expansion of small-scale growth method to include all five adjuvants

The fourth growth trial built upon the third growth trial, with the addition of all five adjuvants and a modification to the way in which the plant material was harvested. . The control was treated only with deionised water. The trays were prepared and treated as per the methodology outlined in section 6.3. Again, Zn treatments, ZnO, ZnSO₄ and Zn(NO₃)₂ were prepared to 50 ppm. Adjuvant was included at 15% of the Zn content, in accordance with the way in which seed treatments were formulated. For this experiment adjuvants A-E were selected.

On day 10, the plant material was harvested by cutting the mustard as close to the base of the stem as possible, therefore only retaining the shoot and leaves of the plant but eliminating any variation to the dry mass caused by incomplete removal of the silver sand growth mixture, and any effect of repeated washing with deionised water.

The plant material was then dried in a freeze drier to constant mass and the total dry mass of the plant material from each treatment was recorded.

The material was ground using an electric coffee grinder for 120 seconds, until a homogenous powder was formed. This powder was then randomly sampled in triplicate for digestion and analysis by ICP-MS.

6.8.2. Results for expansion of small-scale growth method to include all five adjuvants

In this trial, all 5 adjuvants (A-E) were individually included as treatments in addition to ZnO. As before, once the plant material was harvested and dried, dry mass data was recorded. The dry mass data is shown in table 25 and figure 57.

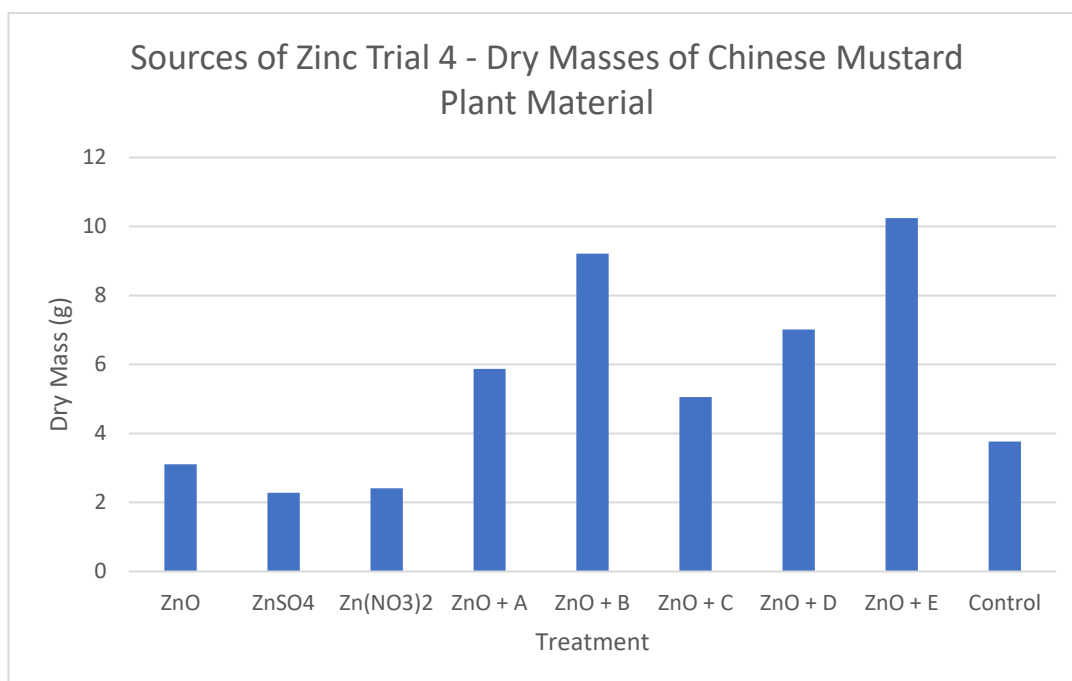


Figure 57: Graphical representation of the dry masses of Chinese Mustard plant material generated as part of the fourth small-scale growth trial, featuring all of the adjuvants as treatments.

Treatment	Dry Mass (g)
ZnO	3.1
ZnSO ₄	2.3
Zn(NO ₃) ₂	2.4
ZnO + A	5.9
ZnO + B	9.2
ZnO + C	5.1
ZnO + D	7.0
ZnO + E	10.2
Control	3.8

Table 25: Dry masses (g) of the plant material grown as part of the fourth small-scale growth trial which encompassed all 5 adjuvants as treatments.

The dry mass of the ZnO, ZnSO₄ and Zn(NO₃)₂ was lower than the dry mass of the control treatment during this trial, which broadly aligns with the results of the second growth trial, as if the presence of adjuvant-free Zn treatment reduces plant growth to some degree. A similar pattern was observed in the third growth trial with ZnSO₄, although this was not the case with Zn(NO₃)₂ or ZnO. Although the results for dry mass from this trial are not directly comparable to the results

of the previous trials due to the change in the way in which the plants were harvested, with the plants from this trial being cut at the base of the stem as opposed to harvesting the complete shoot and root and attempting to separate the root material from the silver sand.

In this experiment, in all cases the inclusion of adjuvant gave rise to an increase in plant dry mass when compared to the adjuvant-free ZnO solution. One possible explanation for this may be that all of the adjuvants, to varying degrees gave rise to increased water retention in the growth matrix, or silver sand in this case. The increased water availability may have increased plant growth by ensuring that there was no drought stress present which would lead to reduced plant yield. Due to the porosity of the silver sand and the trays in which the experiment was carried out, it is possible that the water control sand may have drained and dried out very quickly following irrigation. This may also account for the reduction in plant yield for the ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$ treatments in this experiment. Inhibition of plant growth is a known effect of drought stress in plants.^{192,193}

As before, the plant material was digested, and the Zn content was analysed by ICP-MS. The elemental analysis results are given in figure 58 and table 26.

Zn Content in Chinese Mustard Treated with a Range of Zn Sources - by ICP-MS

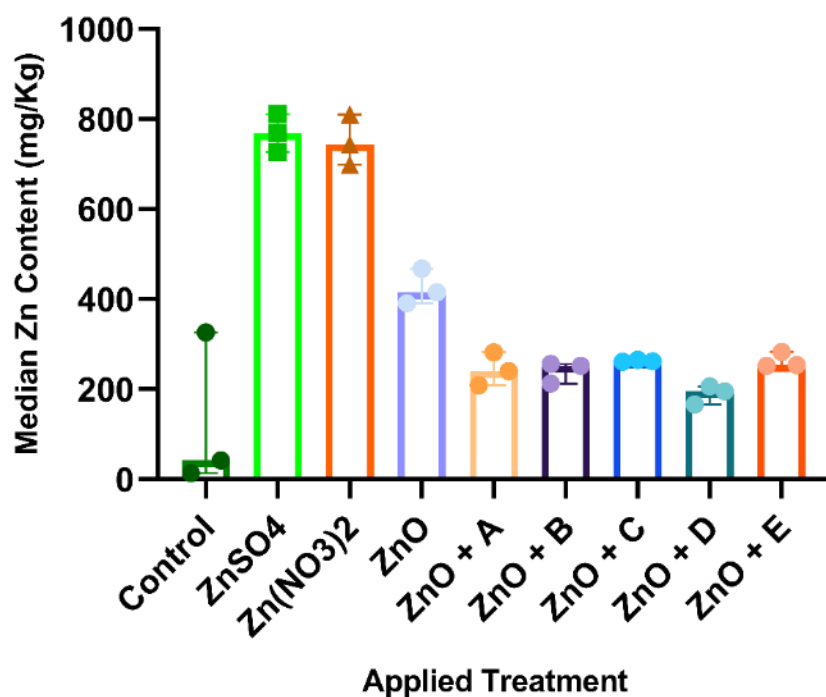


Figure 58: Zn content of Chinese Mustard plant material (mg/Kg), grown during the fourth growth trial, analysed by ICP-MS.

Treatment	Median Zn Content (mg/Kg)
ZnO	415.3
ZnSO ₄	769.6
Zn(NO ₃) ₂	744.9
ZnO + A	240.7
ZnO + B	252.1
ZnO + C	262.0
ZnO + D	195.1
ZnO + E	254.0
Control	42.7

Table 26: Zn Content (mg/Kg) of digested Chinese mustard plant material, grown during the fourth growth trial, analysed by ICP-MS.

The results compare favourably with published work on *Brassica juncea* as a remediation vehicle for Zn contaminated soils. Chaudhry et al used *Brassica juncea* to study Zn uptake with different treatment levels of ZnSO₄ (0-160 mg/L) using a similar digestion method and Atomic Absorption Spectroscopy (AAS). They reported Zn contents of between 80 and 1100 mg/Kg, depending on the Zn treatment level and whether the plants were grown in soil or in sand.¹⁷⁵

These results were in the same order as the results generated during this study, adding credence to the results presented within this chapter.

As with the previous trials detailed in this chapter, the Zn content data was normalised to account for the differences in plant mass between the different treatments. These results are given in figure 59 and table 27.

Uptake of Zn from a Range of Sources in Chinese Mustard - by ICP-MS

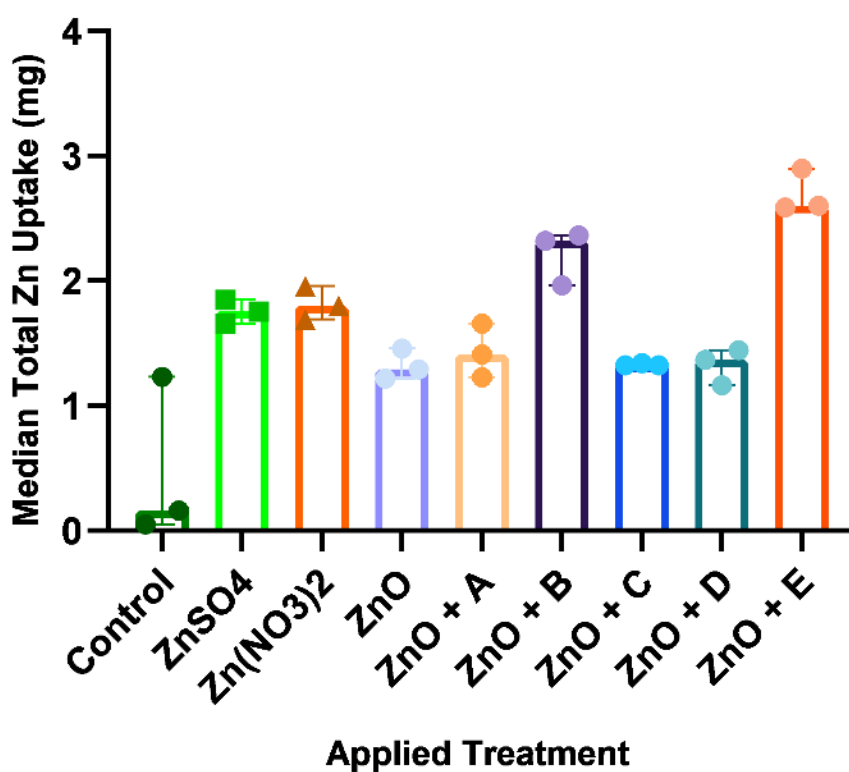


Figure 59: Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the fourth growth trial, analysed by ICP-MS.

Treatment	Median Zn Uptake (mg)
ZnO	1.3
ZnSO ₄	1.8
Zn(NO ₃) ₂	1.8
ZnO + A	1.4
ZnO + B	2.3
ZnO + C	1.3
ZnO + D	1.4
ZnO + E	2.6
Control	0.16

Table 27: Zn uptake normalised to dry mass of Chinese Mustard plant material, grown during the fourth growth trial, analysed by ICP-MS.

The results were once again broadly consistent with the previous trials, with the soluble Zn sources resulting in greater Zn uptake than the control or ZnO treated mustard plants. As before, adjuvant E had a beneficial effect on Zn uptake and was significantly higher in Zn uptake than the ZnO treated plants. So, too, was the adjuvant B treated plant material. Adjuvants A, C and D showed no significant benefit in this case.

Reported studies on ZnO often focus on the uptake of ZnO nanoparticles as opposed to the microparticles studied in this work. Studies often report increased plant Zn content with ZnO nanoparticles which is attributed to the smaller particle size increasing uptake when compared to ZnO microparticles. Studying Zn uptake using ICP-OES in *Prosopis juliaflora-velutina* (velvet mesquite), Hernandez-Viezcas et al. reported that plants analysed by ICP-OES which had been treated with ZnO nanoparticles showed increased Zn content. They also showed using X-ray absorption near edge structure analysis (XANES) that the Zn in the tissues of the treated plants was present in the form of Zn^{2+} and not still in the form of ZnO nanoparticles. This suggests that either ZnO NPs are converted into Zn^{2+} prior to plant uptake or after uptake and before translocation to the plant tissues. Due to regulatory and environmental concerns, the manufacturers of crop enhancement products strongly prefer to use ZnO microparticles. This finding suggests that if the adjuvant was able to increase ZnO microparticle uptake, there may also be an increase in tissue Zn^{2+} concentration in a similar way to a soluble Zn source such as ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$.¹⁹⁴

6.9. LA-ICP-MS Analysis of plants from the small-scale growth trials

To support the bulk analysis of Chinese mustard plant material, shoots were isolated, post-treatment for imaging by laser ablation inductively coupled plasma mass spectrometry.

6.9.1. Method for semi-quantitative analysis of Chinese mustard shoots

LA-ICP-MS analysis was carried out using an imageBIO266 laser ablation system (Elemental Scientific Lasers, Bozeman, MT, USA) coupled to a NexION 350X ICP-MS system (PerkinElmer, Waltham, MT, USA).

6.9.2. Mounting of shoots for LA-ICP-MS analysis

Shoots were harvested following treatment as described in 2.2.

Shoots were selected to be as similar in size, and by extension growth stage as possible. The shoots were then adhered to glass slides using double-sided adhesive before being sandwiched with another microscope slide to ensure the sample remained flat and placed in a vacuum desiccator for 48 hours to ensure the sample was dry. The slides were then mounted in the sample holder of the ImageBIO266 laser ablation system.

6.9.3. LA-ICP-MS parameters for imaging of Chinese mustard shoots

Leaves were imaged using a square spot size (XYR mode) of 25 μm , a scan speed of 70 $\mu\text{m}/\text{sec}$, a laser power of 40% and a repetition rate of 200 Hz.

The ICP-MS was set to acquire for the following isotopes: ^{63}Cu , ^{56}Fe , ^{55}Mn , ^{66}Zn , ^{25}Mg , ^{44}Ca , ^{111}Cd for 8 sweeps per reading, with each reading taking 0.341 seconds (duty cycle).

6.9.4.LA-ICP-MS image generation

Images were generated using iolite v4 software (Elemental Scientific Lasers, Bozeman, MT, USA), using the 'ion' colour scheme on the scale 0 – 1,000,000 counts per second.

6.9.5.Results for semi-quantitative analysis of Chinese mustard shoots

The results of the semi-quantitative analysis of Chinese mustard shoot leaves are shown in figure 60.

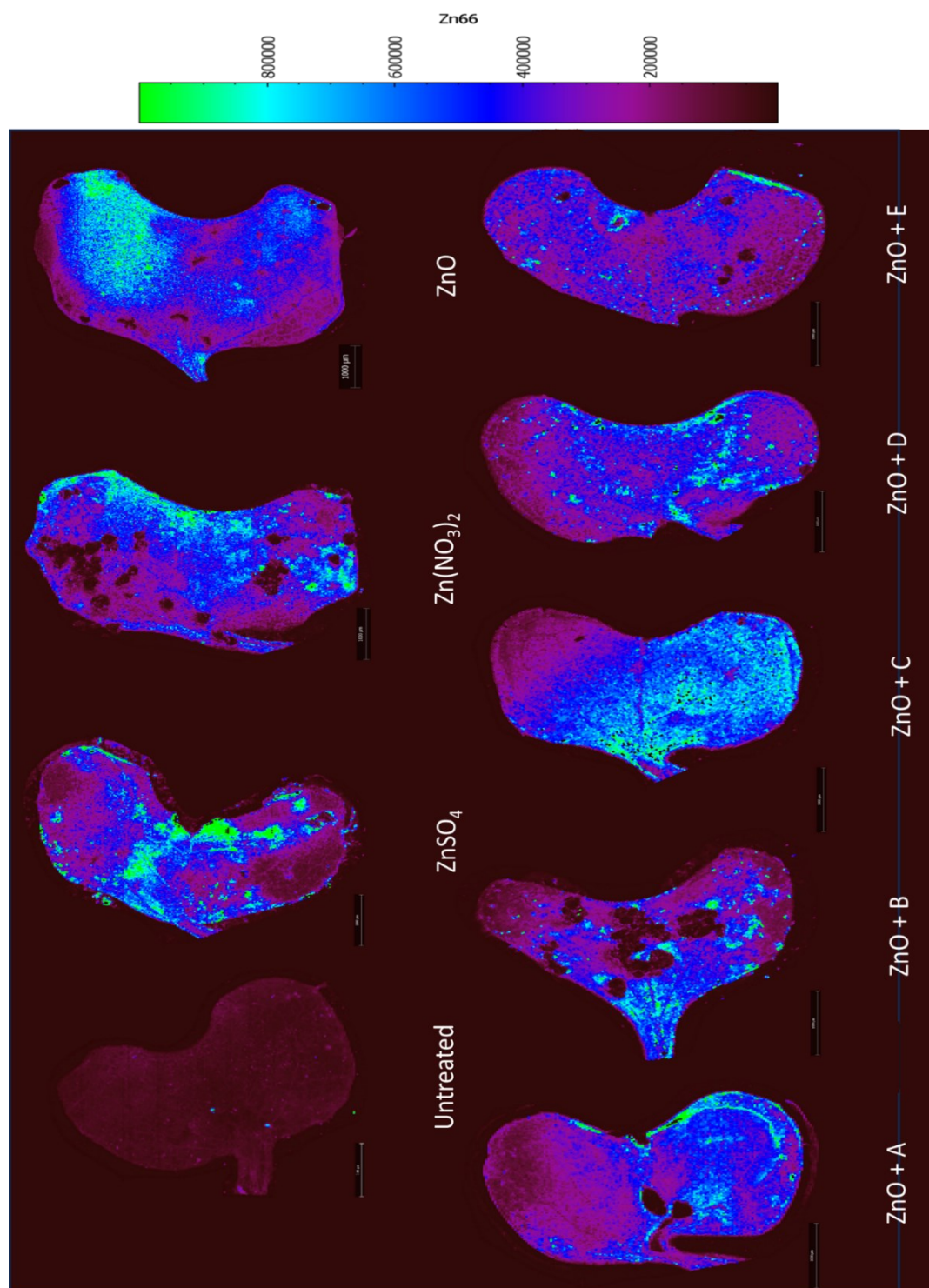


Figure 60: Semi-quantitative LA-ICP-MS images of Chinese Mustard Leaves treated with a range of Zn sources. All images were generated on the same 0-1,000,000 counts per second (CPS) scale. From top left to bottom right the treatments are: Control, ZnSO₄, Zn(NO₃)₂, ZnO and ZnO+A-E.

The LA-ICP-MS images indicate some differences in the distribution of Zn in Chinese mustard leaves with different Zn containing treatment. All of the treatments show a noticeable difference in signal intensity across the leaf image, when compared with the untreated control. The signal intensity trend was in line with the bulk analysis by ICP-MS data in this regard. The two treatments which show higher total Zn uptake were ZnO + B and ZnO + E. These differences were not immediately apparent by looking at the semi-quantitative images given in figure 16. Of course, the bulk analysis gave a measure of the total Zn based on representative samples of the whole population, whilst the imaging was only carried out on individual leaves. Whilst every effort was taken to choose leaves of a similar size and stage of maturity, there is always scope for variation between individual leaves within a population. The data does indicate that there may be some subtle differences in the localisation of Zn within the leaf tissue, with different Zn sources applied. The soluble Zn sources appeared to give rise to Zn in hotspots within the leaf tissue, whilst the ZnO based Zn sources, most notably ZnO, ZnO + C and ZnO + E appear to show a more homogenous distribution of the Zn within the leaf tissue. Interestingly, ZnO + B has a similar distribution profile to the ZnSO₄ treated leaf, with hotspots which appear to be randomly distributed throughout the leaf, whilst ZnO + D shows a similar profile to the Zn(NO₃)₂ treated leaf, with both hotspots and a localisation at the growing edge of the leaf visible in both cases. These were the two treatments which performed the strongest in the bulk analysis so perhaps this suggests the presence of those adjuvants has some effect on the mobility and uptake mechanisms of Zn within the leaves.

6.10. Large-scale greenhouse sources of Zn trial

A larger scale growth trial in winter wheat was carried at Plant Impact plc (Harpenden, UK) in the glasshouses of Rothamsted Research (Harpenden, UK), to translate the initial findings from the small-scale trials in fast-growing Chinese mustard into the winter wheat – the crop of interest throughout this work. Researchers at Rothamsted grew the plants following protocols developed as part of this thesis. The author harvested all of the plants and completed all of the analysis. Due to time and space limitations, three adjuvants were chosen to take forward to the growth trial, based upon the results from the fourth small scale growth experiment in Chinese mustard, described in 5.2. Adjuvants B and E were selected as they were shown to have a beneficial effect on the Zn content of Chinese mustard when applied with ZnO. Adjuvant C was selected to act as a negative control, as the previous work had shown no beneficial effect of its inclusion alongside ZnO. The aim of this section of the work was to correlate the findings of the small-scale work in Chinese mustards shoots, with larger scale conditions, using wheat grown in pots in the greenhouse, as per the previous growth trials carried out in chapter 5 of this thesis.

6.10.1. Sources of Zn glasshouse trial method

Sixty 4” plant pots were prepared with a sand and perlite mix, two parts coarse sand to two parts fine sand and one part perlite to act as a ‘nutrient-free’ growth medium. Six seeds were sewn per pot and arranged in a randomised order in the

glasshouse. The greenhouse temperature was set to 21 °C daytime temperature and 19 °C night-time temperature, with 14 hours of supplementary lighting. Plants were removed from the randomised structure and irrigated three times per week. The control pots were treated only with reverse osmosis (RO) water. The Zn treatments were made up in RO water to 50 ppm Zn with 15% adjuvant on the Zn content. Plants were watered to excess with the same volume of solution to reduce the differences of water availability across treatment groups. Treatments were prepared and applied in different watering cans to prevent cross-contamination of treatments and adjuvants.

The plants were grown for four weeks, before being harvested. Plants were cut at the base of the stem, as close as possible to the sand and perlite mix, the plants were then weighed as quickly as possible using a 4-figure balance. Each plant was cut immediately before being weighed to minimise any moisture loss between harvest and weighing which may have affected the results. Fresh masses of plant material were recorded on the day of harvest, plants were then placed into individual brown paper bags before being dried in an oven, in the plant drying facility at Rothamsted Research, at 90 °C overnight.

6.10.2. *Preparation of plant material for analysis by ICP-MS*

Once dried, plant material was ground using an electric grinder (Morphy Richards, Swinton, UK) to a homogeneous powder. The powdered plant material was weighed out to 0.2 g $\pm$ 0.005 g in triplicate, then microwave digested in 5 mL 69% HNO₃ and 2 mL 30% H₂O₂ using the 'plant material' one-touch method on

the CEM Mars 6 (CEM Corporation, Matthews, NC, USA). The digested material was then made up to 50 mL in deionised water, before being diluted a further two-fold with deionised water for analysis by ICP-MS.

6.10.3. Results of sources of Zn glasshouse trial

The fresh mass results from the Zn glasshouse trial are given in figure 61 with all of the results from the trial summaries in table 28.

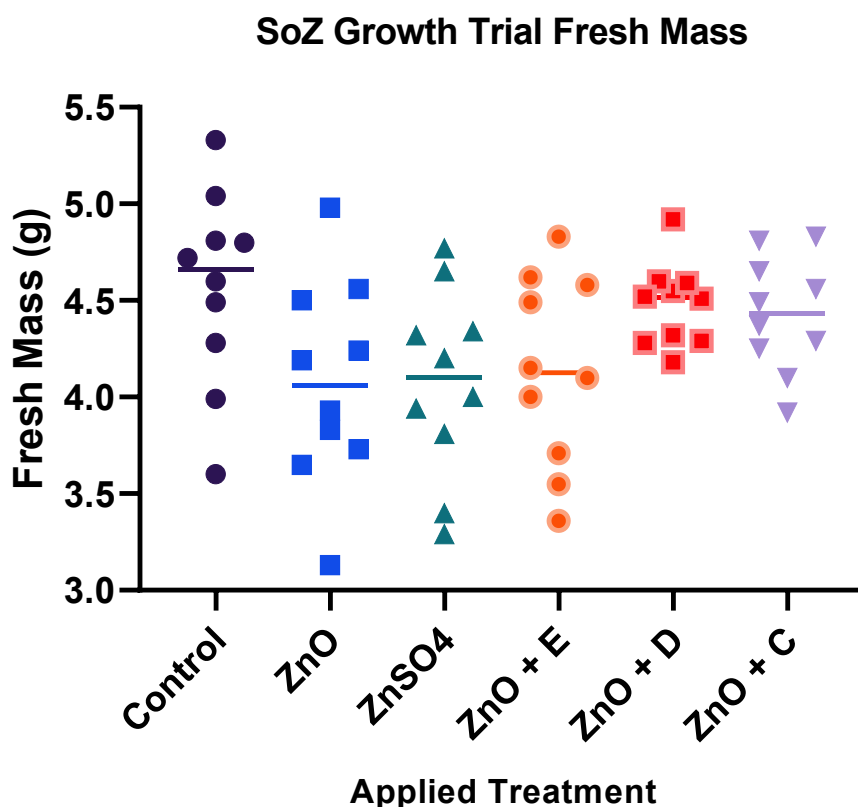


Figure 61: Fresh mass of winter wheat plant material recorded during the harvest of plants from the sources of Zn glasshouse growth trial, The horizontal line represents the median value whilst the individual points denote individual plants.

The results varied quite widely within each treatment, meaning that there were no statistically significant differences between the fresh masses. However, the median results for ZnO, ZnSO₄ and ZnO with the inclusion of adjuvant E were noticeably lower than the results for the control, and adjuvants D and C. Values are given in table 28. This suggests that, compared with the water only treated control, the application of ZnO, ZnSO₄ and ZnO + E had a negative effect on plant

growth. ZnO + D and ZnO + C, however, appeared to reverse this trend, giving no observable difference in plant mass when compared with the control.

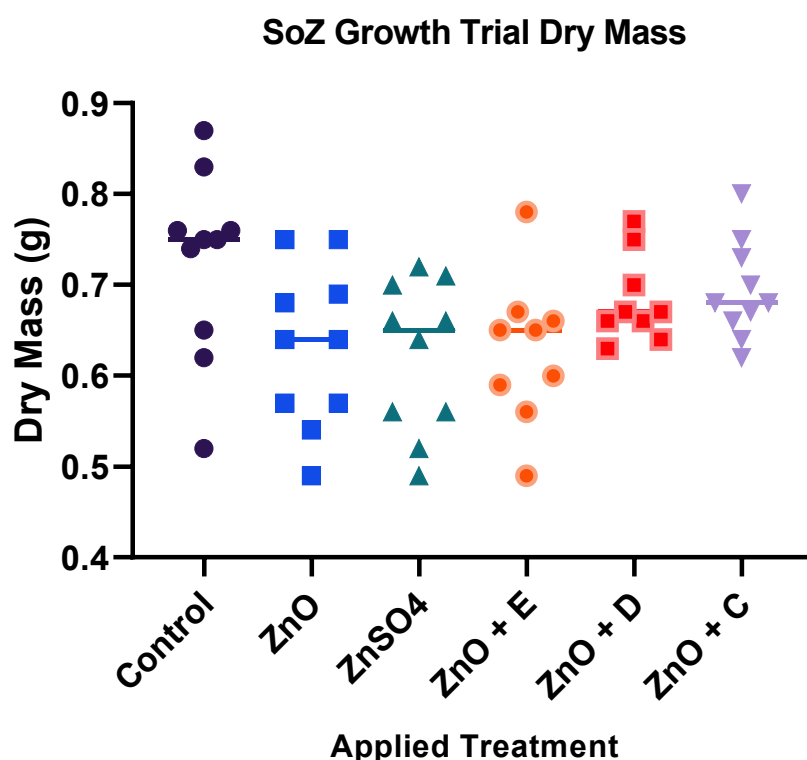


Figure 62: Dried mass of winter wheat plant material recorded after drying the plants from the sources of Zn glasshouse growth trial, The horizontal line represents the median value whilst the individual points denote individual plants.

The dry mass results followed the trend of the fresh mass results. The control treatment plants were higher in dry mass than the treated ones, with ZnO + D and ZnO + C coming closest to closing the gap. One difference with the dry mass data is that the difference between Zn treatments appears less pronounced than the differences in the fresh mass results. This result corresponds with the findings of Watts-Williams et al. In their study of various Zn treatments on tomato plants grown in a glasshouse, they reported no significant difference in plant biomass

between Zn treatments. Their treatments included ZnO and ZnSO₄, but they did not include Zn(NO₃)₂ or a water control, as were included in this study.¹⁷⁷

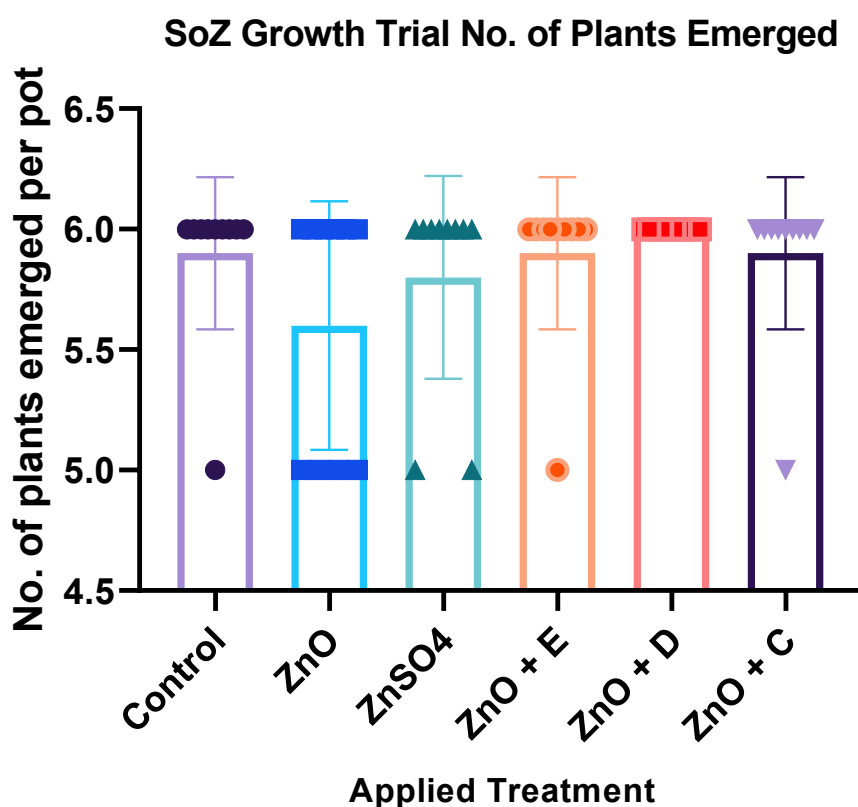


Figure 63: Graphical representation of the number of plants which emerged per pot, out of a total of 6 by treatment. The horizontal top of the bar gives the median value per treatment, whilst each point represents one pot.

The total number of plants to emerge from each pot was monitored throughout the trial, there were 6 seeds sewn per pot. ZnO + D had the strongest results in this test, with all 6 plants emerging in each of the pots, the control, ZnO + E and ZnO + C were in second place with only one of the seeds in one of the pots failing to germinate. The ZnSO₄ treated plants were in third place with two seeds failing

to germinate. The interesting result from this experiment was that the ZnO treated pots had a much higher failed germination rate than the other treatments, with approximately half of the pots having a seed which failed to germinate.

The results from the number of plants per pot to emerge study, presented in figure 63 are reflected in figure 64, which gives the percentage emergence by treatment.

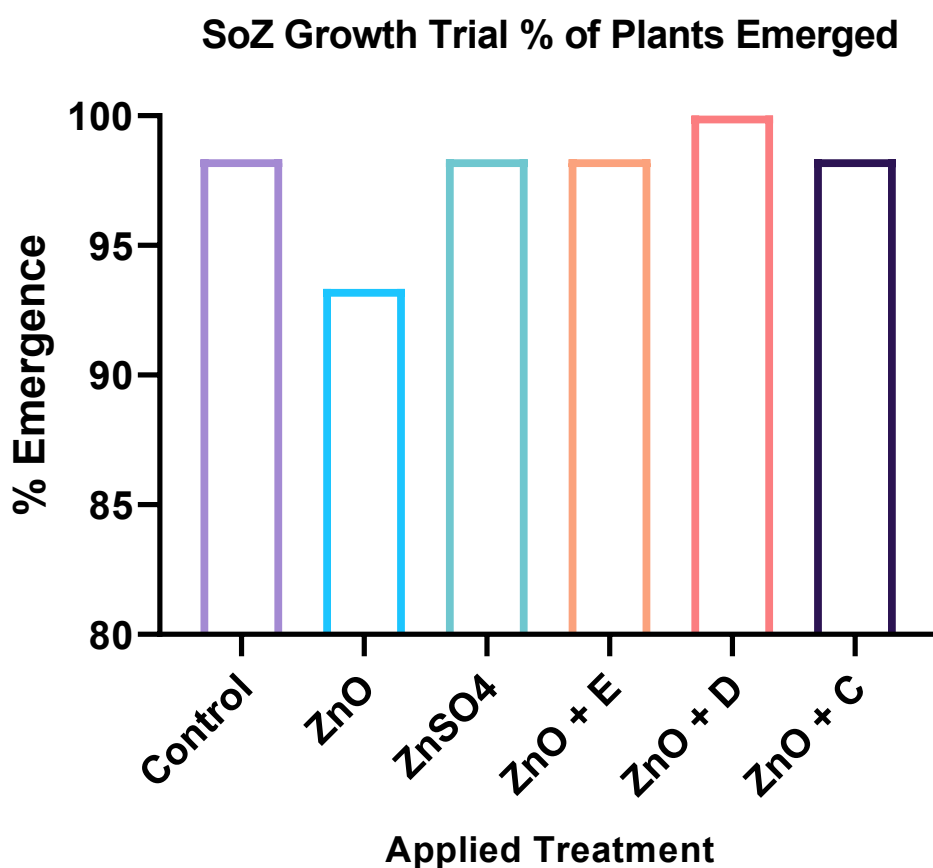


Figure 64: Graphical representation of the percentage of plants which emerged by treatment, during the sources of zinc glasshouse growth trial.

The ZnO treated plants performed noticeably worse than the rest of the treatments, with a 93% emergence rate. Control, ZnSO₄, ZnO + E and ZnO + C had a 97 % emergence rate, whilst ZnO + D had the strongest performance with a 100 % emergence rate. A recent study by Tymoszuk et al looked at the effect of ZnO and ZnO nanoparticle application on germination and seedling growth in Onion, they too reported some reduction in germination and seedling viability with ZnO application especially at higher concentrations. They found that there was no difference in this effect between micro and nano particle Zn, suggesting it was the concentration of Zn causing the reduction in viability, rather than the particle size.¹⁹⁵ Since this effect was only observed in the ZnO treated plants in this work, and not with the treatments including adjuvant or the water control, perhaps this suggests that the adjuvants may act in some way to limit the cytotoxicity of applied ZnO for a given application concentration.

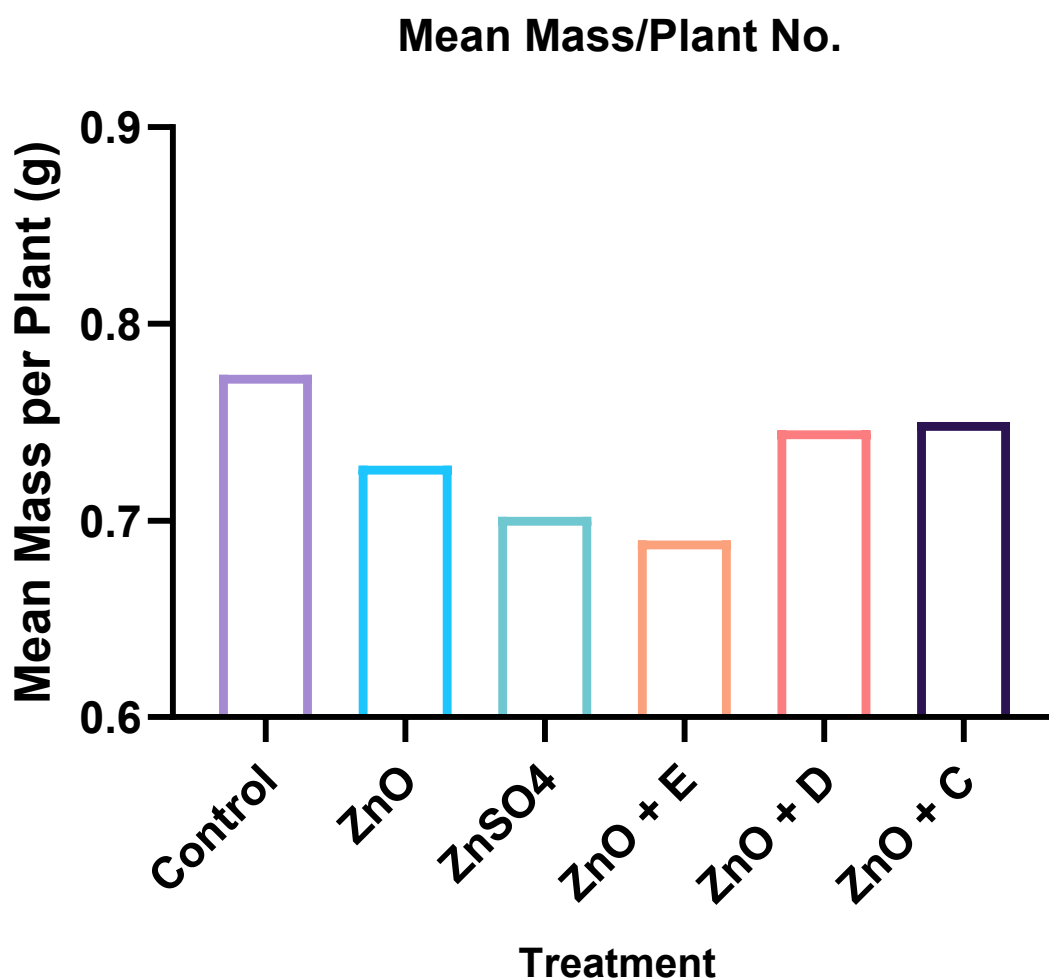


Figure 65: Graphical representation of the mean plant mass per number of plants harvested by each treatment, in the sources of Zn glasshouse growth trial.

The mean plant mass per plant was calculated, the results again broadly follow the fresh and dry mass data. The control plants performed marginally better than the ZnO + D and ZnO + C treated plants, with the ZnO treated plants in third place. ZnSO₄ was slightly lower than ZnO, with ZnO + E slightly lower again.

Once the plant material was microwave digested and diluted, the Zn content was analysed using ICP-MS, this data is presented in figure 66.

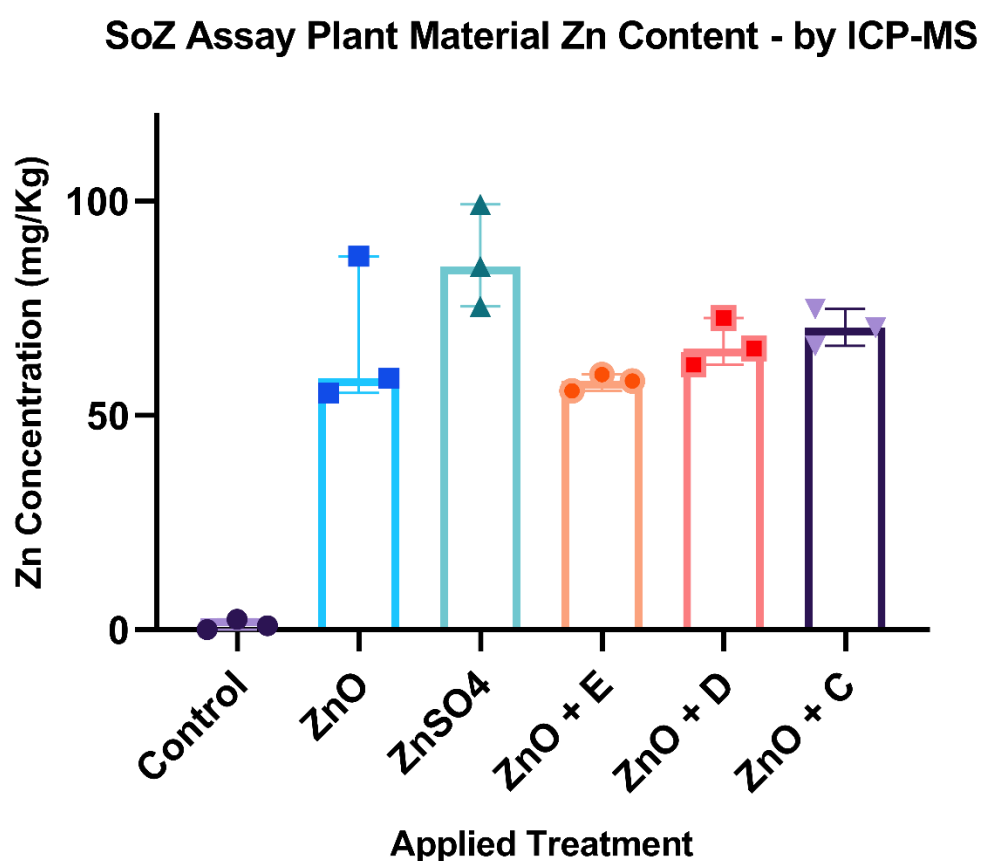


Figure 66: Zn content (mg/Kg) of digested winter wheat plant material harvested following the sources of Zn glasshouse growth trial, analysed by ICP-MS.

Once the plant material was microwave digested and diluted, the Zn content was analysed using ICP-MS. The results show a significant difference between the water control and all of the Zn treated plants, with all of the Zn treatments causing an increase of the Zn content of the resulting plants. ZnSO₄ had the strongest performance with a median Zn content of 84.8 mg/Kg, ZnO + C treated plants

had the second highest Zn content at 70.5 mg/Kg, closely followed by ZnO + D at 65.7 mg/Kg. ZnO and ZnO + E treated plants had almost identical Zn contents at 58.6 and 58.0 mg/Kg, respectively..

As per previous trials, the Zn content data was normalised to the plant mass, to account for differences in plant growth and give a value for the total Zn taken up in µg. This data is given in figure 67.

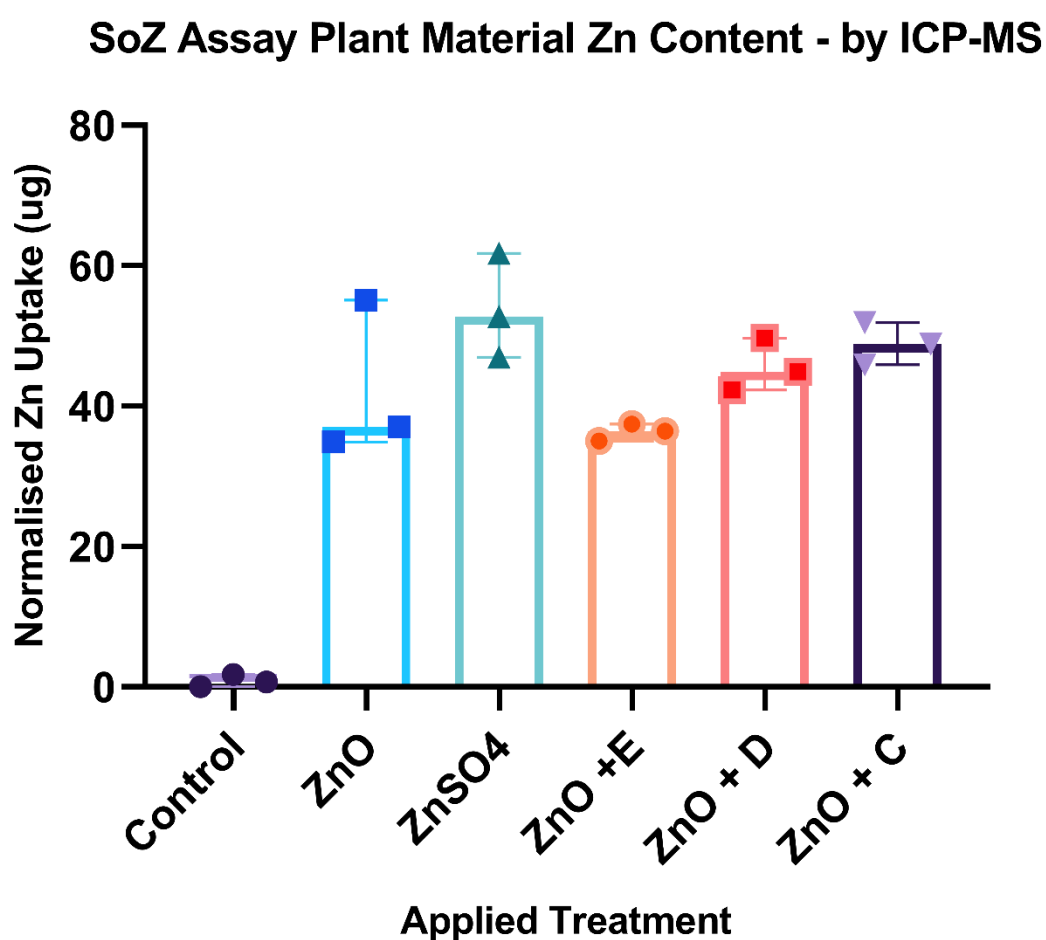


Figure 67: Zn uptake (µg) normalised to dry mass of Winter Wheat plant material, grown during the sources of Zn glasshouse growth trial, analysed by ICP-MS.

As per previous trials, the Zn content data was normalised to the plant mass, to account for differences in plant growth and give a value for the total Zn taken up in μg . The results followed the same trend as the mg/Kg data presented in 23, this is unsurprising since there was no significant difference between the dry masses of the treated plants.

The data presented in figures 61-67 was collated into a table, giving the values for each of the columns on the graphs presented. This data is given in table 28.

Treatment	Median Fresh Mass (g)	Median Dry Mass (g)	No. of plants emerged	% of Plants Emerged	Mean Mass/Plant (g)	Median Zn Content (mg/Kg)	Median Uptake of Zn (mg)
Control	4.66	0.75	5.9	98	0.77	0.9	0.7
ZnO	4.06	0.64	5.6	93	0.73	58.6	37.0
ZnSO4	4.10	0.65	5.8	98	0.70	84.8	52.7
ZnO + E	4.13	0.65	5.9	98	0.69	58.0	36.4
ZnO + D	4.52	0.67	6	100	0.75	65.7	44.9
ZnO+ C	4.43	0.68	5.9	98	0.75	70.5	48.8

Table 28: Results by treatment for each of the measurements carried out during the sources of Zn full scale glasshouse trial.

Direct comparison of the Zn content results with published work is challenging. Studies are often carried out on plants grown in soil, rather than in an inert matrix, as per this study and often focus on the grain Zn content of fully grown wheat as

opposed to the Zn content of plant material during the early stages of plant growth. Nonetheless, a study by Liu et al. which focused mainly on grain Zn content, did report the Zn content of winter wheat straw of mature plants grown in soil. The reported straw Zn content ranged from 11.7 mg/Kg for crops grown in soil with no additional Zn application to 64 mg/Kg Zn content in the straw of wheat crops treated with 34.1 kg/ha ZnSO₄ based fertiliser.¹⁹⁶ These values are of a similar order to those reported following the glasshouse growth trial where the ZnSO₄ treated plants contained a median Zn concentration of 84.8 mg/Kg.

6.11. Sources of Zn study conclusion

In conclusion, throughout this chapter, a method was developed to provide a small-scale assay to assess the effect of varied sources of Zn, as well as the effect of ZnO in combination with a range of adjuvants on the Zn content of *Brassica juncea*, a Zn hyperaccumulator. This assay was combined with both bulk solution mode ICP-MS analysis to study the Zn content of plant material, alongside spatially resolved elemental information in the form of LA-ICP-MS. This also enabled the localisation of Zn within the leaf tissue to be investigated, giving some visibility as to the translocation mechanism of the Zn within the plant tissue. The method was refined throughout the chapter, resulting in an experiment which encompassed all of the adjuvants being investigated as part of the wider study. The results of this experiment were used to inform the study design for the larger, more resource intensive glasshouse trial which was carried out to translate the findings in the mustard plants to the crop of interest – in this case winter wheat. The small assay results showed significant Zn uptake benefit of the inclusion of adjuvants B and E, when compared to the application of ZnO alone. This was supported by LA-ICP-MS imaging which showed a similar distribution of Zn within the leaf tissue between ZnO treatments including adjuvants and the soluble Zn sources, whilst ZnO alone appeared to have a different localisation pattern. This goes to suggest that the inclusion of adjuvant facilitates the uptake of Zn in a Xylem transportable form, which is not observed with the application of ZnO alone. To the best of our knowledge, this is the first reported study on the effect of adjuvant inclusion on the uptake of ZnO microparticles in plant species, as well

as the first use of LA-ICP-MS to investigate the effect of adjuvant on ZnO microparticle uptake in plants.

There was some evidence from the sources of Zn growth trial that the application of ZnO-containing solution to winter wheat had a negative effect of seed viability. Seeds irrigated with ZnO had a higher germination failure rate than control seeds or seeds treated with Zn treatments using soluble Zn sources. Another interesting observation in this experiment was that the inclusion of adjuvant appeared to mitigate this trend. ZnO + D treated plants had a 100 % germination rate in this trial, whilst ZnO + C and ZnO + E came in at 98 %. This is in comparison with 93 % for plants treated with ZnO alone. An interesting future experiment following on from this finding would be to assess the effect of Zn solution concentration on this reduction of seed emergence and to investigate the mechanism by which the adjuvant counteracts this effect.

Page left intentionally blank

7.0. Conclusions

This thesis presents a body of research investigating the effect of adjuvants on the uptake of zinc from seed film coatings by wheat.

In chapter 4, a method was developed for the washing of seed coatings from the seed surface and the bulk analysis of seed material by ICP-MS and ICP-OES. Methods have also been developed for the LA-ICP-MS imaging of treated and untreated wheat seed sections, and for the imaging of whole wheat seeds by micro-CT. The results showed that there was no diffusion of seed-applied Zn treatment into the endosperm, irrespective of adjuvant inclusion in the seed treatment formulation or the length of time the seeds were stored for following treatment. Although micro-CT is well established in life sciences for the analysis of bone and related tissues, there are far fewer studies of its application for the analysis of plants. This thesis presents for the first time, the imaging of ZnO microparticles on the surface of treated wheat seeds, with the identification of the Zn-containing particles supported by SEM-EDX elemental imaging. The LA-ICP-MS images of seeds which have been treated then washed with nitric acid, appear to show a hotspot, or localisation of Zn in the germ of the seed following washing. This is particularly evident with the application of ZnO seed coating including adjuvant E. This suggests that adjuvant E may have an effect of encouraging Zn uptake into the germ during the imbibition process. The SEM and EDX data presented in chapter 6 strongly supported the findings of the LA-ICP-MS analysis, all of which showed that prior to exposure to moisture, the applied ZnO-containing seed coating remained on the surface of the seed as solid microparticles, irrespective of the period of storage prior to analysis.

In chapter 5, a controlled glasshouse growth trial protocol was developed and coupled with elemental analysis using ICP-MS which was able to identify Zn deficiency in young plants, investigate the effect of Zn-containing seed treatments on winter wheat plants, and to investigate the effects of adjuvant inclusion on Zn-containing seed treatments. The developed methods were able to determine a zinc deficiency in plants grown in inert soil when irrigated with Zn-free Hoagland's when compared to plants irrigated with Zn-containing Hoagland's solution. The growth trial was expanded to compare winter wheat seeds with no coating compared to seeds treated with the ZnO-containing reference seed coating used throughout this work. The results showed an increase in plant height, fresh mass and the plant Zn content of seed-treated plants compared to untreated plants. The most striking finding of the growth trial study was a clear effect of adjuvant E, when included in the ZnO-containing seed treatment formulation. There was an increase in Zn content of the adjuvant E treated plants when compared with the other treatments. Cu and Mn were also analysed, these were found in the Hoagland's treatment, but not in the seed treatment, there was no increase in the Cu or Mn content of the plant irrespective of treatment, the colocalization of the nutrient of interest and the adjuvant was necessary to see the beneficial effect. We propose that the mechanism of action is that the adjuvant holds Zn and moisture close to the seed to which they are applied as it grows, increasing the concentration of Zn in the root zone, thus leading to the increased uptake of Zn during the early stages of plant growth.

In chapter 6, taking advantage of the rapid growth, and Zn hyperaccumulation properties of Chinese mustard (*Brassica juncea*), a laboratory scale assay was

developed which, when combined with digestion and ICP-MS analysis, alongside imaging by LA-ICP-MS enabled rapid screening of the effect of various Zn treatments on Zn-uptake. The study was able to demonstrate a significant increase of Zn uptake with the inclusion of adjuvants B and E, when compared to ZnO alone. The LA-ICP-MS imaging showed a similar distribution of Zn in the leaves treated with ZnO with adjuvant, when compared with the application of water-soluble Zn sources, suggesting that the inclusion of adjuvant promoted the uptake of Zn in a Xylem transportable form. To the best of our knowledge, this is the first reported study on the effect of adjuvant inclusion on the uptake of ZnO microparticles in plant species, as well as the first use of LA-ICP-MS to investigate the effect of adjuvant on ZnO microparticle uptake in plants.

Greenhouse scale studies on the effect of different Zn sources when applied to winter wheat, showed some negative effect on irrigation of plants with ZnO treatment. The inclusion of adjuvant appeared to reduce, in the case of adjuvant C and E, or completely reverse this effect, in the case of adjuvant D. There are no previously reported studies on the effect of adjuvant inclusion alongside Zn treatment on either Zn uptake in wheat or on the effect of adjuvant inclusion with Zn treatment on seed viability.

The combination of both the laboratory scale mustard assay and the larger greenhouse trial with ICP-MS and LA-ICP-MS analysis presented is a powerful workflow for the examination of the effect of treatment formulation on nutrient uptake in plants. This could prove a very useful workflow with enormous scope to be applied across endless other crops, treatments, and additive regimes.

8.0. *Recommendations for future work*

An interesting area for further work following on from the work outlined in chapter 4 would be to capitalise on the non-destructive and low prep nature of μ -CT. Since the seed is not destroyed during the analysis, it would theoretically be possible to generate several images of the same seed as it moves through the stages of germination and early seedling development. The localisation and presence of the Zn particles at each stage of the process would give enhanced insight into the mechanism by which the Zn is removed from the seed surface and taken up by the seedling.

It would also be interesting to develop a method by which elemental analysis could be carried out on the root zone, this would enable the mapping of nutrients in space and would prove extremely insightful as to how the adjuvants increase Zn uptake in plants, it is likely that they act by holding moisture and nutrients in the root zone as the plant begins to grow. A soil elemental imaging methodology would be able to shed light on this. There would be challenges to this, namely a nutrient-free growth medium would be required which would mimic plant growth in soil, whilst allowing the root zone to be prepared for elemental analysis. This would most likely be possible using an aqueous soil mimic such as a hydrogel or agar type material which could then be frozen and sectioned for analysis by LA-ICP-MS. The higher throughput capabilities of modern sample chamber designs would make a method such as this much more feasible than it would have been previously.

A worthwhile follow up to the work presented in chapter 5 would be to experiment with different levels of Zn in the Hoagland's solution which was used to irrigate

the plants. This would enable the sensitivity of the assay to be determined and the magnitude of change in Zn concentration which could be detected using the assay. This could then be related to the amount of Zn included in the film coatings. This would require a large amount of development as the loss of nutrient from Hoagland's which passes through the pot and is lost would be difficult to quantify, and so the comparisons to the seed treatment would have to be made very carefully.

Building upon the data presented in chapter 5, and any future growth trials which include the controlled application of nutrients would benefit from the development of a more representative nutrient-free soil mimic. A growth medium which was nutrient-free and could be tailored to closely match the type of soil the studied plants are to be grown in would help to make the results as representative as possible of real-world conditions whilst still being a controlled environment. The texture, particle size distribution, pH and moisture retention properties of the soil mimic should be considered carefully and could even be adapted for different soil types.

The data presented in chapter 6 proved interesting and warrants further investigation. A worthwhile avenue would be to explore different concentrations of Zn and applied adjuvant, to determine whether the dose response is linear, or whether there is an optimum concentration and nutrient to adjuvant ratio to boost nutrient uptake. It would also be of interest to study different particle sizes of ZnO, whilst it is known that ZnO nanoparticles are more readily taken up compared to ZnO micro particles, it would be interesting to see whether the addition of adjuvant has any effect on this relationship.^{197–199}

The growth trials presented in chapter 6 could be replicated using a more soil-like growing medium. A medium with similar moisture retention characteristics to soil, may retain moisture more effectively than the sand and perlite mix, which may cause the adjuvants to have a more pronounced effect on nutrient uptake.

Page left intentionally blank

9.0. References

- 1 N. Natasha, M. Shahid, I. Bibi, J. Iqbal, S. Khalid, B. Murtaza, H. F. Bakhat, A. B. U. Farooq, M. Amjad, H. M. Hammad, N. K. Niazi and M. Arshad, Zinc in soil-plant-human system: A data-analysis review, *Science of The Total Environment*, 2022, **808**, 152024.
- 2 I. Cakmak and H. Marschner, Effect of zinc nutritional status on activities of superoxide radical and hydrogen peroxide scavenging enzymes in bean leaves, *Plant Soil*, 1993, **155–156**, 127–130.
- 3 M. Hambidge, Zinc and Health: Current Status and Future Directions Human Zinc Deficiency 1, *J Nutr*, 2000, **130**, 1344–1349.
- 4 A. F. López-Millán, D. R. Ellis and M. A. Grusak, Effect of zinc and manganese supply on the activities of superoxide dismutase and carbonic anhydrase in *Medicago truncatula* wild type and *raz* mutant plants, *Plant Science*, 2005, **168**, 1015–1022.
- 5 I. Cakmak, Possible roles of zinc in protecting plant cells from damage by reactive oxygen species, *New Phytologist*, 2000, **146**, 185–205.
- 6 K. B. Disante, D. Fuentes and J. Cortina, Response to drought of Zn-stressed *Quercus suber* L. seedlings, *Environ Exp Bot*, 2011, **70**, 96–103.
- 7 L. Bucker-Neto, A. L. S. Paiva, R. D. Machado, R. A. Arenhart and M. Margis-Pinheiro, Interactions between plant hormones and heavy metals responses, *Genet Mol Biol*, 2017, **40**, 373–386.
- 8 M. Sillanpaa, in *FAO Soils Bulletin No. 63*, FAO, Rome, 1990.

- 9 C. Zou, X. Gao, R. Shi, X. Fan and F. Zhang, in *Micronutrient Deficiencies in Global Crop Production*, Springer Netherlands, Dordrecht, 2008, pp. 127–148.
- 10 B. J. Alloway, *Micronutrient Deficiencies in Global Crop Production*, Springer Netherlands, Dordrecht, 2008.
- 11 I. Cakmak, in *Micronutrient Deficiencies in Global Crop Production*, Springer Netherlands, Dordrecht, 2008, pp. 181–200.
- 12 Y. Ishimaru, K. Bashir and N. K. Nishizawa, Zn Uptake and Translocation in Rice Plants, *Rice*, 2011, **4**, 21–27.
- 13 F. G. Viets, L. C. Boawn and C. L. Crawford, Zinc contents and deficiency symptoms of 26 crops grown on a zinc-deficient soil, *Soil Sci*, 1954, **78**, 305–316.
- 14 B. J. Alloway, Soil factors associated with zinc deficiency in crops and humans, *Environ Geochem Health*, 2009, **31**, 537–548.
- 15 W. L. Lindsay, in *Advances in Agronomy*, 1972, pp. 147–186.
- 16 L. Kiekens, in *H. Marschner, in Zinc in Soils and Plants*, ed. A. D. Robson, Kluwer Academic Publishers, Dordrecht, 1st edn., 1993, pp. 59–77. *Heavy metals in soils*, ed. B. J. Alloway, Blackie Academic and Professional, London, 2nd edn., 1995, pp. 284–305.
- 17 H. Marschner, in *Zinc in Soils and Plants*, ed. A. D. Robson, Kluwer Academic Publishers, Dordrecht, 1st edn., 1993, pp. 59–77.
- 18 B. J. Alloway, in *Micronutrient Deficiencies in Global Crop Production*, Springer Netherlands, Dordrecht, 2008, pp. 1–39.

- 19 N. Gupta, H. Ram and B. Kumar, Mechanism of Zinc absorption in plants: uptake, transport, translocation and accumulation, *Rev Environ Sci Biotechnol*, 2016, **15**, 89–109.
- 20 V. Romheld, The role of phytosiderophores in acquisition of iron and other micronutrients in graminaceous species: An ecological approach, *Plant Soil*, 1991, **130**, 127–134.
- 21 S. Shojima, N.-K. Nishizawa, S. Fushiya, S. Nozoe, T. Irifune and S. Mori, Biosynthesis of Phytosiderophores, *Plant Physiol*, 1990, **93**, 1497–1503.
- 22 K. Alberts, B. Johnson, A. Walter, P. Lewis, J. Raff, M. Roberts, *Molecular biology of the cell*, Taylor & Francis, New York, 5th edn., 2007.
- 23 M. Lou Guerinot, The ZIP family of metal transporters, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 2000, **1465**, 190–198.
- 24 M. Lou Guerinot and D. Eidet, Zeroing in on zinc uptake in yeast and plants, *Curr Opin Plant Biol*, 1999, **2**, 244–249.
- 25 V. Demidchik, H. C. Bowen, F. J. M. Maathuis, S. N. Shabala, M. A. Tester, P. J. White and J. M. Davies, Arabidopsis thaliana root non-selective cation channels mediate calcium uptake and are involved in growth, *The Plant Journal*, 2002, **32**, 799–808.
- 26 G. H. Yu and P. J. Kramer, Radial Transport of Ions in Roots, *Plant Physiol*, 1969, **44**, 1095–1100.
- 27 J. Claus, A. Bohmann and A. Chavarría-Krauser, Zinc uptake and radial transport in roots of Arabidopsis thaliana: a modelling approach to understand accumulation, *Ann Bot*, 2013, **112**, 369–380.

- 28 E. Steudle, Water uptake by roots: effects of water deficit, *J Exp Bot*, 2000, **51**, 1531–1542.
- 29 H. Q. Yang and Y. L. Jie, Uptake, and transport of calcium in plants., *Journal of plant physiology and molecular biology*, 2005, **31**, 227–234.
- 30 T. E. Sondergaard, A. Schulz and M. G. Palmgren, Energization of Transport Processes in Plants. Roles of the Plasma Membrane H⁺ - ATPase, *Plant Physiol*, 2004, **136**, 2475–2482.
- 31 A. Pich and G. Scholz, NicotJanamine and the Distribution of Iron into Apoplast and Symplast of Tomato (*Lycopersicon esculentum* Mill.): II. Uptake of Iron by Protoplasts from the Variety Bonner Beste and its Nicotianamine-less Mutant Chloronerva and the compartmentation of Iron, *J Exp Bot*, 1991, **42**, 1517–1523.
- 32 P. J. Rudall, *Anatomy of Flowering Plants*, Cambridge University Press, Cambridge, 2007.
- 33 A. Longnecker, Nancy. Robson, in *Zinc in Soils and Plants*, ed. A. D. Robson, Springer, Dordrecht, 1993, pp. 79–91.
- 34 H. Thomas, Senescence, ageing and death of the whole plant, *New Phytologist*, 2013, **197**, 696–711.
- 35 N. Bouain, Z. Shahzad, A. Rouached, G. A. Khan, P. Berthomieu, C. Abdelly, Y. Poirier and H. Rouached, Phosphate and zinc transport and signalling in plants: toward a better understanding of their homeostasis interaction, *J Exp Bot*, 2014, **65**, 5725–5741.

- 36 A. G. L. Assunção, D. P. Persson, S. Husted, J. K. Schjørring, R. D. Alexander and M. G. M. Aarts, Model of how plants sense zinc deficiency, *Metallomics*, 2013, **5**, 1110.
- 37 A. G. L. Assunção, E. Herrero, Y. F. Lin, B. Huettel, S. Talukdar, C. Smaczniak, R. G. H. Immink, M. Van Eldik, M. Fiers, H. Schat and M. G. M. Aarts, Arabidopsis thaliana transcription factors bZIP19 and bZIP23 regulate the adaptation to zinc deficiency, *Proc Natl Acad Sci U S A*, , DOI:10.1073/pnas.1004788107.
- 38 A. J. Karley and P. J. White, Moving cationic minerals to edible tissues: potassium, magnesium, calcium, *Curr Opin Plant Biol*, 2009, **12**, 291–298.
- 39 A. Kabata-Pendias, Soil-plant transfer of trace elements – an environmental issue, in *Geoderma*, 2004.
- 40 M. Tkalec, P. P. Štefanić, P. Cvjetko, S. Šikić, M. Pavlica and B. Balen, The effects of cadmium-zinc interactions on biochemical responses in tobacco seedlings and adult plants, *PLoS One*, DOI:10.1371/journal.pone.0087582.
- 41 G. Igrejas and G. Branlard, in *Wheat Quality for Improving Processing and Human Health*, Springer International Publishing, Cham, 2020, pp. 1–7.
- 42 B. Shiferaw, M. Smale, H.-J. Braun, E. Duveiller, M. Reynolds and G. Muricho, Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security, *Food Secur*, 2013, **5**, 291–317.

- 43 O. Erenstein, M. Jaleta, K. A. Mottaleb, K. Sonder, J. Donovan, and H.-J. Braun, in *Wheat Improvement*, Springer International Publishing, Cham, 2022, pp. 47–66.
- 44 Croda, Adjuvants, <https://www.crodacropcare.com/en-gb/applications/adjuvants>, (accessed March 2024)
- 45 Croda, Croda Life Sciences, <https://www.croda.com/en-gb/our-markets/life-sciences>, (accessed 1 February 2024).
- 46 Croda, Croda Product Functions, <https://www.crodacropcare.com/en-gb/product-finder>, (accessed 5 January 2024).
- 47 S. Pedrini, D. J. Merritt, J. Stevens and K. Dixon, Seed Coating: Science or Marketing Spin?, *Trends Plant Sci*, 2017, **22**, 106–116.
- 48 D. Harris, A. Rashid, G. Miraj, M. Arif and H. Shah, ‘On-farm’ seed priming with zinc sulphate solution—A cost-effective way to increase the maize yields of resource-poor farmers, *Field Crops Res*, 2007, **102**, 119–127.
- 49 G. Matthews, R. Bateman and P. Miller, *Pesticide Application Methods*, John Wiley & Sons, Oxford, 2014.
- 50 I. W. Callan, Achievements and limitations of seed treatments, *Outlook Agric*, 1975, **8**, 271–274.
- 51 T. J. Wood and D. Goulson, The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013, *Environmental Science and Pollution Research*, 2017, **24**, 17285–17325.
- 52 R. Schmuck and G. Lewis, Review of field and monitoring studies investigating the role of nitro-substituted neonicotinoid insecticides in the

- reported losses of honeybee colonies (*Apis mellifera*), *Ecotoxicology*, 2016, **25**, 1617–1629.
- 53 A. W. Schaafsma, V. Limay-Rios, T. S. Baute and J. L. Smith, Neonicotinoid insecticide residues in subsurface drainage and open ditch water around maize fields in southwestern Ontario, *PLoS One*, 2019, **14**, e0214787.
 - 54 C. Hitaj, D. J. Smith, A. Code, S. Wechsler, P. D. Esker and M. R. Douglas, Sowing Uncertainty: What We Do and Don't Know about the Planting of Pesticide-Treated Seed, *Bioscience*, 2020, **70**, 390–403.
 - 55 A. P. Chirag and M. Amir, Seed coating technology: A sustainable approach for improving seed quality and crop performance, *The Pharma Innovation Journal*, 2023, **12**, 970–978.
 - 56 Vantage Market Research, Seed Coating Market, <https://www.vantagemarketresearch.com/industry-report/seed-coating-market>, (accessed 15 March 2024).
 - 57 I. Rocha, Y. Ma, P. Souza-Alonso, M. Vosátka, H. Freitas and R. S. Oliveira, Seed Coating: A Tool for Delivering Beneficial Microbes to Agricultural Crops, *Front Plant Sci*, , DOI:10.3389/fpls.2019.01357.
 - 58 E. Gornish, H. Arnold and J. Fehmi, Review of seed pelletizing strategies for arid land restoration, *Restor Ecol*, 2019, **27**, 1206–1211.
 - 59 I. Afzal, T. Javed, M. Amirkhani and A. G. Taylor, Modern Seed Technology: Seed Coating Delivery Systems for Enhancing Seed and Crop Performance, *Agriculture*, 2020, **10**, 526.

- 60 H. J. Hill, Recent Developments in Seed Technology, *Journal of New Seeds*, 1999, **1**, 105–112.
- 61 N. R. Konkol, C. J. McNamara, K. Bearce-Lee, H. Kunoh and R. Mitchell, Novel method of micronutrient application increases radish (*raphanus sativus*) and shirona (*brassica rapa* var. *Pekinensis*) biomass, *J Plant Nutr*, 2012, **35**, 471–479.
- 62 G. P. Gomes, R. A. de Queiroz and L. S. A. Takahashi, Physiological potential of corn seeds treated with different dosages of molybdenum and potassium, *Semin Cienc Agrar*, 2021, **42**, 411–418.
- 63 M. U. Haider, M. Hussain, M. Farooq, and A. Nawaz, Optimizing zinc seed priming for improving the growth, yield and grain biofortification of mungbean (*Vigna radiata* (L.) wilczek), *J Plant Nutr*, 2020, **43**, 1438–1446.
- 64 M. Rizwan, S. Ali, B. Ali, M. Adrees, M. Arshad, A. Hussain, M. Zia ur Rehman and A. A. Waris, Zinc and iron oxide nanoparticles improved the plant growth and reduced the oxidative stress and cadmium concentration in wheat, *Chemosphere*, 2019, **214**, 269–277.
- 65 M. M. A. Mohammed and E. Pekşen, Impact of different zinc application strategies on yield, yield component, and chlorophyll content of wheat under drought and zinc-deficiency stress conditions, *J Plant Nutr*, 2020, 1–18.
- 66 N. Mitter and K. Hussey, Moving policy and regulation forward for nanotechnology applications in agriculture, *Nat Nanotechnol*, 2019, **14**, 508–510.

- 67 M. Ihara and K. Matsuda, Neonicotinoids: molecular mechanisms of action, insights into resistance and impact on pollinators, *Curr Opin Insect Sci*, 2018, **30**, 86–92.
- 68 S. M. Ensley, in *Veterinary Toxicology*, ed. R. Gupta, Elsevier, 2018, pp. 521–524.
- 69 R. Blake, Eu Neonicotinoid Ban Removes Vital Tools in Global Fight Against Pests, *Outlooks on Pest Management*, 2018, **29**, 197–200.
- 70 A. Fairbrother, J. Purdy, T. Anderson and R. Fell, Risks of neonicotinoid insecticides to honeybees, *Environ Toxicol Chem*, 2014, **33**, 719–731.
- 71 J. P. Staveley, S. A. Law, A. Fairbrother and C. A. Menzie, A Causal Analysis of Observed Declines in Managed Honeybees (*Apis mellifera*), *Human and Ecological Risk Assessment*, 2014, **20**, 566–591.
- 72 W. Aktar, D. Sengupta and A. Chowdhury, Impact of pesticides use in agriculture: their benefits and hazards, *Interdiscip Toxicol*, 2009, **2**, 1–12.
- 73 J. L. Shamshina, A. Kelly, T. Oldham and R. D. Rogers, Agricultural uses of chitin polymers, *Environ Chem Lett*, 2020, **18**, 53–60.
- 74 O. Hassan and T. Chang, Chitosan for Eco-friendly Control of Plant Disease, *Asian J Plant Pathol*, 2017, **11**, 53–70.
- 75 L. Orzali, B. Corsi, C. Forni and L. Riccioni, in *Biological Activities and Application of Marine Polysaccharides*, InTech, 2017.
- 76 W. Wang, H. Gao, S. Jin, R. Li, and G. Na, The ecotoxicological effects of microplastics on aquatic food web, from primary producer to human: A review, *Ecotoxicol Environ Saf*, 2019, **173**, 110–117.

- 77 J. P. G. L. Frias and R. Nash, Microplastics: Finding a consensus on the definition, *Mar Pollut Bull*, 2019, **138**, 145–147.
- 78 S. Pedrini, A. Balestrazzi, M. D. Madsen, K. Bhalsing, S. P. Hardegree, K. W. Dixon and O. A. Kildisheva, Seed enhancement: getting seeds restoration-ready, *Restor Ecol*, , DOI:10.1111/rec.13184.
- 79 M. Wagner, R. F. Pywell, T. Knopp, J. M. Bullock and M. S. Heard, The germination niches of grassland species targeted for restoration: effects of seed pre-treatments, *Seed Sci Res*, 2011, **21**, 117–131.
- 80 J. J. James, T. J. Svejcar and M. J. Rinella, Demographic processes limiting seedling recruitment in arid grassland restoration, *Journal of Applied Ecology*, 2011, **48**, 961–969.
- 81 M. H. M. Menz, K. W. Dixon and R. J. Hobbs, Hurdles and Opportunities for Landscape-Scale Restoration, *Science (1979)*, 2013, **339**, 526–527.
- 82 M. D. Madsen, S. J. Kostka, A. Hulet, B. E. Mackey and A. Matthew, Surfactant Seed Coating – a Strategy to Improve Turfgrass Establishment on Water Repellent Soils, *International Symposium on Adjuvants for Agrochemicals*, 2013.
- 83 S. Pedrini, A. Balestrazzi, M. D. Madsen, K. Bhalsing, S. P. Hardegree, K. W. Dixon and O. A. Kildisheva, Seed enhancement: getting seeds restoration-ready, *Restor Ecol*, 2020, **28**, S3.
- 84 J. B. Taylor, K. L. Cass, D. N. Armond, M. D. Madsen, D. E. Pearson, and S. B. St. Clair, Deterring rodent seed-predation using seed-coating technologies, *Restor Ecol*, 2020, **28**, 927–936.

- 85 D. E. Pearson, M. Valliant, C. Carlson, G. C. Thelen, Y. K. Ortega, J. L. Orrock and M. D. Madsen, spicing up restoration: can chili peppers improve restoration seeding by reducing seed predation?, *Restor Ecol*, 2019, **27**, 254–260.
- 86 Thomas. W. May and R. H. Wiedmeyer, A table of polyatomic interferences in ICP-MS, *At Spectrosc*, 1998, **19**, 150–155.
- 87 N. Yamada, Kinetic energy discrimination in collision/reaction cell ICP-MS: Theoretical review of principles and limitations, *Spectrochim Acta Part B At Spectrosc*, 2015, **110**, 31–44.
- 88 N. I. Rousis, I. N. Pasias and N. S. Thomaidis, Attenuation of interference in collision/reaction cell inductively coupled plasma mass spectrometry, using helium and hydrogen as cell gases – application to multi-element analysis of mastic gum, *Anal. Methods*, 2014, **6**, 5899–5908.
- 89 R. E. Russo, Laser ablation research and development: 60 years strong, *Applied Physics A*, 2023, **129**, 168.
- 90 J. F. Ready, Effects Due to Absorption of Laser Radiation, *J Appl Phys*, 1965, **36**, 462–468.
- 91 D. Lichtman and J. F. Ready, Laser Beam Induced Electron Emission, *Phys Rev Lett*, 1963, **10**, 342–345.
- 92 J. C. Miller, 1994, pp. 1–10.
- 93 R. E. Honig and J. R. Woolston, Laser-induced emission of electrons, ions, and neutral atoms from solid surfaces, *Appl Phys Lett*, 1963, **2**, 138–139.

- 94 I. Konz, B. Fernández, M. L. Fernández, R. Pereiro and A. Sanz-Medel, Laser ablation ICP-MS for quantitative biomedical applications, *Anal Bioanal Chem*, 2012, **403**, 2113–2125.
- 95 A. Hanć, A. Piechalak, B. Tomaszewska and D. Barańkiewicz, Laser ablation inductively coupled plasma mass spectrometry in quantitative analysis and imaging of plant's thin sections, *Int J Mass Spectrom*, 2014, **363**, 16–22.
- 96 D. A. Frick, C. Giesen, T. Hemmerle, B. Bodenmiller, and D. Günther, An internal standardisation strategy for quantitative immunoassay tissue imaging using laser ablation inductively coupled plasma mass spectrometry, *J Anal At Spectrom*, 2015, **30**, 254–259.
- 97 T. Luo, Z. Hu, W. Zhang, D. Günther, Y. Liu, K. Zong and S. Hu, Reassessment of the influence of carrier gases He and Ar on signal intensities in 193 nm excimer LA-ICP-MS analysis, *J Anal At Spectrom*, 2018, **33**, 1655–1663.
- 98 R. Thomas, A beginner's guide to ICP-MS, in *Spectroscopy Tutorial*, 2001, vol. 16, pp. 38–55.
- 99 J. Chen, R. Wang, M. Ma, L. Gao, B. Zhao and M. Xu, Laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS)–based strategies applied for the analysis of metal-binding protein in biological samples: an update on recent advances, *Anal Bioanal Chem*, 2022, **414**, 7023–7033.

- 100 R. Khan, Sharib, Babita. Sharma, A. Chawla, Pooja, and Rohit. Bhatia, Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES): a Powerful Analytical Technique for Elemental Analysis, *Food Anal Methods*, 2022, **15**, 666–688.
- 101 C. P. Lienemann, S. Dreyfus, C. Pecheyran and O. F. X. Donard, Trace Metal Analysis in Petroleum Products: Sample Introduction Evaluation in ICP-OES and Comparison with an ICP-MS Approach, *Oil & Gas Science and Technology - Revue de l'IFP*, 2007, **62**, 69–77.
- 102 A. Technologies, Elemental analysis by ICP-OES or ICP-MS, <https://www.agilent.com/en/product/atomic-spectroscopy/icp-oes-vs-icp-ms>, (accessed February 2024).
- 103 W. Zhou, R. Apkarian, Z. L. Wang, and D. Joy, in *Scanning Microscopy for Nanotechnology*, Springer New York, New York, NY, 2006, pp. 1–40.
- 104 D. Stokes, in *Principles and practice of variable pressure/environmental scanning electron microscopy*, Wiley, 1st edn., 2008, pp. 1–60.
- 105 M. Abd Mutalib, M. A. Rahman, M. H. D. Othman, A. F. Ismail, and J. Jaafar, in *Membrane Characterization*, Elsevier, 2017, pp. 161–179.
- 106 C. Seaman, PhD Thesis, Sheffield Hallam University, 2017.
- 107 J. D. Ready and C. Seaman, in *Imaging Mass Spectrometry*, 2nd edn., 2023, pp. 123–133.
- 108 S. Pedrini, D. J. Merritt, J. Stevens and K. Dixon, Seed Coating: Science or Marketing Spin?, *Trends Plant Sci*, 2017, **22**, 106–116.

- 109 T. Adhikari, S. Kundu and A. S. Rao, Zinc delivery to plants through seed coating with nano-zinc oxide particles, *J Plant Nutr*, 2016, **39**, 136–146.
- 110 M. J. L. Castro, C. Ojeda, and A. F. Cirelli, in *Green Materials for Energy, Products and Depollution*, Springer, Dordrecht, 2013, pp. 287–334.
- 111 K. Richards, KWS ZYATT Variety overview, <https://www.kws.com/gb/en/products/cereals/wheat/variety-overview/kws-zyatt/>, (accessed 1 February 2024).
- 112 P. Bénézech, D. A. Palmer and D. J. Wesolowski, The solubility of zinc oxide in 0.03 m NaTr as a function of temperature, with in situ pH measurement, *Geochim Cosmochim Acta*, 1999, **63**, 1571–1586.
- 113 H. Isaak Reuter, L. Rodriguez Lado, T. Hengl and L. Montanarella, Continental-scale digital soil mapping using European soil profile data: soil pH, *Hamburger Beiträge zur Physischen Geographie und Landschaftsökologie*, 2008, **92**, 885–888.
- 114 T. Hengl, J. M. de Jesus, R. A. MacMillan, N. H. Batjes, G. B. M. Heuvelink, E. Ribeiro, A. Samuel-Rosa, B. Kempen, J. G. B. Leenaars, M. G. Walsh and M. R. Gonzalez, SoilGrids1km — Global Soil Information Based on Automated Mapping, *PLoS One*, 2014, **9**, 1–17.
- 115 P. Panagos, M. Van Liedekerke, P. Borrelli, J. Köninger, C. Ballabio, A. Orgiazzi, E. Lugato, L. Liakos, J. Hervas, A. Jones and L. Montanarella, European Soil Data Centre 2.0: Soil data and knowledge in support of the EU policies, *Eur J Soil Sci*, 2022, **73**, 1–18.

- 116 Y. H. Kim, K. A. Kwak, T. S. Kim, J. H. Seok, H. S. Roh, J. Lee, J. Jeong, E. H. Meang, J. Hong, Y. S. Lee and J. S. Kang, Retinopathy Induced by Zinc Oxide Nanoparticles in Rats Assessed by Micro-computed Tomography and Histopathology, *Toxicol Res*, 2015, **31**, 157–163.
- 117 L. Gargiulo, C. Leonarduzzi and G. Mele, Micro-CT imaging of tomato seeds: Predictive potential of 3D morphometry on germination, *Biosyst Eng*, 2020, **200**, 112–122.
- 118 S. R. Tracy, J. F. Gómez, C. J. Sturrock, Z. A. Wilson and A. C. Ferguson, Non-destructive determination of floral staging in cereals using X-ray micro computed tomography (μ CT), *Plant Methods*, 2017, **13**, 1–12.
- 119 D. Yamauchi, D. Tamaoki, M. Hayami, M. Takeuchi, I. Karahara, M. Sato, K. Toyooka, H. Nishioka, Y. Terada, K. Uesugi, H. Takano, Y. Kagoshima and Y. Mineyuki, Micro-CT observations of the 3D distribution of calcium oxalate crystals in cotyledons during maturation and germination in *Lotus miyakoijimae* seeds, *J Electron Microsc (Tokyo)*, 2013, **62**, 353–361.
- 120 C. Han and P. Yang, Studies on the molecular mechanisms of seed germination, *Proteomics*, 2015, **15**, 1671–1679.
- 121 T. D. Q. Le, C. Alvarado, C. Girousse, D. Legland and A.-L. Chateigner-Boutin, Use of X-ray micro computed tomography imaging to analyze the morphology of wheat grain through its development, *Plant Methods*, 2019, **15**, 84.

- 122 Y. Takaku, S. Takehara, C. Suzuki, H. Suzuki, M. Shimomura, and T. Hariyama, in situ elemental analyses of living biological specimens using 'NanoSuit' and EDS methods in FE-SEM, *Sci Rep*, 2020, **10**, 14574.
- 123 J. Tian, L. Chen, J. Dai, X. Wang, Y. Yin and P. Wu, Preparation, and characterization of TiO₂, ZnO, and TiO₂/ZnO nanofilms via sol–gel process, *Ceram Int*, 2009, **35**, 2261–2270.
- 124 J. Iqbal, B. A. Abbasi, T. Yaseen, S. A. Zahra, A. Shahbaz, S. A. Shah, S. Uddin, X. Ma, B. Raouf, S. Kanwal, W. Amin, T. Mahmood, H. A. El-Serehy and P. Ahmad, Green synthesis of zinc oxide nanoparticles using *Elaeagnus angustifolia* L. leaf extracts and their multiple in vitro biological applications, *Sci Rep*, 2021, **11**, 20988.
- 125 M. B. McDonald, C. W. Vertucci and E. E. Roos, Soybean Seed Imbibition: Water Absorption by Seed Parts, *Crop Sci*, 1988, **28**, 993–997.
- 126 J. Zhao, Y. He, X. Li, X. Weng, D. Feng, J. Ying, and Z. Wang, an integrated RNA-Seq and physiological study reveals gene responses involving in the initial imbibition of seed germination in rice, *Plant Growth Regul*, 2020, **90**, 249–263.
- 127 L. W. Woodstock, Seed Imbibition: A Critical Period for Successful Germination, *Journal of Seed Technology*, 1988, **12**, 1–15.
- 128 J. D. Bewley, K. J. Bradford, H. W.M. Hilhorst and H. Nonogaki, in *Seeds: Physiology of Development, Germination and Dormancy, 3rd Edition*, Springer, Third., 2013, pp. 141–151.

- 129 S. Afsheen, H. Naseer, T. Iqbal, M. Abrar, A. Bashir, and M. Ijaz, Synthesis and characterization of metal sulphide nanoparticles to investigate the effect of nanoparticles on germination of soybean and wheat seeds, *Mater Chem Phys*, 2020, **252**, 123216.
- 130 R. Molina, A. Lalueza, C. López-Santos, R. Ghobeira, P. Cools, R. Morent, N. de Geyter and A. R. González-Elípe, Physicochemical surface analysis and germination at different irrigation conditions of DBD plasma-treated wheat seeds, *Plasma Processes and Polymers*, 2021, **18**, 1-15.
- 131 F. Yasmeen, N. I. Raja, A. Razzaq and S. Komatsu, Proteomic and physiological analyses of wheat seeds exposed to copper and iron nanoparticles, *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 2017, **1865**, 28–42.
- 132 D. Yamauchi, D. Tamaoki, M. Hayami, M. Takeuchi, I. Karahara, M. Sato, K. Toyooka, H. Nishioka, Y. Terada, K. Uesugi, H. Takano, Y. Kagoshima and Y. Mineyuki, Micro-CT observations of the 3D distribution of calcium oxalate crystals in cotyledons during maturation and germination in *Lotus miyakoijimae* seeds, *Microscopy*, 2013, **62**, 353–361.
- 133 G. S. Montanha, E. S. Rodrigues, J. P. R. Marques, E. de Almeida, M. Colzato and H. W. Pereira de Carvalho, Zinc nanocoated seeds: an alternative to boost soybean seed germination and seedling development, *SN Appl Sci*, 2020, **2**, 857.
- 134 National Centre for Biotechnology information, PubChem Compound Summary for CID 23034, 2-Naphthalenecarboxamide, 3-hydroxy-N-(2-

methylphenyl)-4-((2,4,5-trichlorophenyl)azo),

<https://pubchem.ncbi.nlm.nih.gov/compound/23034>, (accessed 12 February 2024).

- 135 A. G. L. Assunção, H. Schat and M. G. M. Aarts, Regulation of the adaptation to zinc deficiency in plants, *Plant Signal Behav*, 2010, **5**, 1553–1555.
- 136 D. Montalvo, F. Degryse, R. C. da Silva, R. Baird and M. J. McLaughlin, Agronomic effectiveness of zinc sources as micronutrient fertilizer, *Advances in Agronomy*, 2016, **139**, 215–267.
- 137 T. M. Muthukumararaja and M. V. Sriramachandrasekharan, Effect of zinc on yield, zinc nutrition and zinc use efficiency of lowland rice, *Journal of Agricultural Technology*, 2012, **8**, 551–561.
- 138 I. Zewide and A. Sherefu, Review Paper on Effect of Micronutrients for Crop Production, *Nutrition and Food Processing*, 2021, **4**, 01–08.
- 139 C. Noulas, M. Tziouvalekas and T. Karyotis, Zinc in soils, water and food crops, *Journal of Trace Elements in Medicine, and Biology*, 2018, **49**, 252–260.
- 140 D.-Y. Zhao, S.-S. Zheng, M. K. Naeem, J.-Q. Niu, N. Wang, Z.-J. Li, H.-L. Wu and H.-Q. Ling, Screening wheat genotypes for better performance on reduced phosphorus supply by comparing glasshouse experiments with field trials, *Plant Soil*, 2018, **430**, 349–360.
- 141 C. Tang, M. Nuruzzaman and Z. Rengel, Screening wheat genotypes for tolerance of soil acidity, *Aust J Agric Res*, 2003, **54**, 445.

- 142 V. Mitter, M. C. Zhang, C. J. Liu, R. Ghosh, M. Ghosh and S. Chakraborty, A high-throughput glasshouse bioassay to detect crown rot resistance in wheat germplasm, *Plant Pathol*, 2006, **55**, 433–441.
- 143 H. J. B. LOWE, Development, and practice of a glasshouse screening technique for resistance of wheat to the aphid *Sitobion avenae*, *Annals of Applied Biology*, 1984, **104**, 297–305.
- 144 M. L. Nguyen, S. Spaepen, P. du Jardin and P. Delaplace, Biostimulant effects of rhizobacteria on wheat growth and nutrient uptake depend on nitrogen application and plant development, *Arch Agron Soil Sci*, 2019, **65**, 58–73.
- 145 N. Gülmezoğlu, N. Köleli, B. Karakaya and H. Dağhan, Impact of titanium dioxide nanoparticles (TiO₂-NPs) on growth and mineral nutrient uptake of wheat (*Triticum vulgare* L.), *Tarla Bitkileri Merkez Arastirma Enstitusu*, 2020, **29**, 69–76.
- 146 A. Lehmann, S. D. Veresoglou, E. F. Leifheit and M. C. Rillig, Arbuscular mycorrhizal influence on zinc nutrition in crop plants – A meta-analysis, *Soil Biol Biochem*, 2014, **69**, 123–131.
- 147 P. Songkhum, T. Wuttikhun, N. Chanlek, P. Khemthong and K. Laohhasurayotin, Controlled release studies of boron and zinc from layered double hydroxides as the micronutrient hosts for agricultural application, *Appl Clay Sci*, 2018, **152**, 311–322.

- 148 W. Du, J. Yang, Q. Peng, X. Liang and H. Mao, Comparison study of zinc nanoparticles and zinc sulphate on wheat growth: From toxicity and zinc biofortification, *Chemosphere*, 2019, **227**, 109–116.
- 149 L. B. Singh, R. Yadav and T. Abraham, Studies on the effect of Zinc levels , and methods of boron application on growth , yield and protein content of Wheat (*Triticum aestivum* L .), *Bulletin of Environment, Pharmacology and Life Sciences*, 2015, **4**, 108–113.
- 150 D. R. Hoagland and D. I. Arnon, The water-culture method for growing plants without soil, *California Agricultural Experiment Station Circular*, 1950, **347**, 32.
- 151 S. D. Hothem, K. A. Marley and R. A. Larson, Photochemistry in Hoagland's Nutrient Solution, *J Plant Nutr*, 2003, **26**, 845–854.
- 152 W. Thorne, Zinc deficiency and its control, in *Advances in Agronomy*, 1957, pp. 31–65.
- 153 M. L. Adams, W. D. Philpot and W. A. Norvell, Yellowness index: An application of spectral second derivatives to estimate chlorosis of leaves in stressed vegetation, *Int J Remote Sens*, 1999, **20**, 3663–3675.
- 154 F. Xing, X. Fu, N. Wang, J. XI, Y. Huang, W. Zhou, L. Ling and L. Peng, Physiological changes, and expression characteristics of ZIP family genes under zinc deficiency in navel orange (*Citrus sinensis*), *J Integr Agric*, 2016, **15**, 803–811.

- 155 A. P. Pant, T. J. K. Radovich, N. V. Hue and R. E. Paull, Biochemical properties of compost tea associated with compost quality and effects on pak choi growth, *Sci Hortic*, 2012, **148**, 138–146.
- 156 G. L. Terman, J. M. Soileau and S. E. Allen, Municipal Waste Compost: Effects on Crop Yields and Nutrient Content in Greenhouse Pot Experiments, *J Environ Qual*, 1973, **2**, 84–89.
- 157 R. Lal, Soil organic matter and water retention, *Agron J*, 2020, **112**, 3265–3277.
- 158 H. Wang and J. Jin, Effects of Zinc Deficiency and Drought on Plant Growth and Metabolism of Reactive Oxygen Species in Maize (*Zea mays* L), *Agric Sci China*, 2007, **6**, 988–995.
- 159 T. Kim and H. Y. Wetzstein, Cytological and ultrastructural evaluations of zinc deficiency in leaves, *Journal of the American Society for Horticultural Science*, 2003, **128**, 171–175.
- 160 T. Huang, Q. Huang, X. She, X. Ma, M. Huang, H. Cao, G. He, J. Liu, D. Liang, S. S. Malhi and Z. Wang, Grain zinc concentration and its relation to soil nutrient availability in different wheat cropping regions of China, *Soil Tillage Res*, 2019, **191**, 57–65.
- 161 C. Noulas, M. Tziouvalekas and T. Karyotis, Zinc in soils, water and food crops, *Journal of Trace Elements in Medicine, and Biology*, 2018, **49**, 252–260.
- 162 A. Kabata-Pendias, *Trace Elements in Soils and Plants*, CRC Press, 2000.

- 163 L. Ozturk, S. Karanlik, F. Ozkutlu, I. Cakmak and L. V. Kochian, Shoot biomass and zinc/cadmium uptake for hyperaccumulator and non-accumulator *Thlaspi* species in response to growth on a zinc-deficient calcareous soil, *Plant Science*, 2003, **164**, 1095–1101.
- 164 M. Wang, F. Kong, R. Liu, Q. Fan and X. Zhang, Zinc in Wheat Grain, Processing, and Food, *Front Nutr*, 2020, **7**, 1–10.
- 165 M. Waseem Abbas, Germination and Seedling Growth of Wheat as Affected by Seed Priming and its Duration, *Agric Res Technol*, 2018, **18**, 27–33.
- 166 Croda, Hydravance 200, https://www.crodacropcare.com/en-gb/product-finder/product/342-Hydravance_1_200, (accessed 3 January 2023).
- 167 Q. Fariduddin, M. Saleem, T. A. Khan and S. Hayat, Zinc as a versatile Element in Plants: and overview on its uptake, translocation, assimilatory roles, deficiency, and toxicity symptoms, in *Microbial Biofertilizers and Micronutrient Availability: The Role of Zinc in Agriculture and Human Health*, 2021.
- 168 M. Wang, F. Kong, R. Liu, Q. Fan and X. Zhang, Zinc in Wheat Grain, Processing, and Food, *Front Nutr*, 2020, **7**, 1–10.
- 169 N. Gupta, H. Ram and B. Kumar, Mechanism of Zinc absorption in plants: uptake, transport, translocation and accumulation, *Rev Environ Sci Biotechnol*, 2016, **15**, 89–109.
- 170 J. J. Hart, W. A. Norvell, R. M. Welch, L. A. Sullivan, and L. V. Kochian, Characterization of Zinc Uptake, Binding, and Translocation in Intact

- Seedlings of Bread and Durum Wheat Cultivars, *Plant Physiol*, 1998, **118**, 219–226.
- 171 N. Rascio and F. Navari-Izzo, Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? *Plant Science*, 2011, **180**, 169-181.
 - 172 M. Sut-Lohmann, M. Grimm, F. Kästner, T. Raab, M. Heinrich and T. Fischer, Brassica juncea as a Feasible Hyperaccumulator of Chosen Potentially Toxic Metals Under Extreme Environmental Conditions, *Int J Environ Res*, 2023, **17**, 38
 - 173 E. Dalyan, E. Yüzbaşıoğlu, B. C. Keskin, Y. Yıldızhan, A. Memon, M. Ünal and B. Yüksel, the identification of genes associated with Pb and Cd response mechanism in Brassica juncea L. by using Arabidopsis expression array, *Environ Exp Bot*, 2017, **139**, 105–115.
 - 174 M. Sut-Lohmann, M. Grimm, F. Kästner, T. Raab, M. Heinrich and T. Fischer, Brassica juncea as a Feasible Hyperaccumulator of Chosen Potentially Toxic Metals Under Extreme Environmental Conditions, *Int J Environ Res*, 2023, **17**, 1-10
 - 175 H. Chaudhry, N. Nisar, S. Mehmood, M. Iqbal, A. Nazir and M. Yasir, Indian Mustard Brassica juncea efficiency for the accumulation, tolerance, and translocation of zinc from metal contaminated soil, *Biocatal Agric Biotechnol*, 2020, **23**, 101489.

- 176 S. N. Whiting, J. R. Leake, S. P. McGrath and A. J. M. Baker, Zinc accumulation by *Thlaspi caerulescens* from soils with different Zn availability: A pot study., *Plant Soil*, 2001, **236**, 11–18.
- 177 S. J. Watts-Williams, T. W. Turney, A. F. Patti and T. R. Cavagnaro, Uptake of zinc and phosphorus by plants is affected by zinc fertiliser material and arbuscular mycorrhizas, *Plant Soil*, 2014, **376**, 165–175.
- 178 T. M. Shaver, D. G. Westfall, and M. Ronaghi, Zinc Fertilizer Solubility and Its Effects on Zinc Bioavailability Over Time, *J Plant Nutr*, 2007, **30**, 123–133.
- 179 K. L. Linge and K. E. Jarvis, Quadrupole ICP-MS: Introduction to Instrumentation, Measurement Techniques and Analytical Capabilities, *Geostand Geoanal Res*, 2009, **33**, 445–467.
- 180 V. Mihaylova, V. Lyubomirova and R. Djingova, Optimization of sample preparation and ICP-MS analysis for determination of 60 elements for characterization of the plant ionome, *Int J Environ Anal Chem*, 2013, **93**, 1441–1456.
- 181 J. Du, Z. Guo, R. Li, A. Ali, D. Guo, A. H. Lahori, P. Wang, X. Liu, X. Wang and Z. Zhang, Screening of Chinese mustard (*Brassica juncea* L.) cultivars for the phytoremediation of Cd and Zn based on the plant physiological mechanisms, *Environmental Pollution*, 2020, **261**, 114213.
- 182 A. Kutrowska, A. Małecka, A. Piechalak, W. Masiakowski, A. Hanć, D. Barańkiewicz, B. Andrzejewska, J. Zbierska and B. Tomaszewska, Effects of binary metal combinations on zinc, copper, cadmium and lead uptake

- and distribution in *Brassica juncea*, *Journal of Trace Elements in Medicine, and Biology*, 2017, **44**, 32–39.
- 183 T. Verdenal, J. E. Spangenberg, Á. Dienes-Nagy, V. Zufferey, J. -L. Spring, O. Viret and C. van Leeuwen, Nitrogen dynamics and fertilisation use efficiency: carry-over effect of crop limitation, *Aust J Grape Wine Res*, 2022, **28**, 358–373.
 - 184 B.-M. Chen, Z.-H. Wang, S.-X. Li, G.-X. Wang, H.-X. Song and X.-N. Wang, Effects of nitrate supply on plant growth, nitrate accumulation, metabolic nitrate concentration and nitrate reductase activity in three leafy vegetables, *Plant Science*, 2004, **167**, 635–643.
 - 185 T. M. Shaver, D. G. Westfall, and M. Ronaghi, Zinc Fertilizer Solubility and Its Effects on Zinc Bioavailability Over Time, *J Plant Nutr*, 2007, **30**, 123–133.
 - 186 K. R. Raghupathi, R. T. Koodali and A. C. Manna, Size-Dependent Bacterial Growth Inhibition and Mechanism of Antibacterial Activity of Zinc Oxide Nanoparticles, *Langmuir*, 2011, **27**, 4020–4028.
 - 187 S. J. Watts-Williams, T. W. Turney, A. F. Patti and T. R. Cavagnaro, Uptake of zinc and phosphorus by plants is affected by zinc fertiliser material and arbuscular mycorrhizas, *Plant Soil*, 2014, **376**, 165–175.
 - 188 F. Goodwin, in *Encyclopedia of Chemical Technology*, ed. Kirk-Othmer, John Wiley & Sons, New York, NY, 2006, vol. 26.
 - 189 D. J. Wesolowski, P. Bénézech and D. A. Palmer, ZnO Solubility and Zn²⁺ Complexation by Chloride and Sulfate in Acidic Solutions to 290°C with In-Situ pH Measurement, *Geochim Cosmochim Acta*, 1998, **62**, 971–984.

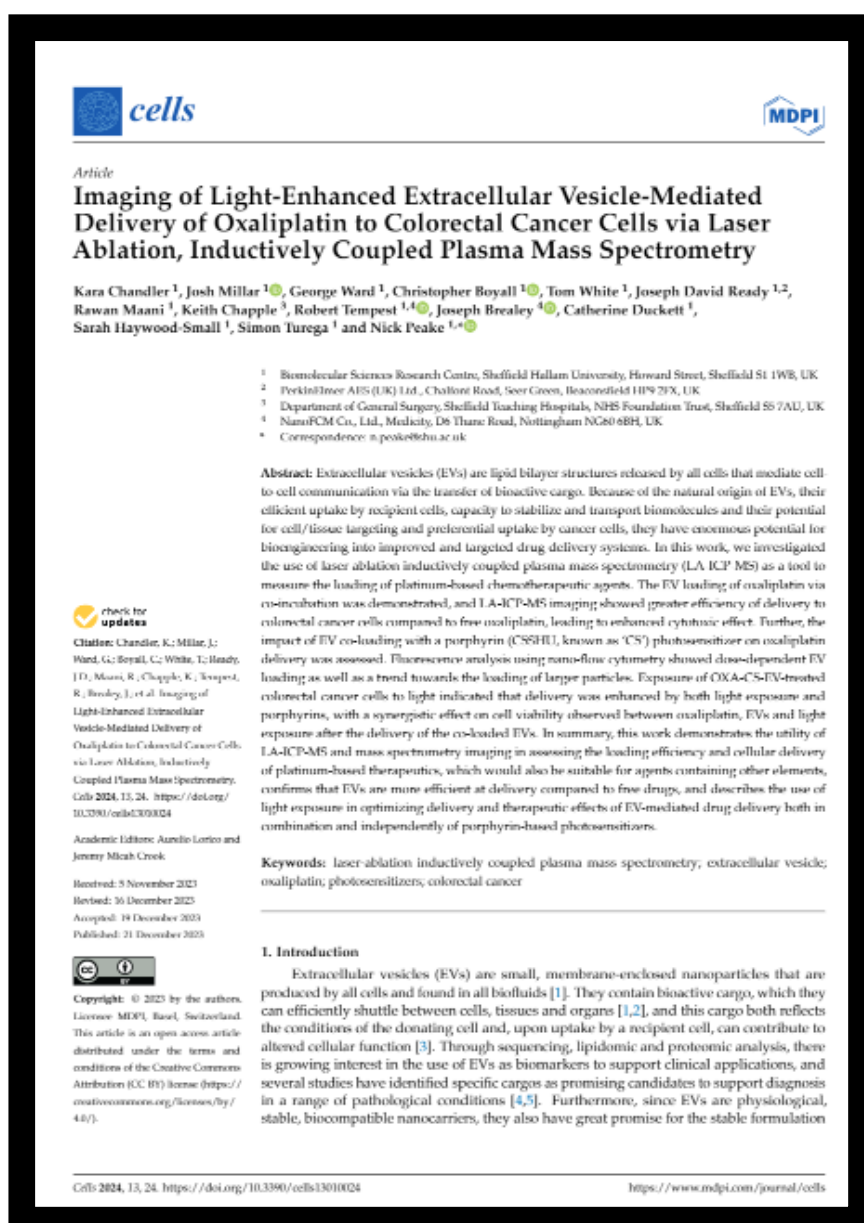
- 190 H. L. Clever, M. E. Derrick and S. A. Johnson, The Solubility of Some Sparingly Soluble Salts of Zinc and Cadmium in Water and in Aqueous Electrolyte Solutions, *J Phys Chem Ref Data*, 1992, **21**, 941–1004.
- 191 H.-X. Yang, L.-H. Zhao, J.-X. Gao, W. Liu, and B. Li, Bioimaging and Distribution of Cd, P, S, K, Ca, Cu and Zn Elements in Indian Mustard Stem, *Chinese Journal of Analytical Chemistry*, 2014, **42**, 355–359.
- 192 O. Ahluwalia, P. C. Singh and R. Bhatia, A review on drought stress in plants: Implications, mitigation and the role of plant growth promoting rhizobacteria, *Resources, Environment and Sustainability*, 2021, **5**, 100032.
- 193 G. Kaur and B. Asthir, Molecular responses to drought stress in plants, *Biol Plant*, 2017, **61**, 201–209.
- 194 J. A. Hernandez-Viezcas, H. Castillo-Michel, A. D. Servin, J. R. Peralta-Videa and J. L. Gardea-Torresdey, Spectroscopic verification of zinc absorption and distribution in the desert plant *Prosopis juliflora-velutina* (velvet mesquite) treated with ZnO nanoparticles, *Chemical Engineering Journal*, 2011, **170**, 346–352.
- 195 A. Tymoszuik and J. Wojnarowicz, Zinc Oxide and Zinc Oxide Nanoparticles Impact on In Vitro Germination and Seedling Growth in *Allium cepa* L., *Materials*, 2020, **13**, 2784.
- 196 D.-Y. Liu, Y.-M. Liu, W. Zhang, X.-P. Chen and C.-Q. Zou, Zinc Uptake, Translocation, and Remobilization in Winter Wheat as Affected by Soil Application of Zn Fertilizer, *Front Plant Sci*, 2019, **10**, 426–426

- 197 V. Mishra, R. K. Mishra, A. Dikshit and A. C. Pandey, in *Emerging Technologies and Management of Crop Stress Tolerance*, Elsevier, Amsterdam, 2014, pp. 159–180.
- 198 Z. Lv, H. Sun, W. Du, R. Li, H. Mao, and P. M. Kopittke, Interaction of different-sized ZnO nanoparticles with maize (*Zea mays*): Accumulation, biotransformation and phytotoxicity, *Science of The Total Environment*, 2021, **796**, 148927.
- 199 C. García-Gómez, A. Obrador, D. González, M. Babín and M. D. Fernández, Comparative effect of ZnO NPs, ZnO bulk and ZnSO₄ in the antioxidant defences of two plant species growing in two agricultural soils under greenhouse conditions, *Science of The Total Environment*, 2017, **589**, 11–24.

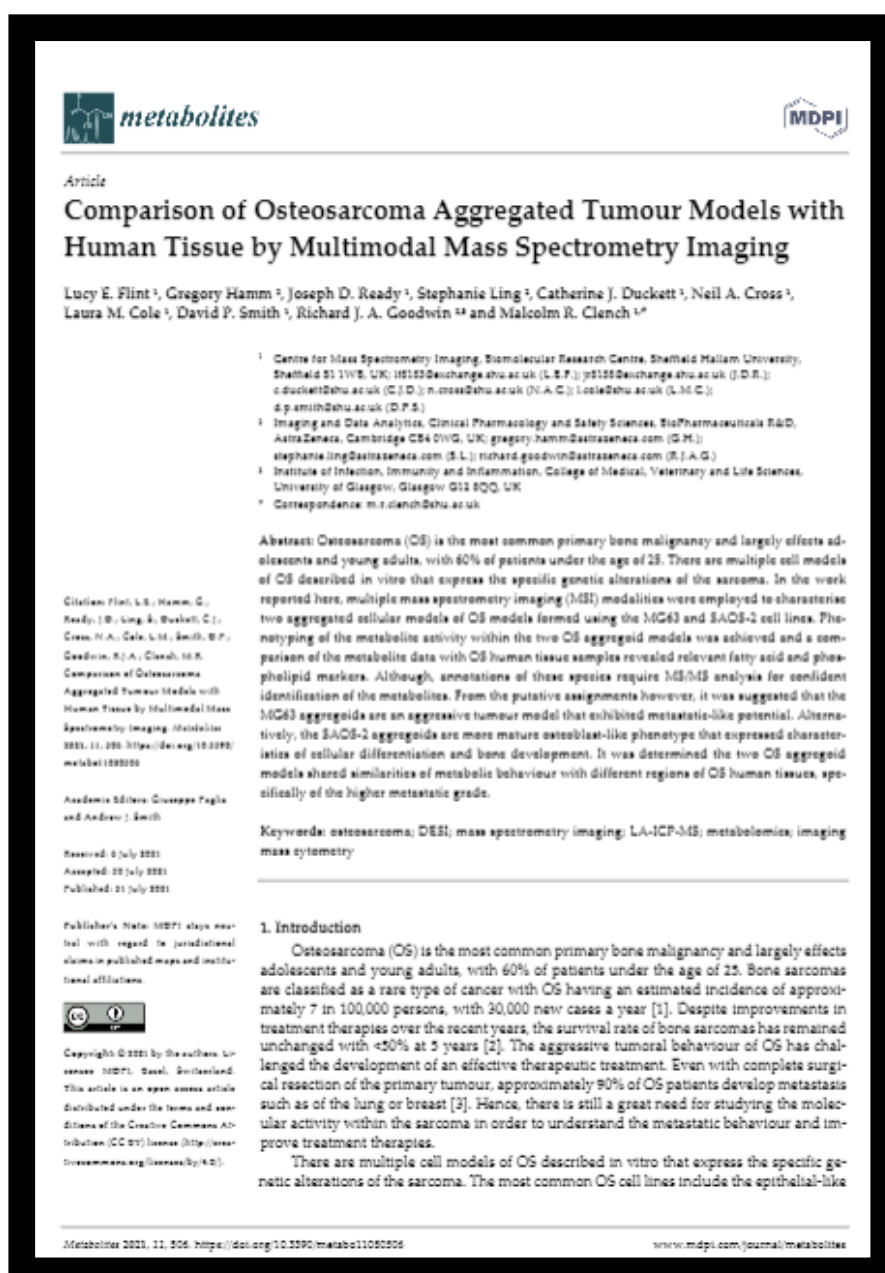
Page left intentionally blank

10.0. Other research contributions whilst undertaking this research.

Chandler, K.; Millar, J.; Ward, G.; Boyall, C.; White, T.; **Ready, J.D.**; Maani, R.; Chapple, K.; Tempest, R.; Brealey, J.; et al. Imaging of Light-Enhanced Extracellular Vesicle-Mediated Delivery of Oxaliplatin to Colorectal Cancer Cells via Laser Ablation Inductively Coupled Plasma Mass Spectrometry. **Cells**, 2024, 13, 24. <https://doi.org/10.3390/cells13010024>.



Flint, L.E.; Hamm, G.; **Ready, J.D.**; Ling, S.; Duckett, C.J.; Cross, N.A.; Cole, L.M.; Smith, D.P.; Goodwin, R.J.A.; Clench, M.R. *Comparison of Osteosarcoma Aggregated Tumour Models with Human Tissue by Multimodal Mass Spectrometry Imaging*. **Metabolites** 2021, 11, 506. <https://doi.org/10.3390/metabo11080506>.



Flint, L.E.; Hamm, G.; **Ready, J.D.**; Ling, S.; Duckett, C.J.; Cross, N.A.; Cole, L.M.; Smith, D.P.; Goodwin, R.J.A.; Clench, M.R. *Characterization of an Aggregated Three-Dimensional Cell Culture Model by Multimodal Mass Spectrometry Imaging*. *Analytical Chemistry*, 2020, 92, 18, 12538-12547, <https://doi.org/10.1021/acs.analchem.0c02389>.



pubs.acs.org/ac

This is an open access article published under a Creative Commons Attribution (CC-BY) license, which permits unrestricted use, distribution and reproduction in any medium, provided the author and source are cited.

ACS AUTHORCHOICE

Article

Characterization of an Aggregated Three-Dimensional Cell Culture Model by Multimodal Mass Spectrometry Imaging

Lucy E. Flint, Gregory Hamm, Joseph D. Ready, Stephanie Ling, Catherine J. Duckett, Neil A. Cross, Laura M. Cole, David P. Smith, Richard J. A. Goodwin, and Malcolm R. Clench*

Cite This: *Anal. Chem.* 2020, 92, 12538–12547

Read Online

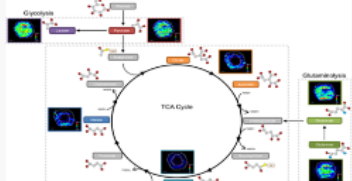
ACCESS |

Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: Mass spectrometry imaging (MSI) is an established analytical tool capable of defining and understanding complex tissues by determining the spatial distribution of biological molecules. Three-dimensional (3D) cell culture models mimic the pathophysiological environment of *in vivo* tumors and are rapidly emerging as a valuable research tool. Here, multimodal MSI techniques were employed to characterize a novel aggregated 3D lung adenocarcinoma model, developed by the group to mimic the *in vivo* tissue. Regions of tumor heterogeneity and the hypoxic microenvironment were observed based on the spatial distribution of a variety of endogenous molecules. Desorption electrospray ionization (DESI)-MSI defined regions of a hypoxic core and a proliferative outer layer from metabolite distribution. Targeted metabolites (e.g., lactate, glutamine, and citrate) were mapped to pathways of glycolysis and the TCA cycle demonstrating tumor metabolic behavior. The first application of imaging mass cytometry (IMC) with 3D cell culture enabled single-cell phenotyping at 1 μm spatial resolution. Protein markers of proliferation ($Ki-67$) and hypoxia (glucose transporter 1) defined metabolic signaling in the aggregated model, which complemented the metabolite data. Laser ablation inductively coupled plasma (LA-ICP)-MSI analysis localized endogenous elements including magnesium and copper, further differentiating the hypoxia gradient and validating the protein expression. Obtaining a large amount of molecular information on a complementary nature enabled an in-depth understanding of the biological processes within the novel tumor model. Combining powerful imaging techniques to characterize the aggregated 3D culture highlighted a future methodology with potential applications in cancer research and drug development.



Mass spectrometry imaging (MSI) is a sophisticated technology capable of simultaneously mapping a variety of molecules within a biological sample. The benefits of conventional MSI techniques compared to other imaging modalities are the abilities to detect ionizable compounds in both targeted and untargeted methods without the use of specific labeling reagents.¹ The spatial localization of a molecule can determine the interplay of biological functions and interactions within a tissue. This also enables a greater biological understanding of cellular phenotypes and their structural organization, in addition to the surrounding microenvironment. MSI has therefore had a major influence on cancer research and drug development by utilizing spatial localization of biomarkers, therapeutics, their active metabolites, and cellular responses.¹

Depending on the biological sample and the molecules of interest, different MSI techniques can be employed for optimum analysis. Matrix-assisted laser desorption/ionization (MALDI) is the most widely used MSI technique across many applications due to its high spatial resolution and speed of acquisition. MALDI-MSI can detect a wide range of analytes in an untargeted manner including metabolites, lipids, peptides,

and proteins.^{2–4} Desorption electrospray ionization (DESI)-MSI is also widely used due to the minimal sample preparation requirement of this ambient ionization methodology.⁵ The combination of DESI with Orbitrap and QTOF type mass spectrometers has generated images with high mass specificity for metabolites and small molecule therapeutics in tissue samples.^{6,7} The requirement for the study of trace elements or metal isotope distribution in tissues has also seen the development of laser ablation-inductively coupled plasma (LA-ICP)-MSI,^{8,9} a technique that has been applied to the analysis of metal-containing therapeutics such as cisplatin.^{10,11} Advancements of LA-ICP-MSI have evolved to imaging mass cytometry (IMC), a novel multiplex method capable of tissue phenotyping, and imaging biological processes at a high spatial

Received: June 4, 2020

Accepted: July 29, 2020

Published: July 29, 2020





© 2020 American Chemical Society

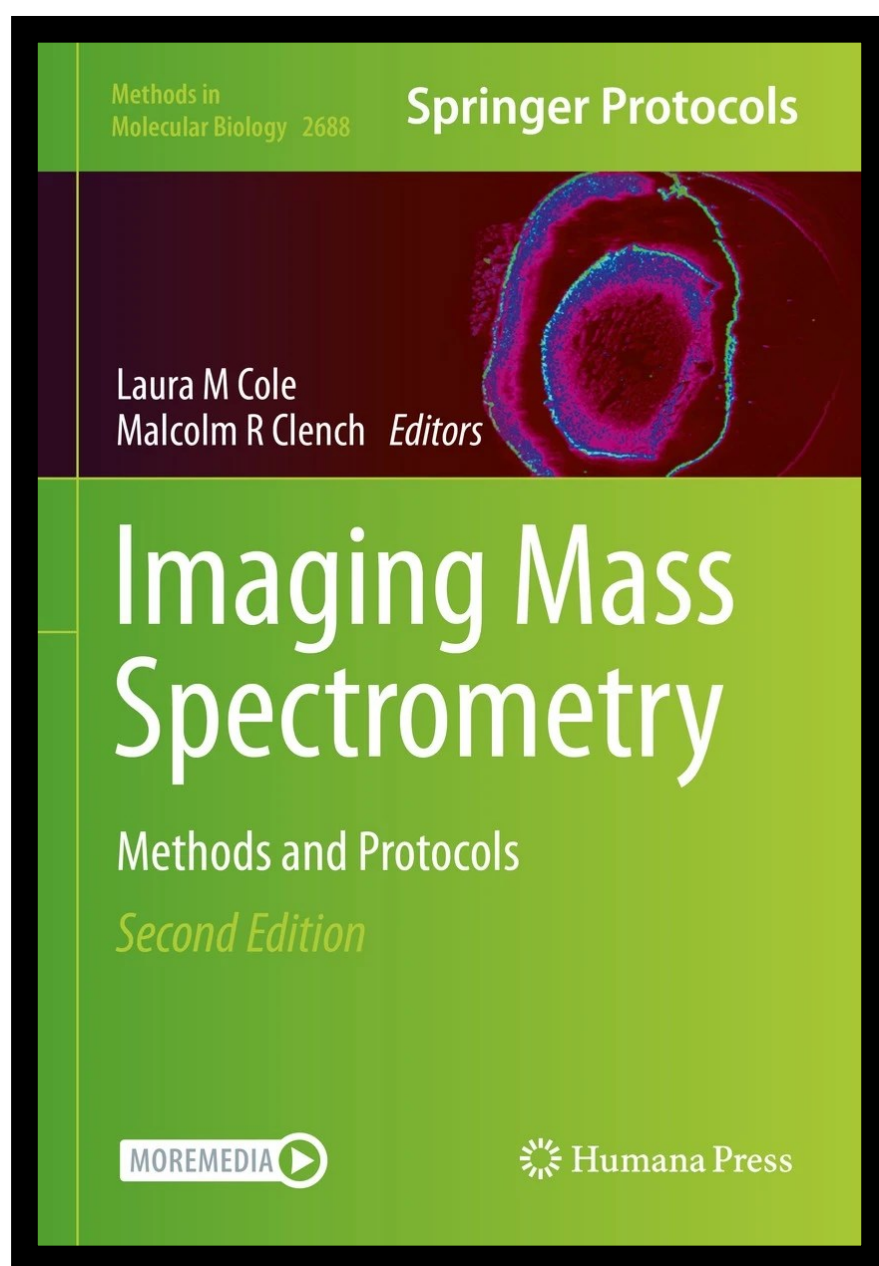
12538

<https://doi.org/10.1021/acs.analchem.0c02389>

Anal. Chem. 2020, 92, 12538–12547

271

Ready, J., Seaman, C. *Laser Ablation Inductively Coupled Plasma Mass Spectrometry Imaging of Plant Materials*. In: Cole, L.M., Clench, M.R. (eds) *Imaging Mass Spectrometry*. **Methods in Molecular Biology**, 2023, vol 2688. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-3319-9_11



11.0. Appendix

11.1. ICP-MS seed washing zinc analysis method validation.

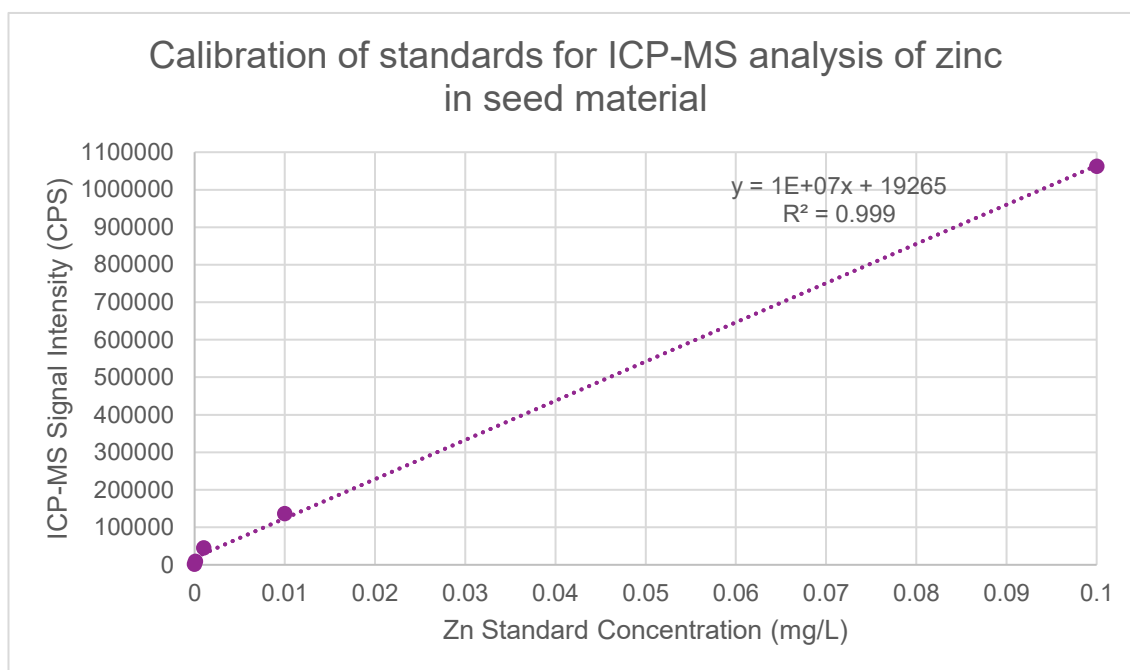


Figure 68: Calibration graph for Zn standards by ICP-MS – standards used to quantify the Zn content of digested seed material throughout Chapter 4 of this thesis, R2 value is shown, and the equation of the straight line is used to calculate the limit of detection for the analysis (LOD) below.

R^2 Value = 0.999

Magnitude of treated washed samples (CPS) = ~ 70,000 CPS.

Magnitude of treated (Ref control) samples = ~ 500,000 CPS.

Midpoint of calibration = ~500,000 CPS.

LOD at 3.3 x stdev blank = 0.0013 ppm

Mean untreated = 0.023 ppm.