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Virtual Fertilizer Research Center

Summary

Plants can absorb nano- and micro-size particles containing nutrients, opening the possibility for instantaneous uptake of nutrients supplied through roots or leaves. Here, we studied the uptake by lettuce (*Lactuca sativa* L.) of particulate nutrients from a micnobit (mixture of nanoparticles producing different nano and micro-scale sizes) formulation composed of ZnO, CuO, Fe_3O_4, MnO, and B_2O_3 used as fertilizers, in comparison to a similar mixture of their ionic equivalents from salts of Zn, Cu, Fe, Mn and boric acid at levels optimal for growth, yield, metabolism and nutritional quality attributes in food crops. In the case of Zn, the effect of a double dose compared to the basic fertilization was also studied to evaluate the possibility of increasing Zn levels in vegetable crops, as a potential strategy for alleviating Zn deficiency in human/animal diets.

A greenhouse pot experiment was conducted using a sandy soil (pH 7.1) poor in most nutrients, including micronutrients but rich in calcium, and with very low content of organic matter to study the effects of micronutrient fertilization on lettuce. For application of the micronutrients, four fertilizer treatments were used: i) traditional (ionic) fertilizer; ii) micnobit particle coated seeds, iii) micnobit foliar application (spraying treatment), and iv) micnobit soil application. The lettuce plants were grown for 73 days and harvested periodically to study fresh and dry weight production and leaf greenness by SPAD measurements over time. Shoot or root materials from the final harvest was used to determine contents of micronutriënts, leaf chlorophyll and biomolecules (vitamins, flavonoids, phenolics, and antioxidants).

Values from SPAD measurements significantly differed between the harvests and showed an increase with time up to 59 days after sowing. Differences between the treatments were less distinct, but showed the highest values between the Priming and Control treatments. Chlorophyll contents did not significantly differ between the treatments. The lettuce plants showed S-shaped growth under all treatments, although the foliar applications, both with micnobits and ions, caused necrosis at the leaf edges in the long run. Also, Foliar application with micnobits caused deposition and showed black spots on the leaves that could not be removed by rinsing methods described in the literature. Growth analysis, i.e. production of fresh weight, dry weight and root-total weight ratio over time, showed that for both shoot and root, the Control treatment resulted in the best growth. Priming of seeds resulted in similar growth as at the Control treatment. Addition of micronutrients to soil or leaves, both applied as ions or as micnobits, even decreased fresh weight and dry weight production of lettuce. Moreover, the shoots and roots of plants in the Control treatment also showed accumulation of micronutrients, while no micronutrients had been applied. No significant differences were found between the Foliar application and Soil treatments. Moreover, even no significant differences were found between application of micnobits or application of ions. Thus, we concluded that the soil - although very poor with a low content of micronutrients - was not lacking in the tested nutrients and the low availability of micronutrients in the soil was already sufficient for an optimal growth.

The determination of micronutrient contents showed that ions and micnobits were taken up by both roots (Soil treatments) and by leaves (Foliar application). However, the amounts of micronutrients found in the leaves appeared to be higher than those described in literature for plant shoots with adequate growth. The contents of the micronutrients other than Fe varied between the treatments in both leaves and roots, suggesting excessive uptake that could have inhibited biomass production in these treatments. Foliar application with ions resulted in a higher micronutrient content than foliar application with micnobits. The fact that all treatments, whether applied to the leaves or to the soil or via priming, resulted in an accumulation of micronutrients in both shoots and roots, proved that the micronutrients had been transferred through the plants from the shoot to the roots (Foliar

application) and from the roots to the shoot (Soil application). However, it could not be determined in which form, i.e. whether as ions, micnobits or metabolically processed, the micronutrients were transported through the plant. The presence of micnobits could only be detected for Fe_2O_3, CuO and MnO_3. However, due to a high noise level in the ICP-MS sample analyses, it was not possible to quantify the content of the elements properly.

Vitamin C content in leaves was highest in plants from the Control treatment, but not significant different from other treatments. No clear distinction could be made between the Soil and Foliar application, nor between treatments with ions or with micnobits. LCMS profiles of the lettuce leaves showed a relative intensity of 257 compounds present in the leaves. In the Soil and Foliar application, 80 out of the 237 metabolites (25%) had been changed significantly, although not annotated, from which 66 of them revealed a more than 2-fold difference.

We conclude that this study with composite nanoparticles could not endorse the hypothesis that micnobits will be taken up more efficiently than ions, or that they will enhance growth in lettuce as reported in the literature for other crops. Furthermore, this study also clearly demonstrates the need for plant tissue testing as an important yardstick for supporting soil-based nutrient testing, prior to fertilizer recommendations.

Contents

List of Tables

List of Figures

List of acronyms and abbreviations

B	boron
B_2O_3	boron oxide
B.V.	Besloten Vennootschap (*Dutch*), meaning: Private company
C	carbon
Ca	calcium
$Ca(NO_3)_2$	calcium nitrate
Cl	chloride
Cu	copper
CuO	copper oxide
$CuSO_4$	copper sulfate
DM	Dry Matter
EDTA	Ethylenediaminetetraacetic acid
H_3BO_3	boric acid
ICP-MS	Inductively Couple Plasma Mass Spectroscopy
Fe	iron
FeNa-EDTA	EDTA ferric sodium salt
Fe_3O_4	iron oxide
G	gram
GC	Growth Cycle
K	potassium
Kg	kilogram
KH_2PO_4	monopotassium phosphate
KNO3	potassium nitrate
L	litre
LCMS	Liquid chromatography – mass spectrometry
N	nitrogen
Mg	magnesium
$MgSO_4$	magnesium sulfate
mg	milligram
µg	microgram
mmol	millimol
Mn	manganese
MnO	manganese oxide
$MnSO_4$	manganese sulfate
Mo	molybdenum
Na	sodium
Na_2MoO_4	sodium molybdate
nm	nanometer
P	phosphorus
P_2O_5	phosphorus oxide
S	sulfur
SPAD	Soil Plant Analysis Development

T	Temperature
VFRC	Virtual Fertilizer Research Center
Zn	zinc
ZnO	zinc oxide
$ZnSO_4$	zinc sulfate

1   Introduction

1.1 Background

The conventional form in which plants accumulate mineral nutrients from the soil is the ionic form. However while ionic nutrients are readily soluble in the rhizosphere and, therefore, should be bioavailable to the plant, they are also prone to the confounding influence of soil chemistry such as pH, cations, anions, as well as the presence of compounds such as phosphates and carbonates, with which they react to form insoluble chemical precipitates. Ionic nutrients may also interact with clay colloids, organic matter and other mineral complexes, rendering them unavailable to the crop. As such, a strategy that reduces the contact of ionic nutrients with such edaphic hindrances may permit a better use efficiency of nutrients by plants (Bindraban *et al.*, 2015).

Bioenvironmental nanoscience, the study of the interaction of nanomaterials with the living environment (in this case plants and the rhizosphere), has revealed that plants may also accumulate nutrients as particles of both nano and/or micro sizes (e.g. Zhu *et al.*, 2008; Dimkpa *et al.*, 2012; Wang *et al.*, 2012; Dimkpa *et al.*, 2013; Wang *et al.*, 2013b, c; Bandyopadhyay *et al.*, 2015). See Figure 1-1.
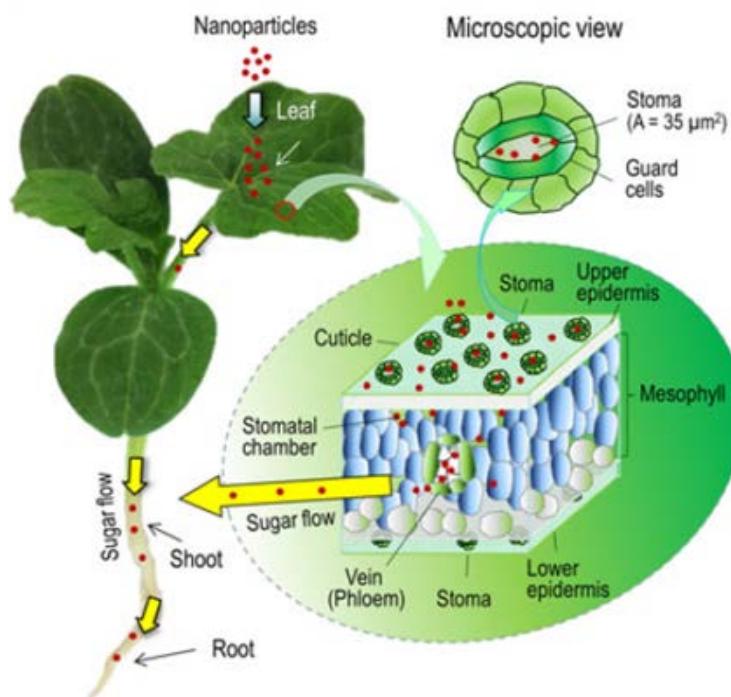
Figure 1-1. Schematic diagram of nanoparticle transport inside watermelon plants (Wang et al., 2013c)

There also is evidence suggesting that the intact particles can:
(i)   Be transported from root to shoot (Dimkpa *et al.*, 2012; Wang *et al.*, 2012; Dimkpa *et al.*, 2013; Bandyopadhyay *et al.*, 2015);
(ii)   Be transported from shoot to the root (Wang *et al.*, 2012; Wang *et al.*, 2013b, c);

(iii)   Liberate ions in the rhizosphere, and possibly, inside the plant (Wang *et al.*, 2012; Lv *et al.*, 2014); and that

(iv)   the released ions can form complexes with metabolic constituents of the plant (Dimkpa *et al.* 2013; Wang *et al.*, 2013 b,c; Lv *et al.*, 2014; Bandyopadhyay *et al.*, 2015).

As recently reviewed by Bindraban *et al.* (2015), different kinds of nanomaterials, including nanoforms of micronutrients such as Zn, Fe and Mn, have been demonstrated as being able to improve crop growth and/or content of these elements (Wang *et al.*, 2013a, b, c; Siddique and Al-Whaibi 2014; Servin *et al.,* 2015; Subbaiah 2016). In some cases, the positive effects of nanoparticles (NPs) on crop growth occur to a greater extent than the effect of the equivalent dose of the same mineral nutrient presented in ionic (salt) or bulk form (Alidoust and Isoda 2013; Pradhan *et al,.* 2013; Zhao *et al.,* 2013; Kim *et al.,* 2014; Subbaiah 2016); and when applied at the same concentration at relatively high doses, the toxicity threshold is lower with ions than with NPs (Dimkpa *et al,.* 2012; Pradhan *et al.,* 2013; Kim *et al.,* 2014).

Hence, a new nomenclature, 'micnobits', was proposed by Dimkpa and Bindraban (2016), to describe tiny (micro and nano sized) particulate micronutrient fertilizer formulations capable of being taken up intact by plants through various avenues, including pores or via endocytosis, for the release of the active ingredients in planta. Recently, a composite of micnobits of ZnO, CuO AND B_2O_3 NPs was shown to mitigate drought stress in soybean via promoting growth, yield and uptake of several nutrients under drought stress that inhibited these parameters (Dimkpa et al. in press). To evaluate the effects of micnobit-type fertilizers on a vegetable plant growth and development, the current work evaluated different application techniques (seed priming, soil fertilization and foliar application) of micnobits micronutrient fertilizers were compared with traditional (ionic) micronutrient fertilizers. Additionally, in order to evaluate whether the accumulation of Zn in plants can provide adequate Zn to potentially alleviate Zn deficiency problems in some human diets, we studied the effects of a higher Zn application rate on the nutritional content of the crop produce. For this purpose, we used lettuce, a vegetable crop with a relatively short growth cycle and a direct yield component. Lettuce is eaten in most parts of the world, being the most important species of the group of leafy vegetables generally with a dry matter (DM) content of 5-6%, a harvest index (HI) of 0.90 and growth cycle (GC) of 65-75 days (Sosa *et al.*, 2012). In addition, lettuce is known to have a low nutrient use efficiency (Greenwood *et al.*, 1986) and nutrient recovery depends, among other agronomy factors, on the form in which it is supplied to the plant (Abu-Rayyan *et al.*, 2004). Furthermore, dependent on form and dose, trace elements may modulate nutrition related metabolic products in the plant (e.g., Zhao et *al.,* 2014). Accordingly, metabolomics was used to study possible effects of the micnobits on lettuce metabolic quality (Mannina *et al.*, 2012: Pereira *et al.*, 2014).

As with soil and foliar application methods, on-farm seed priming with water is an often-used agricultural practice (Harris *et al.*, 1999), especially in semi-arid zones. This technique has been proven to be a low cost and low risk intervention that results in direct benefits including faster emergence, more vigorous plants, earlier harvest, better yields and better drought tolerance. In the case of Zn deficiency, priming of wheat seeds with 0.3% Zn has been reported to increase mean shoot dry mass of wheat significantly and increased mean grain yield of wheat with 14% (Harris *et al.,* 2008).In addition to increasing grain yield, priming also significantly increased grain zinc concentration by 12% (Harris *et al.*, 2008).

For the current studies, we hypothesized that:
1)   Nutrients supplied as micnobit particles will be more efficiently taken up by a vegetable crop compared to nutrients supplied as traditional ionic fertilizers, and that

2) Growth, development and quality of a vegetable crop would not be negatively affected, but rather be enhanced, if nutrients are provided as micnobit fertilizers at doses relevant for crop nutrition.

1.2 Objectives

In this project, we studied the uptake of intact micnobit fertilizers by plants, and the form in which they are present in planta. To this end, we compared composites of micnobits of ZnO, CuO, Fe_3O_4, MnO, and B_2O_3 in water suspension, with a solution of their ionic equivalents derived from salts of Zn, Cu, Fe, Mn and boric acid, at levels optimal for growth, yield, metabolism and nutritional quality attributes in food crops (Vijayan, 2014). In the case of Zn, we also studied the effect of a double dose than in the conventional fertilizer, to evaluate the possibility of increasing Zn levels in lettuce.

The concentrations of the nutrients supplied to the lettuce crops as ions or micnobits are used at rates that will provide the amounts in the shoot as determined for lettuce (Hartz and Johnstone, 2007; US Department of Agriculture, 2015) and are relevant to their nutritional requirements.

2 Materials and Methods

A greenhouse pot experiment with lettuce was conducted using a nutrient-poor, calcium-rich sandy soil (pH 7.1), with very low organic matter and micronutrients contents, especially of Zn.

To this end, the micronutrients were applied as follows:

A. Two different forms: ionic (salts)or micnobit particles;
B. The application techniques: seed treatment, soil fertilization, or foliar application;
C. Two levels of Zn: Zn1 (to accumulate 50 mg/kg dry matter as natural value in lettuce (Hartz and Johnstone (2007) and Zn2 as double dose (for calculations: see Annex II)

2.1 Soil characteristics

Soil used for the study was obtained from the Netherlands. As most soils in the Netherlands are rich in micronutrients (especially with Zn) we looked for nutrient poor sandy soils from the coastal region of the country and obtained 3 m^3 from Fa Grondbalans B.V., Heerhugowaard. The soil information indicated a calcium rich soil (pH 7.1), with very low content of organic matter and micronutrients contents (see BLGG-analysis report, see Annex I).

2.2 Plant Growth

Seeds of lettuce (*Lactuca sativa* L cv Hilde II) were directly sown in pots for germination and placed in a greenhouse (GH) with supplemental lightning conforming with common horticultural practice in wintertime in the Netherlands. Artificial light (Philips SON-T lamps, 100 µmol m^{-2} s^{-1}) was switched on when outside radiation was below 250 W m^{-2} during the 16 h preceding sunset. The lamps switch off when the radiation exceeded 400 W m^{-2}. The relative humidity inside the GH was controlled by a high-pressure fogging system with a set point of 85% in order to keep humidity above 85%.

2.3 Fertilizer treatments

All chemicals were sourced commercially, from Sigma-Aldrich, Zwijndrecht, the Netherlands. The nutrients were added at rates that are assumed to provide the amounts in the shoots as determined for lettuce by Hartz and Johnstone (2007) and also included in the National Nutrient Database for Standard Reference (US Department of Agriculture, 2015). These values for nutrient levels on dry weight (DW) basis are: N 50 mg.g^{-1}, P 6 mg.g^{-1}, K 46 mg.g^{-1}, Ca 6 mg.g^{-1}, Mg 3 mg.g^{-1}, S 3 mg.g^{-1}, Na 1 mg.g^{-1} and in a lower range B 35 mg.kg^{-1}, Zn 50 mg.kg^{-1}, Mn 50 mg.kg^{-1}, Fe 160 mg.kg^{-1}, Cu 10 mg.kg^{-1}. Used stock solutions and application procedure for macro and micronutrients are described in Annex II.

Aqueous solutions with micronutrients in ionic form are transparent and homogeneous, but aqueous suspensions of nanoparticles are emulsions, for which the stability had to be tested. For determination of the stability of suspensions of micnobits, see Annex II, 6.2.1.

For administration of the micronutrients, plants were exposed to 11 different treatments as shown in shown in

Table 2-1 and Figure 2-2, with 4 replicates per treatment. Plants were positioned in randomized blocks from which one single plant was harvested at any time per treatment per block. Periodically, water was supplied to compensate for the transpiration of the plants. Therefore, all pots were individually weighed and water was replenished to the required weight (i.e. 80% of field capacity).

For determination field capacity, see Annex II, 6.3.2.
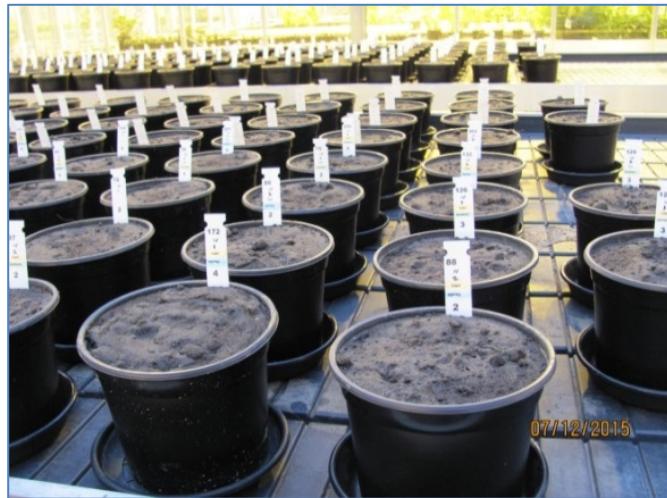
Figure 2-1. Treatments were executed per pot individually

Table 2-1. Overview treatments

Nr	Applied to	Application form micronutrients	Zn-level
1	-	No micronutrients (Control)	
2	Soil	Ionic form (Common Agricultural Practice)	Zn1
3	Soil	Ionic form	Zn2
4	Soil	Micnobits	Zn1
5	Soil	Micnobits	Zn2
6	Foliar	Micnobits	Zn1
7	Foliar	Micnobits	Zn2
8	Foliar	Ionic form	Zn1
9	Foliar	Ionic form	Zn2
10	Seed	Priming with micnobits	Zn1
11	Seed	Priming with ionic form	Zn1

2.3.1 Soil fertilization

In the treatments 2-5 (see Table 2-1), suspensions with micronutrients were prepared individually, and then physically mixed to form the composite 'ionic fertilizer' or 'micnobit fertilizer'. The treatments were applied in the form of suspensions of micnobit particles or solutions of ionic micronutrients, supplied to the soil in two portions and times. The first portion was supplied in diluted form (1:15, i.e. 13.5 ml Stock solution + 186.5 ml demineralized water) at 3 weeks after sowing (at the same time with the first foliar spraying – see below), while the second portion was supplied in diluted form (1:15) at 5 weeks after sowing (at the same time with the second

spraying). The soil and foliar micronutrient treatments received equal amounts of water, to exclude differences in soil water content.

2.3.2 Foliar application

In treatments 6-9 (see Table 2-1), foliar application was executed biweekly (which stopped 2 weeks before harvest) by spraying the leaves with a suspension of micnobit particles or with a solution of micronutrients in ionic form, supplied from a stock solution. During spraying the bottles were shaken intermittently to prevent aggregation of the particles. The concentrations of the micronutrients in the suspension or solution were based on the composition in foliar sprays used in Common Agricultural Practice, nr 2 in Table 2-1 (1% of each component). The quantity sprayed was based on the expected fresh weight production with time and the average concentrations of mineral nutrient in plant shoot dry matter sufficient for optimal growth. For details of the spraying procedure: see Annex II.

As the suspended micnobits have a tendency to precipitate rapidly, we tested possibilities to remove the residues from the leaves, see Annex II, 6.2.3. However, the micnobit residues of the could not be efficiently removed from the leaves following the protocols published by Hong *et al.* (2014). Therefore, after harvest sprayed leaves were separated from new leaves that developed after spraying was doneBoth parts were weighed (FW and DW), but only newly formed leaves were used for the analyses of micronutrient content and metabolomics.

2.3.3 Seed priming

To evaluate the effects of seed treatment with micronutrients in relation to soil and foliar applications, seeds were primed (see Figure 2-2) with an emulsion of micnobits or a solution of micronutrients in ionic form prior to sowing. Concentrations of both micnobits or ions were the same as in the foliar application.
Seeds were soaked in 50 ml Nalgene tubes for 3 hours in an emulsion with the micnobit particles suspension or ionic solution. The soaking was done for 3 hours because seeds of lettuce generally germinate after 4 hours. The tubes were turned around on a rollerbank (40 rpm). Thereafter, the seeds were filtrated and used for sowing. The micnobits colored the seeds black (Figure 2-2 D).
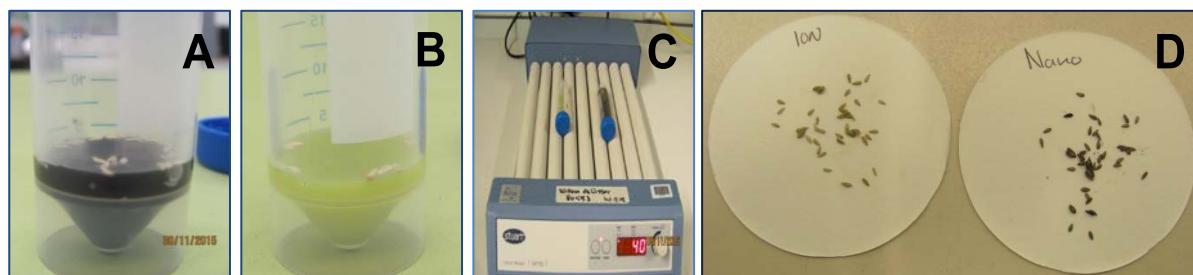
Figure 2-2. Priming of seeds. A. Soaking in an emulsion of micnobits, B. soaking in a solution of micronutrients, C. rotating on a rollerbank, D. after priming.

2.4 Plant data

Periodical observations on lettuce roots and shoots were made in 4 replicates (blocks) per treatment at 2-weekly intervals, starting 3 weeks after sowing until crop maturity. In total, plants were harvested 4 times starting at day 35 after sowing. Prior to harvest, leaf greenness of the intact leaves was measured with a SPAD 502 Plus

Chlorophyll Meter (Figure 2-3). Leaf punches (∅ = 1 cm; Figure 2-4 A) were taken and directly frozen in liquid nitrogen for determination of chlorophyll content (Figure 2-4 B). For description of the chlorophyll analysis see below.

Figure 2-3. SPAD measurement

Figure 2-4. Leaf punches for chlorophyll determination. A. Cut from the leaves and B. stored in liquid nitrogen.

After harvest, leaves and roots were separated, and roots (Figure 2-5) were rinsed to get rid of the soil. After determining root and shoot fresh weight, the samples from harvests 1-3 were dried for 8 hours at 70 ℃. The leaf samples from harvest 4 were partly dried, partly frozen at – 80 ℃.

Samples were used to analyze:

- Dried materials for contents of micronutrients in roots and shoots in the form total nutrients in ionic forms, as well as micnobit particles (all in dried material). In this case, samples of 5 plants with the same treatment per block were mixed and analyzed for nutrient content and – as far as possible - its form (micnobits or ions).
- Frozen leaves for content of chlorophyll and for metabolic analyses (i.e. vitamins, flavonoids, phenolics, and antioxidants).

Figure 2-5. Cross section of potted soil showing lettuce roots

2.4.1 Analysis of total element content of metal oxides by ICP-MS in Lettuce

Analytical instrumentation was subsequently used to (i) quantify total nutrient uptake, and to (ii) determine and

quantify the form of the nutrients from the micnobits that are present in the shoot and roots, where possible. For both quantifications, inductively couple plasma mass spectroscopy (ICP-MS) was used. First, the total amount of individual elements was quantified, and then the presence of the micnobits was determined and quantified using single particle ICP-MS. To this end, sample preparation and measurement were performed according to the modified Standard Operating Procedures, A1057 (Rikilt; procedure accredited) and A1120 (A1057: Microwave destruction sample preparation for elemental analysis; A1120: Analysis of elements using ICP-MS). Briefly, dried lettuce leaves and roots were transferred to 50ml centrifuge tubes and cut into small pieces with a pair of ceramic scissors. For each sample 300mg of dry leaf/root material was weight into a 50ml centrifuge tube. Acid leaching was performed with 10 ml of 70% HNO_3, followed by microwave destruction. Prior to analysis, samples were diluted 1000, 200 and 5 times in MQ. Calibration lines were prepared in MQ and acid through dilutions steps of the ICP-MS multi-element ionic standards (BIO SCIENCE) and single element standards for B, Zn and Fe in higher concentrations (Merck). As internal standard Rh was used. Samples, as well as standards were measured on Perkin Elmer Nexion 350D ICP-MS instrument in a KED mode and expressed in mg/kg dry material. Recorded m/z 11, 55, 56, 65, 66, 98 for B, Mn, Fe, Cu, Zn and Mo, respectively, were measured as a single event. Samples were processed using in-house Excel VBA Macro "Data file processor SOP-A-1120 Nexion_v1" to check the quality assurance parameters in the measurements.

2.4.2 Determination of the presence of nanoparticles in lettuce leaves by spICP-MS

Lettuce leaves, and in case of foliar application, the newly developed leaves after spraying, were transferred to 50ml centrifuge tubes and cut into small pieces with a pair of ceramic scissors. Approximately 100mg of dry leaf material was weight into 10 ml centrifuge tube. Nanoparticles were released from leaf material by adding 4 ml 20% TMAH (tetramethylammonium hydroxide, Sigma-Aldrich) according to the modified Perkin Elmer Application Note "Analysis of Nanoparticles in Biological Tissues using spICP-MS" [1]. The mixture was vortexed and ultrasonicated for 15 min, 4 ml of H_2O (total volume 8 ml) was added, and the mixture was further vortexed and ultrasonicated for 15 min. Prior to analysis, samples were diluted in MQ, 25 times for Zn, B and Mo oxides, 125 times for Mn and Cu oxides, and 125 and 250 times for Fe oxides. Matrix matched calibration standards were prepared through dilution steps of the spICP-MS ionic standards (MERCK) separately for each element. Samples as well as standards were measured on Nexion 350D ICP-MS instrument. Data was acquired for 60 sec with the dwell time set up to 3 ms in a KED mode. Recorded m/z 11, 55, 56, 65, 66, 98 for B, Mn, Fe, Cu, Zn and Mo respectively measured as separate events. Transport efficiency of particles in the nebulizer was determined based on the measurement of 60nm 50ngL AuNPs (NIST certificated standard). Samples were processed using RIKILT single particle calculation tool version 2.

2.4.3 Chlorophyll content determination

Chlorophyll was extracted in dimethylformamide (DMF) in the dark at -20°C. The absorbance of the extract was measured in the range 400–750 nm using a Cary 4000 spectrophotometer (Varian Instruments, Walnut Creek, CA, USA), and the chlorophyll a, b and c contents were calculated using the equations provided by Wellburn (1994).

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[1] https://www.perkinelmer.com/lab-solutions/resources/docs/APP_NexION-350Q-Silver-Nanoparticles-in-Bio-Tissues-011803_01.pdf

2.4.4 Metabolomics

For nutritional quality attributes at maturity we analyzed growth data to select a subset for LCMS metabolomics analysis to quantify micronutrients effects on the profile of biomolecules, including vitamins, flavonoids, phenolics, and antioxidants (De Vos *et al.*, 2007; Wahyuni *et al.*, 2013).

2.4.4.1   Vitamin C

Vitamin C (total ascorbate) was analyzed by HPLC with a photodiode array (PDA) detector, as described previously (Helsper et al., 2003). Briefly,, 300 mg FW leaf was mixed with 1.2 ml 5% metaphosphoric acid containing 1 mM DTPA. The slurry was sonicated for 15 min, centrifuged, filtered and 10 µl of the clear supernatant was injected. Vitamin C was detected at 262 nm, and its level in the samples calculated using a calibration series with the authentic standard.

2.4.4.2   LCMS profiling

Metabolite analysis was performed as described in De Vos et al. (2007). After harvest, leaves were snap frozen in liquid nitrogen and ground to a fine powder. Subsequently, 300 mg (+/–2 mg) of powder was extracted with 1.2 mL of methanol containing 0.1% formic acid. Extracts were sonicated for 15 min, centrifuged at 12500×g for 10 min, and then filtered through 0.45 µm filters (Minisart SRP4, Biotech GmbH, Germany). An Acquity High Pressure Liquid Chromatography system with a photo diode array (HPLC-PDA; Waters) coupled to an LTQ Ion Trap-Orbitrap Fourier Transformed Mass Spectrometer (FTMS; Thermo) hybrid system was used to detect, identify and quantify compounds (van der Hooft et al., 2012). A LUNA 3 µ C18 (2) 150 × 2.00 mm column (Phenomenex, USA) was used to separate the extracted metabolites, with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) in MQ water as solvents. A linear gradient from 5 to 95% B at a flow rate of 0.19 ml/min was used. The FTMS was set at a mass resolution of 60,000 HWHM and a mass range of m/z85-1200, using electro spray ionization in negative mode. Identification of detected compounds was based on retention time, accurate masses of both the parent and fragment ions, in combination with any PDA absorbance spectra (recorded at 240–600nm). The following replicated treatments were analyzed (total 24 samples):

- Control
- Soil: ion Zn1, ion Zn2 and nano Zn1
- Spray: ion Zn1 and nano Zn1

2.5   Data analyses

Plant growth and plant tissue nutrient content data were statistically analyzed to determine significant differences among the treatments by a GENSTAT analysis (VSN International (2015). Genstat for Windows 18th Edition (VSN International, Hemel Hempstead, UK. Web page: Genstat.co.uk). Analysis of Variance was carried out on the following data: shoot and root FW and DW, SPAD measurements, contents of micronutrients in shoots and roots and contents of chlorophyll in leaves. To obtain approximate normality the data were log10 transformed. A mixed model was used that comprised nested random effects for replication, days and units. The fixed effects were main effects and interactions for days and treatments. The treatment factor had 11 levels that were specific combinations of ion, nano in two concentrations, and three delivery mechanisms (soil, leaf and seed). Measurements were collected at four harvests with time intervals of 14 days, according to a randomized complete block design with four replications. Means were compared pairwise with Fisher's protected LSD method.

The LCMS profiles were statistically evaluated by a Multivariate Analysis (PCA; Worley and Powers, 2016) to visualize similarities and differences between samples. Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) are powerful statistical modeling tools that provide insights into separations between experimental groups based on high-dimensional spectral measurements from NMR, MS or other analytical instrumentation.

In this study a Monte Carlo analysis of PCA group separations and OPLS-DA cross-validation metrics was performed on NMR datasets with statistically significant separations in scores-space. A linearly increasing amount of Gaussian noise was added to each data matrix followed by the construction and validation of PCA and OPLS-DA models.

3 Results

3.1 Growth

By visual observation, the lettuce plants showed normal growth pattern under all treatments (see overview in Table 2-1) and started to head at about 40 days after sowing. Figure 3-1 provides an impression of different time points during growth of the plants. The ionic and micnobits spraying treatments caused necrosis at the leaf edges in the long run. In addition, spraying with micnobits caused deposition of dark spots on the leaves that could not be removed (Figure 3-1 I). In contrast, no such dark spots were found on the leaves of soil-treated or seed-primed plants.
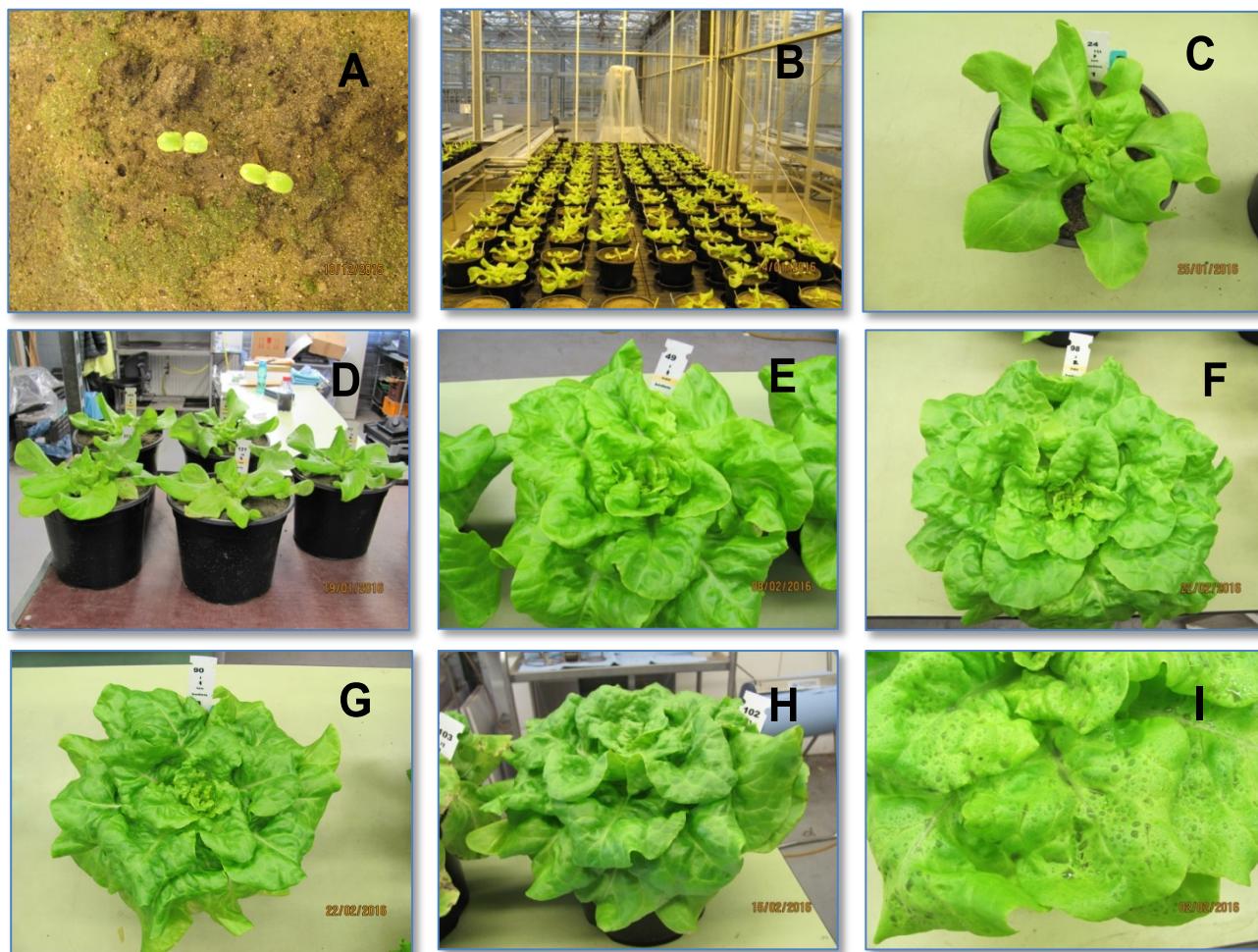
Figure 3-1. Growth of lettuce plants in the greenhouse. A. 4 days after sowing, B. 31 days after sowing; C. 35 days after sowing (ion-soil treatment); D. 36 days after sowing (nano-spray treatment); E. 49 days after sowing (nano-soil treatment); F. 63 days after sowing (nano-soil treatment); G. 63 days after sowing (ion-soil treatment) H. 70 days after sowing (ion-seed priming treatment); I. 50 days after sowing (nano-spray treatment).

Plants were harvested four times during the growth period. Shoot and root growth over time are shown in Figure 3-2 for the Control treatment (growth curves for all treatments are shown in Annex IV for shoots and roots, respectively). The figures show that shoot growth with time followed the normal S-shaped curves for all treatments, although the growth of plants treated with Ion Spraying and Ion Addition to the Soil showed a

decrease of plant production at the fourth harvest, probably due to leaf drying at the end of the growth period. This suggests that these treatments show an earlier onset of senescence than other treatments. However, this did not result in an earlier initiation of growth in these treatments (see Annex IV).
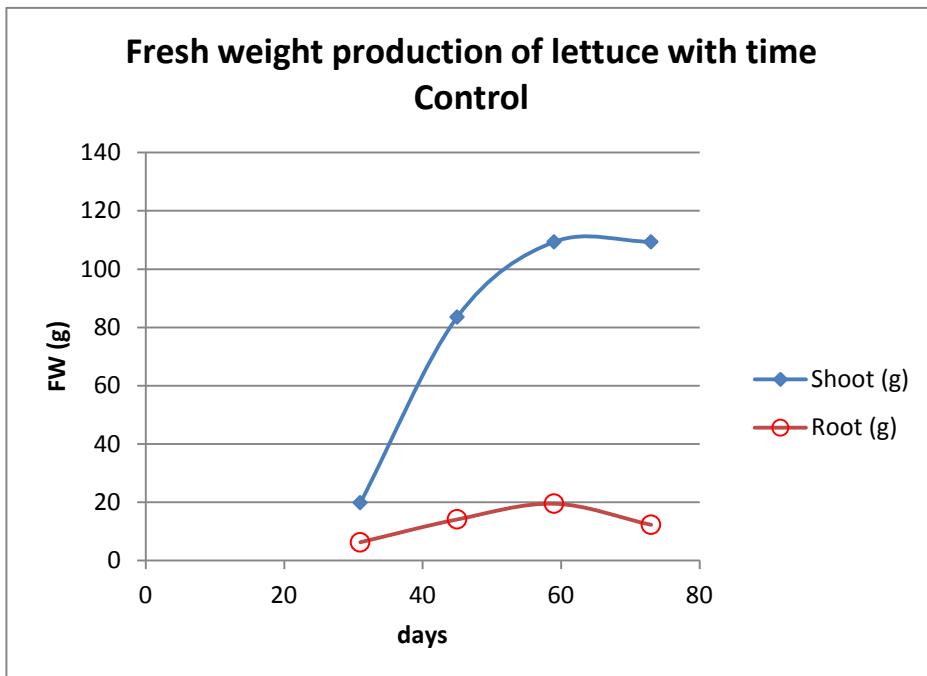
Figure 3-2. Shoot and root fresh weight production (g) of lettuce with time. Control treatment.

To further evaluate the effect of the treatments on shoot growth, S-shaped trend lines for **Yield** vs **time** were fitted through the growth data for each treatment, using following equation:

$$Y = Y_{max} / (1 + \exp(- \alpha * time + \beta)$$

In which:

Y = yield

time = day at growth curve

α = slope parameter

β = time parameter

From these trend analyses describing growth, values were derived for Ymax and for the time needed to reach 95% of Ymax, shown in Figure 3-3. The plants in the treatments Control, Priming (both ion and nano) and Ion Spray with high Zn-concentration showed the best growth, while plants treated with Ion spray exhibited lowest final weight. The time needed to reach 95% of Ymax was for almost all treatments between 55 – 60 days, except for Ion Spray with high Zn-concentration, where the growth rate was slower.

Root growth data is also presented in Figure 3-2 for the Control treatment and for the other treatments in Annex IV, showing growth increase until the third harvest. At the fourth harvest, fresh weight values appeared to be lower for the treatments Control, Priming and Foliar application, possibly due to senescence of old root material. However, in all soil treatments root growth continued until the end of the experiment.
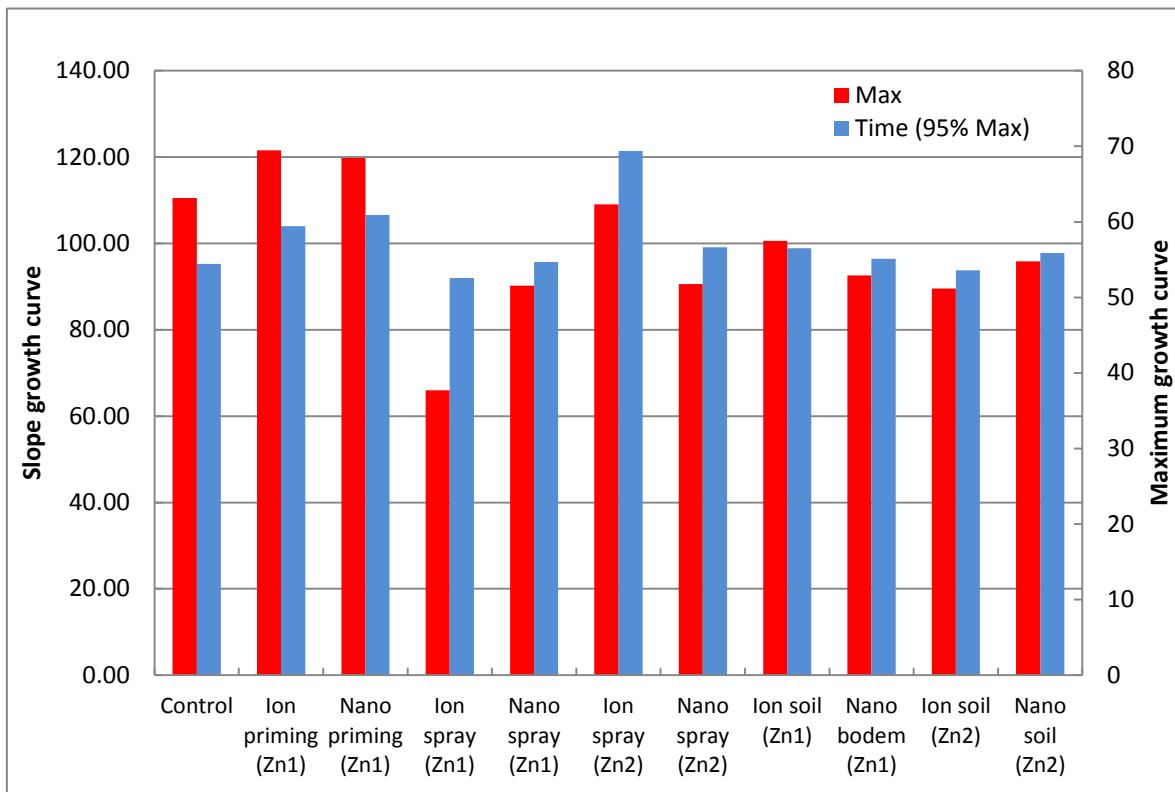
Figure 3-3. Calculated maximum yield (Max) and time to reach 95% of this maximum at different treatments, based on fresh weight production over time
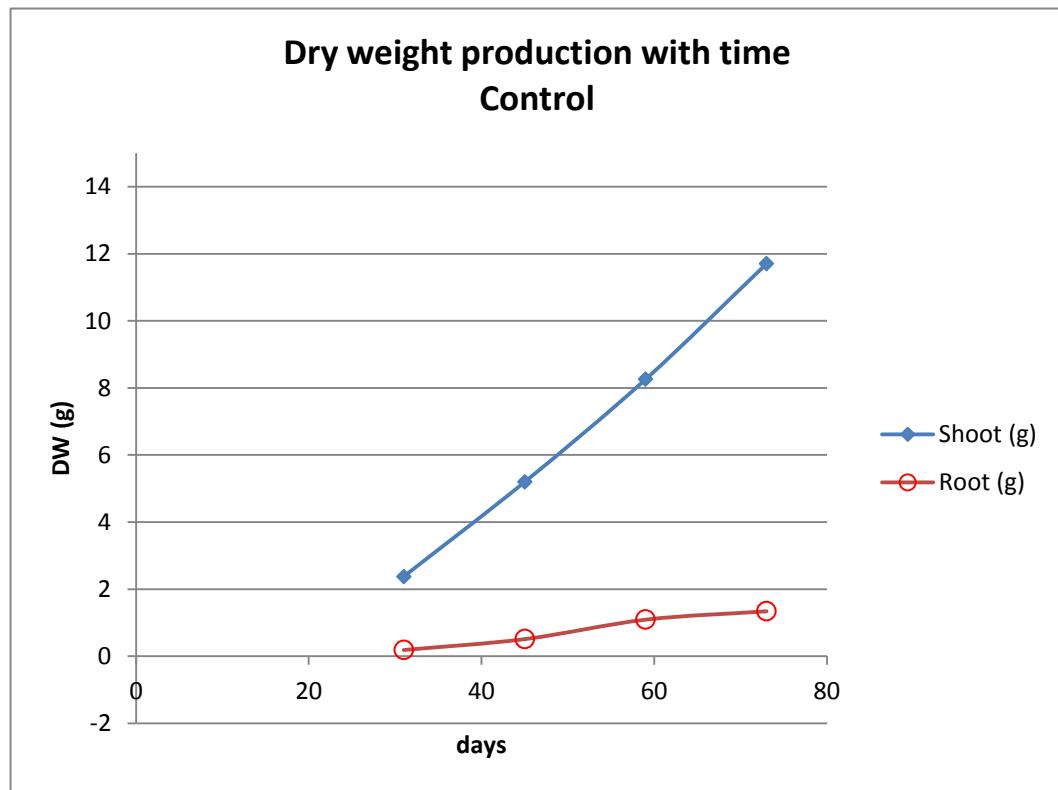
Figure 3-4. Shoot and root dry weight production (g) of lettuce with time. Control treatment.

In Figure 3-4 dry weight production for both shoot and root with time is shown for the Control treatment (dry matter production for all treatments is shown in Annex IV for shoots and roots, respectively). Dry weight production does not show the S-shaped curve as exhibited for fresh weight production. This indicates that dry matter percentage of the shoot continuously increased, especially at the end of growing period.

To gain insight into the effects of the treatments on root weight - total plant weight ratio (RWR) was calculated for all treatments. The RWR for FW in the Control treatment is shown in Figure 3-5 and for all treatments in Annex IV for fresh weight at two different Zn-concentrations. In the Control treatment, RWR decreases with time in accordance with results generally observed during plant growth. In the soil treatments, the RWR levels off at the end of the growth period for both Ion and micnobit treatments, suggesting that the roots were starting to feel stressed under these treatments.
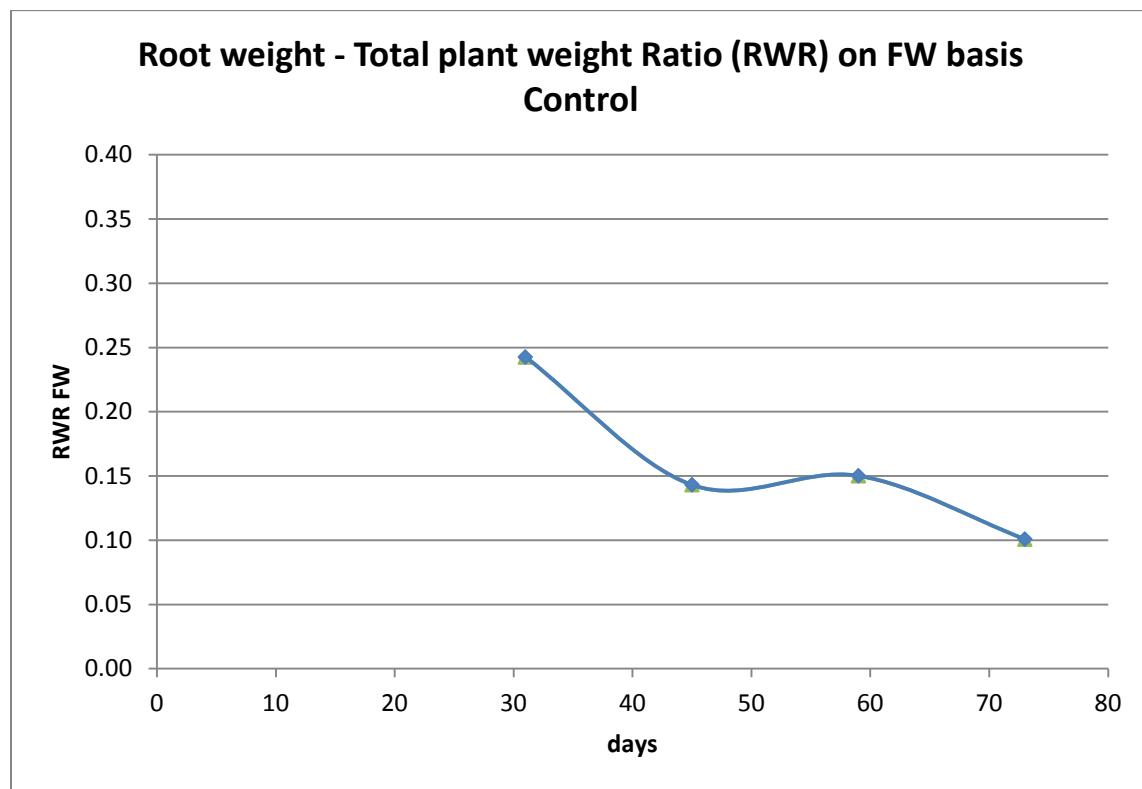
Figure 3-5. Root weight -Total plant weight Ratio (RWR) on FW basis. Control treatment.

The RWR for DW in the Control treatment is shown in Figure 3-6 and for all treatments in Annex IV. In the control, the RWR levels off already at the second harvest, indicating a quicker increase in DW of the root than in the shoot. The differences between the treatments are much bigger for this feature. Priming and Spraying show a decrease of RWR during the whole growth, while for the soil treatments RWR only increases from the first harvest.

For statistical analyses, all data for FW and DW had to undergo a log-transformation to enable the performance of an ANOVA. In Table 3-1 means of shoot and root fresh and dry weight production are summarized and followed by letters to show the significance of differences between the treatments at $p < 0.001$.
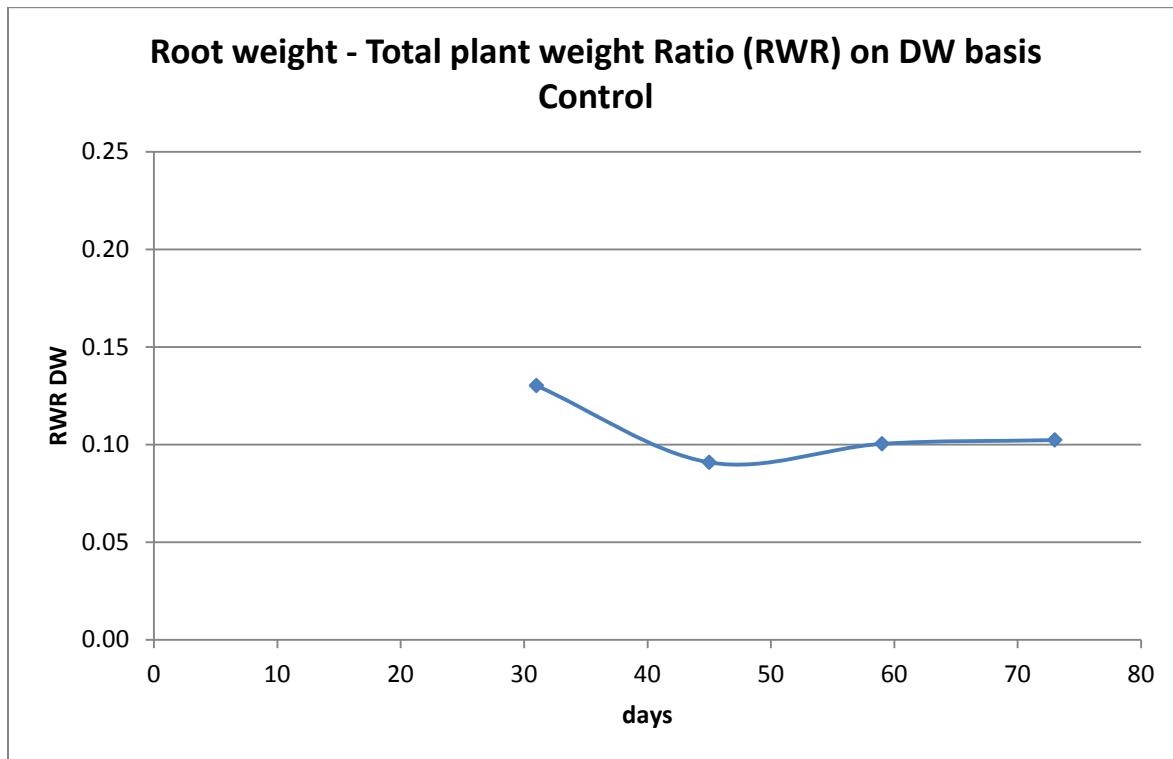
Figure 3-6. Root-Total plant weight Ratio based on DW. Control treatment.

Table 3-1. Means of total shoot and root fresh and dry weight production of lettuce grown at different treatments after log-transformation. Means followed by different letters are significantly different at p < 0.001.

Treatment			FW (g)				DW (g)			
			Shoot		Root		Shoot		Root	
Control			66.07	e	11.72	f	5.65	e	0.59	e
Ion	soil	Zn1	46.88	cd	7.40	bcd	3.52	abc	0.35	c
Nano	soil	Zn1	47.42	cd	8.04	cd	3.56	abc	0.35	c
Ion	priming	Zn1	59.98	de	10.76	ef	4.39	cde	0.49	de
Nano	priming	Zn1	59.70	de	10.91	ef	4.74	de	0.55	de
Ion	spray	Zn1	35.40	ab	5.36	a	3.11	ab	0.21	a
Nano	spray	Zn1	44.98	bc	9.40	def	3.56	abc	0.41	cd
Ion	soil	Zn2	38.11	abc	6.14	ab	2.79	a	0.31	bc
Nano	soil	Zn2	40.64	abc	6.58	abc	2.85	a	0.32	bc
Ion	spray	Zn2	33.96	a	6.12	ab	3.16	ab	0.25	ab
Nano	spray	Zn2	48.31	cd	8.61	de	4.00	bcd	0.36	c
Significance treatment			<0.001		<0.001		<0.001		<0.001	
Interaction effect (day*treatment)			n.s.		<0.001		n.s		<0.001	

In the case of fresh weight production, it can be seen from Table 3-1 that Control performed best. Priming achieved second best and not significantly different from the Control. The other treatments did significantly differ from the Control. There was no significant difference between the Spraying and Soil treatment. The roots showed

the same pattern. Dry weight production exhibited more or less similar trend as fresh weight production. Here, high Zn-concentrations resulted in low dry weight production, especially when added in the soil. For both shoot FW and DW, no significant interaction effects were shown. However, the results did show interaction effects for the roots, but the interaction between time (different harvests) and different treatment was marginal.

3.2 Uptake of micronutrients

The levels of micronutrients (B, Mn, Fe, Cu, Zn and Mo) in the plants are shown in Tables 3-2 and 3-3. For statistical analyses the values were log-transformed and listed in the tables as mean values of the micronutrients, accumulated in the shoots and roots of the lettuce plants grown under the different treatments. The values are followed by letters indicating the significance of the differences among the treatments at $p < 0.001$. For comparison, values determined by Hartz and Johnstone (2007) are also listed.

Remarkably, the shoots and roots of plants in the Control treatment showed accumulation of micronutrients, whereas no micronutrients had been applied, and the analysis report on the composition of the soil (see Annex I) suggested low values, for at least Mn, Fe, Cu, Zn and Mo. Also, both leaves and roots of the priming treatment contained micronutrients with levels similar to those in the control treatment. For B and Zn, the levels in the shoots turned out to be even higher than the values considered to accumulate under natural circumstances, as published by Hartz and Johnstone (2007). Compared to the control treatment, the micronutrient levels in the shoots increased significantly by the soil treatment and foliar application for B, but only by the foliar application for B in the roots and for Mn, Cu, Zn and Mo for both shoots and roots. For Fe no treatment significantly differed from the control.

For B, the levels of the micronutrients in the shoots were approximately two times higher than in the roots for all treatments, while the levels in shoots and roots did not differ much for Mn and Zn (except for Foliar application). For Fe and Cu, however, the levels of the micronutrients were much higher in the roots than in the shoots for all treatments. For Mo, the levels were higher in the roots than in the shoots in the soil treatment and in the foliar application.

When the applications with ions were compared with those with micnobits (indicated as 'Nano' in the tables), no significant differences were found for B, Fe and Mo in the shoots, for all treatments. However, the levels differed between ion and micnobit application for Mn in shoots in the foliar application for both Zn-concentrations; for Cu in the shoots in soil treatment (Zn1); foliar application with ions for both Zn-concentrations; and for Zn levels in the shoots in foliar application for both Zn-concentrations.
For the roots, no differences were seen for levels of B and Fe between Priming, Soil treatments (both Zn1 and Zn2) and Foliar application at Zn1. Also, for Mn, Cu, Zn and Mo, the levels did not significantly differ between the treatments, except for the foliar ion application. Remarkably, the roots contained much higher amounts of Mn, Cu, Zn and Mo in the Spray treatment than in the soil treatment.

Based on sp ICP-MS, the presence of micnobit particles could only be detected for Fe_2O_3, CuO and MnO_3 in the shoots. However, due to a high noise level in the ICP-MS analyses in the leaves from both the Soil and Foliar application (newly developed leaves), as well as from the priming of seeds, it was not possible to quantify the content of the elements properly.

Table 3-2. Mean contents of the different microelements in shoot and root of lettuce calculated over all harvests. Means followed by different letters are significantly different at p < 0.001.

Treatment			B (mg kg^{-1})				Mn (mg kg^{-1})				Fe (mg kg^{-1})			
			Shoot		Root		Shoot		Root		Shoot		Root	
Values determined by Hartz and Johnstone (2007)			35				50				160			
Control			48.08	a	29.92	bc	13.00	a	11.09	ab	126.18	-	475.34	-
Ion	soil	Zn1	64.12	bc	28.18	ab	12.08	a	16.22	de	138.36	-	498.88	-
Nano	soil	Zn1	60.95	b	30.90	bc	12.68	a	15.85	de	146.89	-	567.54	-
Ion	priming	Zn1	47.75	a	29.72	abc	11.19	a	10.26	a	125.31	-	423.64	-
Nano	priming	Zn1	46.99	a	26.92	a	10.47	a	12.33	abc	128.82	-	445.66	-
Ion	spray	Zn1	71.94	c	41.30	fg	48.64	c	17.06	e	133.97	-	545.76	-
Nano	spray	Zn1	67.14	bc	37.84	ef	31.05	b	13.52	bcd	137.40	-	473.15	-
Ion	soil	Zn2	65.31	bc	28.84	ab	12.82	a	14.45	cde	146.89	-	505.82	-
Nano	soil	Zn2	64.86	bc	32.36	cd	13.24	a	11.75	abc	137.72	-	400.87	-
Ion	spray	Zn2	70.79	c	42.36	g	48.64	c	17.50	e	149.97	-	539.51	-
Nano	spray	Zn2	71.78	c	35.56	de	32.14	b	13.21	bcd	142.23	-	505.82	-
Significance treatment			<0.001		<0.001		<0.001		<0.001		n.s.		n.s.	
Interaction effect (day*treatment)			<0.001		n.s		<0.001		<0.001		<0.001		<0.001	

Table 3-3. Mean contents of different microelements in shoot and root of lettuce calculated over all harvests. Means followed by different letters are significantly different at p < 0.001.

Treatment			Cu (mg kg^{-1})				Zn (mg kg^{-1})				Mo (mg kg^{-1})			
			Shoot		Root		Shoot		Root		Shoot		Root	
Values determined by Hartz and Johnstone (2007)			10				50				Not reported			
Control			8.26	ab	26.30	cd	69.34	a	73.28	ab	3.34	a	1.26	a
Ion	soil	Zn1	8.89	b	20.75	ab	59.98	a	66.68	a	2.67	a	8.81	b
Nano	soil	Zn1	6.21	a	23.39	bcd	58.48	a	66.37	a	2.44	a	8.95	b
Ion	priming	Zn1	7.01	ab	21.63	bc	61.38	a	70.96	ab	2.67	a	1.34	a
Nano	priming	Zn1	6.90	ab	20.89	b	62.52	a	69.50	a	2.44	a	1.34	a
Ion	spray	Zn1	39.90	e	49.20	e	129.12	cd	89.54	c	23.12	c	60.12	de
Nano	spray	Zn1	26.92	cd	27.86	d	92.90	b	93.54	c	23.07	c	45.81	d
Ion	soil	Zn2	7.13	ab	22.34	bcd	66.68	a	78.34	b	2.55	a	7.52	b
Nano	soil	Zn2	6.75	ab	16.60	a	66.99	a	73.28	ab	2.92	a	9.86	b
Ion	spray	Zn2	32.81	de	57.54	e	149.28	d	122.18	d	18.84	bc	78.52	e
Nano	spray	Zn2	22.49	c	25.12	bcd	108.89	bc	91.62	c	15.00	b	27.42	c
Significance treatment			<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
Interaction effect (day*treatment)			<0.001		<0.001		<0.001		<0.05		<0.001		<0.05	

3.3 SPAD measurements

SPAD measurements were performed in 3 well developed leaves per plant at harvest. For all treatments, SPAD values showed a slight increase with time up to 59 days (data not shown). Data for SPAD are presented in Table 3-4 for the different treatments, where it can be seen, that the differences among the treatments were not very distinct, but the control treatment was significantly better than ion soil treatment and the foliar ion and micnobit application (in this case for both Zn1 and Zn2). Among the fertilizer treatments themselves the effects were less distinct.

Table 3-4. Means of values from SPAD measurements at different treatments. Means followed by different letters are significantly different at p < 0.001.

Treatment			Means the SPAD values	
Control			21.68	ef
Ion	soil	Zn1	19.68	bcd
Nano	soil	Zn1	21.23	def
Ion	priming	Zn1	20.65	cdef
Nano	priming	Zn1	21.98	f
Ion	spray	Zn1	18.16	a
Nano	spray	Zn1	19.59	abc
Ion	soil	Zn2	20.28	bcde
Nano	soil	Zn2	20.09	bcde
Ion	spray	Zn2	20.32	bcde
Nano	spray	Zn2	19.10	ab
Significance treatment			<0.001	

Table 3-5. Means contents (µl/l) of chlorophyll a, b and c of the lettuce leaves at different treatments

			Chl a		Chl b		Chl c	
Control			6.333	-	1.560	abc	1.702	-
Ion	soil	Zn1	6.628	-	1.632	cd	1.764	-
Nano	soil	Zn1	6.212	-	1.567	abc	1.752	-
Ion	priming	Zn1	6.209	-	1.537	abc	1.664	-
Nano	priming	Zn1	6.956	-	1.784	d	1.827	-
Ion	spray	Zn1	6.098	-	1.400	a	1.599	-
Nano	spray	Zn1	6.253	-	1.544	abc	1.715	-
Ion	soil	Zn2	6.382	-	1.510	abc	1.683	-
Nano	soil	Zn2	6.086	-	1.587	bcd	1.695	-
Ion	spray	Zn2	6.305	-	1.419	ab	1.64	-
Nano	spray	Zn2	6.03	-	1.484	de	1.612	-
Significance treatment			n.s.		<0.011		n.s.	

The values for chlorophyll a, b and c contents in the leaves are shown in Table 3-5. For Chlorophyll a and c no significant differences were found and for Chlorophyll b values the differences among the treatments were small and showed strong overlap.

3.4 Vitamin C

In Figure 3-7 values for vitamin C are shown. It can be seen that plants from the Control treatment show the highest levels, while no clear distinction between soil or spraying can be made, nor between a treatment with ions or with micnobits.
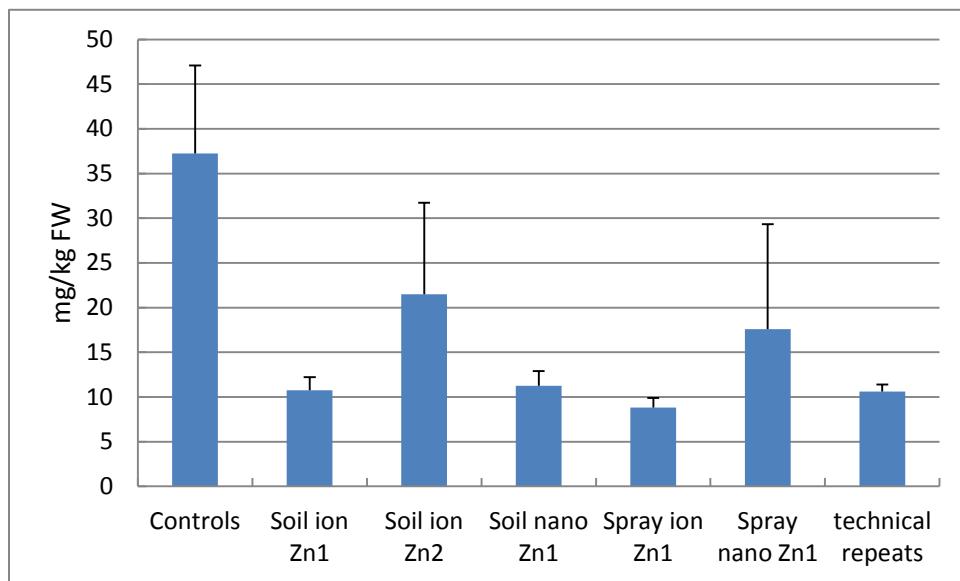
Figure 3-7. Vitamin C levels in lettuce leaves 73 days after sowing and grown under different micronutrient application treatments

3.5 LCMS profiles

The LCMS profiles of the lettuce leaves are shown in Figure 3-8, while the relative abundance of each of the compounds are presented in Figure 3-9. These Figures indicate that each leaf sample generated 257 compounds, based on their relative intensity. A Principle Component Analysis (PCA) was performed on these compounds to visualize similarities and differences among leaf samples and to evaluate the effect of different treatments on the global metabolome of the plant. The result of the PCA is shown in Figure 3-10, where it can be seen that the production of 80 out of the 257 metabolites (25%) was significantly altered. Out of those, 66 showed more than 2-fold difference. The metabolites are not yet annotated and further analysis and data processing are currently ongoing.
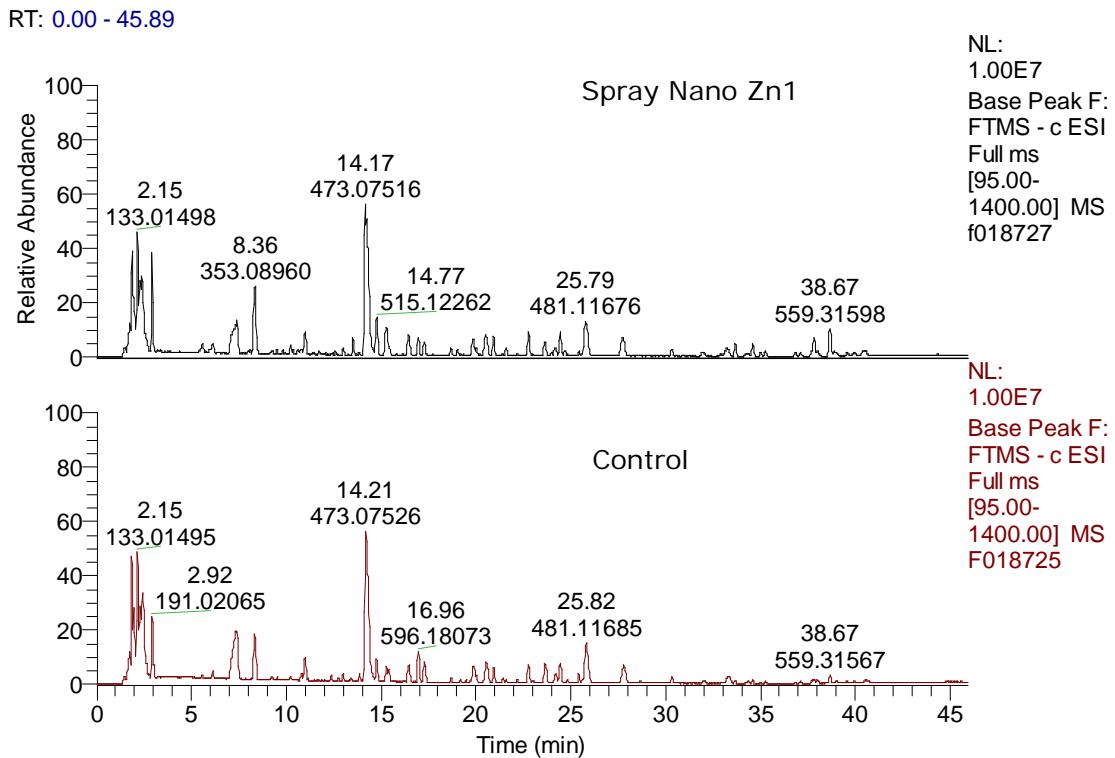
Figure3-8. LCMS profiles in lettuce leaves spray with micronutrients as micnobits and control
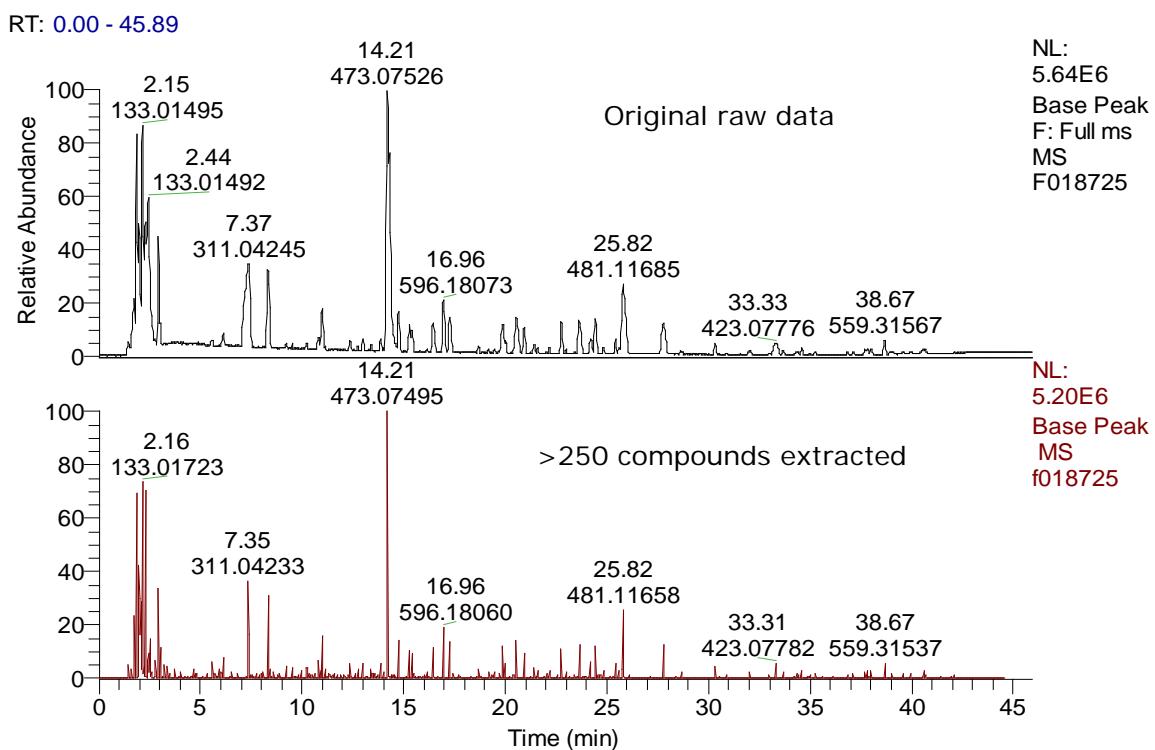
Figure 3-9. Unbiased LCMS profiles in lettuce leaves, original raw data and after analyses of the number of compounds

Group		Members
■ (yellow)	● technical repeats	4
■ (green)	○ controls	4
■ (blue)	■ bodem ion Zn1	4
■ (purple)	□ bodem ion Zn2	4
■ (cyan)	◆ bodem Nano Zn1	4
■ (red)	✳ spray ion Zn1	4
■ (orange)	⊘ spray Nano Zn1	4

Component	Relative	Cumulative
X= 1	23.6%	23.6%
Y= 2	16.4%	40.0%
Z= 3	14.0%	54.0%
4	8.1%	62.2%
5	5.4%	67.6%
6	4.7%	72.2%
7	3.5%	75.8%
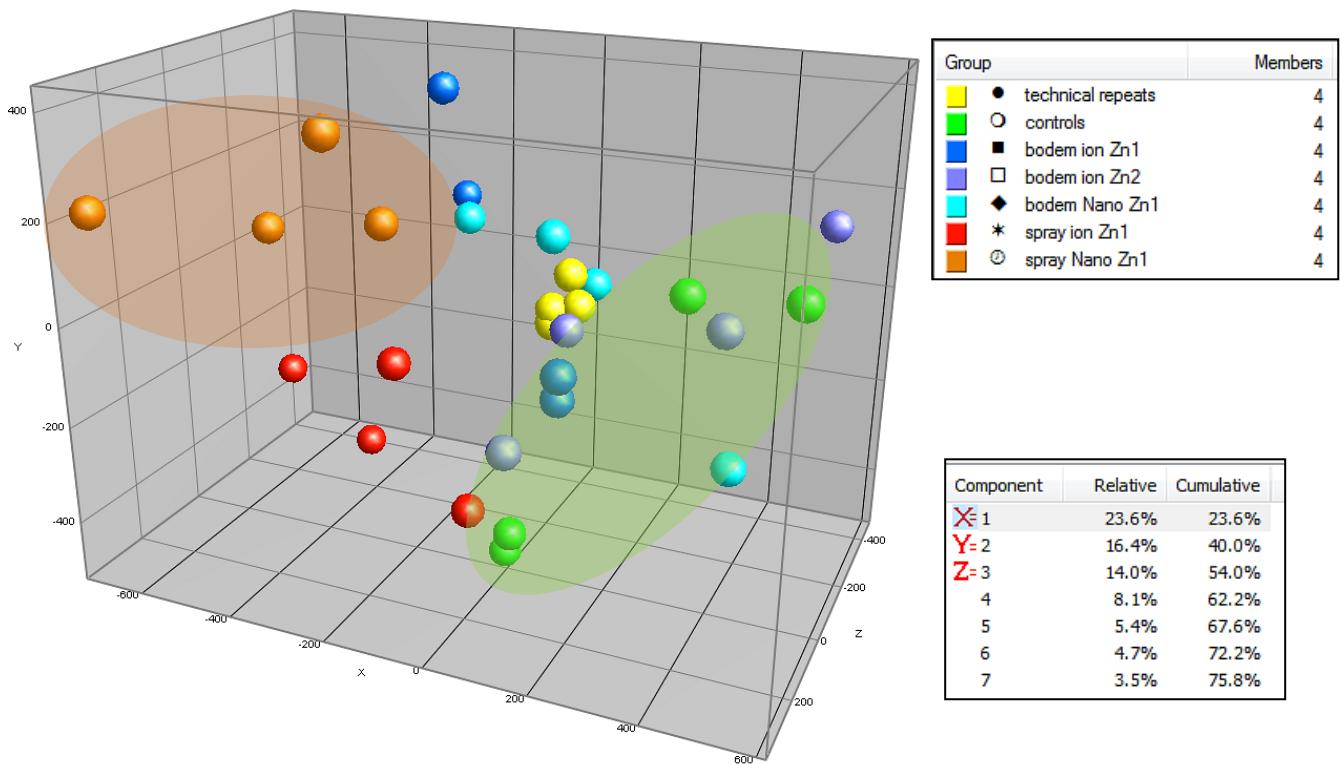
Figure 3-10. Principle Component Analysis on the 257 compounds identified in lettuce leaves in relation to the different treatments during growth. P<0.05; n=4 (Note: 'bodem' means soil).

4   Discussion

All lettuce plants showed normal S-shaped growth curves and had similar extent of leaf greenness, indicating that micronutrients can be supplied both as micnobit particles and traditional (ionic) fertilizers, allowing for the use of different nutrient forms, as suggested by Bindraban et al. (2015). However, the results on fresh and dry weight production clearly showed that the lettuce plants grew best in the Control treatment. Addition of micronutrients to soil or leaves, applied as ions or as micnobits actually decreased fresh weight and dry weight production of lettuce. Moreover, the shoots and roots of plants in the Control treatment also showed accumulation of micronutrients, while no micronutrients had been applied. Thus, the question arises whether at all the plants of the Control treatment suffered from micronutrient deficiency. Although for this experiment a nutrient-poor sandy soil from the coastal region in the Netherlands with a low content of micronutrients (except for B) and organic matter had specifically been used, the plants still appeared to be able to take up micronutrients, even from very low soil contents. In fact, plants are known to be capable of coping with micronutrient deficiencies in their environment, using different mechanisms. For example, Hernandez-Apaolaza (2014) discusses in her overview that silicon - which is sufficiently available in our soil (see Annex I) - can protect plants against the effects of micronutrient deficiency. The same author mentions that for Fe, deficiency stress is especially a problem in calcareous soils (high bicarbonate content;, pH 7,5 – 8,5) and the lower Fe threshold concentration for optimal plant growth is 10^{-8} M. In our soil the pH is lower than 7.5, and the threshold level given by Hernandez-Apaolaza (2014) is below the content in the soil. Petrazzini et al. (2014) discusses that deficiency stress in lettuce is mainly shown for B and less for Zn. In our soil B was sufficiently available. In the case of Mn deficiency,, plant species and cultivars differ considerably in their susceptibility to Mn deficiency. Oat, wheat, soybean or peaches are very susceptible, whereas maize and rye are much less susceptible. For lettuce, no threshold concentrations are reported. For Zn, Hernandez-Apaolaza (2014) discusses that deficiency stress is not related with a low Zn content in the soil, but rather with the soil characteristics (like high pH, presence of $CaCO_3$ and organic matter) that control the plant availability of this element. Only at shoot Zn levels of 15-20 mg/kg dry weight do plants start to show deficiency symptoms. In our study, the shoots and roots in the Control treatment contained at least 60 mg/kg Zn, significantly more than the limit value. Apparently, the soil was not lacking in the tested nutrients, based on their reported soil critical levels (Dimkpa et al., 2016), and thus the low availability of micronutrients in the soil was already sufficient for optimal growth.

The priming of seeds slightly enhanced growth rate and productivity, although not differing significantly from the Control treatment. Stimulation of growth in wheat, a 14% increase in grain yield, and a 12% increase in grain Zn concentration by seed priming with Zn was reported by Harris et al. (2008). They used the same priming procedure as we did in this study, although the soaking of the wheat seeds lasted longer (8 hours) than was permitted for the lettuce seeds, based on its germination time of 4 hours. The effect of priming will probably not be explained by a substantial increase of the micronutrient level within the seed, as that will not cover the demand for total growth. Nautiyal and Shukla (2012) showed that seed priming with Zn has an enhancing effect on the uptake rate rather than increasing the Zn content of the seed. Priming of seeds in chickpea has been shown to effectively improve the Zn status of seedlings and their establishment in Zn deficient conditions by increasing dry mass, seed Zn, Zn uptake and translocation at low Zn relative to hydro-primed seeds at normal Zn (Nautiyal and Shukla, 2012). This is also shown for mung bean by Shah et al (2012). As the availability of micronutrients might have been sufficient already in the experimental soil (as discussed above), the priming of seeds only slightly enhanced growth rate.

The levels of micronutrients found in leaves and roots, show that both ions and micnobits were taken up by the roots from soil, and by the leaves from foliar spray. For B and Zn, the levels in the shoots even exceeded the values considered as adequate for optimal growth (Hartz and Johnstone, 2007). This finding, together with the observed decrease in in biomass production upon supply of micronutrients by the Soil and Foliar treatments, raises the question of whether the concentrations of micronutrients may (temporally) have exceeded toxic levels, causing periods of stress with reduction of growth as a result. Tripathi et al (2015) reported in their overview on micronutrients the following toxicity threshold levels for micronutrients accumulated in vascular plants: B > 80 mg/kg, Mn > 200 mg/kg, Zn > 120 mg/kg, Cu > 25 mg/kg and Fe > 2000 mg/kg. For Mo no threshold levels have been reported. In our study, most levels of micronutrients both in leaves and roots did not exceed the toxic threshold levels. The only levels that exceeded the thresholds were for Cu in leaves and roots in the foliar application (both Nano and ion); for Zn in the leaves and roots in foliar ion application for Zn1 and Zn2, and for roots in foliar ion application for Zn2.

When evaluating the efectiveness of the different forms in which the micronutrients were applied, we did not find significant differences between applications with ions and those with micnobits. In addition, different Zn concentrations did not show clear differences in growth characteristics either. Due to the unfavorable characteristics of the soil and adequate growth of plants in the Control treatment, we did not obtain clear insights to support prior reports on the increase in the effectiveness of micronutrients when applied as micnobits than as ions (Dimkpa *et al,.* 2012; Pradhan *et al.,* 2013; Kim *et al.,* 2014). Nevertheless, a recent study with composite formulations of micnobits or ions comprising of ZnO, CuO and B_2O_3 or their salt equivalents when applied as foliar treatments also did not indicate clear differences in affecting crop (soybean) productivity, although both types of formulation performed significantly better than the control (Dimkpa et al, in press).

In accordance with the observations of different authors (Dimkpa *et al.*, 2012; Wang *et al.*, 2012; Dimkpa *et al.*, 2013; Bandyopadhyay *et al.*, 2015; Wang *et al.*, 2012; Wang *et al.*, 2013b, c), this study confirms that when micnobits were applied, micronutrients (either in the form of micnobits or as ions) are transferred from root to shoot (elements are found in leaves in the Soil treatments) and from the shoot to the root (elements are found in the roots in the Spray treatments). It was also shown that the roots contained much higher amounts of Mn, Cu, Zn and Mo in the Spray treatment than in the soil treatment, which indicated that either translocation of these ions from the leaves is more efficient, or the micronutrients are faced with more competition at the root surface. Translocation of micronutrients from leaf to root, either in the form of ions or as micnobits, is evidence that they travel by the phloem transport mechanism. This is in accordance with findings for maize by Wang et al. (2012), and for watermelon by Raliya et al. (2016), the latter who showed that gold nanoparticles were transported both by apoplast and by symplast. Unfortunately, we cannot conclude on the form in which the micronutrients are taken up and/or transferred in this study, as the quantity of micnobits could not be determined properly due to the high noise level in the in the ICP-MS analyses. This needs further research and better detection methods such as Xray absorption spectroscopy among others.

Remarkably, in the foliar application the contents of Cu and Mo differ dramatically from those in the soil and seed priming treatments. This phenomenon may indicate a competition for uptake of both micronutrients at the root surface, a phenomenon which may play a lesser rolewhen the micronutrients are sprayed at the leaves. Antagonism has been described by Bailey and Nelson (2014) between Fe (high soil level) and Mn and Zn (low plant level) or Mn (high soil level) and Zn and Fe (low plant level) or between Cu (high soil level) and Zn (low plant level) and Zn (high soil level) and Cu (low plant level). If competition for uptake is involved in our study, it is even more remarkable that no differences have been found between the contents of ions and those of micnobits. Either

the uptake transporters of ions resemble the ones of micnobits, or micnobits are decomposed already when they enter the root, as can occur at neutral pH (between 7.4 – 7.6) as described by Bian *et al.* (2011).

In contrast to previous nano studies (Dimkpa *et al.,* 2015,a,b; Taran *et al.*, 2014), there appears not to be the same kind of significant negative interactions between the nutrients for uptake via the soil. Competition between Cu and Mo at the root surface in the Soil treatment was shown, but the soil application of Zn at high levels (Zn2) did not diminish root and shoot levels of B, Cu, Mn, Fe and Mo relative to the control treatment. Similarly supplying the Zn at the high dose via the leaves did not inhibit nutrient uptake from the root, compared to the control. Thus, the notion of nutrient ratio imbalance playing a role in nutrient antagonism (Bindraban *et al.,* 2015) had a different outcome for lettuce, in this study.

The SPAD measurements as indication of greenness from the chlorophyll in the leaves showed the highest values for the Control treatment, indicating that the plants performed best under this treatment. However, when comparing the differences in the chlorophyll contents, the effects of the treatments were not significant for chlorophyll a and c. However, the results from the vitamin C analyses distinctly showed the highest levels in the Control treatment. The reduction in the Vitamin C levels of the plants treated with ionic or micnobit micronutrients from the root or leaves could suggest a trade-off between different produce quality criteria (in this case mineral nutrients versus nutrition-related metabolites). In previous work, Zhao *et al.* (2014) reported a significant increase in cucumber starch content, with a trend towards increases in globulin, albumin and prolamin contents by ZnO micnobits at high Zn uptake. It is unclear what the nature of the seeming antagonistic interaction between biomolecules and mineral nutrients is, but it represents significant ramifications for the overall nutritional quality of edible crop produce, especially given that the nutrient elements were not present in the plant leaf biomass at toxic levels as indicated by their levels in the control versus soil treatments. Similar to vitamin C modulation, the effect of the nutrient treatments could also been seen with other metabolites. Although these compounds have yet to be annotated, their up or down regulation by the nutrients likely reflects a response to stress induced possibly by the deposition of the minerals on the leaf surface that could reduce the photosynthetically active leaf area – seemingly evident in the SPAD readings, but unclear with the actual chlorophyll measurement - or other stress-related outcomes of metal-plant interactions on biomolecules (Schutzendubel and Polle, 2002; Mustafa and Komatsu, 2016). Unfortunately, the scope of the study does not permit the disentangling of the effects from individual nutrients in the fertilizer composite, in light of the fact that not all of them (e.g., boron) were metallic in nature.

Differences in the LCMS profiles among the different treatments indicate that the treatments do differ in their impact on plant metabolism, but due to a lack of detailed information on the annotation of the metabolites, we could not at this point generate any additional insights into the changes of plant quality.

Thus, the full extent of discussion for this report is difficult to be wrapped up at this point, with a number of key findings still very preliminary. The LCMS metabolites are still unknown, coupled with the fact that the spICP-MS analysis did not clearly determine/quantify whether the nutrients found in the shoot and roots existed in forms that included micnobits. In our analyses, Fe_2O_3, CuO and MnO_3 were detected as micnobits in the shoots, but necessary information on the presence of these elements in the roots and on the other applied micnobits, important to conclude about mechanisms and to make clearer distinctions among the different treatments are still lacking, and need further research.

Innovative in this study is the use of composite (mixed) nanoparticles (micnobits), in contrast to individual ones to investigate the effect of nanoparticles in a leafy vegetable crop. To date, such studies with leafy vegetables are not reported. The rationale for using mixed micnobits was in the context of balanced fertilization regimes as

practiced in conventional agriculture, where more than NPK are used to fertilize crops. In addition, the use of different nanoparticles in very many industrial processes means that there will be the simultaneous presence in the environment of different nanoparticles, either as co-contaminants or co-additives. However, unlike the known and anticipated reactions in the environment of individual micnobits with specific chemistry (aggregation, dissolution or stabilization), little is known about the chemical reactions that may occur among the micnobits applied in a mixture. Such complex interactions may aggravate aggregation of the micnobits on the surface, lowering their dissolution. Where they do dissolve, however, the resultant ions may form insoluble metal-metal complexes. These unknown outcomes might explain the very noticeable deposition of micnobits on the leaf surface of the foliar applications to the extent that has not been reported or noted to be of major concern in previous micnobit foliar studies involving single micnobits (Hong et al., 2016; Torabian et al. 2016; Dimkpa et al, in press), and that could not be removed by published washing procedures (Hong et al, 2014).

Our data taken together, we conclude that this study, as reported, could not confirm the hypothesis that nutrients provided as micnobits will be taken up more efficiently than when provided as traditional ionic fertilizers, nor that they enhance growth, development and quality of a vegetable crop as mentioned in literature for individual micnobits (Alidoust and Isoda 2013; Pradhan *et al,.* 2013; Zhao *et al.,* 2013; Kim *et al.,* 2014). It should be emphasized that the spray of the nanoparticles could not be removed properly from the leaves, making it still not suitable for practical use in leafy vegetables.

Acknowledgements

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6   Annexes

6.1 Annex I. BLGG analysis report on the composition of the soil from Fa Grondbalans B.V., Heerhugowaard

In this analysis report, provided by BLGG on August 5th 2015 and based on samples taken at July 21th 2015, soil contents are shown of macro-elements (in Dutch: 'hoofdelement'), microelements (in Dutch: 'sporenelement'), physical properties (in Dutsch: 'fysisch') and biological actrivity ('biologisch'). Titles of the columns: Unity (in Dutch: 'eenheid'), Value (in Dutch: 'resultaat'), Mean (in Dutch: 'Gem'), Target path (in Dutch: 'streeftraject'), Low (in Dutch: 'laag'), rather low (in Dutch: 'vrij laag'), Acceptable (in Dutch: 'good'), rather high (in Dutch: 'vrij hoog'), High (in Dutch: 'high'). At the physical properties percentages are provided for Clay conteny (in Dutch: 'klei'), Silt and Sand content (in Dutch: 'zand').

Onderzoek	Onderzoek-/ordernr: 810111/003612466	Datum monstername: 21-07-2015	Datum verslag: 05-08-2015						

Resultaat hoofdelement		Eenheid	Resultaat	Gem.*	Streeftraject	laag	vrij laag	goed	vrij hoog	hoog
	N-totale bodemvoorraad	mg N/kg	1640							
	C/N-ratio		11	11	13 - 17					
	N-leverend vermogen	kg N/ha	85	116	93 - 147					
	S-totale bodemvoorraad	mg S/kg	650							
	C/S-ratio		28		50 - 75					
	S-leverend vermogen	kg S/ha	43	32	20 - 30					
	P plant beschikbaar	mg P/kg	2,7	2,1	1,0 - 2,4					
	P-bodemvoorraad (P-Al)	mg P$_2$O$_5$/100 g	68	54	27 - 47					
	Pw	mg P$_2$O$_5$/l	44							
	K plant beschikbaar	mg K/kg	120		70 - 110					
	K-bodemvoorraad	mmol+/kg	4,8		2,5 - 3,6					
	Ca plant beschikbaar	kg Ca/ha	550		215 - 502					
	Ca-bodemvoorraad	kg Ca/ha	4610		4620 - 6935					
	Mg plant beschikbaar	mg Mg/kg	84	127	50 - 85					
	Na plant beschikbaar	mg Na/kg	22	30	35 - 50					
sporenelement	Si plant beschikbaar	µg Si/kg	17460		6000 - 32000					
	Fe plant beschikbaar	µg Fe/kg	< 2030		2500 - 4500					
	Zn plant beschikbaar	µg Zn/kg	< 100		500 - 750					
	Mn plant beschikbaar	µg Mn/kg	< 250		1000 - 1300					
	Cu plant beschikbaar	µg Cu/kg	25		40 - 65					
	Co plant beschikbaar	µg Co/kg	< 2,5		25 - 50					
	B plant beschikbaar	µg B/kg	284		77 - 122					
	Mo plant beschikbaar	µg Mo/kg	6		100 - 5000					
	Se plant beschikbaar	µg Se/kg	2,5		3,5 - 4,5					
fysisch	Zuurgraad (pH)		7,0	7,1	> 5,9					
	C-organisch	%	1,8							
	Organische stof	%	3,7	5,6						
	C-anorganisch	%	0,21							
	Koolzure kalk	%	1,2		2,0 - 3,0					
	Klei	%	6	21						
	Silt	%	15							
	Zand	%	74							
	Klei-humus (CEC)	mmol+/kg	99	204	> 89					
	CEC-bezetting	%	100	89	> 95					
biologisch	Bodemleven	mg N/kg	87		60 - 80					

* Dit zijn regiogemiddelden. Meer informatie staat bij onderdeel Gemiddelde.

6.2 Annex II. Application of macro- and micronutrients

For the calculations of the amounts to be supplied to the plants, following assumptions were made:

1. Plants will reach a maximal final fresh weight of 400 gram
2. Final DW% = 12% Thus, maximal final dry weight is 50 gram
3. When dividing the growth period in 6 intervals of 2 weeks, fresh weight production per interval is as follows:

Interval	1	2	3	4	5	6
% Growth	5%	15%	30%	30%	15%	5%

N.B. The experiment has lasted until the fourth harvest. Then the expereiment had been stopped as plants had reached their final weight and symptoms of senescence became visible.

4. Nutrients will accumulate to values as values reported by Hartz and Johnstone (2007).

Macronutrients are individually supplied as follows:

Element	Expected demand mg /plant	g/l (stock solution)	ml applied per interval (from Stock solution)					
			1	2	3	4	5	6
N	2500	25.0	5.00	15.00	30.00	30.00	15.00	5.00
P	300	3.0	5.00	15.00	30.00	30.00	15.00	5.00
K	2300	23.0	5.00	15.00	30.00	30.00	15.00	5.00
Ca	300	3.0	5.00	15.00	30.00	30.00	15.00	5.00
Mg	150	1.5	5.00	15.00	30.00	30.00	15.00	5.00

Micronutrients are prepared in a mixture stock solutions as follows :

Elements	Expected demand mg /plant	Stock solutions (mmol/l)	
		For foliar application	For soil treatment
Zn1	3.00	15.29	1.74
Zn2	(6.00)	30.59	3.48
Mo	Not reported	10.42[2]	0.52[2]
Mn	3.00	18.20	11.83
Cu	0.45	15.74	0.31
B	1.50	92.51	43.67
Fe	8.75	17.91	20.00

From stock solution following amounts (ml) were used (and diluted) for spraying:

	ml applied from stock solution per interval					
	1	2	3	4	5	6
Foliar application	1.50	4.50	9.00	9.00	4.50	1.50
Soil treatment	1.32	3.96	7.92	7.92	3.96	1.32

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[2] Based on concentration used in a usual Hoagland nutrient solution.

6.3 Annex III. Tests

6.3.1 Stability aqueous suspensions of micnobits

Generally, aqueous solutions with micronutrients in ionic form are transparent and homogeneous, but aqueous suspensions of micnobits are emulsions, which will precipitate quickly (Figure 6-1). To be sure that we use the correct concentrations during handling (i.e. taking samples, dilution of stock solutions, foliar application, etc.), we needed to have insight in the stability of emulsions with time. To study the stability with time, we ultra-sonicated the emulsion with micnobits after preparation for 3 hours in a table top Sonicor (Figure 6-1 D). Immediately after ultra-sonication, we took samples of the emulsion at 3 different heights (Figure 6-1 E). Differences in colour between the samples demonstrated that sonication only was not sufficient to get a homogenous emulsion. Herewith we concluded, that shaking of the suspensions before every handling is necessary.
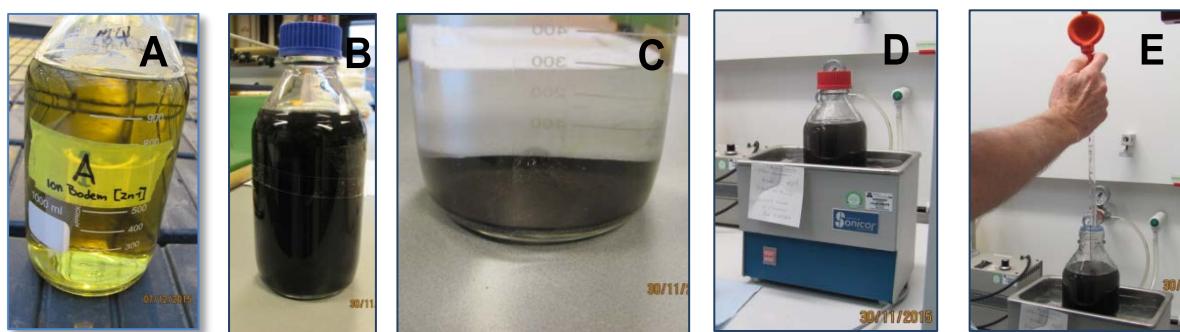
Figure 6-1. Aqueous solutions of micronutrients. A. In ionic form, B. suspension of micnobits just after shaking, C. suspension of micnobits after precipitation, D. suspension of micnobits during ultra-sonication and E. taking samples from micnobit suspension just after sonication

6.3.2 Determination field capacity pots

During growth, periodically water need to be supplied to compensate for the transpiration of the plants and to keep the soil at field capacity. To determine the field capacity, all pots were saturated with water and gravity drained for 2 days under a plastic sheet (Figure 6-2). Thereafter, all pots were weighed individually to determine total weight of the pots with soil and water content at field capacity. During the growth period all pots will be weighed individually and water will be supplied to the required weight (i.e. 80% of field capacity).

Figure 6-2. Determination field capacity the pots (see text for explanation)

6.3.3 Micnobit residues after foliar application

As the aqueous solutions of micnobits are emulsions, which precipitate quickly, the foliar applications with micnobits caused deposition of dark spots on the leaves. In order to get rid of the residues before analyses of the micronutrient content of the leaves, we followed published protocols to remove any particles that might be absorbed on the leaf or root surface (Hong *et al.*, 2014). However, the spray could not be removed from the leaves with KOH a week after spraying (Figure 6-3) or with water. Therefore, after harvest sprayed leaves were separated from newly developed leaves since the last spraying. Both parts were weighed (FW and DW), but only newly formed leaves were used for the analyses of micronutrient content and metabolomics.

Figure 6-3. Assessing the removal of micnobit spray. A) Rinsing of an old and a freshly sprayed leaf with KOH and B) Rinsing as a function of time.

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6.4 Annex IV. Plant growth

6.4.1 Fresh weight production with time – Different treatments at Zn1-concentration

## 6.4.2 Fresh weight production with time - Different Zn concentrations

Blom-Zandstra *et al.*, VFRC Report 2016/x
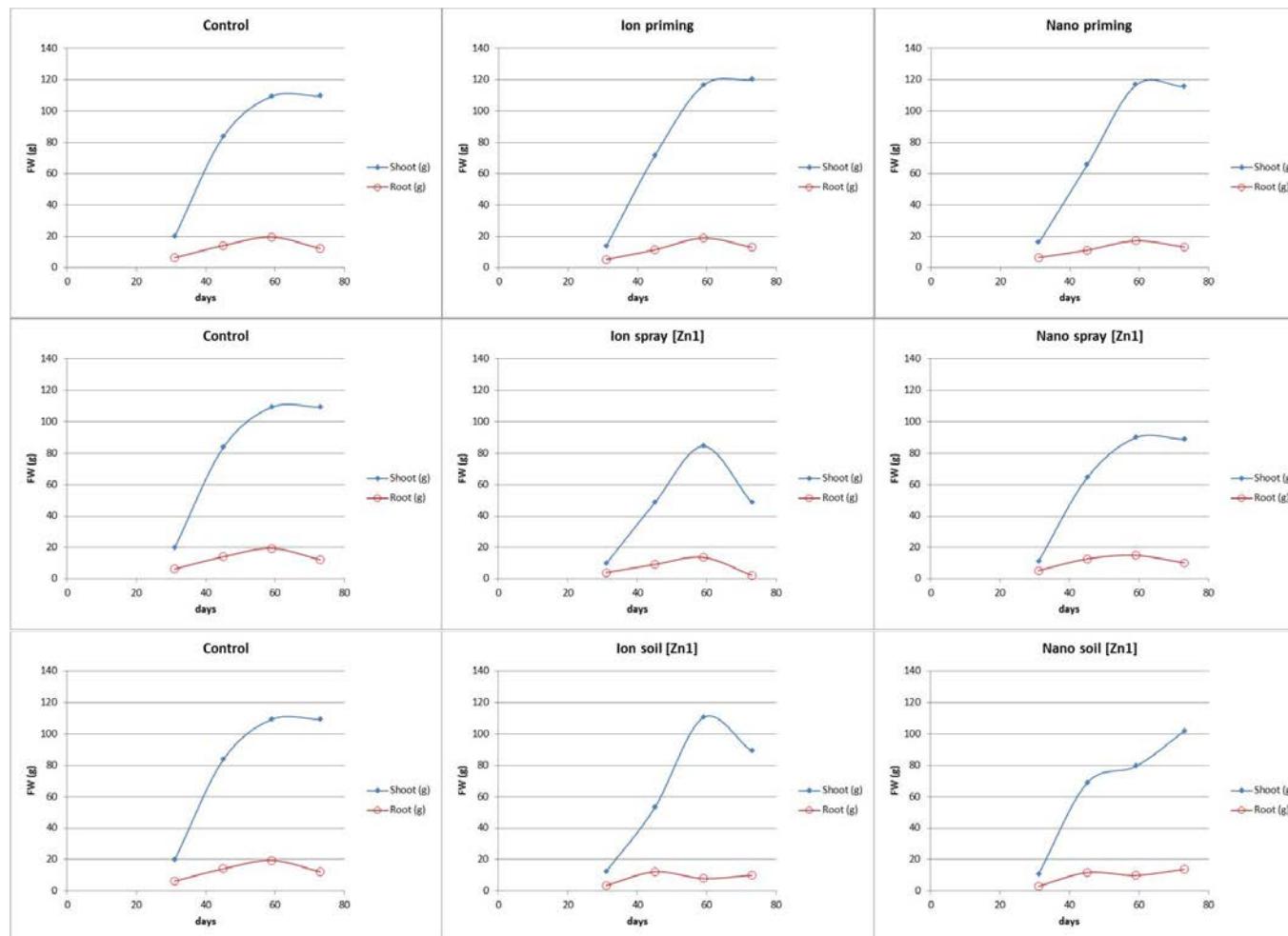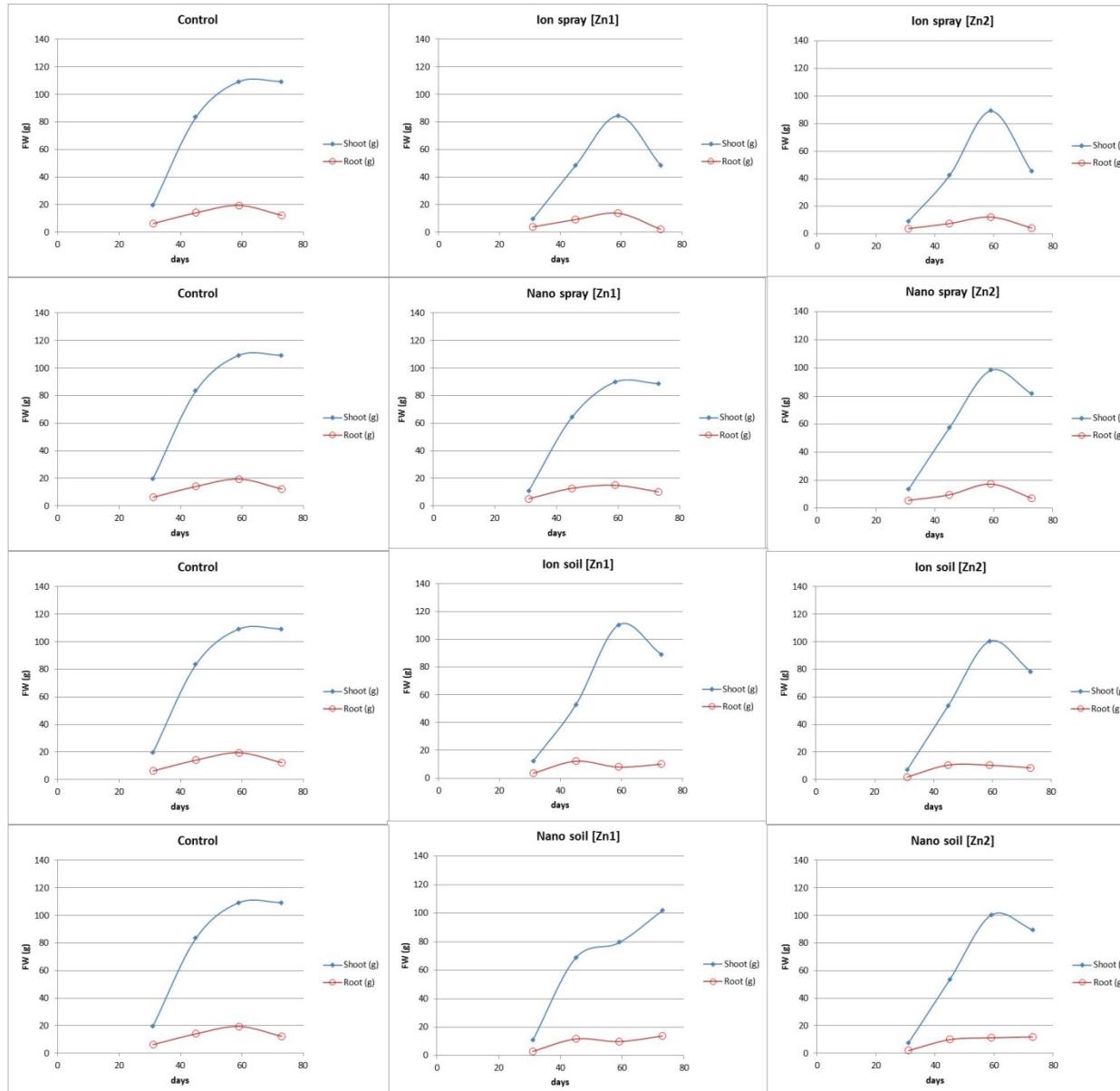
## 6.4.3 Dry weight production with time – Different treatments at Zn1-concentration
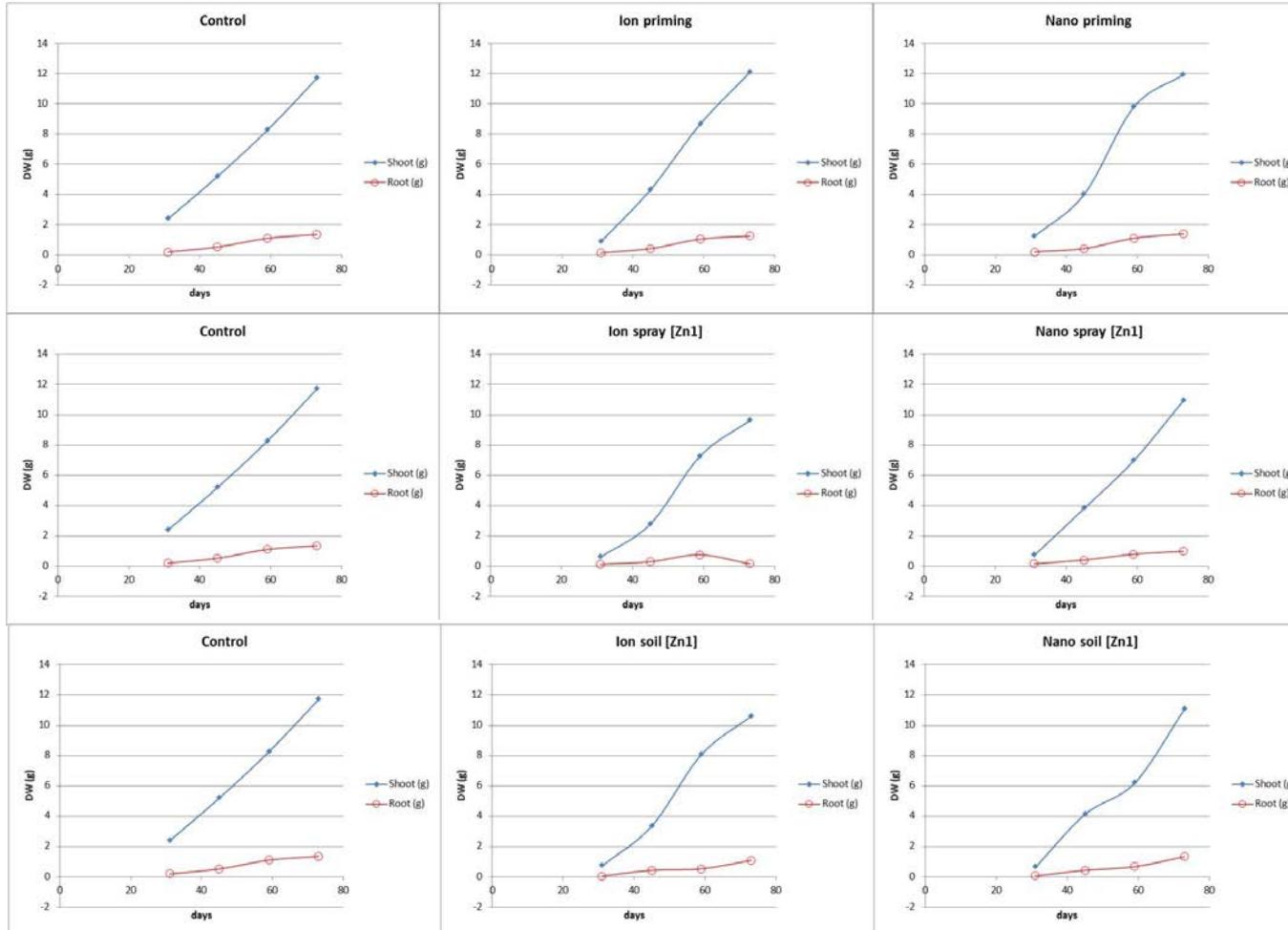
## 6.4.4 Dry weight production with time - Different Zn concentrations
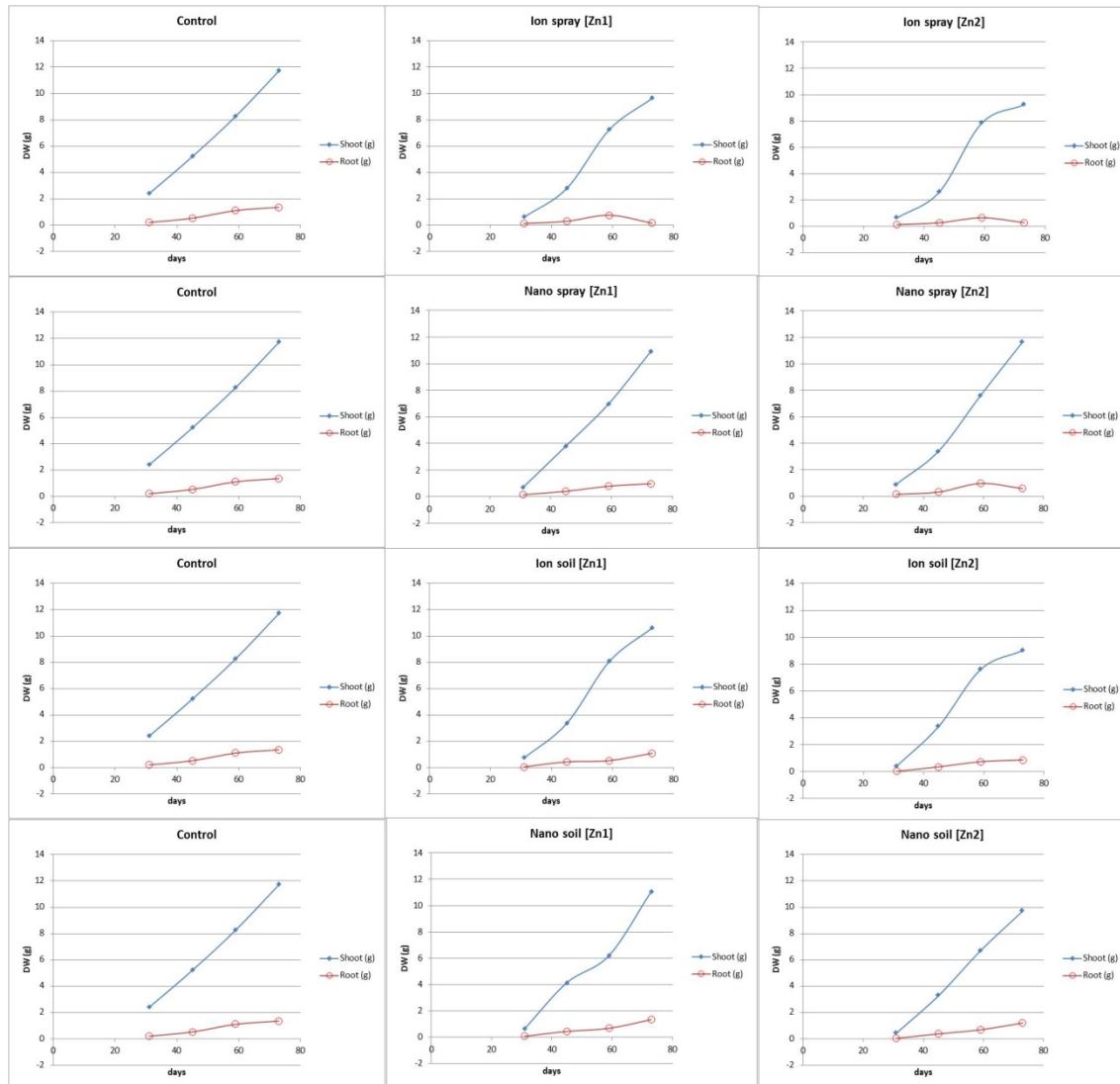
6.4.5 Root weight – Total plant weight ratio for different treatments based on fresh weight at Zn1 concentration.


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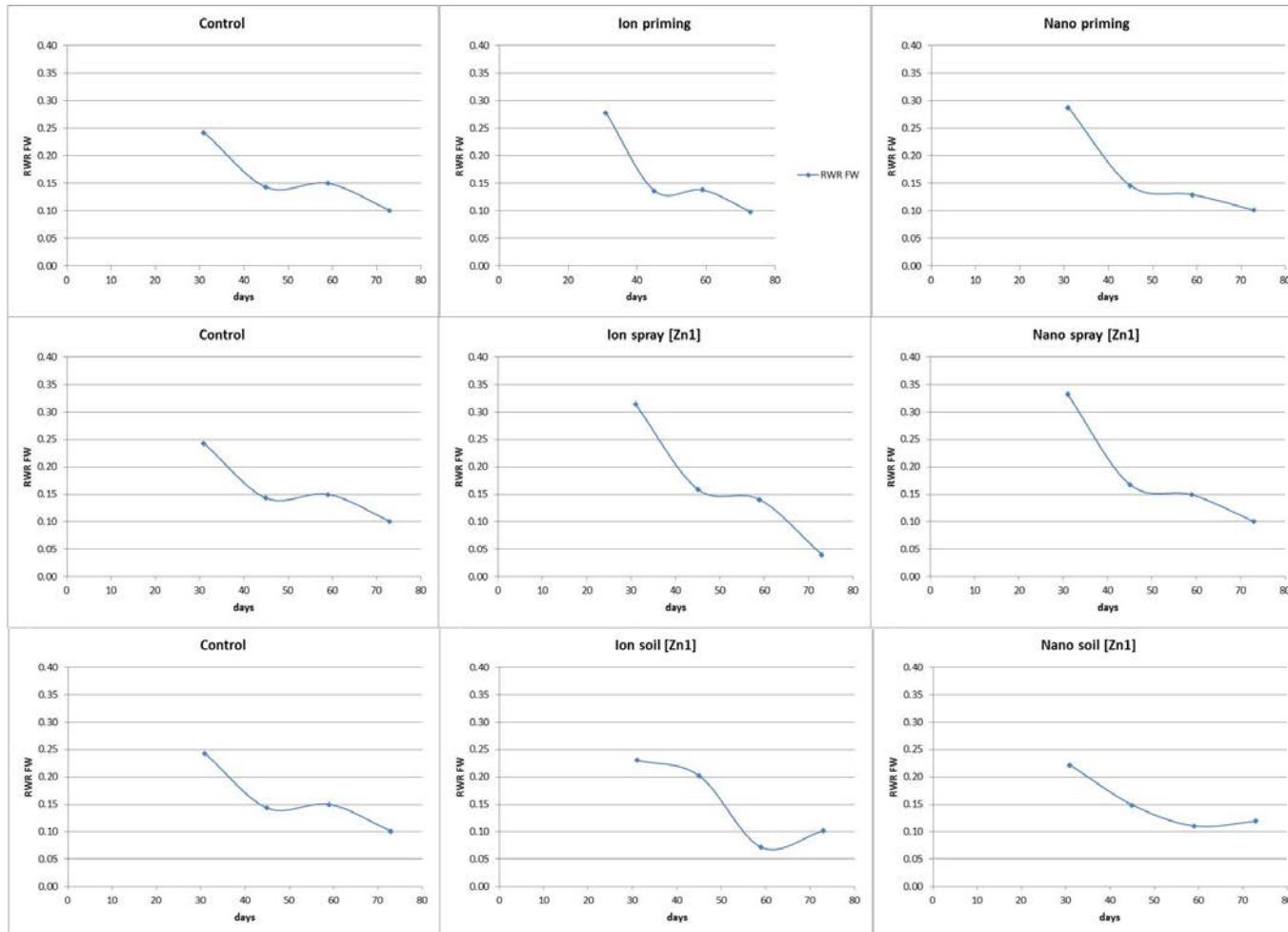
6.4.6 Root weight – Total plant weight Ratio for different Zn-concentrations based on fresh weight
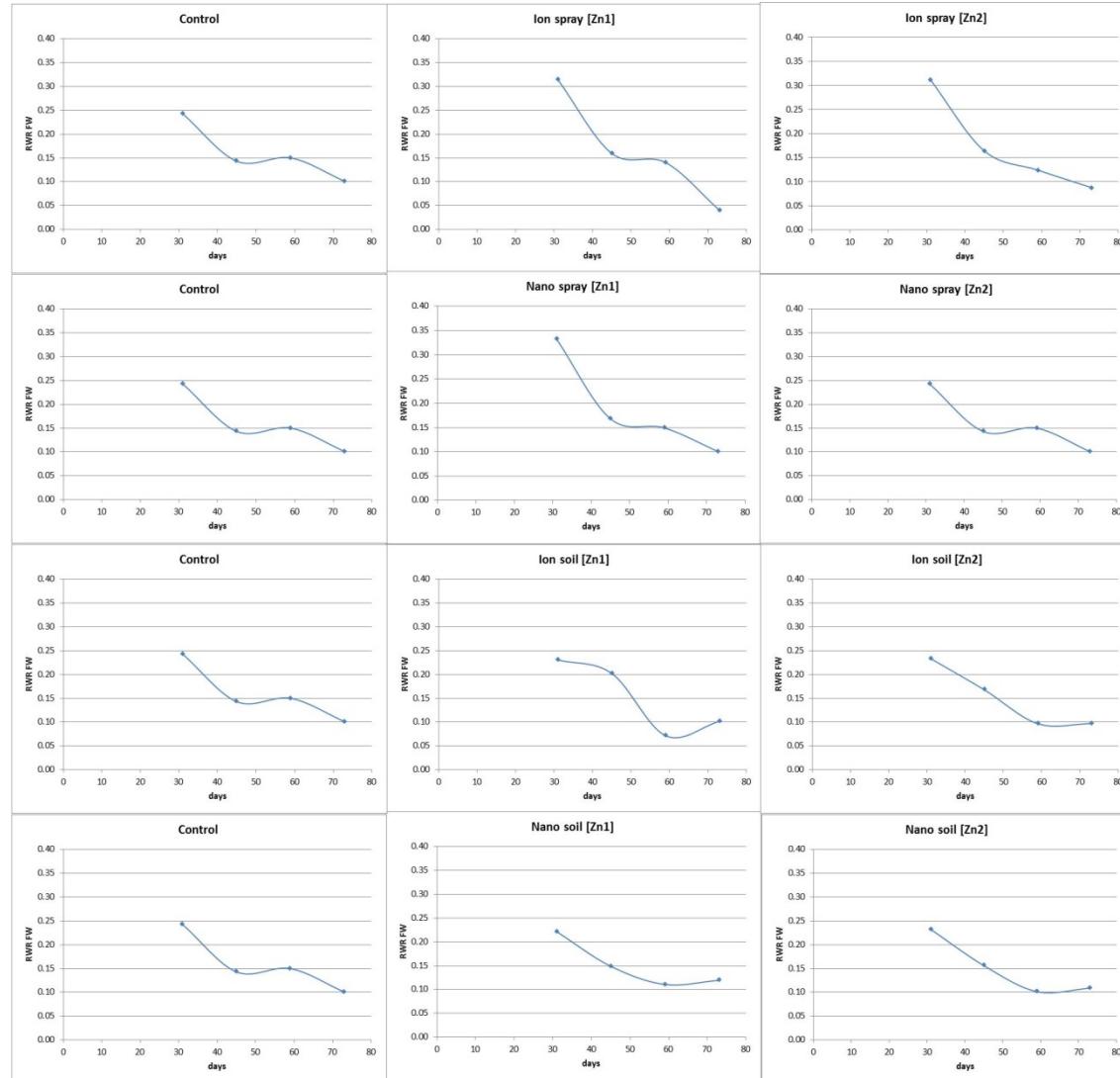
6.4.7 Root weight – Total plant weight Ratio for different treatments based on Dry Weight at Zn1 concentration.

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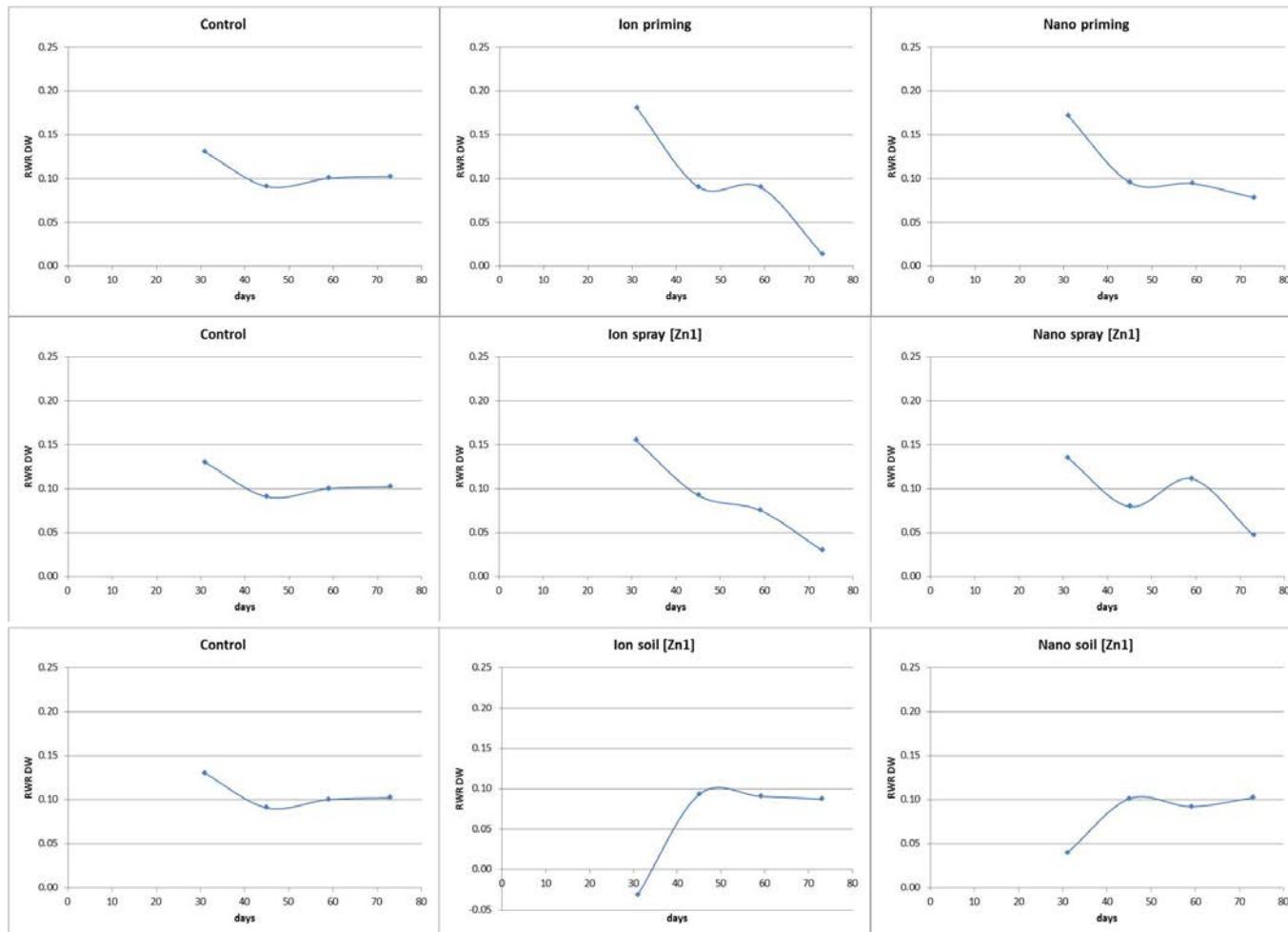
6.4.8 Root weight – Total plant weight Ratio for different Zn-concentrations based on Dry Weight
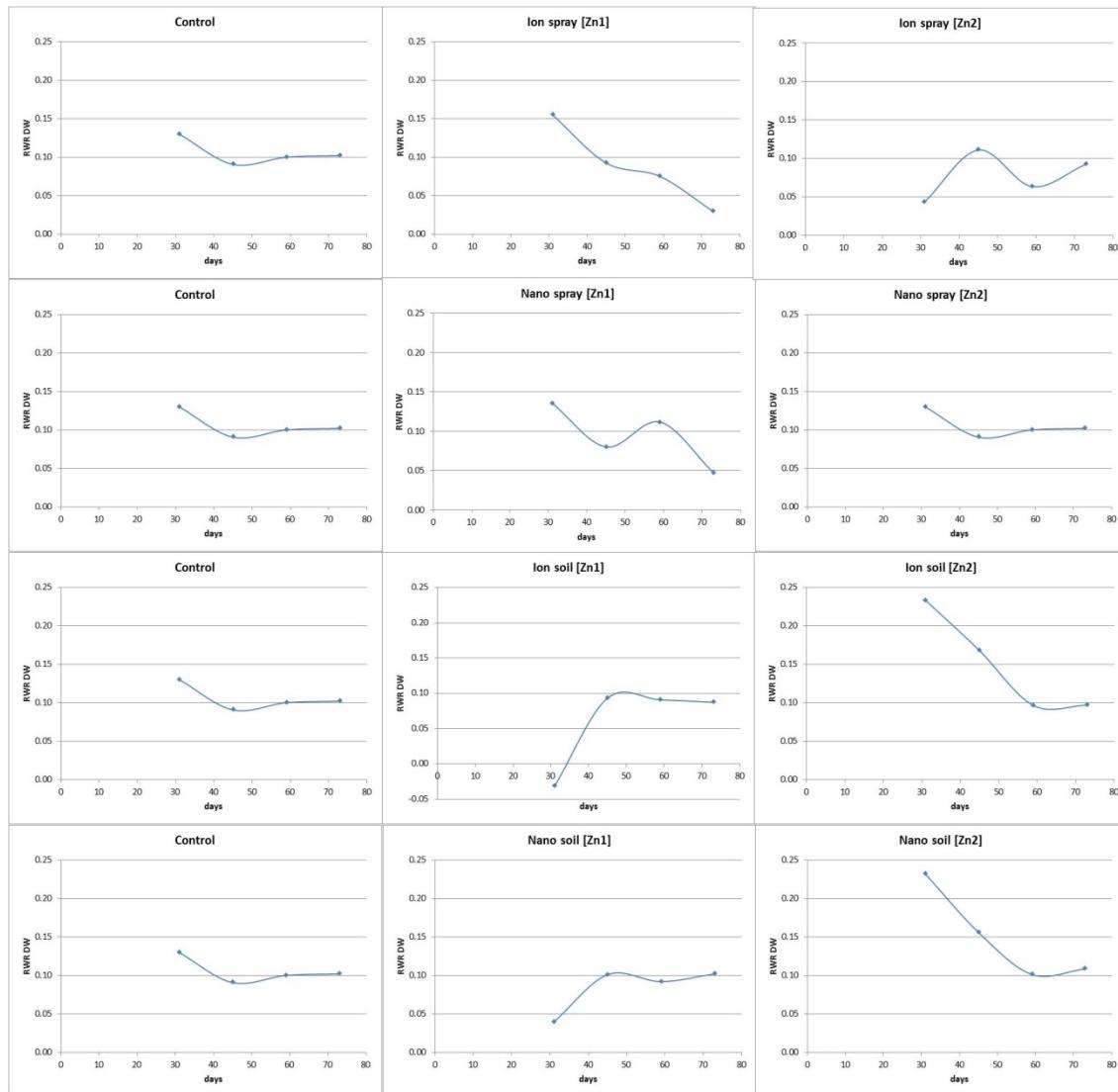
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