ALUMINUM TOLERANCE, GROWTH, AND ORGANIC ACID EXUDATION IN RAMBUTAN (*Nephelium lappaceum*)

by

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ABSTRACT

A randomized complete block design experiment with six aluminum (Al) concentrations was carried out to evaluate the effect of aluminum on nutrient content, dry matter (DM) production and Al-induced organic acid exudation in rambutan (*Nephelium lappaceum*). Rambutan cultivar cv. Jitlee was grown in nutrient solution at pH 4.0 with 1.0, 2.3, 4.1, 6.7 and 10.2 mM Al and without Al. The results revealed that all growth variables were significantly reduced with increasing concentration of Al in the nutrient solution. Nevertheless, even at an aluminum concentration of 3.5 mM, rambutan plants grew relatively well, confirming that this crop is highly tolerant to high Al as compared to other crops. Analyses of root exudates for the presence of organic acids showed no detectable amounts in solution. Accumulation of Al in leaves, stems and roots suggests the existence of an Al- sequestration mechanism in rambutan which may involve an Al-ligand complex which translocates from roots to shoots, where it may accumulate in leaf vacuoles.

RESUMEN

Un estudio con diseño de bloques completos aleatorizados con seis concentraciones diferentes de aluminio (Al) se llevó a cabo para evaluar el efecto del aluminio en el contenido de nutrientes, producción de materia seca y la exudación de ácidos orgánicos por inducción de Al en rambután (*Nephelium lappaceum*). La variedad de rambután cv. Jitlee se cultivó en una solución nutritiva a pH 4.0 con 1.0, 2.3, 4.1, 6.7 y 10.2 mM de Al y sin Al. Los resultados revelaron que todas las variables de crecimiento se redujeron significativamente mientras se aumentaba la concentración de Al de la solución nutritiva. Sin embargo, incluso en una concentración de aluminio de 3.5 mM, las plantas de rambután crecieron relativamente bien, lo que confirma que este cultivo es altamente tolerante a Al, en comparación con otros cultivos. Los análisis de los exudados de raíces para la presencia de ácidos orgánicos no mostraron cantidades detectables en solución. La acumulación de Al en las hojas, tallos y raíces sugiere la existencia de un mecanismo de secuestración de Al en el rambután que puede envolver complejos Al-ligando para translocarlos de las raíces a los tallos y subsecuentemente acumularlo en las vacuolas de las hojas.

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To God, my family, friends and co-workers.

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INTRODUCTION

Rambutan (*Nephelium lappaceum*) is a medium-sized tropical tree in the Sapindaceae family. The non-climateric fruit of this tree is appreciated for its juicy and sweet flavor (Martínez-Castellanos et al., 2009). Recent work conducted by Goenaga (2011) suggests that rambutan is highly tolerant to acid soils with high concentration of soil aluminum (Al).

Al toxicity is considered to be a serious constraint limiting crop production in acid soils (pH below 5.5) (Mora et al., 1999; Langer et al., 2009). Plants growing in acid soils also suffer from deficiencies of nitrogen, phosphorous, potassium, calcium, magnesium and toxicity of manganese (Samac and Tesfaye, 2003). Aluminum can inhibit root cell elongation and division causing a reduction in root growth, and limiting water and nutrient uptake (Samac and Tesfaye 2003; Langer et al., 2009).

Plants have evolved two major mechanisms for Al tolerance: 1) internal tolerance and 2) Al exclusion from the root apex (Barceló and Poschenrieder, 2002; Kochian et al., 2005; Langer et al., 2009). Several studies suggest that Al-tolerant plants exclude Al from roots by exudation of organic acids to the rhizosphere or by creating Al-ligand complexes for translocation from roots to shoots, and subsequent accumulation in the leaves (Watanabe and Osaki, 2002; Samac and Tesfaye, 2003).

The objective of this work is to study the mechanism conferring Al tolerance in rambutan. It is hypothesized that this tolerance is brought about by synthesis of organic acids which may involve an Al-ligand complex that makes Al non toxic in the rhizosphere.

LITERATURE REVIEW

In recent years, research on aluminum tolerance has been conducted to study the mechanism conferring Al-tolerance in crops.

Goenaga (2011) studied four cultivars of rambutan grown on an acid Ultisol and found that leaf, petiole, stem and root dry matter significantly increased at soil Al concentrations between 0.67 cmol/kg and 11.0 cmol/kg. Soil Al concentrations higher than 11.0 cmol/kg resulted in a significant reduction in dry weight of all plant parts. The increase in plant dry weight, plant height and stem diameter with increasing levels of soil Al up to 11 cmol/kg of soil Al indicates that rambutan is highly tolerant to high soil Al. Leaf N concentration was not affected by soil Al but P, Ca, Mg, and Mn in leaves declined significantly with increases in soil Al. Leaf K concentration increased with increments in soil Al. Al and Fe concentration in leaf tissue declined with increments in soil Al and then increased at exactly the same soil Al concentration (11cmol/kg) in which dry weights commenced to decline. The author concluded that rambutan is highly tolerant to acid soils and suggests that tolerance may involve an Al and Mn exclusion mechanism (Goenaga, 2011).

Langer et al., (2009) studied four alfalfa cultivars at two pH levels (4.5, 6.0) and found that Al caused a considerable reduction in dry weight, particularly when grown at pH 4.5 and an Al concentration of 0.050 mM . Organic acid exudation was affected by pH and Al treatments independently. Citrate and succinate exudation increased with high Al concentrations in nutrient solution at pH 4.5. However, no relationship between pH and carboxylate exudation was observed at pH 6.0. Concentration of P in shoots was not affected

by Al treatments at both pH levels. Conversely, concentrations of Al and P in roots increased in plants exposed to high Al. Precipitation of Al and P in root tissues is one mechanism of reducing Al toxicity suggesting the existence of an exclusion mechanism for Al in alfalfa. The authors found that the exudation of citrate increased significantly in response to Al stress and that succinate was not capable of forming strong complexes with Al to reduce its toxicity.

Mimmo et al., (2009) studied the influence of Al on phosphate (P_i) uptake in two bean species, *Phaselous vulgaris* and *Phaselous lunatus*. The two species were treated first with solutions of Al at different concentrations (0, 0.025, 0.050 and 0.10 mM, pH 4.5) and then with solutions of P_i (0.15 mM) at pH 4.5. The higher the Al concentration the higher Al concentration adsorbed in the roots by the plants but *P. vulgaris* adsorbed significantly more Al than *P. lunatus.* Both species released organic acids: *P. vulgaris* released fumaric acid and *P. lunatus* fumaric and oxalic acids which could have hindered further Al uptake.

Matiello et al., (2008) studied two *Coffea canephora* coffee clones (Mtl 25 and Mtl 27) and the coffee variety Amarelo (*Coffea Arabica*) and found that Amarelo was less sensitive to Al than the *C. canephora* clones and that Mtl 25 was less Al sensitive than Mlt 27. A higher Al concentration (2.0 mM) resulted in a reduction of the P and Ca content in the leaves and roots, especially in clones Mtl 25 and Mtl 27. The authors concluded that Al accumulation in the root system along with transport to the shoot are important processes in conferring Al tolerance of coffee plants.

Tian-Rong et al., (2007) studied the effect of soil Al and cadmium (Cd) on Al and mineral nutrient concentrations in plant and on Al-induced organic acid exudation in two barley cultivars with diverse Al tolerance. Cultivar Shang 70-119 had significantly higher Al

concentration and accumulation in the shoot than Al tolerant cultivar Gebeina, particularly when subjected to low pH (4.0) and Al treatments (0.10 mM Al and 0.10 mM Al + 0.010 mM Cd). At low pH, Al treatments caused a reduction in Ca and Mg concentrations in all plant parts, P and K concentrations in shoots; Fe, Zn and Mo in leaves, Zn and B in shoots, and Mn concentrations both in the roots and shoots. Malate, citrate, and succinate were found in significantly higher concentrations in plants exposed to 0.10 mM Al relative to the control, and the Al-tolerant cultivar had a significantly higher exudation of these organic acids than the Al-sensitive one, indicating that Al-induced exudation of these organic acids is linked to Al tolerance in barley.

Wang et al., (2006) studied the variation of wheat root exudates under aluminum stress and found that increasing the Al concentration of the nutrient solution gradually stimulated the exudation of organic acids in the rhizosphere. Under high Al concentration, wheat roots exuded malic and citric acids after being treated with Al for 0.5 h (malic acid) and 2-4 h (citric acid). The citric acid exuded from wheat roots increased when the external Al concentrations ranged from 0 to 0.185 mM, and decreased when the external Al concentrations ranged from 0.185 mM to 1.48 mM. Al accumulation in wheat roots under normal growth (under 0.93mM Al) was moderately low (0.37 cmol/kg). However, when Al concentration was higher than 1.48 mM, Al accumulation in roots increased to 2.2 cmol/kg. The results showed that the citric and malic acids exuded from wheat roots increased within the range of 0 to 0.185mM Al. These organic acids can play a significant role in complexing the soil Al to ameliorate aluminum toxicity.

Liao et al., (2006) studied the effects of Al and P interactions on soybean root growth and root organic acid exudation using homogeneous (uniform Al and P distribution in bulk solution) and heterogeneous nutrient solutions. Analysis of root exudates indicated that: 1) Al toxicity induced citrate exudation; 2) P deficiency triggered oxalate exudation; and 3) malate exudation was induced by both experiments. Citrate and malate were the two main organic acids induced by Al toxicity and/or low P-availability, while oxalate appeared to be mainly induced by P deficiency. Al-induced citrate exudation decreased significantly when roots were also grown at high-P levels, demonstrating that there is a clear Al x P interaction for root citrate exudation. Conversely, moderately low levels of citrate exudation induced by low-P status occurred only during the first 6 h of the exudation period. This demonstrated that the Al-activated citrate exudation is a relatively long-term response while low P-induced citrate exudation is transient.

Zheng et al., (2005) studied the accumulation of Al and P in two buckwheat cultivars, Jiangxi (Al resistant) and Shanxi (Al sensitive). Their results suggest that the immobilization of Al in roots by precipitation with P might contribute to reduce Al toxicity. This hypothesis is also supported by the fact that accumulation of P in roots increases in plants exposed to higher levels of Al. Ca and Mg concentrations in the roots were reduced by Al treatment, whereas Al and P concentrations increased. Interestingly, the concentration of P and Al in Jiangxi was twice the concentration of these elements in Shanxi.

Samac and Tesfaye (2003) in their review explain that exposure to Al causes stunting of the primary root and inhibition of lateral root formation. Affected root tips were found to be stubby due to inhibition of cell elongation and division. The root system was impaired for

nutrient and water uptake, making the plant more susceptible to drought stress. Aluminum toxicity is also associated with alterations in a number of physiological processes and biochemical pathways after cessation of cell elongation.

Kochian et al., (2002) studied the mechanisms of metal (aluminum and heavy metals) tolerance in plants and proposed two classes of mechanisms to account for Al tolerance: 1) mechanisms that allow the plant to tolerate Al accumulation in the symplasm (Al tolerance), and 2) those which exclude Al from the root apex (Al exclusion). Recent experimental evidence has been presented supporting the role of organic acid anion exudation from the root apex as a major mechanism of Al exclusion. Al resistance by exclusion appears to be mediated by Al- activated release of organic acid anions such as malate, oxalate, or citrate, which chelate Al in the rizhosphere and prevent its entry into the root apex.

Osaki et al., (2003) analyzed the relationship between the concentration of Al and that of other minerals in the leaves of different plant species growing in various types of soils distributed in tropical and temperate regions and found that Al concentration in the leaves had a negative relationship with concentrations of several elements regardless of growth conditions, indicating that Al accumulator species restrict the accumulation of other mineral in their leaves, whereas non-accumulator species tend to accumulate other minerals in their leaves.

Delisle and Houde (2001) studied the characterization of oxalate oxidase and cell death in Al-sensitive and tolerant wheat roots and proposed a tolerance mechanism in wheat based on accelerated development of root epidermal cells. Within 8 h of exposure to Al, they

observed cell death in the root epidermis in the tolerant variety. They suggested cell death is aimed at replacing epidermal cells intoxicated with Al while maintaining root growth.

Watanabe and Osaki (2001) studied the influence of aluminum and phosphorous on growth and xylem sap composition in *Melastoma malabathricum*. Based on analysis of organic acids and Al in the xylem sap of seedlings grown in nutrient solution with or without 0.2 mM Al for three weeks they found that the translocation form of Al was an Al-citrate complex and the aluminum concentration was 3 mM (15 times higher than the Al concentration in the medium) at pH 5.0.

The range of Al tolerance in various plant species could be remarkably high. For example, Watanabe et al., (1997) studied *Melastoma malabathricum* and found that this plant can accumulate 37.1 cmol/kg Al in seedlings grown in nutrient solution containing 0.5mM Al for 6 weeks. Britez et al. (1997) studied *Faramea marginata* and found that this plant can accumulate 59.3 cmol/kg Al. Working with *Miconia albicans* de Medeiros and Haridasan (1985) found that this plant can accumulate 40.8 cmol/kg Al in old leaves. Finally, Matsumoto et al. (1976) studied the localization of aluminum in tea leaves and found that tea plant is an Al accumulator species accumulating more than 111.2 cmol/kg Al in older leaves, although only 2.2 cmol/kg Al in young leaves. Watanabe and Osaki (2002) suggested that these species have two mechanisms to alleviate Al toxicity: 1) formation of a complex between an organic acid anion and the Al ion, and 2) intracellular and tissue compartmentalization.

OBJECTIVES

The objective of this study is to determine the mechanism conferring Al tolerance to rambutan.

MATERIALS AND METHODS

This study was conducted at the USDA-ARS, Tropical Agriculture Research Station in Mayagüez, Puerto Rico. Seed of rambutan cultivar Jitlee were germinated on washed sand in a greenhouse and seedlings allowed to grow for about 4 weeks. Seedlings at the 6-leaf stage were transferred to 30 cm x 18.5 cm x 10.5 cm plastic containers containing 3.6 liters of a modified Magnavaca's nutrient solution (Piñeros et al., 2005) at pH 4.0 (Figure 1). The nutrient solution contained the following macronutrients (mM): $Ca(NO_3)_2 \cdot 4H_2O$, 3.53; NH_4NO_3, 1.30; KNO_3, 0.56; KCl, 0.58; $Mg(NO_3)_2 \cdot 6H_2O$, 0.86; KH_2PO_4, 0.04 and micronutrients (µM): Fe-*Sprint 138*, 180; $MnCl_2 \cdot 4H_2O$, 9.1; H_3BO_3, 25; $ZnSO_4 \cdot 7H_2O$, 2.3; $CuSO_4 \cdot 5H_2O$, 0.63; and $Na_2MoO_4 \cdot 2H_2O$, 0.83

Figure 1. Experimental layout of treatments in the greenhouse

Nutrient solutions were renewed every seven days. Before renewal, the volume of each container was determined and a sample collected for determinations of macro and micronutrients. Six aluminum treatments at a concentration of 0, 1.0, 2.31, 4.12, 6.7 and 10.2 mM and with activity of 0, 0.250, 0.500, 0.750, 1.00 and 1.25 mM, respectively were added in the form of aluminum chloride ($AlCl_3$). The pH was kept constant at 4.0 by addition of 0.1 M hydrochloric acid (HCl) and 0.1 M potassium hydroxide (KOH) as required (Langer et al., 2009). Treatments were arranged in a randomized complete block design with five replications. The experiment was replicated twice over time. Average solar radiation, maximum and minimum temperature during the first trial were 5.04 MJ, 37.4°C and 19.7°C respectively and 4.06 MJ, 37.3°C and 22.3°C in the second trial.

Organic acid determination

Collection of exudates for organic acid determination was performed according to Langer et al., (2009) and Piñeros et al., (2002). After 15 days of growth, a seedling from each Al treatment and replication was carefully removed from the plastic container. The root system of this plant was immediately placed in a solution having the same Al concentration where the seedling was growing plus 4.39 mM $CaCl_2$ (Figure 2). After 24 hours, the plant was removed from the vial and the solution stored at -20 °C for subsequent determination of organic acids.

Figure 2. Rambutan collection of exudates for organic acid analyses

Organic acids (citrate, succinate, malate and oxalate) were determined using HPLC (Agilent® HP 1100) (Appendix A). For this purpose, root exudates were lyophilized and the residue re-dissolved in 5 mL deionized water and filtered through a 45 µm filter. To obtain optimum analytical conditions, separation was achieved using a two 150 x 4 mm reverse-phase column in series at 25^0C. Sample solutions (10 µL) were injected into the column, and a 1% ortho-phosphoric acid at pH 2.1 and methanol (0.89:0.11 ratio respectively) were used for isocratic elution at a flow rate of 0.5 mL/min with UV detection at 210 nm (Figure 3).

Standard solutions of oxalic, citric, malic and succinic acids at concentrations of 0.50, 1.00, 2.00 3.00 and 4.00 µg/g each, were used for analyses. The malic acid standard used in this study was malic acid-DL (Dextrorotatory and Levorotatory enantiomers); for this reason we obtained two peak signals in chromatograms of the organic acid. The standard solutions were injected thrice. Calibration curves were constructed for the four organic acids (Appendix B). The

equations, detection limits and quantification limits are shown in Table 1. Preliminary studies with organic acid standards indicated a recovery of about 95%.

Figure 3. Chromatogram of organic acids

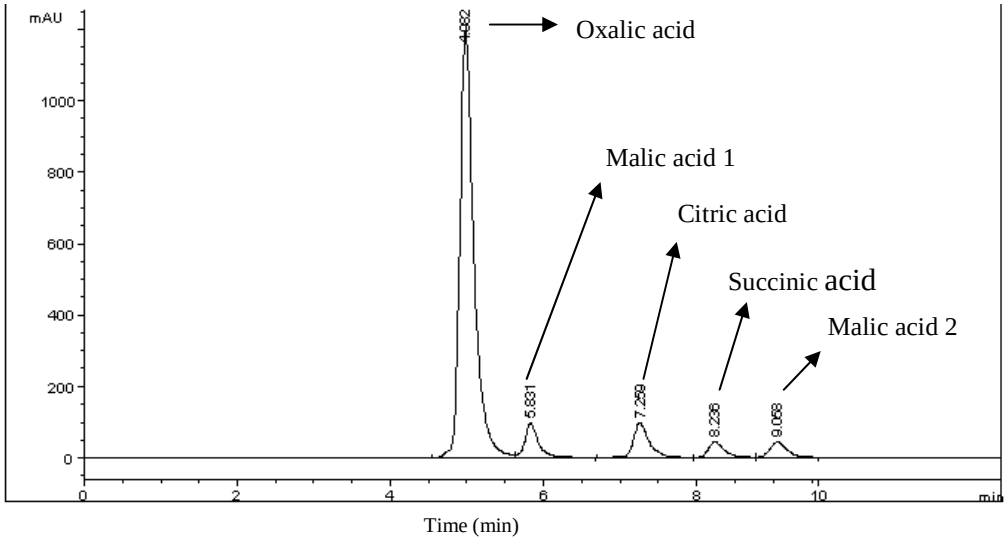


Table 1. Calibration curves, detection limits (LOD) and quantification limits (LOQ) and useful ranges of the organic acids

Organic acid	Regression equation	R^2	LOD (µg/g)	LOQ (µg/g)	Useful range (µg/g)
Oxalic acid	Y=-51.9+1607.7x	0.9999	0.048	0.16	(0.048, 4.00)
Citric acid	Y=-12.7+144.8x	0.9980	0.22	0.72	(0.22, 4.00)
Malic acid 1	Y=-8.6+122.4x	0.9954	0.33	1.1	(0.33, 4.00)
Malic acid 2	Y=-13.0+77.8x	0.9996	0.099	0.33	(0.099, 4.00)
Succinic acid	Y=-12.2+71.5x	0.9985	0.19	0.63	(0.19, 4.00)

Plant Growth and Nutrient Composition

Plants were harvested 60 days after being transferred to nutrient solutions. At harvest, number of leaves, leaf area, plant height and stem diameter were measured. Leaf area was measured by scanning leaves using a LI-3100 Area meter (LI-COR, Inc., Lincoln, NE), plant height with a ruler and, stem diameter with a digital caliper. Plants were then separated into roots, stems and leaves and dried to constant weight at 70º C for dry matter determination (Figure 4). In addition, fallen leaves from each Al treatment during the 60-day experimental period were also collected for dry matter determination. A root tolerance index (RTI) was calculated by dividing root dry weight of plants grown with Al by the root dry weight of plants grown without Al.

Figure 4. Separation of plants into leaves, stems and roots

Plant parts were ground using a Wiley mill and analyzed for P, K, Ca, Mg, Fe, Mn, Zn, Mo, B and Al concentration using recommended digestion procedures (Perkin-Elmer, 1994). For this purpose, tissue samples were incinerated in crucibles at 500 ºC for four hours, and allowed to cool overnight. The incinerated samples were digested with 20 mL 33% HCl until 10 mL of solution remained in the crucible. After digestion was completed, each sample was filtered through Whatman No. 541 filter paper into a 100 mL volumetric flask. After the sample was cooled the solution was used for nutrient determination using inductively coupled plasma-optical emission spectroscopy (Perkin Elmer® 7300 DV) (Appendix C).

The emission signal of samples was obtained by developing calibration curves (Appendix D) at the concentrations shown in table 2. The results, detection limits and quantification limits are shown in table 3. Standard reference material (peach leaves, Standard Reference Material 1547, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA) with certified concentrations of elements indicated a recovery within the range of certified values.

Table 2. Standard calibration curves for various elements analyzed by ICP-OES

Element	Std 0 (µg/g)	Std 1 (µg/g)	Std 2 (µg/g)	Std 3 (µg/g)	Std 4 (µg/g)	Std 5 (µg/g)	Std 6 (µg/g)
P	0.0	3.5	7.0	9.0	13.0	16.0	20.0
K	0.0	50.0	100.0	140.0	200.0	240.0	300.0
Ca	0.0	25.0	50.0	105.0	150.0	160.0	200.0
Mg	0.0	7.5	15.0	21.0	30.0	37.0	47.0
Fe	0.0	0.5	1.0	2.0	3.0	5.0	6.0
Mn	0.0	0.15	0.30	0.40	0.60	0.80	1.0
Zn	0.0	0.10	0.20	0.30	0.40	0.60	0.80
B	0.0	0.15	0.30	0.40	0.60	0.80	1.0
Mo	0.0	0.10	0.20	0.30	0.40	0.60	0.80
Al	0.0	0.5	1.0	1.5	2.0	3.0	4.0

Table 3. Regression equations, detection limits (LOD), quantification limits (LOQ) and useful ranges for minerals analyzed by ICP-OES

Mineral	Regression equation	R^2	LOD (µg/g)	LOQ (µg/g)	Useful range (µg/g)
P	Y=623.0+1180x	0.9966	0.92	3.1	(0.92, 20)
Ca	Y=129023+20182x	0.9964	9.7	32.5	(9.7, 200)
Mg	Y=260862+208889x	0.9953	3.1	10.2	(3.1, 47)
Fe	Y=1764+14756x	0.9984	0.18	0.59	(0.18, 6)
Mn	Y=3182+191980x	0.9987	0.03	0.09	(0.03, 1)
Zn	Y=134.2+4457x	0.9965	0.03	0.12	(0.03, 0.8)
B	Y=-244.4+38141x	0.9974	0.04	0.13	(0.04, 1.0)
Mo	Y=-47.5+4266x	0.9967	0.03	0.11	(0.03, 0.8)
Al	Y=-91.9+102283x	0.9995	0.06	0.21	(0.06, 4.0)

Total nitrogen was determined by a modification of the microkjeldahl method (Foss, 2002). For this purpose, 0.2 g of tissue was weighed and transferred to a Kjeldahl tube. The following compounds were added to each tube: Hengar granules for smooth boiling; one catalyzing tablet (5g K_2SO_4 + 0.15g $CuSO_4$); 5 mL concentrated H_2SO_4 and 3 mL 30% H_2O_2. Samples were digested in a digestion block for 2 hrs at 380º C (Appendix E).

RESULTS AND DISCUSSION

Organic acid exudation and Al accumulation

The Al-dependent efflux of organic acids into the rhizosphere has been widely described as an important mechanism in plant species to minimize the toxic effects of Al in acid soils (Langer et al., 2009; Kochian et al., 2004; Barceló and Poschenrieder, 2002). In this study, analyses of root exudates for the presence of organic acids showed no detectable amounts in solution suggesting another type of tolerance mechanism in rambutan. The HPLC instrument used in this research had a low detection limit of 0.048, 0.099, 0.19, 0.22, 0.33 µg/g for oxalic, malic 2, succinic, citric and malic acid 1, respectively. These low detection limits indicated that the method used for organic acid determination can detect trace amounts that are lower than those reported for similar studies (Langer et al. 2009; Mimmo et al. 2009; Tian Rong et al. 2007; Wang et al. 2006).

Figure 5 shows a chromatogram of root exudates for organic acids at 4.12 mM Al. The peak in the chromatogram corresponds to $AlCl_3$ which was present in the root exudates solution. Since we found no evidence of production of organic acids, we suggest that rambutan tolerates high concentration of Al in the rhizosphere by producing an Al-ligand complex which translocates from roots to shoots, and subsequent accumulation in leaf vacuoles or by intracellular and tissue compartmentalization (Ma et al., 2001; Watanabe and Osaki, 2002).

Figure 5. Chromatogram of root exudates for organic acids at 4.12 mM Al.
Peak corresponds to $AlCl_3$

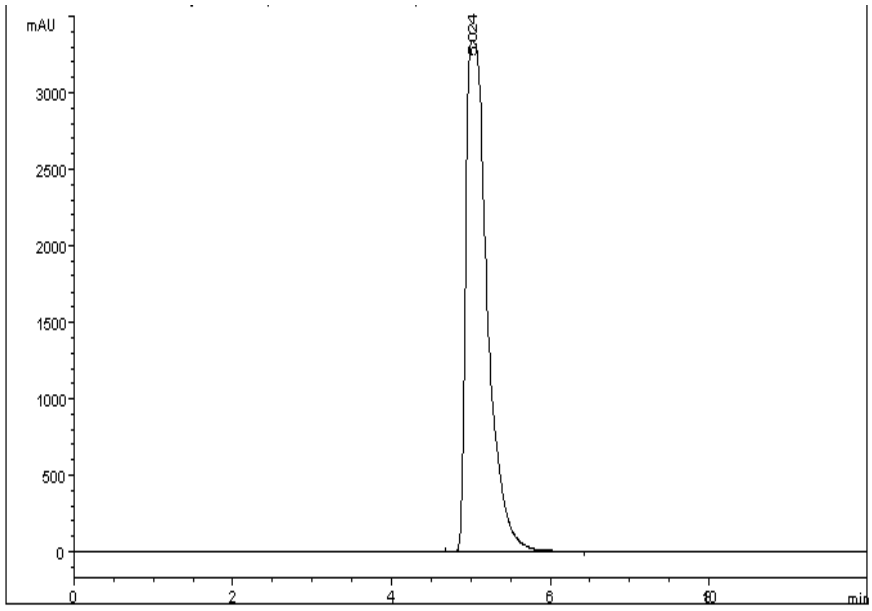
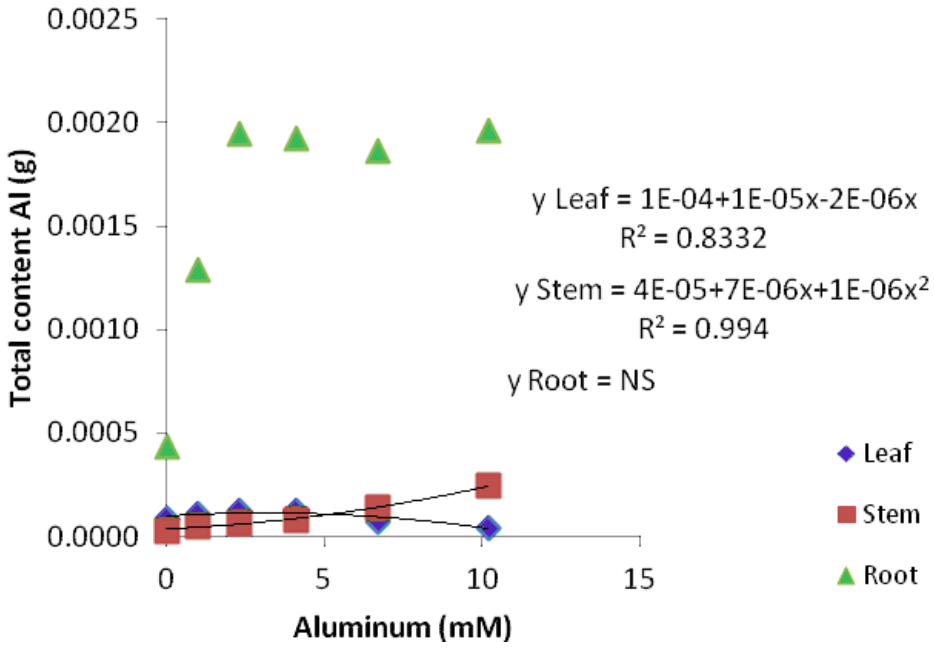


Figure 6. Total Al content in various plant parts of rambutan as influenced by soil aluminum. Absence of curve fitting denotes lack of a significant response.



Total content of leaf Al (Figure 6) increased until 4.1 mM Al and then declined. These results agree with those of Goenaga and Smith (2002) and Duncan et al. (1980) who found that the concentration of leaf Al increased significantly with increases in soil Al. Studies with other crops (Wang et al., 2006) showed that Al accumulation in the root system of wheat increased significantly with increases in Al concentration in the rhizosphere. Piñeros et al. (2002) found that in maize, the Al content in root tips increased with increments in Al concentration of the nutrient solution. At 0.22 mM Al, the Al content in the maize root tip reached 2,951 µg/g. Matiello et al. (2008) found that the Al content was higher in coffee roots than in leaves with Al concentrations higher than 8,244 µg/g in high Al treatments suggesting an internal tolerance mechanism to support this high content of Al. In our study, the concentration of Al in stems and roots was 960 µg/g and 8,580 µg/g respectively, in the 10.2 mM Al treatment. Further studies are needed to assess the development and concentration of potential Al-ligand complexes which may be loaded into the xylem vessels of rambutan roots for export and sequestration in the shoots.

Some highly tolerant species can accumulate high concentrations of Al in above-ground plant parts without showing symptoms of Al toxicity presumably by detoxifying or compartmentalizing internal aluminum (Ma et al., 2001). Al-tolerant species known to posses internal tolerance mechanisms to withstand high Al include hydrangea and buckwheat which detoxify accumulated Al with organic acids. This mechanism allows these plant species to accumulate Al in their leaves to high levels in hydrangea (3000 µg/g) and moderately high levels in buckwheat (450 µg/g) as compared to plant species like wheat, which employ Al exclusion mechanisms in roots and accumulate less than 50 µg/g Al in their leaves (Kochian et al., 2002).

Other plant species that can accumulate Al in their leaves include *Melastoma malabathricum* (10,000 µg/g), *Miconia albicans* (11,000 µg/g), *Faramea marginata* (16,000 µg/g) and the tea plant (*Camellia sinensis)* which can accumulate 30,000 µg/g in older leaves and 600 µg/g in younger leaves (Watanabe and Osaki, 2002). In this study, the concentration of Al in rambutan leaves reached 1,040 µg/g when exposed to 10.2 mM Al for two months. Watanabe and Osaki (2002) classified Al accumulator plants as those exceeding a concentration of 1,000 µg/g in their leaves.

Rambutan growth

Aluminum treatments had a significant effect ($P \leq 0.01$) on total, leaf, stem and root dry weights. Increasing Al concentration from 0 to 10.2 mM resulted in a decrease in leaf, stem, root and total dry weight. Leaf dry weight of fallen leaves (ie., leaves that abscised as a result of treatment effects) increased with increments in Al levels in the nutrient solution (Figure 7).

Miyasaka et al., (1991) found that Al at 0.15 mM significantly reduced shoot and root dry weights in Al sensitive cultivar 'Romano' and Al tolerant cultivar 'Dade' snapbeans. In our study, significant reductions in dry weights of rambutan plant parts were observed at Al concentrations higher than 3.5 mM. Matiello et al., (2008) found a significant reduction in shoot and root dry weights in three different coffee cultivars at Al actvivities ranging from 0.0027 to 0.33 mM. In our study, a significant reduction in total dry matter occurred after an Al activity of 0.50 mM was reached confirming that rambutan is highly tolerant to acid soils.

As with dry matter, leaf number, leaf area, plant height and stem diameter significantly decreased with increases in Al concentration (Figure 8 A-D). These results agree with similar

studies conducted with other crops. Langer et al., (2009) studied four alfalfa cultivars and found that Al treatments (0.050 and 0.10 mM) caused a significant reduction in dry weight and reduced plant growth. The fact that rambutan seedlings grew relatively well even at an Al concentration of about 3.5 mM demonstrates that this crop is highly tolerant to Al. Similar nutrient culture studies with other crops such as alfalfa (Langer et al., 2009), soybean (Liao et al., 2006), barley (Tian-Rong et al., 2007) and wheat (Wang et al., 2006) showed significant reductions in dry matter production at much lower Al concentrations (0.05 mM to 1.48 mM) than those used in this study. Goenaga (2011) working with rambutan found that dry matter production, stem diameter and plant height were unaffected until very high levels of soil Al.

Figure 7. Dry weight of plant parts of rambutan at different aluminum concentrations

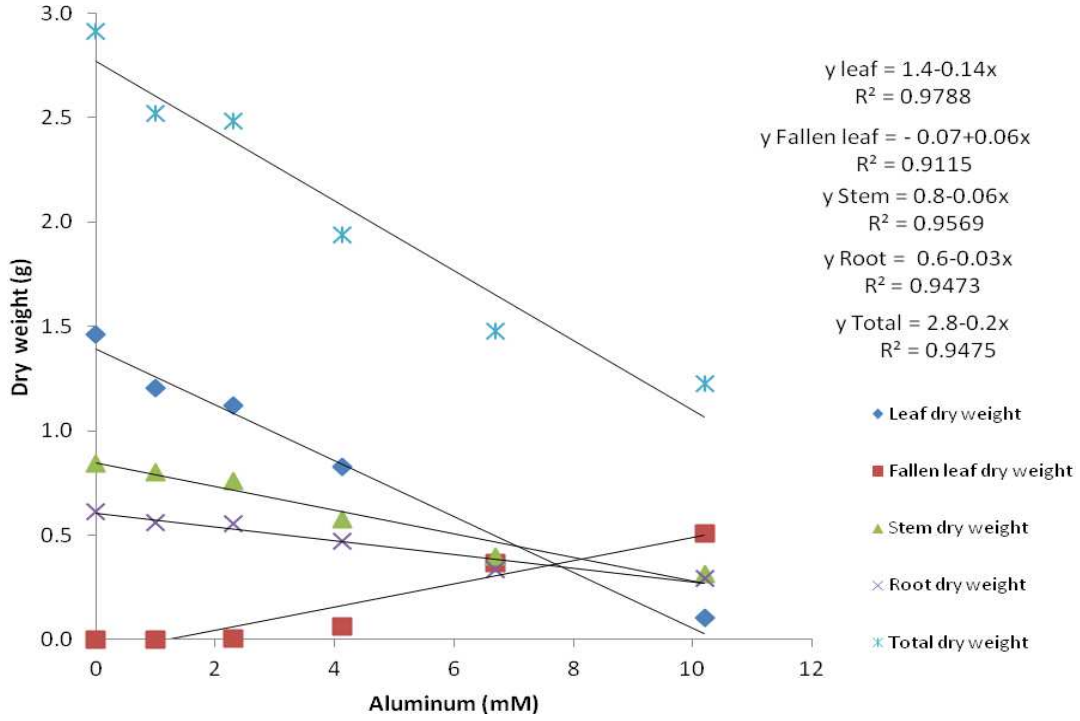
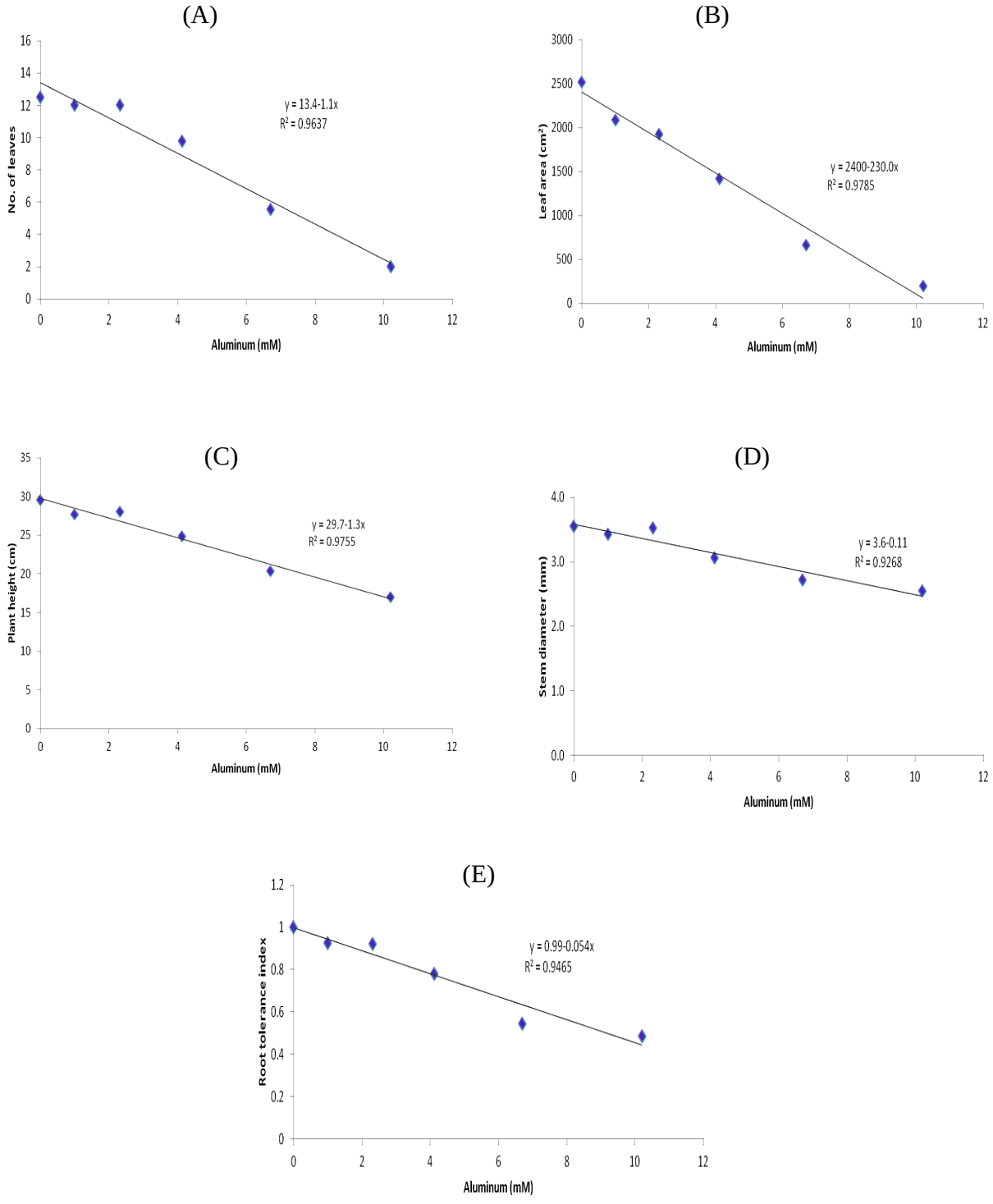


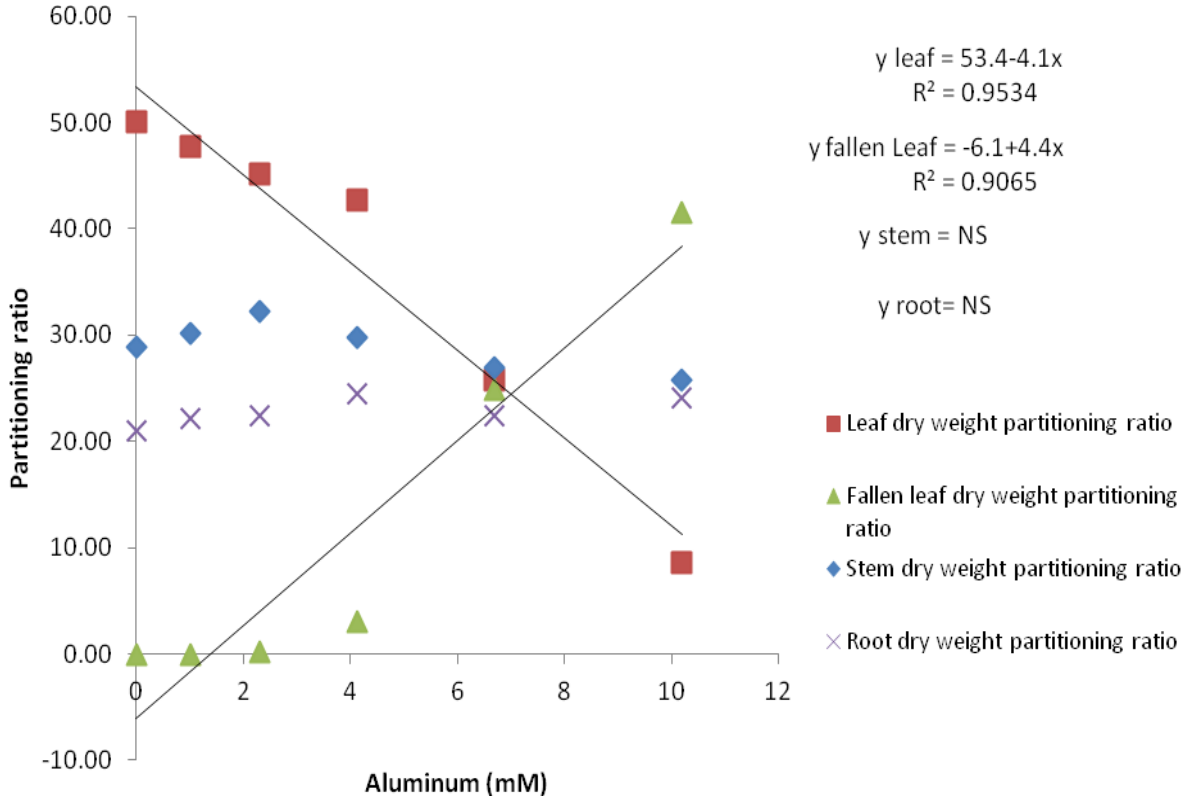
Figure 8. Plant growth parameters of rambutan at different aluminum concentrations



The root tolerance index remained relatively constant (1.00 to 0.85) in Al treatments ranging from 0 to 3.5 mM, but it declined significantly in Al treatments higher than 4.12 mM (Figure 8E). These results indicate that rambutan is highly tolerant to Al concentrations ranging from 0 to 3.5 mM. Taylor and Foy (1985) suggested that an RTI of 0.85±0.03 defines Al tolerance in wheat cultivars.

Ratios of dry matter partitioning to leaves, stems and roots as a fraction of total plant dry matter are presented in Figure 9. At Al concentrations ranging from 0 to 4.0 mM, plants allocated a greater percentage of their dry matter to leaves with this organ accounting for over 40% of the total dry matter in plants. At higher Al concentrations, the partitioning ratio decreased significantly for leaves. It is noteworthy that at Al concentration higher than 4.0 mM, total dry matter of fallen leaves increased significantly whereas the partitioning ratio of roots and stems was not significantly affected. This response may indicate a mechanism whereby plants reduced their leaf area as a way to minimize sink strength for carbohydrates to favor survival of other plant organs (e.g. roots).

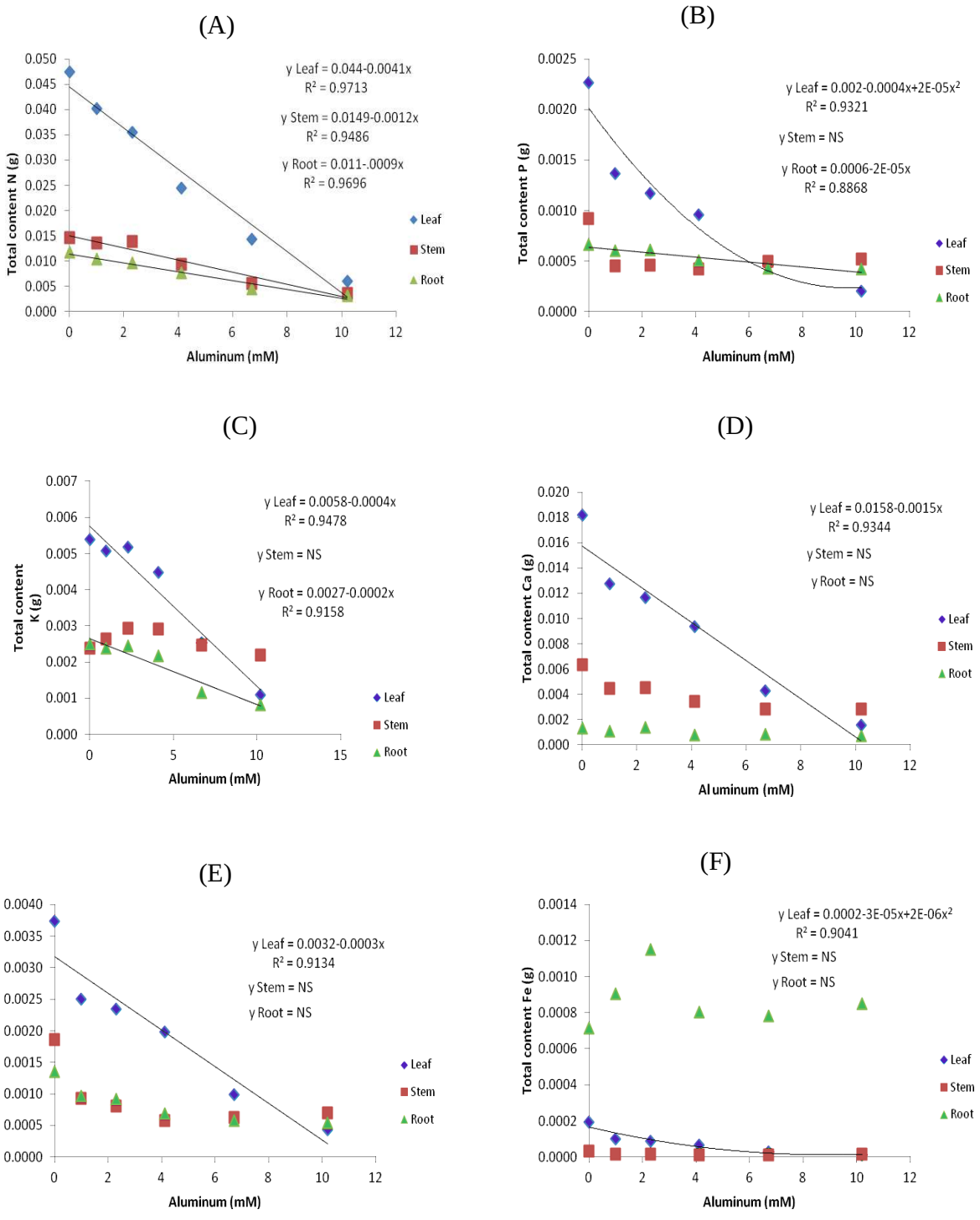
Figure 9. Relationship between dry matter partitioning of rambutan plant organs at different aluminum concentration. Absence of curve fitting denotes lack of significant response.



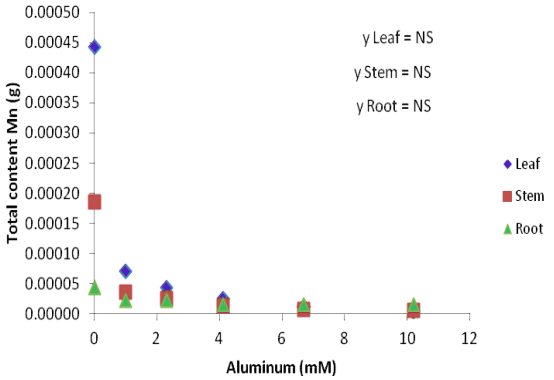
Rambutan mineral content and concentration

Figure 10 shows total content of nutrients in leaves, stems and roots collected at the end of the experimental period. As expected, increasing the concentration of Al resulted in significant reductions in total content of leaf N, P, K, Ca, Mg, Fe, Zn, B and Mo, particularly in leaf tissue. It is noteworthy that even though the content of most nutrients declined with increases in Al, the concentration of P in most tissues was generally significantly higher in high Al treatments but lower than in the control (0 mM) treatment (Tables 4 to 7). A similar response was observed for K and Ca for stem tissue. Al treatments did not have a significant effect on root Ca concentration even though Al is known to inhibit Ca uptake by blocking Ca channels in the plasma membrane (Huang et al., 1992). Goenaga and Smith (2002) found that adaptation of beans to acid soils may require efficient uptake and utilization of these nutrients, particularly calcium. Jun-ping et al. (2006) working with barley found that high Al causes deficiencies of essential nutrients like calcium, magnesium, iron and molybdenum and decreased availability of phosphorous result in overall stunting, dark green leaves, late maturity, purpling of stems, leaves and leaf veins, yellowing and death of leaf tips and thickened and distorted root systems. In this study some of these symptoms were visible when Al treatment concentration exceeded 4.12 mM (Figure 11).

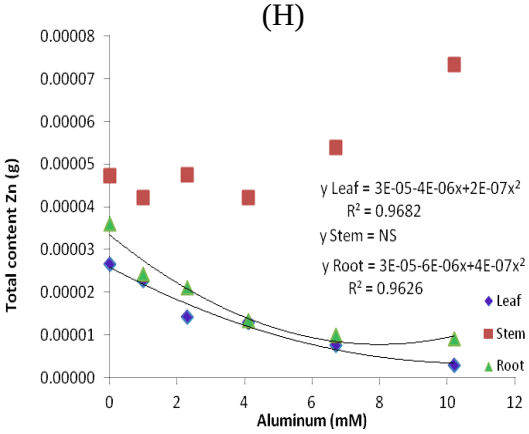
Figure 10. Total nutrient content in various plant parts of rambutan as influenced by soil aluminum. Absence of curve fitting denotes lack of a significant response.



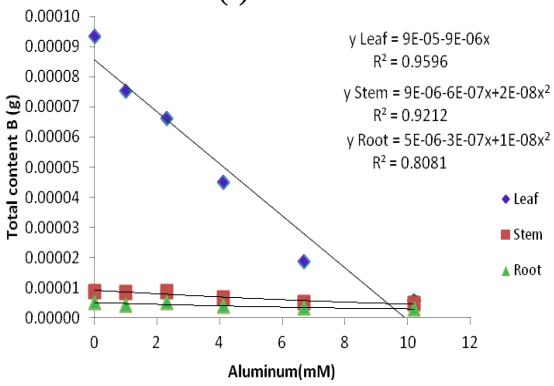
(G)


y Leaf = NS
y Stem = NS
y Root = NS
Leaf
Stem
Root
Total content Mn (g)
Aluminum (mM)

(H)


y Leaf = 3E-05-4E-06x+2E-07x²
R² = 0.9682
y Stem = NS
y Root = 3E-05-6E-06x+4E-07x²
R² = 0.9626
Leaf
Stem
Root
Total content Zn (g)
Aluminum (mM)

(I)


y Leaf = 9E-05-9E-06x
R² = 0.9596
y Stem = 9E-06-6E-07x+2E-08x²
R² = 0.9212
y Root = 5E-06-3E-07x+1E-08x²
R² = 0.8081
Leaf
Stem
Root
Total content B (g)
Aluminum(mM)

(J)

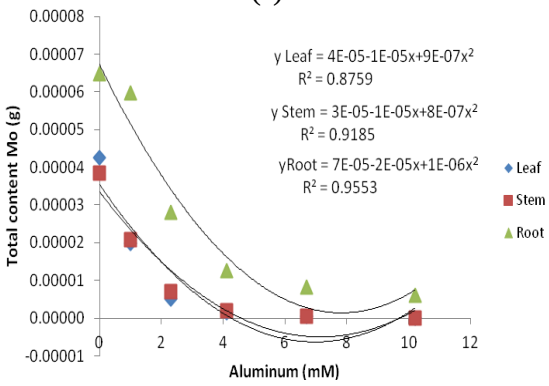

y Leaf = 4E-05-1E-05x+9E-07x²
R² = 0.8759
y Stem = 3E-05-1E-05x+8E-07x²
R² = 0.9185
yRoot = 7E-05-2E-05x+1E-06x²
R² = 0.9553
Leaf
Stem
Root
Total content Mo (g)
Aluminum (mM)

Table 4. Rambutan leaf nutrient concentration at various aluminum concentrations in nutrient solution

Al treatment (mM)	N %	P %	K %	Ca %	Mg %	Fe %	Mn %	Zn %	Al %	B %	Mo %
0.00	3.25	0.16	0.37	1.24	0.25	0.013	0.031	0.0018	0.0059	0.0064	0.0029
1.00	3.35	0.12	0.43	1.06	0.21	0.0083	0.0057	0.0018	0.0090	0.0062	0.0016
2.31	3.17	0.11	0.47	1.04	0.21	0.0080	0.0039	0.0013	0.011	0.0059	0.00044
4.12	2.97	0.12	0.54	1.13	0.24	0.0078	0.0030	0.0015	0.015	0.0055	0.00016
6.70	2.92	0.12	0.65	0.99	0.23	0.0062	0.0024	0.0016	0.025	0.0059	0.000083
10.20	2.76	0.15	0.76	1.01	0.29	0.0068	0.0026	0.0021	0.028	0.0038	0.000017
HSD	0.29	0.015	0.15	0.23	0.05	0.003	0.002	NS	0.006	0.003	0.0004

Note: Data presented as the mean over Al treatment and experiments (N=10). HSD is the minimum significant difference according to Tukey's Studentized Range Test. NS is not significant at $P \leq 0.05$.

Table 5. Rambutan fallen leaf nutrient concentration at various aluminum concentrations in nutrient solution

Al treatment (mM)	N %	P %	K %	Ca %	Mg %	Fe %	Mn %	Zn %	Al %	B %	Mo %
0.00	-	-	-	-	-	-	-	-	-	-	-
1.00	-	-	-	-	-	-	-	-	-	-	-
2.31	-	0.16	0.21	1.19	0.19	0.0072	0.0025	0.0055	0.016	0.0024	-
4.12	1.96	0.18	0.57	1.21	0.23	0.0078	0.0026	0.0022	0.016	0.0023	-
6.70	1.87	0.18	0.55	1.36	0.27	0.0083	0.0029	0.0024	0.037	0.0019	-
10.20	1.80	0.18	0.58	1.30	0.27	0.0078	0.0029	0.0031	0.068	0.0017	-
HSD	NS	NS	0.09	NS	NS	NS	NS	NS	0.04	0.0007	-

Note: Data presented as the mean over Al treatment and experiments (N=10). HSD is the minimum significant difference according to Tukey's Studentized Range Test. NS is not significant at $P \leq 0.05$.

Table 6. Rambutan stem nutrient concentration at various aluminum concentrations in nutrient solution

Al treatment (mM)	N %	P %	K %	Ca %	Mg %	Fe %	Mn %	Zn %	Al %	B %	Mo %
0.00	1.72	0.11	0.29	0.76	0.22	0.0039	0.0022	0.0058	0.0035	0.0011	0.0046
1.00	1.79	0.06	0.35	0.59	0.12	0.0020	0.0048	0.0057	0.0064	0.0011	0.0028
2.31	1.73	0.06	0.37	0.57	0.10	0.0021	0.0031	0.0060	0.0084	0.0011	0.00089
4.12	1.61	0.07	0.51	0.60	0.10	0.0021	0.0024	0.0073	0.014	0.0012	0.00035
6.70	1.37	0.13	0.63	0.73	0.16	0.0029	0.0019	0.015	0.038	0.0013	0.00011
10.20	1.11	0.17	0.70	0.89	0.22	0.0048	0.0018	0.024	0.079	0.0015	0.000015
HSD	0.20	0.02	0.06	0.10	0.04	0.002	0.002	0.007	0.02	0.0002	0.0005

Note: Data presented as the mean over Al treatment and experiments (N=10). HSD is the minimum significant difference according to Tukey's Studentized Range Test. NS is not significant at $P \leq 0.05$.

Table 7. Rambutan root nutrient concentration at various aluminum concentrations in nutrient solution

Al treatment (mM)	N %	P %	K %	Ca %	Mg %	Fe %	Mn %	Zn %	Al %	B %	Mo %
0.00	1.92	0.11	0.41	0.21	0.22	0.12	0.0071	0.0060	0.07	0.00085	0.011
1.00	1.86	0.11	0.43	0.19	0.17	0.16	0.0039	0.0044	0.23	0.00076	0.011
2.31	1.73	0.11	0.44	0.25	0.16	0.20	0.0039	0.0037	0.35	0.00087	0.0050
4.12	1.61	0.11	0.46	0.17	0.14	0.16	0.0031	0.0028	0.40	0.00079	0.0027
6.70	1.34	0.13	0.35	0.25	0.17	0.24	0.0043	0.0030	0.57	0.0010	0.0025
10.20	1.07	0.15	0.27	0.24	0.18	0.29	0.0049	0.0032	0.68	0.0010	0.0020
HSD	0.16	0.01	0.06	NS	0.06	0.15	0.003	0.002	0.10	NS	0.001

Note: Data presented as the mean over Al treatment and experiments (N=10). HSD is the minimum significant difference according to Tukey's Studentized Range Test. NS is not significant at $P \leq 0.05$.

Figure 11. Symptoms of Al toxicity in rambutan seedlings. A) death of leaf tips; B) overall stunting; C) thickened and distorted root systems

(A)

(B)

(C)

The reduction in leaf P and Ca agree with Matiello et al. (2008) who found that at an Al concentration of 2.0 mM phosphorus concentration decreased significantly when compared to an Al concentration of 1.0 mM and that Ca in roots is not significantly affected by increasing concentration of Al.

Root elongation depends on adequate concentration of Ca for optimum growth. Increased concentration of Ca in the root rhizosphere, have been shown to ameliorate Al toxicity (Jun-ping et al. 2006). In this study, total content of Ca (Figure 10D) in roots was not significantly affected. The decrease in total content of Ca in leaves can be attributed to Al in roots inhibiting Ca uptake and translocation to the leaves (Delhaize and Ryan, 1995).

CONCLUSIONS AND FUTURE WORK

The results of this study revealed that even at an aluminum concentration of 3.5 mM, rambutan plants grew relatively well, confirming that this crop is highly tolerant to high Al as compared to other crops. The hypothesis that rambutan tolerates high concentration of Al by exudation of organic acids (Al exclusion mechanism) is rejected. Accumulation of Al in leaves, stems and roots in this study suggests the existence of an Al-sequestration mechanism in rambutan which may involve an Al-ligand complex which translocates from roots to shoots, where it may accumulate in leaf vacuoles. Future studies should be directed toward finding the ligand and mechanism responsible for conferring high Al tolerance in rambutan. This mechanism may be transferrable to Al-sensitive crops through genetic manipulation.

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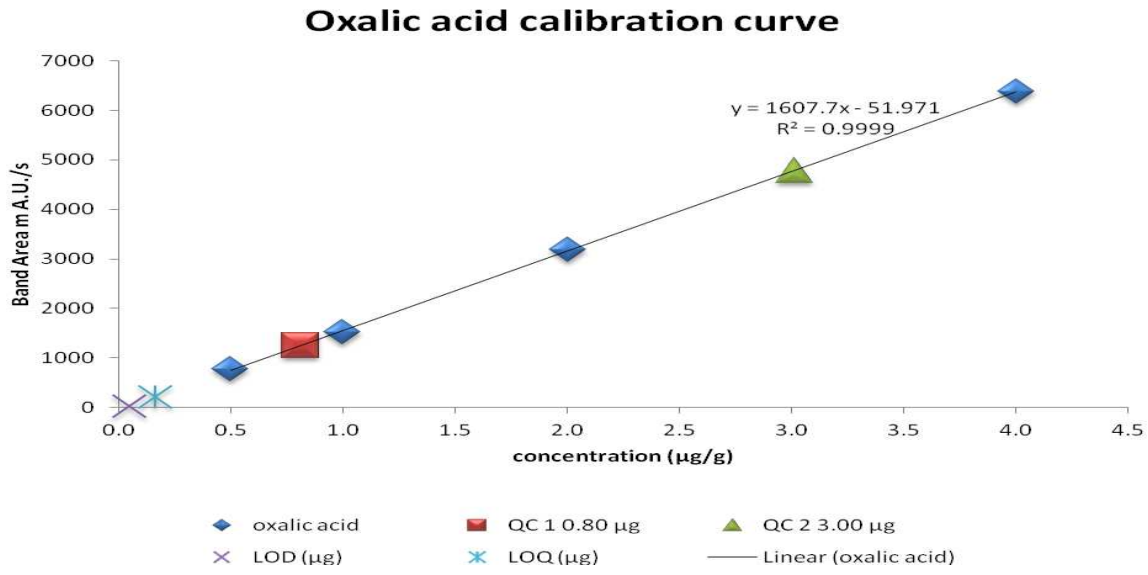
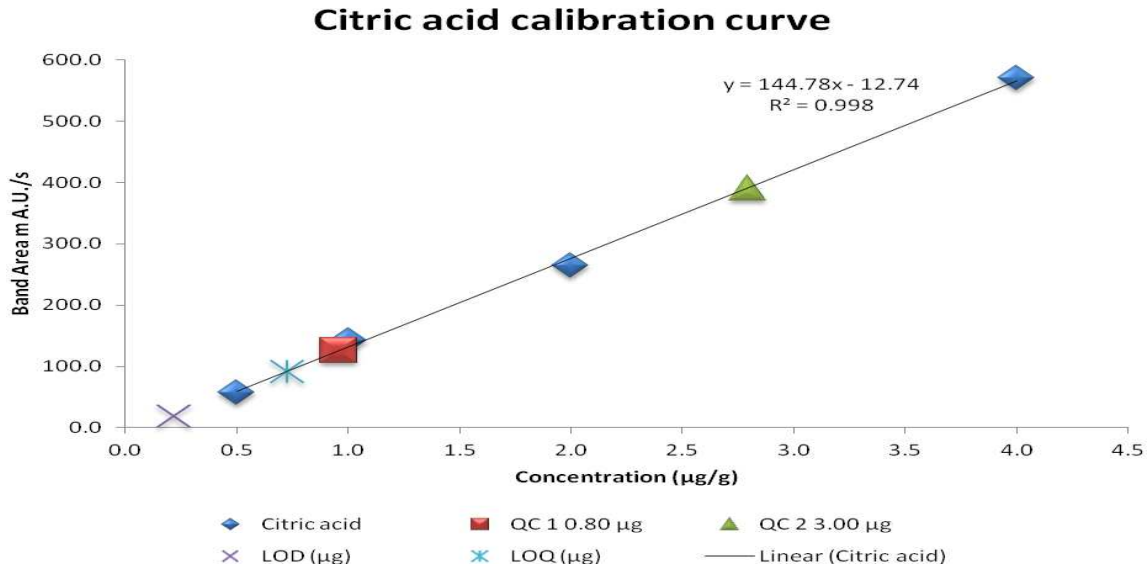
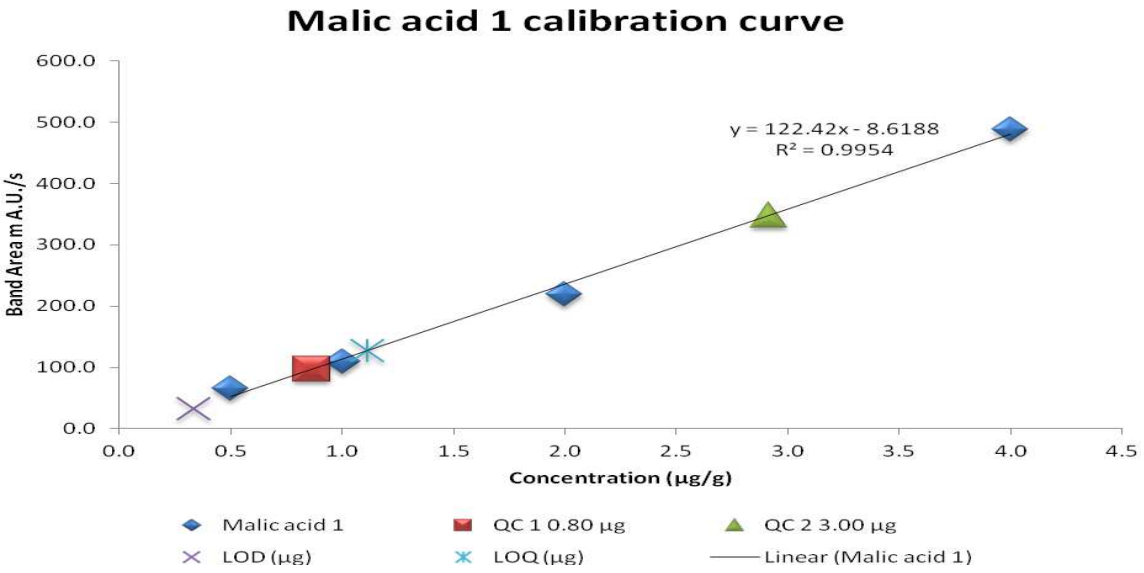
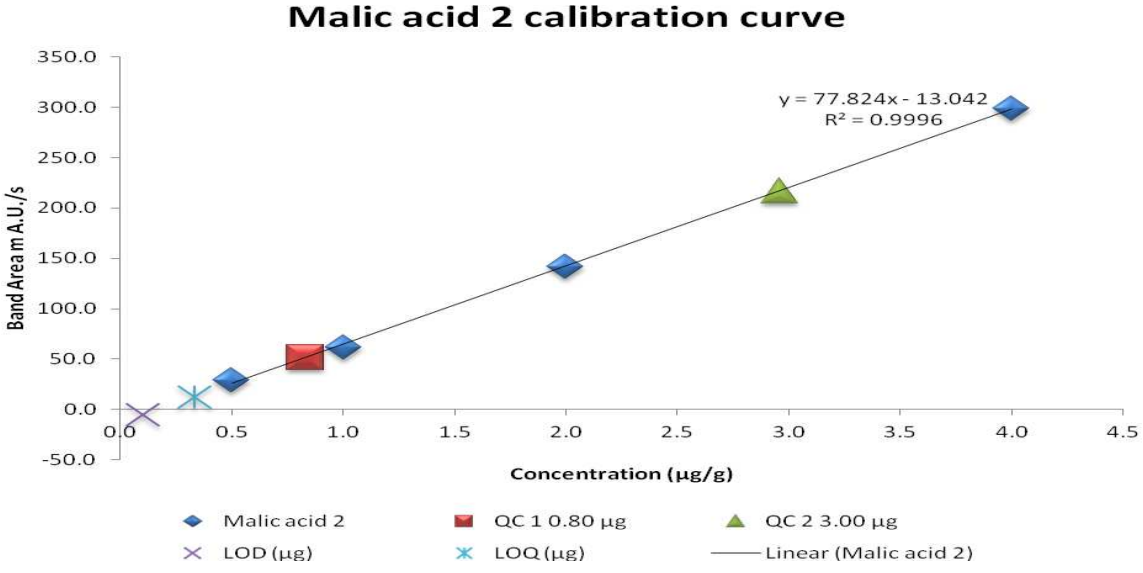
Appendix A. HPLC instrumentation used for organic acid analysis

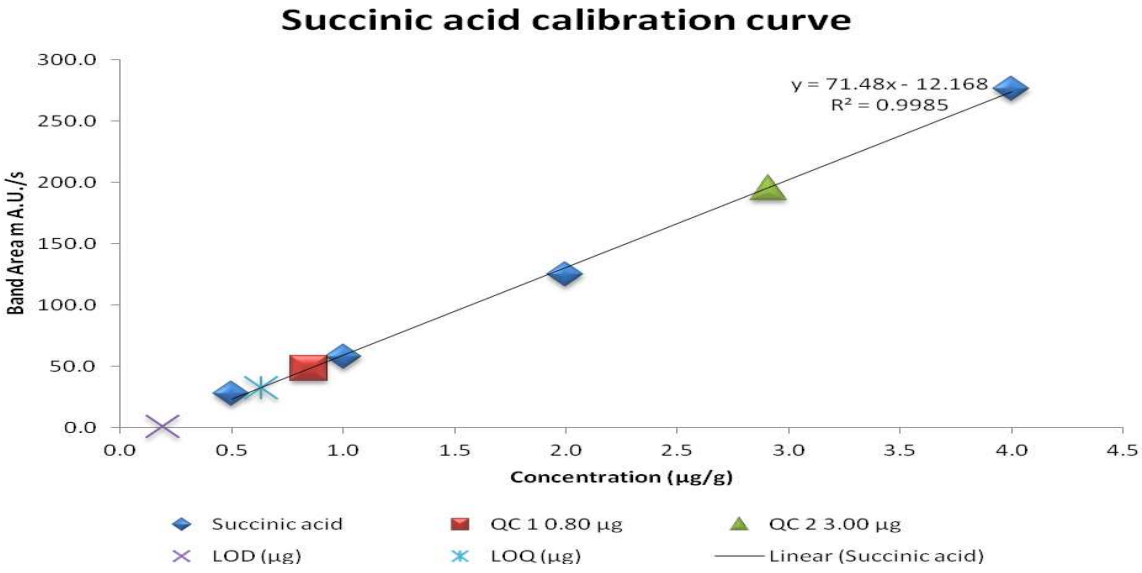
Appendix B. Calibration curves of organic acids



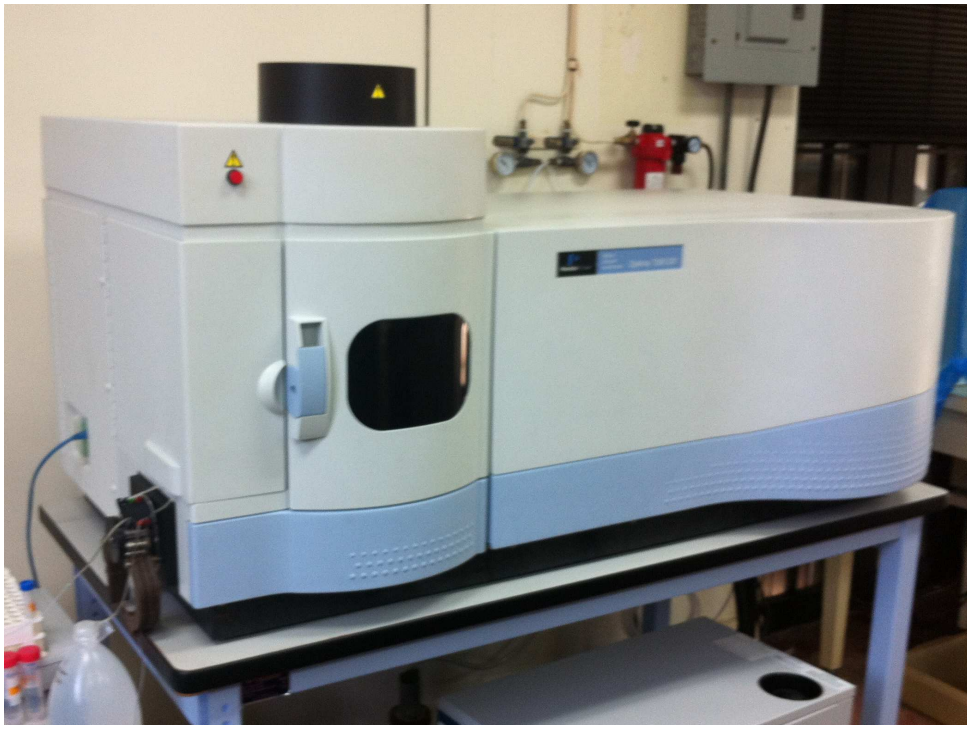



Malic acid 1 calibration curve
y = 122.42x - 8.6188
R² = 0.9954
Band Area m A.U./s
Concentration (µg/g)
Malic acid 1
QC 1 0.80 µg
QC 2 3.00 µg
LOD (µg)
LOQ (µg)
Linear (Malic acid 1)

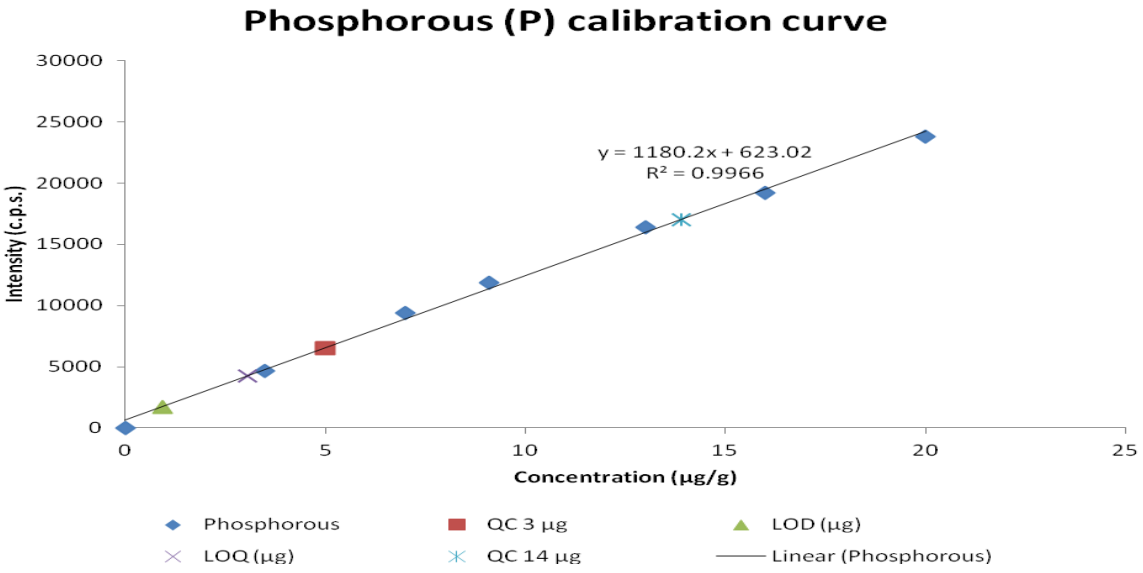

Malic acid 2 calibration curve
y = 77.824x - 13.042
R² = 0.9996
Band Area m A.U./s
Concentration (µg/g)
Malic acid 2
QC 1 0.80 µg
QC 2 3.00 µg
LOD (µg)
LOQ (µg)
Linear (Malic acid 2)

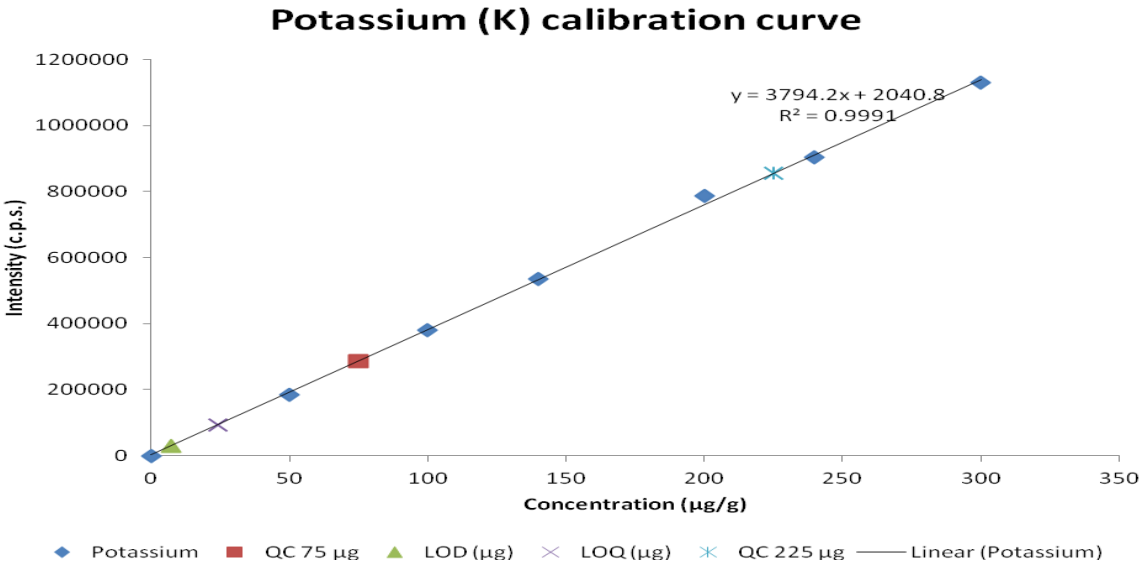

Succinic acid calibration curve
y = 71.48x - 12.168
R² = 0.9985
Band Area m A.U./s
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Concentration (µg/g)
Succinic acid
QC 1 0.80 µg
QC 2 3.00 µg
LOD (µg)
LOQ (µg)
Linear (Succinic acid)

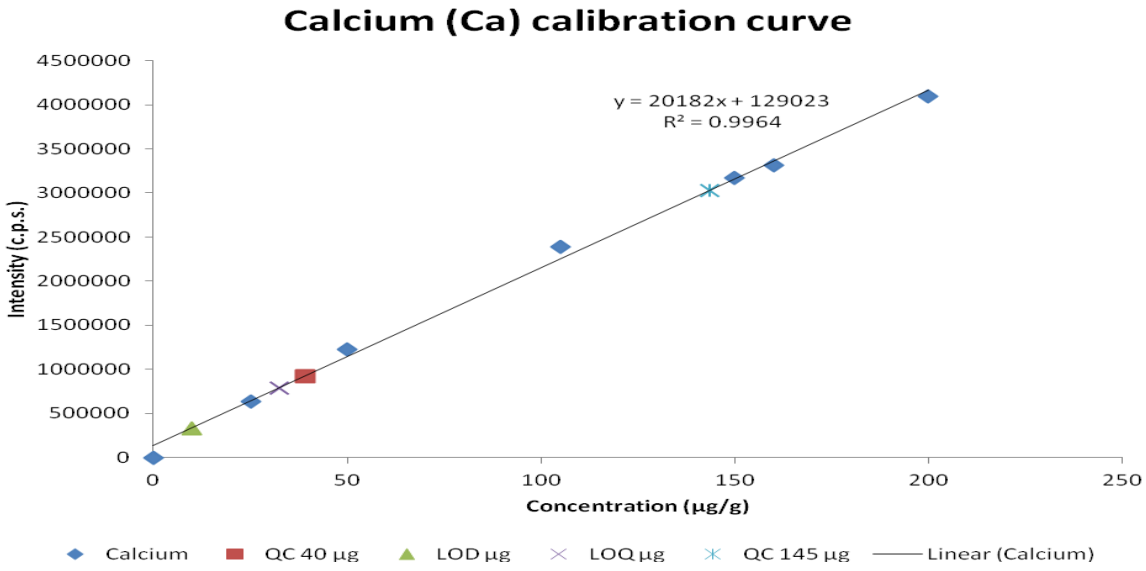
Appendix C. ICP-OES 7300 DV instrument used for mineral content analysis

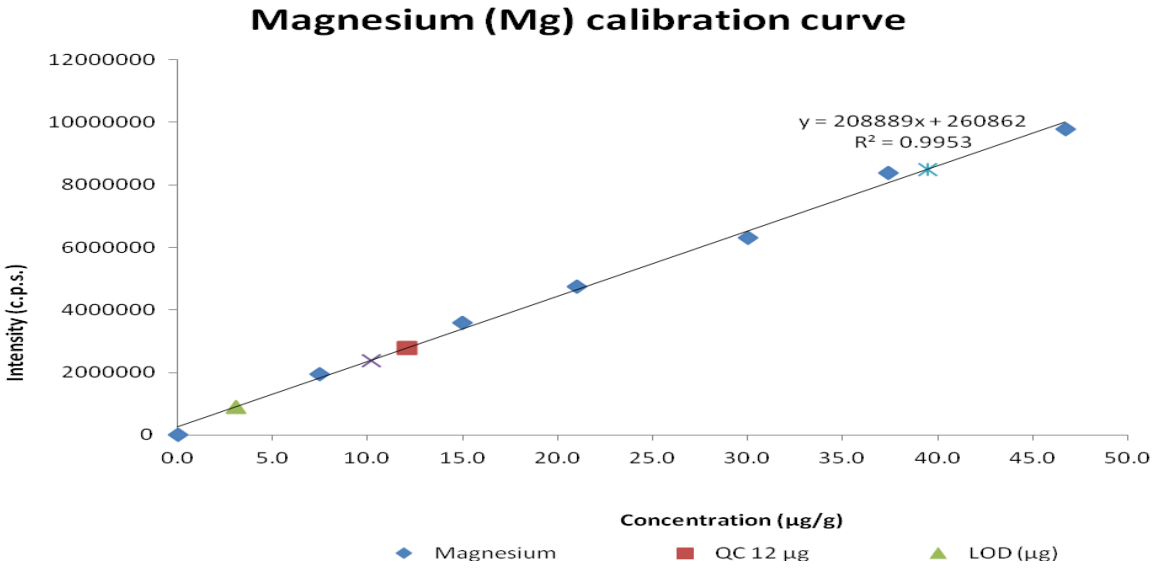


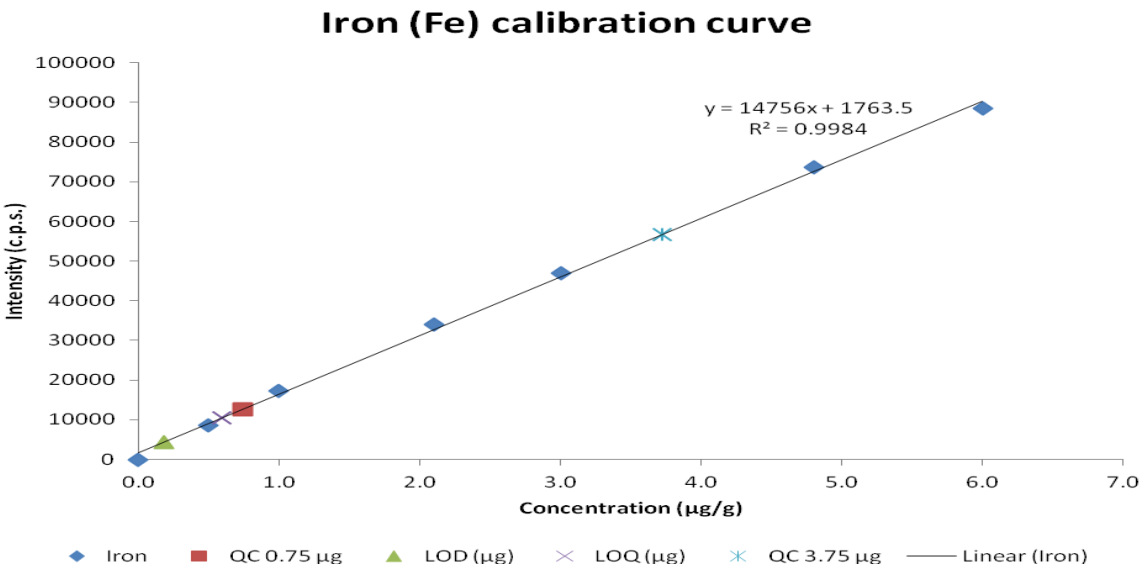
Appendix D. Calibration curves for various elements determined in the study by ICP-OES

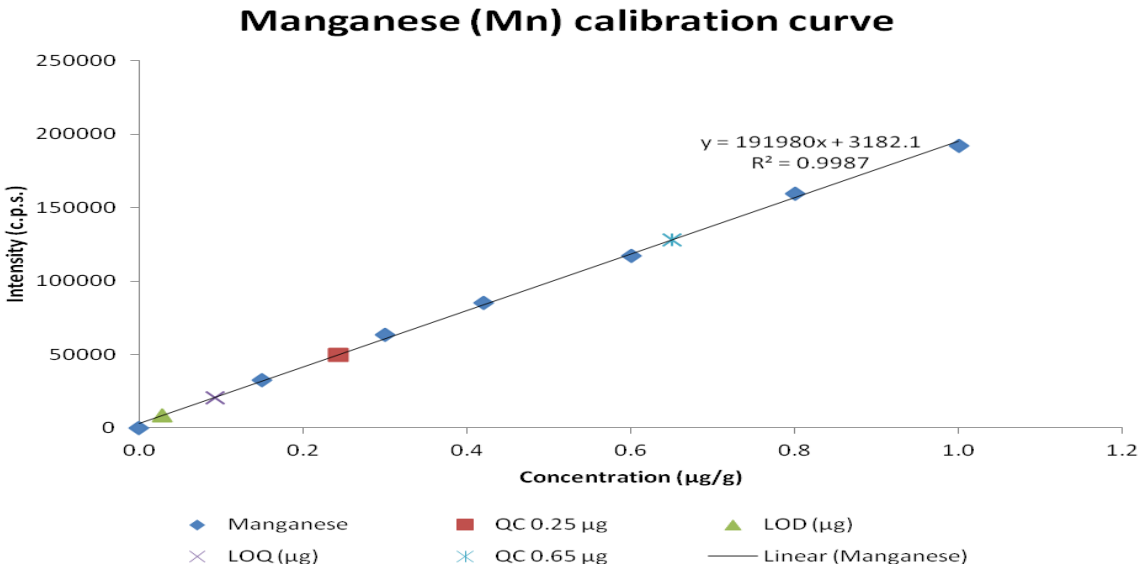


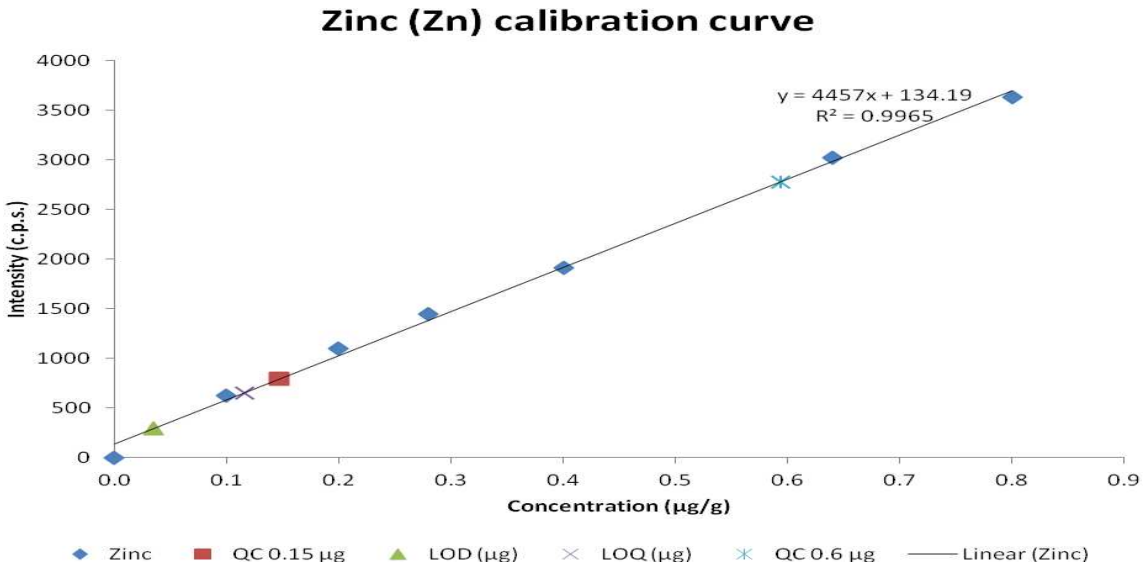


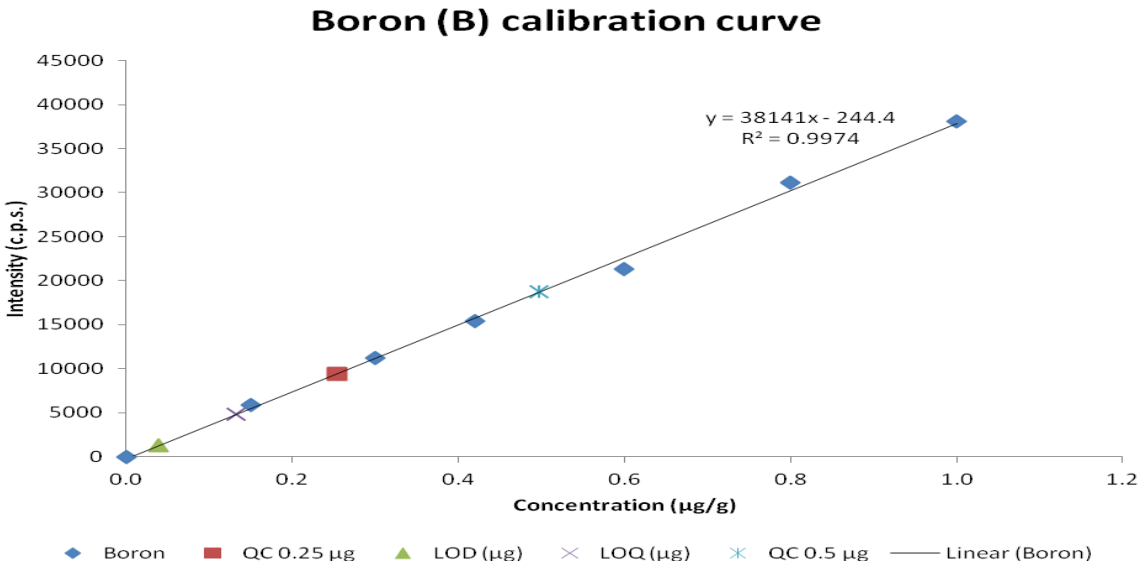

Calcium (Ca) calibration curve
y = 20182x + 129023
R² = 0.9964
Intensity (c.p.s.)
Concentration (µg/g)
Calcium
QC 40 µg
LOD µg
LOQ µg
QC 145 µg
Linear (Calcium)

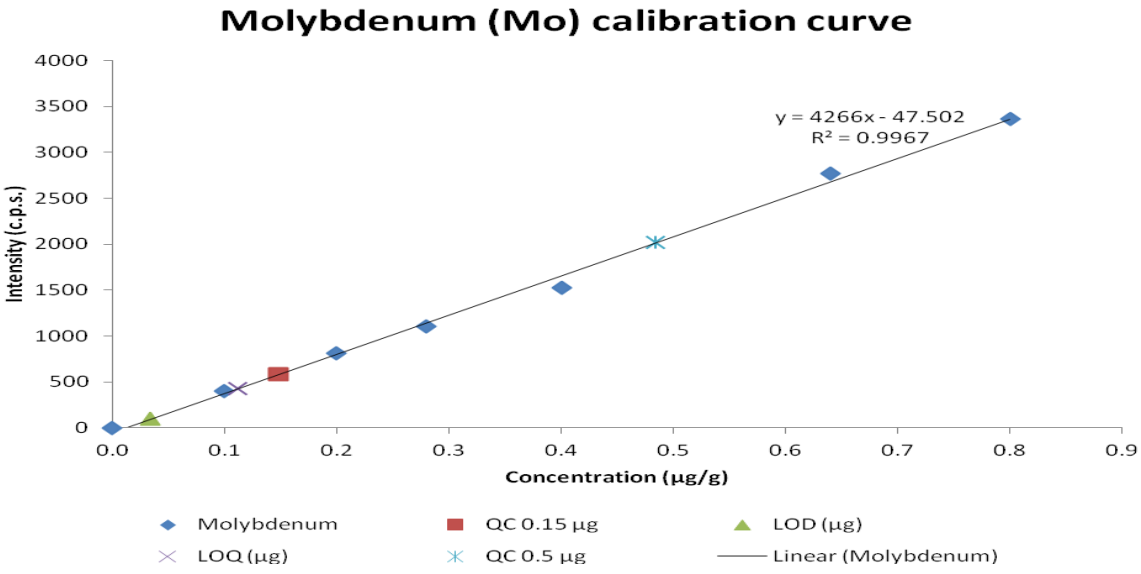

Magnesium (Mg) calibration curve
y = 208889x + 260862
R² = 0.9953
Intensity (c.p.s.)
Concentration (µg/g)
Magnesium
QC 12 µg
LOD (µg)

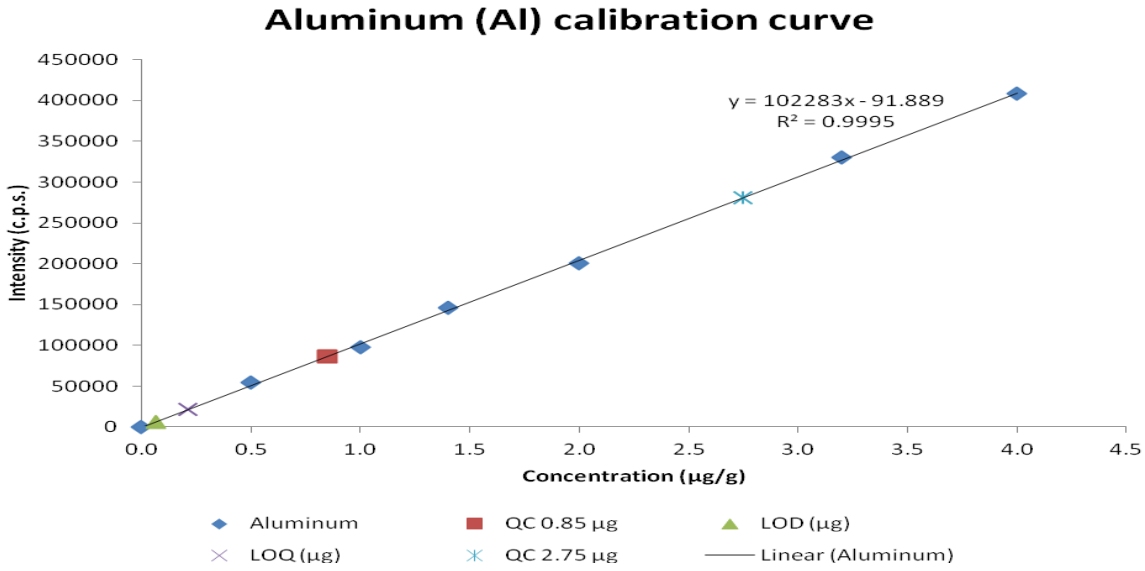

Iron (Fe) calibration curve
y = 14756x + 1763.5
R² = 0.9984
Intensity (c.p.s.)
Concentration (µg/g)
Iron
QC 0.75 µg
LOD (µg)
LOQ (µg)
QC 3.75 µg
Linear (Iron)


Manganese (Mn) calibration curve
y = 191980x + 3182.1
R² = 0.9987
Intensity (c.p.s.)
Concentration (µg/g)
Manganese
QC 0.25 µg
LOD (µg)
LOQ (µg)
QC 0.65 µg
Linear (Manganese)


Zinc (Zn) calibration curve
y = 4457x + 134.19
R² = 0.9965
Intensity (c.p.s.)
Concentration (µg/g)
Zinc
QC 0.15 µg
LOD (µg)
LOQ (µg)
QC 0.6 µg
Linear (Zinc)


Boron (B) calibration curve
y = 38141x - 244.4
R² = 0.9974
Intensity (c.p.s.)
Concentration (µg/g)
Boron
QC 0.25 µg
LOD (µg)
LOQ (µg)
QC 0.5 µg
Linear (Boron)


Molybdenum (Mo) calibration curve
y = 4266x - 47.502
R² = 0.9967
Intensity (c.p.s.)
Concentration (µg/g)
Molybdenum
QC 0.15 µg
LOD (µg)
LOQ (µg)
QC 0.5 µg
Linear (Molybdenum)


Aluminum (Al) calibration curve
y = 102283x - 91.889
R² = 0.9995
Intensity (c.p.s.)
Concentration (µg/g)
Aluminum
QC 0.85 µg
LOD (µg)
LOQ (µg)
QC 2.75 µg
Linear (Aluminum)

Appendix E. Block digestion and distillation unit Kjeltec 2100 used for total nitrogen analysis