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A Preliminary Study on Expression of Zinc Transporter Gene of Cassava Grown in Nutrient Solutions with some Physiological and Biochemical Responses

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ABSTRACT

Cassava growth can be affected by either zinc (Zn) deficiency or excess supply throughout the crop cycle. In this study, cassava plants (cv. KU50) were grown in Enshi's nutrient solutions combined with three Zn levels (0, 1.0 and 10.0 μM) in a plastic house. Procedures covering sampling and replacement with fresh nutrient solutions were conducted weekly. The results indicated that the Zn application at 10.0 μM produced a significant increase in shoot and total dry matter. The dry weight shoot-root ratio showed a significant increase at all Zn levels. Thus, Zn nutrient promoted shoots more than roots during the early growth stage of cassava. An increased Zn supply increased the cassava Zn accumulation and Zn uptake, but decreased the Zn utilization efficiency. Under different Zn conditions, the partial *MeZIP* gene was observed in all plant parts. At a Zn-deficient level, the transcription patterns of the partial *MeZIP* showed high expression in the roots rather than in the stems and leaves. The differential expression of the *MeZIP* gene corresponded to the different Zn levels. This implied that the partial *MeZIP* gene reflected plant performance to overcome the Zn deficiency.

Keywords: cassava**,** zinc deficiency, zinc accumulation, zinc uptake, zinc transporter

INTRODUCTION

Cassava is one of the staple food crops in the tropics and is a major economic crop being the third most important source of calories after rice and maize (Howeler *et al*., 2013). In Thailand, cassava production is utilized as ethanol (5%), pellets for domestic consumption (8%), pellets for export (32%), starch for domestic consumption (19%) and starch for export (36%) according to Poramacom *et al.* (2013), indicating that 68% of cassava products in Thailand are for export. Thus, cassava cultivation is expanding rapidly with the major planted areas being 55% in northeastern, 20% in northern and 25% in central areas of the country, respectively (Office of Agricultural Economics, 2012). Normally, cassava can be grown in many areas of Thailand where there is a sandy-loam to loamy-sand soil texture and a pH in the range 4.5–6.0 (Ratanawaraha *et al*., 2001) However, some cassava planted in northeastern Thailand has been reported to exhibit Zn deficiency

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symptoms in calcareous soil (Havlin *et al*., 2005; Thanimmarn, 2011; Takrattanasaran *et al*., 2013). Alloway (2008) reported that crop production on calcareous soil tends to be Zn deficient because of the low levels of organic matter and available nitrogen whereas the pH and calcium carbonate content in this soil are high. Zn is an essential micronutrient which is involved in physiological plant growth and metabolic processes, including enzyme activity, protein synthesis and the metabolism of carbohydrates, lipid, auxins and nucleic acid (Rehman *et al*., 2012). The average concentration of Zn required for optimal plant growth varies between 25 and 150 mg.kg^{-1} and Zn can limit plant growth when it is present in either low or excessive concentrations (Marschner, 1995). In cassava, Zn deficiency is characterized by chlorosis in the interveinal region of the upper leaves (Howeler, 2002) which can cause a reduction in net photosynthesis by 50–70% due to a major decrease in the chlorophyll content and the abnormal structure of the chloroplasts (Alloway, 2008). In cereals, Zn deficiency decreased the shoot dry matter production (Cakmak *et al*., 1998) while reduced shoot, root weight and main stem and root lengths were reported in *Arabidopsis thaliana* (Talukdar and Aarts, 2008).

Alloway (2008) reported that toxic symptoms can develop when the Zn concentration threshold of toxicity was greater than 150 mg Zn. kg^{-1}. In kidney bean, increasing the Zn accumulation (200–500 µM) decreased the fresh mass and leaf area, net photosynthetic rate, transpiration, stomatal conductance, rate of apparent photosynthetic electron transport and isoprenoids accumulation (Vassilev *et al*., 2011). Similarly, in poplar, a Zn concentration at 1,000–5,000 µM resulted in a reduction in leaf area and thickness, biomass, photosynthesis, Fe and chlorophyll content and an increase in the leaf density (Fernàndez *et al*., 2012).

In plants, Zn is absorbed from the rhizophere via cation transporters and it is potentially involved in metal ion homeostasis (Mäser *et al*., 2001). Zn translocation was found in the xylem and phloem as the divalent form and an organic acid bond (Kochian, 1991) and was regulated by a ZIP (zinc-regulated, iron regulated transport-like protein) (Li *et al*., 2013). The ZIP gene family has been identified and its functions investigated in several plants, such as *Arabidopsis thaliana*, *Medicago truncatula*, *Lycopersicon esculentum*, *Glyzine max*, *Oryza sativa*, *Hordeum vulgare*, *Thlaspi japonicum* and *Thlaspi caerulescens* (Pedas *et al*., 2009). Grotz *et al*. (1998) reported that under a Zn deficiency, the Arabidopsis *ZIP1* and *ZIP3* are expressed primarily in roots whereas *ZIP4* is induced in both the shoots and roots. In cassava grown in tissue culture, the *MeZIP* gene was highly expressed in young leaves and stems when compared with fibrous roots and the expression level of this gene was higher in low Zn-treated plants (Bamrungsetthapong *et al*., 2010). In barley under Zn-deficient conditions, the *HvZIP7* gene was strongly induced and found expression in the vascular tissues of roots and leaves. In addition, when moderately high concentrations of Zn were supplied, the *HvZIP7* gene was observed and its over expression in barley increased the Zn uptake (Tiong *et al*., 2013).

Thus, the effect of Zn levels on the physiological performance of cassava growth, Zn accumulation, Zn uptake and the expression of the *MeZIP* gene of cassava during the early growth stage were evaluated to provide improved understanding of the Zn uptake mechanism in cassava.

MATERIALS AND METHODS

Plant materials and nutritional treatments

Cassava (*Manihot esculenta* Crantz cv. KU50) stem cuttings were pre-grown in sand culture for 2 wk before transferring to Enshi's nutrient solution (Shinohara and Suzuki, 1988) in a hydroponic culture system. Cassava stems with sprouts were grown hydroponically with

six plants per 15 L container aerated with the basic Enshi's nutrient solution containing 0, 1.0 and 10.0 μM Zn as $ZnSO_4.7H_2O$, respectively. The fresh nutrient solution was replaced weekly. Throughout the experiment, the plants were exposed to natural light intensity, temperature, humidity and photoperiod in the plastic house.

Sampling procedures

The plant growth parameters for the plantlets were sampled at 7 and 14 d after transplanting (DAT) and the agronomic characteristics of stem height, root length and root number were measured. The three plant parts of leaves, stems and roots were separated and determined for fresh weight and then oven dried at 65 °C for 72 hr to obtain constant weight measurements.

Biochemical analysis

Plant samples were separated into leaves, stems and roots and then oven dried and ground. The samples (about 0.5 g) were digested in 4 mL HNO_3 and 2 mL HCl in a microwave digestion oven (Multiwave^{TM} 3000; Graz, Austria). After digestion, each solution was cooled, filtered through Whatman No. 0 filter paper, and brought to a final volume of 25 mL with deionized water. The filtered extracts were preserved under refrigeration (4 °C) until analysis. Zn concentrations were determined using atomic absorption spectrophotometry (AAS; Perkin-Elmer Analyst 300; Waltham, Massachusetts, USA). The accuracies of the elemental analyzes for Zn were confirmed by carrying standard reference materials through the digestion and analytical process, as well as one blank sample for quality control. The Zn uptake and Zn utilization efficiency were calculated according to El-Nasharty *et al*. (2013).

Semi-quantitative polymerase chain reaction analysis

Total RNA was extracted from the plant parts of cassava using the modified CTAB method (Doyle and Doyle 1990). The total RNA quality and quantity were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA). The cDNA was synthesized from the oligo $(dT)_{18}$ primer with 800 ng of total RNA using M-MuLV RT (Fermentas; Burlington, Ontario, Canada). PCR reactions were assembled with Taq polymerase according to the manufacturer's recommendation. The partial *MeZIP* gene was amplified by using the forward primer ZpF 5'-CACTCTGTGATAATAGGAATCTCTTTAGG-3' and the reverse primer ZpR 5' -CTCTTCTT GTTGGAGCA-3'. Sequencing revealed that the partial *MeZIP* gene was 399 bp in length. The polymerase chain reaction (PCR) cycle was: 1 cycle at 94 °C for 2 min, 40 cycles at 94 °C for 30 sec, 58 °C for 1 min and 72 °C for 1 min, followed by 1 cycle at 72 °C for 10 min. The PCR products were analyzed by electrophoresis on 1% agarose gel in 1X TAE buffer at 100 V for 30 min, stained with ethidium bromide and visualized under ultra violet light. The expression level of the *MeZIP* gene was assessed by measuring the intensity of the bands using the imageJ software (Schneider *et al*., 2012).

Experimental design and statistical analysis

The experiment was conducted using a completely randomized design (CRD) with three replications. Data were subjected to analysis of variance appropriate for a CRD. Separated analysis of variance was performed for each measurement. Mean comparisons were accomplished using a least significant difference test at the 5% level.

RESULTS

Cassava growth performance

The effects of Zn levels on cassava growth are shown in Table 1. It was found that the dry weight of shoots and roots increased with increasing Zn concentrations. In particular, the dry weight of shoots showed significant differences

among the different Zn levels with the highest shoot weight observed in the plants treated with a high Zn level (10.0 μM). Similarly, the shoot-root ratio was significantly different among different Zn levels. In the 10.0 μM Zn treatment, there was a higher shoot-root ratio compared to the control (0 μM Zn) as shown in Table 1, which reflected an increased shoot dry weight with increasing Zn concentrations. The total dry matter was significantly increased with increasing Zn levels and the highest weight was observed at the high level of Zn. The 1.0 μM Zn and 10.0 μM Zn levels increased the total dry matter by 7% and 70%, respectively, compared to the control (Table 1). The dry weight analysis showed a higher weight partition to shoots than to roots with increasing Zn levels. The dry weight was higher in shoots than in roots with a 6% shoot dry weight increase, while the root dry weight decreased approximately 26% when compared with the control (Figure 1a).

At 14 DAT, the root number and root length were significantly increased in the Zn-treated plants. In particular, the Zn level of 1.0 μM produced the highest root number and greatest root length but these growth parameters reduced at the high Zn level (10.0 μM Zn) as shown in Figures 1b and 1c. The stem height did not differ significantly with Zn level (Figure 1d).

Zn accumulation and use efficiency

The Zn accumulation in all plant parts (roots, stems and leaves) at 14 DAT was determined using AAS. It was found that Zn accumulation in all plant parts increased when the Zn concentration increased (Figure 2). Compared to the control, Zn accumulation in the roots increased by 68% and 620% at 1.0 μM Zn and 10.0 μM Zn, respectively; in the stems, Zn increased by 7% and 40% at 1.0 μM Zn and 10.0 μM Zn, respectively; and in the leaves, Zn increased by 29% and 60% at 1.0 μM Zn and 10.0 μM Zn, respectively. Thus, Zn accumulation was greater in the roots than in the leaves and stems, respectively.

The Zn uptake (mg per plant) increased with increasing Zn concentrations. The Zn uptake at each Zn concentration level approached that of the high Zn concentration at 10.0 μM (Figure 3a). Linear regression indicated that Zn uptake accounted for 94% of the variation in the shoot dry weight (Figure 3b). The Zn utilization efficiency (measured in grams dry weight per milligram of Zn uptake) decreased with increasing Zn concentrations (Figure 3c) while linear regression indicated that the Zn utilization efficiency accounted for 52% of the variation in shoot dry weight (Figure 3d).

***MeZIP* gene expression responding to the availability of Zn**

The differential expression of partial *MeZIP* cDNA was conducted on the cassava plants grown in the presence of different concentrations of Zn in the nutrient solution at 14 DAT. All plant parts (leaves, stems and roots) were harvested for

Table 1 Effects of different Zn levels on dry weight of shoots, roots, total dry matter (TDM) and shoot-root ratio at 14 d after transplanting.

Zn levels (μM)	Shoot	Root	TDM	Shoot-root ratio
	(g per plant)			
0	3.55[b]	0.95	4.50[b]	3.73[b]
1	3.55[b]	1.30	4.85[b]	2.73[b]
10	6.45[a]	1.20	7.65[a]	5.37[a]
F-test	*	ns	*	**

Means within a column with the same letters are not significant at $P < 0.05$ based on a least significant difference test.
* = Significant at $P = 0.05$; ** = Significant at $P = 0.01$; ns = Not significant.

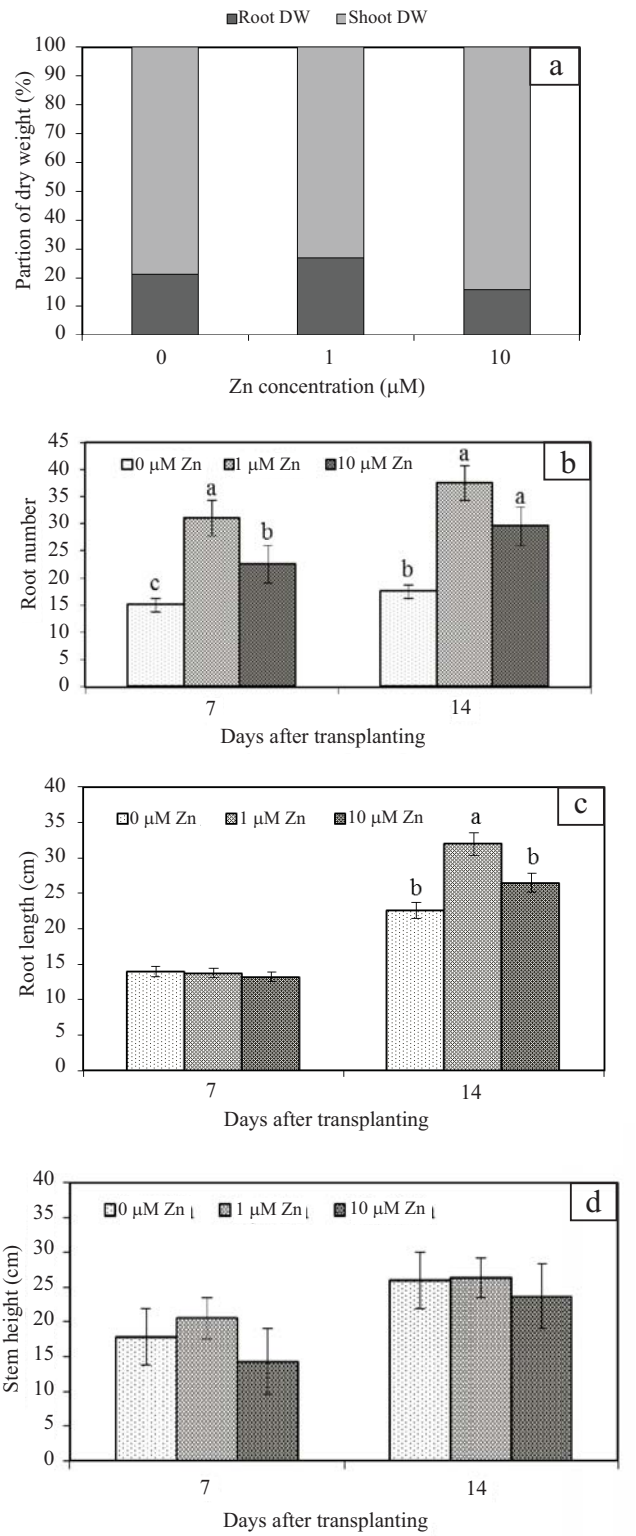

Figure 1 Effect of different Zn levels on: (a) Partition of dry weight (DW) at 14 d after transplanting (DAT); and at 7 and 14 DAT on (b) Root number; (c) Root length; and (d) Stem height.

total RNA extraction. Semi-quantitative PCR was used to determine the expression of the partial *MeZIP* gene and partial cassava 18S rRNA was used as a reference. The results showed that the partial *MeZIP* gene can be detected in all cassava plant parts but was more highly expressed in the roots compared with the leaves and stems (Figure 4).

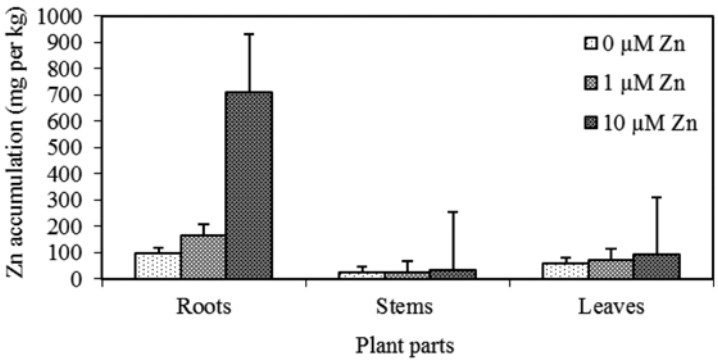

Figure 2 Effect of Zn levels on Zn accumulation in roots, stems and leaves of cassava plant grown in nutrient solution for 14 d after transplanting.

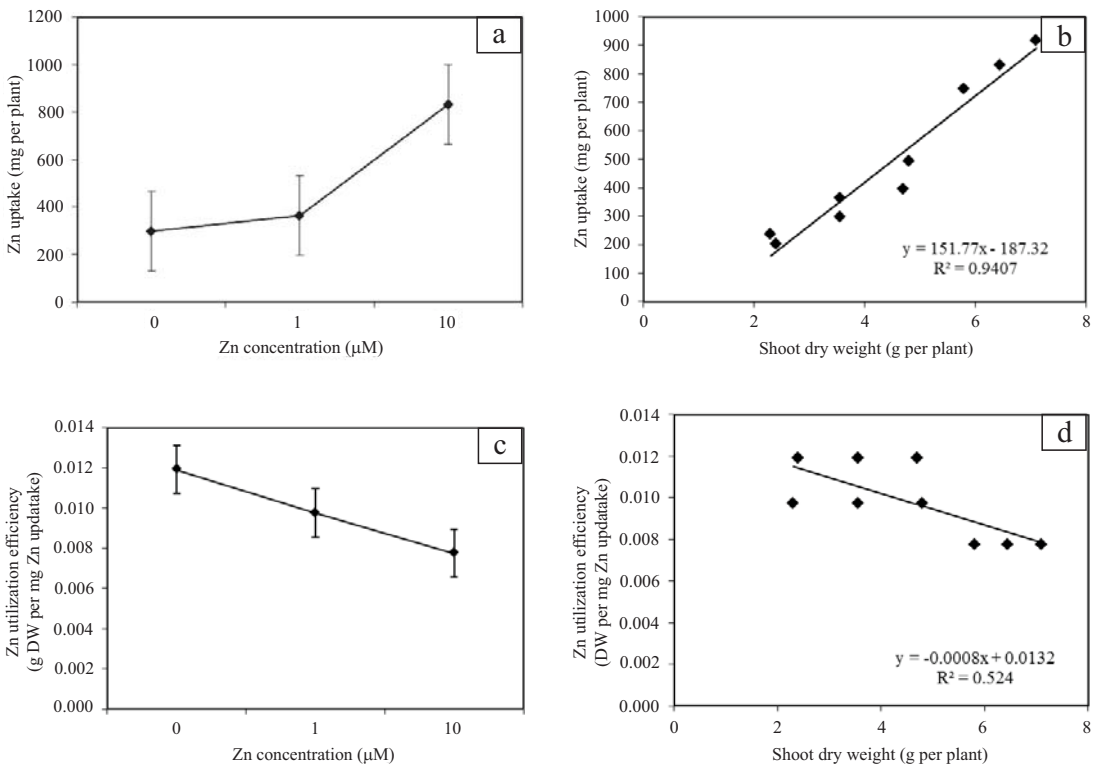

Figure 3 Effect of different Zn levels on: (a) Zn uptake; (b) Interrelationship between shoot dry weight (DW) with Zn uptake; (c) Zn utilization efficiency; and (d) Interrelationship between shoot dry weight with Zn utilization efficiency. R^2 = Regression co-efficient. Error bars indicate mean ± SE.

The investigation of the partial *MeZIP* gene in cassava plants showed differential expression corresponding to various Zn concentrations. In leaves and stems, the partial *MeZIP* was highly expressed at the concentration of 0 µM Zn and then gradually decreased with increasing Zn concentrations (Figures 4a and 4b).

The band density was detected by the imageJ software which indicated the relative expression level of the *MeZIP* gene. When compared to the 1.0 µM Zn, it was found that the relative expression of the *MeZIP* gene in Zn deficiency (0 µM Zn) was increased 1.02-, 1.77- and 1.02-fold in leaves, stems and roots, respectively. In contrast, at the 10.0 µM Zn concentration, the relative expression of the *MeZIP* gene was decreased 0.27-, 0.24- and 0.97-fold in leaves, stems and roots, respectively (Figure 5).

DISCUSSION

Zn is an essential nutrient for plants and plays an important role in the physiological process of growth and development of plants. The current results found that a Zn application increased the total dry weight of cassava. This result was consistent with the previous studies of Ma and Uren (2006) and Tahir *et al*. (2009) which reported that the application of Zn produced a significant increase in the dry weight of the shoots and the whole plant in maize. Additionally, the increased Zn concentration in cassava plants also increased the shoot-root ratio which indicated that Zn could promote shoot growth by using a higher Zn level as a main source for supplying food in the early growth stage of cassava. Reduced biomass production with Zn deficiency (0 µM Zn) was also observed. The reduction of plant growth as a result of Zn deficiency could have been due to a decrease in the growth hormones in which Zn is essential for tryptophan synthesis—a prerequisite for auxin formation; therefore the amount of auxin decreases with a Zn deficiency (Mousavi, 2011).

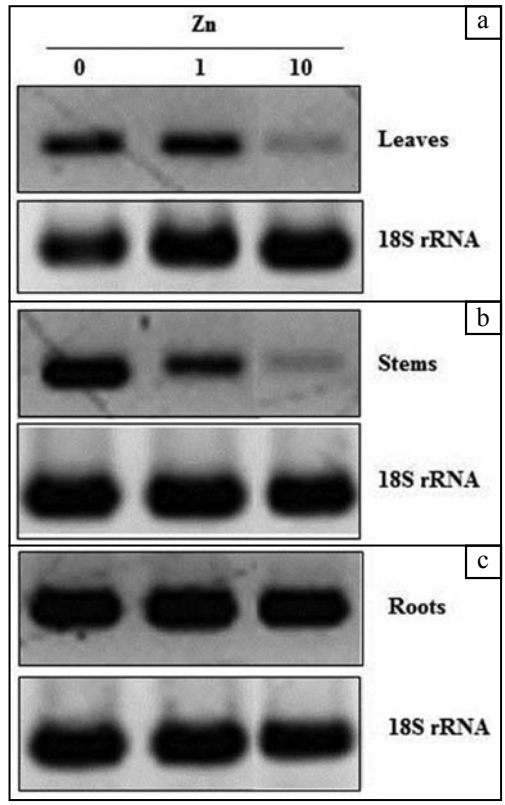

Figure 4 Differential transcriptional response of partial *MeZIP* gene toward different Zn treatment levels in cassava plants at 14 d after transplanting using partial cassava 18S rRNA as a reference: (a) Leaves; (b) Stems; and (c) Roots.

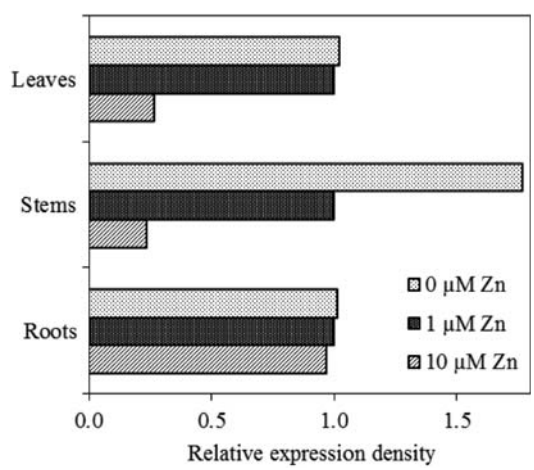

Figure 5 Relative expression density of partial *MeZIP* gene at 14 d after transpiration.

However, Gomes *et al*. (2013) reported that Zn had both stimulatory and deleterious effects on plant biomass production. Their research found that the root number, root length and stem height decreased under low and high Zn concentrations. Fernàndez *et al*. (2012) reported that the number of young leaves, the stem length and stem diameter declined with increasing Zn concentration (5,000 µM Zn). Similarly, biomass production was reduced under Zn deficient conditions and high Zn concentrations (Bamrungsetthapong *et al*., 2010; Fernàndez *et al*., 2012; Gomes *et al*., 2013).

Zn accumulation was increased with increasing Zn levels and accumulated to greater levels in the roots than in the leaves and stems, respectively. Talukdar and Aarts (2008) reported that under all tested condition, the Zn concentration accumulated more in the roots than in the flowers, leaves, stems and siliques, respectively. The Zn uptake observed in the current research shows an increase in uptake with increased Zn concentration. During crop growth, the Zn uptake increased with an increased rate of Zn concentration (Chaudhary and Singh, 2007). The increase in Zn uptake was due to the increased application and hence availability of the applied Zn in the root zone and the role of roots in the growth and development of the plant. However, Zn utilization efficiency decreased at a high Zn concentration. El-Nasharty *et al*. (2013) reported that increasing the Zn application in wheat decreased Zn use efficiency and Zn utilization efficiency. Similarly, in rice, the increasing Zn use efficiency was due to decreased rates of Zn application (Fageria *et al*., 2011). This was due to the inverse relationship often observed between utilization and the rate of application (Muthukumararaja and Sriramachandrasekharan, 2012).

The relationship between the availability of Zn in nutrient solutions and the transcriptional pattern of the partial *MeZIP* gene was evaluated in cassava cv. KU50. The expression of the partial *MeZIP* gene was observed in all parts of cassava with high expression in the roots compared with the leaves and stems. In addition, the differential expression of the partial *MeZIP* gene was detected in the leaves and stems which corresponded to different Zn levels. However, the partial *MeZIP* gene was always highly expressed in the roots under all Zn conditions. Bamrungsetthapong *et al*. (2010) reported that under tissues culture conditions, the *MeZIP* gene was highly expressed in young leaves and stems when compared with fibrous roots and the level of *MeZIP* transcription was higher under Zn deficient conditions. In rice, *OsZIP* was highly expressed in the shoots and roots under conditions of Zn deficiency (Ishimaru *et al*., 2011). In *A. thaliana*, under Zn deficient conditions, the *ZIP1* and *ZIP3* genes were expressed primarily in the roots whereas *ZIP4* was induced in both the shoots and roots (Grotz *et al*., 1998). Thus, the results suggested that the partial *MeZIP* was spatially expressed and the highest level of the partial *MeZIP* gene in the roots can be associated with transporting Zn from the external environment into the plant. Moreover, the high expression level of *MeZIP* in Zn deficiency could promote the supply of Zn as a micronutrient required for the growth and development of cassava.

CONCLUSION

Different Zn concentrations induced a response in cassava growth, with the biomass of cassava increased with increasing Zn concentrations. The shoot-root ratio was increased significantly with the highest ratio at 10.0 µM Zn. An increased Zn concentration could promote shoot growth more than root growth when compared to a control without Zn. The Zn accumulation and Zn uptake were increased corresponding to the increased Zn concentrations. In contrast, the Zn utilization efficiency was decreased when the Zn concentrations were increased. The *MeZIP* gene was expressed in all plant parts with the highest expression in the roots compared with the leaves and stems. The highest expression of the *MeZIP*

gene was found in the treatment without Zn which proves that the higher level of expression of the *MeZIP* gene reflects the plant's performance to overcome the Zn deficiency.

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