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Citrate Root Exudation under Zn and P Deficiency

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ABSTRACT

Zinc and phosphorus are essential nutrients with low bioavailability in calcareous soils. Some plants exude organic acids to increase the solubility of these two nutrients. The objective of this study was to examine citrate exudation rates of different lupin (Feodora and Energy) and rapeseed (Dunkeld, Yickadee and Rainbow) cultivars under deficiencies of Zn and P. The plants were cultivated into three different nutrient solutions (complete, -Zn, and -P) with pH around 7. Under Zn deficiency, rapeseed cultivars lost about 80% of its shoot fresh weight, but the roots did not exude any organic acids such as citrate, malate or oxalate. Both lupin and rapeseed cultivars exuded citrate only under phosphorus deficiency. The exudation rates of Feodora and Energy were 3.89 μmol g^{-1} RDW h^{-1} and 3.45 μmol g^{-1} RDW h^{-1}, respectively, while that of Dunkeld was 15.1 μmol g^{-1} RDW h^{-1}. The results indicated that lupin and rapeseed lost their production under Zn deficiency but they did not exude organic acid, while under P deficiency both plants exuded citrate.

Keywords: Citrate, deficiency, exudation rate, lupin, phosphorus, rapeseed, Zn

INTRODUCTION

Zinc deficiency is a widespread problem in plant cultivation. It is estimated that half of cereal crops worldwide are grown on Zn deficient soils (Cakmak 2008). Some cereal crops (rice, wheat, etc.) which serve as staple food, are deficient in Zn (Fageria et *al*. 2002). This trace element is essential for crop productivity and nutritional food quality. Soils do not supply sufficient Zn which result in a yield reduction and poor plant quality. Plants with Zn deficiency exhibit intervenal chlorosis and necrotic spots on the leaves (Alloway 2008). Moreover, low concentrations of Zn can cause leakage of cations, *e.g.* K^+ from the root system, so Zn deficiency can disturb the operating efficiency of the root system (Pinton *et al.* 1993).

Zinc concentration in plant tissue is determined by the bioavailability of Zn in soil and plant uptake. Soil parameters such as soil pH, CEC, soil organic matter content, clay contents, redox potential and total Zn are considered as important parameters for Zn bioavailability in soil solution. Calcareous soils which have a high pH are more prone to Zn shortage (Mehrotra *et al.* 1986) , since the solubility of Zn is limited at high soil pH due to Zn binding to the soil matrix (Van Breemen and Castro 1980) and to precipitation. However, organic amendments can enhance trace element availability in alkaline soils, and correct Zn and Fe deficiency (Bohn *et al.* 2001). Zinc in solution is found in the form of free zinc (Zn^{2+}) or is associated within organic ligands. These soluble Zn species are available to plants. Zinc forms soluble complexes with low molecular weight organic acids and inorganic compounds (chloride, phosphate, nitrate) then contributes to total soluble concentration in soil (Alloway 2008) .

Phosphorus (P) is an essential macronutrient for plants and animals. Almost 90% phosphate rock worldwide is used for food production (Schröder *et al.* 2010). In agriculture, P fertilizer is the main source of phosphorus. Phosphate rock, such as mineral apatite, is the primary raw material for producing P fertilizer. However, P availability is low in calcareous soil, because P can be bound by calcium and forms Ca-P mineral. Therefore, in alkaline soils P is deficient for plants. In order to improve P solubility, microorganism can be used to solubilize phosphate (Salih *et al.* 1989). Some plants also exude some organic acids to increase the bioavailability of P. For example, rapeseed has physiological adaptation when grown under P deficiency by exuding citrate and malate in the root zone. Citrate has a capability to mobilize P in soil with high Ca-P content (Jones and Darrah 1995) .

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Some plant roots exude some low molecular weight organic acids (LMWOA) to increase the bioavailability of micronutrients such as Fe and Zn under deficiency of Fe and Zn (Jones and Darrah 1995) . LMWOAs such as citric acid are engaged in the nutrient acquisition process (Jones and Darrah 1993) by complexing with metal ions and mobilizing those ions to the root surface (Cakmak 2008). Some researches related to the citrate as the mobilizator of Zn and P had been done. Citrate exudation rate by rice plant was correlated with minus zinc and phosphorus condition (Hoffland *et al.* 2006). Moreover, oxalate and citrate were more efficient than malate in extracting P under calcareous soils, as it was found that malate was degraded faster than oxalate and citrate. White lupin and rapeseed were able to extrude low molecular organic acid when responding to nutrient stress condition. The youngest proteoid rootlets of white lupin exuded citrate, which an exudation rate 6.1 nmol h^{-1} cm^{-1} root, to acidify the rhizosphere under P deficiency (Keerthisinghe *et al.* 1998). Lupin formed more cluster roots and exuded more proton as well as citrate and acid phosphatase under minus P than sufficient P treatment (Shen *et al.* 2005).

Another finding also suggested that lupin might excrete organic acid when the plant took up more anions than cations in order to keep the internal electroneutrality of the plant (Loss *et al.* 1993) . Furthermore, *Brassica napus* also exuded citrate under P deficiency (Hoffland *et al.* 1992) . Some cultivars of lupin and rapeseed, however, had not been identified for its citrate exudation rates; while it was assumed that different cultivars had different tolerance on Zn and P shortage. For instance, CSIRO-I , which is a genotype of *Brassica juncea*, was reported as a superior genotype in transporting Zn from soil to plant and also significant in acidifying pH rhizhosphere under low Zn availability (Grewal *et al.* 1997) . Therefore, study about concentration rates of different lupin and rape cultivars under Zn and P deficiency and their relation to Zn, Fe and P availability in alkaline soil was needed. Thus, this study would provide information that might be useful for further investigation to solve Zn and P deficiency problem in alkaline soils.

The first objective of this study was to investigate citrate exudation rates of lupin and rapeseed grown under Zn and P deficiency with high pH. The second was to study influence of cluster root of lupin and rapeseed on citrate exudation rate.

MATERIALS AND METHODS

Plant

White lupin (*Lupinus albus* L.) and rapeseed (*Brassica napus* L.) were used for nutrient solution experiment which was conducted at Wageningen University. White lupin can acidify the rhizosphere by exuding organic acids. It is a good plant reference for studying root exudation. Two white lupin cultivars (Energy and Feodora) and three rapeseed cultivars (Dunckeld, Yickadee and Rainbow) were used in the nutrient solution experiment. Dunkeld was a Zn efficient genotype, while the other two genotypes were inefficient (Grewal *et al.* 1997). Those seeds were germinated in moist quartz sand for a week until the second cotyledon emerged. The plants were grown in the climate chamber with temperature control 25/18 °C (day/night) and the light intensity was 525 μM m^{-2} s^{-1}.

Nutrient Slution Experiment

The nutrient solution experiment aimed to determine the exudation rate of citrate that was exuded by different plant cultivars under different levels of zinc and phosphorus. After the seeds were sown for a week in quartz sand, seedlings were transplanted to 50 L containers containing continuously aerated nutrient solution. The composition of nutrient solution is shown in Table 1. Three different boxes (A, B, C) were treated with three different treatments: complete, minus zinc (-Zn) and minus phosphorus (-P), respectively. In the complete treatment, all nutrients were applied, while for -Zn or –P treatments, $ZnSO_4$ or KH_2PO_4 were omitted.

Four seedlings of each rapeseed and lupin cultivars were planted in the containers, but only two plants were chosen for further analysis. The plants were grown in the climate chamber with temperature control 25/18 °C (day/night) and the light intensity was 525 µM m^{-2} s^{-1}. After 21 days, plants were harvested for collecting the LMWOA released by roots. The pH of the solution was maintained at around pH 7.3 during the cultivation, as this value represents pH of calcareous soils where located in tropical area. Calcareous soil is usually low in Zn and P availability. Result of exudation rates from the solution experiment were used in soil extraction experiment, which used soil from tropical (Saudi Arabia) and sub tropical (Turkey) areas.

The method for collecting root exudation was based on Gao (Gao *et al.* 2009) . Plants were harvested and the roots were rinsed with deionized

Table 1. Nutrient composition on nutrient solution.

Nutrients	Concentration (µ*M*)
KH_2PO_4	200
$Ca(NO_3)_2 .4 H_2O$	2,000
$MgSO_4 .7 H_2O$	1,000
K_2SO_4	440
KCl	625
H_3BO_3	6.25
$MnCl_4 .4H_2O$	0.375
$ZnSO_4 .5H_2O$	0.8
$CuSO_4 .5H_2O$	0.2
$(NH_4).6Mo.7O_{24}.4H_2O$	0.1
FeHBED	5

Souce: (Jaitz *et al.* 2011).

water. Afterwards, each plant was put individually in a petridish containing about 30 ml of 0.001 *M* $CaCl_2$ solution for 2 h. Calcium chloride solution was used for maintaining the osmotic pressure of solution surrounding the roots. Subsequently, 10 ml of exudation solution were filtered using 0.45 µm (Ø) filter and added with 50 µl of $CHCl_3$ to avoid microbial degradation. The clean samples were analyzed directly using capillary electrophoresis (CE) according to Galli (Galli *et al.* 2003). After collecting root exudates, roots were cut to determine surface area using root scanner and WinRHIZO program. Subsequently, both shoot and root were dried in oven at 70°C for 72 h and weighed.

Low Molecular Weight Organic Acid Analysis (LMWOA)

Capillary electrophoresis (CE) (Waters Corp., Milford, MA, USA) was used to analyze LMWOA (citrate). Method of Westergaard was used to analyze LMEO. Ions were detected by using UV detector at 254 nm. The separation was performed in a fused-silica capillary of 75 mm. The voltage was -25 kV and capillary was placed at 20°C. The column was rinsed with water (0.5 min), 0.1 M sodium hydroxide solution (1 min), and rewatered for 0.5 min followed by preconditioning with the background electrolyte for 3 min. The background electrolyte consisted of 3 mM TMA (Trimellitic acid) and 0.02% v/v DETA (Diethylene triamine) with the pH adjusted to 5.8 with sodium hydroxide was used (Westergaard *et al.* 1998).

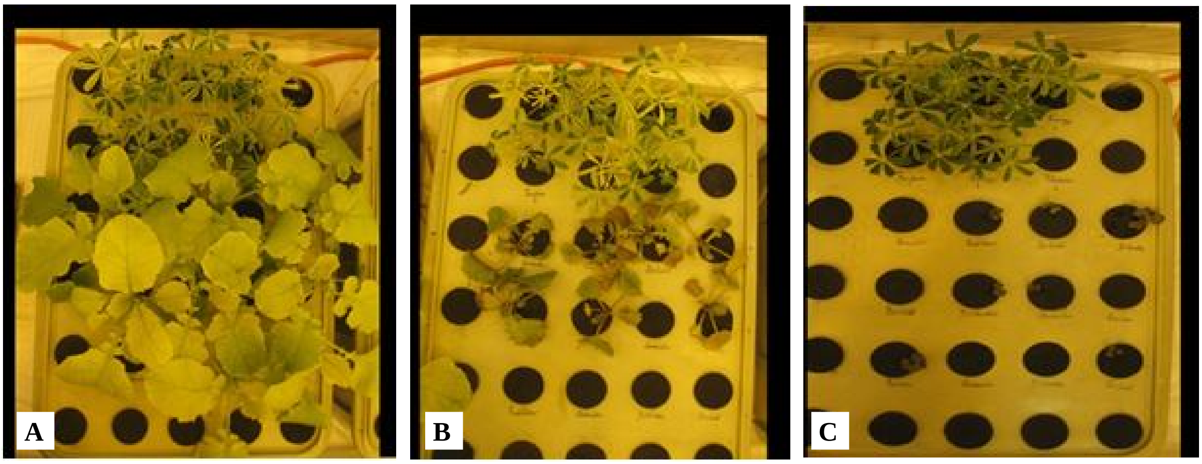
Statistical Analysis

A two way analysis of variance (ANOVA) was performed to see the main effect of the treatments on root dry weight, and root surface area. The data analyses were performed with SPSS 19 program.

RESULTS AND DISCUSSION

Plant Symptoms

Three cultivars (Dunkeld, Yickadee, Rainbow) of rapeseed exhibited a severe phosphorus deficiency after three weeks growing under -P condition. The plants were small and the leaves were purple. Rapeseed lost about 99% and 88% of its shoot fresh weight when grown under P and Zn deficiency, respectively. Based on visual observation, the growing of lupin cultivars (Feodora and Energy) showed a visible symptom of Zn deficiency, which was yellowing of leaves in the –Zn treatment. Both rapeseed and lupin thrived well in the complete nutrient treatment as they had bigger green leaves and denser roots (Figure 1).



Complete - Zn - P

Figure 1. Rapeseed and lupin plants in the aerated nutrient box solution at climate chamber.

Root Dry Weight

The main effect of nutrient supply was significant on the root dry weight of rapeseed. The root dry weight of Dunkeld, Rainbow and Yickadee were higher in the complete nutrient treatment than those in the -Zn and the –P treatments. In the –Zn treatment, the root dry weight of rapeseed was also higher than that the –P. The root dry weight was about 8-fold and 4-fold higher in the complete and –Zn treatments, respectively than that in the –P treatment. On the other hand, the effect of cultivar and the interaction were not significant on the root dry weight of the three rapeseed cultivars (Figure 2). In terms of lupin cultivars, the effect of nutrient supply, cultivars and also the interaction were significant on the root dry weight. It was almost 2-fold higher in the complete and –P treatments compared to the -Zn for Feodora cultivar. Whereas, for Energy cultivar, the root dry weight was only high in the –P treatment, while in the complete and –Zn, the root dry weight was not significantly different. The root dry weight of Feodora in the three different nutrient supplies was higher than that the Energy (Figure 3).

Root Surface Area

The main effect of nutrient supply was significant on the root surface area of three rapeseed cultivars (Dunkeld, Rainbow, and Yickadee). The complete nutrient supply increased the root surface area of rapeseed cultivars compared to both –Zn and –P treatments. The root surface area in the complete nutrient condition was about 1.3-fold and 6-fold higher than in the –Zn and –P treatments, respectively. The root surface area of those rapeseed cultivars also was higher (4-fold) in the –Zn compared to the –P treatment. The cultivar treatment and the interaction between both treatments had no significant effect on the root surface area. The root surface area of Dunkeld, Rainbow and Yickadee was not significantly different in the three different nutrient supplies (data unpublished), except in the -Zn treatment, root surface area of Dunkeld was slightly higher compared to the other rapeseed cultivars. The effect of nutrient supply was also significant on the root surface area of lupin cultivars, while the cultivar and the interaction effect were not significant. The complete and -P treatments increased the root surface area of Feodora. Similarly to Feodora, the root surface area of Energy was high under –P treatment, but it was not different in the complete and –Zn treatments. The root surface area of Energy was almost double in the –P compared to the others treatments (data unpublished). In addition, the formation of cluster roots was visible under deficiency of P. The Energy cultivar grew a greater amount of cluster roots in the –P compared to the other two treatments (Figure 4).

Citrate Exudation Rate

Both rapeseed and lupin cultivars exuded citrate only in the condition of minus P, while at complete and –Zn nutrient supplies, the citrate concentration might be below the detection limit (10 µM) of CE (Capillary electrophoresis). There was no other ligand such as oxalate or malate that could be detected by the CE. The exudation rate of Dunkel was about 6-fold higher (15.1 µmol g^{-1} RDW h^{-1})

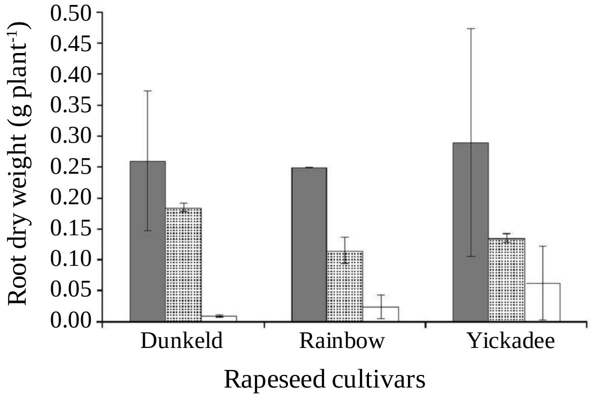


Figure 2. Root dry weight of three Rapeseed cultivars under different nutrient supplies, n = 2. The error bars show standard deviation. Fertilizer effect P (0.002); cultivar effect P (0.751); interaction effect P (0.877).

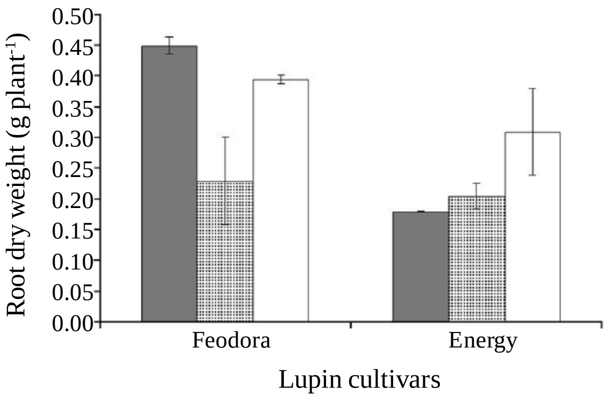


Figure 3. Root dry weight of two Lupin cultivars under different nutrient supplies, n = 2. The error bars show standard deviation. Fertilizer effect P (0.010); cultivar effect P (0.002); interaction effect P (0.015).

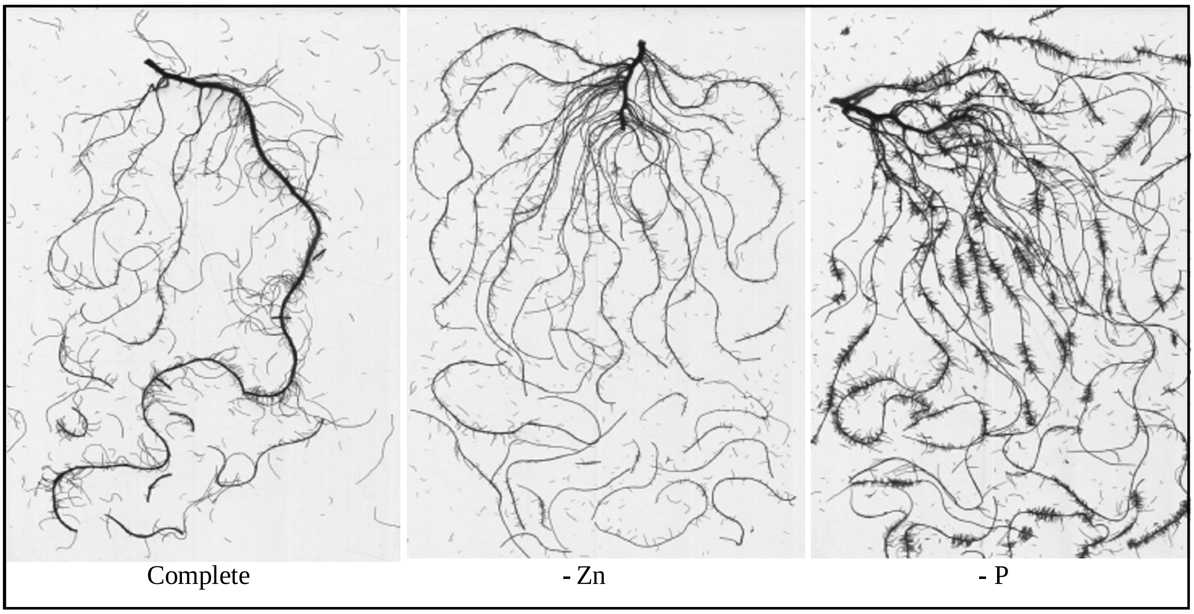


Figure 4. Cluster root formation of the Energy cultivar under three different nutrient supplies. The fresh roots were scanned and analyzed using WinRHIZO program. The circle in the - Zn and - P treatment is the example of cluster root.

compared to Yickadee (2.43 µmol g^{-1} RDW h^{-1}), while Rainbow exuded below the detection limit. For lupin cultivars, Feodora had a slightly higher exudation rate than Energy cultivar, their rate was about 3.89 µmol g^{-1} RDW h^{-1} and 3.45 µmol g^{-1} RDW h^{-1} respectively (Figure 5).

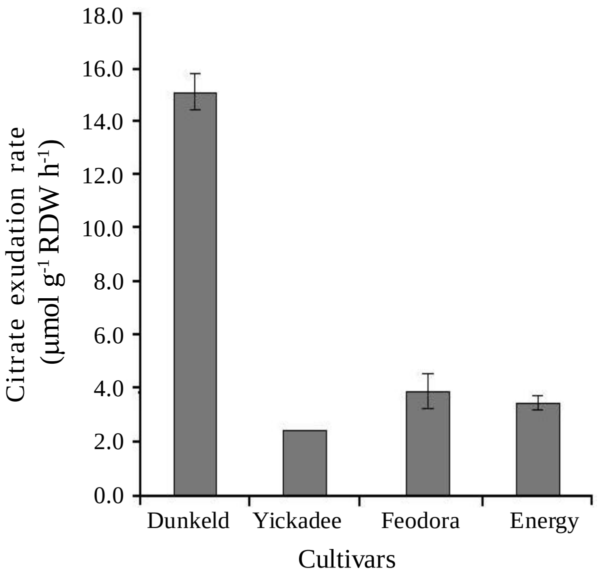


Figure 5. The exudation rate of different plant cultivars under - P tretment, n = 2. The error bars show standard deviation. RDW = Root dry weight. Dunkeld and Yickadee are lupin cultivars, while Feodora and Energy are rapeseed cultivars.

Discussion

The objective of this study was to investigate citrate exudation rate by different cultivars of lupin and rapeseed. The present results exhibited that both Feodora and Energy exuded citrate only under phosphorus stress. This was in agreement with previous finding that 90 % of citrate and proton exudation from root of lupin was depended on P supply (Sas *et al.* 2001). The exudation rate of Feodora was slightly higher (3.89 µmol g^{-1} RDW h^{-1}) than Energy (3.45 µmol g^{-1} RDW h^{-1}) (Figure 5). In terms of rapeseed cultivars; Dunkeld and Yickadee also released citrate under depletion of phosphorus (Figure 5). This finding was in line with Hoffland *et al.* (1992) that rapeseed synthesized citrate in shoot and was exuded by root under P shortage; though the shoot dry weight (data unpublished) and the root surface area (data unpublished) of both cultivars were significantly low under minus phosphorus. The exudation rate of Dunkeld was six times higher than Yickadee, as the root dry weight of Dunkeld was also six times lower than Yickadee under minus P (Figure 2). In addition, Dunkeld cultivar was a Zn efficient cultivar when tested in the pot and field experiments (Grewal *et al.* 1997). The efficient cultivars produced high plant biomass and had high root exudation rate under P deficiency (Zhang *et al.* 2009). On the other hand, Shu *et al.* (2007) argued that citrate exudation rate was significantly higher under supplemented P compartment than without P application. The discrepancy might be due to different source of P applied and also different of media growth. Furthermore, in the -Zn supply, both rapeseed and lupin exuded citrate and other LMWOA under the detection limit of CE. This result was different with previous research which measured citrate exudation rate (1.13 nmol g^{-1} root dw h^{-1}) of rice under Zn depletion (Hoffland *et al.* 2006). The disagreement might be due to different crop used. As different crops might have different sensitivity to Zn deficiency. For example, rice is more sensitive than lettuce and soybean (Alloway 2008). Thus we suggested that these two plants (lupin and rapeseed) might be less sensitive to Zn deficiency compared to rice, so they exuded low amount of citrate. Moreover, the sodium phosphate (KH_2PO_4) concentration that used in the current study was lower (0.2 m*M*) than the previous finding (1.5 m*M*).

Root surface area of Feodora and Energy was higher in phosphorus depletion (-P treatment) (data unpublished). This result proved that phosphorus deficiency stimulated the formation of cluster roots of lupin cultivars (Figure 4). This was in agreement with Sas (Sas *et al.* 2001), that the development of cluster root of white lupin which grown under minus P in the culture solution was high. Therefore, this current study suggests that those cluster roots increased the root surface area. Since, cluster roots are characterized by increasing of root surface area to enhance the uptake of nutrient (Lamont 2003). Furthermore, less amount of cluster roots formation in the minus Zn treatment in the current study might be due to less auxin metabolism in the plants. As Zn are required for auxin synthesis (Alloway 2008). Auxin is a growth regulating compund and it promotes the cluster roots formation (Neumann and Martinoia 2002). Feodora shoot did not grow well under insufficient nutrient condition, this was shown when the shoot dry weight of Feodora was lower in the –Zn and –P treatment compared to complete nutrient treatment, as the plant might have inadequate nutrient to complete its metabolism proses. Moreover, the decreasing of shoot dry weight was not caused by the cluster root formation. For the formation of cluster roots did not influence the shoot dry weight or plants dry matter yield (Keerthisinghe *et al.* 1998). Keerthisinghe found that plants which form high amount (> 50%) of proteoid roots, have adequate P concentration in their tissue for optimum grow. It means that under complete nutrient condition, the cluster root can be formed, this also occurred in our current study that Feodora and Energy also formed cluster roots under complete nutrient condition but not as much as in minus P. Another finding also agrees that root formation did not expense from the growing shoot, since the root formation started earlier before the shoot growth (Sas *et al.* 2001). Energy cultivar exhibited no difference in plant biomass under both adequate and inadequate nutrient condition. The root dry weight of Energy was even higher in –P treatment than in sufficient condition. This indicated that cluster roots formation enhanced the root dry weight of Energy.

CONCLUSIONS

To conclude, under P deficiency lupin dan rapeseed exuded citrate, which Dunkeld cultivar had the highest exudation rates. Meanwhile, under Zn deficiency, both lupin and rapeseed only showed reduction on their shoot fresh weight and root dry weight, whereas citrate exudation rates of those plants under Zn deficiency were below the detection limits. Furthermore, P deficiency stimulated formation of cluster roots.

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