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FULL PAPER

Effect of zinc-solubilizing bacteria and organic acids on zinc uptake and growth of rice applied with zinc sulfate

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Abstract

An investigation on the influence of zinc-solubilizing bacteria (ZSB) inoculation, Zn fertilizer and organic acids on growth of rice was done at Microbiology lab, Universiti Putra Malaysia. Commercial oxalic acid (BDH), malic acid (SIGMA), citric acid (SIGMA), succinic acid (SIGMA) was used to solubilise Zn oxide, Zn carbonate and Zn sulfate at four different rates (0, 0.1, 1.0 and 10 mM). Selected organic acid and its rate was then combined with ZSB and Zn sulfate using sand culture experiment for rice plant treatment. Highest Zn sulfate solubilisation activity at 0.421 mg/L was found using citric acid as a solubilizer. Similar observation found for malic acid and oxalic acid in Zn sulfate at 0.331 mg/L and 0.249 mg/L respectively. Then, selected organic acids were malic acid and citric acid for plant treatment with Zn-solubilizing bacteria (ZSB) and Zn sulfate. Highest plant biomass was found in inoculated plants treated with 0.1 mM malic acid in the presence of Zn sulfate at 135.67 mg/3 plants. The same treatment was also observed for the highest plant height at 29.57 cm. With the addition of malic acid, the population of the rhizosphere, endosphere and non-rhizosphere varied, but higher than control treatment Application of ZSB inoculation, with Zn sulfate at 0.2 mg/L worked best with malic acid at 0.1 mM concentration due to high increase of rice plant growth parameters, Zn uptake and Zn concentration. Bacterial populations also varied due to different organic acids and their rate.

1. Introduction

Organic acids have good potential to solubilise Zn because both proton and ligand-mediated mechanisms of mobilization play an integral role in the chemical attack on mineral's surface (Fomina *et al*., 2005). Plant roots release organic acids into the rhizosphere. Some of these organic acids like citric acid and malic acid are intermediates in the tricarboxylic (TCA) cycle for energy production while malic acid, malonate acid and oxalic acid are primarily present in cells for cation charge (Uren, 2000). In rice, greater exudation of malic acid was associated with increased Zn efficiency (Hajiboland *et al*., 2005). Meanwhile, solubilisation of zinc sulfate is dependent on exudation of citrate from rice in a small amount. It also appears to be dependent on proton excretion and pH reduction (Duffner *et al*., 2012). Organic acids are more advantageous than high-molecular-weight compounds as they are mobile and readily assimilated by a wide range of microorganisms like zinc-solubilizing bacteria (ZSB) (Szmigielska *et al*., 1985; Robert, 1986). The ZSB are able to produce 2-ketogluconic acid and oxalic acid during Zn solubilisation process (Simine and Gadd, 1998; Fasim *et al*., 2002). Previous studies showed that ZSB produced high organic acids during zinc sulfate solubilisation but it varied during the plant growth period.

To date, very little work has been directed on the utilization of organic acid to solubilize Zn (Ebrahimian and Bybordi, 2012). Many researchers also found inconsistent results of Zn solubilisation through the production of organic acids by bacteria and plants (Hajiboland *et al*., 2005; Shakeel *et al*., 2015). The inconsistent results were due to many factors. The factors are, plant species itself, competition with indigenous bacteria, different types and amounts of organic acid production during growth. Thus, it is very

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important to determine the effects of ZSB inoculation, organic acids and rates of organic acids on Zn uptake and plant growth of rice using zinc sulfate fertilizer. Hence, the present study objectives are to determine the effects of different types and rates of organic acids application on Zn solubilisation and to determine the effects of ZSB inoculation, types and rates of organic acids on growth and Zn uptake of the rice plant.

2. Materials and methods

2.1 Solubilization of zinc source by different organic acids

Laboratory studies were conducted to test the solubility of Zn by two types of organic acids at four rates. Commercial malic acid (SIGMA) and citric acid (SIGMA) was used to solubilise zinc oxide, zinc carbonate and zinc sulfate at four different rates (0, 0.1, 1.0 and 10 mM). The tests were carried out using Petri dishes with 20 mL of modified minimal salts medium agar amended with different Zn sources. Each of the organic acid (0.1 mL) was added into the wells in the agar with a diameter of 7 mm. Halozone formation around the well indicated positive results then clearing zone around the well was measured. About 0.1 mL of each organic acid was also pipetted to the medium amended with insoluble Zn and analysed for the availability of Zn using atomic absorbance spectrophotometer (AAS) (Perkin-Elmer 400). This method was modified from Fomina *et al*. (2005).

2.2 Growth chamber study of the effects of ZSB inoculation, organic acids types and rates on rice plant

2.2.1 Experimental design for sand culture under controlled condition

The experiment was arranged in a completely randomized design. The treatments were arranged in a 2 x 2 x 4 factorial design, with three replications. The total samples were forty-eight samples. The first factor consisted of two inoculation types which were, inoculated with ZSB *Acinetobacter* sp. (TM56) and non-inoculated. The second factor was two treatments derived from different sources of organic acids which were malic acid and citric acid. While the third factor consisted of four different rates of organic acids (0, 0.1, 1.0 and 10 mM). Organic acids were weighed and put into four 1 L sterilized distilled water to make the concentration (0, 0.1, 1.0 and 10 mM) as required. During inoculation, individual organic acid solution (1 mL) was applied to each planting unit according to treatments. Zinc sulfate at 0.2 mg/L used in Hoagland nutrient solution. Plants were grown for 40 days in a growth chamber with 12 hr light/dark cycle at 29±1^{0}C temperature.

2.2.2 Seed surface sterilization

The seed surface sterilization was determined based on Amin *et al*. (2004). Rice seeds were dehusked and shaken in 70% ethanol for five mins. The ethanol was then discarded and the seeds were agitated in hypochlorite solution comprising 3% of Clorox (2.6% NaOCl). Next, the seeds were washed using sterilized distilled water. After three rinses of sterile water, the seeds were rinsed with sodium thiosulphate solution (2%) to neutralize the chloramine residue on the seeds. Lastly, the efficacy of sterilization was checked by germinating seeds on nutrient agar.

2.2.3 Preparation of sand culture

Sands were sieved with 2.0 mm sieve and soaked overnight with 0.1 M of hydrochloric acid (HCl). Subsequently, the sands were washed several times with distilled water until pH 6.0 obtained. The sand was then air-dried and sterilised using autoclave at 15 psi and 121° C for 15 mins. Prior to transplanting, the sand was soaked with 200 mL of sterile Zn free plant Hoagland growth medium. Zinc was supplied using Zn sulfate and Zn oxide. The treatment rates were 0, 0.2 and 0.4 mg/L for each Zn source. The nutrient solution was applied weekly to the plants until 40 days of growth. Plants were grown in the growth chamber with 12 hr light/dark cycles at 28°C (Naher *et al*., 2009).

2.2.4 Preparation of bacterial inoculums and inoculation of rice seedlings

Isolate *Acinetobacter* sp. (TM56) were grown for 48-72 hrs in nutrient broth. The bacterial cells were harvested by centrifugation at 13500 × *g* for 10 mins in an Eppendorf tube and were further washed using phosphate buffer saline (PBS).

2.3 Determination of plant growth parameters

Plant height was recorded after 40 days of growing period. While, for plant biomass, the plant samples were dried in an oven at 70°C for five days until a constant weight was obtained and then the weight was recorded.

2.4 Zinc tissue analysis

Plant samples were dried in an oven at 70°C for five days. Next, the dried plants were ground finely and 100 mg of the plant tissue was placed in a 100 mL digestion tube containing 10 mL nitric acid (HNO_3) and perchloric acid ($HClO_4$) at 9:4 ratios. The tubes were placed on digestion block, and the entire plant material was digested at 300^{o}C until it turned colourless and left to cool in a fume hood. Then, the extract was put into 100 mL volumetric flask, and distilled water was added until the volume reached 100 mL. These samples were used for the estimation of Zn concentration and Zn uptake. Zn concentration in the plant was estimated using AAS (Perkin-Elmer, 400). Zinc uptake by the plant was determined as Zn concentration multiplied by the plant dry weight (Goteti *et al*., 2013).

2.5 Bacterial population of inoculated treatments

2.5.1 Determination of non-rhizosphere

After 40 days of growth, about 10 g of sand was taken from each sampling unit and added into 90 mL sterile distilled water and shaken using a vortex. A series of 10-fold dilution up to 10^{11} was prepared. Aliquots of 0.1 mL from each dilution were dropped onto nutrient agar and populations were determined using the drop plate count method (Somasegaran and Hoben, 1985).

2.5.2 Determination of rhizosphere

During harvesting, around 1.0 g of plant roots were washed gently using sterile water and placed in a conical flask containing 99 mL distilled water. The content in the flask was shaken for 15 mins, and a series of 10-fold dilutions were prepared, and bacterial populations were determined as prescribed previously.

2.5.3 Determination of root endosphere

Approximately 1.0 g of roots were washed, and surface sterilized with 70% ethanol for 5 mins. Subsequently, it was treated with 3% Clorox for 1 min. After several washes with sterile distilled water, the roots were meshed using a sterilized mortar and pestle (Gyaneswar *et al*., 2001). Then, the roots were checked for the efficacy of surface sterilization. A 10-fold series of dilution was prepared up to 10^{-10} and the diazotrophic populations were measured as described previously.

2.6 Data analysis

Data for Zn solubilization by commercial organic acids consisted of different Zn source and rates of organic acids. The data were subjected to ANOVA and post hoc Tukey's HSD test for mean comparison. Similarly, for growth chamber experiment, data for the treatment ZSB inoculation factor consisted of inoculated and non-inoculated, treatments factor of organic acid with malic acid and citric along with four rates of organic acids (0, 0.1, 1.0 and 10 mM) were subjected to ANOVA and treatment means were compared using Tukey's HSD test ($p<0.05$). All data were statistically analysed using the SAS Software program (Version 9.4).

3. Results and discussion

3.1 Zn solubilisation by different types of organic acids

Organic acids could solubilize different forms of Zn sources used in the study (zinc oxide, zinc sulfate, and zinc phosphate) (Figure 1). Interaction of Zn sources, types of organic acid and rates of organic acid significantly affected Zn solubilisation at $p < 0.05$. A significant increase of Zn solubilization was recorded in malic acid at 10 mM. The highest increase of Zn solubilization was zinc sulfate with 0.79 mg/L compared to zinc oxide and zinc carbonate. A similar observation was recorded for citric acid. The higher rates of citric, the higher zinc oxide, zinc carbonate and zinc sulfate that were solubilized (Figure 1). Whereby high Zn solubility was in zinc sulfate at 0.50 mg/L at 10 mM of citric acid. Overall, the solubility of Zn in mineral salts media was directly proportional to the concentration of the organic acid. Increasing concentration of organic acid showed the trends of increasing Zn solubilisation activity.

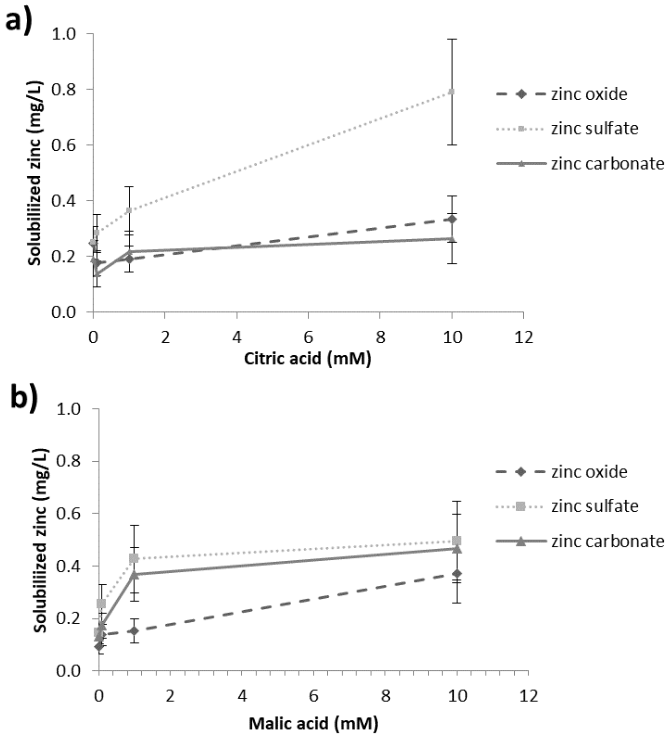

Figure 1. Zn solubilisation using organic acids in liquid media with a) citric acid and b) malic acid. Bars indicate standard error, n=3

3.2 Effect of ZSB inoculation, types of organic acids and its rates on growth of rice

Plant biomass was significantly affected by ZSB inoculation, types of organic acids and rates of organics acids at $p<0.05$ (Table 1). In inoculated treatment, plant biomass that was applied with malic acid showed a significant increase from 0 to 0.1 mM, then plant biomass was significantly decreased from 1.0 to 10 mM (Figure 2). While, for citric acid, plant biomass

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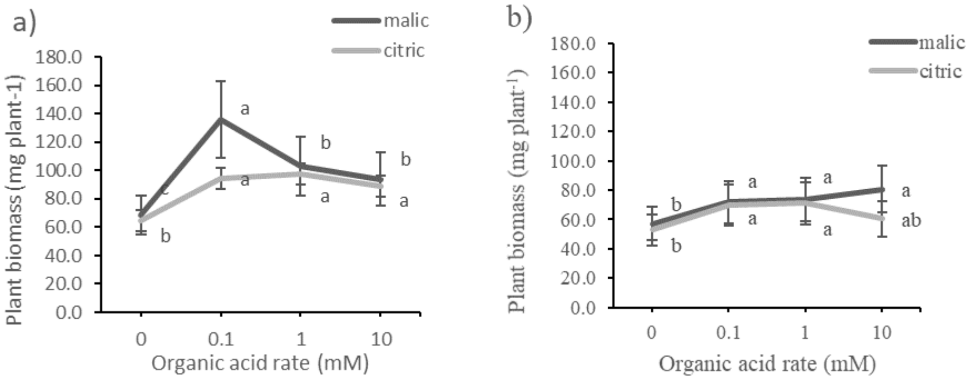

Figure 2. Effects of ZSB Inoculation, types of organic acid and its rates on plant biomass of rice plant: a) inoculated and b) non-inoculated.
Bars indicated standard error. Means with the same letter within rates of malic acid and citric acid are not significantly different at P>0.05

significantly increased from 0 to 0.1 mM. However, there was no further increase from 1.0 to 10 mM of citric acid. In non-inoculated treatment, plant biomass for plants receiving the application of malic acid and citric acid were significantly increased from 0 to 0.1 mM. Then, there were no further increased at 1.0 and 10 mM. The highest (135.67 mg) plant biomass was recorded from inoculated treatment with malic acid at 0.1 mM compared to without application of organic acids.

Table 1. Effects of ZSB inoculation, organic acid types and rates on plant biomass

Treatment factor	Plant biomass (mg plant^{-1})
ZSB inoculation	
Non-inoculated	93.33^{a}
Inoculated	67.13^{b}
Organic acid	
Malic acid	85.54^{a}
Citric acid	74.92^{b}
Organic acid rate (mM)	
0	60.75^{c}
0.1	92.92^{a}
1	86.25^{b}
10	81.00^{b}
Significance level	
ZSB	***
Organic acids	***
ZSB*Organic acids	*
Rates of organic acid	***
ZSB*Rates of Organic acid	***
Organic acids*Rates of organic acids	**
ZSB*Organic acids*Rates of organic acid	***
CV	6.51

P < 0.05; **P < 0.01; ***P<0.001, ZSB= Zinc-solubilizing bacteria *Acinetobacter* sp (TM56)
Values with the same superscript among ZSB inoculation, organic acid types and organic acid rates are not significantly different at P>0.05.

There was a significant interaction for ZSB inoculation, organic acids and organic acids rates for plant height at p<0.001 (Table 2). In inoculated treatment, plant height was significantly increased when applied with malic acid from 0 to 0.1 mM (Figure 3). However, plant height decreased when malic acid reached 1.0 and then 10 mM. While for citric acid, plant height decreased from 0 to 0.1 mM. There was no significant increase of 1.0 mM and 10 mM. In non-inoculated treatment, malic acid showed an insignificant plant height from 0 mM to 1.0 mM. Then, plant height decreased at 10 mM. While, for citric acid treatment, plant height significantly increased from 0 to 0.1 mM, then there was no significant increased at 1.0 to 10 mM.

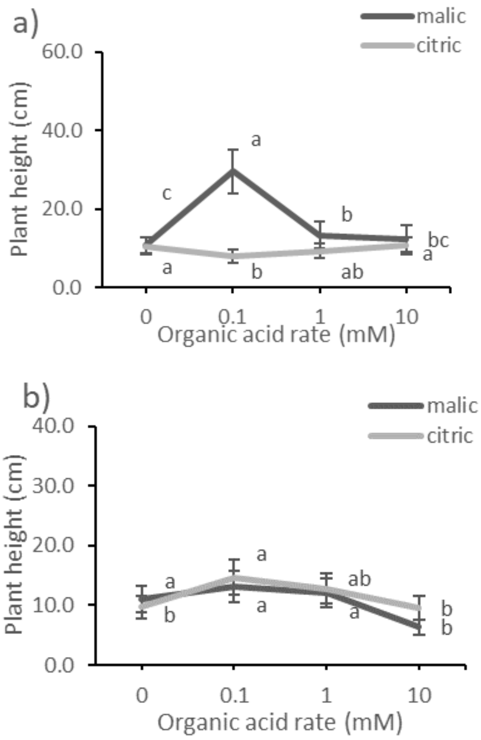

Figure 3. Effects of ZSB Inoculation, types of organic acids and its rates on plant height of rice plant: a) inoculated and b) non-inoculated rice plant. Bars indicated standard error. Means with the same letter within rates of malic acid and citric acid are not significantly (NS) different at P>0.05

Among all, the highest (29.57 cm) plant height was recorded for inoculated treatment with 0.1 mM compared to control.

Table 2. Effects of ZSB inoculation, organic acid types and rates of organic acids on rice plant height

Treatment factor	Plant height (cm)
ZSB inoculation	
Non-inoculated	11.16^{b}
Inoculated	13.10^{a}
Organic acid	
Malic acid	13.60^{a}
Citric acid	10.65^{b}
Organic acid rate (mM)	
0	10.49^{c}
0.1	16.33^{a}
1	11.93^{b}
10	9.76^{c}
Significance level	
ZSB	***
Organic acids	***
ZSB*Organic acids	***
Rates of organic acid	***
ZSB*Rates of Organic acid	***
Organic acids*Rates of organic acids	***
ZSB*Organic acids*Rates of organic acid	***
CV	9.68

*P < 0.05; **P < 0.01; ***P<0.001, ZSB= Zinc-solubilizing bacteria Acinetobacter sp (TM56)
Values with the same superscript among ZSB inoculation, organic acid types and organic acid rates are not significantly different at P>0.05.

3.3 Effect of ZSB inoculation, organic acid types and rates of organic acids on relative chlorophyll contents in rice plant leaves

Relative of chlorophyll content of plant leaves was significantly affected by the interaction of ZSB inoculation, organic acids and organic acid rates at p<0.001 (Table 3). For inoculated treatment, relative chlorophyll contents increased with the application of malic acid from 0 to 0.1 mM (Figure 4). However, the amount decreased when malic acid concentration reached 1.0 to 10 mM. For citric acid, there was also a significant increase in plant biomass from 0 to 0.1 mM. Then, there was a significant decrease at 1.0 mM followed by a significant decrease at 10 mM. For non-inoculated treatment, there was a similar increase from 0 to 0.1 mM of malic acid and citric acid. However, there was no further increase in relative chlorophyll content at 1.0 and 10 mM of citric acid. While, for malic acid, there

Table 3. Effects of ZSB inoculation, organic acid types and rates of organic acids on relative chlorophyll content in rice plant.

Treatment factor	Relative chlorophyll content
ZSB inoculation	
Non-inoculated	15.45^{b}
Inoculated	20.44^{a}
Organic acid	
Malic acid	19.31^{a}
Citric acid	16.58^{b}
Organic acid rate (mM)	
0	15.95^{b}
0.1	22.63^{a}
1	16.87^{b}
10	16.33^{b}
Significance level	
ZSB	***
Organic acids	***
ZSB*Organic acids	***
Rates of organic acid	***
ZSB*Rates of Organic acid	***
Organic acids*Rates of organic acids	***
ZSB*Organic acids*Rates of organic acid	***
CV	4.83

*P < 0.05; **P < 0.01; ***P<0.001, ZSB= Zinc-solubilizing bacteria Acinetobacter sp (TM56)
Values with the same superscript among ZSB inoculation, organic acid types and organic acid rates are not significantly different at P>0.05.

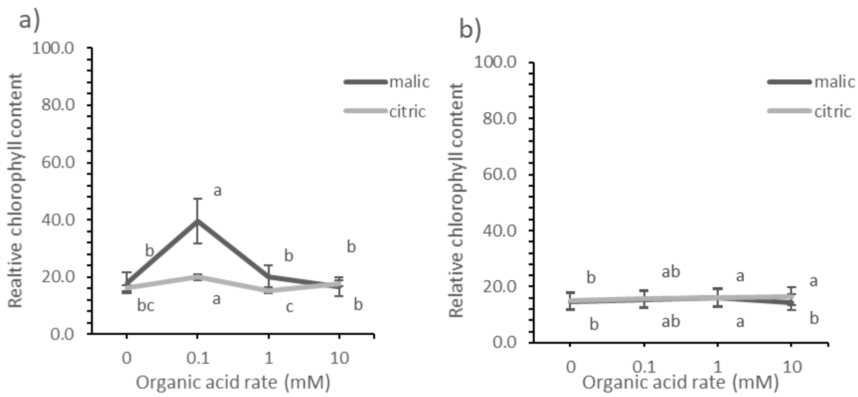

Figure 4. Effects of ZSB Inoculation, Organic Acids and its rates on relative chlorophyll content in rice plant: a) inoculated and b) non-inoculated. Bars indicated standard error. Means with the same letter within rates of malic acid and citric acid are not significantly (NS) different at P>0.05

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was a significant decrease at 10 mM of its application. Among all, the application of malic acid at 0.1 mM showed the highest (39.57) relative chlorophyll content compared to without malic acid applied to the plant.

3.4 Effects of ZSB inoculation, organic acid types and rates of organic acids on Zn concentration in rice

A significant interaction of ZSB inoculation, organic acid types and the organic acid rate was detected for Zn concentration at $p<0.001$ (Table 4). In inoculated treatment, Zn concentration was significantly increased with the application of malic acid from 0 to 0.1 mM (Figure 5). Then, Zn concentration significantly decreased at 1.0 mM and no further decrease at 10 mM. While for citric acid, there was a significant decrease from 0 to 1.0 mM. However, there was a significant increase at 10 mM of citric acid application. In non-inoculated treatment, malic acid showed a significant increase in Zn concentration from 0 to 1.0 mM. No further increase was observed at 10 mM. While for citric acid, there was a significant increase from 0 to 0.10 mM. Then, there was a significant decrease at 1.0 mM and 10 mM. Highest (52.20 mg kg^{-1}) Zn concentration was recorded for inoculated treatment which applied with malic acid at 0.1 mM compared to no application of organic acids.

Table 4. Effects of ZSB inoculation, organic acid types and rates on Zn concentration in rice

Treatment factor	Zn concentration (mg kg^{-1})
ZSB inoculation	
Non-inoculated	26.69[b]
Inoculated	26.99[a]
Organic acid	
Malic acid	26.73[a]
Citric acid	22.94[b]
Organic acid rate (mM)	
0	21.83[b]
0.1	32.70[a]
1	23.98[b]
10	20.83[b]
Significance level	
ZSB	**
Organic acids	**
ZSB*Organic acids	*
Rates of organic acid	***
ZSB*Rates of Organic acid	***
Organic acids*Rates of organic acids	ns
ZSB*Organic acids*Rates of organic acid	***
CV	16.37

*P < 0.05; **P < 0.01; ***P<0.001, ZSB= Zinc-solubilizing bacteria Acinetobacter sp (TM56)
Values with the same superscript among ZSB inoculation, organic acid types and organic acid rates are not significantly different at P>0.05.

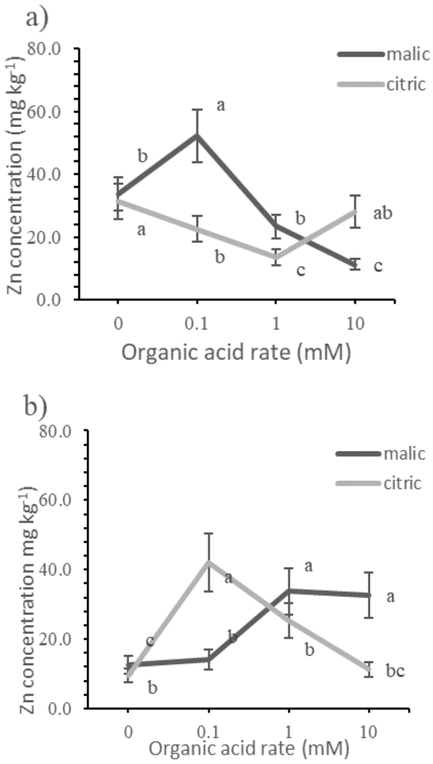

Figure 5. Effects of ZSB Inoculation, types of organic Acids and its rates on Zn concentration of rice plant: a) inoculated and b) non-inoculated
Bars indicated standard error. Means with the same letter within rates of malic acid and citric acid are not significantly (NS) different at P>0.05.

3.5 Effect of ZSB inoculation, organic acid types and rates of organic acids on Zn uptake in rice plant

Interaction of ZSB inoculation, organic acids and rate of organic acids affected Zn uptake at $p<0.001$ (Table 5). In inoculated treatment, there was a significant increase of Zn uptake from 0 to 0.1 mM of malic acid application, then, a significant decrease from 1.0 mM to 10 mM. While for citric acid application, there was a significant decrease from 0 to 1.0 mM. However, at 10 mM there was a significant increase of Zn uptake detected (Figure 6). In non-inoculated treatment, malic acid showed a similar increase from 0 to 0.1 mM. However, at 1.0 mM, there was a significant increase then no further increase observed at 10 mM. While for citric acid, a significant increase was observed from 0 to 1.0 mM. However, the application of citric acid showed a significant decrease of Zn uptake from concentration 1.0 to 10 mM. Highest (777.64 mg/3 plants) Zn uptake was recorded for inoculated treatment with malic acid at 0.1 mM compared to non-malic acid application to the plants.

3.6 Effect of ZSB, organic acid types and rates of organic acid to root development of rice plant

There was an interaction of variable ZSB, types of

Table 5. Effects of ZSB inoculation, organic acid types and organic acid rates on Zn uptake of rice

Treatment factor	Zn uptake (mg/ 3 plants)
ZSB inoculation	
Non-inoculated	157.64[b]
Inoculated	269.16[a]
Organic acid	
Malic acid	253.46[a]
Citric acid	173.34[b]
Organic acid rate (mM)	
0	134.68[c]
0.1	347.33[a]
1	199.28[b]
10	172.31[bc]
Significance level	
ZSB	***
Organic acids	***
ZSB*Organic acids	***
Rates of organic acid	***
ZSB*Rates of Organic acid	***
Organic acids*Rates of organic acids	***
ZSB*Organic acids*Rates of organic acid	***
CV	18.33

*P < 0.05; **P < 0.01; ***P<0.001, ZSB= Zinc-solubilizing bacteria Acinetobacter sp (TM56)
Values with the same superscript among ZSB inoculation, organic acid types and organic acid rates are not significantly different at P>0.05.

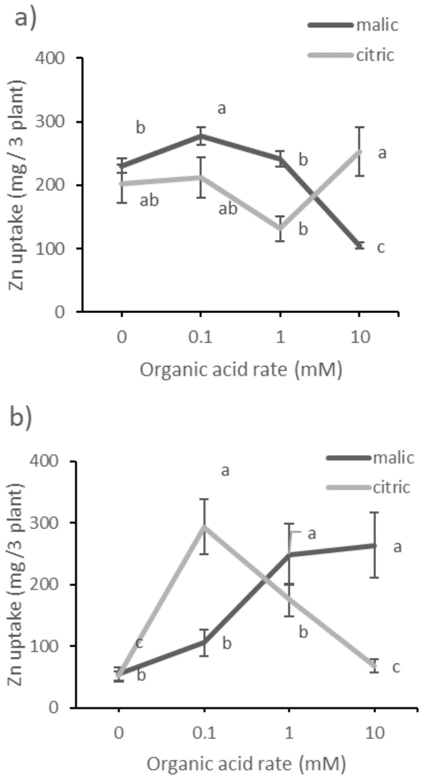

Figure 6. Effects of ZSB inoculation, types of organic acids and its rates on Zn uptake of rice plant: a) non-inoculated and b) inoculated.
Bars indicated standard error. Means with the same letter within rates of malic acid and citric acid are not significantly (NS) different at P>0.05

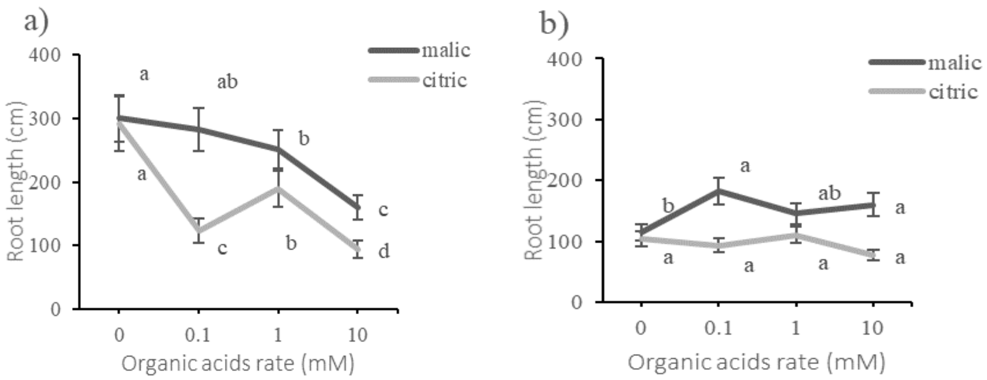

Figure 7. Effects of ZSB inoculation, organic acids types and rates on root length for rice plant; a) inoculated and b) non-inoculated.
Bars indicated standard error. Means with the same letter within rates of malic acid and citric acid are not significantly (NS) different at P>0.05

organic acids and organic acid rates at p<0.01 (Table 6). For inoculated treatment, malic acid showed a significant decrease from 1.0 to 10 mM of root length (Figure 7). While for citric acid, there was a significant decrease from 0 to 0.1 mM, then root length significantly increased at 1.0 mM before decreased again at 10 mM (Figure 30). For non-inoculated treatment, malic acid showed a significant increase from 0 mM to 0.1 mM. There was no further increase of 1.0 mM and 10 mM. While for citric acid, there was no significant increase from 0 to 1.0 mM. However, there was a significant decrease in root length when applied with citric acid at 10 mM. Root volume was affected by the interaction of ZSB inoculation, organic acid types and organic acids rates at p<0.01 (Table 6). For inoculated treatment, only malic acid affected root volume where at 0.1 mM root volume was significantly increased and then decreased when the rate approached 1.0 mM and 10 mM (Figure 8). However, for citric acid, similar root volume was observed for all of the rates. Highest (0.408 cm^3) root volume was detected from treatment consisted of malic acid at 0.1 mM with ZSB inoculation compared to non-

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Table 6. Effects of ZSB, organic acids and rates of organic acids on root development of rice plant

Treatment factor	Root length (cm)	Root volume (cm^3)	Root diameter (cm)	Root area (cm^2)
ZSB inoculation				
Non-inoculated	123.29^b	0.12^b	0.33^a	0.12^b
Inoculated	211.18^a	0.20^a	0.32^a	0.20^a
Organic acid				
Malic acid	199.14^a	0.19^a	0.33^a	0.19^a
Citric acid	135.33^b	0.12^b	0.32^a	0.11^b
Organic acid rate				
0	202.54^a	0.14^b	0.38^a	0.13^a
0.1	169.99^b	0.20^a	0.33^{ab}	0.23^a
1	173.77^b	0.15^b	0.31^{bc}	0.12^a
10	122.65^c	0.13^b	0.27^c	0.14^a
Significance level				
ZSB	***	***	ns	*
Organic acids	***	***	ns	*
ZSB*Organic acids	*	**	ns	ns
Rates of organic acid	***	**	***	ns
ZSB*Rates of Organic acid	***	**	ns	ns
Organic acids*Rates of organic acids	***	**	ns	ns
ZSB*Organic acids*Rates of organic acids	**	**	ns	ns
CV	8.73	26.6	15.22	41.48

*P < 0.05; **P < 0.01; ***P<0.001, ns = not significant, ZSB= Zinc-solubilizng bacteria *Acinetobacter* sp (TM56)
Values with the same superscript among ZSB inoculation, organic acid types and organic acid rates are not significantly (NS) different at P>0.05

inoculated control. There was a significant difference in organic acid rates for root diameter at p<0.05 (Table 6). Similar high root diameter was detected at rates 0.1, 1.0 and 10 mM of organic acid rates compared to without organic acid application. A significant difference in organic acid types and inoculation types also detected for root area at p<0.05 (Table 6). Malic acid showed a significantly higher (0.193 cm^2) root area compared to citric acid. While for inoculation types, the inoculated treatment showed the highest (0.20 cm^2) root area compared to non-inoculated.

3.7 Effects of ZSB inoculation, organic acid types and rates on bacterial population

There was no significant difference between organic acid types and rates at p<0.01 for rhizosphere population and non-rhizosphere population (Table 7). While, A significant differences between malic acid and citric acid was detected only for endosphere population at p<0.01. Malic acids showed higher (8.13 log 10 CFU mL^{-1}) endosphere population than citric acid.

Table 7. Effects of organic acid types and rates on rhizosphere, endosphere and non-rhizosphere population of inoculated treatments.

Treatment	Rhizosphere population (CFU mL^{-1})	Endosphere population (CFU mL^{-1})	Non-rhizosphere population (CFU mL^{-1})
Organic acid types			
Malic acid	7.89^a	8.14^a	6.05^a
Citric acid	7.53^a	7.43^b	5.88^a
Organic acid rates (mM)			
0	7.46^a	7.83^a	5.70^b
0.1	7.80^a	7.73^a	6.38^a
1	7.50^a	7.86^a	5.78^b
10	8.06^a	7.70^a	6.00^{ab}
Significance level			
Organic acid types	ns	**	ns
Organic acids rates	ns	ns	**
Organic acid types*organic acid rates	ns	ns	ns
CV	8.14	6.11	5.77

**P < 0.01; ns = not significant
Values with the same superscript among ZSB inoculation, organic acid types and organic acid rates are not significantly (NS) different at P>0.05

3.8 Effects of ZSB inoculation, organic acid types and rates on pH of sand culture

There were significant differences between ZSB inoculation at p<0.01 (Table 8). Inoculated treatment showed significantly higher (6.61) pH than non-inoculated treatment. There was a similar pH detected between malic acid and citric acid and also among rates of organic acid tested.

Table 8. Effects of ZSB inoculation, organic acid types and rates on pH of sand culture

Treatment factor	pH
ZSB inoculation	
Non-inoculated	6.45[b]
Inoculated	6.61[a]
Organic acid	
Malic acid	6.54[a]
Citric acid	6.51[a]
Organic acid rate	
0	6.52[a]
0.1	6.55[a]
1	6.52[a]
10	6.52[a]
Significance level	
ZSB	**
Organic acids	ns
ZSB*Organic acids	*
Rates of organic acid	ns
ZSB*Rates of Organic acid	ns
Organic acids*Rates of organic acids	ns
ZSB*Organic acids*Rates of organic acids	ns
CV	1.75

*P < 0.05; **P < 0.01; ***P<0.001, ns = not significant, ZSB = Zinc-solubilizng bacteria *Acinetobacter* sp (TM56)
Values with the same superscript among ZSB inoculation, organic acid types and organic acid rates are not significantly (NS) different at P>0.05

4. Discussion

The results of Zn solubilisation by malic acid and citric acid revealed the influence of the type of organic acids and its concentration on Zn solubilisation. There was a significant effect of Zn types and organic acids rates on Zn solubility. This is because citric acid presents a high metal complexation strength with Zn (Madhaiyan *et al*., 2006; Huang *et al*., 2008; Labanowski *et al*., 2008). These observations were supported by Khademi *et al*. (2009), in which strong binding of citric acid at 1.94 µmol have been proven to be effective in mobilising Zn. Organic compounds can dissolve tightly bound forms of heavy metals which caused a decrease in the concentration of metals in any forms (Elkins and Nelson, 2002; Kos and Lestan, 2004). Zinc solubilisation activity relied on the concentration of organic acid. The concentration of malic acid at 0.047 µM and 0.080 µM of citric acid are directly proportional to the proton action produced which results in higher acidity and positive hydrogen ion that leads to higher Zn solubilisation (Krishnamurti *et al*., 1997). The results showed that the application of malic and citric acids highly affected the solubilisation of Zn from zinc sulfate both in the presence and in the absence of ZSB inoculation. Strength of Zn complexation with organic acids increased when organic acids become protonated (Lindsay, 1979; Impa and Johnson-Beebout, 2012). These weakening associations temporarily increase the free Zn in the solution, making it more available for plant uptake or for other competing reactions. However, at higher organic acid concentration, the plant growth decreased because an elevated level of Zn in plants may suppress the metabolism and translocation of reserve material in the plants (Gupta and Sinha, 2005).

The combination of ZSB with malic acid at 0.1 mM has increased plant growth parameters (plant height, plant biomass and relative chlorophyll content), Zn uptake, Zn concentration and root volume. From the results, malic acid inoculated with ZSB showed a rapid increase in plant growth parameters with addition rates of organic acids compared to control. Plant growth, Zn uptake, Zn concentration and root volume increased when the availability of Zn became higher due to dissolution of Zn by organic acids. Zinc bioavailability to plants might be caused by secretion of root exudates which contains an organic acid (Hajiboland *et al*., 2005). However, a small amount of organic acids secreted by plants can be increased by introducing commercial organic acids like usual phytoremediation method. The method usually used for contaminated soil. But using suitable rates of organic acids, it might help for the increment of plant growth and Zn nutrition to the plant. Another factor that helped for the affected significant interaction on plant growth parameters is ZSB. Zinc-solubilizing bacteria were found positive for IAA production, a phytohormone responsible for increasing the length of the root hairs which could promote better absorption of nutrients from the soil and lead to better Zn uptake by plants (Vaid *et al*., 2014). However, in this study, a higher concentration of organic acids than 1 mM caused a reduction in plant growth parameters and root morphology. This is due to the increase in the concentration of organic acids as a longer chain of organic acid like citric acid caused increasing phytotoxicity to the plants' growth (Takijima, 1964). In rice, root elongation of rice seedlings had increased at 1 mN and 5 mN but then decreased at 10 mN acid concentrations due to the toxicity to the rice roots (Rao and Mikkelsen, 1977). Overall, there was a significant increase of pH during plant growth with ZSB inoculation because of their intrinsic system buffering potential by

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ZSB and insoluble zinc (Franz *et al*., 1991). Any addition of organic acids also had not affected the interaction between organic acids types and rates on bacterial population among the treatments during the planting period.

5. Conclusion

There was a significant effect of organic acids rate and Zn types on zinc solubilisation. Malic acid and citric acid showed a significant increase in Zn solubilisation when its concentration increased. Interaction of ZSB inoculation, organic acids and rates of organic acids was also significant for plant height, plant biomass, relative chlorophyll content, Zn uptake, Zn concentration, and root length and root volume. Among all, application of ZSB inoculation worked best with malic acid at 0.1 mM concentration compared to control for plant growth and Zn uptake.

Conflict of interest

The authors declare no conflict of interest.

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