Pak. J. Bot., 46(1): 383-387, 2014.

THE IMPACT OF SIDEROPHORE SECRETION BY *PSEUDOMONAS STUTZERI* TO CHELATING Cu METAL IN SOLUTION CULTURE

RAFIA AZMAT

Department of Chemistry, University of Karachi, Karachi-75270, Pakistan
Corresponding author's e-mail: rafiasaeed200@yahoo.com

Abstract

This article discuss the interaction of siderophores (which are low molecular weight, secreted metabolites) of microorganisms (*Pseudomonas stutzeri*) separately and simultaneously with Cu metal in the solution culture on the roots of 4d old seedlings of *Vigna radiata.* Naturally occurring bacteria play an important role in plant growth due to the release of pigment. It is a coloring compound (siderophore) showed high chemical oxygen demand (COD), lowers the pressure of oxygen. This lowers the nutrient uptake by the roots due to which reduced plant growth with metabolic disorder was observed in the whole plant. A wide range of phenomena from simple to complex interactions was observed between microorganisms and Cu metal in relation with plant root growth such as adsorption, oxidation/reduction of pigment, solubilization. Results showed that microbial strain showed a significant effect on weight of root in aqueous culture whereas nutrient medium support the root growth. Biochemical analysis reflects that lipids were main target of both abiotic and biotic stress that may utilizes to overcome the stress due to which protein contents were seemed to be unaffected at highest concentration of metal or it may also be related with the degradation of lipids due to the biotic and abiotic stress.

Introduction

Among heavy metals, some are essential as trace elements, for living organism such as plants, microorganism but at high levels they become toxic (Quariti *et al*.,1997). For example, copper occurs naturally in rock, soil, water, air, and also in plants and animals. The copper ion is a metal cofactor of various enzymes including tyrosinase, ascorbic acid oxidase, and butyryl Co-A dehydrogenase. It is one of the metals concerned with photosynthesis (Ozounidou, 1994). While copper is an essential micronutrient, exposure to excess copper has a detrimental effect on plant growth, especially on root growth and morphology (Azmat & Khan 2011), inhibits root and shoot growth, affects nutrient uptake and homeostasis, and often accrued by the roots (Azmat & Riaz, 2012). As principle effect of Cu toxicity is on root growth. The study of Cu toxicity in a soil environment is difficult. Solution culture provides a model of the interaction between the plant and the soil solution and allows examination of root growth and morphology. The effect of bacterial inoculation on plant growth and copper uptake by maize (*Zea mays*) and sunflower (*Helianthus annuus*) was investigated using one of the isolates (*Pseudomonas* sp. TLC 6-6.5-4) with higher IAA production and phosphate and metal solubilisation, which resulted in a significant intensification in copper accumulation in maize and sunflower, and an increase in the total biomass of maize (Li and Ramakrishna, 2011 ; Kefeng & Wusirika, 2011). They also reported that multiple metal-resistant bacterial isolates characterized in having potential applications for remediation of metal contaminated soils in combination with plants and metal contaminated water. Siderophores are other important natural chelating agents which are synthesized by microorganisms and some plants to solubilize external iron (Belimova, 2005). The siderophores are secondary metabolites secreted under iron-deficient conditions, binding to Fe(III) with high affinity. Siderophores are low molecular weight, secreted metabolites of microorganisms and plants with a high affinity for ferric iron and they are capable to metal scavenging through specific receptors/transporters on the cell surface. There are numerous studies on complexation of metal ions with organic acids such as citric acid, acetic acid, and oxalic acid which may exist in the soil for a short period of time (Chakraborty *et al*., 1990; Feistner *et al*., 1993; Gledhill, 2001).

Our aim of current study was to investigate the effect of siderophore in relation with Cu metal and root growth *In vivo* in 4 days old seedlings of *Vigna radiata* grown in essential metal nutrient medium with *Pseudomonas stutzeri*, strains which might be useful to increase plant biomass production under unfavorable environmental conditions. The study of such metal contaminated environment with plant-microbe associations is aimed in improving the efficiency of phytoremediation of heavy metal infested soils.

Material and Method

The seeds of (4 to 5) V*igna radiata* were washed and grown in optimal conditions in seven different Petri plates containing water, Hoagland solution, while Cu was added viz., 0, 50 ppm,100ppm. In order to accomplish the goal of this experiment 20ml solution were poured into the each petri plate at the time of germination. The procedure was performed for 4 days in triplicate and analysis of physical and biochemical parameters including enzymatic activity were performed. The data obtain were subjected to statistical analysis.

Microbial identification: Microorganism was isolated from garden soil and identified morphologically and biochemically. The isolated microorganism was identified as gram negative short rods which can grow on MacConky's agar. Biochemical tests have been also done showing that the microorganism grow on Triple sugar iron agar (TSI), Simon citrate agar, and giving positive oxidase test, which was purely gram negative. The identification of the specie's name of that gram negative bacterium *via* QTS-NE KIT protocol was done (provided by Desto laboratories, Pakistan) and the name identified was *Pseudomonas stutzeri* (Hayyat *et al.*, 2012).

Biochemical parameters: Protein, glucose, and lipids were estimated by the RANDOX test kit (Randox Laboratories Ltd, 55 Diamond Road, Crumlin, Co. Antrim, United Kingdom, BT29 4QY), according to manufactures s instructions. Biomolecules activities were expressed as a specific activity (mg/dl) and all experiments were performed in triplicate.

Enzymes activity: The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) using a semi-automatic biochemical analyzer (Technicon, RA-XT, USA) with the Randox test kit (Randox Laboratories Ltd., 55 Diamond Road, Crumlin, Co. Antrim, United Kingdom, BT29 4QY), according to manufacturer's instructions. Enzyme activities were expressed as specific activity (mg/dl) and all experiments were performed in triplicate (Azmat *et al*., 2013).

Results

Root length promotion of *Vigna radiata* under the inoculation of strain of identified *Pseudomonas stutzeri* in absence and presence of Cu were shown in the Table 1. Results showed that in an aqueous medium without Cu root elongation was severely effected as compared to nutrient medium therefore all experiments were performed in nutrient medium. The length and weight of root (Table 1) in aqueous medium was 9.715 and 0.206 respectively while it was 8.915 and 0.216 respectively in Hoagland nutrient medium without microbial strain *P. stutzeri* which was due to that of blue pigment (siderophore) release by the *Pseudomonas* sp. (Marschner *et al*., 1987; Azmat *et al*., 2013). Results showed that the root length was highest in presence of Cu at 50 ppm and reduced in presence of microbial strain. On the other hand an increase in concentration of Cu (100 ppm) showed the reduction in growth parameters while inoculation of strain at this stage causes slight increase in root length when compared with 50ppm Cu and strain (Table 1). Biochemical analysis showed that at 50ppm of Cu, glucose, protein and lipids were found to be decreases but growth was stimulated (Table 1) which may be related with the expense of protein, glucose and lipids due to free radical generation by oxidative stress.

The valuation of glucose, lipids and protein is also in accordance of results in Table 1 and reflects that glucose contents were adversely affected by the Cu at 50ppm and 100 ppm (80 & 79 mg/dl) whereas increases in presence of *P. stutzeri* (Table 2).There is an overall reduction in lipid concentration of root at all applications of Cu and bacteria when compared with control with no significant difference in protein contents

Enzymes activity reflects the environmental stress, used as biomarkers for monitoring soil heavy metal pollution with microorganism. AST, ALT and LDH are all intracellular enzymes and are generally treated as signs of bacterial damage (Korzeniewski & Callewaert, 1983). Decrease or increases in the enzyme activity represents the metabolic burden due to stress in any organism (Feistner *et al*., 1993). Three biomarkers was analyzed in biotic and abiotic stress separately and simultaneously in nutrient medium (Table 3). It was found that the effect of toxicants on enzymatic activity was one of the bioindicator to reflect the situation. LDH enzyme activity was 668U/L in aqueous medium while it was 688 U/L at 100ppm of Cu with strain and maximum alone with bacteria . ALT and AST were highest at 100 ppm of Cu with reduced LDH.

Table 1. Effect of Cu and microbial strain on growth of root of *Vigna radiate*.

Sample	Average root length (cm)	Weight of root (gm)	L/W ratio
H_2O	9.575 ± 0.09	0.206 ± 0.08	46.48
H_2O + Ps	1.23** ± 0.09	0.031** ± 0.08	39.67
HS	8.675* ± 0.39	0.216 ± 0.07	40.16
HS + Ps	4.44 ± 0.09	0.175 ± 0.06	25.37
HS + 50ppm Cu	12.520 ± 0.08	0.253 ± 0.09	49.48
HS + 50ppm Cu + Ps	2.900* ± 0.07	0.142 ± 0.09	20.42
HS + 100ppm Cu	7.000 ± 0.07	0.086** ± 0.06	81.39
HS + 100ppm Cu + Ps	3.500 ± 0.04	0.122 ± 0.02	28.68

N=3, p<0.05, * = Significant,** = Highly significant

Table 2. Effect of Cu and microbial strain on biochemical parameters of root of *Vigna radiate*.

Sample	Glucose (mg/dl)	Lipid (mg/dl)	Protein (gm/dl)
H_2O	104 ± 14	1043 ± 122	7 ± 0.09
H_2O + Ps	109 ± 14	783** ± 119	4 ± 0.08
HS	105 ± 13	866 ± 113	7 ± 0.09
HS + Ps	96 ± 12	782 ± 111	6 ± 0.08
HS + 50 ppm Cu	80* ± 11	760** ± 114	5 ± 0.07
HS + 50 ppm Cu + Ps	104 ± 12	900 ± 118	4* ± 0.09
HS + 100 ppm Cu	79** ± 10	845 ± 119	6 ± 0.08
HS + 100 ppm Cu + Ps	111 ± 10	855 ± 119	5 ± 0.09

N=3, p<0.005, * = Significant,** = Highly significant

Table 3. Effect of Cu and microbial strain on enzymatic activity of root of *Vigna radiate*.

Sample	LDH (U/L)	AST (U/L)	ALT (U/L)
H_2O	668 ± 87	81 ± 9.0	15 ± 2-0
H_2O + Ps	1433** ± 213	64 ± 8.2	62 ± 8.7
HS	271 ± 11	84* ± 7.9	76 ± 15
HS + Ps	1191 ± 234	31 ± 8.6	1079* ± 118
HS + 50 ppm Cu	706 ± 28	18 ± 2.5	296 ± 18
HS + 50 ppm Cu + Ps	891 ± 29	76 ± 7.9	182 ± 9.8
HS + 100 ppm Cu	107** ± 8.1	125 ± 14	1062** ± 155
HS + 100 ppm Cu + Ps	688 ± 23	17** ± 2.9	68* ± 11

N=3, $p<0.005$, * = Significant,** = Highly significant

Discussion

This study identified many complex and multiple phenomena of siderophore in relation with root growth in aqueous and nutrient medium in presence and absence of microbial strain. There was a positive correlation between bacterial effects on root length of Cu treated and untreated plants. Because copper tends to accumulate in the root tissue with little translocated to the shoots (Azmat & Khan, 2011; Azmat & Riaz, 2012).

Biophysical parameters: The damaging effect on roots due to secretion of pigment by the bacterial strain may be related with an increase in high chemical oxygen demand (COD), lowers air pressure due to which nutrient uptake by the root was effected consequently inhibitory effect on root elongation was observed while inoculation of bacteria in nutrient medium represent that extra nutrient may support growth of seedlings. The maximum root length-promoting effect on Cu-treated plants was at 50 ppm. Stimulation on root length and weight was 12.52g & 0.253g respectively whereas inoculations of microbial strain decreased the growth of root markedly (2.9g & 0.142g). This clearly indicates binding capability of siderophore to the metal which binds the Cu which results in inhibition of root growth. Inhibition of root elongation by the bacteria was more pronounced with Cu treated plants at100ppm of Cu (Table 1) as compared to Cu and *pseudomonas* treated plants separately. This showed that Cu was toxic at high concentration (100pm) separately but when applied simultaneously with bacterial strain, surprisingly it showed some promoting effects on root growth at 100ppm of Cu with demise of all microorganism. This may be attributed to that, Cu is immobilized in presence of dead mass of *P. stutzari*. No life at 100ppm concentration showed that Cu was toxic at higher concentration to the bacteria with no pigmentation (Hayyat *et al*., 2012). This may be attributed to that of adsorption of Cu metal on microbial strain dead mass which was definitely take place as reported earlier (Azmat *et al*., 2013) due to which stimulation in root growth was observed, since the principle achievement of Cu toxicity is on root growth, the study of Cu toxicity in a soil location is difficult (Tank & Saraf 2009). Aqueous nutrient medium provides a model of the interface between the plant and the soil solution and permits assessment of root growth and morphology and can be helpful as a bio-tool for measurement of air pressure and biological oxygen demand (BOD).

Biochemical parameters: Biochemical analysis i-e lipids, glucose, and proteins of root showed that lipid (Table 2) was the main targeted contents separately and simultaneously of Cu and siderophore toxicity, produced approximately the same deleterious effect on lipids as on biophysical parameters (Table 1). Cu has been found to decline neutral lipid and total lipid contents in tomato seedling, and this reduction was observed in the lipid levels of membranes of organelles such as the chloroplast as reported earlier (Quariti *et al*., 1997; Ozounidou, 1994). This indicates that there was a positive relation between lipids and growth in early stage of development of seedlings. Results reported in the Table 2 showed that complex of Cu and siderophore caused effects on proteins as well on lipid contents (35% decline) and slight increase in glucose contents which may be the results of conversion or oxidation of lipid into glucose at the time of oxidative stress for survival while in Hoagland solution protein and glucose were same as in aqueous medium. The low lipid may be due to peroxidation, owing to the production of free radical (Quartacci *et al*., 2001). Our data on Cu accretion in roots are dependable with what is recognized about divergences between plant tissues in the mobility and binding properties of this micronutrient. The decrease in lipids may be related with the oxidative stress due to the metal binding capacity as reported earlier (Azmat & Khan, 2012) but in this study it can also be related with the pigment released by the bacteria (Table 2) to prevail over the both stresses that converted lipids into the glucose contents in energy for survival. As Cu is essential nutrient but when in excesses (100ppm), is an efficient inhibitor of vegetative augmentation and induces general indications of senescence (Table 2). Inoculation of microbial strain *P.stutzeri* at high (100ppm) concentration of Cu showed improvement in glucose, lipid and proteins contents where no life of microorganism with stimulation in growth of roots was reported. This may be related with immobility of Cu due to the adsorbing capability of microbial dead mass (Qiaoyun *et al*., 2005) which results in an effective growth and high biochemical contents of seedlings. It can also be related with the elevated toxicity of Cu to the plants has led to the development of a number of defensive strategies. In comparison with the control, only the excess of Cu caused a decline in the lipid to protein ratio as well as a change in the glucose contents. Biophysical disorder in the plants growth is largely due to the maximum amount of Cu absorbed by plants is retained in the roots (Foy *et al*., 1978).

Enzymes activity under stress: The enzymatic studies in presence of stress showed that it may be increased or inhibited due to the active site being either denatured or distorted. Guo *et al*., (2005) reported that under physiological conditions, little or no intracellular enzymes are released from the cell, except for physical injury to the cell wall and/or a change in the permeability of the cell membrane. Therefore, the data obtained for the intracellular enzyme activities of bacteria and Cu treated plant suggested that the permeability of the cell membranes changed significantly and roots of the seedlings suffer drastically. LDH activity was generally associated with cellular metabolic activity which acts as a pivotal enzyme between the glycolytic pathway and the tricarboxylic acid cycle commonly; transform the pyruvic acid into lactic acid under metal stress. This may be monitored in the roots as a strategy adopted by the plants in its root tissues. Thus, elevation of LDH activity as compared to control seedlings (Fig. 1) may suggest a bias towards the anaerobic glycolytic pathway. LDH activity was less to that of aqueous medium. Highest LDH activity in presence of bacterial stress showed the effect of siderophore on physically on root growth and biochemically on lipids (783). While in nutrient medium it was decreased sharply with slight increase in lipid contents. At this stage non utilization of glucose may be related with lower oxygen available as bacterial strain requires oxygen which results in the anaerobic glycolytic pathway. When strain was inoculated with nutrient medium LDH activity increased, compared with activity in nutrient medium with approximately same amount of lipid in aqueous medium but less glucose contents showed aerobic glycolytic pathway. Addition of 50ppm and 100ppmof Cu showed inhibitory effect on root germination related to the decline contents in lipids, glucose and proteins in both abiotic and biotic stresses or the decrease in contents was a direct consequence of oxidative stress produced by the metal. Inoculation of microbial strain gives slight positive effects on lipid and glucose contents with the decrease in protein contents. An increase in glucose and lipids contents may also be related with the two important enzymes i.e., AST and ALT. Activity of both enzymes were found to be alter in presence and absence of Cu and microbial strain which indicate the denaturing of enzymes in stress condition or alteration may be related with strategies for survival to safe the macromolecules like glucose, lipids and proteins in favors of growth that was observed at 100 ppm of Cu with no life of microorganism at this concentration and non-significant change in protein at 100ppm of Cu separately and simultaneously (Fig. 2) .

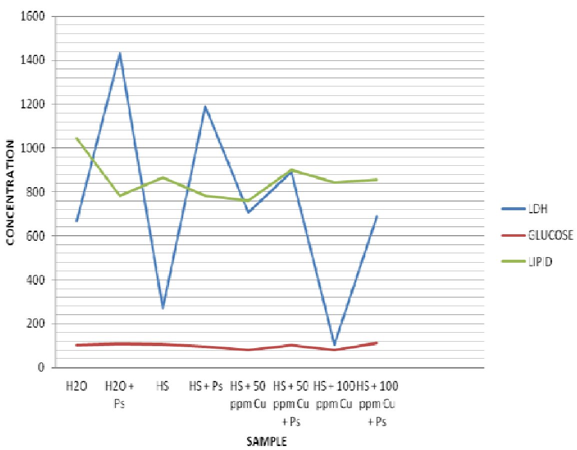

Fig. 1 Effect of LDH activity on glucose and lipid of root of *Vigna radiata* under Cu and microbial strain.

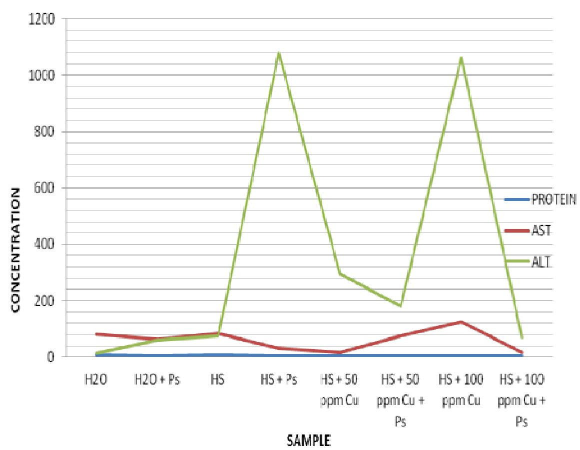

Fig. 2. Effect of ALT and AST activities on protein contents of root of *Vigna radiata* under Cu and microbial strain.

Conclusion

A wide range of phenomena from simple to complex interactions was observed between microorganisms and Cu metal in relation with plant root growth such as adsorption of Cu on dead biomass, oxidation-reduction, complex formation with pigment, solubilisation. Changes in the enzymes activity may be related with the resistant against the biotic and abiotic stress or it could be indirect regulation for survival. It was suggested that microbial dead mass at highest concentration of metal act as a good biosorbent where Cu was adsorbed and normal growth parameters were recorded. Also siderephore of the florescent bacteria can act as a bio-tool for metal, detection, complex formation and its deactivation in soil.

Acknowledgement

Author 1s very thankful to her research associate from Department of Microbiology Ms. Aliya Hayyat for detection of Microbial life and Dean Faculty of Science for Financial Assistance

References

Azmat, R. and N. Khan. 2011. Nitrogen metabolism as a bio indicator of Cu stress in *Vigna radiata*. *Pak. J. Bot.*, 43(1): 515-520.

Azmat, R. and S. Riaz. 2012. The inhibition of polymerization of glucose in carbohydrate under Cu stress in *Vigna radiata*. *Pak. J. Bot.*, 44(1): 95-98.

Azmat, R., A. Hayat, F. Aziz and M. Qadri. 2013. Function of enzyme lactate dehydrogenase in relation with lipid and glucose in biotic and abiotic stresses in the seedlings of *Vigna radiata*. *African J. Microbiolo. Research*, *7*(4): 290-297.

Belimova, A.A., N. Hontzeasb, V.I. Safronovaa, S.V. Demchinskayaa, G. Piluzzac, S. Bullittac and B.R. Glick. 2005 Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.) *Soil Biolo & Biochem*., 37: 241-250.

Chakraborty, R.N., H.N. Patel and S.B. Desai**.** 1990. Isolation and partial characterization of catechol-type siderophore from *Pseudomonas stutzeri* RC-7. *Current Microbiolo*., 20: 283-286.

Feistner, G.J., D.C. Stahl and A.H. Gabrik. 1993. Proferrioxamine siderophores of *Erwinia amylovora*. A capillary liquid chromatographic/electrospray tandem mass spectrometric study. *Journal of. Mass Spectrometry*, 28: 163-175.

Foy, C.D., R.L. Chaney and M.C. White. 1978. The physiology of metal toxicity in plants. *Ann. Review Plant Physio*., 29: 511-566.

Gledhill, M. 2001. Electrospray ionization-mass spectrometry of hydroxamate siderophores. *Analyst*, 126: 1359-1362.

Guo, T., Y. Ma, P. Guo and Z. Xu. 2005. Antibacterial effects of the Cu(II)-exchanged montmorillonite on *Escherichia coli* K88 and *Salmonella choleraesuis*. *Veterinary Microbiolo*., 105(2): 113-122.

Hayyat, A., R. Azmat and F. Aziz. 2012. Effect of Cu on pigmentation and survival of *Pseudomonas stutzeri*. *J. Biomed. and Pharmacolo.* 5(1): 51-56.

Kefeng Li and R. Wusirika. 2011 Effect of multiple metal resistant bacteria from contaminated lake sediments on metal accumulation and plant growth. *J. Hazards. Mat*., 189(1-2): 531-539.

Korzeniewski, C. and D.M. Callewaert. 1983. An enzyme-release assay for natural cytotoxicity. *J. Immunolo. Methods*, 64(3): 313-320.

Li, K. and W. Ramakrishna. 2011. Effect of multiple metal resistant bacteria from contaminated lake sediments on metal accumulation and plant growth. *J. Hazards. Mat.*, 189(1-2): 531-9.

Marschner, H., V. Romheld and M. Kissel. 1987. Localization of phytosiderophore release and of iron uptake along intact barley roots. *Physiologia Plantarum*., 71: 157-162.

Ozounidou, G. 1994. Root growth and pigment composition in relationship to element uptake in *Silene compacta* plants treated with copper. *J. Plant Nut*., 17: 933-943 .

Qiaoyun, H., C. Wenli and X. Leihui. 2005. Adsorption of copper and cadmium by Cu- and Cd-resistant bacteria and their Composites with soil colloids and Kaolinite. *Geomicrobiolo. J*., 22(5): 227-236

Quariti, O., N. Boussama, M. Zarrouk, A. Cherif and M.H. Ghorbal. 1997. Cadmium and copper-induced changes in tomato membrane lipids. *Phytochemis*, 45: 1343-1350.

Quartacci, M.F., E. Cosi and F. Navari-Izzo. 2001. Lipids and NADPH-dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. *J. Exp. Boy*., 52(354): 77-84.

Tank, N. and M. Saraf. 2009. Enhancement of plant growth and decontamination of nickel-spiked soil using PGPR. *J. Basic Microbiolo*., 49(2): 195-204.

(Received for publication 2 April 2012)