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Full Length Research Paper

Function of enzyme lactate dehydrogenase in relation with lipid and glucose in biotic and abiotic stresses in the seedlings of *Vigna radiata*

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Soil contamination by heavy metals has caught our consideration to assess and determine the cumulative effect of isolated bacterial strain of *Pseudomonas stutzeri* with copper (Cu) as a bioactive element in seed germination of four days old *Vigna radiata*. Effects of varying concentrations of essential trace metal on the germination of seeds (bioassay) were also taken into consideration. An important function of the enzyme lactate dehydrogenase (LDH) was observed in the shoots of four days old seedlings of *V. radiata,* cultivated in a series of experiments in the presence and absence of biotic and abiotic stresses simultaneously and separately. The data suggested that *P. stutzeri* separately inhibited the seed germination possibly due to the marked decrease in lipid contents whereas high LDH activity and high glucose contents as compared to the control ones indicated that high LDH activity was linked with elevated energy demand to overcome the stress for germination of seeds. Unexpectedly, germination of seeds was favoured with increasing concentrations of Cu (100 ppm) with *P. stutzeri* simultaneously, although both biotic and abiotic seemed to be toxic separately. No microbial life at the highest concentration of Cu showed that it was toxic to the bacterial strain, but normal growth of seedlings suggested that dead mass of *P. stutzeri* was effective for the adsorption of the Cu on their surface due to which Cu mobility was checked resuming the normal activity of LDH and glucose contents that believed to be at the cost of lipid contents. A suitable mechanism consistent with the finding has been proposed.

Key words: *Pseudomonas stutzeri*, Cu, lactate dehydrogenase (LDH), lipid, glucose.

INTRODUCTION

The impact of pollution from urbanization and industrialization wastes has reached a disturbing level. Environmental contaminants are widely distributed in the soils, thereby having effect on the trophic chain, plants, animals and men. These pollutants can remain in soil for a long time. Trace metals (non-biodegradable) are naturally present in the biological world in acceptable quantities, but since the last century, an increase of these through anthropogenic contributions, has been known to affect microbial growth, numbers, survival, biomass and abundance (Sokhn et al., 2001).

In the same way as aquatic environments, soils are the target of thousands of contaminants that vary in composition and in concentration. These contaminants enter the system as a result of a wide range of actions such as intentional applications, inadequate residue disposal, accidental wastes and inappropriate use (Knaebel et al., 1994). The toxic waste include inorganic

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compounds as nitrates, phosphates and perchlorates (Nozawa-Inoue et al., 2005); a range of herbicides such as diuron, linuron and chlorotoluron (Fantroussi et al.,1999); heavy metals (Glick, 2003); explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Kitts et al.,1994); and by mono aromatic hydrocarbons like benzene, toluene, ethylbenzene and xylene (known as BTEX) (Rooney-Varga et al., 1999)]; polycyclic aromatic hydrocarbons (Wang et al.,1990)

In the case of soil remediation, there are several factors that should be taken into consideration. The soil, as any other ecosystem, is a vital habitat to thousands of organisms associated like a wide variety of fungi, actinobacteria, algae, protozoa and different types of bacteria that differ in physiology. These microorganisms can occur in association to clay particles or organic matter and in the rhizosphere of plants essential to their metabolism in synergism with plant roots; this term can be defined as the three units interacting: the plant, the soil and the microorganisms.

Metal contaminations in the soils not only lead to the changes of the soil microorganisms and their activities and result in soil fertility deterioration but also directly affect the change of physiological indices and, furthermore, results in yield decline (Cheng, 2003).

Teitzel and Parsek (2003) reported that the bio- layer and live culture were tested for toxic metal tolerance through rotating-disk biofilm reactor and found that biofilm was more resistant to heavy metal as compared to live microbial cell. Biofilm was capable to concentrate the bacteria in it.

Regarding bacteria, there are numerous reports on siderophore production by *Pseudomonas spp*. (Abdallah, 1991; Budzikiewicz, 2001; Chakraborty et al., 1990). More than 50 structurally related siderophores, such as pyoverdins, produced by the fluorescent *Pseudomonas spp*., especially *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* have been characterized (Budzikiewicz, 2001). All pyoverdins emit yellow fluorescent light due to the presence of a 5-amino-2,3-dihydro-8,9-dihydroxy-1-H-pyrimido-quinolinecarboxylic chromophore, to which a peptide chain and a carboxyl chain are attached (Abdallah, 1991; Budzikiewicz, 2001; Meyer and Abdallah,1980).

Several researchers reported the activities of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) in plant stress condition. Transaminases catalyse the transfer of the amino groups of amino acids to 2-oxoacids. In plants, transaminases very efficiently contribute in transformations of nitrogen compounds. They are imperative for the biosynthesis of amino acids from oxo-acids in the citrate cycle and for other crucial biochemical pathways, such as synthesis of chlorophyll. Alkaline phosphatase is a ubiquitous enzyme in all organisms and catalyses hydrolysis of orthophosphate monoesters; their physiological functions remain unclear, but they are considered to play a role in phosphate uptake (Krystofova et al., 2009). Betsche (1981) proposed a function of leaf L-lactate dehydrogenase as a constituent of the systems regulating the cellular pH and/or controlling the concentration of reducing equivalents in the cytoplasm of leaf cells. Lactate dehydrogenase catalyzes the inter conversion of pyruvate and lactate with concomitant inter conversion of NADH and NAD^+. At elevated concentrations of lactate, the enzyme exhibits feedback inhibition, and the rate of conversion of pyruvate to lactate is decreased.

The aim and objective of this investigation was to check the siderophore interaction with Cu metal and their simultaneous and separate affects on growth of seedlings in aquaculture. This article discusses the function of enzyme lactate dehydrogenas (LDH) in relation with lipid and glucose in biotic and abiotic stresses in the seedlings of *Vigna radiata* and remediation of Cu metal by bacteria.

MATERIALS AND METHODS

Effects of bioactive element Cu metal with microbial strain *P. stutzeri* were investigated in relation with the legume species *V. radiata*. The four to five surface sterilized seeds of *V. radiata* were grown in optimal conditions in seven different Petri plates, restrained water, Hoagland solution, in which Cu was added namely 0, 50 and 100 ppm. In order to accomplish the goal of this experiment, 20 ml solution were poured into the each Petri plate at the time of the germination. The experiments were carried out for 4 days in triplicate. After four days, seedlings were subjected to physical and biochemical analyses including lipid, glucose, protein and enzymatic activity. The data obtain were subjected to statistical analysis.

Microbial identification

P. stutzeri strains were isolated from garden soil and identified morphologically and biochemically. The isolated microorganism was identified as gram negative short rods. The organism was grown on MacConkey's agar (Oxoid) (gm/L) (peptone, 20.0; lactose, 10.0; Bile salts, 5.0, Sodium chloride, 5.0; Neutral red, 0.075; agar, 12.0, pH 7.4 ± 0.2), triple sugar iron agar (Oxoid) (gm/L) (Lab-Lemco' powder, 3.0; yeast extract, 3.0; peptone, 20.0; sodium chloride, 5.0; lactose, 10.0; sucrose, 10.0; glucose, 1.0; ferric citrate, 0.3; sodium thiosulphate, 0.3, phenol red, 0.024; agar 12.0, pH 7.4 ± 0.2) and Simmon citrate agar (Merck) (gm/L) ($MgSO_4 .7H_2O$ 0.2, $NH_4H_2PO_4$ 1, K_2HPO_4 1, sodium citrate 2, NaCl 5, agar 15, Bacto bromothymol blue 0.8).

For oxidase test, filter paper strip were soaked in N,N,N^1,N^1-tetramethyl-p-phenylene diamine dihydrocholride (50.0 mg/tube). A loopful culture of isolated organism was spread on filter paper strip for the appearance of deep purple color. Other biochemical tests were performed for the identification of organism using QTS-NE Kit (Table 1) (DESTO –laboratories, Pakistan) (Hayat et al., 2012).

Biochemical parameters

Lipids were estimated by the method described by Frings and Dunn (1970) at a wave length of 540 nm using sulfophosphovanillin reaction and determined by formula.

Table 1. Identification of microorganism by QTS-NE KIT.

Test	Name of reaction	Result	Organism identified
IND	Indole	Negative	"Pseudomonas stutzeri"
GLU (Acid)	Acid from Glucose Nitrate reduction	Positive	
ADH	Arginine dihydrolase	Negative	
URE	Urea hydrolysis	Negative	
ESC	Esculin hydrolysis	Positive	
GEL	Gelatin hydrolysis	Positive	
ONPG	O, nitrophenyl β D galactosidase	Negative	
GLU	Glucose assimilation	Positive	
MAL	Maltose assimilation	Negative	
MNE	Mannose assimilation	Positive	
NAG	N-acetyl-glucose amine-assimilation	Negative	
CIT	Sodium citrate assimilation	Positive	
CO Strip	Spot test cytochrome oxidase	Positive	

$$\text{Total lipid concentration (mg/dl)} = \frac{\text{A sample}}{\text{A standard}} \times 800$$

Protein and glucose were estimated by the RANDOX test kit (Randox Laboratories Ltd, 55 Diamond Road, Crumlin, Co. Antrim, United Kingdom, BT29 4QY), according to the manufacturer's instructions. Bio-molecular activities were expressed as a specific activity (UL-1) and all experiments were performed in triplicate.

Enzymes activity

The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were determined 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). All experiments were performed in triplicate.

Statistics

Data was subjected to statistical analysis. The values reported were the average of three replicates ± standard error obtained from the one-way analysis of variance followed by Tukey's honestly significant difference analysis performed with the statistical package SPSS Version 12.0 (SPSS, Chicago, IL, USA.).

RESULTS AND DISCUSSION

Cu has been shown to reduce or inhibit the seed germination and, the extent of the effect depended on concentration in the solution and the bacterial species concerned. The effects were assessed for bacterial growth in culture and in symbiotic parameters such as biomass production and analysis of some biochemical parameters in seedlings of *V. radiata*. Cu was found to have detrimental effect in both biotic and abiotic conditions in all its concentrations on bacterial strain and plant. But effect was reduced at high concentration of Cu on plant growth simultaneously that was related with adsorption of Cu on dead mass of bacteria. Application of Cu was found inhibitory for bacterial strains at all concentrations separately. Specific growth rate of bacteria in culture was found to decrease from 50 ppm while all microorganisms experienced death at 100 ppm of Cu. Similarly, Cu was observed to have a negative effect in all concentration on the growth of the plant.

The following aspects of metal and pigment interaction on plants growth was discussed as: (i) heavy metal effects on plants growth and bacterial pigment separately as a abiotic and biotic stress; (ii) both biotic and biotic stress simultaneously on early stage of germination of seedlings and (iii) detoxification of both metal and pigment pollution.

Growth and biomass yield

Effect of Cu at both concentration namely 50 and 100 ppm in culture of *Pseudomonas* strains and plant seedlings were observed separately and simultaneously. Bacterial growth and seedling growth rate were adversely affected (Figure 1). Results of biotic and abiotic stress on weight of seedlings are presented in Figure 2. The results related to the uptake of metals in this study, suggests that roots of *V. radiata* was an efficient obstacle to Cu and pigment of *P. stutzeri* to the above ground aerial part of the plant which was connected to a chemiosmotic process across the membrane of intact root (Mitchell, 1979; Harmens et al., 1993). Some physicomorphological parameters like weight of whole seedling and length were recorded and some remarkable results were observed

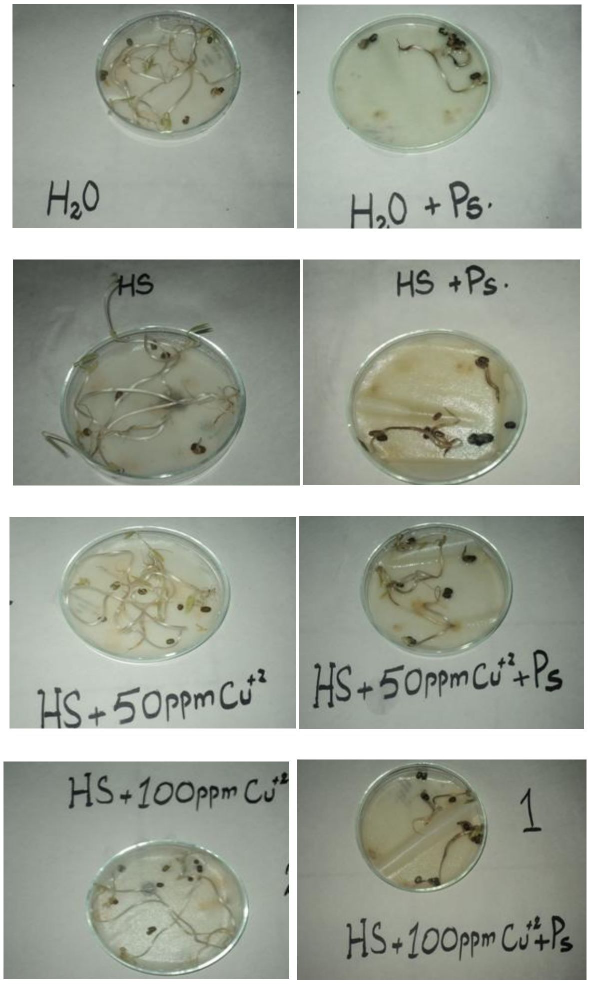

Figure 1. Seed germination in the presence of pseudomonas and two different concentration of copper.

related with the weight of whole seedlings as compared to seeds germinated in water and nutrient medium. It was observed that roots were significantly affected as compared to shoot because of direct link with the toxicant and roots. Seed which were germinated in the Petri dish inoculated with the microbial strain showed a drastic decrease in the weight and length as compared to those of water and nutrient medium which may be due to the toxic or non promoting action of bacteria on growth which showed inhibition in seed germination. Seed germinated with the 50 ppm of Cu had remarkable effects on weight as compared to non Cu medium which may be attributed to Cu as a bioactive inorganic elements required in the early stages of growth as an important nutrient element but with *P. stutzeri,* it again showed a depletive effect on weight regarded to germination whereas there was an increase in the concentration of Cu; inhibition in the seed germination was observed but sample seeds grown in the high concentration of Cu with *P. stutzeri* showed a good germination. This may be related with complex formation of Cu with the pigments secreted by bacteria due to which Cu mobility in the medium was controlled; excess concentration of Cu resulted in the inhibition of activity of *P. stutzeri* and dead mass of bacterial strain adsorbed the Cu on their surface which resulted in better growth of seedlings at higher concentration of Cu. The decrease in weight of seedlings with *P. stutzeri* showed that pigment released by bacteria was toxic and it directly interacted with the seed coats membrane (Sharma et al., 2003).

Biochemical pathway under biotic and abiotic stress

The LDH activity was monitored related to the lipids and glucose contents in two stresses. The results are reported in the Tables 2 and 3 which showed adverse affects on biochemical profile of shoot of the *V. radiata.* Results reported in the Tables 2 and 3 showed that protein contents of seedlings reflects no significant depletion while worse lipid contents in the shoot indicate that it was the main source of energy in biotic and abiotic stresses. Increase in glucose contents may also be related to the facts that protein and glycerol from fat can be converted into glucose under stress condition as lipids are those compounds which dissociated as primary metabolites in stress condition to overcome the energy demand by the plant for growth. The decrease in lipids contents may be related with the toxicity of pigment and Cu because fat helps the body in giving energy at the time of emergency that is if the energy supplied by carbohydrates is not enough or carbohydrate fails to supply enough energy for survival. Results reported in Table 2 show that in stress, Cu and *P. stutzeri* glucose was increased and lipid decreased severely (Boopathy, 2000). This may be attributed to the conversion of lipid in glucose to minimize the stress or it may be that lipids can be characterized in relation to bio-energy for continued existence (Figure 3).

Results of enzyme activity reported in the Table 3 show that activity of enzymes of seedlings in aqueous medium were high in comparison to nutrient medium where AST, ALT, were decreases while ALP and LDH were increases (Figure 4). Enzymes activity of plants in the nutrient medium along with *P. stutzeri* was significantly affected which may be due to the pigment secreted by bacterial strain. This can be associated also to that of absorption of oxygen by pigment and Cu and may be a factor of growth inhibition of seedling while AST, ALT ALP at 50 ppm of Cu separately increased significantly with significant decreased in LHD activity while higher activity was observed in all enzymes at higher concentration of Cu (100 ppm) but at 50 ppm in both aboitic and biotic stress, enzymes activity were higher. An interesting results of all studied enzymes of shoot of *Vigna radiata* in the present investigation at 100 ppm of Cu with *Pseudomonas stutzeri* simultaneously showed no significant change in the LDH activity. This clearly support

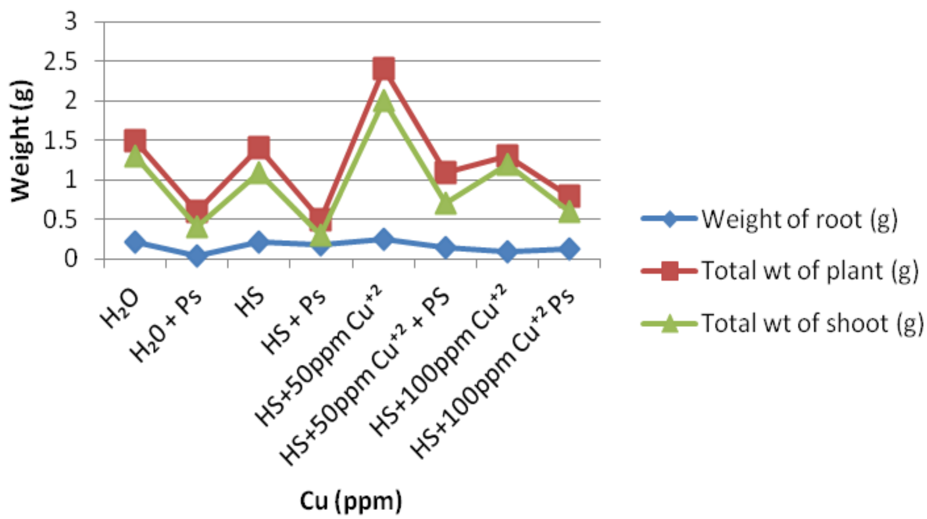

Figure 2. Weight of *Vigna radiata* under biotic and abiotic stresses.

Table 2. Biochemical Profile of *Vigna radiata* under biotic and abiotic stresses.

Sample	Total protein (mg/dl)	Total lipid (mg/dl)	Glucose (mg/dl)
H_2O	7.00	1232	110
H_2O+ Ps	7.00	341**	124**
HS	6.00	747**	130
HS + Ps	6.00	758**	108
HS+50ppm Cu^{2+}	7.00	801**	104*
HS+50ppm Cu^{2+} +PS	6.00	792**	125
HS+100ppm Cu^{2+}	5.00	825**	134**
HS+100ppm Cu^{2+} Ps	7.00	740**	109

N=3, *significant;**highly significant at p<0.05, HS, Hoagland Solution.

Table 3. Enzymes alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) of *Vigna radiata* under biotic and abiotic stresses.

Sample	AST (U/L)	ALT (U/L)	ALP (U/L)	LDH (U/L)
H_2O	26	19	11	242
H_2O + Ps	58**	40**	22	287**
HS	19	12*	15	262
HS + Ps	69**	44*	71**	181
HS + 50ppm Cu^{+2}	54**	53**	41**	132**
HS + 50ppm Cu^{+2} + Ps	40**	66**	31**	281**
HS + 100ppm Cu^{+2}	35**	44**	69**	271**
HS + 100ppm Cu^{+2} +Ps	24	16	8**	239

N=3, *significant; **highly significant at p<0.05, HS, Hoagland Solution, PS = *Pseudomonas stutzeri.*

our present mechanism of adsorption of Cu on dead mass of bacteria due to which healthy growth of the host plant was observed with approximate same values of protein and glucose with decline lipids contents which was believed to serve as potential bio-energy provider at the time of stress (Amora-Lazcano et al., 2010).

The possible inhibition in seed germination may also be related with pigment secreted by *Pseudomonas stutzeri*

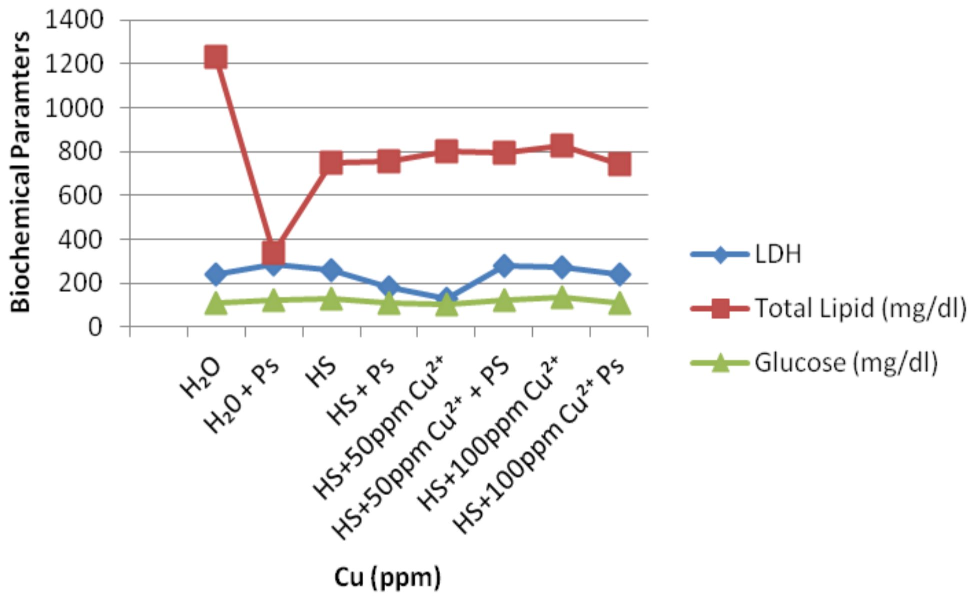

Figure 3. Function of LDH in relation with protein and glucose of *Vigna radiata* under biotic and abiotic stresses, HS = Hoagland Solution, PS = *Pseudomonas stutzeri.*

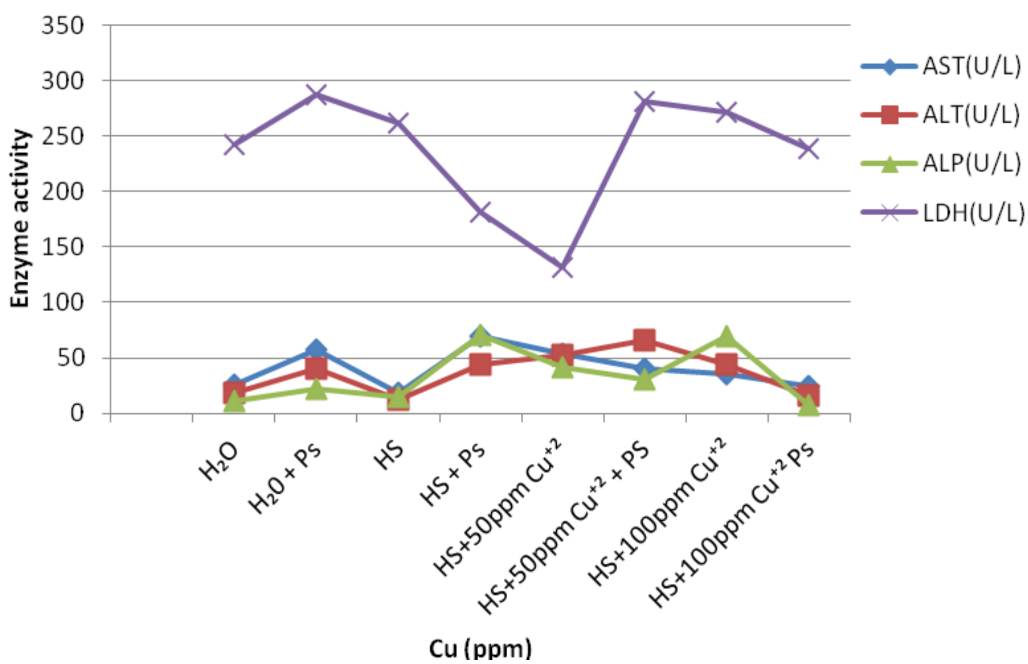

Figure 4. Enzymatic activities of *Vigna radiata* under biotic and abiotic stresses; HS, Hoagland solution, PS, *Pseudomonas stutzeri.*

separately that can cut the light and effect on absorption of oxygen or possibly due the marked decrease in lipid contents whereas high LDH activity and high glucose contents as compared to control ones indicate that high LDH activity was linked with elevated energy demand to overcome the stress for germination of seeds. Unexpectedly germination of seeds was favoured with increasing concentrations of Cu (100 ppm) with *Pseudomonas stutzeri* simultaneously although both biotic and biotic seems to be toxic separately under investigation. No microbial life at highest concentration of Cu showed that highest concentration of Cu was toxic to the bacterial strain but normal growth and germination at high concentration (100 ppm) suggests that dead mass of *Pseudomonas stutzeri* was effective in adsorption of the Cu on their surface due to which Cu mobility was checked and normal growth of the seedlings were observed with approximately normal activity of LDH and glucose contents. This may be at the cost of lipid contents (Figure 3).

Mechanisms of rhziobacteria influencing heavy metal accumulation

Results show that heavy metal influence the natural bacterial populations which resulted to the inhibition of growth and death of microbes. Dead mass of microbes adhered the metal on its surface and control the activity of toxic metal. There are several possible explanations for this observation. The concentration-dependent toxicity of a heavy metal can be reduced by various means: through complex-formation with siderophore (Milanovich and Wilson, 1975; Benes et al., 1976), through bacterial incorporation and accumulation (Doyle et al., 1975) and through biological metal-transformation (Brunker and Bott, 1974; Holm and Cox, 1975). Thus, in the surface of micro layer, the toxic effects of heavy metals could possibly be reduced or masked through the formation of complexes of the metals with siderphore or pigment or organic substances.

Pseudomonas stutzeri is a vital bacterium playing a significant role due to secretion of pigments which exhibit a major role related to the mechanisms of metal chelating agents called siderophore, have a central role in the acquisition of several heavy metals and other aspects which was observe in present investigation of this bacteria. It was observed that, after experiencing the death at high concentration of heavy metal Cu, their dead mass became an effective surface site that was able to absorb excesses of metal on their surface and become supportive in controlling the mobility of metal to avoid the toxic effects of Cu on seedling growth that results in good growth even at higher concentration of metal.

Conclusion

Cu metal is a necessary component of some physiologically important compounds such as enzymes and other cofactors. The application of high dose of Cu inhibits growth of bacteria and host plant both. Thus, excessive accumulation of this heavy metal in soils must be prevented. It was concluded that application of bacterial strain with high concentration of Cu results in the death of microbial life which were found to be favourable for plant growth due to the accumulation of Cu on the dead surface of microbes. This resulted in good plant growth.

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