Oxidative Damage and Antioxidative Defense Mechanisms of *Nostoc muscorum* in Response to Copper (Cu^{2+}) Stress

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MIZORAM UNIVERSITY, AIZAWL

2016

Oxidative Damage and Antioxidative Defense Mechanisms of *Nostoc muscorum* in Response to Copper (Cu^{2+}) Stress

By

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BOTANY DEPARTMENT

submitted in partial fulfillment of the requirement of the degree of

Doctor of Philosophy

in

Botany

Of

Mizoram University, Aizawl

2016

DECLARATION BY THE CANDIDATE

MIZORAM UNIVERSITY

APRIL, 2016

I, **Yengkhom Mrinamala Devi**, hereby declare that the subject matter of this thesis entitled **"Oxidative Damage and Antioxidative Defense Mechanisms of *Nostoc muscorum* in Response to Copper (Cu^{2+}) Stress"** is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for degree of Doctor of Philosophy in Botany.

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I have great pleasure in forwarding the thesis entitled **"Oxidative Damage and Antioxidative Defense Mechanisms of *Nostoc muscorum* in Response to Copper (Cu^{2+}) Stress"** submitted by Yengkhom Mrinamala Devi for the Ph. D degree of Mizoram University. Yengkhom Mrinamala Devi has put in the prescribed number of terms of research work under my supervision. The data incorporated in the thesis are based on her own independent observations.

(S. K. MEHTA)

Supervisor

Preface

Heavy metal pollution of water bodies due to indiscriminate disposal of industrial and domestic wastes has become a global problem. These pollutants continually pose a serious threat to aquatic organisms by persisting in the sediment of water bodies for a very long period of time. *Nostoc muscorum* is an important nitrogen fixing cyanobacterium of rice fields. Any harmful effect of heavy metals on nitrogen fixing cyanobacteria affects seriously the nitrogen economy of the aquatic ecosystem. The present thesis describes the uptake and toxicity of copper in *N. muscorum*. Efforts were also made to understand the antioxidant responses of test organism to sublethal doses of copper. An understanding of effect of heavy metal on photosynthesis is another topic covered in this study.

The thesis is organized into seven chapters with 'General Discussion' at the end. Chapter 1 'General Introduction' reviews the current status of heavy metals in the environment and their toxicity effects. Chapter 2 deals with the methods used in the present study. Chapter 3 deals with the kinetics of adsorption and uptake of Cu^{2+} by the test alga. This is followed by investigations on effect of pH, influence of cations and metal ions on adsorption and uptake of test metal. The next chapter, (Chapter 4) deals with toxicity and antioxidative defense systems in *N. muscorum.* Chapter 5 describes with the effect of Cu^{2+} on the processes of photosystems and potential target sites in ETR where copper mostly acts. The next chapter, Chapter 6, is focused on nitric oxide accumulation and its possible roles in metal toxicity and tolerance. Although each chapter includes discussion, a brief 'General Discussion' has also been included as chapter 7. Summary of the thesis is given after general Discussion. A list of papers cited in the text is given at the end as 'Reference'. I feel sorry that some repetitive statements in certain parts of the thesis may appear as minor irritants to some readers. Despite my all efforts, some repetition could not be avoided as each chapter includes Introduction and Discussion besides General Introduction and General Discussion.

Acknowledgements

I would like to take this opportunity to thank all the people behind the success of my research work. It would not have been possible to finish this study without their help and support.

First and foremost, all the thanks to Almighty God for being with me always, whose blessings have enabled me to accomplish my thesis work successfully.

Secondly I am expressing my profound sense of respect, deep gratitude and indebtedness to my supervisor Dr. S.K. Mehta, Department of Botany, Mizoram University, Mizoram for his kind cooperation, invaluable guidance and encouragement throughout the course of the present investigation. His suggestions and painstaking supervision gave me the strength to overcome the hard times during the entire course of the study.

I am highly thankful to the Head, Department of Botany, Mizoram University for providing necessary laboratory facilities. I express gratefulness to all the distinguished faculties of Botany Department for helping and giving me suggestions whenever in need. I also extend my immense pleasure to thank all non-teaching staffs of the Botany Department for their kind cooperation.

I am thankful to my labmates- Lalrinkimi, Joelle, Mirabai, John and Augustine for helping me in various ways during experiments and during the compilation of my thesis. I also express my thanks to the Coordinator, Central Instrument Laboratory, MZU for providing Dual PAM-100 system and AES (Agilent).

I express gratefulness to Dr. N. Rokendro Singh, Department of Commerce, Mizoram University and his family for being with me all the time and guiding me throughout my stay in Mizoram. I am also extremely thankful to my youngers (Jaya and Rebina) for taking care of my daughter and helping me all the time.

I acknowledge the blessings, affection and inspiration of my parents, my in-laws and other family members for their guidance and constant encouragement which enabled me to take the challenging task successfully in life.

This study has been financially supported by University grants Commission, the government funding agency of India under Rajiv Gandhi National Fellowship (RGNF).

I am very grateful to all those whom I didn't mentioned their names, but gave me encouragement and helped me directly or indirectly during the course of this study.

And, the last but not the least, I would like to thank my husband, Premchandra and my daughter, Chingkheilembi for their love, support, understanding, and for being the strongest motivational forces in my life. I dedicate this work to both my parents and my husband.

Aizawl, April 2016 ***Yengkhom Mrinamala Devi***

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Abbreviations

µM	Micromole
$^1O_2$	Singlet Oxygen
AA	Ascorbic acid
AL	Actinic light
AOS	Active oxygen species
APX	Ascorbate peroxidase
BSA	Bovine serum albumin
CAR	Carotenoid
CAT	Catalase
CDF	Cation-diffusion facilitator
CEF	Cyclic electron flow
Chl a	Chlorophyll a
Cu/Zn SOD	Copper /zinc Superoxide dismutase
DHAR	Dehydroreductase
DTNB	Dithiobis nitrobenzoic acid
EC	Electrical conductivity
EL	Electrolyte leakage
ETR	Electron transport rate
ETR I	Apparent electron transport in PS I
ETR II	Apparent electron transport in PS II
ETR_{max} (I)	Maximal electron transport rate in PS I
ETR_{max} (II)	Maximal electron transport rate in PS II
F	Steady state fluorescence
F_0	Minimal fluorescence after dark- adaptation
Fe- SOD	Iron- Superoxide dismutase
F_m	Maximum fluorescence after dark-adaptation

F'_m	Maximum fluorescence signal
F^o	The fluorescence yield briefly before application of a SP
$F^{o\prime}$	The minimal fluorescence yield of illuminated sample with all PS II centers open
F_v/F_m	Maximum yield of PSII
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSH	Oxidized glutathione
HbO_2	Oxyhemoglobin
HR	Hypersensitive response
K_d	Dissociation constant
K_m	Concentration of Cu^{2+} at which rate of intracellular uptake is half of $V_{max.}$
LC_{50}	Lethal concentration
LEF	Linear electron flow
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
Met Hb	Methaemoglobin
ML	Measuring light
Mn-SOD	Manganese- Superoxide dismutase
MTs	Metallothioneins
NADPH	Nicotinamide adenine dinuleotide phosphatase
NBT	Nitroblue tetrazolium
NiR	Nitrite reductase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP-SH/ NPT	Non-protein thiol

NR	Nitrate reductase
Nramp	Natural resistance associated macrophage
O_2^-	Superoxide radical ion
OD	Optical density
OH	Hydroxyl radical
P	The P700 signal
P_0	The minimum level of P700 signal
PAR	Photosynthetic active radiation
PC	Phycocyanin
PCs	Phytochelatins
Pcy	Plastocyanin
P_m	Maximal change in P700 (Far red light is applied)
P'_m	Maximal change in P700 (no far red light applied)
PS I	Photosystem I
PS II	Photosystem II
qN	Coefficient of non-photochemical fluorescence quenching
qP, qL	Coefficient of photochemical quenching, measure of the overall "openness" of PS II
ROS	Reactive oxygen species
SAR	Systematic acquired resistance
SDS-PAGE	Sodium dodecyle sulphate- polyacryl amide gel electrophoresis
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SP	Saturation pulse
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
T-SH	Total thiol
U_{max}	Maximum adsorption of metal ions on the surface of the cell
UV	Ultra violet

V_{max}	Maximum rate of intracellular metal uptake
Y(CEF)	Quantum yield of cyclic electron flow
Y(CEF)/ Y(II)	Ratio of quantum yield of CEF to that of LEF.
Y(CEF)/Y(I)	Indicated contribution of CEF and LEF to the yield of PS I.
Y(I)	Effective photochemical quantum yield of PS I
Y(II)	Effective photochemical quantum yield of PS I
Y(II)/Y(I)	Distribution of quantum yield between two photoststems.
Y(LEF)	Quantum yield of linear electron flow driven by PS II and PS I
Y(NA)	Quantum yield of non-photochemical energy dissipation of reaction centres due to acceptor side limitation.
Y(ND)	Quantum yield of non-photochemical energy dissipation of reaction centres due to donor side limitation.
Y(NO)	Quantum yield of non-light induced non-photochemical fluorescence quenching.
Y(NPQ)	Light induced non-photochemical fluorescence quenching

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria (also known as Blue-green algae, blue-green bacteria, and Cyanophyta) are a phylum of bacteria that obtain their energy through oxygenic photosynthesis. They possess the ability to synthesize chlorophyll-a. They belong to the Division: Cyanophyta, which is a small group comprising of about 2,500 species placed under the genera. All of them are included in a single class Cyanophyceae or Myxophyceae. The members of this class are considered the simplest, living autotrophic plant. Individually all the cyanobacteria are microscopic, both fresh water and marine. In cyanobacteria the cells constituting the thallus are prokaryotic. The photosynthetic pigments are located in the thylakoids, which lies free in the cytoplasm. The flagella are entirely lacking, even the reproductive cells are non-flagellated. Locomotion is of gliding or jerk type. The phycobillin pigments unique to this class are blue C-phycocyanins and red C-phycoerythrin, in addition to chlorophyll-a, B-carotene, mycoxanthin and mycoxanthophyll. There are membrane bound chromatophores. The unique food storage compounds in blue green algae are the myxophycin starch and a protenacious material cyanophycin. Many filamentous blue green possess specialized cells of disputed function known as the heterocysts. Sexual reproduction is completely absent but indication of

genetic recombinations, which fulfill the function of sex, is known in some members. One general attribute of most cyanobacteria is that they are morphologically (cell structure, heterocysts, reproduction) quite conservative but metabolically (nitrogen fixation, photosynthetic metabolism) versatile. In this respect, they contrast with most eukaryotic algae which tend to be structurally versatile but metabolically conservative.

The first appearance on the Earth

The Earth was formed about 4.5 billion years ago (Halliday, 2001) and the early life on it was dominated by microbes (Carroll, 2001). Cyanobacteria, also known as "blue green algae", belong to one of the oldest organisms on the Earth (Schopf and Packer, 1987). They are very primitive in evolutionary prospective. Earlier claims for the existence of cyanobacteria were 3.5 (Awranik, 1992; Schopf, 1993) and may be as early as 3.7 billion years ago (Des Marias, 2000: Kopp et al., 2005). However, chemical evidence and recent fossil findings indicate that cyanobacteria existed only about 2.5-2.6 billion years ago (Oslon and Blankenship, 2004). They have played fundamental roles in driving much of the ocean carbon, oxygen and nitrogen fluxes from the time to present.

Cyanobacteria as architects of the early Earth's atmosphere

Cyanobacteria are considered as a key to any understanding of Earth's early biological and environmental history (Tomitani et al., 2006). Photosynthesis was well established on Earth at least 3.5 billion years ago, and it is widely believed that these ancient organisms had similar metabolic capacities to modern cyanobacteria (Blankenship, 2002). The evolution of cyanobacteria was a major turning point in

biogeochemistry of the Earth. Prior to the appearances of these organisms, all photosynthetic organisms were anaerobic bacteria that used light to couple the reduction of carbondioxide to the oxidation of low free energy molecules, such as H_2O. Transition from an anoxic to an oxic atmosphere occurred nearly 2.3 billion years ago (Farquhar et al., 2000) and the atmosphere started to accumulate significant quantities of molecular oxygen for about 2.2-2.4 billion years ago during Archaean and Proterozoic Era (Farquhar et al., 2000; Line, 2002; Catling and Zahnle, 2002). The O_2- producing cyanobacteria use water as a terminal reductant and transformed Earth's atmosphere to a suitable one for the evolution of aerobic metabolism and complex life forms (Dismukes et al., 2001).

Oxygen itself is not only toxic for anaerobes but also toxic for cyanobacteria themselves. The adaptation capability of cyanobacteria to oxygenic atmosphere is quite remarkable because nitrogenase, the enzyme responsible for reducing N_2 is deactivated (Kasting and Siefert, 2002) or irreversibly inhibited by O_2 or reactive oxygen species (ROS) (Berman- Frank et al., 2003). Thus, cyanobacteria have evolved a set of complex mechanisms (structural, physiological and biochemical) for protecting their nitrogenase (Kasting and Siefert, 2002). The filamentous *Anabaena spp*. fixes nitrogen at night and photosynthesizes by day (Kasting and Siefert, 2002).

Cyanobacterial diversity

Cyanobacteria are often considered to have been the organisms responsible for the early accumulation of oxygen in the earth's atmosphere. As a result of their long

evolutionary history, cyanobacteria have adopted to almost every conceivable habitat, ranging from ocean to freshwater, from bare rock to soil. They are able to survive at the extreme environmental conditions and are often abundant in freshwater, sea and the terrestrial environments (Fogg et al., 1974). Cyanobacteria occur most abundantly in the tropical and sub-tropical regions. They particularly inhabit the low land rice fields which provide an ideal environment for their luxuriant growth (Singh et al., 2000). In terrestrial habitat cyanobacteria are abundantly found in paddy field because of alkaline pH, flooded conditions and shade provided by Crop canopy.

There are more than 2000 cyanobacterial species that have already been identified. Cyanobacteria are classified into five orders (Van den Hoek et al., 1995)

i. Chroococcales
i. Pleurocapsales
ii. Oscillatoriales
iii. Nostocales
iv. Stigonematales

Under environmental stress like cold, desiccation, nutrient deficiency, etc. they form akinetes to survive (Herdman, 1987; Van den Hoek et al., 1995). Little (1973) reported the occurrence of *Gloeocapsa*, *Nostoc* and *Lyngbya* in the supralittoral zone of marine shores. Cyanobacteria along with certain bacteria occur in alkaline hot springs such as those of the yellow stone and various other parts of the world, where they live at maximum temperature of 73-74°C. A number of blue-green algae grow in association

with other organisms thus, *Gleocapsa* and *Nostoc*, among others, are the phycobionts of lichens, while others like *Nostoc* and/or *Anabaena* occur within the plant bodies of certain liverworts (Duckett, et al., 1975), Cycads (Grilli-Caiola, 1975; Nathanielsz and staff 1975) and angiosperms (Silvester and McNamara, 1976) where they have been called "Cyanelles". About 14 species of Oscillatoriaceae are found in the digestive and respiratory tracts of various vertebrates.

Ecological role of Cyanobacteria

Cyanobacteria represent important components of both aquatic and terrestrial ecosystems and possess a key position in the nutrient cycling due to their unique capacity to fix atmospheric nitrogen into NH_4^+, a form through which nitrogen enters into the food chain. N_2-fixing cyanobacteria form a prominent component of microbial populations in wetland soils, especially in paddy fields, where they significantly contribute as a natural bio-fertilizer (Sinha and Haeder, 1998; Sinha et al., 1998). Cyanobacteria represent only about 1% of marine biomass but they are the main organisms responsible for nitrogen fixation in the ocean (Tyrrell, 1999) and serve as global nitrogen budget (Capone et al., 1997; Karl et al., 2002).

All organisms need nitrogen but only certain bacteria and cyanobacteria are capable of breaking the strong triple bond between atoms of atmospheric nitrogen. Cyanobacteria also play a vital role in the maintenance of soil fertility (Roger and Watanabe, 1986). Plant cyanobacterial symbiosis was considered as a potential bionitrogen fertilizer for paddy rice (Sinha and Haeder, 1998). Cyanobacteria occur as

free- living nitrogen fixers (*Anabaena, Calothrix*, and *Nostoc*) or symbiotic nitrogen fixers (*Gumera-Nostoc*, *Azolla-Anabaena*). Special symbiotic association offers a powerful nitrogen fixation, reaching up to 0.5 kg nitrogen per hectare in the rice field in the case of *Azolla- Anabaena* symbiosis (Taiz and Zeiger, 2006). Cyanobacteria are not only playing an important role in natural ecosystems but they also have a great potential in medical research. Cynavirin- N, a protein extracted from *Nostoc ellipsosporum* is the first cyanobacterial compound with anti- microbial activity against HIV (Boyd et al., 1997).

Toxic cyanobacteria

Although cyanobacteria have a great contribution to life on the earth (primary producer for oxygen and nitrogen), toxic cyanobacterial secondary metabolites are source of nuisance for many other living organisms including human (Duy et al., 2000). During cyanobacterial blooming, cyanobacterial toxin concentrations are relatively high and, in some cases become toxic for human and animals. Physiologically, there are basically two types of cyanobacterial toxins (cyanotoxins): hepatotoxins and neurotoxins (Harada, 2004; Dittmann and Wiegand, 2006). The hepatotoxins are inhibitors of protein phosphatases and affect the animal by causing bleeding in the liver. The neurotoxins are alkaloids (nitrogen-containing compounds of low molecular weight) that block transmission of the signal from neuron and neuron to muscle in animals and man. The cyanotoxins function as anti-herbivore chemicals by inhibiting invertebrate grazers in the aquatic environment. Cyanotoxins also can inhibit the growth of other algae.

Cyanobacteria and nitrogen fixation

Cyanobacteria are able to grow in nearly all illuminated environments on earth and play key roles in the carbon and nitrogen economy of the biosphere. In general, cyanobacteria are able to utilize a variety of inorganic and organic sources of combined nitrogen, like nitrate, nitrite, ammonium, urea or some amino acids. Several cyanobacterial strains are also capable of diazotrophic growth, an ability which may have been present in their last common ancestor in the Archaean. In nitrogen fixation, N_2 from the atmosphere is fixed by the enzyme nitrogenase in to ammonium using ATP as a source of energy. The 2-oxoglutarate has turned out to be the central signaling molecule reflecting the carbon/nitrogen balance of cyanobacteria. Nitrogen fixation by cyanobacteria in coral reef can fix twice the amount of nitrogen than on land - around 1.8 kg of nitrogen is fixed per hectare per day.

Cyanobacteria include unicellular and colonial species. Colonies may form filament, sheets or even hollow balls. Some filamentous colonies show the ability to differentiate into different cell types: vegetative cells, the normal photosynthetic cells that are formed under favorable growing conditions; akinetes, the climate-resistant spores that may form when environmental conditions become harsh; and thick-walled heterocyst, which contain the enzyme nitrogenase, vital for nitrogen fixation. Heterocyst may also form under the certain environmental conditions (anoxic) when fixed nitrogen is scarce. Heterocystous species are specialized for nitrogen fixation and are able to fix nitrogen gas into ammonia (NH_3), nitrate and nitrite which can be absorbed by plants and converted to protein and nucleic acids.

1.2 Heavy-metal pollution

The term heavy metal refers to any metallic element that has a relatively high density and is toxic even at low concentration (Lenntech Water Treatment and Air Purification 2004). Heavy metals is a general collective term, which applies to the group of metals or metalloids with atomic weight ranging between 63.54 to 200.59 and density greater than 4 g/cm^3, or 5 times greater than water (Hawkes, 1997).

There are 90 naturally occurring elements present in the Earth's crust. Among these, 21 are non- metals, 16 are light metals and the remaining 53 (with As included) are heavy metals (Weast 1984, Nies D.H. 1999). Based on the solubility function under physiological conditions, 17 heavy metals are biologically active. Out of these metals Fe, Mo and Mn are important as micronutrients; Zn, Ni, Cu, V, Co, W and Cr are toxic elements with high to moderate importance as trace elements. As, Ag, Sb, Cd, Hg, Pb and U have limited beneficial function and are considered as toxic to animals, plants and microbes (Godbold and Hüttermann, 1985; Nies, 1999; Sebastiani et al., 2004).

Essential metals are required by plants for their normal growth and metabolism and hence cannot be substituted by others. For example, Cu^{2+} is an essential heavy metal in higher plants and algae, particularly for photosynthesis (Mahmood and Islam, 2006; Chatterjee et al., 2006). Cu^{2+} is a constituent of primary electron donor in photosystem I of plants. Because Cu^{2+} can readily gain and lose an electron, it is a cofactor of oxidase, mono- and di-oxygenase and other enzymes involved in the elimination of superoxide radicals. Several enzymes such as carbonic anhydrase, alcohol dehydrogenase,

superoxide dismutase and RNA polymerase contain Zn^{2+}. Zn^{2+} is also involved in the metabolism of carbohydrates, proteins, phosphate, auxins, in RNA and ribosome formation in plants (Nagajyoti et al., 2010). The good functioning of the metabolisms of humans and bacteria is also dependent on above two metals (Adriano, 2001). Trace metal ions form unspecific complex compounds in the cell as soon as their concentration exceeds a specific threshold value, which leads to toxic effects (Sharma and Dietz, 2006). These metals are present in the environment in a wide range of oxidation states and coordination numbers and these differences are related to their individual properties.

Heavy metal pollution can originate from both natural and anthropogenic sources. Activities such as mining and smelting operations and agriculture have contaminated extensive areas of world mostly by heavy metals such as Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cr^{2+} and Ni^{2+}. Heavy metals originate within the Earth's crust; hence their natural occurrence in soil is simply a product of weathering process. These metals are leached out and in sloppy areas are carried by rain water downstream or run off to the sea, rivers and streams. They may then be stored in river bed sediments or trickle in the underground water thereby contaminating underground water sources particularly wells.

Heavy metals are usually present at low concentrations in oceanic surface waters and arrive there through atmospheric transport and upwelling (Table 1.1). Higher levels occur in coastal waters because of river runoff. Close to urban centers, pollution is associated with sewage outlets (Wickfors and Ukeles 1982; Rebhun and Amotz, 1984), but levels are also elevated near extensive areas of industry (Cotte-Krief et al., 2000; Bu-

Olayan et al., 2001; Esser and Volpe, 2002). Interestingly, nutrient availability (in particular, nitrogen) dramatically increases the ability of algae to accumulate heavy metals (Wang and Dei, 2001a, b) suggesting the agricultural runoff into fresh water and coastal areas will greatly increase the entry of heavy metals into the food chain. The annual discharge into the ocean from different sources is given in Table 1.1.

Table 1.1: Metal pollution

Metal	Production (ton year^{-1})	Discharge (tons year^{-1})	Oceanic water (ng ml^{-1})	Polluted seawater (ng ml^{-1})	Potential volume of contaminated seawater (m^3)	Growth inhibition, EC_{50} (ng ml^{-1})
Hg	2×10^3	30	0.001	>0.01	3×10^{12}	>0.4
Cd	1×10^4	60	0.02	>1	0.06×10^{12}	>25
Pb	3.5×10^3	2350	0.03	>5	0.5×10^{12}	>250
Cu	9×10^6	4500	0.10	>2	2×10^{12}	>10

(Source: Davies, 1978; Hollibaugh et al., 1980; Kennish, 1996; Mason et al., 1996; Morel et al., 1998; Faganeli et al., 2003; Hylander and Meili 2003).

1.3 Copper in the environment

Copper occurs naturally in rock, soil, water, sediment, and, at low levels, air. Its average concentration in the Earth's crust is about 50 ppm. Copper can enter the environment through releases from the mining of copper and other metals, and from factories that make or use copper metal or copper compounds. Copper can also enter the environment through waste dumps, domestic waste water, combustion of fossil fuels and

wastes, wood production, phosphate fertilizer production, and natural sources. The concentration of copper in air ranges from a few nanograms to 200 ng/m^3. The concentration of copper in lakes and rivers ranges from 0.5 to 1,000 ppb. Soil generally contains between 2 and 250 ppm copper, although concentrations close to 17,000 ppm have been found near copper and brass production facilities. (source: www.atsdr.cdc.gov/toxprofiles). According to status report of Central Water Commission, Ministry of Water Resources, Government of India (2014) many rivers like Rapti, Ganga, Gomti, Ramganga have copper concentrations above permissible level (50 µg/l). Maximum level of copper as 180 µg/l has been detected in river Rapti. Vanita et al. (2014) have reported copper content in agricultural soil as 8.4-24 mg/Kg soil. However, a study conducted in paddy field of China has shown copper content in paddy field as 78.4 mg/Kg soil (Wu et al., 2010).

1.4 Heavy-metal uptake and accumulation

There are two phases of accumulation of heavy metal ions: first a passive rapid phase called adsorption, and second metabolically active and relatively slow intracellular metal uptake (Mehta et al., 2002). Algal as well as cyanobacterial cell walls have several metal binding groups as carboxylic, hydroxyl, sulfhydryl, aldehydes, phosphate etc. These groups possess high affinity for binding of heavy metal cations. The relative concentrations of these functional groups on surface of cell contribute to differential accumulation of metal ions in the cells. Ion exchange and covalent bonding are mainly involved in adsorption of metal ions on to the surface. Reports indicated that carboxyl

group in algal cell biomass is mainly responsible for binding of various ions (Gardea-Torresdey et al., 1990). Intracellular polyphosphates found in algae participate in metal sequestration and the algal extracellular polysaccharides serve to chelate metal ions (Zhang and Majidi, 1994).

Most of metals enter in to cell as cationic elements (e.g. Zn^{2+}, Cu^{2+}) whereas others cross the plasma membrane as anionic groups or included in small organic compound (e.g. CH_3 Hg^{+}). These species cross membrane by simple diffusion but also via transporters. Different families of metal transporters have been reviewed by Williams et al. (2000) and Mendoza-Cozatl and Moreno Sanchez (2005). The mechanism of metal transport, however, is still not fully known. A common trans-membrane transporter was found for Cd^{2+}, Cu^{2+} and Ni^{2+} (Clarkson and Luttge, 1989). Transport among organelles mostly occurs via CPx-type ATPases which pump essential and non-essential metals across the plasma membrane. The Nramp family (natural resistance associated macrophage proteins) transporters are implicated in the transport of divalent metal ions, the CDF family (cation-diffusion facilitator) transporters are involved in Cd^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} transport and the ZIP family transporters for Fe^{2+}, Mn^{2+}, Cd^{2+} & Zn^{2+} . These transporters are partly found in organelle membranes (Dietz et al., 1998; Ferro et al., 2003; Mendoza-Cozatl and Moreno-Sanchez, 2005).

Plants growing in metal enriched substrate take-up metal to varying degrees in response to external and internal factors, which control the availability of metal ions in soil. In any soil where there is an abundance of organic matters, there is likely to be

interactions among organic matters and heavy metals. Most of the organic matter in soil is comprised of humic substances. The interaction between heavy metals and humic substances has been characterized as chelation, complexation and adsorption. In general the metal- humic substances stability constant is as follows:

$$Pb^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Cd^{2+} > Fe^{2+} > Mn^{2+}$$

Many plants have been used to predict metal availability from soils to plants but with a limited success (Lindsay, 1979). Estimates of heavy metal retention time vary considerably. Lead has one of the largest retention time of any heavy metals. Retention for Cu^{2+} and Zn^{2+} are much shorter due, presumably to preferential uptake by vegetation (Friedland, 1990).

1.4 Heavy-metal toxicity

During the last three decades several studies have been conducted to study the phytotoxicity of heavy metals in algae as well as in higher plants (Gadd and Griffiths, 1978; De Filippis and Pallaghy, 1994; Andrade et al., 2010; Yadav, 2010; Chen et al., 2012). Algal cells have remarkable ability to take up and accumulate heavy metals from their external environment (De Filippis and Pallaghy, 1994). Higher concentrations of metals exert toxic effects on metabolic machinery of algae. Algae are able to tolerate certain concentrations of heavy metals through either of the following general mechanisms; exclusion from cells (Hall, 1981), and intracellular detoxification. The intracellular metal detoxification mechanism comprises binding with metal-binding

peptides and proteins (Gekeler et al., 1988), binding and precipitation within the cytoplasm and/or vacuole (Reed and Gadd, 1990) and sequestration with electron-dense polyphosphate granules (Jensen et al., 1982). Some algae may convert mercuric or phenyl mercuric ions into metallic mercury which is then volatilized out of the cell and from the solution (De Filippis, 1978; De Filippis and Pallaghy, 1994).

Damage of the cell membrane, especially of plasma membrane, is one of the primary events in the heavy metal toxicity effects in plants, algae and cycanobacteria (Harwood, 1995; Janicka et al., 2008). Excessive heavy metal contents induce a sharp increase in plasmamembrane permeability and hence ionic imbalance and subsequent breakdown of cell metabolism. The plant plasma membrane may be regarded as the first 'living' structure that is a target for heavy metal toxicity. Plasma membrane function may be rapidly affected by heavy metals as seen by an increased electrolyte leakage from cells in the presence of high concentration of metals, particularly of Cu^{2+}. It has been shown that Cu^{2+}, but not Zn^{2+}, caused increased K^{+} efflux from excised root of *Agrostis capillaries* (Wainwright and Woolhouse, 1977). Similarly, others concluded that damage to the cell membrane, monitored by ion leakage, was the primary cause of Cu^{2+} toxicity in roots of *Silene vulgaris*, *Mimulus guttatus* and wheat, respectively (De Vos et al., 1991).

Damage to plasma membrane could results from oxidation and cross-linking of protein thiols, inhibition of key membrane proteins such as the H^{+}-ATPase, or changes to the composition and fluidity of membrane lipids. Heavy metals lead conversion of

unsaturated fatty acids of cell membrane into small hydrocarbon fragments such as malondialdehyde (Tappel, 1973; Kappus, 1985). Metal ions can also replace calcium ions at its essential sites on the membranes (Breckle and Kahle 1991). Reactive oxygen species (ROS) are regarded as the main source of cell membrane damage under several biotic and abiotic stresses (Mittler, 2002). Being highly reactive, ROS react with lipids, proteins, nucleic acid; these can cause lipid peroxidation, protein denaturation and DNA mutation (Mittler, 2002; Quiles and López, 2004).

The photosynthetic responses to heavy metals like Cd^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Al^{3+} have extensively been examined. Heavy metals interfere with a variety of ways in the photosynthetic electron transport at multiple sites (Mohanty and Mohanty, 1988; Yuan et al., 2008). Although the metals have specific targets in the chloroplast, PSII is more susceptible to heavy metal induced damages compared to that of PSI. The oxidizing and reducing sides of PSII are affected by different metal ions (Mostowska, 1997). Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} or Zn^{2+} can replace Mg^{2+} from chlorophyll (Kupper et al., 1996) leading to a breakdown in photosynthesis.

Heavy metals, particularly Hg^{2+}, affect photosynthetic electron flow in plants. The Hg^{2+} interrupts the flow of electron at multiple sites, such as oxidizing site of photosystem PSII, plastocyanin (Pcy) (Samson and Papovic, 1990), the P700, the reaction center of PSI (Kojma et al., 1987). The damage of PSII by heavy metals may also be attributed to the action of metal ions at the level of water splitting complex. Although Krupa et al. (1987) have demonstrated that Cd- induced reduction in the rate of

accumulation of extrinsic proteins and antenna proteins of PSII. The effect of Cu^{2+} on electron transport in PSII has been demonstrated by Hsu and Lee (1988). The Cu^{2+} inhibits the acceptor side of PSII. On the other hand, Ranganathan and Bose (1989) has shown Cu-induced loss of chlorophyll-a fluorescence and suggested binding of the metal to the RC II complex.

1.5 Heavy metal-induced ROS and oxidative stress

The toxic effects of heavy metals are related to production of reactive oxygen species (ROS) and the resulting oxidative stress. The reactive oxygen species (ROS) are partially reduced forms of molecular oxygen (O_2) and are formed either from excitation of O_2 to form singlet oxygen or from transfer on 1, 2 or 3 electrons to O_2 to form, respectively, superoxide anion, hydrogen peroxide or hydroxyl radical. Production of ROS is a fundamental consequence of aerobic metabolism and plays direct or indirect roles in plant defense.

Many potential sources of ROS in plants have been shown (Mittler, 2002). Sources producing ROS are localized in almost every cell compartment, including chloroplast, mitochondria, peroxisomes, apoplasm, plasma membrane and cell wall (Mittler, 2002). ROS are produced as unavoidable byproducts in reactions of normal metabolism, such as photosynthesis and respiration, Other sources of ROS belongs to pathways enhanced during abiotic stresses, such as photorespiration, UV radiation, heavy metal pollution etc. However, NADPH oxidases, amine oxidases and cell-wall bound

peroxidases have been identified as potential source of ROS (Mittler, 2002). The above enzymes are tightly regulated and participate in production of ROS during the programmed cell death and pathogen defense (Mittler, 2002).

Singlet oxygen is formed when excitation energy is transferred to oxygen, for example, formation of singlet oxygen in chloroplast. Superoxide Radicals are toxic by-products of oxidative metabolism i.e. mitochondrial electron transport, photosynthesis and flavin dehydrogenase reactions but their toxicity has been attributed to their interaction with H_2O_2 to form highly reactive hydroxyl radicals

The hydroxyl radical constitutes the chemically most reactive species of `activated oxygen' formed by successive monovalent reduction of O_2 in cell metabolism, and is primarily responsible for the cytotoxic effects of oxygen in plants, animals and micro-organisms, living in an oxygenic atmosphere. The short-lived $\bullet OH^-$ molecule unspecifically attacks biomolecules in a diffusion-limited reaction and is thus able to crack, for instance, polysaccharides, proteins and nucleic acids located less than a few nanometres from its site of generation. Hydroxyl radicals can be produced from O_2 under a variety of stress conditions and are involved in numerous cellular disorders such as inflammations, embryo teratogenesis, herbicide effects, cell death and killing of micro-organisms in pathogen-defense reactions. There is evidence that these toxic effects can be traced back to damage by $\bullet OH^-$ of DNA, proteins, or membrane lipids (reviewed in Sharma et al., 2012).

The •OH is produced in biological systems from H_2O_2 by the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH^-$$

The Fe^{2+} is generated through the oxidation by superoxide anion:

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2$$

The combination of above two equations is known as Haber-Weiss reaction

$$O_2^{\bullet -} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + O_2$$

1.6 Tolerance to heavy-metals

Tolerance to heavy metals in plants may be defined as the ability to survive in a soil that is toxic to other plants, and is manifested by an interaction between a genotype and its environment, although the term is frequently used more widely in literature to include changes they may occur experimentally in the sensitive response to heavy metals. Tolerance mechanisms are of primary importance for hyper accumulator plant species, as these plants accumulate large amount of metal ions and sequester them appropriately without incurring/ cellular tissue damage. Important tolerance mechanism identified to date include (i) exclusion of metal ions from root, (ii) intracellular metal compartmentalization to avoid damage and/ or disturbances to basic cellular structures and processes (iv) modulation of cellular metabolism to alleviate damage due to accumulation of toxic metals, and (v) modification of membrane structure.

Exclusion

Cellular exclusion of heavy metals is an important adaptive strategy for heavy metal tolerance in plants. A large fraction of heavy metals in plant roots are found in the

apoplastic space. For example, at equal external Al^{2+} concentrations, a sensitive wheat cultivar had more symplastic Al^{2+} than tolerant cultivar suggesting an exclusion mechanism (Tice et al., 1992). However work with grasses has shown that the total amount of metal present in apoplast, while increasing with external Zn^{2+} supply, was similar in tolerant and sensitive plants (Brookes et al., 1981). Heavy metal transporter proteins are potentially involved in the exclusion of toxic heavy metal ions from the symplastic to the apoplastic space.

Phytochlatins and metallothioneins

Natural chelating compounds viz., amino acid and their derivatives, citric acid, metallothioneins (MTs) and phytochelatins (PCs) are produced by plants to alleviate metal-induced toxicity symptoms. The family of proteins called metallothioneins (MTs) also binds metal ions to closely spaced cysteine thiol groups. The role of MTs and PCs in imparting metal tolerance in plants is well established and has been reviewed in depth by Robinson et al. (1994) and Rauser (1990). MTs are unique family of thiol-containing metal binding polypeptides. These polypeptides are synthesized in plants in response to treatment with metals, and their biosynthesis is linked with glutathione metabolism rather than to ribosomal protein synthesis (Rauser, 1990). Among the metals, Cd^{2+} is the strongest inducer of MT while higher concentration of Zn^{2+} also elicits a similar induction response.

The phytochelatins (PCs) has been widely studied in plants in relation to Cd^{2+} tolerance (Cobbett, 2000; Goldsbrough, 2000). The PCs are family of metal complexing

peptides that have a general structure $(\gamma\text{-Glu-Cys})_n$-Gly where n=2-11, and are rapidly induced in plants by heavy metal treatments (Zenk, 1996; Cobbett, 2000; Goldsbrough, 2000). PCs are synthesized non-transitionally using glutathione as a substrate by PC synthase (Grill et al., 1989), an enzyme that is activated in the presence of metal ions (Cobbett, 2000). The genes for PC synthase have been identified in *Arabidopsis* and yeast (Clemens et al., 1999). Evidence has been presented both for and against the role of PCs in heavy metal tolerance (Ernst et al., 1992; Zenk, 1996; Cobbett, 2000; Goldsbrough, 2000). However, a clear role of Cd^{2+} detoxification has been supported by a range of biochemical and genetic evidences. Howden et al. (1995) isolated a series of Cd^{2+} sensitive mutants of *Arabidopsis* that varied their ability to accumulate PCs; the amount of PCs by the mutants correlated with the degree of sensitivity to Cd^{2+}. In *Brassica juncea*, it has been shown that PC content is theoretically sufficient to chalets all Cd^{2+} taken up; this protects photosynthesis but did not protect a decline of transpiration rate (Haag-Kerwer et al., 1999).

Vacuolar compartmentalization of heavy metal

Significant differences have been reported between tolerant and non-tolerant of the same species, in the distribution of metal ions in their tissues (Baker, 1978). At the sub-cellular level, the central vacuole often contains high concentration of heavy metals: Zn^{2+} and Ni^{2+} (Brookes et al., 1981), Cu^{2+} and Pb^{2+} (Mullins et al., 1985) or Cd^{2+} (Rauser and Ackerley, 1978). Altered compartmentalization patterns at the sub-cellular level, due to

changes in tonoplast transferase system, could likely play an important role in different tolerance to heavy metals in plants (Ernst and Verkleij, 1990).

Alteration of cellular metabolism

Hyper accumulator plant species survive in metal-rich environment by avoiding damage to metal sensitive metabolic processes by activation of alternative pathways or through over production of cyto-solutes, which detoxify the metal ions. Alternatively, enhanced metabolism and increased levels of metabolic energy for metal sequestration represent a potential mechanism involved in the survival of hyper-accumulator plants. An increased production of metal sensitive enzymes could also counteract the metal inactivation of these critical enzymes, thereby, maintaining biosynthetic processes and normal growth.

Alteration of membrane structure

Evidence of metal ion induced changes in membrane structure originates from studies of quantifying ion leakage from roots or cells exposed to variety of metal ions. Several ions including Ni^{2+}, Co^{2+}, and Zn^{2+} enhance K^{+} efflux from *Zea mays* root segments, while Cd^{2+} enhances solute leakage from leaf discs of *Phaseolus vulgaris* (Cumming and Taylor, 1990). Cu^{2+} induced potassium loss has been observed in *Agrostis capillaries* (Woolhouse, 1983). These reports provide circumstantial evidence for heavy metal induced alterations in plasma membrane.

1.7 Tolerance to oxidative stress

Plants have a range of potential mechanisms at the cellular level that might be involve in the detoxification and thus tolerance to heavy metal stress (Hall, 2002). The antioxidant network consists of enzymatic and non-enzymatic components. Algae including cyanobacteria respond to heavy metals by induction of several antioxidants, including diverse enzymes such as O_2^- scavenging by superoxide dismutase (SOD), H_2O_2 decomposition by ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and the synthesis of low molecular weight compounds such as carotenoids and glutathione.

Cell wall is sometime considered as the site of primary action of plant peroxidases. Cell wall- bound-peroxidases are involved in growth regulation and different biochemical pathways. Increased rate of peroxidase activity has been reported in many plant systems in response to reduced growth rate (Lee and Lin, 1995; Chen and Kao, 1995). Therefore, peroxidases are believed to be putative wall lignification enzymes (Cosgrove, 1997) and are directly in lignin biosynthesis.

The antioxidative defense mechanisms have two components: antioxidant enzymes and antioxidant compounds.

Antioxidant enzymes

SOD: SOD destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by catalase or glutathione peroxidase reactions. SOD converts the

highly reactive superoxide radical to the less reactive H_2O_2. There are three distinct types of SOD classified on the basis of the metal cofactor: the copper/zinc (Cu/Zn SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) isoenzymes (Bannister et al., 1987). Prokaryotic cells and many eukaryotic algae contain only the Mn-SOD and Fe-SOD isoenzymes and are believed to be more ancient.

CAT: Catalase is a common enzyme found in living organisms. It is known for being the first enzyme to be isolated in a highly purified state. Catalase is a tetrameric heme-containing enzyme which is responsible for the dismutation of hydrogen peroxide (H_2O_2) into water and oxygen.

APX: Ascorbate peroxidase is thought to play the most essential role in scavenging ROS and protecting cells in higher plants, algae, euglena and other organisms. These enzymes reduce H_2O_2 to monodehydroascorbate and water using ascorbate as the specific electron donor. Monodehydroascorbate spontaneously generates ascorbate and dehydroascorbate. Dehydroascorbate reductase uses glutathione to reduce dehydroascorbate to ascorbate (Shigeoka et al., 2002).

GR: Glutathione reductase is a NADPH flavoprotein found in both prokaryotes and eukaryotes which reduce GSSG to GSH at the expense of oxidizing NADPH. It is necessary for maintaining high GSH/GSSG ratios in cells (Williams, 1976). The GR is a key enzyme of glutathione-ascorbate cycle and functions in peroxide scavenging and protection against other oxidative processes.

Antioxidant compounds

Ascorbic Acid: Ascorbic acid is water soluble antioxidant to prevent or in minimize the damages caused by ROS in plants. It has been detected in the majority of plant cells types, organelles and in the apoplast. The ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes AA the main ROS-detoxifying compound. AA can directly scavenge superoxide, hydroxyl radicals and singlet oxygen and reduce H_2O_2 to water via ascorbate peroxidase reaction (Noctor and Foyer, 1998).

Glutathione: Glutathione is ubiquitous tripeptide γ-glutamyl-cysteinyl glycine, found in most plants, microorganisms including cyanobacteria which is considered as most important intracellular defense against ROS induced oxidative damage. Glutathione exists in two forms: thiol-reduced (GSH) and disulfide oxidized (GSSG). It occurs abundantly in reduced form in plant tissues and is localized in all cell compartments and plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics and the expression of stress- response genes.

Carotenoids: Carotenoids act as a competitive inhibitor for the formation of singlet oxygen. Carotenoids can protect the photosystems (i) by reacting with lipid peroxidation products to terminate chain reactions, (ii) by scavenging singlet oxygen and dissipating the energy as heat, (iii) by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen and (iv) by the dissipation of excess excitation energy through the xanthophylls cycle.

1.8 Heavy metal-induced nitric oxide (NO) accumulation

Nitric oxide is a small, highly diffusible gas and a ubiquitous bioactive molecule which reacts rapidly with molecular oxygen. Nitric oxide is a signaling molecule and is involved in stress- induced physiological responses in plants. Plants, as well as animals, respond to ambient levels of nitric oxide (NO), and also generate NO themselves via various enzymatic and non-enzymatic pathways (Yamasaki, 2000; Neill et al., 2003; Rio et al., 2004). NO is formed in subcellular organelles, e.g., chloroplast peroxisomes, amyloplast, and in nearly all tissues. NO arises from at least four enzymatic sources ((Yamasaki et al., 2001; Durzan and Pedroso, 2002):

i. Nitrate reductase (NR) activity, where nitrite serves a substrate for NO in the presence of NADH.
ii. Nitrite reductase (NiR), leading to nitrite accumulation.
i. Ni-NO reductase, the conversion of nitrite to NO.
ii. Nitric oxide synthase (NOS) activity

NO can also be generated by non-enzymatic mechanisms from nitrite depending on pH and oxygen availability and the presence of reducing agents.

It has been shown that NO has multiple physiological functions in plants. NO reacts with hemes and thiols to produce other transient compounds whose functions are unknown. It is suggested that NO is involved in plant growth, development and responses to environmental factors affecting root growth, shoot formation, leaf expansion, stomatal

closer, pollen tube growth (Salmi et al., 2007). Further, NO is involved in plant defense responses to a wide range of biotic and abiotic stresses including heavy metals, salinity, pathogen attack, wound and mechanical injury. The protective role of NO against copper toxicity has been demonstrated in green alga, *Chlorella vulgaris* and rice leaves (Singh et al., 2004; Yu et al., 2005). The decreased Cd-induced oxidative damage by exogenous NO has been shown in rice leaves (Hsu and Kao, 2004), sunflower leaves (Laspina et al, 2005) and wheat roots (Singh et al., 2008). However, due to lack of mutants for altered NO production, the knowledge on role of NO and its synthesis in plants is still limited.

1.9 Need and importance of present study

N. muscorum is very important component of microbial population in wetland soils, especially in Indian rice paddy fields, where they significantly contribute to building up soil fertility as a natural biofertilizer. Contamination of soil with toxic heavy metals adversely affects the microbial population of rice fields. Copper at low concentrations is essential for normal growth of microorganisms. However, higher concentrations of copper may cause ultrastructural changes, alteration in electron transport rate and damage to thylakoid membrane. Being redox active, copper may enhance the ROS level in cell, thereby, may lead to an irreversible damage to macromolecules and cell membrane. Copper was chosen as test metal due to its high aquatic toxicity at environmentally relevant concentrations.

The aim of the present study was to further investigate the mechanisms of copper toxicity and tolerance in agriculturally important prokaryotic organism. The present study has following major objectives:

i. To study the mechanisms of copper uptake, accumulation and toxicity in *N. muscorum*.

ii. To study copper-induced reactive oxygen production and ROS-mediated damages in the test organism.

iii. To study various defense mechanisms and their relative contribution in overall oxidative stress tolerance of *N. muscorum*.

iv. To study the role of nitric oxide in metal toxicity and tolerance of *N. muscorum*.

The findings of the present study will further enrich our knowledge on metal accumulation and tolerance in cyanobacteria. Microorganisms with an enhanced tolerance to heavy metals can find potential applications in bioremediation of polluted waters and soil as well as in development of biosensors. Because fundamental cellular processes of cyanobacteria and higher plants are largely similar, the results of this study can be equally applied to develop metal tolerant crop plants as well as metal resistant plant species for phytoremediation of heavy metal contaminated soil.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Organism and Growth condition

The test cyanobacterium *Nostoc muscorum* was obtained from Banaras Hindu University, Vanarasi. It was grown and maintained axenically in Laboratory of Algal Biology and Biochemistry, Mizoram University in Chu-10 (without N_2) medium in an air conditioned culture room illuminated by fluorescent tubes under 8 h photoperiod (Gerloff et al.,1950). The pH of the medium was maintained with 1N HCl and 1N NaOH to bring at 7.5± 0.2. The cultures were grown in 250-ml sterilized flasks. The cultures were hand shaken at least 2-3 times daily. Copper treatment was given from the stock solutions of copper prepared in Milli-Q water using analytical grade salts $CuCl_2.2H_2O$ (Merck, India). For every 10 ml of algal culture, a 0.1 ml stock solution of Cu^{2+} was added to obtain the selected concentrations (0.5, 1.5, 2.5, 5, 7.5 and 10 µM).

2.2 Measurement of Survival and growth

Growth was determined by measuring the optical density of cyanobacterial culture at 663 nm in a UV/ VIS spectrophotometer (Systronics, India). To study the effect of copper on growth of *N. muscorum*, the sample was transferred in o test tubes and test metal (Cu^{2+}) was added. Metal solutions having various concentrations were sterilized by autoclaving

before adding to the algal cultures. The specific growth rate (μd^{-1}) was calculated for control and treatment after 96 h of treatment. Specific growth rate was calculated using the equation:

$$Specific\ growth\ rate\ (\mu d^{-1}) = \frac{\ln\ (n_2 - n_1)}{t_2 - t_1}$$

where, μ stands for specific growth rate and n_1 and n_2 are absorbance of culture suspension at the beginning (t_1) and the end (t_2) of the selected time interval. Lethal concentration (LC_{50}) was determined using data of specific growth rate of the cyanobacterium under the stress as mentioned in Guillard (1973).

Table 2.1: Composition of Chu-10 medium used to grow *Nostoc muscorum*.

Macronutrients	g l^{-1}	Micronutrients	mg l^{-1}
KNO_3	0.04	$MnCl_2.4H_2O$	0.5
K_2HPO_4	0.01	$Na_2MoO_4.2H_2O$	0.01
$MgSO_4.7H_2O$	0.04	H_3BO_3	0.5
Na_2SiO_3	0.02	$CuSO_4.5H_2O$	0.02
Ferric citrate	0.003	$CoCl_2$	0.04
Citric acid	0.003	$ZnSO_4$	0.05

2.3 Determination of Photosynthetic pigments

For extraction of photosynthetic pigments, 5 ml of cyanobacterial culture was centrifuged at 10,000 rpm for 20 min and the pellet was suspended in 5 ml 95% ethanol.

After overnight incubation at 4^{o}C, the suspension was centrifuged and the supernatant was used for quantifying the chlorophyll a (Chl-a) and carotenoid (CAR) contents. For estimation of pigments, absorbance were recorded with the help of a UV/VIS spectrophotometer at 665 nm 649 nm and 470 nm for Chl-a and CAR, respectively.

The contents of Chl-a and CAR were calculated using the following equations (Li, 2000).

$$\text{Chl. a} = 13.7A_{665} - 5.76A_{649}$$

$$\text{Carotenoid} = (1000A_{470} - 0.474A_{652}) / 5.34$$

For determination of phycocyanin culture were re-suspended in 5 ml of 0.05 M PBS (PH 7.8) and then exposed to freezing and thawing five times. The absorbance of the extracts was measured at 615 and 652 nm, using the following equation to determine phycocyanin content (Myers and Kratz, 1955).

$$\text{Phycocyanin} = (A_{615} - 0.474A_{652}) / 5.34$$

2.4 Determination of Protein

The protein content was measured using the method given by Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard. 0.2 ml of algal sample was mixed with 2 ml of alkaline solution [(a) 50 ml of 2% Na_2CO_3 mixed with 50 ml of 0.1N NaOH and (b) 10 ml of 1.56% $CuSO_4$ and 10 ml of 2.37% sodium potassium tartarate. Mixed (a) and (b) in the ratio 50:1]. After 10 min digestion in boiling water bath it was cooled down. To this 0.2 ml of Folin's reagent was added and kept for 30 min for the

development of blue colour. Absorbance was recorded at 660 nm against a blank without sample. The protein content was quantitatively estimated using the standard curve.

2.5 Determination of heterocyst frequency

Heterocyst frequency was determined in algal cultures grown in medium with and without N and also with different concentrations of metal for 5 days. Total number of cells (vegetative cells+heterocysts) and total number of heterocysts were counted under microscope and the percentages of heterocysts were calculated using total number of heterocysts and total number of cells.

2.6 Copper uptake and accumulation

The intracellular Cu^{2+} content of the pellet was determined following the method of Bates et al. (1982). To remove extracellular metal bound on the cell surface, cells were washed with 10 ml of EDTA (2 mM) for 10 min and washed twice with 10 ml of Millipore water. The EDTA-washed pellet was transferred to 10 ml of digestion solution containing 70% HNO_3, 30% H_2O_2 and H_2O in a 1:1:3 ratios. Digestion was carried out on a hot plate at 80º C until a clear solution of about 2 ml was left. The residue was taken up in 2% (v/v) HNO_3 and the final volume was adjusted to 5 ml. The metal content of the solution was determined using an atomic emission spectrophotometer (Agilent MP-AES). From EDTA filtrate the extracellular metal binding was determined (Bates et al., 1982).

2.7 Determination of photosynthetic oxygen evolution

Gross photosynthesis in terms of photosynthetic O_2 evolution was measured using a Clark-type O_2 enclosed in an airtight vessel connected to a digital oxygen analyzer (Rank Brothers, UK). Measurement of O_2 evolution was done at 26ºC ± 1 under an illumination of 300 µM m^{-2} s^{-1} PAR. The reaction vessel containing 4 ml of Cu^{2+} treated- and control culture of *Nostoc* was taken and the reading was recorded after every 5 min for light and dark, respectively. Reading recorded in the light was used for O_2 evolution and the reading taken in the dark was for O_2 consumption.

2.8 Determination of chlorophyll fluorescence: PSI and PSII

Simultaneous measurement of quantum yields and fluorescence quenching parameters of PS I and PS II

PS I and PS II activities of *N. muscorum* were measured simultaneously using a Dual-PAM- 100 system (Heinz Walz GmbH, Effeltrich, Germany). All samples were dark-adapted for 15 min prior to recording of fast fluorescence induction curves. F_o, the minimum fluorescence, was detected by a measuring light (ML) at low intensity. A 300 ms saturating pulse of 10,000 µmol photons m^{-2} s^{-1} was applied to determine the maximum fluorescence (F_m). Maximum quantum yield of PS II was calculated as F_v/F_m, where $F_v = F_m-F_o$. The maximal change in P700 signal (P_m) was measured using a saturation pulse after far-red light (Klughammer and Schreiber, 2008).

After determination of F_o, F_m and P_m, the fast light- induction curves were recorded with the routine induction protocol of the Dual-PAM-100 software. Rapid light-response curves were obtained using a light curve method, consisting of sixteen consecutive 30 s steps with an increasing AL intensity, and an SP was applied at each AL intensity to obtain the maximal fluorescence yield of a light-adapted sample (F_m'). The coefficient of photochemical quenching (qP), a measure of the overall "openness" of PS II was obtained as $qP=(F_m'-F^o)/F_m'-F^{o\prime})$, where F^o is the fluorescence yield briefly before application of a SP, and $F^{o\prime}$ is the minimal fluorescence yield of illuminated sample with all PS II centers open (Schreiber et al., 1986). The coefficient of photochemical fluorescence quenching (qL) assuming interconnected PS II antennae was calculated as $qL=qP \times (F_o'/F^o)$ (Kramer et al., 2004). The effective quantum yield of PS II (Y(II)) was determined as $Y(II) = (F_m'-F^0)/F_m'$. The quantum yield of regulated energy dissipation in PS II (Y(NPQ)) was calculated according to Kramer et al. (2004) using equation: $Y(NPQ) = (F_m-F_m')/F_m'$. The quantum yield of non-regulated energy dissipation, Y(NO), in PS II is calculated according to Kramer et al. (2004) as $Y(NO)= 1/(NPQ+1+qL(F_m/F_o-1))$.

Y(I), the effective photochemical quantum yield of PS I, represents the fraction of overall P700 that in a given state is reduced and not limited by the acceptor side. Y(I) is calculated as $Y(I) = (P_m'-P)/P_m$. Y(ND) (the non-photochemical quantum yield of PS I representing fraction of overall P700 that is oxidized in a given state) is calculated from the P700*red*. parameter as Y(ND) = 1-P700*red*. The P700*red.* represents the fraction of overall P700 that is reduced in a given state determined after application of a SP and is

calculated from P_m. Y(NA), the quantum yield of non-photochemical energy dissipation of reaction centers due to PS I "acceptor" side limitation. It represents the fraction of overall P700 that cannot be oxidized by a SP in a given state due to lack of acceptors. Y(NA) is calculated as $Y(NA) = (P_m-P_m')/P_m$.

Determination of electron transport rates and RLC parameters in PS I & PS II:

The electron transport rates in PS I (ETR(I)) and PS II (ETR(II)) were recorded during the slow induction curve and calculated using Dual PAM software. The responses of electron transport in PS I and PS II to increasing irradiation were measured by the recording of rapid light curves (RLCs). The RLC consists of electron transport responses to eleven increasing irradiances (PAR 0, 30, 37, 46, 77, 119,150, 240, 363, 555 and 849 nm) for 30 seconds. The following RLC parameters were derived using the exponential functions: α, the initial slop of RLC of ETR(I) or ETR(II) indicated the quantum yield of PS I or PS II, respectively; $ETR_{max,}$ the maximal electron transport rates in PS I or PS II; I*k*, the index of light adaptation of PS I or PS II (determined from the interception point of the alpha value with maximum photosynthetic rate), was calculated as ETR_{max}/α.

Determination of cyclic electron flow (CEF) and linear electron flow (LEF)

The relationship between CEF and LEF was determined by the estimation of Y(CEF)/Y(I), Y(CEF)/Y(II) and Y(II)/Y(I). Y(CEF) is the quantum yield of CEF and is calculated by differences between Y(I) and Y(II) (Y(CEF) = Y(I)-Y(II)). Y(CEF)/Y(I)

shows the contribution of CEF to Y(I), Y(II)/Y(I) indicates the contribution of LEF to Y(I); Y(CEF)/Y(II) shows the ratio of quantum yield of CEF to LEF. The distribution of quantum yield between PS I and PS II is shown by the ratio of Y(II)/Y(I).

2.9 Toxicity and ROS level determination

Estimation of Lipid peroxidation

Oxidative damage of lipid was measured in terms of the total content of 2-thiobarbituric acid-reactive substances (TBA) and expressed as equivalent of MDA (malondialdehyde) using method of De Vos et al. (1989). Treated algal cells were harvested by centrifugation at 10,000 rpm for 10 min and the resulting pellet was treated with 0.25% (w/v) thiobarbituric acid (TBA) in 10% (w/v) trichloroacetic acid (TCA). After heating at 95^{o}C for 30 min, the mixture was cooled in an ice bath and again centrifuged at 10000 rpm for 10 min. The absorbance of the supernatant at 532 nm was recorded and corrected for unspecific turbidity by subtracting the value at 600 nm. The concentration of the total MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The following formula was used to calculate MDA content:

$$MDA\ (mM\ mg^{-1}\ protein) = (A_{532}-A_{600})/\ 155$$

Measurement of relative intactness of plasma membrane

The relative intactness of the plasma membrane was measured as percent leakage of electrolytes as described by Gong et al. (1998). Pellets were washed three times with

Milli Q water to remove any extracellularly adsorbed Cu^{2+}. These were then suspended in 25 ml of Milli Q water and incubated in water bath at 30ºC for 2 h. The suspension medium was used for measuring the initial electrical conductivity (EC_1) by digital conductivity meter. The samples were then boiled at 100ºC for 15 min to release all the electrolytes, cooled and the final electrical conductivity (EC_2) was measured. The percent leakage of electrolytes was calculated using the formula

$$\% \text{ Electrolyte Leakage (EL)} = (EC_1/EC_2) \text{ X } 100.$$

Estimation of H_2O_2

Level of H_2O_2 in the algal sample was determined by adopting the method as described in Sagisaka (1976). The pellet was transferred in 5 ml of 50% TCA and after 10 min sample were centrifuged. Pellet was discarded and H_2O_2 was measured in the supernatant. To 1.6 ml of supernatant, 0.4 ml of 50% TCA, 0.4 ml 10 mM ferrous ammonium sulphate and 0.2 ml of 125 mM potassium thiocyanate were added. The solution was shaken and the absorbance was recorded at 480 nm using spectrophotometer. The peroxide content was quantitatively estimated using the standard curve made on the basis of H_2O_2 decay.

Estimation of superoxide radical

Superoxide radical (O_2^-) generation was determined by the method of Achary et al. (2012). Cells were immersed in the reaction mixture containing 50 mM Tris-HCl buffer (pH 6.4), 0.2 mM nitrobluetetrazolium (NBT), 0.2 mM NADH and 250 mM sucrose, vacuum-infiltrated after 10 min and filtrate was illuminated at 200 µM m^{-2} s^{-1} for 24 h to

developed colour as a result of reduction of NBT. The absorbance of blue monoformazan formed in the reaction mixture was measured at 480 nm. O_2^- content was calculated using an extinction coefficient of 12.8 mM^{-1} cm^{-1} and was expressed as µM mg^{-1} protein.

2.10 Extraction and assay of enzymatic antioxidants

Extraction of antioxidative enzymes

The cell pellets were suspended in cell lysis buffer [potassium phosphate buffer (pH 7.0), 1m M EDTA and 1% (w/v) PVP] and subjected to sonication (350 mA for 2 min with six intervals of 20 sec each) in ice-cold condition (4^oC). However, the above buffer additionally contained 1 mM ASA for APX assay. The sonicated sample was centrifuged at 12,000 rpm for 30 min at 4^oC, and the resulting supernatant containing antioxidant enzymes was used for further assay.

Superoxide dismutase (EC 1.15.1.1)

The total SOD activity was assayed by monitoring the inhibition of reduction of nitroblue tetrazolium according to the method of Robert et al. (1980). A 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT (nitroblue tetrazolium), 2 µM rivoflavin, 0.1 mM EDTA, and 500 µl of enzyme extract. The reaction mixture was illuminated for 20 min at high light intensity. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition in NBT reduction monitored at 560 nm. Blank contains no enzyme and was kept in the dark

where as control contains no enzyme but was exposed to light. The result was expressed in Unit SOD (U SOD) mg^{-1} protein.

Catalase (EC 1.11.1.6)

Catalase activity was estimated using the method of Jiang and Zhang (2001) by measuring the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 1 min. Make a 10 mM stock of H_2O_2 in 50 mM potassium phosphate buffer (pH 7). Add 2.8 ml of this solution followed by 200 µl of enzyme extract (in the cuvette). The result was expressed in mM mg^{-1} protein min^{-1}.

Ascorbate peroxidase (EC 1.11.1.11)

APX activity was determined according to the method of Jiang and Zhang (2001) by measuring the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min in 1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 1.5 mM ASA, 0.3 mM H_2O_2, and 200 µl of enzyme extract. The reaction was started by adding enzyme extract. Corrections were made for low, non-enzymatic oxidation of H_2O_2. The result was expressed in mM mg^{-1} protein min^{-1}.

Glutathione reductase (EC 1.6.4.2)

GR activity was determined by measuring the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 5 min in 2 ml of assay mixture according to the method of Schaedle and Bassham (1977) containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM GSSG (glutathione oxidized),

and 200 µl of enzyme extract. The reaction was initiated by adding NADPH. Absorbance of the assay mixture without NADPH at 340 nm was used for background correction. The result was expressed in mM mg^{-1} protein min^{-1}.

2.11 Estimation of non-enzymatic antioxidants

Total thiols (T-SH)

Total thiols were assayed by the method of Sedlak and Lindsay (1968). Algal pellets were mixed with extraction buffer containing 1.0 M Tris-HCl, pH-8.2, 5% SDS and 10 mM DTNB (in absolute methanol). Mixture was sonicated for 3 min at an interval of 30 sec and incubated at room temperature for 20 min with intermittent stirring. After addition of 3 ml chilled methanol it was centrifuged at 10000 rpm for 10 min and the concentration of total thiols was determined in the clear supernatant by recording the absorbance at 412 nm using UV-Visible spectrophotometer (Systronics). Total thiol was calculated by using reduced glutathione as standard and expressed as µM mg^{-1} protein.

Non protein thiols (NP-SH)

Treated algal pellets were sonicated for 3 min giving an interval of 30 sec with 4 ml of 5% (W/V) sulfosalicylic acid and was centrifuged at 10,000 rpm at 4ºC for 10 min. The 1 ml of supernatant was mixed with 3 ml of 0.2 M Tris-HCl (pH=8.5) and 0.1 ml of 10 mM DTNB (in absolute methanol). This was incubated at room temperature for 2 min and absorbance was recorded at 412 nm by using UV-Visible spectrophotometer (Systronics-117) (Sedlak and Lindsay, 1968). Non-protein thiol was calculated from the

standard curve made by using reduced glutathione (GSH) and expressed as µM mg^{-1} protein.

Glutathione

Reduced glutathione was determined by the method of Moron et al. (1979). 1 ml of cyanobacterial sample was treated with 1ml of 10% TCA. The precipitate was removed by centrifugation at 10000 rpm for 15 min. To the supernatant, 4 ml of phosphate buffer and 0.5 ml of DTNB reagent were added. The colour developed was read at 420 nm using UV-Visible spectrophotometer (Systronics-117). For the standard, 10 mg of reduced glutathione was dissolved in 100 ml of distilled water and the result was expressed as µg mg^{-1} protein.

Proline content

Proline estimation was done by the method of Bates et al. (1973). To determine proline content, cell pellets were transferred to 3% (W/V) sulfosalicylic acid and cells were disrupted with an ultrasonicator at 200 mA for 2 min giving an interval of 20 sec. The cell debris was then separated from cell extract by centrifugation at 4000 rpm for 20 min. 2 ml of supernatant was mixed with 2 ml of acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) solution and 2 ml of glacial acetic acid. The mixture was incubated in boiling water bath at 100°C for 30 min. Reaction was terminated by placing tubes in ice bath. Afterward 4 ml of toluene was added. The sample was vortexed and chromophore partitioned in toluene layer was separated using

separating funnel. The absorbance of separated chromophore was recorded at 520 nm. The amount of proline was calculated by using a standard curve made by L-proline.

2.12 In-Gel assay of SOD and CAT

Glass plates were clean with methanol. Gel casting was done using separating gel (12% for SOD and 8% for CAT) and 5% stacking gel. 12% separating gel (H_2O, 30% Acryl-Bis, Tris -separating buffer, TEMED, 10% APS) solutions were added to the glass plate assembly about 1 cm from the top of apparatus. A layer of water was added slowly over the running until it was entirely covered. Wait 20-30 min for polymerization. 5% stacking gel (30% Acryl-Bis, Tris- stacking buffer, 40% sucrose, 0.004% riboflavin, TEMED) was added to the surface of running gel, filling the glass assembly to the top with stacking gel. 1.5 mm comb was added by avoiding air bubbles. Gel apparatus was placed under a fluorescent light for 15-30 min. Comb was removed from the gel and gel assemblies from the casting stand. Gel assembly was attached to the electrode assembly for electrophoresis. Running buffer (Tris base and glycine, pH 8.3) was added to the reservoir and chamber. Sample was loaded on the clean wells using loading buffer (stacking gel buffer, glycerol, 5% bromophenol blue solution) in 1:1 ratio of sample and loading buffer. Gel was run for 4-5 h (40 mA, 4ºC). It was stopped once the dye front reaches the bottom.

SOD

Gel was carefully taken out from the glass plates and it was stained with SOD native gel stain (H_2O, 2.34 mM NBT, 28 mM TEMED, 53 mg rivoflavin in 1ml in 50 mM phosphate buffer, pH 7.8) for 20 min. The gel was rinsed with water twice. Enough water was added to cover the gel. It was place under fluorescent light for 2 h or until the gel begins to turn purple and clean bands appeared. It was washed for three times after bands have appeared. The gel was kept at room temperature for 18-24 h in water for further band development. The bands were intensifying over the next 4- 24 h. SOD bands appear colorless against a purple background. Isozymes were detected by the method of Beyer and Fridovich (1987). The gels were cut and soaked in 2 mM of H_2O_2 or with 2 mM KCN for 10 min prior to soaking in NBT. FeSOD is inactivated by H_2O_2; Cu-ZnSOD is inactivated by KCN while MnSOD remains unaffected by these compounds.

CAT

After taken out the gel from the glass plates, it was washed with distilled water for three times. Gel was incubated in 0.003% H_2O_2 for 10 min and was rinsed twice with water for 5 min. Water was decanted and stain (2% ferric chloride + 2% potassium ferricyanide) was added to the gel for 5-10 min. The stain was pour off when green bands began to form. It was again rinsed with excess water and was imaged.

2.13 Protein separation by SDS-PAGE

Gel casting was done using 10% resolving gel and 5% stacking gel. 10% resolving gel (H_2O, 30% Acryl-Bis, 1.5 M Tris-buffer (pH 8.8), 10% SDS, 10% APS, TEMED)

solutions was added to the glass plate assembly about 1 cm from the top of apparatus. A layer of water was added slowly over the running until it was entirely covered. Placed for 20-30 min for polymerization. 5% stacking gel (30% Acryl-Bis, 0.5 M Tris-buffer (pH 6.8), 10% SDS, 10% APS, TEMED) was added to the surface of running gel, filling the glass assembly to the top with stacking gel. 1.5 mm comb was added by avoiding air bubbles. Gel apparatus was placed under a fluorescent light for 15-30 min. Comb was removed from the gel and gel assemblies from the casting stand. Gel assembly was attached to the electrode assembly for electrophoresis. Running buffer (Tris base, glycine and SDS) was added to the reservoir and chamber. 20 µl of total protein extract and 20 µl of loading buffer (0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, 0.1% bromophenol blue, β- mercaptoethanol) was boiled for 5 min and immediately placed on ice for 1 min. From this 30 µl of sample was loaded on the clean wells. Run the sample on Tarson SDS gel apparatus at 100V (Biorad power supply) for 1 h. After the run was finished, it was destained (10% acetic acid, 30% methanol, 60% H_2O) for 30 min. It was visualized using Biorad Gel Doc imager.

2.14 Nitric oxide (NO) determination

Nitric oxide production by the test alga was studied by differentiating intracellular NO from NO released into the external medium. Exponentially growing cultures was concentrated. To the concentrated cell suspension Cu^{2+} was added to give final concentrations of 2.5 µM. After adding the flask were sealed to check NO escape. At different time interval 10 ml suspension was removed and centrifuged at 10,000 rpm for

20 min. The NO pellet (intracellular NO) and filtrate (NO released) was determined. To determine the intracellular NO level cells were disrupted in 10 ml homogenization buffer (pH 6.0) containing 0.1 M sodium acetate, 1.0 M NaCl and 1% (w/v) ascorbic acid. The homogenate was centrifuged with screw-caped centrifuged tubes at 10,000 rpm for 20 min at 4ºC. NO was determined in filtrate immediately after discarding cell debris. In other set of experiment the alga was treated with different concentrations of Cu^{2+}, and NO generation was monitored in cells as well as in the external medium as described above.

Hemoglobin-trapping technique, based on conversion of ferrous form of hemoglobin (oxyhemoglobin, or HbO_2) into ferric form, methemoglob in (mteHb), by nitric oxide (Murphy and Noack, 1994) was used for the determination of NO in cell homogenate and external medium. Oxyhemoglobin was prepared from bovine crystallized hemoglobin as follows. Sodium dithionite of excess molar concentration was added to the buffer (1-2 mg in 1 ml of sodium acetate buffer), followed by 1 ml of hemoglobin (10^{-6} equivalents ml^{-1}, one equivalent equals to 16125 g Hb, and which contains 1 g atom of Fe and combine with 1 g molecule of O_2). Continuous O_2 was provided to this flask. The conversion of metHb to HbO_2 was monitored by the change of colour from brown to purple as the metHb was reduced by the sodium dithionite to HbO_2 (deoxyhemoglobin) and then from purple to bright-red as deoxyhemoglobin reacted with oxygen contained in the sodium acetate buffer when passed through the column (Sephadex G-25). This HbO_2 was stored on ice in dark for NO determination.

For further measurement of nitric oxide production 10 µM HbO_2 from stock was added to the above assay sample buffer. After enough time the assay solution was collected and the conversion of HbO_2 to metHb was quantified by taking its absorbance at 401 and 421 nm. NO concentration was determined by using an extinction coefficient = 77 $mM^{-1}cm^{-1}$

2.15 Statistical analysis

Graphical representation and statistical analysis of the data was done using Sigma plot 11.0 version (Chicago, IL) software. All the experiments were conducted in triplicates. The mean values of different parameters were analyzed separately and difference between the control and treated cells were interpreted by one-way analysis of variance (ANOVA) taking $P<0.05$ as significance level according to Duncan's new multiple range test (DMRT).

CHAPTER THREE

COPPER UPTAKE, ADSORPTION AND TOXICITY IN *Nostoc muscorum*

3.1 Introduction

Copper is an essential heavy metal for higher plants and algae including cyanobacteria, particularly for photosynthesis (Mahmood and Islam, 2006; Chatterjee et al., 2006). Cu^{2+} is a constituent of primary electron donor in photosystem I of plants. Because Cu^{2+} can readily gain and lose an electron, it is a cofactor of oxidase, mono- and di-oxygenase (e.g., amine oxidases, ammonia monoxidase, ceruloplasmin, lysyl oxidase) and of enzymes involved in the elimination of superoxide radicals (e.g., superoxide dismutase and ascorbate oxidase). At least thirty copper containing enzymes are known, all of which function as catalysts. (e.g., cytochrome oxidase, nitrate reductase) or dioxygen carriers (e.g., haemoglobin) (Weser et al., 1979). Elements designated as trace elements each comprise less than 0.1 mg Kg^{-1} of the earth's crust. Cu^{2+} is classified as heavy metal with density greater than 5 gm/cm^3 (Förstner and Whittman, 1979). Cu^{2+} plays essential roles in metal homeostasis and normal metabolism in plants. It is involved in wide range of biological processes. Although Cu^{2+} is an essential element, at elevated levels Cu^{2+} becomes toxic in most of the organisms.

The total copper content in soil varies from 2-100 mg/ Kg soil, and the content in plants is 1-10 mg/ Kg (Pais and Jones, 1997). Copper tends to be very strongly bound to soil organic matter non-toxic to plants. The levels of biologically accessible Cu^{2+} in the environment are rising as a result of human activities that increase its abundance by application and emission in water. The activities by which Cu^{2+} enters water bodies include smelting (Beavington, 1977), mining (Lopez and Lee, 1977), industrial (metal plating, steelwork, refineries) and domestic waste emission (Förstner and Whittman, 1979) and application of fertilizers, sewage sludge (Barkay et al., 1985), algicides (Mackenthum and Cooley, 1952; Elder and Horne, 1978) and fungicides (Yamamoto et al., 1985). The Cu^{2+} levels in soil and water also results from weathering of Cu^{2+} containing parent rock.

The best-characterized metal transport pathway in photosynthetic cells is that for Cu^{2+}. Cu^{2+} is required as a co-factor in the thylakoid lumen electron transport protein plastocyanin. However, the pathway of Cu^{2+} uptake into and trafficking inside plants and cyanobacteria is still partially understood; the current knowledge about this was reviewed by Williams et al., 2000; Cavet et al., 2003; Cobbett et al., 2003; Hall and Williams, 2003 and recently by Huertas et al., 2014. In cyanobacteria, Cu^{2+} is transported through the plasma membrane by a P-type ATPase. A second P-type ATPase is required for the transport of Cu^{2+} across the thylakoid membrane. Inside the cyanobacterial cell, Cu^{2+} is chaperoned by a small, soluble protein that contains the signature CXXC Cu-binding motif (Tottey et al., 2005). In the cyanobacterium *Synechocystis*, the CPx-ATPases, CtaA and PacS, which are located in the plasma and thylakoidal membranes, respectively

seems to transport Cu^{2+} into the cell. These two proteins are assisted by a small cytosolic soluble copper metallochaperone, Atx1. Cu^{2+} import inside the cell is mediated by CtaA and then it is transferred to PacS, which finally transports it inside the thylakoid membrane. In addition, transporters of ZIP family (ZIP="ZRT, IRT-like proteins") also seem to be involved in Cu uptake into the cell, in particular under Cu-deficiency stress.

A number of reviews on metal toxicity of algae and cyanobacteria have been published (De Filippis and Pallaghy, 1994). Excess of Cu^{2+} may replace other metals in metalloproteins or may interact directly with –SH groups of proteins. Excess of Cu^{2+} inhibits a large number of enzymes and several aspects of plant process including photosynthesis, (Vavillin et al., 1995), pigment synthesis (Chettri et al., 1998), permeability of plasma membrane, decrease in oxygen evolution, etc. Cu^{2+} is a most effective inducer of lipid peroxidation. Cu^{2+} may exert its toxicity in sub-cellular organelles, interfering with mitochondrial electron transport, respiration, ATP production and photosynthesis in the chloroplast. Copper ions are also likely to be transported to chloroplast via cytoplasm where it inhibits photosynthesis by uncoupling electron transport to $NADP^{+}$ (Stauber and Florence, 1987). It is also participates in electron transport reaction of photosynthesis in the form of plastocyanin. However, at high level of copper become strongly phytotoxic to cell and causes inhibition of growth or even death of the organism (Chen et al., 2000). Copper in plant cells may activate molecular oxygen and generate reactive oxygen species (ROS) within subcellular compartments (Mocquot et al, 1996; Chen et al., 2000; Wang et al., 2004). Cu-induced generation of hydrogen peroxide, hydroxyl radicals and other ROS has been directly correlated with

damage to membrane lipids and proteins (Murphy and Taiz, 1997; Wang et al., 2004). Cu^{2+} in soil plays a cytotoxic role which induces stress and causes injury to plants. This leads to plant growth retardation and leaf chlorosis (Lewis et al., 2001). Cu^{2+} toxicity affected the growth of *Alyssum montanum* (Ouzounidou, 1994). Neelima and Reddy (2002) reported that Cu^{2+} and Cd^{2+} in combination have affected adversely the germination, seedling length and number of lateral roots in *Solanum melongena*.

Although many studies have been carried out to study the heavy metal uptake and accumulation in plants, there is always lack of data for explaining physiological mechanisms of heavy metal uptake, transport and accumulation in cyanobacteria. In this study we have investigated the Cu^{2+} accumulation by distinguishing the intracellular copper uptake from extracellular adsorbed fraction on the surface. We have also investigated the effect of other mono-and divalent cations on the intracellular metal uptake and adsorption of Cu^{2+}. The results of this study can give important information regarding Cu^{2+} uptake, accumulation and toxicity mechanisms in *N. muscorum.*

3.2 Material and Methods

Exponentially growing algal cultures were subjected to Cu^{2+} treatments for various experimental setups. For every 10 ml of algal culture, a 0.1 ml stock solution of Cu^{2+} was added to obtain the selected concentrations (0.5, 1.5, 2.5, 5, 7.5 and 10 µM). Cu^{2+} solution was prepared from analytical grade salts of $CuCl_2$. $2H_2O$ using Milli-Q water.

Intracellular Cu^{2+} uptake and adsorption

10 ml of algal culture (in Milli-Q water) having O.D. of 2.067 at 663 nm was treated with 0.1 ml of different concentrations of Cu^{2+} (0.5, 1.5, 2.5, 5, 7.5 and 10 µM) for 1 h. After the treatment, cells were separated by centrifugation for 30 min at 10,000 rpm. For time course study a single concentration of 2.5 µM Cu^{2+} was chosen. Time-course study of Cu^{2+} uptake and accumulation was performed for 5, 15, 30, 60, 90, 120, 180, 240 and 300 min duration in 10 ml of algal culture having an O.D. of 2.512 at 663 nm.

After incubation with metal, the pellet was washed with 2 mM EDTA as described in Chapter 2. The intracellular metal content was determined from EDTA-washed pellet where as adsorption was estimated by analyzing the metal content of EDTA washing using MP-AES (Agilent).

Effect of different pH on Cu^{2+} uptake and accumulation

Experiments were set to determine uptake and accumulation of Cu^{2+} at different pH (6.5, 6.8, 7.0, 7.2 and 7.5). The culture was treated with 1.5 and 2.5 µM Cu^{2+} for 1 h. pH was maintained in Milli-Q water with HCl and NaOH without adding buffer. 10 ml of algal culture in Milli-Q water having different pH was taken for performing the experiment (O.D. at 663 nm was 1.798). Cu^{2+} contained of treated and untreated cultures in water were determined as per method given in chapter 2.

Uptake and adsorption of Cu^{2+} in presence of other bi- and mono-valiant cations

Experiments were set to determine intracellular uptake and adsorption of Cu^{2+} in presence of zinc (Zn^{2+}), cadmium (Cd^{2+}), potassium (K^{+}) and calcium (Ca^{2+}) in the

external medium. Concentrations of the above mentioned cations were taken identical to Cu^{2+} i.e., 0.5, 1.5, 2.5, 5, 7.5, and 10 µM. 10 ml of algal cultures were treated with Cu^{2+} along with different concentrations of cations for 1 h. Cells were separated by centrifugation after 1 h of incubation and metal contained was determined from the pellet. Metal contained was determined as per method given in chapter 2.

Kinetics of intracellular uptake and adsorption of Cu^{2+}

The exploratory enzyme kinetics based and Michaelis-Menten kinetic was used to determine the inhibition parameters of copper adsorption in intracellular uptake due to other cations. Direct linear plot as described by Comish-Bowden (1999) was used to determine the type of linear inhibition involved. In contrast to Michaelis-Menten kinetics, the direct linear plot considers the parameters V_{max} and K_m as variables and graphs V_{max} vs. K_m. Enzyme kinetics typically graph combinations of velocity V and substrate S against one another.

The direct linear plot is derived by first assuming that Michaelis-Menten kinetics is applicable:

$$V = \frac{V_{max}S}{K_m+S} \qquad (1)$$

Re-writing of equation (1) gives direct linear plot:

$$V_{max} = V + \frac{V}{S}K_m \qquad (2)$$

For the fixed value of V and S the plot is straight line. The Y-intercept ($K_m = 0$) for straight line is V_{max}; and X-intercept ($V_{max} = 0$) is K_m= -S.

Examining the tranjectory of the medians in the direct linear plot as the inhibitor concentration is increased gives information about the type of linear inhibition.

For competitive inhibition the median tranjectory moves horizontal to right in direct linear plot. Diagonal down and to the right movement of tranjectory indicates mixed type of inhibition. In case of non-competitive inhibition the tranjectory moves vertically down. Movement of tranjectory toward the origin suggests uncompetitive inhibition.

The secondary plots were used to determine the type of inhibition and to obtain estimates of the inhibition dissociation constants.

The copper adsorption data were fitted to Michaelis-Menten equation as it is very similar to Langmuir adsorption isotherm. Both describe the saturation kinetics. Table 3.1 represents the Michaelis-Menten kinetics constants.

Growth behavior of N. muscorum

To see nature of growth in Chu-10 medium (without N_2), alga was grown axenically for 37 days illuminated by fluorescent tubes under 8 h photoperiod at pH 7.5. The cultures were hand shaken at least 2-3 times daily. O.D. was taken at 663 nm in a UV/ VIS spectrophotometer (Systronics, India) on every third day by using reference blank of basal culture medium. Nature of algal growth was also determined in the medium having different concentrations of Cu^{2+} for 18 days. The specific growth rate (μd^{-1}), based on absorbance was calculated for control and treated cells after 96 h.

Table 3.1: The Michaelis-Menten kinetic constants and their definitions used in the present study.

Parameter	Unit	Definition
V_{max}	(µg mg^{-1} protein h^{-1})	Maximum rate of intracellular metal uptake
K_m	(µM)	Concentration of Cu^{2+} at which rate of intracellular uptake is half of $V_{max.}$
U_{max}	µg mg^{-1} protein	Maximum adsorption of metal ions on surface of cyanobacterium. An estimate of maximum number of binding sites on the cell surface.
K_d	Dimensionless	Dissociation constant. It shows affinity of adsorption. Higher values show less affinity.

Effect Cu^{2+} photosynthetic pigments and protein content

Photosynthetic pigments like Chlorphyll-a, phycocyanin and carotenoid content in the growth medium was determined for 37 days. Culture were also treated with 0.5, 1.5, 2.5, 5, 7.5 and 10 µM Cu^{2+} for 96 h to determined effect of Cu^{2+} on the photosynthetic pigments and protein content. Estimation of pigments and protein content used in this study has been given in chapter 2.

3.3 Results

Nostoc sp. showed a logarithmic growth during the first 30 days of inoculation and subsequently declines the growth (Fig. 3.1A). Chlorophyll-a, phycocyanin and carotenoid content also followed the same pattern (Fig. 3.1B). Protein content (mg/ml of culture) of the algae also increased till the 30 days cultivation (Fig. 3.1C). Figure 3.2 shows growth pattern of *N. muscorum* grown under different concentrations of Cu^{2+} in the growth medium. Growth of the test organism decreases with increasing concentrations of copper except in 0.5 µM Cu^{2+}.

Table 3.2 shows specific growth rate, chlorophyll-a, carotenoids and phycocyanin contents in *N. muscorum* treated with various concentrations of Cu^{2+} for 96 h. The specific growth rate was significantly decreased ($P<0.05$) with increasing concentrations of Cu^{2+} in the external medium. The untreated culture showed specific growth rate as 0.121± 0.004. Further the above effect was more pronounced at the higher Cu^{2+} concentrations (7.5 µM and10 µM), where there was no growth at all. A 52% inhibition in specific growth rate of *N. muscorum* was observed at 2.5 µM of Cu^{2+} in medium. A metal-induced general reduction in, chlorophyll-a, caroteniods, phycocyanin and protein contents was also observed in a concentration-dependent manner during 96 h of Cu^{2+} treatment (Table 3.2). We also found that compared to pigments, protein content of the culture (mg/ml culture) was less affected. Results showed a decreasing pattern of the above mentioned parameters with increasing concentrations of Cu^{2+} up to 10 µM.

Culture growing without added nitrogen showed heterocyst frequency as 3.23±0.64. When culture was grown in Chu-10 medium with added N, the heterocyst

Table 3.2: Effect of copper on specific growth rate, photosynthetic pigments (chl. a, carotenoids, phycocyanin and protein content of *N. muscorum*) after 96 h of treatment.

Treatment $Cu^{2+,}$ (µM)	Specific growth rate (μd^{-1})	Chlorophyll-a (µg ml^{-1})	Carotenoids (µg ml^{-1})	Phycocyanin (µg ml^{-1})	Protein (mg ml^{-1})
Control	0.121± 0.004	1.971 ± 0.026	0.604 ± 0.016	9.738± 0.306	0.533± 0.150
0.5	0.119± 0.004 *(2.0)*	1.848 ±0.029 *(6.0)*	0.597 ± 0.010 *(1.0)*	8.989± 0.459 *(8.0)*	0.492± 0.010 *(8.0)*
1.5	0.075± 0.007 *(38.0)*	1.506 ± 0.026 *(24.0)*	0.506 ± 0.020 *(16.0)*	7.204± 0.237 *(26.0)*	0.467± 0.010 *(12.0)*
2.5	0.058± 0.004 *(52.0)*	1.305 ± 0.040 *(34.0)*	0.434 ± 0.023 *(28.0)*	6.067± 0.321 *(38.0)*	0.410± 0.006 *(23.0)*
5.0	0.016± 0.008 *(87.0)*	0.960 ± 0.039 (51.0)	0.351 ± 0.020 *(42.0)*	4.357± 0.168 *(55.0)*	0.349± 0.015 *(35.0)*
7.5	NG *(100.0)*	0.754 ± 0.019 (62.0)	0.300 ± 0.010 *(50.0)*	3.296± 0.231 *(66.0)*	0.305± 0.015 *(43.0)*
10.0	NG *(100.0)*	0.555 ± 0.027 (72.0)	0.249 ± 0.011 *(59.0)*	1.960± 0.382 *(80.0)*	0.235± 0.020 *(56.0)*

NG: No growth. Values in parentheses are percent inhibition/decrease compared to control.

frequency decreased by 59% on day five (Table 3.3). The 2.5 µM Cu^{2+} treatment resulted in to 56% reduction in heterocyst frequency. Further, we found that heterocyst frequency

decreased with increasing concentrations of Cu^{2+} in the medium. No cells and no heterocyst were observed on 7.5 and 10 µM Cu^{2+} after treating for 5 days (Table 3.4).

Table 3.3: Effect of N and Cu^{2+} on % heterocyst frequency of *N. muscorum.* Values are mean ±SE three replicates.

Treatment	0 day	2nd day	5th day
Control (-N)	3.172% ± 0.614	3.230% ± 0.641	3.320% ± 0.554
+N	3.172%	2.201% ± 0.441	1.366% ± 0.173
-N+ 2.5 µM Cu^{2+}	3.172%	2.083% ± 0.414	1.462% ± 0.252

Table 3.4: Effect of Cu^{2+} on % heterocyst frequency in *N. muscorum*. Values are mean ± SE three replicates.

Treatments (µM, Cu^{2+})	0 day	5th day
0.0	3.135% ± 0.307	3.512% ± 0.701
0.5	3.135%±0.241	2.762% ± 0.452
1.5	3.135%±0.211	2.058% ± 0.441
2.5	3.135%±0.152	1.587% ± 0.301
5.0	3.135%±0.110	0.847% ± 0.109
7.5	3.135%±0.220	*NC, NH*
10.0	3.135%	*NC, NH*

NC: No cell; *NH*: No heterocyst.

Intracellular uptake and adsorption of Cu^{2+} in relation to time and different concentrations of Cu^{2+} are shown in Fig. 3.3. Time-course study of Cu^{2+} uptake showed that intracellular uptake rate was rapid during first 45 min and then increased slowly till the end of experiment (Fig. 3.3A). It suggested about a biphasic uptake of copper in *N. muscorum.* Intracellular uptake followed the Michaelis-Menten kinetics and is mediated by two transport systems, one with low and other with high affinity. The intracellular metal uptake in relation to increasing concentrations of copper is shown in Figure 3.3B. With increasing concentrations of copper up to 5.0 µM, intracellular metal uptake increased rapidly. Further increase in metal concentrations, intracellular uptake increased at slower rate and finally attained saturation. The above results suggested about involvement of at least two types, high affinity and low affinity metal transporters in *N. muscorum*.

The maximum uptake rate (2.528 x 10^{-3} µg Cu^{2+} mg^{-1} protein h^{-1}) and half saturation constant (K_m = 1.344±0.580 µM) was determined using exploratory enzyme kinetics (Table 3.5).

Table 3.5: Adsorption and uptake of Cu^{2+} by *N. muscorum*. Values are mean ± SE, n=3.

Adsorption			Intracellular uptake		
$U_{max,}$ µg mg^{-1} protein, x 10^{-3}	K_d, µM	R^2	V_{max}, µg mg^{-1} protein h^{-1}, x 10^{-3}	K_m, µM	R^2
10.288±0.509	0.773±0.179	0.980	2.528± 0.292	1.344± 0.580	0.949

Results showed that adsorption of Cu^{2+} were higher than intracellular metal accumulation. Most of the Cu^{2+} was adsorbed within 5 min of treatment indicating about a passive mechanism (Fig. 3.3C). Adsorption of Cu^{2+} increased very rapidly within the first few min, reached equilibrium during next 30 min of exposure. Rate of adsorption was very rapid up to 2.5 µM Cu^{2+}, and then increased slowly (Fig. 3.3D). It suggested about involvement of different kinds functional groups with varying affinity on cell surface. Functional groups with high affinity to Cu^{2+} were involved in initial rapid adsorption of copper at lower concentrations. Functional groups with lower affinity were playing role at higher concentrations of copper.

The calculated maximum binding sites on the surface of cyanobacterium (U_{max} 10.288 x 10^{-3} Cu^{2+} mg^{-1} protein) was determined using direct linear plots of exploratory enzyme kinetics.

The uptake and adsorption of Cu^{2+} by *N. muscorum* were highly dependent on pH of the medium (Fig. 3.4). The uptake of Cu^{2+} by the alga was maximal at pH 7.0; further increase or decrease in pH of the external medium decreased the copper uptake. Adsorption was maximal at pH 6.6 with U_{max} 16.593±0.830 x 10^{-3} µg mg^{-1} protein. However, further rise in pH decreased the adsorption of copper on to *N. muscorum*. The K_d was found to be the lowest at pH 6.6 and 6.8. Table 3.6 shows the kinetic parameters of Cu^{2+} uptake and adsorption at different pH.

Uptake and adsorption of Cu^{2+} under the influence of other heavy metals (Zn^{2+} and Cd^{2+}) was also studied. Zn^{2+} decreased the intracellular uptake (Fig. 3.5) and adsorption (Fig. 3.6) of Cu^{2+} in a concentration dependent manner. Rate of Cu^{2+} uptake

Table 3.6: The Michaelis-Menten constants of adsorption and intracellular uptake of Cu^{2+} at different pH by *N. muscorum*. Values are mean ± SE three replicates.

pH	Adsorption		Intracellular Uptake	
	U_{max}, µg mg^{-1} protein, $x10^{-3}$	K_d, µM	V_{max}, µg mg^{-1} protein h^{-1}, $x10^{-3}$	K_m, µM
6.6	16.593 ± 0.830	0.727 ± 0.036	1.859 ± 0.092	2.147 ± 0.107
6.8	13.00 ± 0.650	0.725 ± 0.036	3.072 ± 0.154	2.269 ± 0.113
7.0	12.429 ± 0.621	0.772 ± 0.039a	2.85 ± 0.143	1.767 ± 0.088
7.2	8.350 ± 0.418	0.754 ± 0.038a	1.871± 0.094	1.480 ± 0.074
7.4	10.497 ± 0.525	0.800 ± 0.040a	1.123 ±0.056	4.069 ± 0.103
7.6	7.894 ± 0.395	1.087± 0.054b	0.725 ± 0.029	4.838 ± 0.242

and adsorption were inhibited by increasing concentrations of Zn^{2+} in the medium. The Michaelis-Menten constants for adsorption and intracellular uptake of Cu^{2+} in presence of Zn^{2+} are given in Table 3.7. The V_{max} was unchanged and K_m increased, showing that Zn^{2+} was a competitive inhibitor of Cu^{2+} uptake. Linear increase in K_m/V_{max} with increasing concentrations of Zn^{2+} showed a competitive inhibition of Cu^{2+} uptake by Zn^{2+} (Fig. 3.5 A,B).

Adsorption of Cu^{2+} by the presence of Cd^{2+} showed a non-competitive type of inhibition. Cd^{2+} caused significant inhibition in the intracellular uptake (Fig. 3.7 A, B) and adsorption of copper (Fig. 3.7 C, D) by *N. muscorum.* Increasing concentrations of

Table 3.7: The Michaelis-Menten constants of adsorption and intracellular uptake of Cu^{2+} in presence of Zn^{2+} by *N. muscorum*.

Zn^{2+}, µM	Adsorption		Intracellular Uptake	
	U_{max}, µg mg^{-1} protein, $x10^{-3}$	K_d, µM	V_{max}, µg mg^{-1} protein h^{-1}, $x10^{-3}$	K_m, µM
0.0	10.27 ± 0.513	0.798 ± 0.040	2.486 ± 0.124	1.278 ± 0.064
1.5	8.090 ± 0.404	0.823 ± 0.043	2.427± 0.111	1.720 ± 0.072
2.5	7.085 ± 0.354	1.149 ± 0.059	2.419 ± 0.103	2.238 ± 0.112
5.0	8.024 ± 0.400	1.06 ± 0.051	2.486 ± 0.100	3.134 ± 0.131
7.5	4.929 ± 0.246	1.01 ± 0.047	2.348 ± 0.117	3.129 ± 0.101
10.0	4.188 ±0.201	1.604 ± 0.061	2.210 ± 0.098	3.205 ± 0.160

Cd^{2+} in external medium resulted in decreased rate of intracellular uptake of Cu^{2+}. The Michaelis-Menten parameters of copper uptake and adsorption in presence of Cd^{2+} are shown in Table 3.8. Decreased V_{max} and unchanged K_m for Cu^{2+} uptake in presence of Cd^{2+} pointed towards the non-competitive type of inhibition. On the other hand, adsorption of Cu^{2+} in presence of Cd^{2+} was found to be a mixed type inhibition, where U_{max} decreased and K_d increased.

Figure 3.9 exhibits inhibition of intracellular accumulation of Cu^{2+} by different concentrations of Ca^{2+}. This cation inhibited uptake of Cu^{2+} in a concentration dependent manner. Direct linear plot of Cu^{2+} uptake in presence of various concentrations of Ca^{2+} is

Table 3.8: The Michaelis-Menten parameters of adsorption and intracellular uptake of copper in presence of Cd^{2+}. Values are mean ±SE three replicates.

Cd^{2+}, µM	Adsorption		Intracellular Uptake	
	U_{max}, µg mg^{-1} protein, $x10^{-3}$	K_d, µM	V_{max}, µg mg^{-1} protein h^{-1}, $x10^{-3}$	K_m, µM
0	10.29 ± .502	0.772 ± 0.384	2.477±0.124	1.377 ± 0.063
1.5	8.173 ± .391	1.093 ± 0.036	2.084 ±0.104	1.421 ± 0.071
2.5	6.35 ± 0.316	1.421 ± 0.072	1.112 ±0.056	1.301 ± 0.065
5	4.202 ±0.210	2.119 ± 0.110	0.822 ± 0.041	1.314 ± 0.062
7.5	2.570 ±0.128	2.214 ± 0.111	0.513 ± 0.025	1.479 ± 0.073
10	1.332 ±0.067	2.012 ± 0.101		

presented in Fig. 3.9 C. Figure 3.9 D shows the fitness of copper uptake data to Michaelis-Menten kinetics. The calculated kinetic parameters are given in Table 3.9. With increasing concentration of Ca^{2+}, V_{max} and K_m were decreased and increased, respectively, suggesting that Ca^{2+} was a mixed-type of inhibitor of Cu^{2+} uptake. Similarly, the adsorption of Cu^{2+} was also inhibited by the Ca^{2+} (Fig. 3.10). As determined by direct linear plot, Ca^{2+} caused mixed-type inhibition of Cu^{2+} adsorption as U_{max} decreased and K_d was increased.

Table 3.9: The Michaelis-Menten constants for adsorption and intracellular uptake of Cu^{2+} at different concentrations of Ca^{2+} by *N. muscorum*.

Ca^{2+}, µM	Adsorption		Intracellular Uptake	
	U_{max} (µg mg protein^{-1}, x10^{-3}	K_d, µM	V_{max}, µg mg^{-1} protein h^{-1}, x10^{-3}	K_m (µM)
0	10.32 ± 0.512	0.771 ± 0.039	2.73 ± 0.137	1.287 ± 0.064
1.5	9.80 ± 0.456	1.18 ± 0.051	1.53 ± 0.075	2.648 ± 0.132
2.5	8.859 ± 0.413	1.515 ± 0.055	0.823± 0.044	2.544 ± 0.112
5	8.278 ± 0.401	1.785 ± 0.087	0.432 ±0.022	3.412 ± 0.172
7.5	8.583 ± 0.421	1.580 ± 0.077	0.212± 0.011	4.872 ± 0.243
10	8.256 ± 0.412	1.615 ± 0.079	0.112 ±0.009	5.072 ± 0.250

Uptake and adsorption of Cu^{2+} were also determined in presence of monovalent K^+ (Figs. 3.11, 3.12). Similar to bivalent cations, K^+ also inhibited the rate of copper uptake in a concentration dependent manner (Figs. 3.10). Kinetic constants V_{max} and the K_m for uptake of Cu^{2+} were decreased and unchanged, respectively, suggesting that K^+ was a non-competitive inhibitor of Cu^{2+} uptake Similarly, adsorption of Cu^{2+} was also inhibited non-competitively by K^+ (Fig. 3.12). The kinetic constants showing inhibition of copper uptake by the K^+ are given in Table 3.10.

Table 3.11 represents the summary of various types of inhibitions in copper uptake and adsorption due to Zn^{2+}, Cd^{2+}, Ca^{2+} and K^+.

Table 3.10: The Michaelis-Menten constants for adsorption and intracellular uptake of Cu^{2+} at different concentrations of K^+ by *N. muscorum*.

K^+, µM	Adsorption		Intracellular Uptake	
	U_{max}, µg mg^{-1} protein, $x10^{-3}$	K_d, µM	V_{max}, µg mg^{-1} protein h^{-1}, 10^{-3}	K_m, µM
0	10.280 ±0.512	0.771 ± 0.039	2.477 ± 0.124	1.430 ± 0.072
1.5	9.549 ± 0.477	0.779 ± 0.042	2.073 ± 0.188	1.375 ± 0.065
2.5	9.611 ± 0.481	0.666 ± 0.031	1.802 ± 0.090	1.317 ± 0.054
5	9.061 ± 0.451	0.828 ± 0.041	0.413 ± 0.021	1.333 ± 0.055
7.5	9.258 ± 0.433	0.795 ± 0.036	0.207 ± 0.010	1.313 ± 0.058
10	8.929 ± 0.411	0.809 ± 0.040	0.214 ± 0.013	1.323 ± 0.066

Table 3.11: Nature of inhibition on Cu^{2+} uptake and adsorption.

Inhibitor	Adsorption	Intracellular uptake
Zn^{2+}	Non-competitive	Competitive
Cd^{2+}	Mixed type	Non-competitive
Ca^{2+}	Mixed type	Mixed type
K^+	Non-competitive	Non-competitive

3.4 Discussions

Toxicity of heavy metals in microorganisms, including cyanobacteria, is regulated by a complex mechanism conforming to physiological status of cells, prevailing environmental conditions and chemical forms of the metal present. Once taken in, such cations adversely affect the cyanobacterial physiology and biochemistry (Singh and Biban, 2013). Intracellular metals inhibit a large number of enzymes and interfere with several metabolic process including photosynthesis, pigment synthesis and membrane integrity.

Cyanobacterium *N. muscorum* showed a series of physiological and biochemical alterations when exposed to various concentrations of Cu^{2+}. Reduction in percent survival and growth of *N. muscorum* at increasing concentration of copper, confirmed the toxic potential of Cu^{2+}. The toxicity of metals may be due to either the disruption of the permeability of the cell membrane or inhibition of photosynthetic pigment and enzyme activities. Similar to present study several reports are available showing the inhibition of growth of algae and cyanobacteria due to Cu^{2+} and other heavy metals (Rai et al., 1994). In the present study it was found that with increasing Cu^{2+} concentration there was reduction in the growth rate of the test algae. Another reason for reduction of growth might be the inhibition of cell division due to binding of Cu^{2+} to sulphydryl groups which is responsible for regulation of cell division in plants.

A significant reduction in Chl-a, phycocyanin, carotenoid and protein content with increasing Cu^{2+} concentrations may be attributed to the inhibition of pigment

synthesis directly by copper or accelerated degradation of pigments due to increased ROS formation at various sites of photosynthetic electron transport chain during stress. Active oxygen species are formed due to leakage of electrons at various sites of photosynthetic and respiratory electron transport chain under stress condition. Copper and other heavy metals are known to inhibit chlorophyll-a biosynthesis particularly by inhibiting – aminolevulinic acid dehydrogenase and photochlorophyllide reductase (Ouzounidou, 1995 and Dubey, 1997). Apart from this, heavy metals have also been reported to replace the central Mg from chlorophyll- a molecules *in vivo* condition. The proteinaceous nature and the exterior localization of phycocyanin on thylakoid membrane could be one of the possible reasons for more damaging effect on this pigment. Finding on inhibition of chlorophyll content by Cu^{2+} is in agreement with those of Tripathi et al. (2003).

Carotenoid has protective roles in restoration of photosynthesis. In present investigation we found decrease in carotenoid content after treatment with copper. Many organisms tend to increase their carotenoid content under diverse kinds of stresses (Mallick and Rai, 1999; Young and Lowe, 2001) However, the present study showed a concentration dependent decline in the level of carotenoids following exposure to Cu^{2+}. Similar results were also observed in some other studies (Tripathi et al., 2003; Rahman et al., 2011).

The present study showed that copper decreased the total protein content, at least at higher doses. It could be suggested that accumulation of protein at low heavy metal concentrations may be one of the ways through which the algae can abolish their toxic effects, or increase respiration leading to the utilization of carbohydrate in favor of

protein accumulation (Osman et al., 2004). Whereas the suppression of protein accumulation may be attributed to shortage of carbon skeleton results from low photosynthetic rate. Such results are in accordance with those of Fathi et al. (2000). However, some authors (Osman et al., 2004; Tripathy and Gaur, 2006) reported that the toxic action of heavy metals on the enzymatic reactions is responsible for decreased protein biosynthesis.

The cells of *N. muscorum* accumulated high levels of Cu^{2+} on the surface as well as inside the cell. In the present study, the surface-bound Cu^{2+} was efficiently extracted from the test organism using 2 mM EDTA. It is likely that such an extraction removes some of the intracellular metal as well. But, Bates et al. (1982) have suggested the EDTA is unable to penetrate cells and, therefore, is only able to react with the surface- bound metal. It is assumed that the values of intracellular metal content reported in the present study are of the fraction firmly bound (non-extractable by EDTA) to the cell components. *N. muscorum* accumulated high concentrations of Cu^{2+}, however, adsorption contributed more percent to total Cu^{2+} accumulation by the test algae. This agrees well with reports by Mehta et al. (2000) demonstrating a greater proportion of metals on the cell surface in comparison to the intracellular fraction.

Time-course study of Cu^{2+} accumulation showed two phases, an initial very rapid adsorption and a slower transport of metal ions. The two phases of metal accumulation have also been demonstrated earlier by several workers (Gadd, 1988; Garnham et al., 1992; Collard and Matagne, 1994; Mehta et al., 2002). The uptake of test metals occurred at an almost constant rate with the passage of time, suggesting slow transport across the

membrane and a carrier-mediated mechanism. The increase in metal uptake with increasing time also explains incorporation of metal ions into cell proteins and possibly other cellular components. *N. muscorum* showed maximum intracellular uptake of Cu^{2+} at 2.528 µg mg^{-1} protein $h^{-1}x\ 10^{-3}$ and maximum adsorption was found to be 10.288 µg mg^{-1} protein $x10^{-3}$.

The present work showed an optimum pH value for both uptake and adsorption of test metal. Uptake and adsorption was maximum at pH 6.6 and pH 7.0, respectively. Similar to present study, Schecher and Driscoll (1985) have also reported that Cu^{2+} uptake increased markedly with increase in pH within the range of 4 to 7 in *Nostoc muscorum.* However, metal uptake and adsorption was decreased with increasing pH. This may be due to denatured carrier molecules at higher pH thereby reducing metal uptake. A reduction in uptake of metal can also cause reduction in metal toxicity (Gadd and White, 1985).

The present work showed that all the tested cations and metal ions strongly influence adsorption and uptake of Cu^{2+} by the test alga. Zn^{2+} increased K_m for Cu^{2+} uptake in the test organism thereby suggesting the lowering affinity of the Cu^{2+} transport system. Therefore Zn^{2+} appears as a competitive inhibitor of Cu^{2+} uptake in *N. muscorum*. Non-competitive inhibition of Cu^{2+} uptake by Cd^{2+} is suggestive of their transport by specific systems. Adsorption of Cu^{2+} was also inhibited by Zn^{2+} and Cd^{2+}. Non-competitive inhibition of Zn^{2+} on Cu^{2+} adsorption suggest that Zn^{2+} did not reduce affinity of the substrate (Cu^{2+}) to bind onto the cell surface (K_d constant), but the binding was reduced. Inhibition by Cd^{2+} on Cu^{2+} adsorption was of mixed type which is a

combination of competitive and non-competitive types of inhibition. It is likely that Cd^{2+} did not act directly on Cu^{2+} transport system. Both adsorption and uptake of Cu^{2+} by the presence of Ca^{2+} showed mixed inhibition. Contrary to the present study, Schecher and Driscoll (1985) and Mallick and Rai (1993) have shown that presence of Ca^{2+} resulted in a decreased metal uptake by competitive inhibition whereas, Mehta et al. (2002) showed non-competitive inhibition of Cu^{2+} uptake by Ca^{2+} in *Chlorella vulgaris*. It is likely that being monovalent, K^{+} did not act directly on the Cu^{2+} uptake system and did not inhibit it competitively. Non-competitive inhibition of uptake and adsorption of Cu^{2+} by K^{+} suggest that Cu^{2+} has specific site on the cell surface for the binding.

CHAPTER FOUR

COPPER-INDUCED OXIDATIVE STRESS AND TOLERANCE MECHANISMS

4.1 Introduction

Photosynthetic organisms can tolerate elevated ROS levels because of endogenous mechanisms that effectively scavenge and remove the toxic products before cellular damage occurs. An array of protective and repair mechanisms in order to combat the menace posed by the presence of ROS has been reported in many organisms including cyanobacteria (Smirnoff, 1993; Potts, 1994; Takeda et al., 1995; Alscher et al., 1997; Foyer et al., 1997).

Cyanobacteria when exposed to abiotic stresses as temperatures, drought, water availability, air pollutants, nutrient deficiency, heavy metals or salt stress show increased level of intracellular ROS e.g., $^1O_2$, $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$. Heavy metal stress invariably induces oxidative stress and antioxidative defense systems. Cyanobacteria have evolved numerous strategies in order to avoid the production of ROS or to scavenge them once produced. The components of antioxidant defense system are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include SOD, CAT, APX, GPX, MDHAR, DHAR and GR and non-enzymatic antioxidants are GSH, AA (both water soluble), carotenoids, tocopherols (lipid soluble) and other compounds capable of quenching ROS (Liu et al., 2009).

SOD acts as the first line of defense against ROS detoxification process which converts O_2^- radicals to H_2O_2 and O_2. Several studies on protective role of SOD in cyanobacteria under diverse kinds of stresses like high light intensities (Kim and Suh, 2005), desiccation (Rajendran et al., 2007), chilling (Thomas et al., 1998), nitrogen starvation (Saha et al., 2003) and heavy metals (Mallick and Rai, 1999; Srivastava et al., 2005) have been reported. CAT is a common enzyme found in nearly all living organisms that are exposed to oxygen. CAT, APX and GPX subsequently detoxify the accumulation of H_2O_2 to H_2O and O_2^-. In contrast to CAT, APX requires an ascorbate and GSH generation system the 'ascorbate-glutathione cycle' where APX reduces peroxides to H_2O so that the accumulation of O_2^- and H_2O_2 is effectively prevented (Parida et al., 2004). Ascorbate is a small, water- soluble antioxidant molecule used as substrate for APX. Ascorbate oxidation always leads to production ascorbyl radical MDHA which is normally converted to oxidized form of ascorbate (dehydroascorbate) (Zhang and Kirkham, 1996) by monodehydroascorbate reductase (MDHAR). DHA is reduced to ascorbate by the action of DHAR using GSH as the reducing substrate. The generation of ascorbate from monodehydroascorbate and dehydroascorbate is catalyzed by NAD(P)H-dependent MDHAR and GSH-dependent DHAR (Zhang and Kirkham, 1996; De Tullio et al., 1998) coupled with GR and glutathione metabolism enzymes operating for the maintenance of GSH levels (Nagalakshmi and Prasad, 2001). Oxidized ascorbate is then reduced by GSH, an intermediary redox metabolite, which is generated from GSSG by GR at the expense of NADPH functioning as co-factor (Arbona et al., 2003). Thus, APX in combination with the effective ascorbate-glutathione cycle, functions to prevent the

accumulation of H_2O_2 (Mittova et al., 2000). GR also plays an important role in protecting against oxidative damage by maintaining a high GSH/GSSG ratio (Foyer et al., 1995; 1997).

Thiol groups, present in plant cells are a major non-enzymatic scavengers of ROS (Douglas, 1987). Thiols are the organic compounds that contain a sulphydryl group. GSH reacts with $^1O_2$, O_2^-, H_2O_2 and OH^- radicals. GSH protects protein from denaturation caused by the oxidation of protein thiols groups under stress. Combined with ascorbate and antioxidative enzymes, it plays important role in protecting the plant from the ROS (John et al., 2007; Arya et al., 2008). GSH is a substrate for glutathione S-transferases, enabling neutralization of potentially toxic xenobiotics (Marrs, 1996). Moreover, GSH is the precursor for phytochelatins.

Non-protein thiols (NP-SH), which contain high percentage of Cys sulphydryl residues in plants, play a crucial role in heavy metal detoxification (Zhu et al., 1999a). The reduced form of glutathione (g-Glu-Cys-Gly, GSH) is one of the most important components of NPT metabolism. Proline accumulation often occurs in algae, cyanobacteria and higher plants when subjected to environmental stresses such as heavy metals (Mehta and Gaur, 1999; Tripathi and Gaur, 2004; Zhang et al., 2008), osmotic stress (Siripornadulsil et al., 2002; Slama et al., 2006), high salinity (Miller et al., 2005) and light-induced stress (Abraham et al., 2003).

To our knowledge, there is limited understanding of Cu-induced molecular mechanisms which triggers oxidative stress by enhancing ROS levels after heavy metal

stress in cyanobacteria. We hypothesized that copper, like other abiotic stresses, induce ROS production and causes oxidative damage in cyanobacteria. To test this hypothesis, we studied the effects of copper causing cellular damage in *N. muscorum* by measuring the levels of ROS as well as antioxidant enzymes (SOD, CAT, APX, GR) and antioxidant compounds. In order to support above hypothesis, other parameters like lipid peroxidation, electrolyte leakage and concentration of proline were also measured in *N. muscorum* exposed to copper.

4.2 Materials and Methods

Cu-induced lipid peroxidation and electrolyte leakage

The lipid peroxidation and % electrolyte leakage, indices of oxidative stress were determined in 10 ml of algal culture (absorbance at 663 nm was 0.573) after 2 h treatment with 0.5, 1.5, 2.5, 5, 7.5, 10 µM Cu^{2+} and with sublethal dose (2.5 µM) of Cu^{2+} for different time periods (1, 2, 3, 4 and 5 h). Culture in the growth medium without any treatment was used as control. Methods as described in Chapter 2 were used to determine the MDA content and electrolyte leakage in samples.

Estimation of Cu-induced ROS formation

The levels of ROS (hydrogen peroxide and superoxide radical) were determined in Cu-treated cells for different time periods (1, 2, 3, 4 and 5 h) as well as in various concentrations (0.5, 1.5, 2.5, 5.0, 7.5, 10.0 µM of Cu^{2+}) for 2 h. Culture in the growth

medium without any treatment was used as control. The level of superoxide radical and hydrogen peroxide were determined by the method as described in Chapter 2.

Assay of Cu-induced antioxidant enzymes

Exponentially growing algal cultures were used for the experiment. $CuCl_2$. $2H_2O$ was added to the medium at concentrations of 0.5, 1.5, 2.5, 5, 7.5, 10 µM for 3 h to study enzymatic (SOD, CAT, APX and GR). A control with Chu-10 medium without Cu^{2+} was taken in all the experiments. For time course study a single concentration of 2.5 µM Cu^{2+} was chosen. Time course study was performed for 60, 120, 180, 240 and 300 min duration. Cultures were centrifuged to separate pellets after the treatment. The cell pellets were suspended in cell lysis buffer and subjected to sonication in ice-cold condition (4^oC). The sonicated sample was centrifuged and the resulting supernatant containing antioxidant enzymes was used for further assay. Assayed of enzyme activities was done as per the method given in chapter 2 (section 2.11).

The different isoforms and their activities of SOD and CAT were assayed on native gel. Cultures were treated with different concentrations of Cu^{2+} and Cd^{2+} (1.5, 5, 10 and 20 µM) for 2 h. In this experiment Cd^{2+} was considered to confirm if response is general or metal specific. Enzymes were extracted as per the method given in chapter 2. The polyacrylamide gel electrophoresis (PAGE) for native gel- assay was carried out under a constant current of 40 mA under non-denaturing conditions. The gel-assay method is described in Chapter 2. The SOD isoezymes were detected by the methods of Beyer and Fridovich (1987) as described in Chapter 2.

Cu- induced antioxidant compounds

Growing algal cultures were treated with 0.5, 1.5, 2.5, 5, 7.5 and 10 µM Cu^{2+} for 2 h and non-enzymatic antioxidant compounds (proline, total thiols, non-protein thiols and glutathione) were determined. For time-course study a single concentration of 2.5 µM Cu^{2+} was chosen. Time-course study was performed for 1, 2, 3, 4 and 5 h. Determination of antioxidant compounds was done as method described in Chapter 2.

SDS -PAGE profiling

Cultures were treated with different concentrations of Cu^{2+} or Cd^{2+} for 2 h to see the effect of metals on proteins profile. Total protein was extracted and separated on 10% polyacrylamide denaturing gels using standard protocols (Laemmli, 1970). The detailed protocol is described in chapter 2.

Protective role of proline against Cu-induced oxidative stress

Effect on Cu^{2+} uptake and adsorption

The effect of proline on subsequent intracellular uptake and adsorption of copper was determined. The following treatments were given:

a. Control (culture without any treatment)

b. Culture + 2.5 µM Cu^{2+}

c. Culture + Proline (10, 100, 1000 or 2000 µM)

d. Proline pretreated culture + 2.5 µM Cu^{2+}

A 10-ml of culture (in Milli-Q water) was treated with the above mentioned concentrations of proline for 1 h under normal culture conditions. After washing, culture was treated with 2.5 µM Cu^{2+} for another 1 h. The Cu^{2+} content of the cells and EDTA washing effluent were determined using atomic emission spectrophotometer as per procedure given in chapter 2.

Effect on Cu-induced lipid peroxidation

In order to elucidate the possible role of proline in Cu^{2+} tolerance, test cyanobacterium was pretreated with different concentrations of proline (10, 100, 1000 and 2000 µM) for a period of 1 h. After incubation in proline-enriched medium, the test cyanobacterium was separated by centrifugation and pellets were re-suspended in to 10 ml of culture medium containing 2.5 µM Cu^{2+} for another 2 h. After 2 h of exposure the cells were removed from incubation medium and lipid peroxidation was determined.

Effect on SOD and CAT activities

The effect of proline pretreatment on Cu-induced modulation of antioxidant enzymes was studied. The following treatments were given and activities of SOD and CAT were determined as described in Chapter 2:

a. Culture (control)

b. Culture + 2.5 µM Cu^{2+}

c. Culture + Proline (10, 100, 1000 or 2000 µM)

d. Proline pretreated culture + 2.5 µM Cu^{2+}

4.3 Results

Plant plasma membrane is regarded as the first living structure that is target for heavy metal toxicity. Heavy metals, particularly Cu^{2+}, increase the membrane permeability and thereby electrolyte leakage due to peroxidation of lipid as well as change in lipid composition in the membrane. We measured the lipid peroxidation in the test cyanobacterium treated with Cu^{2+}. Results showed that Cu^{2+} treatment caused significant increase in lipid peroxidation determined in a time-course study as well as with increasing metal concentrations in the medium. MDA level constantly increased with increasing metal concentrations in medium from 0.5 to 10.0 µM (Fig. 4.1 B). Treatment with 10 µM Cu^{2+} for 2 h resulted in 5- fold increase in MDA level compared to control. Similarly, a maximum of 3.8-fold increase in MDA level over control was observed after 5 h treatment with 2.5 µM Cu^{2+} (Fig. 4.1 A). The effect of increasing concentration of copper on subsequent electrolyte leakage is shown in Fig. 4.1 C. The % electrolyte leakage increased with increasing concentration of copper. *N. muscorum* treated with 2.5 µM and 10.0 µM Cu^{2+} showed 17% and 30% increase in electrolyte leakage, respectively, when compared with control.

The experiments were also performed to measure the levels of superoxide anion and H_2O_2 in *N. muscorum* treated with Cu^{2+}. The H_2O_2 level increased during first two hours of treatment and subsequently decreased till the end of experiment (Fig. 4.2 A). The 2 h and 5 h treatment increased H_2O_2 level by 71% and 34.2%, respectively. In

contrast to time-course study, the level of cellular H_2O_2 increased with increasing concentration of Cu^{2+} in the external medium (Fig. 4.2 B).

The cellular concentration of superoxide radical was found to be dose and time dependent (Fig 4.2 C,D). A 5 h treatment with 2.5 µM Cu^{2+} caused 3.6- fold increase in cellular level of superoxide radical when compared with control.

The highest SOD activity was observed after 3 h treatment and afterward a slight decline in SOD activity was observed (Fig. 4.3 A). Figure 4.3 B shows the modulation in SOD activity with increasing concentration of copper in the medium. The activity of SOD enzyme increased with increasing concentration of copper. The highest SOD activity (1.6-fold of control) was observed with 10.0 µM Cu^{2+} in the medium.

In contrast to activity of SOD, the CAT activity in Cu-treated culture increased with increasing both time of exposure and concentration of copper (Fig. 4.3 A,B). The maximal CAT activity (0.03 µmole mg^{-1} protein min^{-1}) was observed after 5 h treatment with copper.

Similar to CAT, the GR (Fig. 4.4 A,B) and APX activities (Fig. 4.4 C,D) also increased with increasing time and concentration of copper in the external medium. *N. muscorum* treated with 2.5 µM Cu^{2+} for 2 h showed 0.023, 0.098 and 0.440 µmol mg^{-1} protein min^{-1} CAT, GR and APX activity, respectively. However, if compared with the control, the highest change in activity due to 2.5 µM Cu^{2+} for two hour was observed for CAT followed by APX and GR.

In-gel assay of both the Cd^{2+} and Cu^{2+} treated cells showed the prominent activities of Fe-SOD and Mn-SOD enzymes (Plate 4.1, 4.2). The Mn-SOD activity increased with increasing concentrations of Cd^{2+} up to 10 µM and then declined (Plate 4.1). The activity of Fe-SOD declined sharply or remains unchanged in Cd^{2+} treated cells. The above results suggested that Mn-SOD played role against Cd^{2+} stress. On the other hand, both Fe-SOD and Mn-SOD activities increased at 2.5 µM Cu^{2+} (Plate 4.2). At higher concentrations of copper the activity of SOD activity sharply declined. It suggested that higher concentrations of copper inhibit the activities of SOD isoforms.

Activity of CAT increased in Cu^{2+} treated cells with increasing concentrations while it declined with increasing concentrations of Cd^{2+} (Plate 4.3). The above results suggested that activation of different antioxidant enzymes and their isoforms is metal specific.

Experiments were performed to see the effect of copper on SDS- PAGE profile of *N. muscorum* (Plate 4.4). SDS-PAGE analysis of the total protein in *N. muscorum* under Cu^{2+} stress showed the appearance of new bands depending on concentration of copper. In total we have found fourteen new bands in response to copper stress. These bands were not originally detected in the control. The lane/band analysis results are given in Table 4.1 A,B for control and Cu^{2+} (10 µM) treated culture, respectively.

The figure 4.5 shows the changes in non-enzymatic antioxidants of *N. muscorum* in response to copper treatment. Cu^{2+} treatment modulated the contents of total thiol (Fig. 4.5 A,B), NPT (Fig. 4.5 C,D) and GSH (Fig. 4.5 E,F) in time as well as with varying

Table 4.1 A: Lane band and molecular weight analysis of SDS PAGE profile of *N. muscorum* (control).

Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		250.0	0.011	1,180,344	N/A	N/A	10.0	9.6
2		250.0	0.021	3,152,072	N/A	N/A	26.7	25.6
3		191.1	0.050	2,374,764	N/A	N/A	20.1	19.3
4		144.4	0.080	1,022,108	N/A	N/A	8.7	8.3
5		136.7	0.100	995,656	N/A	N/A	8.4	8.1
6		124.5	0.132	502,452	N/A	N/A	4.3	4.1
7		115.9	0.157	60,588	N/A	N/A	0.5	0.5
8		108.6	0.180	25,364	N/A	N/A	0.2	0.2
9		94.8	0.224	50,456	N/A	N/A	0.4	0.4
10		82.9	0.262	78,540	N/A	N/A	0.7	0.6
11		72.2	0.308	47,872	N/A	N/A	0.4	0.4
12		69.5	0.326	65,892	N/A	N/A	0.6	0.5
13		61.4	0.381	399,432	N/A	N/A	3.4	3.2
14		56.6	0.418	92,412	N/A	N/A	0.8	0.7
15		49.0	0.485	90,032	N/A	N/A	0.8	0.7
16		43.8	0.546	204,680	N/A	N/A	1.7	1.7
17		42.2	0.567	74,460	N/A	N/A	0.6	0.6
18		37.5	0.632	21,692	N/A	N/A	0.2	0.2
19		35.5	0.663	35,564	N/A	N/A	0.3	0.3
20		29.2	0.776	22,100	N/A	N/A	0.2	0.2
21		25.5	0.852	198,560	N/A	N/A	1.7	1.6
22		20.4	0.977	861,968	N/A	N/A	7.3	7.0
23		20.0	0.994	245,412	N/A	N/A	2.1	2.0

concentrations of copper in the medium. The cellular concentrations of all examined antioxidants, except total cellular thiol, increased with increasing concentrations of Cu^{2+} and also with increasing time of exposure. Total thiol level increased with time up to first 4 h of treatment and then sharply declined. Total thiol as 2.820 µM mg^{-1} protein (1.8-fold of control) in Cu-treated cells was found in the present study. Similarly, a consistent increase (1.2-, 1.4- and 1.5- fold over control) in total thiol content was found in culture

treated with 1.5, 2.5 and 5 µM Cu^{2+}, respectively, as compared to the control. However, raising the concentration of Cu^{2+} (7.5 and 10 µM) beyond this resulted in a decreased thiol levels.

Time-course study of NPT (Fig. 4.5 C) content in *N. muscorum* showed that there were 1.4- fold increases during the first 2 h (0.299 µM mg^{-1} protein) of Cu^{2+} treatment, compared to control. The NPT content determined in culture treated with various concentrations of copper increased constantly (Fig. 4.5 D). As compared with the control, a 1.7- fold increased in NPT concentration was observed at 10 µM Cu^{2+} in the external medium. Similar to total thiol and NPT levels, a time- and concentration-dependent change in glutathione content was also observed suggesting that a multitude of antioxidants are stimulated in order to combat with copper-induced oxidative stress. Time-course study revealed a continuous increase in GSH content with increasing time. Treatment with 2.5 µM Cu^{2+} enhanced the level of glutathione (GSH) by 2.0- fold during the 5 h of treatment (Fig. 4.5 E). In line with other antioxidant compounds, GSH level also increased with increasing concentrations of copper in the medium. Treatment with 10 µM Cu^{2+} for 2 h resulted 2.4-fold increase in glutathione content (Fig. 4.5 F). Significant increase was observed up to 7.5 µM Cu^{2+} treatments and thereafter was found insignificant increase at 10 µM Cu^{2+}.

The time-course study showed a rapid accumulation of proline in *N. muscorum* (Fig. 4.6 A). The proline level was increased by 4.3- fold during the first 2 h (0.1135 µg

Table 4.1 B: Lane band and molecular weight analysis of SDS PAGE profile of *N. muscorum* treated with 10 µM Cu^{2+}.

Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		250.0	0.011	1,115,404	N/A	N/A	11.1	10.6
2		250.0	0.025	2,109,768	N/A	N/A	21.0	20.1
3		224.5	0.038	1,242,292	N/A	N/A	12.4	11.9
4		167.0	0.059	1,499,332	N/A	N/A	15.0	14.3
5		142.8	0.084	539,716	N/A	N/A	5.4	5.2
6		135.2	0.103	362,100	N/A	N/A	3.6	3.5
7		117.9	0.151	34,272	N/A	N/A	0.3	0.3
8		112.2	0.169	5,644	N/A	N/A	0.1	0.1
9		105.1	0.192	51,136	N/A	N/A	0.5	0.5
10		90.5	0.238	68,272	N/A	N/A	0.7	0.7
11		79.1	0.276	93,976	N/A	N/A	0.9	0.9
12		72.8	0.305	26,792	N/A	N/A	0.3	0.3
13		67.1	0.341	139,060	N/A	N/A	1.4	1.3
14		59.6	0.395	517,208	N/A	N/A	5.2	4.9
15		54.9	0.431	118,864	N/A	N/A	1.2	1.1
16		48.0	0.496	123,216	N/A	N/A	1.2	1.2
17		45.9	0.521	5,100	N/A	N/A	0.1	0.0
18		42.9	0.557	274,788	N/A	N/A	2.7	2.6
19		41.5	0.577	118,796	N/A	N/A	1.2	1.1
20		36.9	0.642	34,612	N/A	N/A	0.3	0.3
21		35.2	0.669	39,168	N/A	N/A	0.4	0.4
22		32.7	0.711	12,512	N/A	N/A	0.1	0.1
23		29.6	0.768	44,336	N/A	N/A	0.4	0.4
24		27.2	0.816	101,592	N/A	N/A	1.0	1.0
25		26.1	0.839	143,480	N/A	N/A	1.4	1.4
26		23.4	0.900	16,184	N/A	N/A	0.2	0.2
27		20.7	0.969	813,348	N/A	N/A	8.1	7.8
28		20.0	0.992	376,992	N/A	N/A	3.8	3.6

mg^{-1} protein) of Cu^{2+} treatment when compared with the control (0.0261 µg mg^{-1} protein). After that time, however, the level gradually declined till the end of the experiment. *N. muscorum* also exhibited a concentration-dependent change in proline content (Fig. 4.6 B). The accumulation of free proline significantly increased at higher

concentrations of Cu^{2+} in the growth medium. Treatment with 10 µM Cu^{2+} (0.1265 µg mg^{-1} protein) resulted in 4.5-fold increase in proline level. A rapid increase in proline level was observed up to 2.5 µM Cu^{2+} (0.108 µg mg^{-1} protein) and slowed down.

In present study we have found that proline pretreatment was effective in protecting *N. muscorum* from Cu-induced lipid peroxidation. 2.5 µM Cu^{2+} caused 1.6-fold (0.0209 µM mg^{-1} protein) higher lipid peroxidation than the control i.e. 0.0134 µM mg^{-1} protein (Fig. 4.7) during 2 h of exposure. Proline pretreatment (1 h) of culture greatly decreased the Cu-induced lipid peroxidation. The inhibition in lipid peroxidation by the proline pretreatment of cultures was dependent on concentration of proline in the incubation medium. Pretreatment with 0.01, 0.1, 1 and 2 mM proline decreased lipid peroxidation by 15.3, 16.3, 23.9 and 30.6%, respectively, when compared to the copper treated culture. However, cell treated with only proline also showed decrease in MDA level, compared to control. The above result suggested that proline plays general protective role and is not copper or metal specific.

Proline pretreatment also modulated the SOD (Fig. 4.8 A) and CAT (Fig. 4.8 B) activities of cells exposed to Cu^{2+}. It was observed that proline pretreatment decreased the activities of SOD and CAT in Cu-treated *N. muscorum*. However, the SOD activity was also inhibited by 13.6, 20.9, 39.0 and 30.6% after pretreatment of culture with proline. Similar result was observed in case of CAT. The CAT activity declined 4.9, 13.2, 15.9 and 34.1%, respectively by external supply (.01, 0.1, 1 and 2 mM) of proline to the untreated culture.

Results showed that proline pretreatment does not inhibit Cu^{2+} uptake, instead intracellular copper uptake was stimulated after pretreatment with proline (Fig. 4.9).

4.4 Discussions

Present study measured the short-term effect of copper on the ROS level and antioxidative defense systems in *N. muscorum* commonly found in rice fields. A range of concentrations of copper i.e. concentrations (below LC_{50}, above LC_{50} and LC_{50}) were used in the present study.

Several mechanisms of metal toxicity in algae, higher plants and cyanobacteria are known (De Filippis and Pallaghy, 1994; Hall, 2002). In this study we found that Cu^{2+} increases the lipid peroxidation in a time and metal concentration dependent manner suggesting that oxidative stress is the main contributor in copper-induced toxicity in the test organism. The present work shows stimulation of lipid peroxidation by Cu^{2+} similar to earlier reports (De Filippis and Pallaghy, 1994; Mehta and Gaur, 1999; Hall, 2002; Tang et al., 2007). Excess of Cu^{2+} increases lipoxygenase activity and hence lipid peroxidation. Srivastava (2010) reported increased lipid peroxidation in *N. muscorum* exposed to salt stress. The prime consequence of lipid peroxidation is a disturbance in membrane fluidity, and thus ion balance, resulting in altered metabolism and ROS production.

Cu^{2+} caused a dose dependent increase in electrolyte leakage in the present study. A marked increase in electrolyte leakage from plasma membrane indicates the degree of

oxidative damage of membrane components and provides a cogent explanation that copper might be sufficient to generate excessive active oxygen species, causing peroxidation of membrane lipids and thus more electrolyte leakage from membrane.

Exposure of organisms including cyanobacteria to external stimuli can generate both radical and non-radical oxidants. In oxygenic photosynthetic organisms such as cyanobacteria, ROS are inevitably generated by photosynthetic electron transport, especially when the intensity of light-driven electron transport outpaces the rate of electron consumption during CO_2 fixation (Latifi et al., 2009). Because different radicals including ROS are known to cause the lipid peroxidation and electrolyte leakage in response to diverse kind of stresses **(**Mehta and Gaur, 1999; Tripathi et al., 2003; Srivastava et al., 2005; Fatma et al., 2007; Choudhary et al., 2007; Zhang et al, 2008; Verma et al., 2008; Zeeshan and Prasad, 2009; Sakthivel et al., 2009), we also measured the cellular levels of superoxide anion (radical) and H_2O_2 (non-radical oxidant) in response to copper stress. Among the different oxidants, H_2O_2 is of great significance owing to its enormous stability (Twiner and Trick, 2000) and role as a secondary messenger to regulate the expressions of defense genes (Yang et al., 2007). The present study showed that level of H_2O_2 significantly increased in *Nostoc sp*. with increasing Cu^{2+} concentrations. Increased levels of H_2O_2 in Cu-treated cells could be attributed to a decreased effectiveness of H_2O_2-scavenging systems under stress conditions (Scandalios, 1994). Similar to the present findings, Srivastava (2010) reported a specific, rapid and concentration dependent accumulation of H_2O_2 in salt treated cells of *Nostoc muscorum.* Increased H_2O_2 content has also been reported in copper treated *Anabaena thaliana*

(Drazkiewicz et al., 2004). The half life of O_2^- is less so it can undergo a dismutation reaction forming H_2O_2, which is relatively stable (Asada, 1994).

Results showed an activation of enzymes SOD, CAT, GR and APX in Cu^{2+} treated cells. It has been demonstrated in many studies that excess copper could promote and stimulate the generation of ROS leading to increase in the activities of antioxidant enzymes (Mittler et al., 2004; Fariduddin et al., 2009; Verma et al., 2011). Increased activity of antioxidant enzymes can be expected to reduce oxidative stress to cyanobacterial cells. In fact, transgenic plants with enhanced activities of antioxidant enzymes have been shown to be tolerant to oxidative stress (Allen et al., 1997). An increase in SOD activity in stressed organisms is indicative of enhanced $O_2^{\cdot-}$ production and oxidative stress tolerance and has been proposed as an important enzyme for plant stress tolerance (Mehta and Gaur, 1999). In agreement with our results, Zhang et al. (2010) report that SOD activity increases under Cu^{2+} stress in *Elsholtzia haichowensis*. Further, in an in-gel assay we have found that Mn-SOD was playing major role as activity of Mn-SOD is increased more than other isoforms of SOD.

We found that activities of CAT, GR and APX increased significantly with increasing concentrations of copper in external medium and also with increase in time of exposure to Cu^{2+}. These enzymes are known to scavenge peroxide radicals in different cell compartments. CAT play an important role in scavenging of H_2O_2 (Wang et al., 2004). However, the response of CAT activity differs broadly depending on the plant species and the metal. Li-men et al. (2000) and Thounaojam et al. (2012) reported that

CAT activity was not affected by Cu^{2+} treatment in rice. A declined activity of CAT after exposure to Cu^{2+} in *Brassica juncea*, has been reported by Song-Hua et al. (2004). Similar to our results, an enhanced CAT activity under Cu^{2+} stress is observed by Wang et al. (2011) in roots of *Medicago sativa* and by Azooz et al. (2012) in early growing stage of wheat respectively.

In present study the activity of APX increased under Cu^{2+} stress. This increase may be attributed to a H_2O_2-mediated induction of the *apx* operon (Vranova et al., 2002). Therefore, it seems that H_2O_2 can be detoxified by a combine effort of both CAT and APX in *N. muscorum*. An increased activity of APX in copper treated cells of *N. muscorum* is in agreement with the study of Cu-induced increased activity of APX in *Anabaena doliolum* (Srivastava et al., 2005). It may worth full to mention that CAT and APX are localized in different cellular compartment in higher plants that may not apply to prokaryotic cyanobacterial cells. It remained unanswered in present study why two antioxidant enzymes are active against H_2O_2 in *N. muscorum*?

NADPH-dependent GR is essential to maintain a high ratio of GSH: GSSG through GSH replenishment, which in turn also acts on H_2O_2 removal in the ascorbate-glutathione cycle. An increased GR level is corroborated with increased APX activity owing to their direct relation in the ascorbate-glutathione cycle. Increase in GR activity following Cu^{2+} stress may be explained by transcriptional activation of *gsh1* and *gsh2* genes coding for GSH (Xiang and Oliver, 1998) which is required for the synthesis of phytochelatin. Increased GR level under copper stress is similar to the findings of

Srivastava (2010). In contrast to our results, a decreased activity of APX and GR in *N. muscorum* is reported by Singh et al. (2012). . The decrease in GR activity as observed by Singh et al. (2012) may be due to exposure of *N. muscorum* to longer period in stressed condition that may have decreased the NADPH levels, a secondary effect of free radicals (Latifi et al., 2009).

In the present investigation non-enzymatic antioxidant compounds; total thiols, non-protein thiols (NP-SH), GSH and proline showed differential responses in time-course study as well as in different Cu^{2+} concentrations. The toxicity of Cu^{2+} is affected by various endogenous antioxidants where the thiol pool of the plant plays an important role (Potters et al., 2002). In present investigation an increase in total thiol content was found in the first 4 h of exposure. The increase in thiol content may be caused by the enhancement of sulfate assimilation due to copper stress (Schützendübel et al., 2001). Increased in exposure period (5 h) and increases the concentration of Cu^{2+} (7.5, 10 µM), the level of total thiol depleted. The depletion in thiol level due to Cu^{2+} stress can be attributed to an increased consumption of glutathione for phytochelatin synthesis (Xiang and Oliver, 1998). However, Singh et al. (1999) have shown fluctuations in total thiol content in a cyanobacterium *N. muscorum* under heavy metal stress.

The concentration of NP-SH, index of phytochelatins and its precursors, in *N. muscorum* increased with the increase in time of exposure and also with increasing Cu^{2+} concentrations. This increase may be due to inactivation (sequestration) of reactive metal by the cytoplasmic detoxification mechanisms (Sanitá di toppi and Gabrielli, 1999).

These thiol-based complexing substances are comprised of several acid-soluble sulfhydryl-components, such as cystein, glutathione, and phytochelatins (De Vos et al., 1992).

Glutathione is well-known antioxidant playing the prominent roles in the defense against ROS (May et al., 1998). Glutathione is a non-protein thiol present in cyanobacterial cells mainly in its reduced form and acts as an intracellular reductant. Reduced glutathione (GSH) together with its disulfide oxidized form (GSSG) acts as free radical scavenger, a defense against oxidizing molecules and harmful metal ions, maintains the cellular redox balance, provides the basis for GSH stress signaling (Peña-Llopis et al., 2001; Blokhina and Fagerstedt, 2010). The present study showed that GSH content gradually increased with increasing time of exposure and also with increasing Cu^{2+} concentrations. The change in GSH concentrations in cyanobacteria under different stresses is controversial. Srivastava et al. (2005) have reported increased GSH concentration with increasing intensity of stresses. Contrary to above study Bhunia et al. (1991) have reported a decrease in GSH concentration. Increased GSH content in the Cu^{2+} treated cells also reflects biosynthesis of phytochelatins (PCs), as GSH is a precursor for PCs (Hall, 2002) which may involve in detoxification and tolerance by chelating the metals (Clemens, 2001). Similar to the present study stimulation of GSH content has been reported in rice (root and shoot) under different concentrations of Cu^{2+} stress after 5 days treatment (Thounaojam et al., 2012). GSH was also shown to mitigate UV-B toxicity (Tyagi et al., 2003) and salt stress (Srivastava et al., 2010). On contrary of the present study, reduced content of GSH has been registered by many earlier studies in

response to metal exposure (Gupta et al., 1998; Hartley-Whitaker et al., 2001; Mallick, 2004; Singh et al., 2012) although other studies have reported an increase or no change in GSH (Gupta et al., 1998; Schat et al., 2002).

Our result showing Cu-induced enhancement of intracellular proline in *N. muscorum* is in agreement with earlier reports (Wu et al., 1995, 1998; Mehta and Gaur, 1999; Mallick, 2004; Fatma et al., 2007). Proline level in Cu-treated cells increased in the first 2 h of treatment and then rapidly decreased. Mehta and Gaur (1999) observed a similar trend on proline accumulation in *Chlorella vulgaris* in response to heavy metals like Cu^{2+}, Zn^{2+}, Cr^{2+} and Ni^{2+}. This is unlike higher plants that require a longer period of exposure to metals to give a similar effect (Bassi and Sharma, 1993a, b). Increase in proline level could be due to its *de novo* synthesis or stress-induced inhibition of oxidative degradation of proline (Mehta and Gaur, 1999). Further, increased accumulation of proline provides osmotic adjustment as well as protection by chelating heavy metals in cytoplasm, scavenging hydroxyl radicals and maintaining water balance, which is often disturb by heavy metals (Hong et al., 2000; Costa and Morel, 1994). However, a rapid decrease in the level of proline at longer time exposure may be due to the inhibitory action in the metabolic pathway of proline biosynthesis caused by prolonged exposure of metal.

In present study we have found that antioxidant enzymes (SOD, CAT, APX, GR) and antioxidant compounds (GSH and proline) were broadly upregulated due to copper stress. It showed that *N. muscorum* has efficient metabolic deactivation pathways and

multi-component antioxidative defense mechanisms to avoid the oxidative stress caused by copper. Further, it is striking that responses of SOD, CAT, APX and GR to increased metal stress were very similar. These enzymes changed in same manner because they act cooperatively to both avoid and revert the oxidative stress (Galhano et al., 2011).

CHAPTER FIVE

COPPER STRESS AND CHLOROPHYLL FLUORESCENCE IN *N. muscorum*

5.1 Introduction

Photosynthesis is very sensitive to environmental pollution including heavy metals. Photosynthetic activities of many species of higher plants and algae including cyanobacteria are inhibited by accumulation of toxic amounts of heavy metals like Cd, Hg, Cu, Zn, Pb, Ni and Al. High levels of Cu^{2+} inhibits photosynthesis in phytoplankton (Küpper et al., 2002) and, therefore, it has been frequently used as an algaecide (Roussel, 2007). Although the metals are shown to have specific target site in the chloroplast, PS II has been found to be the major target of action of metal ions. The oxidizing- and reducing-sites of PS II and PS I are most frequently affected by different metal ions (Mostowska, 1997).

Several mechanisms of metal toxicity to photosynthetic process have been reported. The PS II has been demonstrated as the most sensitive target of metal-induced photo-inhibition. Exposure to heavy metals could lead to inhibition of capture and transfer of energy, quantum yield of photochemistry of PS II, and the synthesis of chlorophyll (Zhang et al., 2006). Photoinhibition is thought to involve at least two mechanisms of PS II inactivation that affect the acceptor and donor sides respectively

(Clijsters and Van Assche 1985; Barber and Andersson, 1992). It is commonly held that electron transport in PS I of plant is less sensitive to Cu^{2+} toxicity than that of PS II. Ouzounidou (1996) found that Cu^{2+} stressed *Thlaspi* leaves maintained an increased capacity for PS I-driven electron flow. On the other hand, the inhibitory effect of metals on reducing side of PS I electron transport system has been reported in algae and plants (Shioi et al., 1978; Baszynski, 1988). Aluminium reduces the photochemical efficiency of PS II and electron transport in several plant species (Chen et al, 2005a,b). The information available for the action of Cu^{2+} to PS I is still very limited, and further studies are needed to understand the effects of Cu^{2+} on the whole chain transport chain from PS II to PS I.

The cyanobacteria are the most primitive and least differentiated group of photosynthetic prokaryotic organisms. In common with photosynthetic bacteria they possess neither chloroplast nor nuclei, and some species, like *Nostoc muscorum*, also are able to fix atmospheric nitrogen. Cyanobacteria are among the very few groups that can perform oxygenic photosynthesis and respiration in the same compartment. Although a good deal of information is available on the action of metals on the photosynthetic electron transport in higher plants and algae, we do not know fully how heavy metals act in photoinhibition in a cyanobacteria where two contradictory metabolic pathways (photosynthesis and respiration) operate simultaneously in the same compartment at the same time and share the same components for the flow of the electrons.

Chlorophyll fluorescence technique has been proved to be useful to study effects of environmental stresses on photosynthesis. During the abiotic stress conditions the

chlorophyll fluorescence characteristics changes and provides important information on the stress mechanisms. Kramer et al. (2004) have proposed a model in which photosynthetic energy is partitioned into three fractions: (i) energy utilized photochemically by PS II; (ii) energy dissipated thermally by regulated quenching mechanisms and (ii) energy dissipated by non-regulated quenching mechanisms. If photochemical capacities are exceeded, as happens during stress conditions, the surplus energy is transferred to O_2 and ROS are produced. Although ROS are important signaling molecules, they can also affect a variety of physiological functions. Even though the amount of chlorophyll fluorescence is very small (approximately 1-2 percent of the total light absorbed), its measurement is straightforward and reliable.

The present Chapter is devoted for understanding of copper toxicity to photosynthetic process including electron transport through PS II to PS I by using Dual-PAM-100 chlorophyll fluorometer (Walz, Germany). The different treatments including various concentrations of copper, proline pretreatments and SNP (a donor of NO) were given to the *N. muscorum* and corresponding fluorescence parameters were determined in order to get information about metal induced inhibition of photosynthesis and role of proline and NO in metal-induced photoinhibition. We showed that there are multiple sensitive target sites in electron flow pathway itself where Cu^{2+} can acts and causes metal-induced photoinhition in *N. muscorum.*

5.2 Materials and Methods

Measurement of O_2 evolution and respiratory O_2 consumption

Exponentially growing cyanobacterium culture (4 ml having an absorbance of 1.213 at 663 nm) were treated with 0.5, 1.5, 2.5, 5, 7.5 and 10 µM of Cu^{2+} for 1 h and dark adapted for 15 min before recording of oxygen evolution. After the treatment, cells were added into the reaction cuvette of a Clark-type oxygen electrode. For time-course study a single concentration of 2.5 µM Cu^{2+} was chosen. Time-course study was performed for 5, 15, 30, 60, 90 and 120 min duration.

Determination of Chlorophyll fluorescence

PS I and PS II fluorescence parameters were determined using Dual PAM-100 (Waltz, Germany).

The fluorescence parameters investigated in present study are listed below:

i.	F_0	Minimal fluorescence after dark- adaptation
ii.	F_m	Maximum fluorescence after dark-adaptation
iii.	F^o	The fluorescence yield briefly before application of a saturation pulse
iv.	$F^{o\prime}$	The minimal fluorescence yield of illuminated sample with all PSII centers open
v.	F'_m	Maximum fluorescence signal
vi.	F	Steady state fluorescence
vii.	F_v/F_m	Maximum yield of PS II
viii.	qP, qL	Coefficient of photochemical quenching, measure of the overall "openness" of PS II
ix.	qN	Coefficient of non-photochemical fluorescence quenching
x.	Y(II)	Effective photochemical quantum yield of PSII Y(NPQ)

		Quantum yield of light induced non- photochemical fluorescence quenching
xi.	Y(NO)	Quantum yield of non-light induced non-photochemical fluorescence quenching
xii.	P_m	Maximal change in P700 (Far red light is applied)
xiii.	P'_m	Maximal change in P700 (no far red light applied)
xiv.	P	The P700 signal
xv.	P_0	The minimum level of P700 signal
xvi.	Y(I)	Effective photochemical quantum yield of PS I
xvii.	Y(ND)	Quantum yield of non-photochemical energy dissipation of reaction centers due to donor side limitation
xviii.	Y(NA)	Quantum yield of non-photochemical energy dissipation of reaction centers due to acceptor side limitation
xix.	Derived parameters of ETR	
	a. CEF	Cyclic electron flow
	b. LEF	Linear electron flow
	c. Y(CEF)	Quantum yield of cyclic electron flow
	d. Y(LEF)	Quantum yield of linear electron flow driven by PS II and PS I
	e. ETR II	Apparent electron transport in PS II
	f. ETR_{max} (II)	Maximal electron transport rate in PS II
	g. ETR I	Apparent electron transport in PS I
	h. ETR_{max} (I)	Maximal electron transport rate in PS I

Effect of Cu^{2+} on chlorophyll fluorescence

Exponentially growing cells (10 ml having an absorbance of 1.396 at 663 nm) were treated with different concentrations of Cu^{2+} (0.5, 1.5, 2.5, 5, 7.5 and 10 µM) for 1 h in light and further dark adapted for 15 min prior to measurement of fluorescence.

Effect of proline and SNP pretreatment on chlorophyll fluorescence

Experiments were set to determine effect of proline and SNP pretreatments separately on chlorophyll fluorescence characteristics. Cultures (10 ml of culture having an absorbance of 2.203 at 663 nm) were pretreated with proline (10, 100, 1000 and 2000 µM) or SNP (5, 50 and 100 µM) for 1 h under normal growing conditions. For this cultures were treated with the above mentioned concentrations of proline or SNP for 1 h under normal growing conditions. After washing cells were treated with 5 µM Cu^{2+} for another 1 h. Experiment was also set to see the effect of only proline and SNP treatment in culture without Cu treatment.

Chlorophyll fluorescence parameters were determined as per method given in chapter 2.

5.3 Results

Photosynthetic O_2 evolution decreased with increasing time of treatment (Fig. 5.1A) as well as with increasing concentrations of Cu^{2+} in the external medium (Fig. 5.1B). However no significant decrease was noticed during early stage of treatment (5 and 15 min). There was a concomitant increase in respiratory O_2 consumption with increasing concentrations of Cu^{2+} as well as treatment time.

Fig. 5.2 shows the relative fluorescence in PS II (*fluo*) and PS I (*P700*) recorded by dual PAM-100 in *N. muscorum* treated with various concentrations of Cu^{2+} for 1 h. Chlorophyll fluorescence in PS II decreased with increasing concentration of copper.

Differing to PS II, the P700 absorbance increased with increasing metal concentration. Further, at the all tested metal concentrations the chlorophyll fluorescence in PS I was always higher than PS II fluorescence.

The F_v/F_m values, which are considered most sensitive parameter of chlorophyll fluorescence, were calculated from fast fluorescence induction curves and results are shown in Fig. 5.3. Up to 2.5 µM Cu^{2+} treatment the F_v/F_m was not altered. However, the F_v/F_m was decreased at concentrations of Cu^{2+} above 2.5 µM. A 40 % decrease in F_v/F_m was found at 5 µM Cu^{2+} (Fig. 5.3).

Treatment with different concentrations of copper for 1 h has affected both PS II and PS I quantum yields and results are shown in Figs. 5.4 and 5.5. Y(II), the effective quantum yield of PS II was not affected when copper level was equal or less than 2.5 µM, but further increase in metal concentration reduced the Y(II) in PS II (Fig. 5.5). Results showed that quenching parameter Y(NO) remained unchanged when metal concentration was 2.5 µM, however, at higher metal concentrations Y(NO) increased. In present study Y(NPQ), the quantum yield of regulated non-photochemical energy dissipation, was not significantly affected by the various concentrations of Cu^{2+}. Copper treatment decreased the quantum yield of PS I on metal concentration dependent manner. Higher concentrations of Cu^{2+} decreased Y(I) more prominently compared to lower concentrations (Fig. 5.5). Y(ND), the quantum yield of non-photochemical energy dissipation in reaction centers due to PS I donor side limitation, increased at lower concentrations (0.5, 1.5 and 2.5 µM), however, at higher concentrations of copper Y(ND) roll back to level of control. At higher concentrations Y(ND) values were higher than

Y(NA) (effective quantum yield of non-photochemical energy dissipation due to acceptor side limitation of PS I reaction centers). Further it was observed that higher concentration of copper was associated with higher values of Y(NA) and decreased of Y(ND).

The effect of increasing concentrations of Cu^{2+} on rates of electron transport of PS II and PS I in *N. muscorum* was determined and results are shown in Fig. 5.6. It was observed that ETR(I) was always higher than ETR(II) even in untreated cells. Further, ETR (II) was more sensitive compared to ETR(I) towards the metal concentrations. The 5.0, 7.5 and 10.0 µM Cu^{2+} in medium greatly decreased the ETR(II) when compared with control. ETR(I) maintained steady levels when the cyanobacterium was treated with various concentrations of Cu^{2+}.

Effects of Cu^{2+} on derived parameters of ETR(II) and ETR(I) are given in Table 5.1. ETR_{max}(II) decreased with increasing concentrations of Cu^{2+} in the medium. ETR_{max}(II) was almost inhibited at 7.5 and 10 µM concentrations of Cu^{2+} in the external medium.

ETR_{max}(I) was initially stimulated at 1.5 and 2.5 µM Cu^{2+} and then significantly ($P<0.05$) decreased at 5.0 and 7.5 µM Cu^{2+}. The 5.0 and 7.5.0 µM Cu^{2+} decreased ETR_{max}(I) by 65 and 85%, respectively. The highest tested concentration (10 µM) of Cu^{2+} in the present study has increased ETR_{max}(I) by 90%.

At lower concentrations of Cu^{2+} α (II) and α (I) were not significantly affected, however, they were decreased at higher concentrations of Cu^{2+} in the medium (Table5.1). The α indicates the quantum yields in PS II and PS I. The constant I*k* (II), an index of the light adaptation of PS II, was almost unaffected at lower concentrations of Cu^{2+}

Table 5.1: The derived parameters of electron transport in PS II and PS I. Parameters were calculated from ETR(II) and ETR(I) curves during rapid light induction curve. The values are mean ± SE of three independent determinations.

Cu^{2+} (µM)	PS II			PS I		
	α (II) (e^{-1} photon^{-1})	ETR_{max}(II) (µmol e m^{-2} s^{-1})	*Ik* (µmol photon m^{-2} s^{-1})	α (I) (e^{-1}photon^{-1})	ETR_{max} (I) (µmol e m^{-2} s^{-1})	*Ik* (µmol photon m^{-2} s^{-1})
Control	0.111 ±0.012	12.80 ±1.02	114.7 ±9.24	0.132 ±0.011	28.60 ±0.014	216.1 ±10.25
0.5	0.109 ±0.011	12.40 ±2.10	114.1 ±7.48	*0.487 ±0.022	*38.80 ±1.50	*79.6 ±9.5
1.5	0.104 ±0.008	11.40 ±1.11	110.0 ±8.25	*0.347 ±0.028	*33.30 ±2.54	*95.7 ±6.20
2.5	0.113 ±0.012	10.40 ±1.00	*91.8 ±5.04	*0.323 ±0.028	*34.60 ±1.26	*107.3 ±5.84
5.0	*0.022 ±0.003	*2.50 ±0.18	114.8 ±5.58	0.143 ±0.014	*8.00 ±1.02	*56.2 ±4.95
7.5	*0.005 ±0.001	*0.50 ±0.03	*93.8 ±4.50	*0.204 ±0.014	*4.20 ±0.54	*20.4 ±1.25
10.0	*0.013 ±0.008	*1.20 ±0.09	*94.9 ±4.02	*0.93 ±0.065	*54.40 ±1.50	*582.5 ±6.25

*Values marked with * are significantly (P<0.05) different compared to control.*

and then slowly decreased at higher concentrations. The I*k* (I) significantly decreased with increasing concentrations of copper till 7.5 µM. At 10.0 µM copper the *Ik* (I) was dramatically increased.

The metal concentration above 2.5 µM Cu significantly decreased the coefficients qP and qL. Concomitantly, the coefficient qN was not affected by copper treatments (Fig.

5.7). Further, our results suggested that decreased qL and qP contributed for decreased ETR as observed at higher copper concentrations.

Y(CEF) (coefficient of cyclic electron flow) decreased with increasing concentrations of copper in the external medium (Fig. 5.8A). At the same time Y(CEF)/Y(I) increased with increasing concentration of copper up to 7.5 µM and then decreased. Further, it was observed that copper treatment decreased the Y(II)/Y(I). We found that Y(CEF)/Y(II) follows the same pattern as Y(CEF)/Y(I), where it increases with increasing concentrations of Cu^{2+} up to 7.5 µM and then decreased (Fig. 5.8B).

Treatment with 5 µM Cu^{2+} increased the F_v/F_m when compared with control (Fig. 5.9). Proline pretreatment has significantly decreased the F_v/F_m in Cu-treated culture. However, this inhibitory effect of proline was free from its concentration. Only proline pretreatment has no effect on F_v/F_m in control. The above results suggested a protective role of proline.

The quantum yield in PS I decreased with increasing concentration of Cu^{2+}. We found that proline pretreatment for 1 h was not effective in amelioration of Cu induced damage to PS I. (Fig.5.10A). It has to be noted that proline itself has no effect on quantum of yield in PS I. Y(ND) was increased in proportion of decrease in Y (I) due to copper stress (Fig. 5.10B). Similar to above results proline has no effect on Y(ND). Similarly Y(NO) was also not affected with proline. The above results suggested that the protective role of proline is not through the interaction of quantum yield that is directly related ETR in PS I. Similar to results obtained for quantum yield of PS I, the proline has no effect over quantum of yield of PS II in copper treated culture (Fig. 5.11 A,B,C).

Instead, at higher proline concentrations the quantum yield in PS II in metal treated cell decreased.

ETR I in *N. muscorum* was increased due to copper treatment (Fig. 5.12). Proline pretreated cells show further decrease in ETR I. However ETR II was greatly inhibited due to Cu^{2+} treatment as was evident above results also. Consistent with decrease in quantum yield of PS II, ETR II was also decreased with proline.

The above results suggested that proline has protective role against Cu^{2+} stress and this is executed in protection of photosynthetic apparatus, and not by protecting from ETR in PS I or PS II.

Y(CEF) determined for proline pretreated cells decreased with increasing concentrations of proline and the decreased was more on the combine treatment of Cu^{2+} and proline (Fig. 5.13). Y(CEF) decreased by 8% and 55 % in the highest tested proline (2 mM) and 2 mM proline + 5 µM Cu^{2+} treatment with respect to control respectively. Y(II)/Y(I) showed similar change of pattern to Y(CEF). Y(II)/Y(I) decreased by 9% at 2 mM proline pretreatment and 42% at 2 mM proline + 5 µM Cu^{2+} treatred cell when compared to control. At the same time, Y(CEF)/Y(II) and Y(CEF)/Y(I) increased when treated with proline and proline +metal. Y(CEF)Y(I) was enhanced by 1.3-fold at 2 mM proline + metal treated cells (Fig. 5.13A). Y(CEF)/Y(II) rose from 1.3 for control to 3.0 for 2 mM proline + metal treated cells (Fig. 5.13B).

The F_v/F_m value was also determined following 1 h incubation with 5, 50 and 100 µM SNP with or without Cu^{2+}, (Fig. 5.14). In respect of F_v/F_m value Cu^{2+} treated cells

showed no significant difference compared to control. Results showed that NO have negative effect on F_v/F_m. in control as well as metal treated *N. muscorum*. It was concluded that NO was not able to mitigate or modulate the F_v/F_m as we observed in case of proline. NO has decreased Y(I) in copper treated cells. Accordingly, NO has increased the Y(NA) but no effect on Y(ND) (Fig. 5.15).

Similar to PS I, parameters of PS II in presence of NO, were broadly unaffected in Cu-treated cells (Fig. 5.16). Consequently, NO has negative effect on ETR I and ETR II determined in copper treated culture. (Fig. 5.17 A,B; Table 5.2).

Y(CEF) was decreased due NO (Figure 5.18 A). At the same time Y(II)/Y(I) was either unaffected or slightly decreased due to SNP. Y(CEF)/Y(II) increases in response to NO (Fig. 5.18B).

5.4 Discussions

In the present study, effects of Cu^{2+} on the photosynthetic oxygen evolution and consumption, activities of PS I, PS II and CEF, the electron transport and energy dissipation in PS I and PS II, and the relation between PS I and PS II under Cu^{2+} treatment, proline and SNP pretreatment were also analyzed.

Present study showed that toxicity of Cu^{2+} increased with its increasing concentrations. PS II is a very sensitive site, as also reported in *Spirulina platensis* (Murthy et al., 1989). Cu-induced inhibition of O_2 evolution may also be attributed to either damage of O_2-evolving complex or alteration in the organization of the thylakoid

membrane. In contrast to this, the increased respiration rate in *N. muscorum* following Cu^{2+} treatment indicated increased energy demands for active exclusion or sequestration of the heavy metal as noticed by Lösch and Köhl (1999).

Table 5.2: The derived parameters of electron transport in PS II and PS I of proline or SNP pretreated cells. Parameters were calculated from ETR(II) and ETR(I) curves during rapid light induction curve. The values are mean ± SE of three independent determinations.

Treatment	PS II			PS I		
	α (II) (e^{-1} photon^{-1})	ETR_{max}(II) (µmol e m^{-2} s^{-1})	*Ik* (µmol photon m^{-2} s^{-1})	α (I) (e^{-1}photon^{-1})	ETR_{max} (I) (µmol e m^{-2} s^{-1})	*Ik* (µmol photon m^{-2} s^{-1})
Control	0.138±0.007	13.5±0.6	97.7±4.9	0.406±0.021	37.8±1.	93.2±4.7
5 µM Cu	0.118±0.003	11.7±0.23	99.6±5.1	0.427±0.034	29.6±2.1	69.2±2.8
10P*+Cu	0.016±0.0008	1.6±0.08	102.0±4.6	0.018±0.009	-73.0±0	0
100P+Cu	0.017±0.0005	1.6±0.04	97.7±3.3	0.215±0.013	3.6±0.17	0
1000P+Cu	0.015±0.0003	1.7±0.06	112.7±6.4	0.170±0.009	8.4±0.25	49.4±2.5
2000P+Cu	0.019±0.0008	2.0±0.09	105.9±5.1	0.128±0.005	6.4±0.19	49.7±2.0
5S*+Cu	0.072±0.004	7.6±0.4	106.3±5.0	0.307±0.024	16.9±0.84	55.0±3.1
50S+Cu	0.092±0.005	10.7±0.53	116.4±6.2	0.239±0.022	19.2±0.92	80.2±4.3
100S+Cu	0.058±0.002	8.3±0.33	145.0±7.1	0.245±0.015	18.4±0.81	75.0±4.5

P*=proline, S*=SNP

Chlorophyll fluorescence in PS II decreased with increasing concentration of copper, showing a strong effect on PS II activity. In contrast to PS II, the P700

fluorescence increased with increasing metal concentration. The increased chlorophyll fluorescence in PS I due to metal stress may be attributed to shifting from linear electron flow towards the cyclic electron flow that may result in to protection of PS II from excessive excitation energy. Lower concentrations of Cu^{2+} (up to 2.5 µM) not altered the F_v/F_m ratio. However, the F_v/F_m was decreased at concentrations of Cu above 2.5 µM. Decreased F_v/F_m at higher metal concentrations may be due to increased proportion of Q_B-non-reducing PS II reaction centers and damage of PS II reaction centers. It can also be concluded that the electron transfer reaction center PS II and the primary acceptor Q_A begins to affect the concentration from 5µM. Decreased of F_v/F_m at higher Cu^{2+} concentrations in the present study was in agreement with the findings of Ahmad-Zakeri and Abu-Bakar (2013), where they have reported that 2 mg L^{-1} Cu^{2+} reduced F_v/F_m of macroalga, *Chondrus lentillifera*. Similarly, Han et al. (2008) observed an apparent decline of F_v/F_m in *Ulva armoricana* (100 µg L^{-1} Cu^{2+}) and *U. pertusa* (250 µg L^{-1}). Further, Deng et al. (2014) reported 73% decreased of F_v/F_m value after treatment with 75 µg L^{-1} Cu^{2+} for 24 h in *Microcystis aeruginosa*.

Increased of Y(ND) at lower concentrations (0.5, 1.5 and 2.5 µM) suggested that copper inhibits the donor side of PS I. Above results suggested that at lower concentration of copper inhibition in PS I activity is due to PS I donor side limitation where as at higher concentrations of Cu^{2+} decrease in quantum yield of PS I was mainly due to limitation of 'acceptor' side of PS I. An increased Y(NA) at higher concentrations of Cu^{2+} may be due to inhibition of CO_2 fixation in dark reaction and decreased rate of charge separation at PS I.

The decreased Y(II) at higher concentration indicated that PS II activity is more affected at higher concentration of metal. Similar to present study, previously it has been shown that PS II is one of the most sensitive target for environmental stress such as low temperature (Pan et al., 2008), high temperature (Yamane et al., 1997) and heavy metals (Zhang et al., 2010; Wang et al., 2013). The simultaneous test of the activities of PS I and PS II confirm the conclusion of some previous studies that PS II is more sensitive than PS I to some environmental stress (Qian et al., 2009; Lípová et al., 2010). The increase of Y(NO) indicated the increase of fraction of 'closed' PS II centers and the inability of PS II to protect itself against damage (Pfündel et al., 2008). It suggests that mechanism of non-photochemical dissipation of energy did not work well and the excessive excitation energy could not be efficiently dissipated into harmless heat at higher concentrations of copper. NPQ protects PS II from stress-induced damage of reaction centers and is dependent on carotenoids content. As shown in chapter 3, copper treatment decreased carotenoids content, thereby, NPQ was not functioning for the protection of PS II from metal induced photo-inhibition by regulated dissipation of energy (Y(NPQ)).

Cu^{2+} caused reduction in ETR of PS I and PS II. However, the highest tested concentration (10 µM) of Cu^{2+} in the present study has increased ETR_{max}(I) by 90% suggesting about shifting of LEF to CEF under the stressful conditions. At 10.0 µM Cu^{2+} the *Ik* (I) was dramatically increased indicating the activation of cyclic electron flow and protection of PS I reaction damage due to acceptor side limitation caused by the higher Cu concentrations.

The metal concentration above 2.5 µM significantly decreased the coefficients qP and qL suggesting that higher metal concentrations decreases the fraction of open' reaction centers in PS II as it was evident by decreased Y(II) at higher concentrations of copper. Concomitantly, the coefficient qN increased at higher concentrations of copper. Further, our results suggested that decreased qL and qP contributed for decreased ETR as observed at higher copper concentrations (Table 5.1).

Y(CEF) (coefficient of cyclic electron flow) decreased with increasing concentrations of copper in the external medium. The CEF plays an important role in protecting PS II and PS I from environmental stresses. In contrast to present study, a stimulation of CEF under Cr(IV) stress has been demonstrated (Wang et al., 2012; Gao and Wang, 2012). At lower concentrations of Cu^{2+}, PS I work normally and electrons from PS I are transferred smoothly to PQ in order to prevent PS I from damage. Therefore, we concluded that Cu^{2+} at higher concentrations disrupted this kind of protective mechanisms around the PS I. Further, it was observed that copper treatment slightly decreased the Y(II)/Y(I) suggesting that copper treatment decreased the contribution of linear electron flow (LEF) to PS I. It has been suggested that reduction of LEF from PS II to PS I prevents PS I from the damage. We found that Y(CEF)/Y(II) increases with increasing concentrations of Cu^{2+} (Fig. 8B). Similar to the present study, Deng et al. (2014) reported decreased of Y(CEF) and Y(II)/Y(I) and increased of Y(CEF)/Y(I) and Y(CEF)/Y(II) in *Microcystis aeruginosa* exposed to increasing concentrations of ofloxacin (antibiotics).

Present study also observed decreased of F_v/F_m when the cells were pretreated with proline and SNP and the decreased was more on the combine treatment of Cu^{2+} along with proline and SNP. This indicates that proline and SNP increases the proportion of closed PS II reaction centers. F_v/F_m decreases by the treatment of proline or SNP in Cu-treated cells that can be due to decline of F_v and increase of F_o levels, which implies structural alterations of light harvesting complex of PS II. Several studies have shown protective roles of proline (Mehta and Gaur, 1999; Mallick, 2004; Fatma et al., 2007) and NO (Laspina et al., 2005; Qian et al., 2009; Xiao et al., 2012; Verma et al., 2013) against abiotic stress in plants, in present study we have not found a consistent protection of photosynthetic apparatus or ETR by the proline.

CHAPTER SIX

ROLE OF NITRIC OXIDE (NO) IN COPPER INDUCED OXIDATIVE STRESS TOLERANCE OF *N. muscorum*

6.1 Introduction

Nitric Oxide is a small, highly diffusible gas and a ubiquitous bioactive molecule which reacts rapidly with molecular oxygen. In prokaryotes, NO is a true intermediate of denitrification and is produced from nitrite by the dissimilatory nitrite reductase. In the course of denitrification, NO is further reduced to nitrous oxide by nitric oxide reductase. Plants, as well as animals, respond to ambient levels of nitric oxide (NO), and also generate NO themselves via various enzymatic and non-enzymatic pathways (Neill et al., 2003; del Rio et al., 2004). Many plants are believed to produce substantial amounts of NO in their natural environments (Wildt et al., 1997). NO mediates various plant physiological responses to biotic and environmental stresses including heavy metals (Wang and Yang, 2004), salinity (Zhao et al., 2004) and chilling (Neill et al., 2003).

Two mechanisms by which NO might abate stress have been proposed. NO functions as an antioxidant by directly scavenging the ROS that are generated by the stressors (Zhang et al., 2003; Hsu and kao, 2004). NO can react rapidly with superoxide radicals to form peroxynitrite (Radi et al., 1991) and this peroxynitrite itself is an

oxidizing agent, therefore it is considered to be less toxic than peroxides and thus NO limits cellular damage. Second, NO may function as a signaling molecule and indirectly modulates ROS levels in plant stress response leading to changes of antioxidative gene expressions (Lamattina et al., 2003). The signaling function of NO includes protein modification by binding to critical cys residues, heme or iron sulfur centres, tyr residues nitration via peroxynitrite formation and through S-nitrosylation of Cys residues of target proteins (Besson-Bard et al., 2008, 2009; Arasimowicz-Jelonek et al., 2011).

In the past years, it has been shown that NO has multiple physiological functions in plants (Delledonne, 2005). NO also reacts with hemes and thiols to produce other transient compounds whose functions remain unknown and needs to study. It is suggested that NO is involved in plant growth, development and responses to environmental factors affecting root growth, shoot formation, leaf expansion, stomatal closer, pollen tube growth (Salmi et al., 2007). The effects of NO, in some cases, are the results of its interaction with ROS. These interactions sometimes can be cytotoxic or cytoprotective depending on the local concentration of the molecule and situation.

The protective role of NO against copper toxicity has been demonstrated in green alga, *Chlorella vulgaris* and rice leaves (Singh et al., 2004; Yu et al., 2005). The decreased Cd-induced oxidative damage by exogenous NO has been shown in rice leaves (Hsu and Kao, 2004), sunflower leaves (Laspina et al., 2005) and wheat roots (Singh et al., 2008). On the other hand, Leshem, 1996 considered NO as a stress-inducing agent. However, the knowledge of the role of NO and its synthesis in plants is still limited due to lack of mutants for altered NO production.

Although it known that NO acts as a signaling molecule and is involved in abiotic stress responses in animals, plants and eukaryotic algae, its role under heavy metal stress in a nitrogen-fixing unicellular prokaryotic cell is unknown. In view of ecological importance of cyanobacteria it is essential understand the relationship among heavy metal toxicity, ROS generation, oxidative damage, antioxidant responses and NO signal. The objectives of this study were to investigate the role of NO signaling molecule on Cu-induced oxidative damage and antioxidant responses in nitrogen-fixing cyanobacterium *Nostoc sp.*

6.2 Materials and Methods

Test organism

The cyanobacterium *N. muscorum* was grown in Chu-10 medium. The exponentially growing cultures were used in all the experiments. In all experiments three replicates were considered.

Determination of Intracellular NO level in response to Cu^{2+}

Intracellular NO content in *N. muscorum* was determined after Cu^{2+} treatment of cyanobacterium for different durations (30, 60, 120, 180 and 240 min). The 10 ml of culture ($O.D._{663nm}$ =1.2) was treated with 2.5 µM Cu^{2+} under light. Cells were harvested by the centrifugation and NO content of pellet and effluent was determined by method as given in Chapter 2. In another set of experiment the effect of varying concentrations of

copper (0.5, 1.5, 2.5, 5, 7.5 and 10 µM) on intracellular levels of NO and NO release in external medium were determined. The incubation time with metal was 1 h.

Effect of donor (SNP) pretreatment studies

Algal cultures were pre-incubated with 5 µM and 50 µM sodium nitroprusside (Himedia), a donor of nitric oxide, for 1 h in the growth medium. Further these cultures were exposed to 2.5 µM Cu^{2+} for 1 h to determine whether NO was able to mediate the biological responses of the test organism to Cu^{2+} stress.

Effect of SNP pretreatment on lipid peroxidation, H_2O_2, O_2^-, proline, SOD and CAT

Cyanobacterium was pretreated with two concentrations of SNP (5 and 50 µM) for a period of 1 h. After incubation in SNP-enriched medium, the test alga was separated by centrifugation and pellets were re-suspended into 10 ml of culture containing 2.5 µM Cu^{2+} for another 2 h. The cells were removed from incubation medium and lipid peroxidation, H_2O_2 level, superoxide radical, proline, SOD and CAT were determined in the cell pellets as per method given in chapter 2. Experiment was also set to see the effect of only SNP treatment without Cu^{2+}.

Effect of NO on Cu^{2+} uptake

The effect of SNP (5, 50 and 100 µM) pretreatment on uptake of Cu^{2+} by *N. muscorum* was studied to test if SNP pretreatment interferes with Cu^{2+} uptake. For this, 10 ml of culture (in Milli-Q water) were treated with the above mentioned concentrations of SNP

for 1 h under normal growing conditions. After washing, the cells were treated with 2.5 µM Cu^{2+} for 1 h. Cu^{2+} contained of treated and untreated cultures were determined as per method given in chapter 2.

6.3 Results

Treatment of *N. muscorum* cells with 2.5 µM Cu^{2+} induced a rapid accumulation of intracellular NO. The intracellular NO increased with the time of Cu^{2+} exposure. The highest level was observed after 1 h of treatment. About 12 -fold increased intracellular NO level was found compared to control. After attaining the maximal level, the intracellular NO gradually declined. Similarly, the amount of NO released into the external medium increased considerably during the first 1 h and was maintained for another one hour (Fig.6.1A). The effect of Cu^{2+} on NO formation in *N. muscorum* was also investigated. Both intracellular and released NO was increased with increasing concentrations of Cu^{2+} in the medium (Fig. 6.1B).

Figure 6.2 shows the effect of SNP-pretreatment on subsequent ROS levels and MDA content in Cu^{2+} -treated culture. Compared to control, 2.5 µM Cu^{2+} treatments for 1 h significantly increased H_2O_2 and O_2^- content in the cells of *N. muscorum* by 1.5 and 1.6 folds, respectively (Fig. 6.2 B,C). However, pretreatment with 5 µM and 50 µM SNP decreased the levels of H_2O_2 by 1.1 fold (5 µM) and 1.3 fold (50 µM), and also O_2^- content by 1.2 fold (5 µM) and 1.3 fold (50 µM), respectively, when compared with Cu treatment alone. Similar trend was also observed in MDA content. 2.5 µM Cu^{2+} treatment for 1 h increased MDA content 1.9 fold higher as compared to control (Fig. 6.2A).

Compared with the Cu^{2+} treatment alone, pretreatment with 5 µM and 50 µM SNP caused a significant decrease in the levels of Cu^{2+}-induced MDA content by 60% and 66% respectively.

To counteract the toxicity of ROS in response to a variety of stresses, plant has ability to activate their antioxidant defense system. In present study the role of NO in activation of antioxidants was studied. SNP pretreatment increased proline content of control (culture without Cu^{2+} treatement) suggesting a relationship between proline metabolism and NO. NO decreased the proline level in Cu^{2+} -treated cells (Fig. 6.3).

Similar changes in the activities of SOD and Catalase were found in the culture treated with Cu^{2+} and SNP. Activities of SOD and CAT in *N. muscorum* increased significantly after 2.5 µM Cu^{2+} exposure, which was found to be 1.7- and 2.1- fold higher than the controls (Fig. 6.4A,B). The SNP plus Cu^{2+} treatment caused decrease in SOD activities by 1.2 times (5 µM) and 1.1 times (50 µM) and also for CAT activities by 1.3 times (5 µM) and 1.2 times (50 µM), respectively, as compared with Cu^{2+} treatment alone.

Pretreatment of the test cyanobacterium with different concentrations of SNP showed no significant difference in the intracellular levels of Cu^{2+} (Fig. 6.5A) suggesting that protective role of NO is not due to reduced metal uptake.

6.4 Discussion

In the present study, we detected the nitric oxide (NO) molecules in control as well as copper treated culture indicating that like animal the cyanobacterial cells also have either enzymatic or non-enzymatic pathways to synthesize NO. In mammals NO is mainly formed by the enzyme NOS (EC 1.14.13.39) which catalyzes a five-electron oxidation of a terminal guanidine nitrogen of L-arginine associated with stoichiometric consumption of dioxygen (O_2) and NADH to form L-citrulline and NO (Palmer et al., 1998). Three major NOS isoforms (neuronal NOS–nNOS-, endothelial NOS–eNOS-, and inducible NOS–iNOS-) have been identified in humans, with different tissue specificity.

In plants, neither the gene or cDNA, nor any protein with high sequence similarity to known NOS, have been found. Data about plant NOS come from its detection using anti-NOS antibodies and the measurement of NOS activity reviewed by Wendenhenne et al. (2001). In biological systems, NO can be generated by enzymatic and/or non-enzymatic reduction of inorganic form of oxidized nitrogen. Non-enzymatic pathway of NO formation in plants involves ascorbic acid and highly acidic pH (4.0). Enzymatic formation of NO in plants is based on activity of enzyme NR which is considered as key points of nitrogen and oxygen metabolisms. The NR activity has been also reported in cyanobacteria (Herrero et al, 1981). Plant cells generate significant amounts of NO under physiological conditions (Wildt et al., 1997; Duboskaya et al., 2007). However, NO generation is strongly enhanced under the action of stress factors (Magalhaes et al., 1999).

The enhanced activity of NO after treatment of Cu^{2+} may be due to increased activity of NR, although other mechanisms cannot be ruled out. Further, we have found that NO level was closely related with levels of other ROS in under the stress condition. The measured levels of superoxide, H_2O_2 and lipid peroxide were decreased after pretreatment with NO donor SNP. Based on results it is suggested that NO in present study functioned as antioxidant and protect from the Cu^{2+} stress by lowering of free radicals. In present investigation we noticed that in response to copper stress, the antioxidant levels were lower when intracellular NO level was higher.

In present study we found that NO has decreased the Cu^{2+} -induced lipid peroxidation in the *N. muscorum* suggesting its antioxidative roles in Cu^{2+} stress. Since its discovery as an endogenous free radical, NO has been proposed to be either cytotoxic or cytoprotective. The cytoprotection is based on NO's ability to regulate the level and toxicity of ROS (Halliwell and Gutteridge, 1984**).** The complex redox chemistry of NO, which is related to changes in the ambient redox milieu, is hypothesized to provide a general mechanism for cell redox homeostasis regulation (Stamler et al., 1992). Thus, NO can exerts a protective action against oxidative stress provoked by an increased concentration of superoxide, hydrogen peroxide, and alkyl peroxides (Wink et al., 1995). In addition, the NO molecule itself possesses antioxidant properties (Kanner et al., 1991). Recent studies have demonstrated that NO can interact with ROS in various ways, in which NO may have an antioxidant function and block ROS-induced lipid peroxidation (Hsu and Kao 2004, Laspina et al 2005, Wang and Yang 2005). NO plays key role in maintaining stability of membrane (Zeng et al., 2011) and NO- inhibitory effect on

peroxidation enzymes (Hogg and Kalyanaraman 1999). There are several other reports advocating for a protective effect of NO against abiotic stresses (Koprya and Gwózdz, 2003; Zhang et al., 2003; Hsu and Kao, 2004).

NO might directly abrogate O_2^- into ONOO (Neill et al., 2003) and consequently protect algae against Cu-induced oxidative stress. Cu induced the synthesis of antioxidants which was manifested in the increase in proline content (Fig. 6.3). In this case, proline may stabilize protein complexes, act as a scavenger of oxygen free radicals or function as a signal for regulation of downstream events (Kavi et al. 2005). However this increase in proline content was fully prevented by the pre-incubation of cultures with 5 µM and 50 µM SNP, even though SNP applied alone had no effect on proline content. Proline is believed to protect cell membranes from salt induced oxidative stress by removing ROS and improving the activities of various antioxidants (Matysik et al., 2002). Present study indicate that cultures treated with SNP and subsequently exposed to 2.5 µM Cu^{2+} treatment exhibit significant changes in antioxidant system as compared with control.

CHAPTER SEVEN

GENERAL DISCUSSION

Elevated levels of heavy metals in the aquatic ecosystem from natural as well as anthropogenic activities are causing a serious threat to aquatic biota, including algae and cyanobacteria. Since algae and cyanobacteria are the principal primary producers of aquatic environment, any harmful effect of metals on algae would affect the whole food chain. Thereby, it is important to study ability of cyanobacteria in metal uptake and accumulation as well as the mechanisms of heavy metal toxicity and tolerance.

Cu^{2+} induced a series of physiological and biochemical alterations to the cyanobacterium *Nostoc muscorum*. Inhibition of growth in the test alga at increasing concentration of copper, confirmed the toxicity of Cu^{2+}. Metal treatment decreased in chlorophyll-a, phycocyanin and carotenoid content may be due to the inhibition on pigment synthesis directly by copper or accelerated degradation of pigments due to increased ROS formation. Maksymiec (1997) reviewed effects of Cu^{2+} on the photosynthetic pigments and the reduction in growth of metal treated cyanobacterium as overall inhibition of photosynthesis. Similarly, reduction in growth and photosynthetic pigments by Cu^{2+} stress has also been documented earlier by several workers (Rai et al., 1994; Ouzounidou et al., 1995; Mallick and Rai, 1999; Mehta and Gaur, 1999). The suppression of protein content in Cu^{2+} stressed cells may be due to shortage of carbon

skeleton resulting from low photosynthetic rate. Such results are in accordance with those of Fathi et al. (2000); Osman et al. (2004); Tripathi and Gaur (2006).

An effort was made to understand the mechanisms of adsorption and intracellular uptake of Cu^{2+} in *N. muscorum.* In this study adsorption was defined as metal ion bound on cell surface and extractable back with EDTA. Intracellular uptake was defined as fraction of metal ion accumulated that cannot be extracted by EDTA. Time-course study of Cu^{2+} accumulation in the test alga showed two phases, an initial very rapid adsorption and a relatively slower intracellular uptake. Similarly two phases of metal accumulation have also been documented earlier by several researchers (Bates et al., 1982; Gadd, 1988; Ting et al., 1989; Wells and Brown, 1990; Collard and Matagne, 1994; Mehta and Gaur, 2002). *N. muscorum* accumulated high concentrations of Cu^{2+}; however, adsorption constituted a large proportion (75%) to the metal accumulated by the test alga. Intracellular uptake of Cu^{2+} at almost constant rate suggested about a carrier-mediated transport of copper in *N. muscorum*. The carrier-mediated transport of metal ions has been reported in a variety of biological systems (Veltrup, 1978; Gadd et al., 1987; Mehta and Gaur, 2002).

Schecher and Driscoll (1985) have reported increased uptake of Cu^{2+} uptake with increase in pH within the range 4 to 7 in *Nostoc muscorum.* Similarly, the present work also showed an optimum Cu^{2+} uptake at pH 7.0. External pH also exerts a strong effect on the adsorption of Cu^{2+}. In the present study, pH 6.6 was most favorable for the adsorption of Cu^{2+}. The pH specificity points out distinct binding sites for the adsorption

of Cu^{2+} on the cell surface. The pH specificity associated with Cu^{2+} adsorption has great applications in bioremediation of wastewaters. The pH specificity may be used for the selective removal of metal contaminants. Microorganisms contain a multitude of binding sites such as phosphate, carboxyl and sulphydryl groups that are ionized at very specific pH (Hunt, 1986; Crist et al., 1990). These groups have distinct dissociation constants (pK_a). Adsorption of Cu^{2+} in pH 6.6 indicated the involvement of functional groups in Cu^{2+} which dissociate(s) at pH 6.6. The presence of many functional groups having different dissociation constants has been also demonstrated by Crist et al. (1988).

Cations and metal ions can interfere with uptake and adsorption of metal(s) of interest. Therefore, effects of competing ions on adsorption and uptake of test metal was examined in the present study. Zn^{2+} caused competitive inhibition of Cu^{2+} uptake and non-competitive inhibition of Cu^{2+} adsorption by *N. muscorum*. This suggests that cyanobacterial membrane has metal protein carriers common to Cu^{2+} and Zn^{2+}. Baker et al. (1994) have proposed a common mechanism for the uptake of several metal ions. In the present study, the uptake of Cu^{2+} was inhibited in the presence of Zn^{2+}. This can be explained that the high concentrations of Zn^{2+} prevent the passage of Cu^{2+} through the membrane (De Filippis and Pallaghy, 1994). Cd^{2+} caused non-competitive inhibition of Cu^{2+} uptake and mixed type inhibition of Cu^{2+} adsorption. Further, Ca^{2+} and K^{+} caused mixed type and non-competitive inhibition respectively for Cu^{2+} uptake and adsorption. All the modes of inhibition (Viz., competitive, non-competitive, uncompetitive and mixed) of uptake and adsorption of test metal by other cations and metal ions have been observed in the present study, but a definite trend was not obtained. In this study we

concluded that uptake and transport of Cu^{2+} through the membrane is influenced by other cations. Divalent cations have stronger inhibitory effect compared to monovalent cation.

Present study showed increase in lipid peroxidation in a time and metal concentration dependent manner, suggesting that oxidative stress is the main contributor in copper-induced toxicity in the test organism. Stimulation of lipid peroxidation by Cu^{2+} finds support from earlier reports of Mehta and Gaur (1999) and Tang et al. (2007). Cu-induced lipid peroxidation in *N. muscorum* also agrees with the finding of Srivastava (2010) exposed to salt stress. Therefore, it is suggested that lipid peroxidation is a common consequence of diverse kind of stresses, and is not specific to copper stress. A marked increase in electrolyte leakage from plasma membrane further indicates the oxidative damage of membrane and provides a cogent explanation of copper toxicity.

As described above we find that oxidative stress is main cause of copper toxicity in *N. muscorum*. Therefore, we determined the level of ROS in Cu^{2+} treated cells. We found increased cellular level of H_2O_2 in Cu-treated cells that could be attributed to a decreased effectiveness of H_2O_2-scavenging systems under stress conditions (Scandalios, 1994). Similar to the present findings, Drazkiewicz et al. (2004) and Srivastava (2010) reported a specific, rapid and concentration dependent accumulation of H_2O_2 in Cu^{2+} and salt treated cells of *Anabaena thaliana* and *N. muscorum* respectively. In oxygen consuming cells, superoxide radical is produced by transport of electron to oxygen. *N. muscorum* exposed to different concentrations of Cu^{2+} and with one metal concentration for different time periods showed considerable increase in the O_2^- formation. The half life

of O_2^- is less so it can undergo a dismutation reaction forming H_2O_2, which is relatively stable.

Like other organisms, cyanobacteria also possess a complex array of enzymatic and non-enzymatic defense systems. It has been confirmed in many studies that excess copper could promote and stimulate the generation of ROS leading to increase in the activities of antioxidant enzymes as a defense system (Mittler et al., 2004; Fariduddin et al., 2009; Verma et al., 2011). Present study showed activation of enzymes SOD, CAT, GR and APX in Cu^{2+} treated cells. Increased activity of antioxidant enzymes can be expected to reduce oxidative stress to cyanobacterial cells. Induction of SOD activity is a requirement of the cell to encounter oxidative damage. In agreement with our results, Zhang et al. (2010) report that SOD activity increases under Cu^{2+} stress in *Elsholtzia haichowensis*. Further, in an in-gel assay we have found that Mn-SOD is playing major role as activity of Mn-SOD is increased more than other isoforms of SOD. We found that activities of CAT, GR and APX increased significantly with increasing concentrations of copper in external medium and also with increase in time of exposure to Cu^{2+}. These enzymes are known to scavenge peroxide radicals in different cell compartments. CAT play an important role in scavenging of H_2O_2 (Wang et al., 2004). Similar to our results, an enhanced CAT activity under Cu^{2+} stress is observed by Wang et al. (2011) in roots of *Medicago sativa* and by Azooz et al. (2012) in early growing stage of wheat respectively. On the contrary, Li-men et al. (2000) and Thounaojam et al. (2012) reported that CAT activity was not affected by Cu^{2+} treatment in rice. Increase of APX activity may be attributed to a H_2O_2-mediated induction of the *apx* operon (Vranova et al., 2002).

Therefore, it seems that H_2O_2 can be detoxified by a combine effort of both CAT and APX in *N. muscorum*. Our result on APX activity is in agreement with the findings of Cu-induced increased activity of APX in *Anabaena doliolum* (Srivastava et al., 2005). An increased GR concentration is corroborated with increased APX activity owing to their direct relation in the ascorbate-glutathione cycle. Increased GR level under copper stress is similar to the findings of Srivastava (2010) in salt stress of *N. muscorum.* Cu-induced GR activity may be due to its product GSH required to maintain cellular homeostasis under Cu^{2+} stress. In contrast to our results, a decreased activity of APX and GR in *N. muscorum* was reported by Singh et al. (2012) after 24 h and 72 h of different concentrations of Cu^{2+} treatment. The decrease in GR activity as observed in above study may be due to exposure of *N. muscorum* to longer period that may have decreased the NADPH levels, a secondary effect of free radicals (Latifi et al., 2009).

The thiol pool of the plant plays an important role (Potters et al., 2002) in homeostasis of metal ions among different cell organelles. There are no cell organelles in cyanobacteria, however, thiol group regulated the availability of free metal ions in the chromoplasm of cyanobacterial cell. In present investigation, we found an increase in total thiol content in the first 4 h of exposure. This increase in thiol content may be due to enhancement of sulfate assimilation due to copper stress (Schützendübel et al., 2001). The depletion in thiol level due to Cu^{2+} stress can be attributed to an increased consumption of glutathione for phytochelatin synthesis (Xiang and Oliver, 1998). Singh et al. (1999) have studied the fluctuations in total thiol content in a cyanobacterium under heavy metal stress. Singh et al. (2005) have studied the effect of temperature shift in *N.*

muscorum. Our data indicated that the tolerance of the test alga to increasing Cu^{2+} concentrations was associated with higher accumulation of NP-SH compounds. Elevated concentration of non-protein thiols indicates about cytoplasmic detoxification mechanisms that further results in reduced oxidative stress (Sanitá di toppi and Gabrielli, 1999).

The change in GSH concentrations in cyanobacteria under different stresses is controversial. Srivastava et al. (2005) have reported increased GSH concentration with increasing salt stress and heavy metal stress. Contrary to above study, Bhunia et al. (1991) have reported a decrease in GSH concentration. The present study showed that GSH content gradually increased with increasing time of exposure and also with increasing Cu^{2+} concentrations. GSH helps in maintaining the reducing environment in cell. We found increased APX activity in copper treated cells. Greater enhancement in GSH content, therefore, is linked with increased APX activity for removal H_2O_2. The above findings suggest that Asada-Halliwell pathway plays major role in oxidative stress tolerance in cyanobacterium. Stimulation of GSH content has been reported in rice (root and shoot) under different concentrations of Cu^{2+} stress after 5 days treatment (Thounaojam et al., 2012). GSH was also shown to mitigate UV-B toxicity (Tyagi et al., 2003) and salt stress (Srivastava et al., 2010). On contrary of the present study, reduced content of GSH has been registered by many earlier studies in response to metal exposure (Gupta et al., 1998; Hartley-Whitaker et al., 2001; Mallick, 2004; Singh et al., 2012) although other studies have reported an increase or no change in GSH (Gupta et al., 1998;

Schat et al., 2002). The above findings suggest that Asada-Halliwell pathway plays major role in copper induced oxidative stress tolerance in cyanobacterium.

Proline accumulation in algae and plants under diverse kinds of stresses is known for several years. It has also been said that abiotic stresses have one common attribute i.e. the production of ROS. Therefore, in this study we investigated the Cu-induced proline accumulation and its role in stress tolerance. Further, studies about proline accumulation in cyanobacteria in response to abiotic stress are scant. Our results showing Cu-induced enhancement of intracellular proline in *N. muscorum* agreed with the reports of Wu et al. (1995, 1998); Mehta and Gaur (1999); Mallick (2004); Fatma et al. (2007). Rapid and initial increase followed by sharp decline in proline concentration in copper treated cells is similar to finding of Mehta and Gaur (1999). This is unlike higher plants that require a longer period of exposure to metals to give a similar effect (Bassi and Sharma, 1993 a,b). Increase in proline level in the stressed cells could be due to its *de novo* synthesis or stress-induced inhibition of oxidative degradation of proline by dehydrogenase (Mehta and Gaur, 1999). Increased accumulation of proline provides osmotic adjustment as well as protection by chelating heavy metals in cytoplasm, scavenging hydroxyl radicals and maintaining water balance, which is often disturb by heavy metals (Hong et al., 2000; Costa and Morel, 1994). However, a rapid decrease in the level of proline at longer time exposure may be due to the inhibitory action in the metabolic pathway of proline biosynthesis caused by prolonged exposure of metal.

Photosynthesis has been shown as most sensitive target of abiotic stresses including heavy metals. Present study analyzed toxic effects of Cu^{2+} on the photosynthetic oxygen evolution and consumption, activities of PS I & PS II, the electron transport rate and energy dissipation. Cu-induced inhibition of O_2 evolution may be ascribed to either damage of O_2-evolving complex or alteration in the organization of the thylakoid membrane. Chlorophyll fluorescence in PS II decreased with increasing concentration of copper, showing a strong effect of metal on PS II activity. The increased chlorophyll fluorescence in PS I due to metal stress indicate shifting from linear electron flow towards the cyclic electron flow that may result in to protection of PS II from excessive excitation energy. The effects of copper on different fluorescence quenching parameters are discussed in Chapter 5. It was concluded that copper toxicity to photosynthesis is due to interference of metal ions at multiple sites of photosynthesis process. Copper reduced rate of photosynthesis by affecting the light harvesting complex, PS II & I activity, and ETR (II) and ETR (I). Similar to photosynthesis in higher plants we observed higher rate of ETR in PS I compared to PS II. Higher metal concentration further increased the ETR in PS I suggesting that shifting of linear electron from to cyclic electron flow.

Being prokaryotic cell, cyanobacteria does not possess chloroplast envelop is true for higher plants and algae. In cyanobacteria thylakoids are easily assessable to free metal ions leading to stronger inhibitory effect on photosynthesis and reduction in overall growth.

In this study we have detected the NO which is considered as signaling molecule and is well documented in mammals. As discussed in Chapter 6, we found a protective role against copper induced oxidative stress. The mechanism of NO synthesis in cyanobacterium is not known in present study. However, enzyme NR has been reported in cyanobacteria. The non-enzymatic formation of NO cannot be ruled out. Pretreatment of *N. muscorum* with NO donor SNP has decreased the level of MDA as well as ROS. It indicates that NO is playing role as ROS sacavenger. There are several reports advocating for a protective effect of NO against abiotic stresses (Koprya and Gwózdz, 2003; Zhang et al., 2003; Hsu and Kao, 2004). Although *N. muscorum* treated with copper showed remarkable concentration of proline, however, we don't find a significant protection against copper toxicity in cyanobacterium. As we have performed experiments with chu-10 medium there was no question of scarcity of water to the cells. In natural rice field conditions desiccation is frequent phenomenon. Therefore, proline may have significant role in oxidative stress tolerance where water scarcity is evident.

Summary

Heavy metals are potential pollutant of aquatic as well as terrestrial ecosystems. Increasing pollution by heavy metals results from their increasing utilization processes. Heavy metals considerably affect the primary producers of aquatic ecosystem. The presence of metals in soil and water bodies exerts toxic effects on higher plants, animals, microorganisms including cyanobacteria. Cyanobacteria, the most primitive and abundant photosynthetic organisms on Earth are attractive models to investigate the interrelations between metal toxicity and oxidative stress, because they perform the two metal-requiring ROS generating processes, photosynthesis and respiration, in the same membrane system.

Although several mechanisms of heavy metal toxicity in algae and cyanobacteria have been demonstrated, oxidative stress is known as principal mechanism of metal toxicity. Being a redox active, heavy metals including Cu^{2+} trigger the generation of various ROS e.g. superoxide anion, hydroxyl radicals and hydrogen peroxide in plant cells. The ROS adversely affect different cellular structure and functions. While a good deal of information is available on heavy metal induced oxidative stress and tolerance mechanisms of plants and some green algae, our knowledge about heavy metal induced oxidative stress and tolerance in nitrogen fixing cyanobacteria is limited. Cyanobacteria with enhanced tolerance to heavy metals may find great applications in bioremediation, biosensors, etc. Further, Cyanobacteria share a wide range of genes in common with

plants. Thus, lessons learned from stress responses in cyanobacteria will also greatly facilitate the understanding of how plant cells face environmental challenges.

In this study an effort was made to understand the basic mechanism of copper uptake and accumulation, toxicity, oxidative damages and antioxidant defense systems of nitrogen fixing cyanobacterium *Nostoc muscorum*. Using chlorophyll fluorescence techniques we also tried to understand the mechanism of photoxidative damages caused by Cu^{2+}. We also investigated accumulation of signaling molecule NO and its role in metal toxicity and tolerance in cyanobacteria. Proline is a most investigated molecule in bacteria to higher plants and animals. In this study we studied the accumulation of proline in response to heavy metal stress. Further, we tested role of proline against copper-induced oxidative stress in test cyanobacterium.

Nostoc muscorum showed a logarithmic growth during the first 30 days of inoculation in Chu-10 medium, and subsequently declines the growth. Specific growth rate (μd^{-1}) was 0.121 in the untreated cells. Excess copper affects growth and metabolism of plants including algae and cyanobacteria. The Cu^{2+} (0-10 µM) treatment caused a concentration dependent reduction in specific growth rate, chl. a, carotenoids, phycocyanin and protein content in *Nostoc muscorum*. A 50% inhibition in specific growth rate was observed at 2.5 µM of Cu^{2+} after 96 h exposure. The growth of test organisms was completely arrested at 10 µM Cu^{2+} during 96 h of exposure. The 10 µM Cu^{2+} treatment decreased the chlorophyll-a, carotenoids, phycocyanin and protein content in *N. muscorum* by 72, 59, 80% and 56%, respectively. Toxicity of Cu^{2+} was also proved by the absence of heterocysts in the higher concentrations of metal tested.

The accumulation of copper in *N. muscorum* comprises of two processes, a fast adsorption on the cell surface and slower intracellular accumulation. In this study we used well known Michaelis-Menten enzyme kinetics to quantifiy the process of intracellular uptake. Since Langmuir adsorption isotherm, a well described model for adsorption, essentially described the saturation kinetic as Michaelis-Menten kinetics, we used Michaelis-Menten parameters to describe adsorption of metal ions. We used V_{max} and K_m as parameters of intracellular uptake, and U_{max} and K_d as parameters of adsorption on the cell surface.

N. muscorum showed maximum uptake rate (V_{max}) of Cu^{2+} as 2.528± 0.292 µg mg^{-1} protein $h^{-1}x10^{-3}$. The number of maximum available Cu^{2+} binding sites (U_{max}) on surface of *N. muscorum* was estimated as 10.288±0.509 µg mg^{-1} protein $x10^{-3}$). As process of metal uptake and accumulation is affected by several factors, in this study we investigated the intracellular uptake and adsorption of copper at different pH of the external medium. The uptake of Cu^{2+} was maximum at pH 7.0 and adsorption was maximum at pH 6.6. It is suggested change in pH affects the metal speciation in the external medium and thereby regulated the available free metal ion in the solution. Secondly, pH affects the metal binding transport in the cell membrane that may affect the rate of metal transport.

In nature single metal ion rarely exists. We studied the effect of others cations on the uptake and adsorption of copper by the test cyanobacterium. Presence of other heavy metal ions (Zn^{2+} and Cd^{2+}), monovalent cation (K^+) and divalent cation (Ca^{2+}) in the

external medium generally inhibited both adsorption and uptake of Cu^{2+} by the test cyanobacterium. All the tested cations inhibited Cu^{2+} uptake and adsorption with increasing concentrations. Inhibition of adsorption and uptake of Cu^{2+} by various cations and metal ions did not explicitly display a trend; however, all modes of inhibition, namely, competitive, non-competitive, uncompetitive and mixed type, were observed. Competitive inhibition of Cu^{2+} uptake was caused by Zn^{2+}. Non-competitive inhibition of Cu^{2+} uptake was caused by Cd^{2+} and K^{+}, and mixed type inhibition of Cu^{2+} uptake was caused by Ca^{2+}. For Cu^{2+} adsorption of Zn^{2+} and K^{+} were non-competitive inhibitor while Cd^{2+} and Ca^{2+} showed mixed type of inhibition. It seems that *N. muscorum* has specific transport systems for Cu^{2+}. It was suggested that uptake and adsorption of copper by *N. muscorum* is influenced by the presence of other cations, and are metal specific.

The lipid peroxidation is an indicator of membrane damage by ROS and free radicals. Lipid peroxidation, electrolyte leakage and ROS (H_2O_2, O_2^-) levels were elevated after exposure to copper. Treatment with 10 µM Cu^{2+} for 2 h caused 5.0-, 1.5-, 2.0- and 4.0- fold increased in MDA, EL, H_2O_2 and O_2^- respectively, while 2.5 µM treatment for 5 h caused 3.8-, 1.3- and 3.6-fold increased in MDA, H_2O_2 and O_2^- respectively.

We investigated the activities of key antioxidant enzymes SOD, CAT, APX and GR in *N. muscorum* exposed to copper stress. The activities of all the studied antioxidative enzymes were significantly increased under Cu^{2+} stress. A 1.6-fold increased activity of SOD was observed in test cyanobacterium after exposure to 10.0

µM. CAT, APX and GR activities increased in time dependent manner up to 5 h of studied. Two isoforms of SOD i.e., Mn-SOD and Fe-SOD were detected from copper treated cells. We found that Mn-SOD was playing a key role against Cu^{2+} stress. We concluded that stimulation of antioxidant system is metal specific. Further, we found that at Cu^{2+} concentrations above 5.0 µM the antioxidative defense system in the test cyanobacterium is suppressed resulting into increased toxicity and oxidative damages as was evident from increased lipid peroxidation, electrolyte leakage and decreased pigment, carotenoid and protein content.

Protein profiling of the test organism under Cu^{2+} stress showed many newly synthesized proteins as well as inhibition of some proteins. 10 µM Cu^{2+} treatment increased GSH (reduced) and NP-SH by 2.4-fold and 1.7-fold, respectively, when compared with control.

We found that due to copper stress *N. muscorum* accumulates intracellular proline several folds higher than control. Further it was examined if proline has any role and confers the protection against copper induced oxidative stress. Pretreatment of *N. muscorum* with proline counteracted Cu-induced lipid peroxidation and inhibition of SOD and CAT activity suggesting protective role of proline under Cu^{2+} stress. Because proline pretreatement did not altered the copper uptake, the protective role of proline cannot be credited to reduced metal uptake due to proline. However, intracellular complexation of copper with proline cannot be ruled out.

Cu^{2+} exposure led to decrease of the activity of photosynthetic O_2 evolution and increase in respiratory O_2 consumption. The above results suggested that copper interfere with process of photosynthesis. The effects of copper (Cu^{2+}) on chlorophyll fluorescence quenching parameters and electron transport rates (ETR) driven by PS I and PS II were measured simultaneously by using novel Dual PAM-100 system in a N_2-fixing cyanobacterium *Nostoc muscorum*. The maximal quantum efficiency (F_v/F_m) decreased at 7.5 and 10.0 µM Cu^{2+} suggesting a large damage to PS II reaction centers. Increased fluorescence in PS I was observed with increasing concentrations of copper indicating a transition from linear electron flow (LEF) towards the cyclic electron flow (CEF) in order to prevent PS II from excess excitation energy or photoinhibition. Chlorophyll fluorescence quenching parameters of PS II (Y(II), Y(NO) and Y(NPQ)) were not altered at lower concentrations of copper, however, higher concentrations of Cu^{2+} decreased Y(II) and increased Y(NO) (non-regulated non-photochemical quenching parameter) suggesting that copper increased the fraction of 'closed' and non-reducing PS II centers. At higher Cu^{2+} levels, the mechanism of non-photochemical energy dissipation, Y(NPQ) did not work as is indicated by unaffected NPQ. An elevated Y(NA) and concomitant decreased Y(ND) at higher concentrations of copper suggested about the limitation of 'acceptor' side of PS I. An increased Y(ND) and decreased Y(NA) at lower concentrations (> 5.0 µM) of copper suggested about 'donor' side limitation in PS I. We have found that 10.0 µM Cu^{2+} increased the ETR_{max}(I) and I*k*(I) indicating about a transition from LEF (linear electron transport flow) to of CEF (cyclic electron flow) and protection of PS I reaction center from damage due to acceptor side limitation caused by

higher Cu^{2+} concentrations. The ETR_{max}(II) was more sensitive to Cu^{2+} than ETR_{max}(I). In conclusion we have found that Cu^{2+}, in a concentration dependent manner, acted at multiple target sites of PS I and PS II resulting in to photo-inhibitory effects and overall reduction in photosynthetic efficiency. Results showed protective role of proline against Cu^{2+} stress on photosynthetic apparatus.

NO was not able to mitigate or modulate the F_v/F_m as we observed in case of proline. NO has negative effects on ETR I and ETR II as determined in copper treated culture.

In the present study, we evaluated Cu^{2+} induced NO formation and its relationship to ROS and antioxidant regulation in *N. muscorum*. A rapid induction intracellular NO as well as NO efflux has been observed. Nitric oxide is a bioactive molecule involved in many biological events that have been reported as prooxidant as well as antioxidant in plants. In this study we observed reduced levels of ROS and MDA content due to NO. It suggested about the antioxidative role of NO in cyanobacterium.

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List of publications

Devi, YM and Mehta SK (2014). Changes in antioxidative enzymes of cyanobacterium *Nostoc muscorum* under copper (Cu^{2+}) stress. Science vision 14(4), 207-214.

Devi, YM and Mehta SK (2014). Effect of Copper (Cu^{2+}) on PS II and PS I Photochemical Activities and Electron Transport as Studied by Chlorophyll Fluorescence in Cyanobacterium *Nostoc muscorum (L.).* Science and Technology Journal 2(2), 81-94.

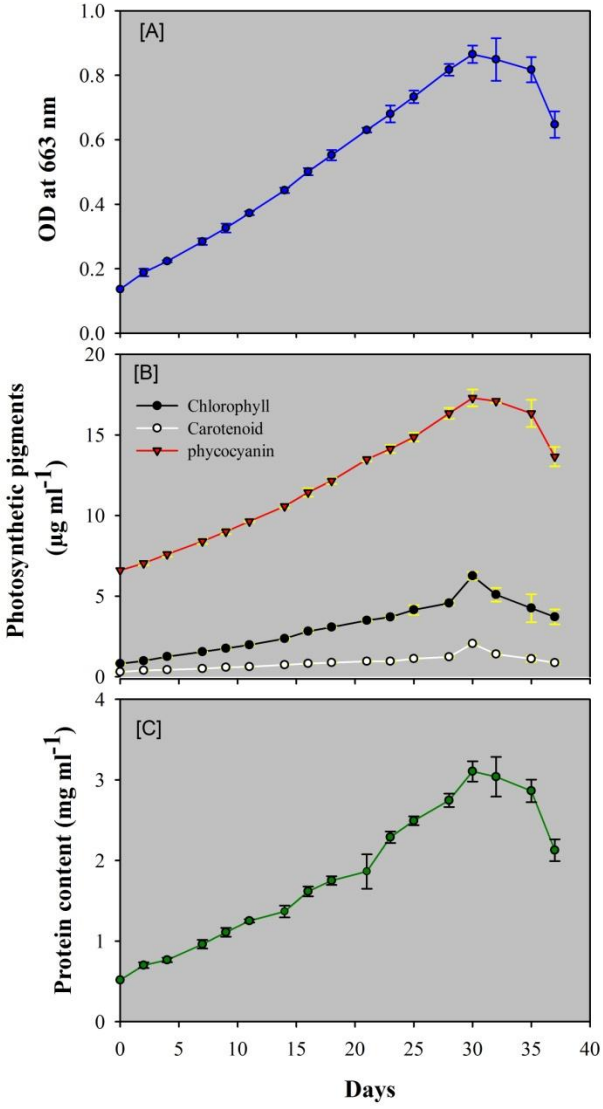


Fig. 3.1: Characterization of *N.muscorum* for growth behaviour [A], pigments [B] and protein [C] contents.

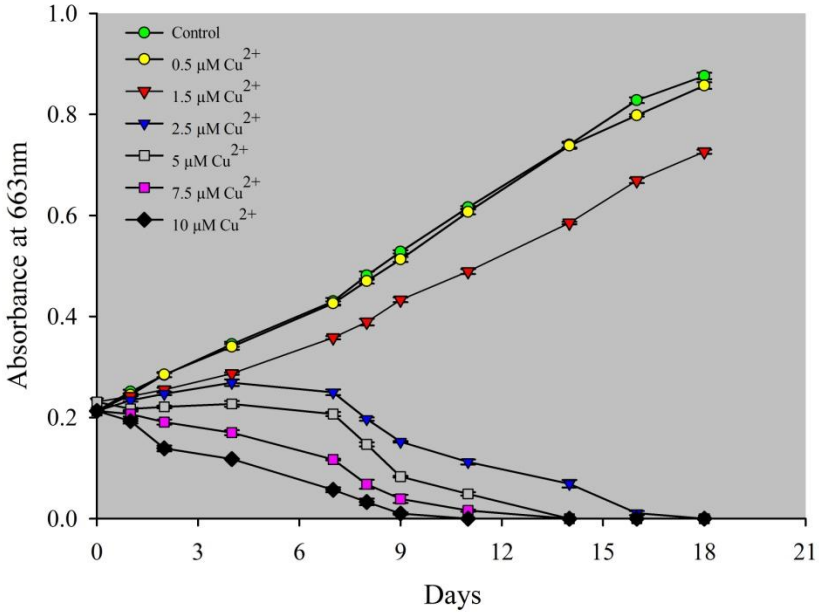


Fig. 3.2: Effect of increasing concentrations of Cu^{2+} on growth of *N. muscorum*. The growth behavior was studied in Chu-10 medium (without N) at pH 7.5. The vaules are mean of three replicates. Vertical bars show ±SE.

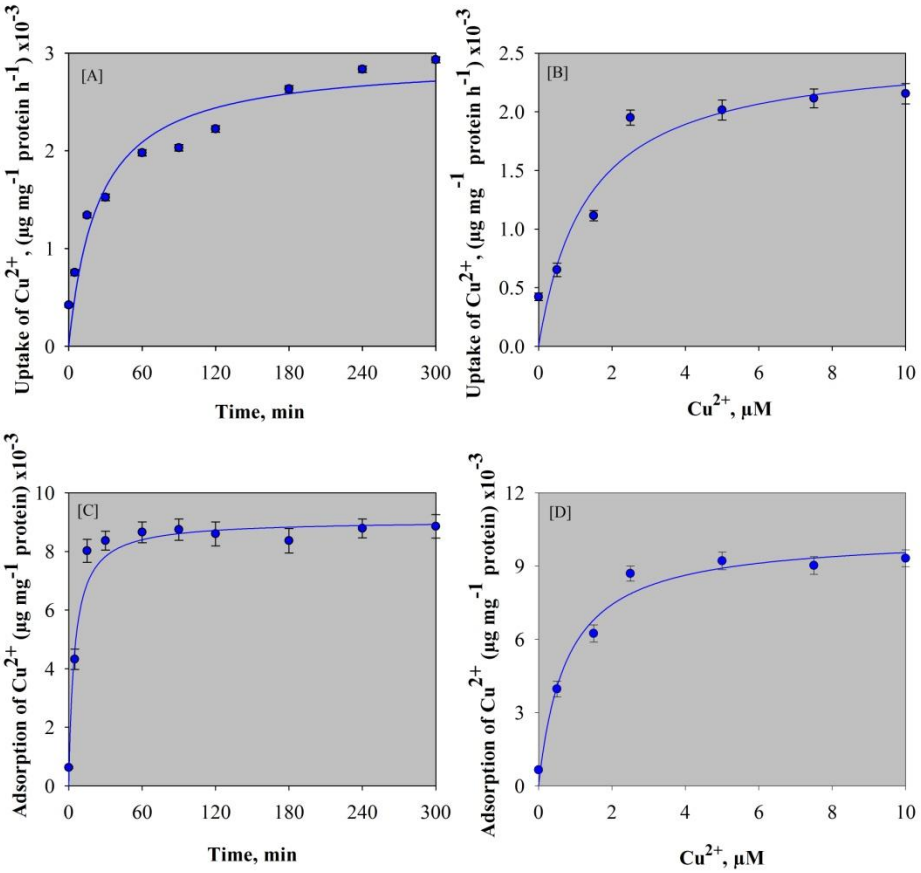


Fig. 3.3: Intracellular uptake and adsorption of copper by *N. muscorum*. [A, C] Time-course study with 2.5 µM Cu^{2+}. [B] uptake and [D] adsorption at varying concentrations of Cu^{2+}. The treatment time was 1 h. The values are mean of three replicates. Vertical bars show ± SE.

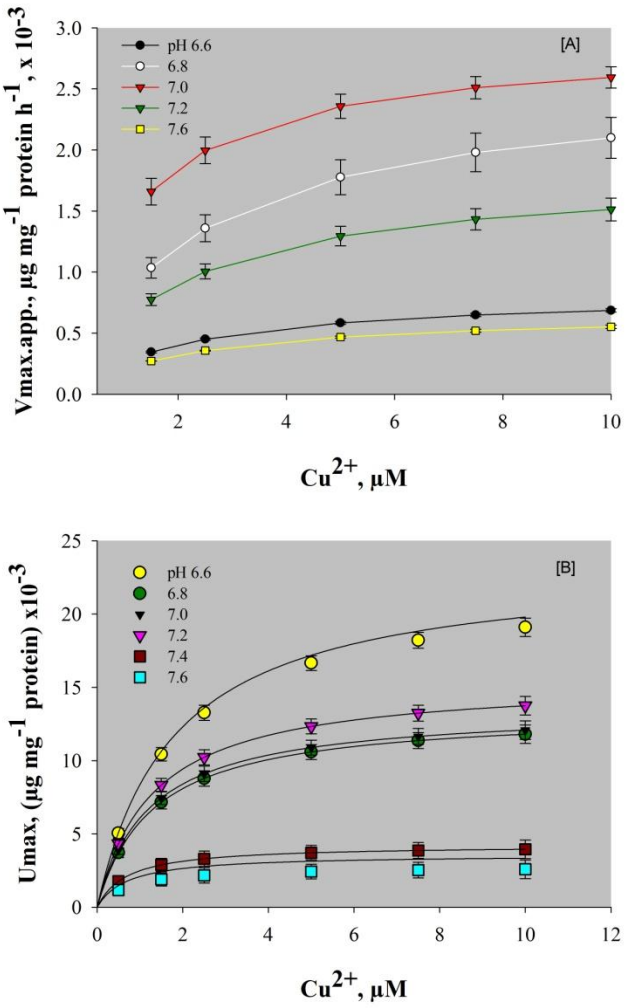


Fig. 3.4: [A] Intracellular uptake, [B] Adsorption of copper by *N. muscorum* at different pH in the medium. The concentration of Cu^{2+} was 2.5 µM and treatment time was 1 h.

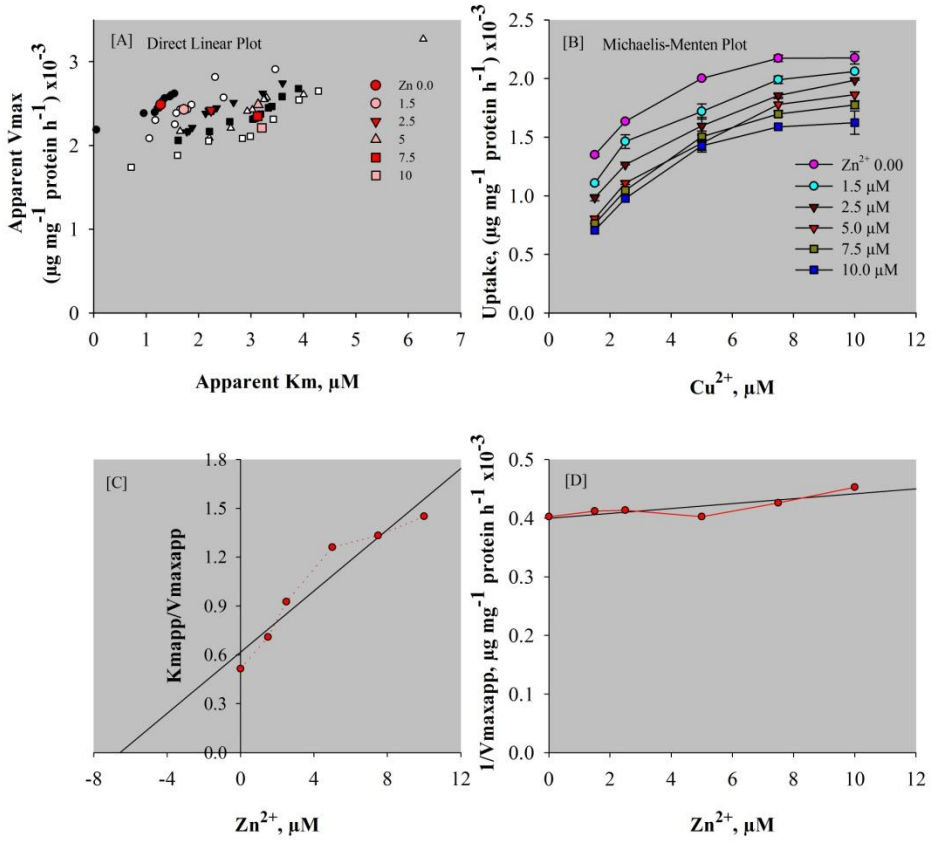


Fig. 3.5: Effect of Zn^{2+} on Michaelis-Menten kinetics of intracellular uptake of copper by *N. muscorum*. [A] The direct linear plot showing horizontal median tranjectory and intersections suggesting a competitive inhibition. [B] The Michaelis-Menten kinetics showing inhibition of Cu^{2+} uptake with increasing concentrations of Zn^{2+} in the medium. [C, D] The secondary plot showing competitive inhibition of Cu^{2+} uptake by the Zn^{2+}. Treatment time with Cu^{2+} and Zn^{2+} was 1 h.

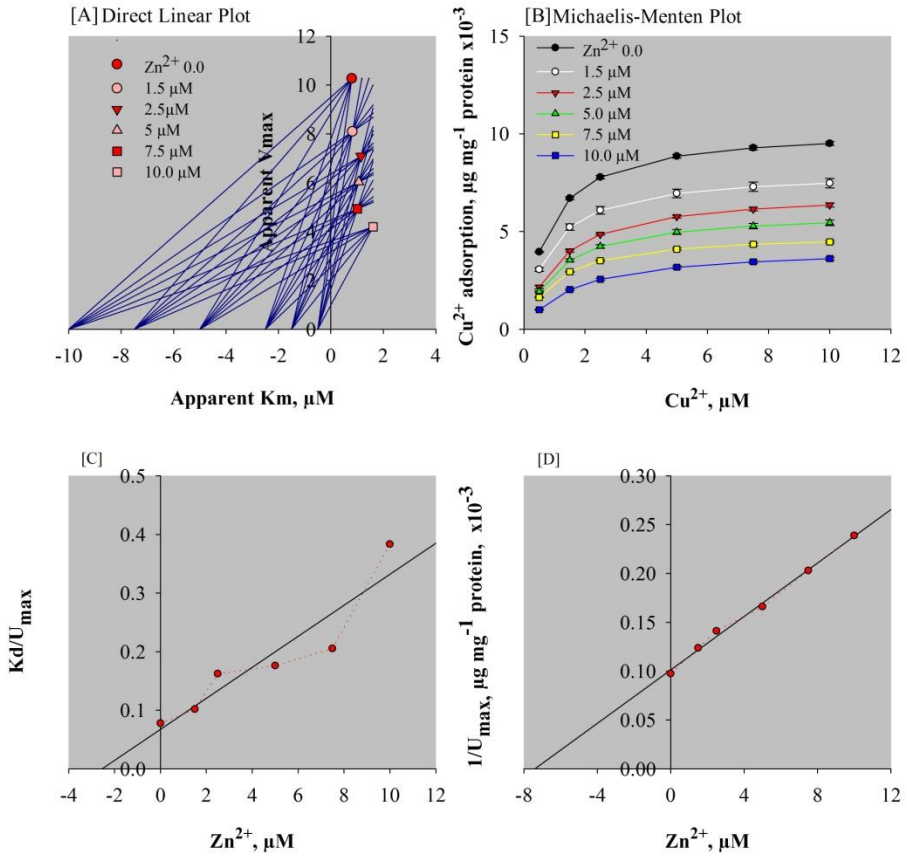


Fig. 3.6: Effect of Zn^{2+} on adsorption of copper by *N. muscorum*. [A] Direct linear plot, [B] Michaelis-Menten plot, and [C, D] Secondary plot showing inhibition of copper adsorption by Zn^{2+}.

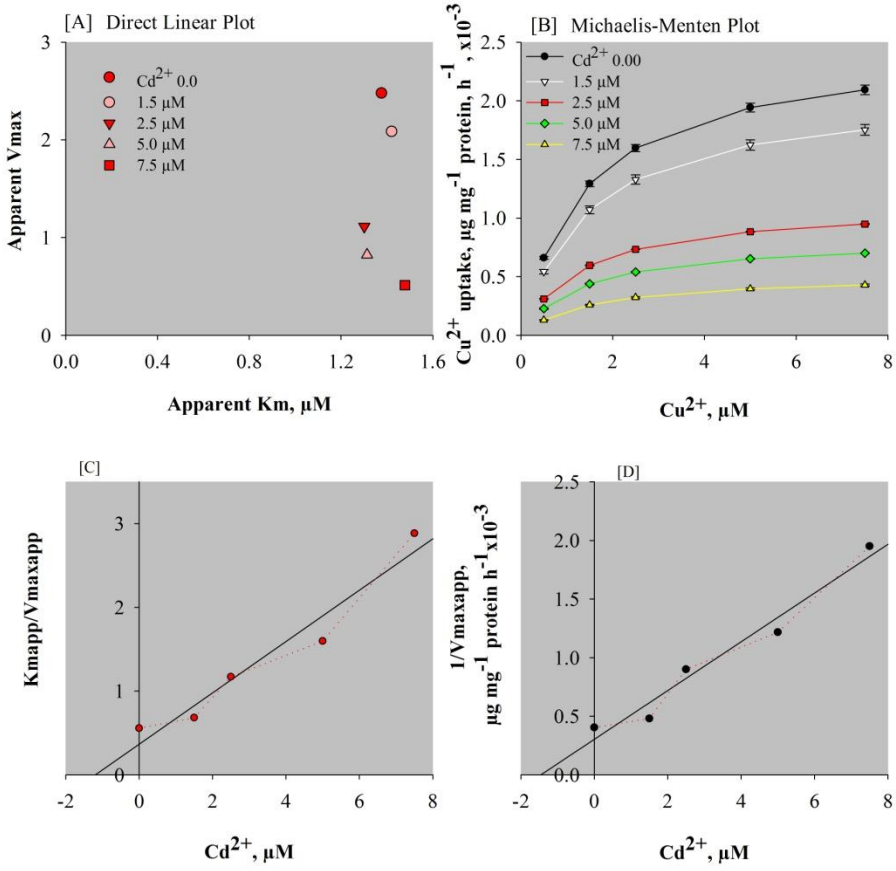


Fig. 3.7: The effect of Cd^{2+} on intracellular uptake of copper by *N. muscorum*. [A] Direct plot, [B] The Michaelis-Menten plot and [C,D] Secondary plots showing non-competitive inhibition of copper uptake by the Cd^{2+}.The values are mean of three replicates.

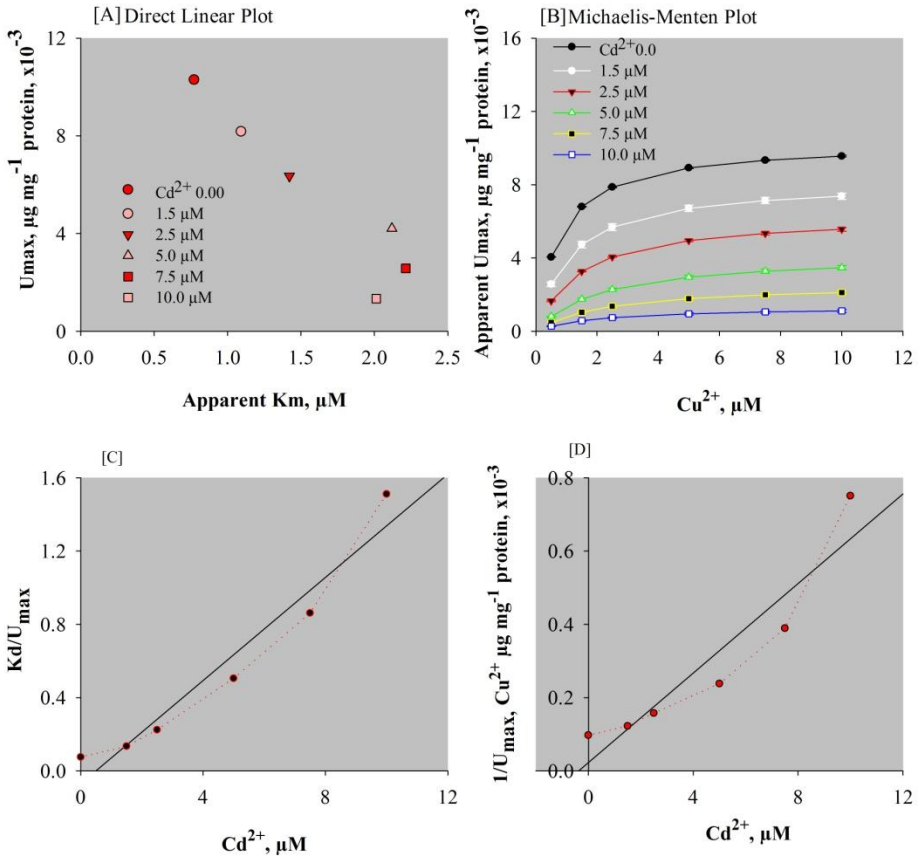


Fig. 3.8: Adsorption of copper in presence of of Cd^{2+} in the external solution. [A] Direct linear plot, [B] Michaelis-Menten plot and [C, D] Secondary plot showing mixed inhibition by the Cd^{2+} in *N. muscorum*. Values are mean of three replicates.

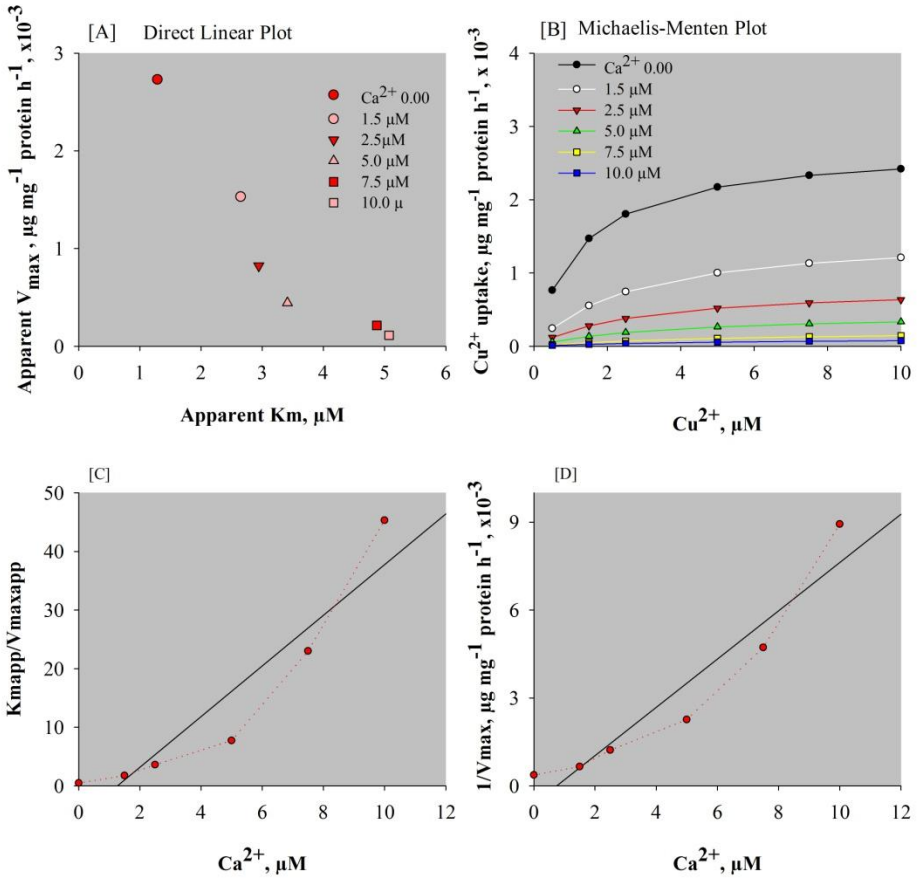


Fig. 3.9: Effect of increasing concentrations of Ca^{2+} on uptake of copper by *N. muscorum*. [A] Direct linear plot, [B] Michaelis-Menten plot and [C, D] Secondary plots showing mixed inhibition. The values are means of three replicates.

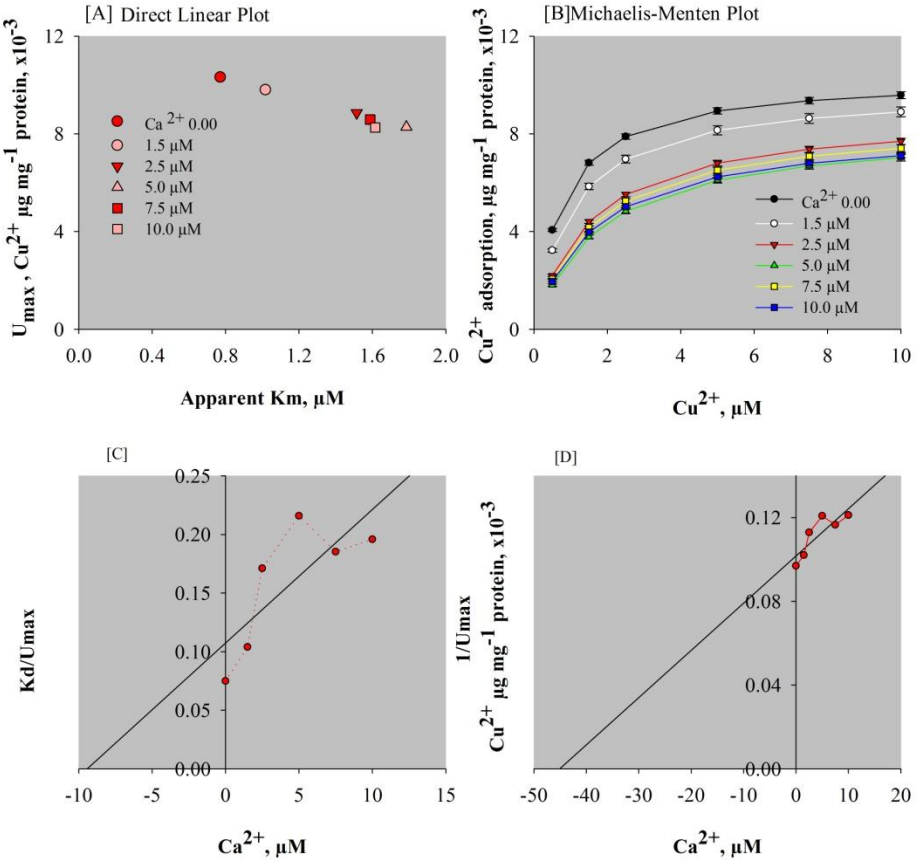


Fig.3.10: Adsorption of copper in presence of various concentrations of Ca^{2+} in the external medium. [A] Direct linear plot drawn using Michaelis-Menten kinetics. [B] Copper adsorption data fitted to Michaelis-Menten equation. [C, D] Derived secondary plots showing effect of Ca^{2+} on copper adsorption.

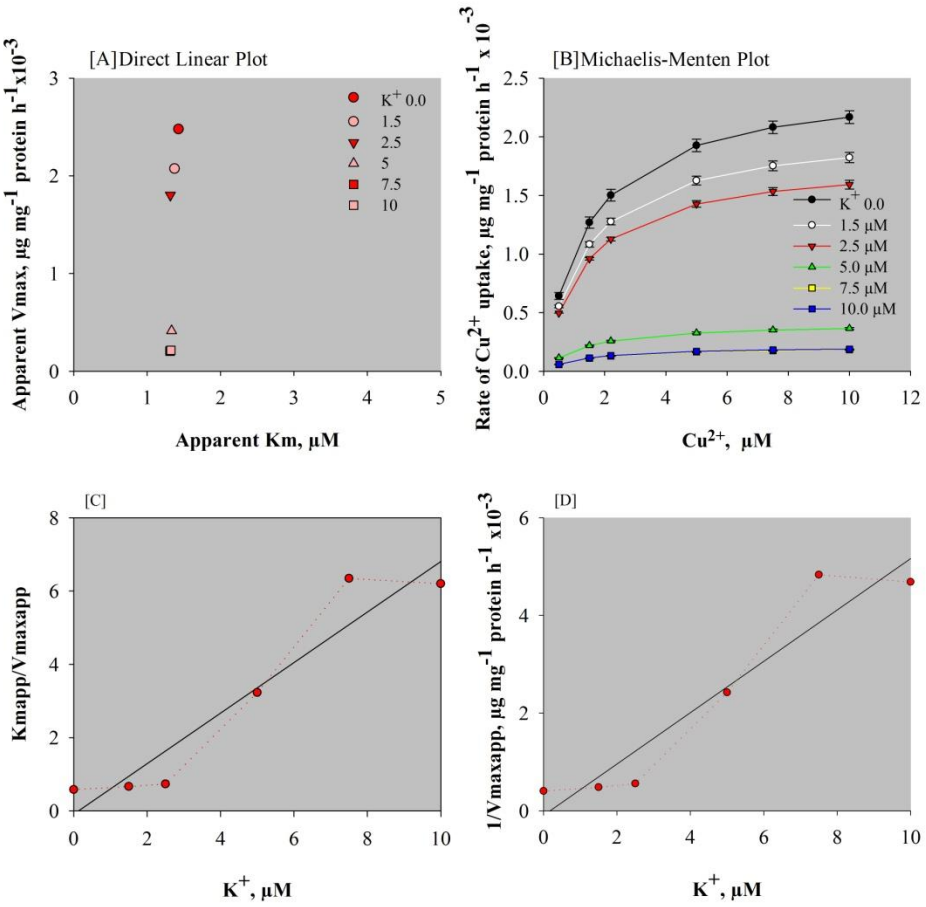


Fig. 3.11: Effect of K^+ on intracelluar uptake of copper by *N. muscorum*. [A] Direct linear plot showing a non-competitive inhibition. [B] The Michaelis-Menten plot and [C, D] Secondary plot showing inhibition of copper by the K^+.

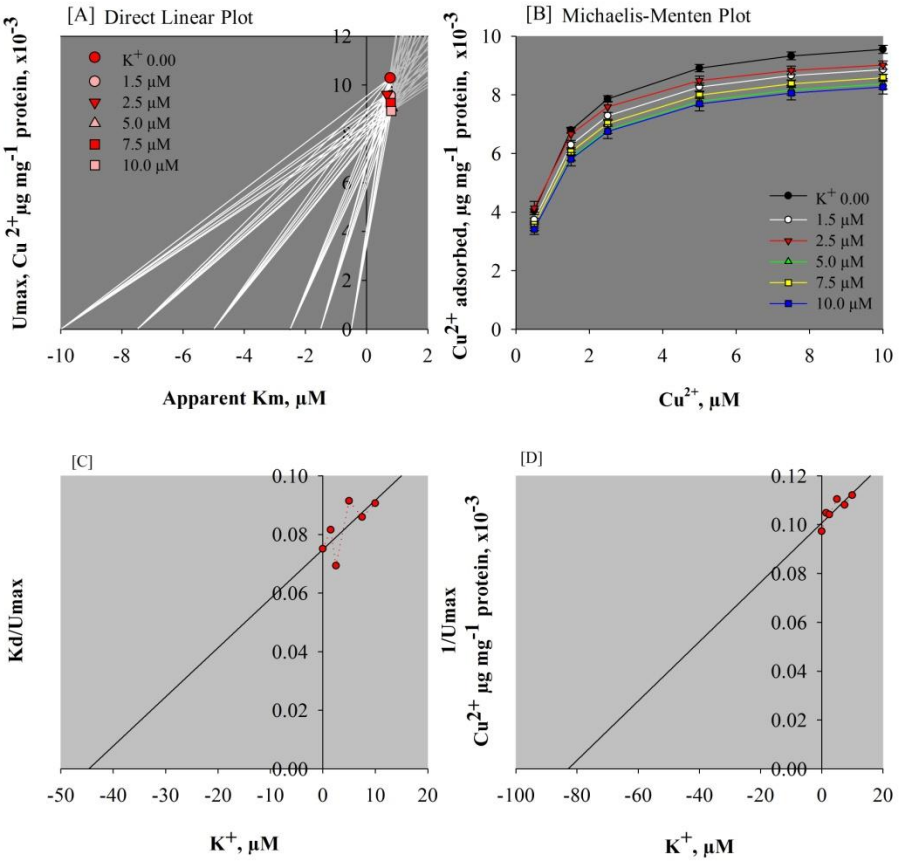


Fig. 3.12: Adsorption of copper at various concentrations of K^+ in the medium by *N. muscorum*. [A] Direct linear plot, [B] The Michaelis-Menten plot and [C, D] Secondary plot showing inhibition of copper adsorption by the effect of K^+.

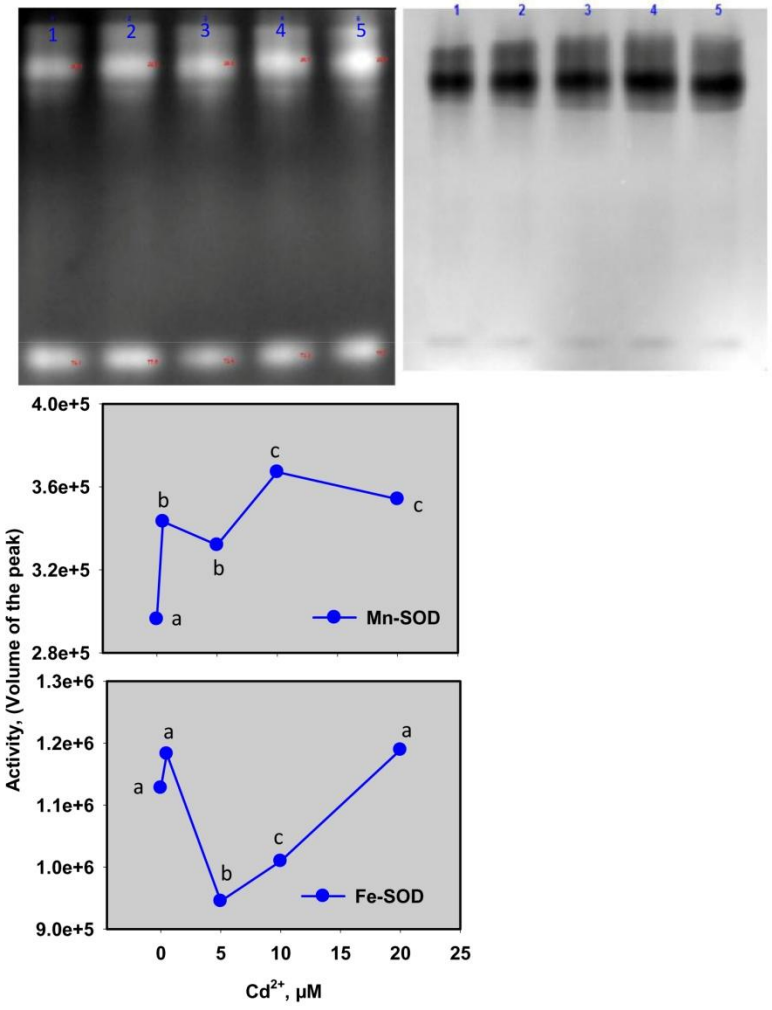
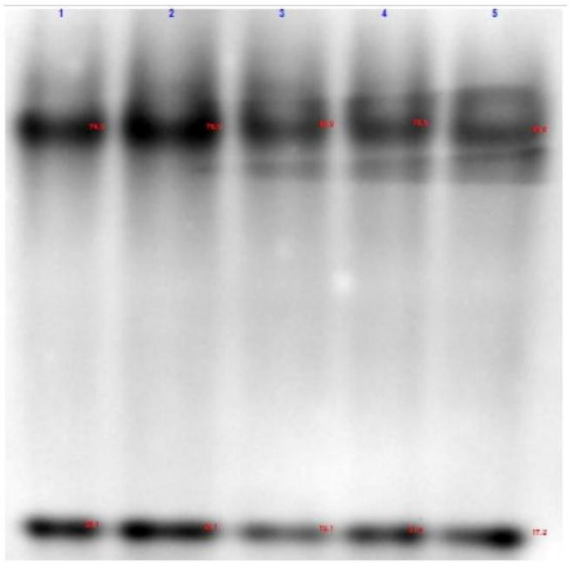


Plate 4.1: Activities of SOD isoforms in Cd^{2+}- treated *N. muscorum*. Upper band : Mn-SOD; lower band : Fe-SOD. In the figure data points indicated by the same letter are not significantly different.



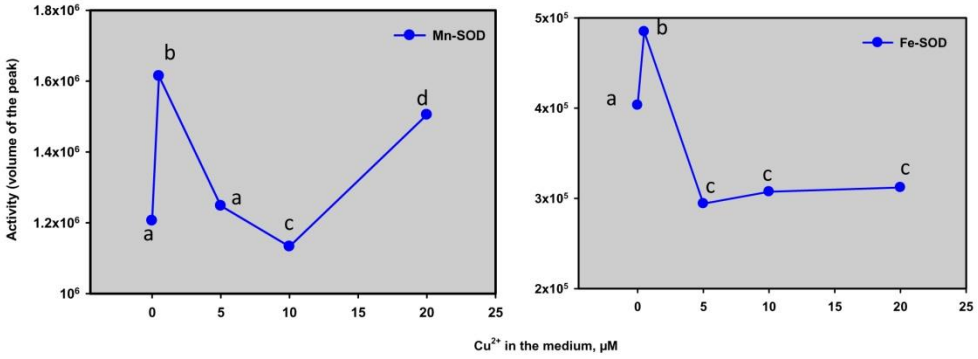
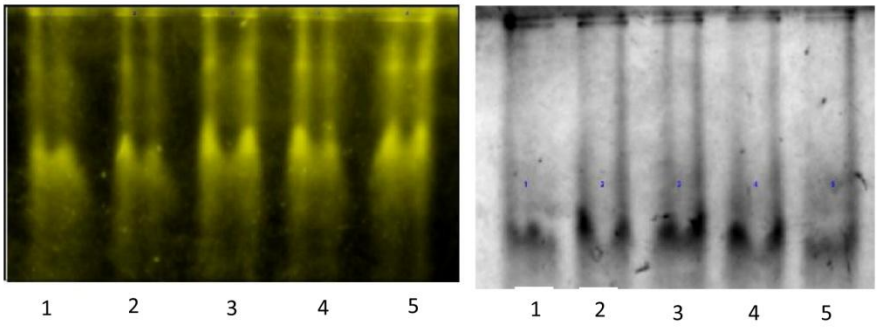


Plate 4.2: In-gel assay of isoforms of SOD in Cu-treated N. muscorum. The data points are mean of three independent estimations. Data points marked with the same letter are not significantly different (P<0.50). [Lane 1. Control; 2.1.5 µM Cu; 3. 5.0 µM Cu; 4. 10.0 µM Cu; 5. 20.0 µM Cu]



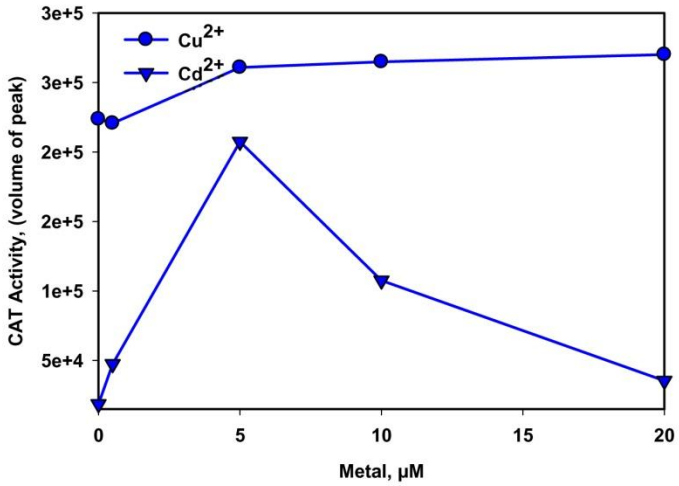


Plate 4.3: In gel-assay of catalase activity in response to copper [A], and cadmium [B]. [C] Relative activity of CAT. [Lane 1. Control; 2. 1.5 µM Cu^{2+} or Cd^{2+}; 3. 5.0 µM Cu^{2+} or Cd^{2+}; 4. 10.0 µM Cu^{2+} or Cd^{2+}; 5. 20 µM Cu^{2+} or Cd^{2+}]

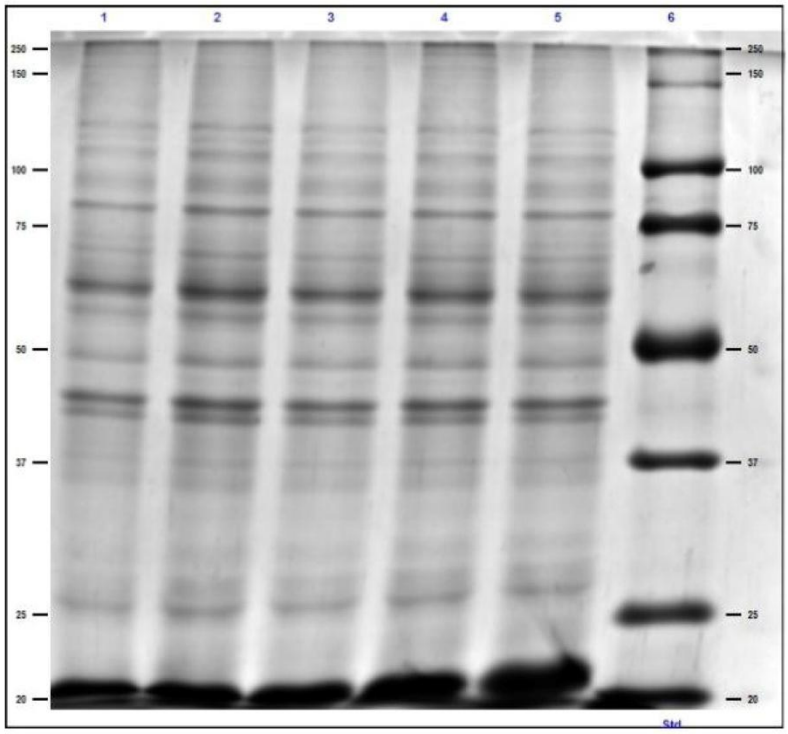


Plate 4.4: Protein profile of *N. muscorum* in response to different concentrations of Cu^{2+} in medium.

[1. Control; 2. 1.5; 3.5.0; 4.10.0; 5. 20.0 µM Cu^{2+}; 6. Protein Mol. Wt. ladder]

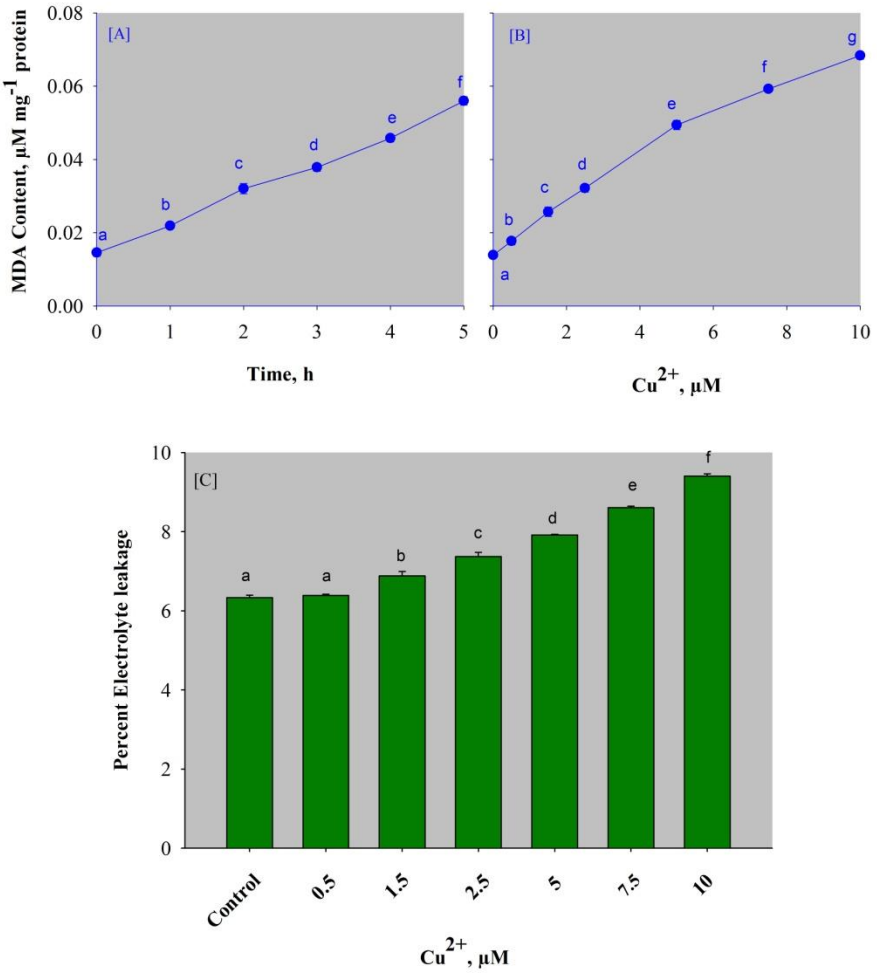


Fig. 4.1: Effect of Cu^{2+} on MDA content and electrolyte leakage in *Nostoc muscorum*. [A] Time-course of lipid peroxidation, [B] Lipid peroxidatiion at varying concentrations of copper in medium, [C] Electrolyte leakage. Vertical bars show standard error of the mean (n=3). Different letters indicate significant difference ($P < 0.05$).

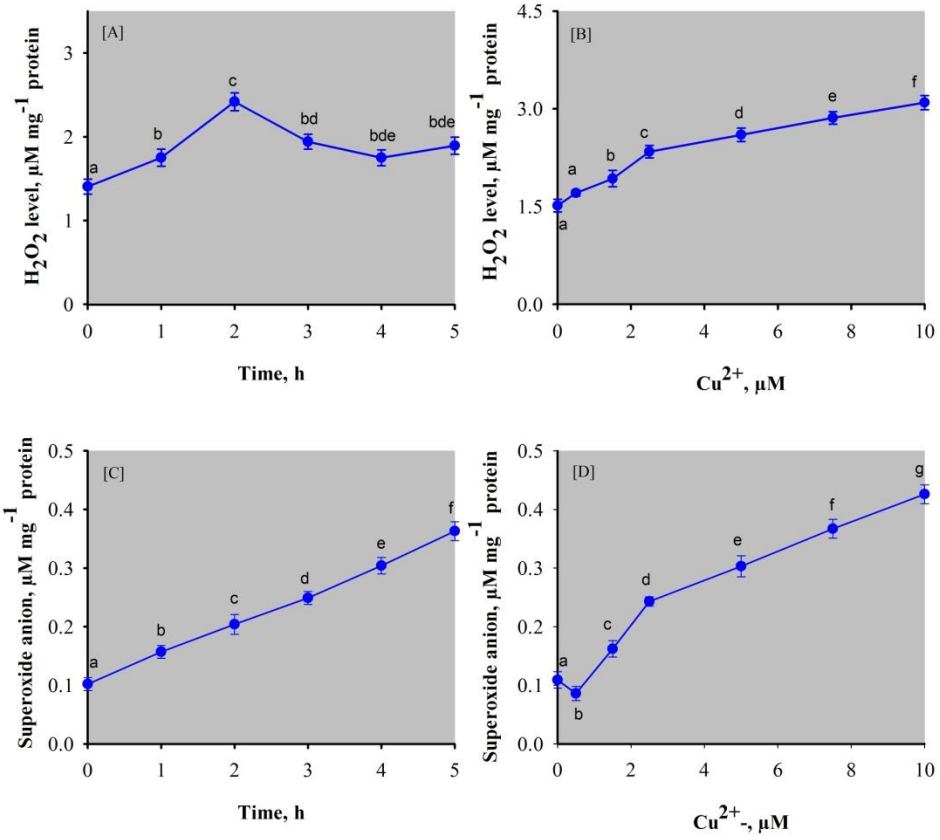


Fig. 4.2: Effect of Cu^{2+} on H_2O_2 level [A, B] and O_2^- generation [C,D] in *N. muscorum*. [A, C] Time-course study, [B,D] Effect at different concentrations of Cu^{2+} on H_2O_2 levels. and O_2^- generation. Vertical bars represent standard error of the mean (n=3).

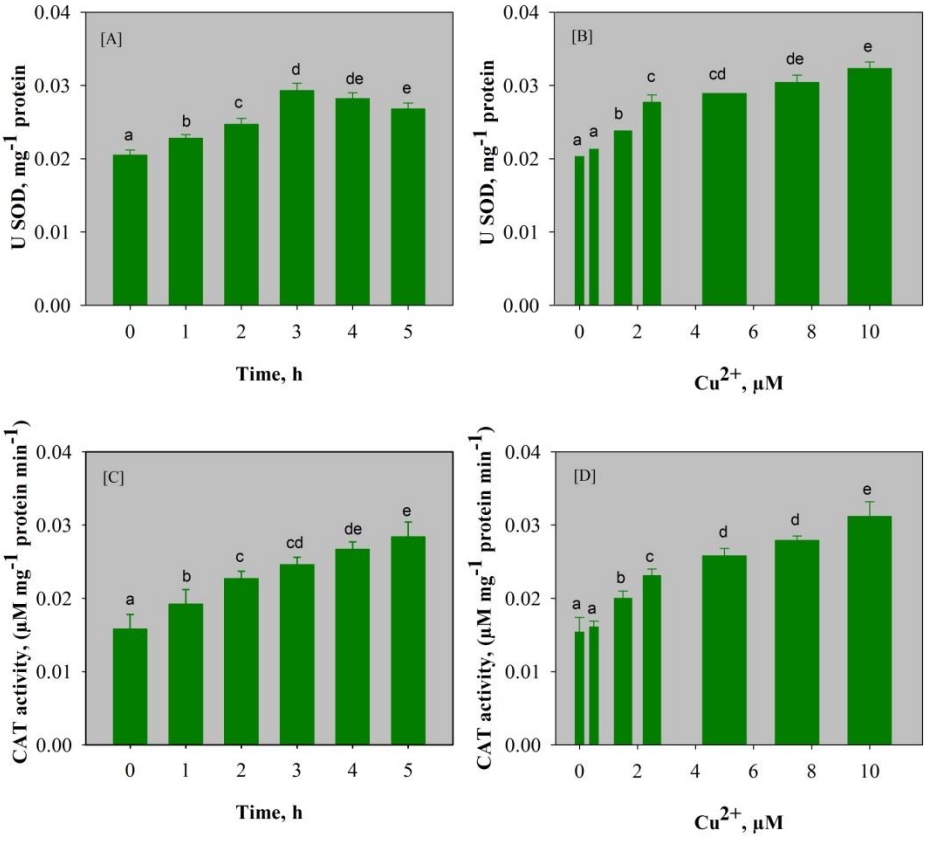


Fig. 4.3: Effect of Cu^{2+} on activities of superoxide dismutase [A, B] and Catalase [C, D]. [A, C] Time-course study and [B, D] At different concentrations of copper in medium. Vertical bars represent standard error of three means (n=3). Bars having different letters are significantly different (P<0.05).

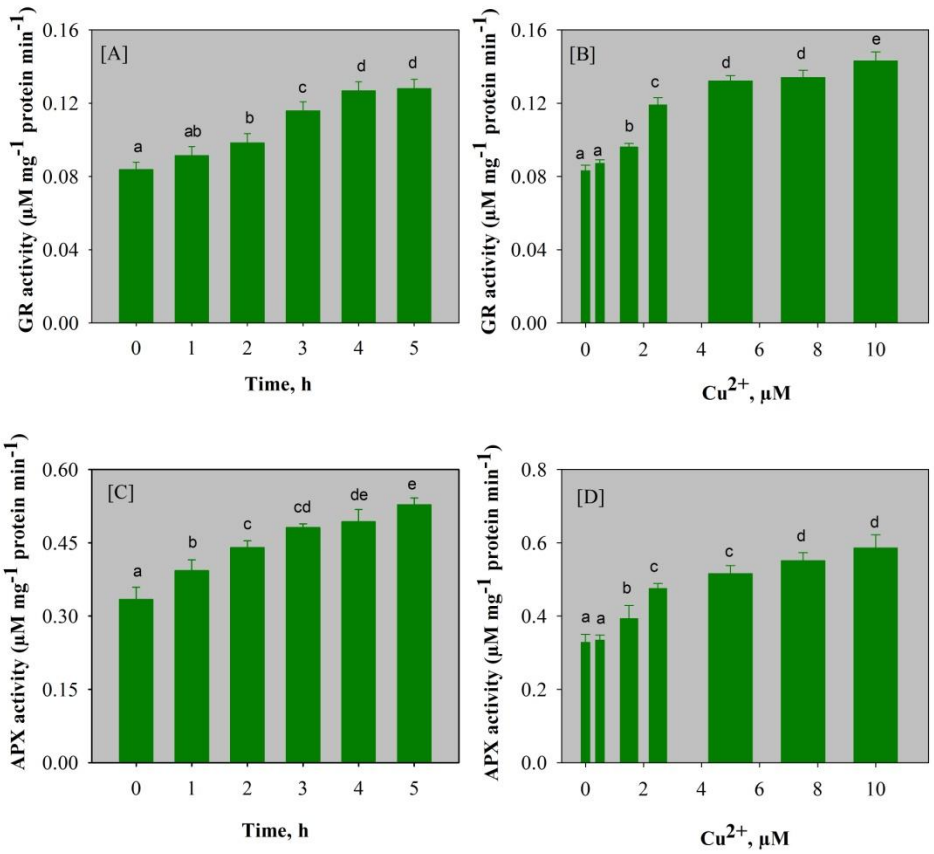


Fig. 4.4: The GR [A, B] and APX [C, D] activities in *N. muscorum* under copper stress. [A, C] Time-course, and [B,D] At different concentrations of copper in the medium. Values are mean of three replicates. Bars with same letter are not significantly different (P<0.05).

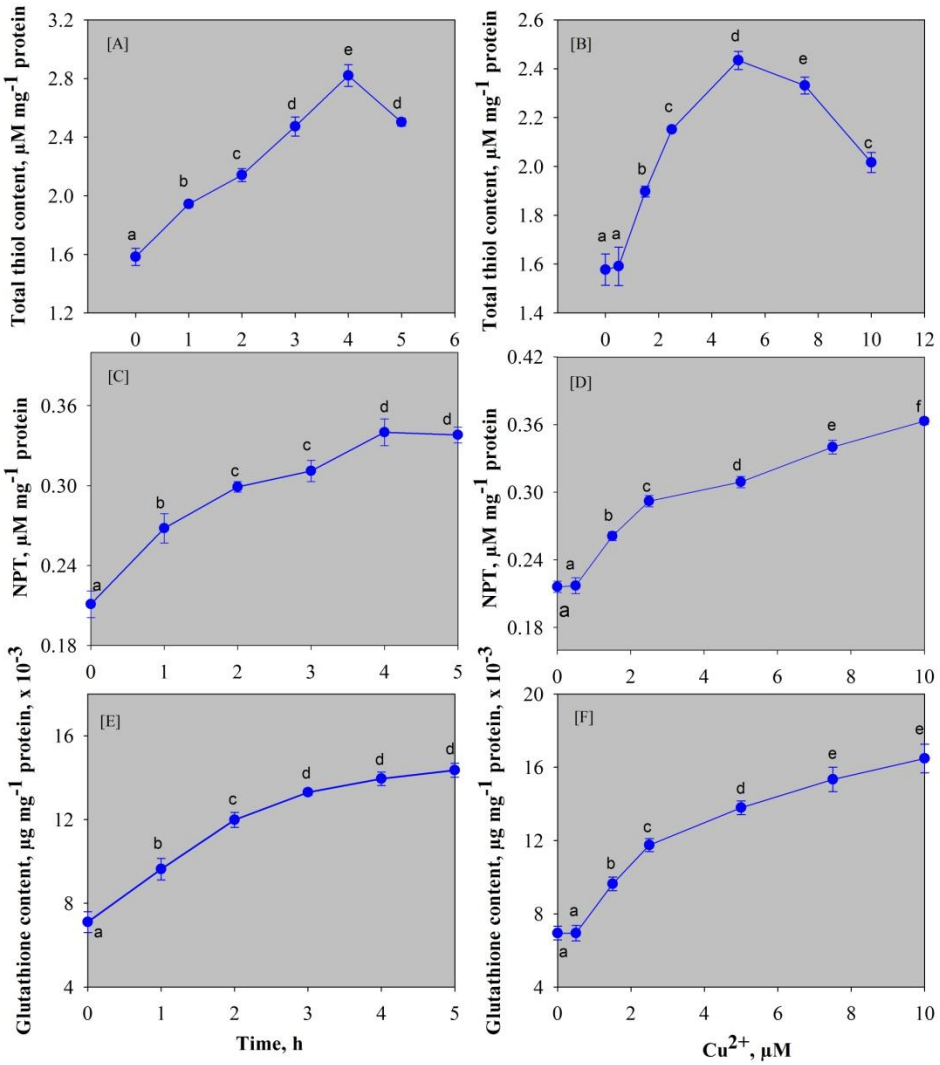


Fig. 4.5: Effect of Cu^{2+} on total thiol [A, B], non-protein thiol (NPT) [C, D] and Glutathione [E, F] content in *N. muscorum*. [A, C, E] Time-course and [B, D, F] At varying concentrations of Cu^{2+} in medium.Vertical bars represent standard error of the mean (n=3). Data points indicated by different letters are significantly different (P< 0.05).

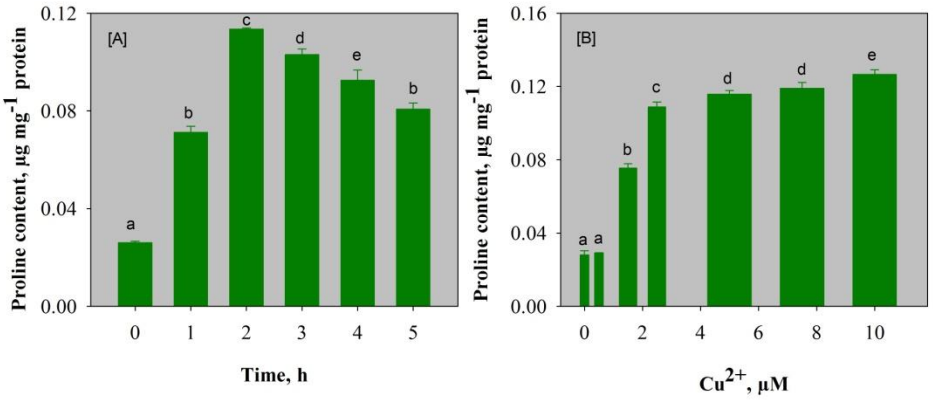


Fig. 4.6: Effect of Cu^{2+} on proline accumulation in *N. muscorum*. [A] Time-course, [B] Proline accumulation at different concentrations of copper. Vertical bars represent the SE (*n*=3).

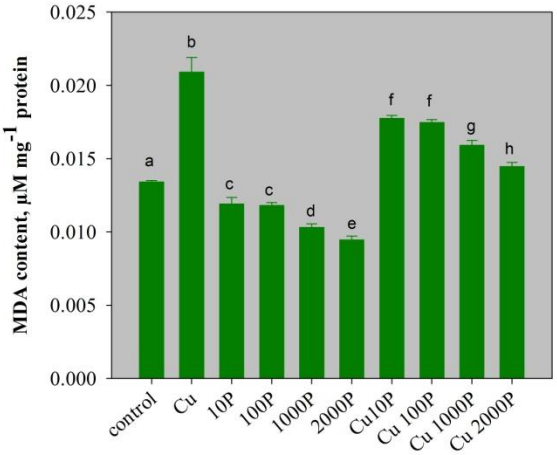


Fig.4.7: Effect of proline pretreatment (1 h) on Cu-induced lipid peroxidation (MDA content) in *N. muscorum*. Values are the mean of three replicates. Error bars represent SE with *n*=3. Cu = Cu^{2+} 2.5 µM, P = proline in µM.

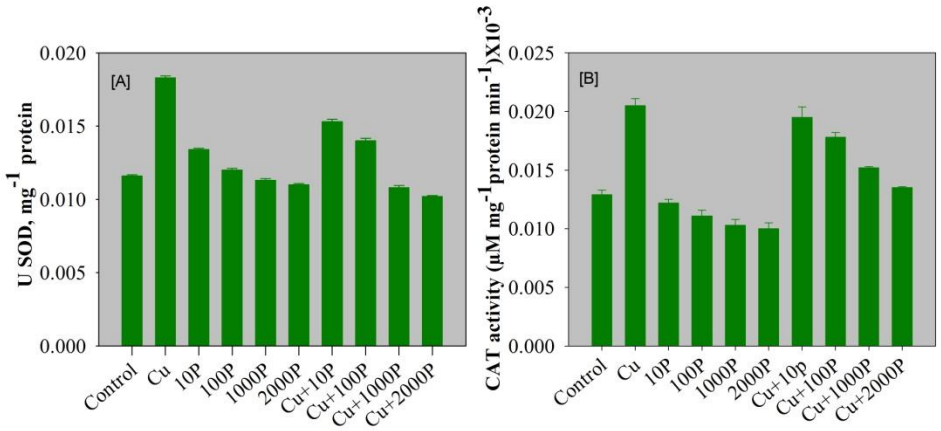


Fig. 4.8: Effect of proline pretreatment on Cu-induced activities of SOD [A] and CAT [B]. Proline pretreatment was given for 1 h. Cu^{2+} concentration was 2.5 µM. Enzyme activity was determined after 2 h of Cu^{2+} treatment. P = proline in µM.

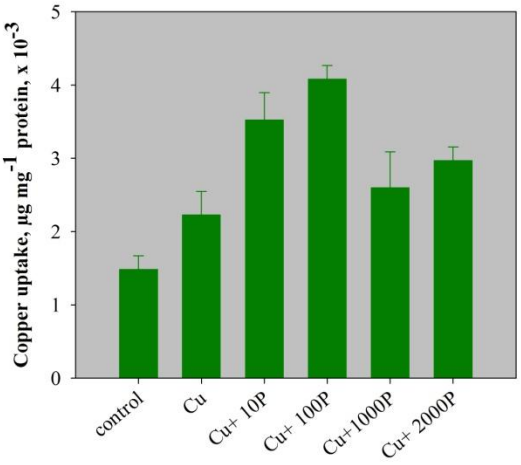


Fig. 4.9: Effect of proline pretreatment (10, 100, 1000 and 2000 µM proline; 1 h) on intracellular copper uptake in *N. muscorum* exposed to Cu^{2+} (2.5 µM) for 1 h.

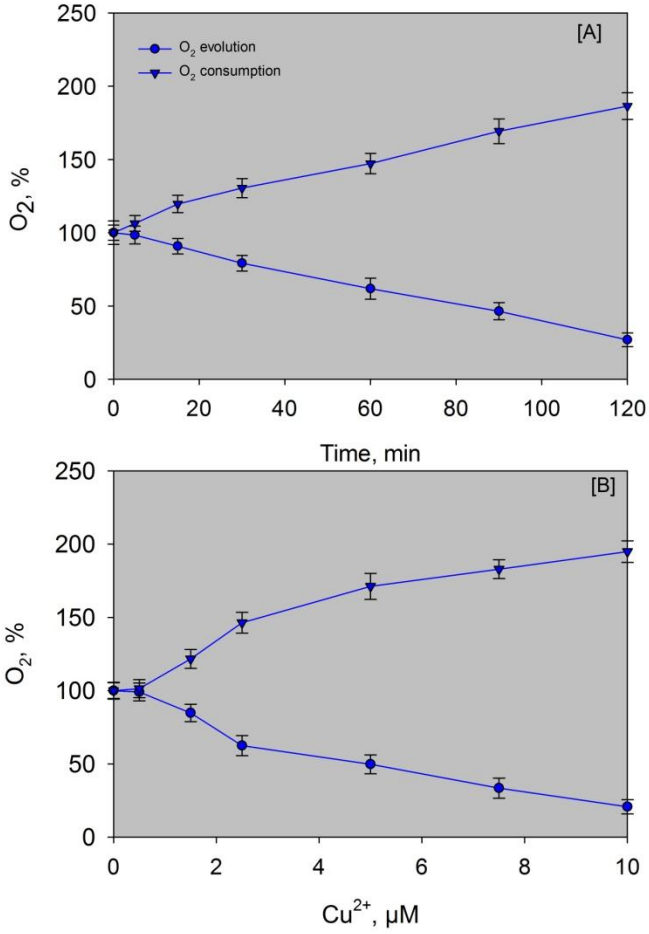


Fig. 5.1: O_2 evolution and consumption in response to copper stress. (A) Time-course study (B) O_2 evolution and consumption at varying concentrations of copper in external medium.

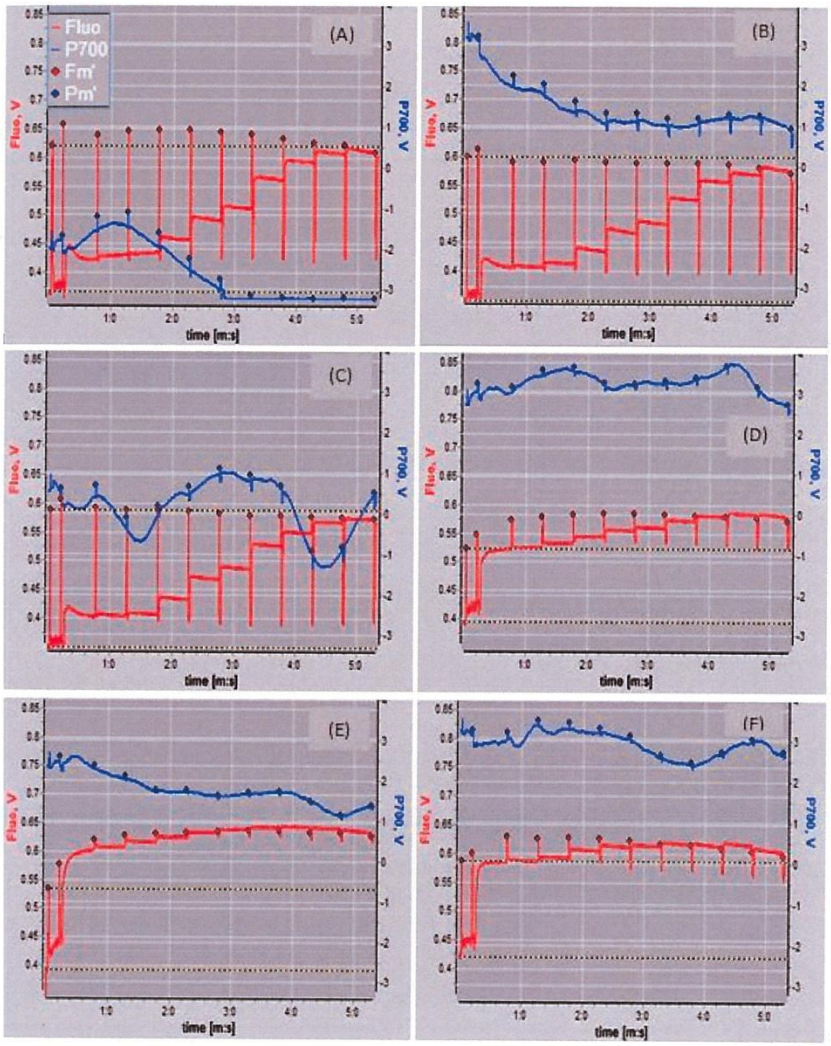


Fig. 5.2: The effect of increasing concentrations of Cu^{2+} in external medium on relative fluorescence yields of PS I & PS II in *N. muscorum.* Fluorescence recording was done after 15 min dark incubation of culture at room temperature. (A) Control, (B) 1.5 µM Cu^{2+}, (C) 2.5 µM Cu^{2+}, (D) 5.0 µM Cu^{2+}, (E) 7.5 µM Cu^{2+}, (F) 10.0 µM Cu^{2+}.

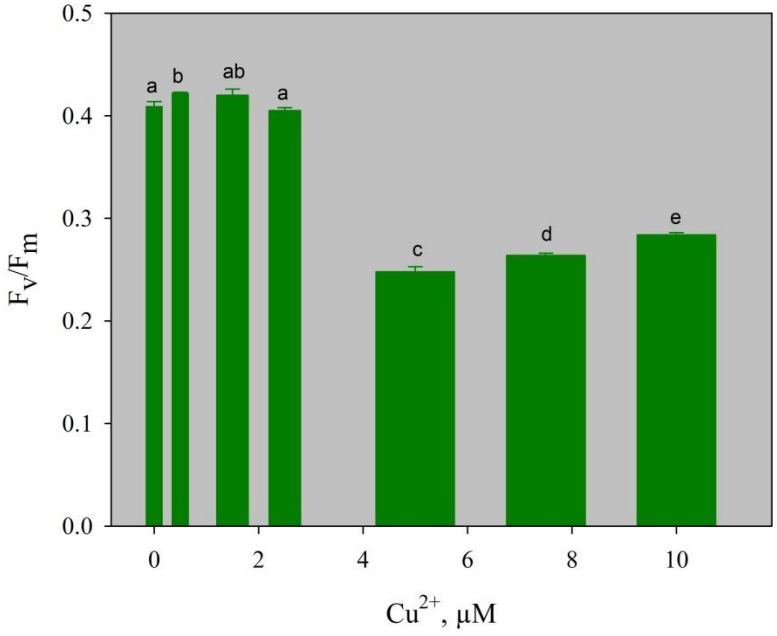


Fig. 5.3: Effect of increasing concentrations of copper on maximum PSII quantum yield (Fv/Fm) in *N. muscorum*. The values are mean of three independent determinations.

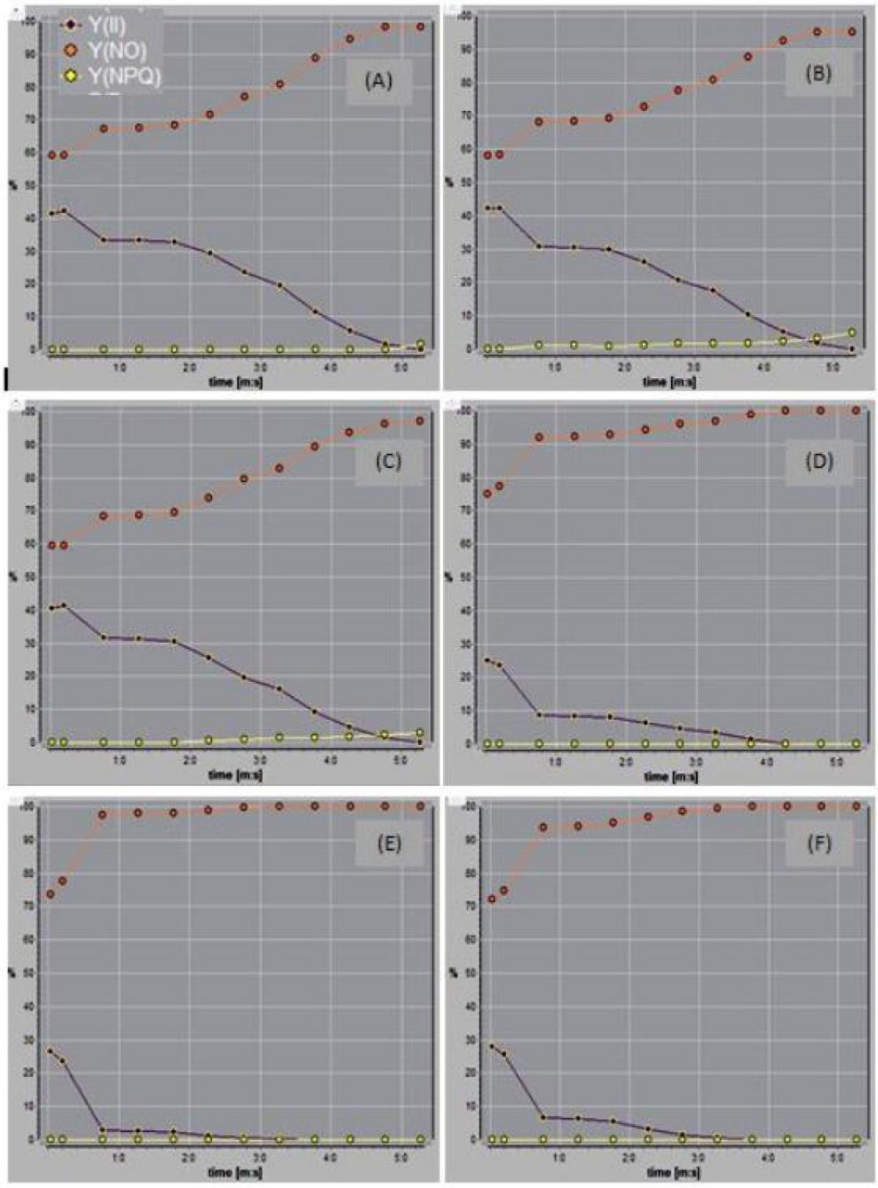


Fig. 5.4: Fluorescence quenching parameters Y(II), *Y(NO)* and *Y(NPQ)* of PS II of *N. muscorum* exposed to various concentrations of Cu^{2+} for 1 h. (A) Control, (B) 1.5 µM Cu^{2+}, (C) 2.5 µM Cu^{2+}, (D) 5.0 µM Cu^{2+}, (E) 7.5 µM Cu^{2+}, (F) 10.0 µM Cu^{2+}.

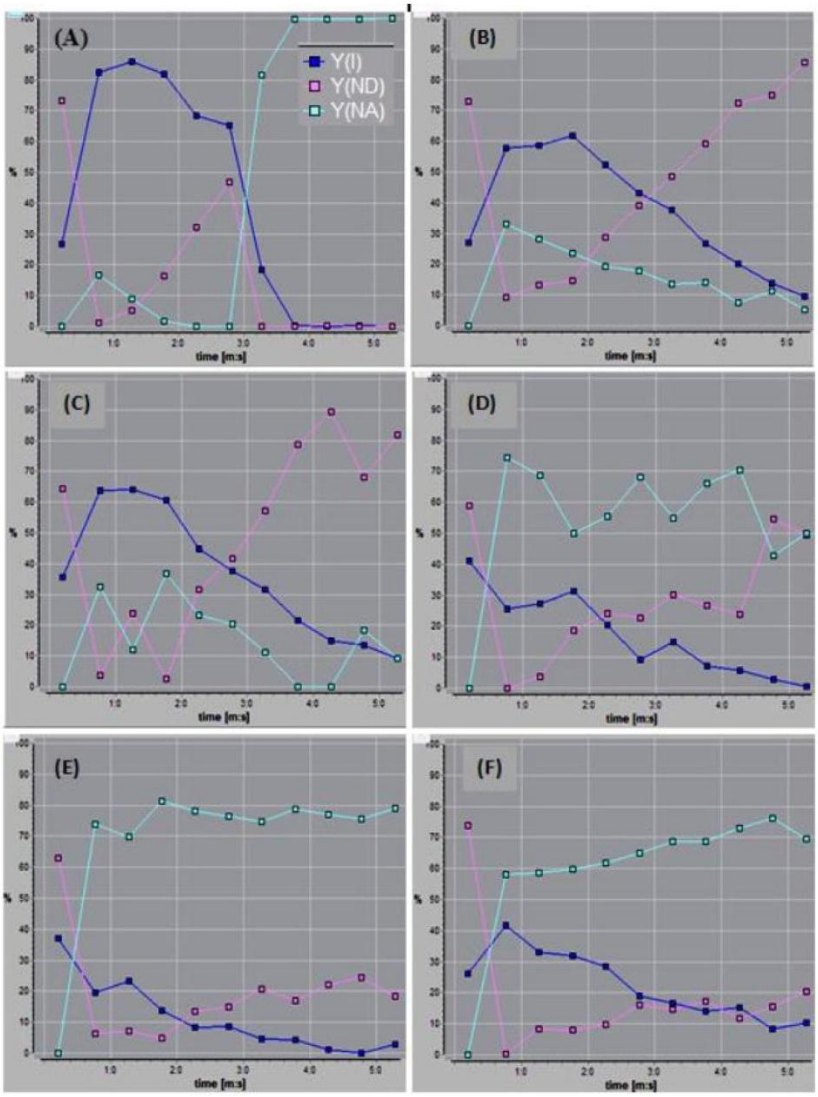


Fig. 5.5: Fluorescence quenching parameters Y(I), Y(ND) and Y(NA) of PS I of *N. muscorum* exposed to Cu^{2+}. (A) Control, (B) 1.5 µM Cu^{2+}, (C) 2.5 µM Cu^{2+}, (D) 5.0 µM Cu^{2+}, (E) 7.5 µM Cu^{2+}, (F) 10.0 µM Cu^{2+}.

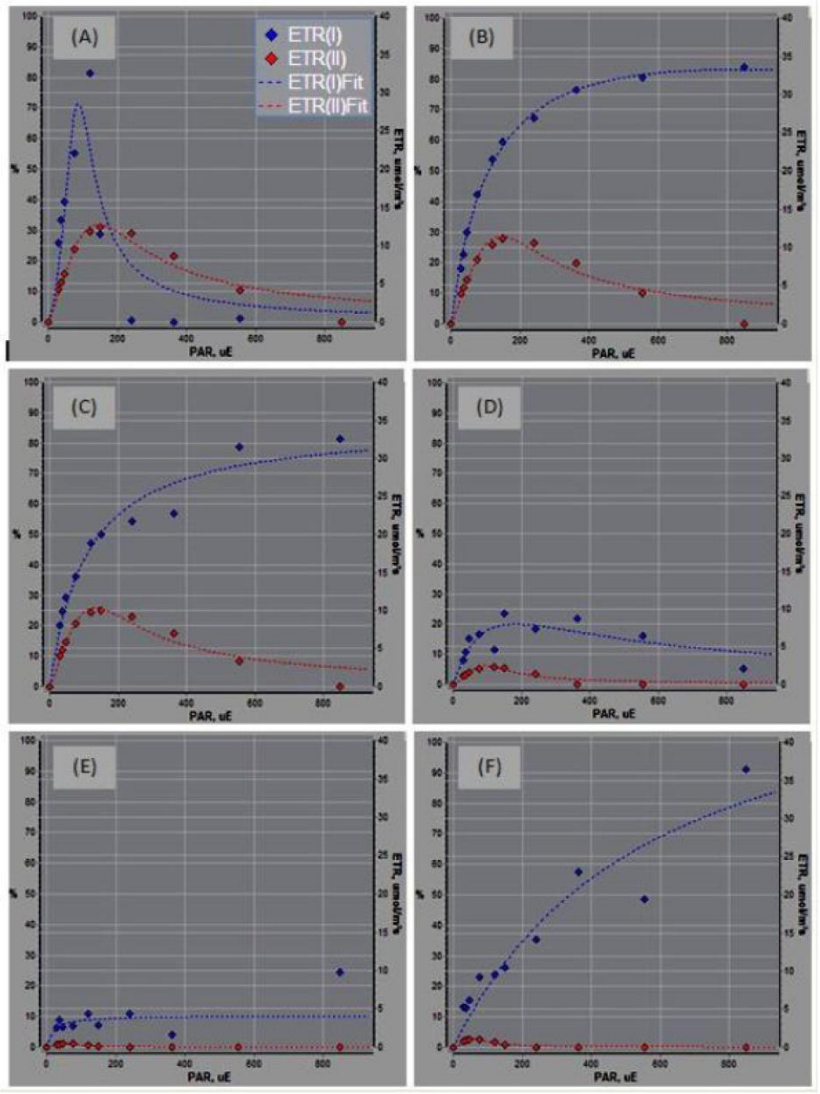


Fig. 5.6: The effect of Cu^{2+} in medium on electron transport rate (ETR) in PS I and PS II in *N. muscorum* recorded during the measurement of the rapid light induction curve. (A) Control, (B) 1.5 µM Cu^{2+}, (C) 2.5 µM Cu^{2+}, (D) 5.0 µM Cu^{2+}, (E) 7.5 µM Cu^{2+}, (F) 10.0 µM Cu^{2+}.

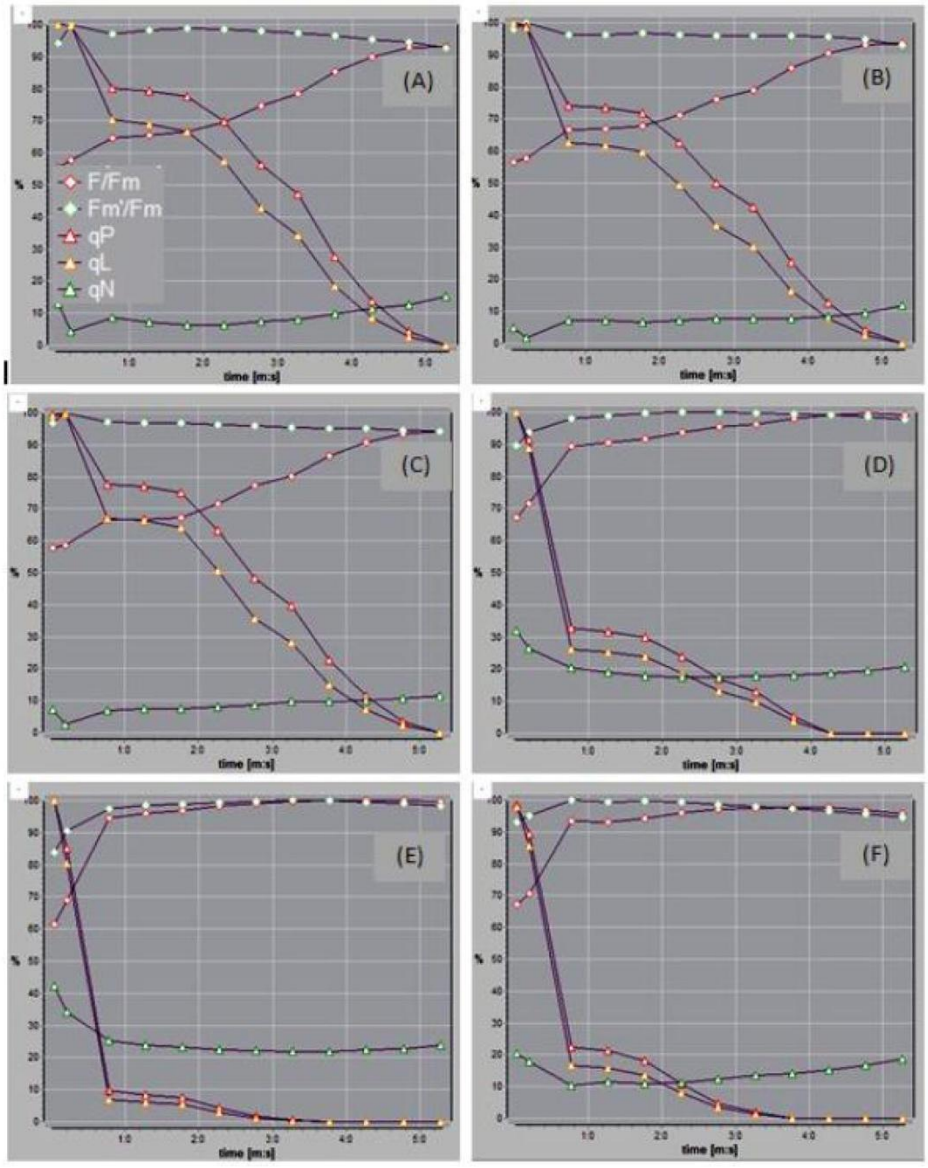


Fig. 5.7: The coefficients of photochemical fluorescence quenching (*qP, qL*) and non-photochemical fluorescence quenching (*qN*) calculated from rapid light response curve in *N. muscorum* treated with various concentrations of Cu^{2+} for 1 h. (A) Control, (B) 1.5 µM Cu^{2+}, (C) 2.5 µM Cu^{2+}, (D) 5.0 µM Cu^{2+}, (E) 7.5 µM Cu^{2+}, (F) 10.0 µM Cu^{2+}.

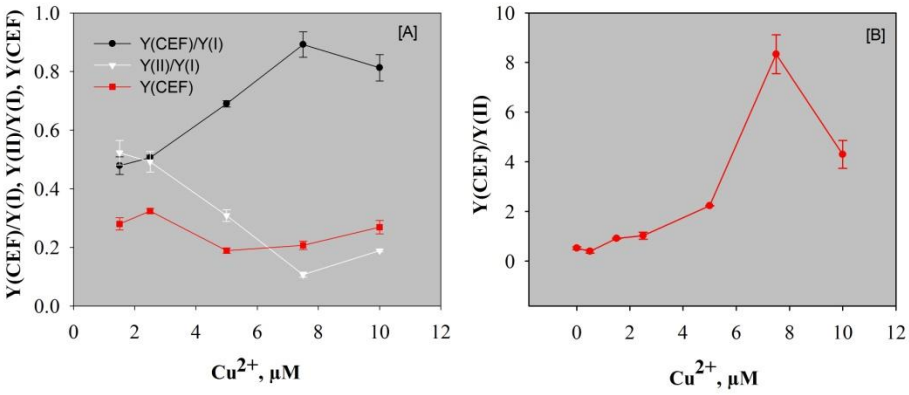


Fig. 5.8: Y(CEF), Y(CEF)/Y(I) and Y(II)/Y(I) in *N. muscorum* treated with various concentrations of Cu^{2+} [A]. Y(CEF)/Y(II) in *N. muscorum* treated with various concentrations of Cu^{2+} [B].

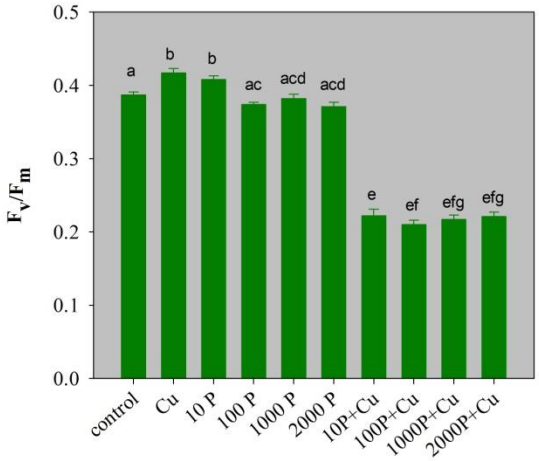


Fig. 5.9: Effect of proline pretreatment on maximum PSII quantum yield (F_v/F_m) of *N. muscorum*. The values are mean of three independent determinations. The vertical bars show S.E. Different letters show significant difference at $P < 0.05$. P = proline (µM), Cu = 5µM Cu^{2+}.

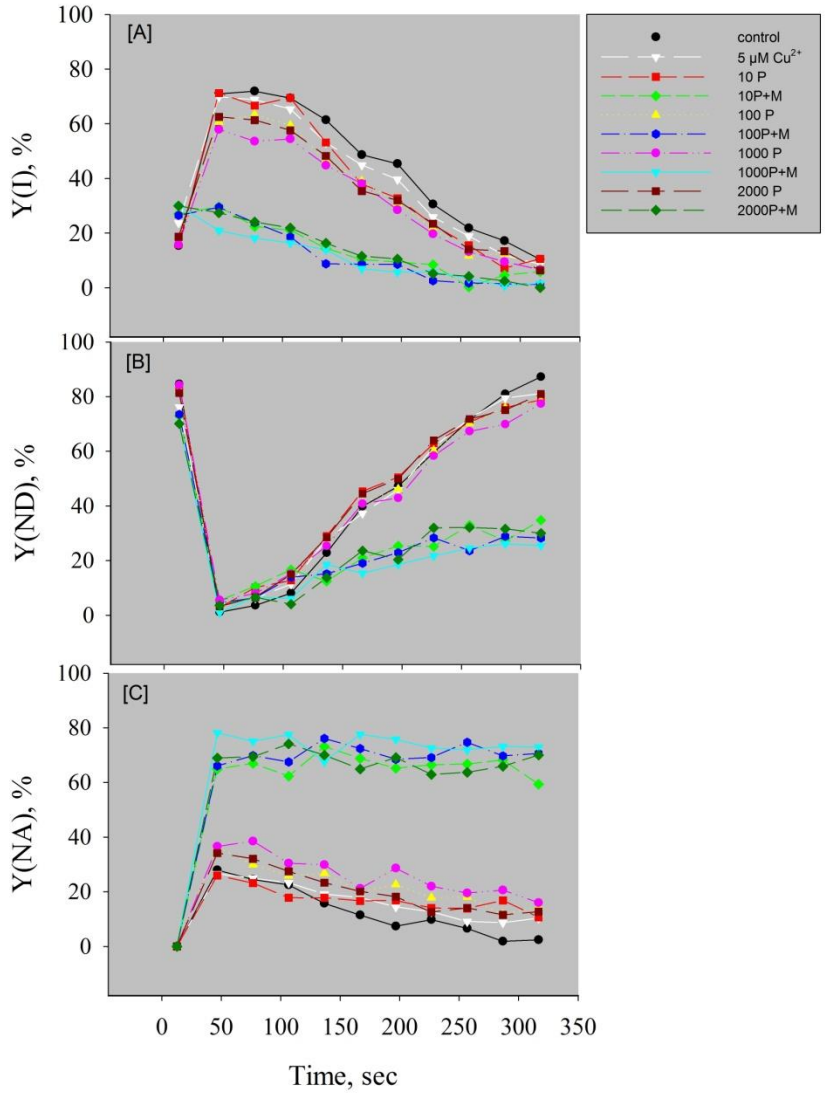


Fig. 5.10: [A] Y(I) (quantum yield of PSI), [B] Y(ND) (non-photochemical enery dissipation due to donor side limitation, and [C] Y(NA) (non-photochemical energy dissipation due to acceptor side limitation of PSI) of *N. muscorum* pretreated to different concentrations of proline (1 h) before treated with 5 µM Cu^{2+} for 1 h. P = proline, M = metal (5 µM Cu^{2+}).

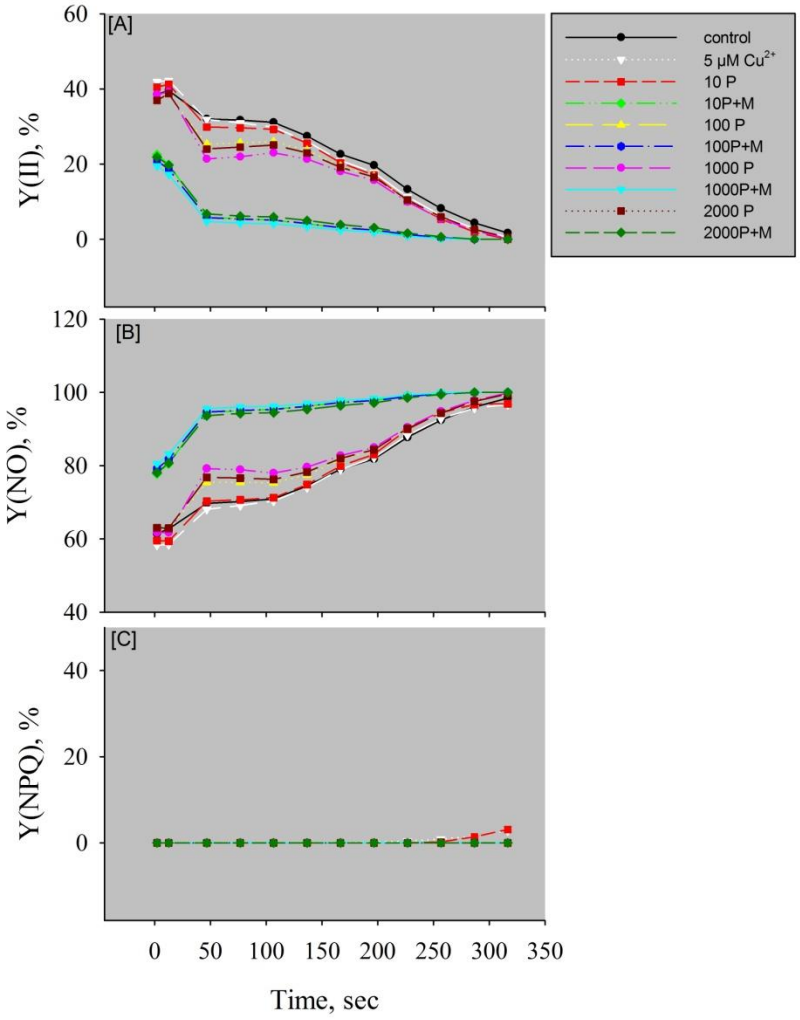


Fig. 5.11: Fluorescence quenching parameters Y(II)- quantum yield of PSII [A], Y(NO)- non-regulated energy dissipation of PSII [B], and Y(NPQ)- regulatedenergy dissipation of PSII [C] of *N. muscorum* pretreated with different concentrations of proline (1 h) before exposing to 5.0 µM Cu^{2+} for 1 h. P = proline (µM), M = metal (5µM Cu^{2+}).

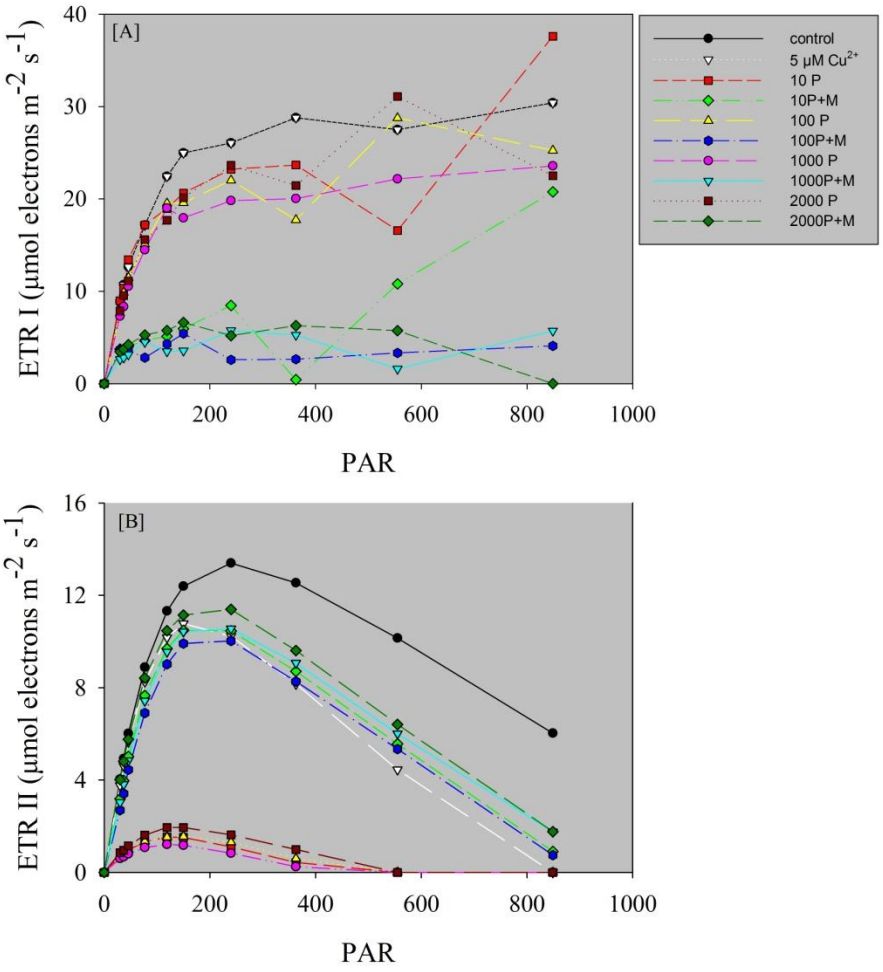


Fig. 5.12: The effect of proline pretreatment of culture on apparent electron transport rate (ETR) in PSI [A] and PSII [B] in *N. muscorum* recorded during the measurement of the rapid light induction curve. P = proline (µM), M = metal (5 µM Cu^{2+}).

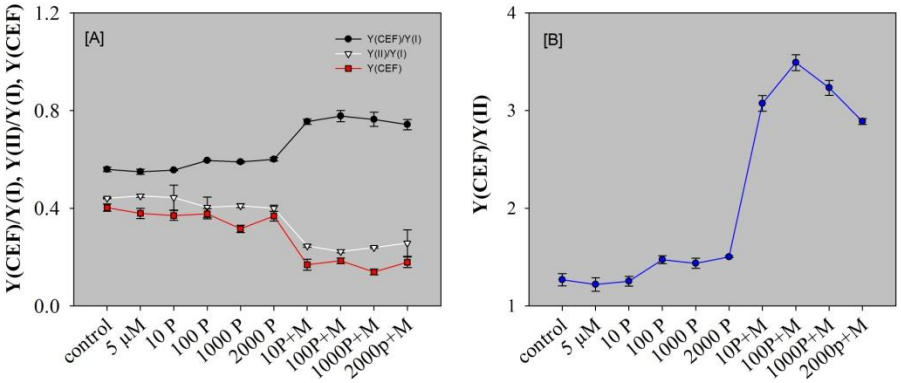


Fig. 5.13: Y(CEF) (the quantum yield of cyclic electron flow), Y(CEF)/Y(I) (ratio of the contribution of cyclic (CEF) to Y(I), and Y(II)/Y(I) (the distribution of quantum yield between PSI and PSII) in *N. muscorum* treated with various concentrations of proline [A]. (CEF)/Y(II) (the ratio of the quantum yield of cyclic electron flow (CEF) to linear electron flow (LEF) in *N. muscorum* treated with various concentrations of proline [B]. P = proline, M = metal (5 µM Cu^{2+}).

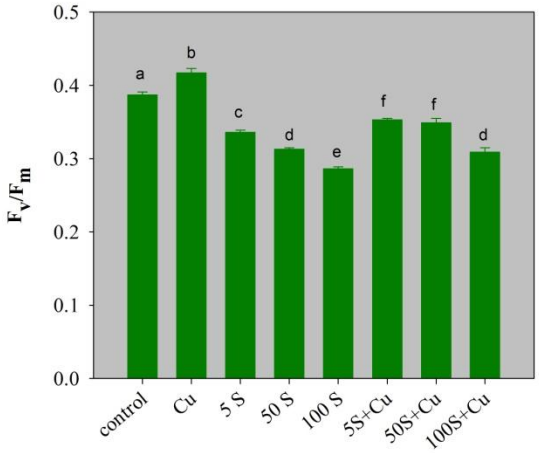


Fig. 5.14: Effect of increasing concentrations of NO donor SNP on maximum PSII quantum yield (F_v/F_m) of *N. muscorum*. The values are mean of three independent determinations. The vertical bars show S.E. Different letters show significant difference at $P< 0.05$. S = SNP (µM), Cu = 5µM Cu^{2+}.

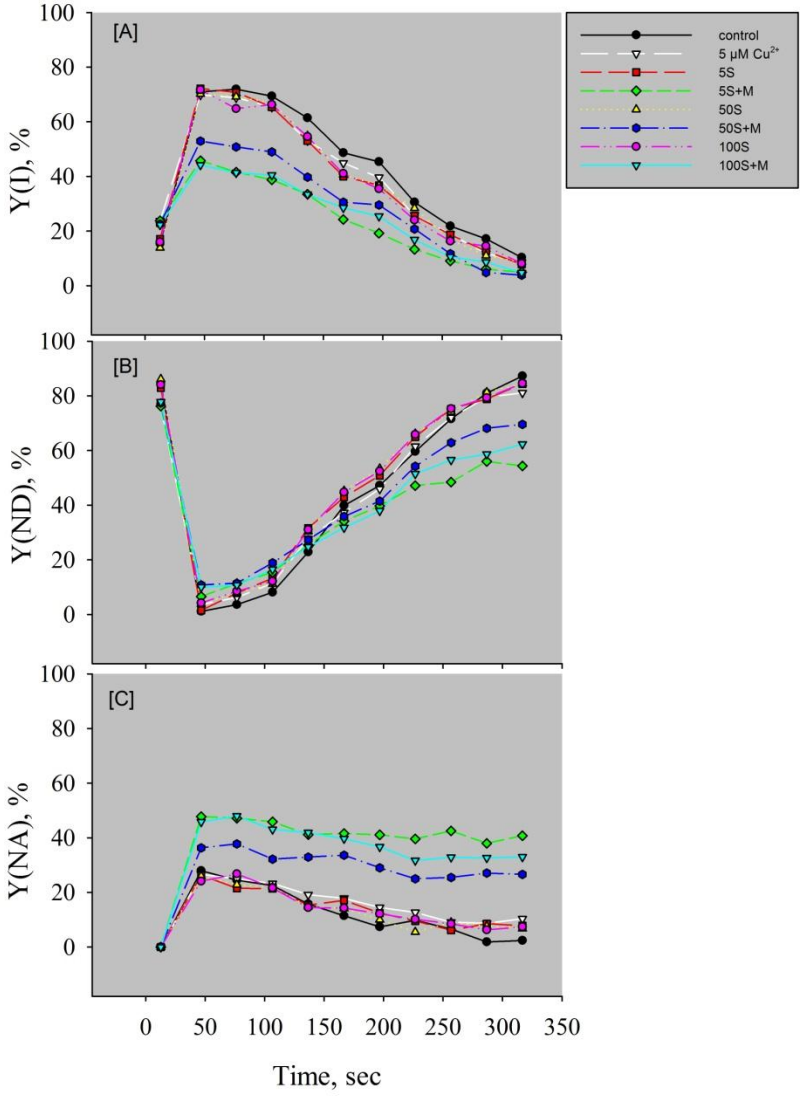


Fig. 5.15: [A] Y(I) (quantum yield of PSI), [B] Y(ND) (non-photochemical energy dissipation due to donor-side limitation), and [C] Y(NA) (non-photochemical energy dissipation due to acceptor side limitation of PSI) of *N.muscorum* pretreated with different concentrations of SNP (1 h) before treating with 5 µM Cu^{2+} for 1 h. S = SNP (µM), M = metal (5 µM Cu^{2+}).

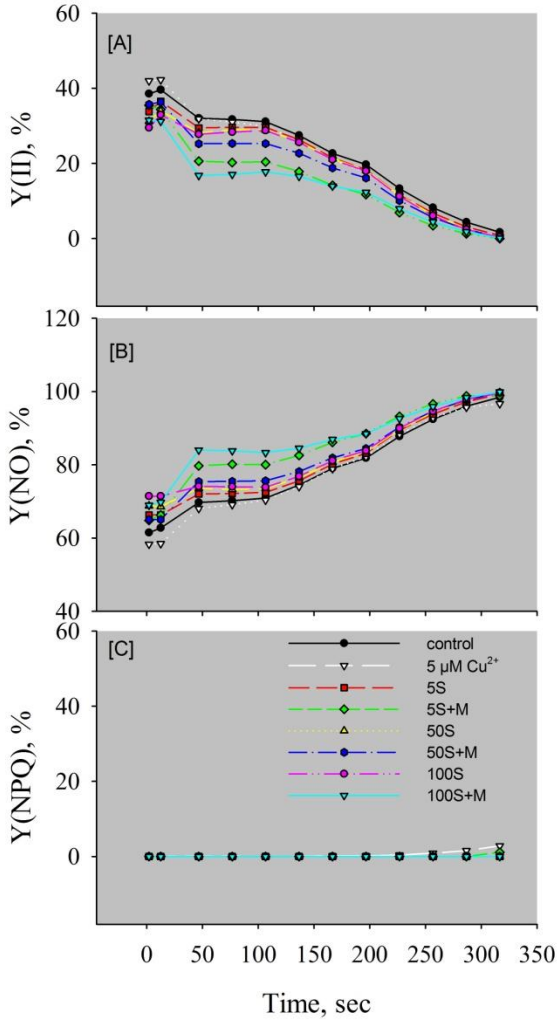


Fig. 5.16: Fluorescence quenching parameters Y(II), Y(NO) and Y(NPQ) in *N. muscorum* pretreated for 1 h to different concentrations of SNP and again treated the cells to 5 µM Cu^{2+} for another 1 h. S = SNP (µM), M = metal (5 µM Cu^{2+}).

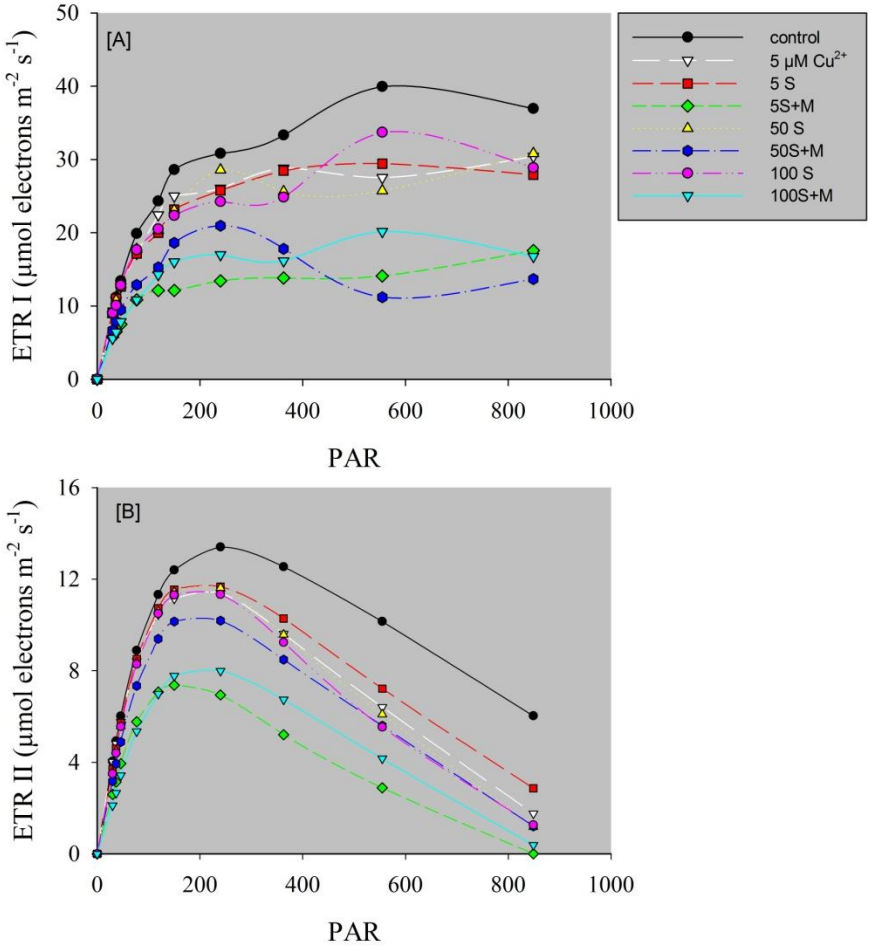


Fig. 5.17: The effect of different concentrations of SNP in the medium on electron transport rate (ETR) in PSI [A] and PSII [B] in *N. muscorum* recorded during the measurement of the rapid light induction curve. S= SNP (µM), M = metal (5 µM Cu^{2+}).

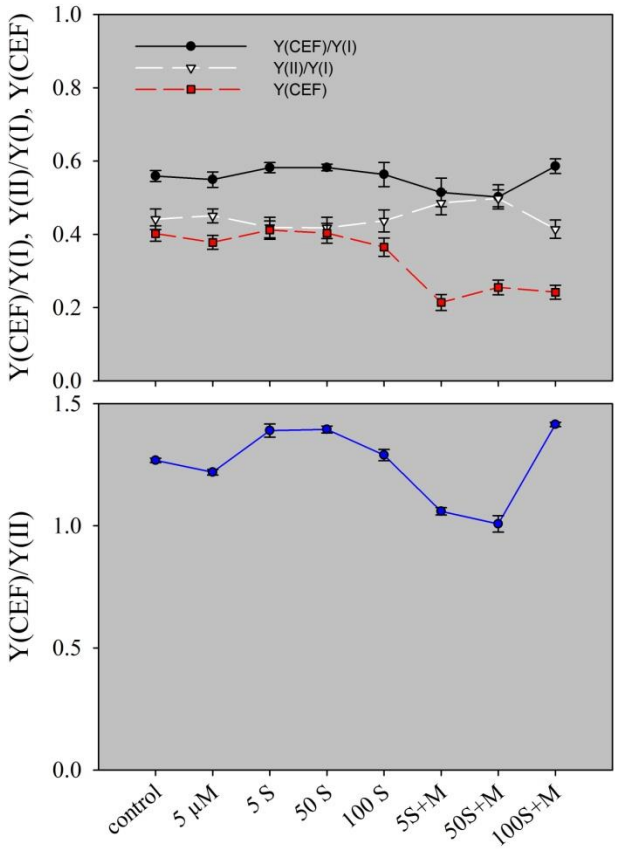


Fig. 5.18: Y(CEF) (the quantum yield of cyclic electron flow), Y(CEF)/Y(I) (ratio of the contribution of cyclic (CEF) to Y(I), and Y(II)/Y(I) (the distribution of quantum yield betweenPSI and PSII) in *N. muscorum* treated with various concentrations of SNP [A]. Y(CEF)/Y(II) (the ratio of the quantum yield of cyclic electron flow (CEF) to linear electron flow (LEF) in *N. muscorum* treated with various concentrations of SNP [B]. S = SNP (µM), M = metal (5 µM Cu^{2+}).

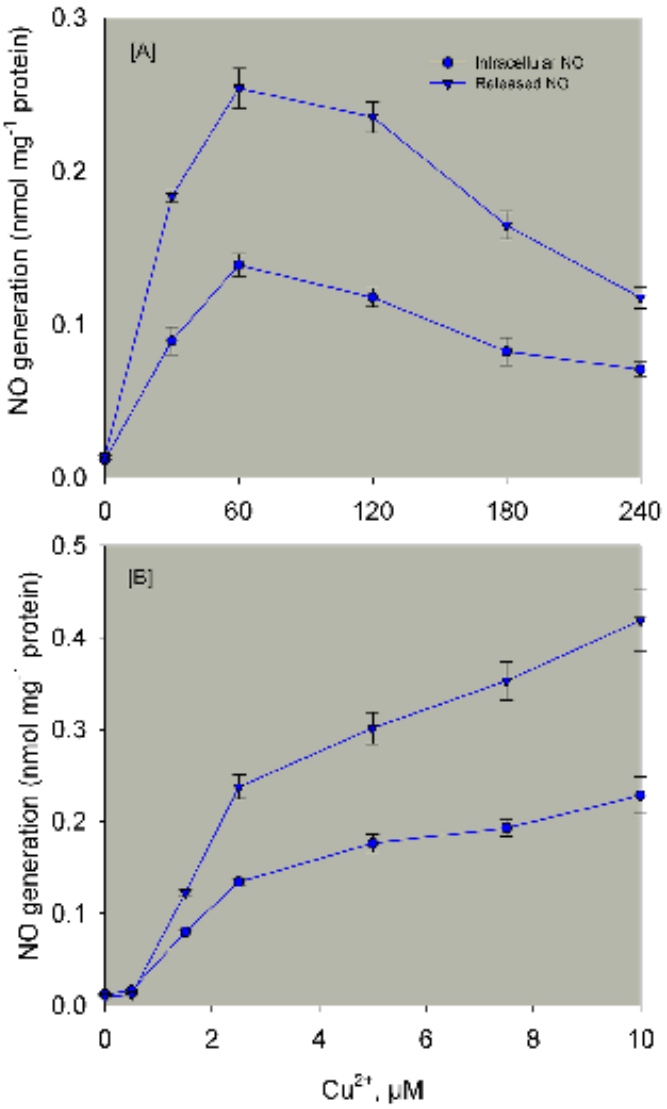


Fig. 6.1: Effect of Cu^{2+}on NO formation and efflux in *N. muscorum* in response to copper stress. NO in the cell extracts (intracellular NO) and NO released in to the medium were measured. [A] time course study, [B] At varying concentrations of copper in medium. The values are mean of three replicates.

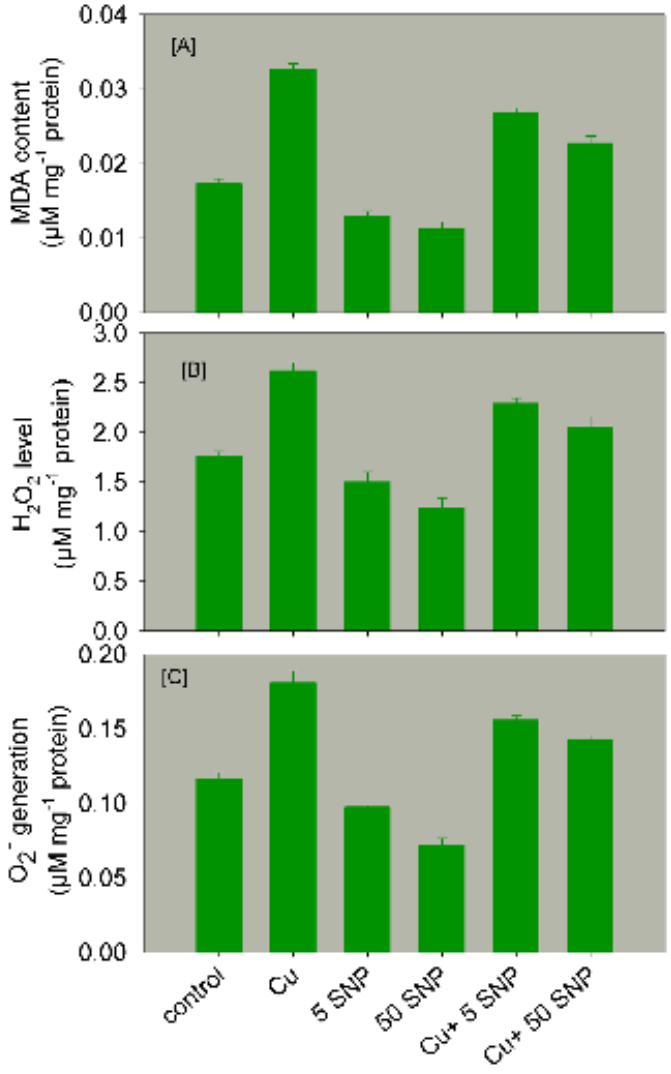


Fig. 6.2: Effect of NO donor SNP on MDA [A], H_2O_2 [B] and O_2^- [C] formation in *Nostoc muscorum* under various treatments. Values are mean ± SE from three independent measurements. Cu=2.5µM Cu^{2+}

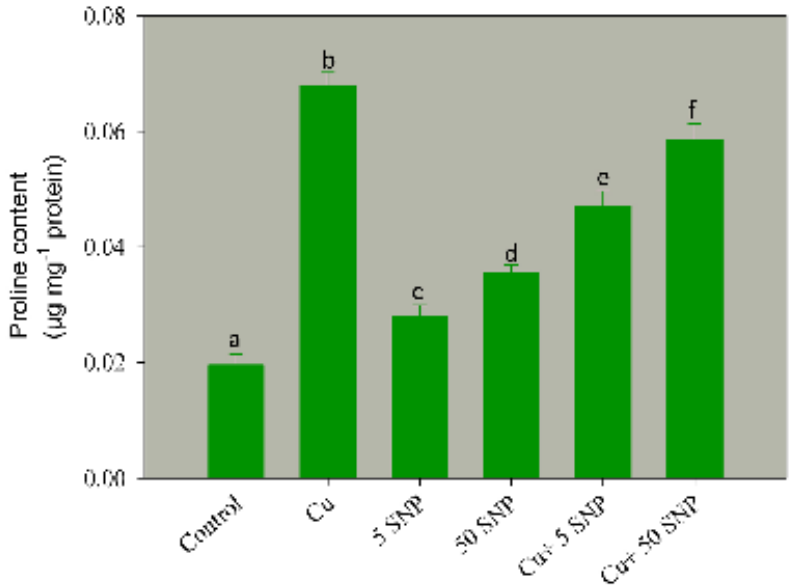


Fig. 6.3: Effect of the NO donor SNP on Cu-induced proline accumulation in *N.muscorum*. Cells were pretreated with 5 and 50 µM SNP for 1 h then exposed to 2.5 µM Cu for 1 h. Values are mean ±SE (n=3) and are statistically significant according to Duncan's multiple range test ($P < 0.05$), within individual Cu treatments. Cu=2.5µM Cu^{2+}

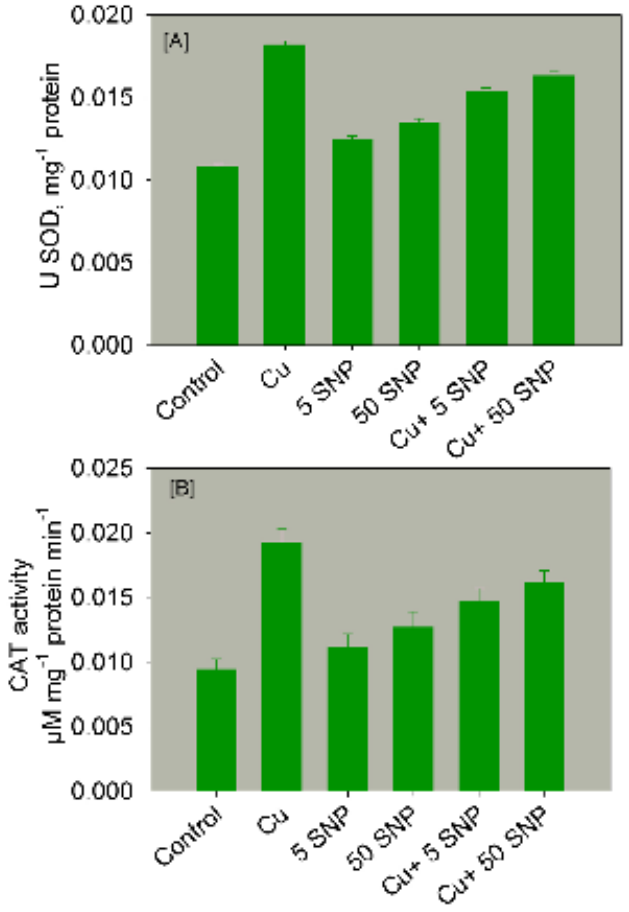


Fig.6.4: Effect of NO donor SNP on SOD [A] and CAT [B] activities in *N. muscorum*. Cells were pretreated with 5 and 50 µM SNP for 1 h then exposed to 2.5 µM Cu^{2+} for 1 h. Values are mean ± SE (n=3).

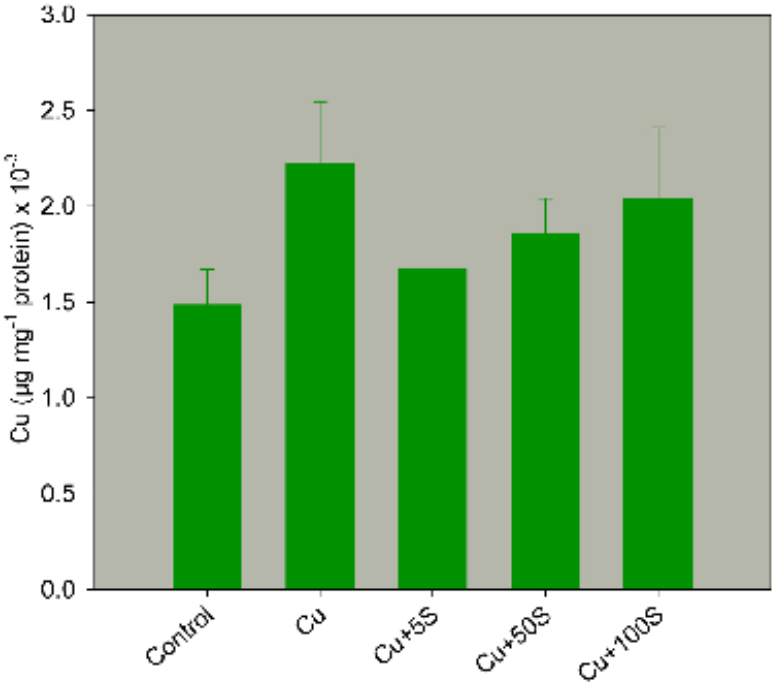


Fig. 6.5: Effect of SNP pretreatment (5, 50 and 100 µM SNP for 1 h) on intracellular uptake of Cu^{2+} by *N. muscorum* . The metal treatment was given for 1 h. Each data point is the mean of three replicates (*n*=3).