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Research Article

EVALUATION OF ACUTE TOXICTIY STUDIES ON COPPER-INDUCED OXIDATIVE STRESS IN *LATHYRUS SATIVUS* L., (VARIETY RATAN) GERMINATING SEEDS: A BIOMARKER BASED RISK ASSESSMENT

Tuhin Ghosh[1], Suman Mukherjee[2], Dipan Adhikari*[3]

[1]*Department of Chemistry (UG &PG), Durgapur Government College (Affiliated to Kazi Nazrul University, Asansol), Durgapur, District- Paschim Bardhaman, West Bengal, India*

[2]*Parasitology and Immunobiology Laboratory, Post Graduate Department of Zoology, Bidhannagar College, EB-2, Sector-1, SaltLake City, Kolkata, West Bengal, India*

[3]*Dept of Botany (UG and PG), Plant Cell and Molecular Genetics Research Laboratory, Hooghly Mohsin College, Chinsurah, Hooghly, West Bengal, India*

**Corresponding author: dipanadhikari@gmail.com*

ABSTRACT

Copper has been assigned to be a heavy metal which occurs most abundantly in agricultural soils owing to its large-scale use in metal industry as-well-as in agriculture as fungicides. Mitotic index, rate and categories of anatelophase chromosome aberrations, as well as the frequency and types of metaphase disturbances were scored in root tip meristems of *Lathyrus sativus* L (variety Ratan) after seed exposure to copper, provided as copper acetate at six concentrations (10, 8, 6, 4, 2, and 1 ppm) respectively. Except the 1 and 2 ppm concentration, all the other concentrations of copper acetate brought forth mito-depressive action. The copper genotoxicity is expressed in the increased levels and rates of chromosome aberrations in mitotic ana-telophase stages including chromosome bridges, laggards and complex aberrations are the most numerous, although multipolarity, fragments and micronuclei are present, but with lower frequency in lower treatments. Metaphases with chromosomes expulsed from equatorial plate are numerically preponderant, followed by C-metaphases. At higher doses (6 ppm onwards) copper exhibited micronucleoli formation and nucleolar disintegration *i.e.,* micronucleoli formation in germinating root tip cells which augmented the fact that although a essential micronutrient, but above suboptimal concentration copper stands out as potential cyto-nuclear poison for plant life which is also proved by inhibition of seed germination percentages, root length inhibition, reduction in total soluble protein and disruption of root metabolic activity by inhibition of dehydrogenase activity. These observations constitute a signal about the risks of the widespread and increasing presence of copper in ecosphere and could be considered for a high throughput evaluation of copper and its effects on other organisms, even on human health, due to large use of copper compounds, inclusively as pesticides and fungicides.

Keywords: Grass pea, Seed germination, Aneugenic effects, Clastogenic action, Genotoxicity, Soluble protein, ROS, Biomarker

1. INTRODUCTION

In the twenty first century high anthropogenic activity [1] brings about large scale heavy metal pollution which pollutes the biosphere in a multidimensional propensity. According to Kabata-Pendias [2] among the most toxic heavy metals reported so far for both higher plants and micro organisms; Copper (Cu), although an essential metal for nutrition, has been an heavy metal evokes a threat for biological world above suboptimal concentrations. As like other bivalent metal cations, excess amount Cu^{2+} comes in agricultural soils and in ecosystem from its use in industry and agriculture as fungicide, algaecide, or bactericides in different countries [3]. Copper has been assigned to be an essential micronutrient for plant growth and has found to impart important role in metabolic systems plants in addition to protein and carbohydrate metabolism, detoxification of free radicals, cell wall lignification, photosynthesis, respiration, seed germination [4] and most importantly in plant disease resistance [2]. Copper as a cofactor controls the catalysis of several essential enzymes such as plastocyanin, cytochrome c, and

Cu/Zn superoxide dismutase (Cu/Zn-SOD) [5]. Different authors had reported that the high concentrations of copper imparted phytotoxic effects inducing leaf chlorosis, root growth suppression and morpho-anatomical malformation in the root architecture [6]. Reports are available related to copper-induced genome alterations in DNA resulting single as well as double strand breaks, cross-linking in two separate DNA strands, protein-DNA cross-links, formation of modified bases, occurrence of intra-strand dimerization of adjacent purine bases, formation of sister chromatid exchange (SCE), resulting in incorrect DNA replication and reduction in mitotic divisions yielding chromosome aberrations. [7-10]. The continuous production and release of chemicals into the environment has asked for judicious explorations to assess their toxic effects in plant lives as the plants happen to be the first members of the ecosystem to counteract the burden of heavy metal poisoning and it requires even a thorough evaluation of their genotoxic potentials on a larger spectrum of different higher plant species (especially commercial crops). Plant systems represent good candidature by offering a battery of lowcost standar-dized assay methods like inhibition of seed germination and retardation in plantlet growth indices [11, 12], mitotic activity, chromosome aberrations [13, 14], nucleolar degradation [15, 9] in monitoring of cytotoxic and genotoxic possibilities of various environmental chemicals and heavy metals, acting as physical stress inducers for plant life. But the results; it so happens that sometimes depending on species, different growth conditions, copper concentration and the types of copper compounds, have been reported to be contradictory (because of many negative as well as false positive findings) with copper [14, 16-18]. Plant systems are largely used for assessing heavy metal bioavailability because they allow the evaluation of visible genotoxic events in root meristems, as the roots of plants accumulate substantially different heavy metals including Cu^{2+}. Different experts have reported the toxic effects of copper (Cu) on seed germination [19, 20] at different statges of plantlet-growth [11, 21] and mobilization of reserve foods in seeds [22]. Well documented reports are available depicting arrest in root growth through the influence on reserve mobilization upon copper exposure during seedling develop-ment [20, 23]. Copper has been reported to arrest mitotic division as in *Helianthus annuus* [23, 24] and *Allium cepa* [25]. Researchers [26] reported that the wastes of copper mine induced some abnormalities including scattered chromosomes in the root tip cells of *Allium cepa*.

Grass pea is a plant which has proven to have some exceptional agronomic properties, such as drought tolerance and can withstand flood and salinity. This plant has a high nitrogen fixation capacity, showing easy cultivation practices as a relay crop with minimal inputs after rice; as fallow harvest, showing greater adaptability to different agro-climatic conditions and soil types [27]. As a legume, it is also a highly nutritious food and fodder crop counted as a life-saver crop for uncounted thousands of people during famines in past. In develop-ping countries like India pulses remain the main source of dietary protein [28]. Apart from other crops in India, the major route could be water absorption from soil or fungicide sprays of copper during cultivation practices. Since most of the water resources in agricultural areas are polluted owing to high anthropogenic activities with different heavy metals, the plants cultivated in these soils or with copper contaminated water would certainly impart antagonistic effect on the plant's metabolic architecture. The present investigation studies the susceptibility of this plant (*Lathyrus sativus* L.) to the genotoxic effect of Cu. After an exhaustive literature search the authors have found out that very few scientific reports are available from Indian cultivated crops, especially in pulses, related to copper toxicity. So the present study possibly enable to throw some light in understanding the total tolerance capacity of copper in germinating *Lathyrus sativus* L**.,** which might induce deleterious effects to deregulate some of the cytogenetic and physiological manifestations upon acute exposure within the cellular environment. For these reasons, it is necessary to deeply know the consequences of action copper on genetic apparatus and probable mechanism of action in this plant. The overall objective of this work is to evaluate and to complete the knowledge on copper genotoxicity coupled with some morphological and biochemical markers, provided as copper acetate, by quantification of cytogenetic damage expressed in reduction in mitosis indices, rate of anatelophase chromosome aberrations and frequency of metaphase disturbances in meristematic cells of *Lathyrus sativus* L**.,** root tips of young seedlings obtained by germination of copper-treated seeds.

2. MATERIAL AND METHODS

2.1. Seed material collection and treatment conditions

Lathyrus sativus L., Ratan seeds were used in the experiments. Ratan is a commercial cultivar of winter

(Locally known as Khesari) obtained from Pulse and Oil seed Research Station, Baharampur, Murshidabad, Ranibagan, Khagra, Berhampore, West Bengal. The seeds were surface disinfected with 1% sodium hypochlorite for 5 min and vigorously rinsed with distilled water. Then they were 6 hours treated with $(CH3COO)_2Cu.H_2O$ (copper acetate monohydrate, SIGMA ALDRICH), molecular weight=199.63 $gmol^{-1}$. Six concentrations (10 ppm, 8 ppm, 6 ppm, 4 ppm, 2ppm and 1 ppm respectively, 24 hrs imbibitions) have been prepared for each copper acetate and were used for seed treatment. Control was set up by seed immersion in distilled water. The treated seeds were placed on moist filter paper in covered Petri dishes and then maintained in dark, at 23°C, in order to germinate. The seeds were then spread over moist cotton kept in Petri-dishes (15 cm diameter) at 24 ± 2°C temperatures for further observation [28].

2.2. Determination of Germination percentage and measurement of radicle length

The germination potential of seeds and radicle (embryonic root) length (measured using a millimeter ruler) were analyzed at every 24 h interval. The experiment was repeated three times under similar conditions. For *Lathyrus sativus* L**.,** the seed germination percentage was measured (after a span of 24 hrs). The germination percentage was measured as Germination %= germinated seed/total seeds x 100 [28].

2.3. Measurement of soluble protein content of root tissue after Cu treatment

Measuring soluble protein content in this investigation was carried out according to Bradford's method [29] using BSA as a standard. The fresh roots from each treatment (6 seedlings) were washed in distilled water, dried and put in a mortar with 5 mL 0.05 M PBS (pH 7.8) at the end of each time interval (2nd day) of the copper treatment. The homogenate was centrifuged at 10,000 × g for 20 min and the supernatant was used for analyzing soluble protein content. The soluble protein content was expressed as mg per g fresh weight.

2.4. Evaluation of metabolic/mitochondrial activity

TTC (2,3,5-Triphenyl tetrazolium chloride) staining is a novel approach to check the viability of cells. The seeds of *Lathyrus sativus* L., were treated with different concentrations of copper salt for 24 h. The same set-up was followed for positive and negative control taken as 0.1% hydrogen peroxide and distilled water, respectively. All the roots were immersed in 0.5% (w/v) TTC stain for 5 h in dark. Subsequently, the roots were washed in distilled water. Absorbance was measured at 490 nm using a spectrophotometer against 95% ethanol as blank. We considered Positive Control (Hydrogen Peroxide O.D.) as 100% metabolic/ Respiratory activity and respective test O.D.s were converted into subsequent activity (in terms of metabolic activity percentage) [30].

2.5. Cytogenetic analysis: Determination of Cytogentoxicity (% of Mitotic inhibition by orcein staining)

To study the cytogenetic changes induced by copper in *Lathyrus sativus* L., plant, the root tips of germinated seeds were used as a source of mitotic cells after the method of Adhikari, 2019 [29]. Mitotic index and chromosomal aberrations in metaphase and anaphase plates were studied using a light microscope under oil immersion. From each slide, minimum of 100 cells were scored and mitotic index was calculated [28]. Chromosomal aberrations such as chromosome fragments (F), precocious separation (SP), stickiness (STC), bridge formation (Br), c-mitosis (C-m), micronuclei, etc were studied in a minimum of 100 cells per slide and expressed in percentage. All stages were examined at 40x and under oil immersion objective using a 100 × eyepiece of a compound microscope (Olympus CH20i microscope) fitted with CMOS Camera (IS 500, 5.0 MP) and its attachment with a computer with the aid of VIEW 7 image analysis software.

Images were acquired and cytotoxic parameters were calculated using the following formulas.

1. Mitotic index % = (Number of dividing cells/Total no of cells) x 100
2. Abnormality mitotic index % = (No of aberrant cells after treatment/Total no of cells counted) ×100.
3. % of Mitotic Inhibition= (Mitotic index in Control-Abnormal Mitotic index after treatment)÷(Mitotic index in Control) X 100

2.6. Statistical Analysis

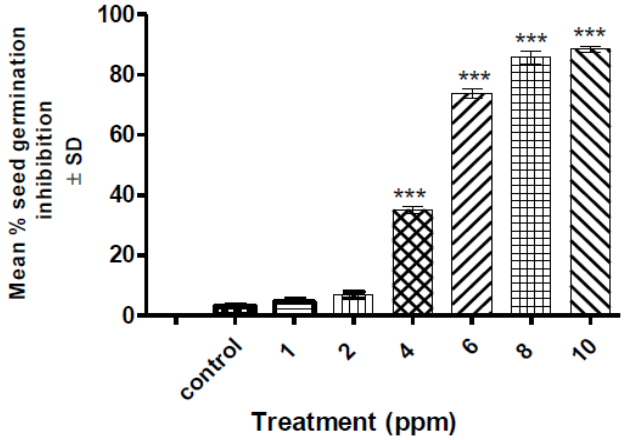
All the values are presented as Mean ± SD (standard deviation, n=6). Statistical analyses were performed with analysis of variance (ANOVA) followed by post-hoc Dunnet's Test. The *p* values less than 0.05 were considered significant. In the statistical analysis, differences between the groups were tested by analysis

of variance (ANOVA) in GRAPH PAD PRIZM-version 6 computer program.

3. RESULTS

3.1. Effect of Cu treatment on seed germination inhibition

At recovery after 72 h of Cu treatment, in comparison to control group, 1 ppm and 2.5 ppm promoted rapid seed germination and could not augment any significant change in seed germination inhibition. However, from 6 ppm onwards *i.e.,* 6, 8 and 10 ppm copper induced significantly inhibition of seed germination were accounted when compared to control ($p < 0.001$). At 10 ppm copper exposure (the highest concentration tested significantly inhibited seed germination ($p < 0.001$) compared to control which was found to be less than 20% (Fig. 1).



*P verses control (***=<0.0001) following ANOVA and Dunnet's multiple comparison test with each treatment with control*

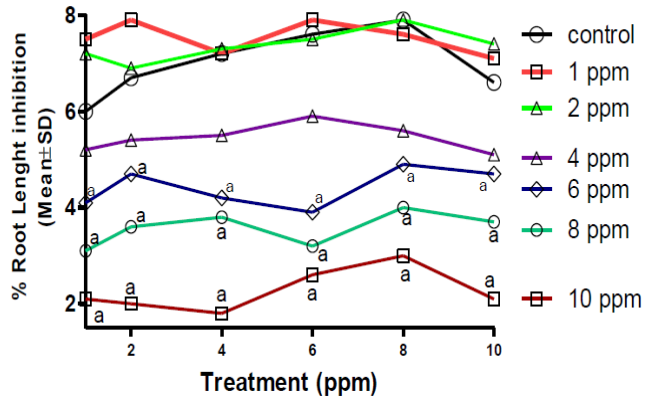
Fig. 1: Determination of seed germination inhibition after 72 hours of recovery from treatment.

3.2. Effect of Cu treatment on root length inhibition

In untreated seeds of *Lathyrus sativus* L., the root length increased with the increase in time after 72 h after the treatment (Figure 2). In comparison to control group, 1 ppm and 2 ppm promoted rapid root length promotion and could not augment any significant change in root length inhibition. A significant decrease in root length were accounted when compared to control ($p < 0.001$) in seeds exposed to 4, 6, 8 and 10 ppm of lead after 48 h in comparison to control (Fig.2).

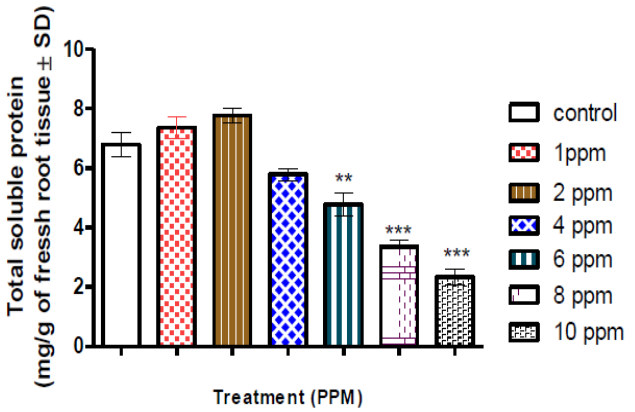
3.3. Effect of Cu treatment on total soluble protein in germinating root tips

Effect of copper in germinating roots tips produced variable results. At lower concentrations *i.e.* 1, 2 and 4 ppm treatments there was slight increase in total soluble protein in germinating root tips. But from 6 ppm onwards there was a significant reduction in the total soluble protein concentrations when compared to control ($p < 0.001$).



P versus control (a= <0.0001) after Dunnet's multiple comparison test with control and all the treatments

Fig. 2: Determination of percentage of root length inhibition in *L. sativus* L. germinating seeds, differences in root growth inhibition in *L. sativus* L. after 72 hours recovery (after treatment) in comparison to control



*P verses control (***=<0.0001, **=<0.001) following ANOVA and Dunnet's multiple comparison test with each treatment with control.*

Fig. 3: Determination of total soluble protein content after copper treatment in germinating roots of *L. sativus* L., showing the total amount of available soluble protein in fresh root tissues of *L. sativus* L. after 72 hours of growth

3.4. Effect of Cu treatment on root metabolic activity (dehydrogenase activity) in germinating root tips

The effect different concentrations of copper on root metabolic activity (dehydrogenase) activity on germinating roots of *Lathyrus sativus* L. TTC staining was employed in the present study as an indicator to evaluate the effect of all biotic and abiotic stressors which amplify the production of ROS, possibly attenuating the mitochondrial metabolic health. In TTC staining 2, 3, 5-triphenyl tetrazolium chloride is reduced to red formazan by mitochondrial enzymes. The result showed a dose dependent decrease in mitochondrial activity as visualized by decrease in staining and absorbance in comparison to positive control ($p< 0.001$) (Fig.5). Positive control remained unstained with minimum absorbance indicating least mitochondrial activity and negative control showed maximum activity. In the case of treatments, it was found that at 2, 4 and 6 ppm there was an increase in root metabolic activity (dehydrogenase activation). But at 8 ppm treatment there was significant lowering of root metabolic activity in comparison to positive control. But at the highest concentration i.e. at 10 ppm treatment there was a total loss of root metabolic activity probably due to cellular poisoning and disruption of dehydrogenase activity (72 hrs).

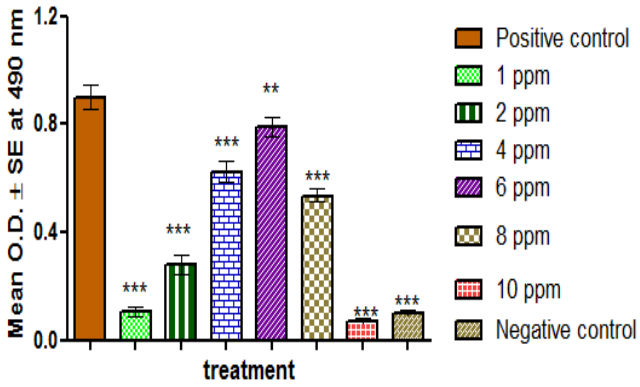


Fig. 4: Determination of root metabolic activity (dehydrogenase activity by TTC staining) after copper treatment in germinating roots of *L. sativus* L.

*TTC staining - Graph showing the effect of different concentrations of copper on mitochondrial activity. (NC) Negative control - Distilled water; (PC) Positive control - 0.1% hydrogen peroxide showing the mean O.D. of formazan produced in root tissues of L. sativus L.after 48 hours of growth due to root dehydrogenase activity. P verses negative control (**=<0.001, ***=<0.0001) following ANOVA and Dunnet's multiple comparison test with each treatment with negative control.*

3.5. Effect of Cu treatment on chromosomal aberration assay (Cytogenetic analysis)

Copper induced a number of mitotic aberrations in *L. sativus* L., (Plates 1-9). Increased incidence of sticky chromosome (STC), c-mitosis (C-m), fragment (F), micronuclei (Mn), early separation cum fragments (Es) and bridge (Br) was observed. Sticky chromosomes, in all phases of mitosis were the most frequent alterations, as they counted for 75-95% of the total number of aberrations, depending upon the copper concentration (4, 6 and 8 ppm). The percentage of c-mitosis and micronuclei formation was higher in 6 ppm concentration of Cu. Other kinds of aberrations, such as fragments, precocious separation, and telophasic bridges were also recorded. (Fig Plates: 1-9, Table: 1). At 10 ppm treatment most of the cells were quiescent, necrotic, highly vacuolated with nuclear atrophy and with very less chromatin materials leading to apoptosis.

4. DISCUSSION

Grass pea (*Lathyrus sativus* L.) with a worldwide cultivation practice has been regarded as one of the cheap protein sources for different dietary supplements for its great nutritive values especially for the consumption for human beings along with as animal fodder in third world countries [31]. It has established itself as an excellent example of an "Orphan Crop" nicely adapted to drought, salinity and very low water use inputs [32, 33]. But very few reports are available [10] related to the action of heavy metal in context to heavy metal stress and its implications in the genomic profile in this plant. In our present study we could find some very interesting findings related to the effect of copper on germinating seeds of *Lathyrus sativus* L. Grass pea is a diploid ($2n = 14$, its big complex 8.2-Gb genome) [34] could be employed as a fantastic model plant to evaluate cytogenotoxic assays in addition to other standardized models like *Allium cepa* L.

In our present study, it was reaffirmed that copper could affect in differential manner affecting significantly the germination of *Lathyrus sativus* L root tips. At lower doses of 1 and 2 ppm respectively copper could act as a promoter of root growth and germination showing no inhibition of seed germination percentage and root length growth in *Lathyrus sativus* L in comparison to control (Fig 1 and 2). From 4 ppm onwards there had been a sharp modulation in the root length growth inhibition and seed germination percentage (Picture 1, Fig 1 and 2). Similar published reports are available related seed germination and seedling growth modulations in *Lens culinaris* (Fabaceae), which depicted

that with increasing concentrations of copper treatment reduced germination percentage (25 ppm significantly; $p<0.05$) along with other declining seedling growth variables i.e. root and shoot length, seedling size, root/shoot ratio and seedlings dry weight with copper treatments as compared to control sets. Tolerance indices and seedling vigor index of *L. culinaris* also decreased with increasing copper treatments (showing the lowest tolerance indices and seedling vigor index at 25 ppm) in contrast to the highest percentage of reduction in seedling tolerance indices of *L. culinaris* (100 ppm of copper treatment) as compared to control [35]. Researchers [36] could report low metal concentrations had least inhibitory effect on seed germination while at high concentrations the inhibiting effect of heavy metals on seed germination could be accounted prominently to reduce the germination percentage. In *Vicia sativa* L. seeds [38] increasing concentrations of $CuBr_2$ altered the germination percentage and root elongation where the lowest copper concentrations ($CuBr_2$ 0.01 and 0.1mM) had no effects on seed germination index and root elongation inhibition while the highest concentrations (1 and 5 mM) exerted significant inhibitory effects on root elongation inhibition after 72h imbibitions. Same results had been established in studies where the reduction in stem and root lengths in *Solanum melongena* L. [39], *Phaseolus vulgaris* L. [40] and *Lolium perenne* [41] by Cu^{2+} treatments. Copper has been widely regarded as very toxic metals to many plants [37] and this statement could be fully confirmed with experiments with *Lathyrus sativus* L germinating seeds (Fig 1 and 2). So we might corroborate that our results are in unison with those earlier reports and could support the established fact that at lower concentrations copper does not impart any effect on root cell division but at higher concentrations (at least in our study with 6, 8 and 10 ppm respectively) there was significant reduction in seed germination percentage and root length growth inhibition.

In this experiment with *Lathyrus sativus* L., seeds after 72 hours of growth (treatments with Cu) we could find that there was an increase in total soluble protein content in the root tissues at 1, 2 and 4 ppm treated roots. But the total soluble protein content significantly decreased in germinating root tips (treated with 6, 8 and 10 ppm treatment) (Fig 3). Total soluble protein content in organisms, an important indicator of reversible and irreversible alterations in metabolism could also be used as a "biochemical biomarker" [39].

In the experiment in eggplant species, authors [39] demonstrated the correlation between total soluble protein content, which was significantly decreased, with increasing Cu^{2+} concentration after 21 days and this inhibition in root growth of eggplant (*Solanum melongena*) seedlings correlated with changes with total soluble protein content as "population biomarkers" suggesting that the extension of the genome damage altering the total soluble protein content must be serious in the actively dividing cells during growth. The resultant decrease in biomass vis-a-vis inhibition of root elongation could be explained by the inhibition of cell elongation and cell division by copper [42]. In our experiment with copper in germinating seeds of *Lathyrus sativus L* we could see that there was a significant decrease in the total no of cells entering in mitotic division (Table 1) and significant inhibition of MI% at different concentrations.

It was propounded that heavy metals like copper can inactivate enzymes by binding to cysteine residues and blocks the essential biological function of enzyme displacing the essential metal ions from catalytic centres after making strong bonds with oxygen, nitrogen and sulphur atoms. Therefore, the displacement of one metal by another would normally also lead to inhibition or loss of enzyme activities [43] in addition to effective binding to SH group and nitrogen containing groups at the catalytically active centres in the enzymes. High concentrations of Cu have been implied to activate oxidative damage and alter cell-membrane properties by lipid peroxidation, thereby demonstrating the inhibitory effect on the enzymes in addition to soluble protein [44]. In this study the high soluble protein content induced by copper (at 1 and 2 ppm treatment) in the germinating roots can be explained by the following aspects. At these lowered concentrations, Cu probably induced the expression of several genes and probably increased the synthesis of several original proteins because copper did not injure the roots heavily and induced protein synthesis in cells [45] as a result of which the soluble protein contents were partially higher than control (Fig 3). Then there was a decreasing trend at 6, 8 and 10 ppm respectively ($p<0.001$). It might so happen that up to 4 ppm root cells could withstand copper toxicity without causing the original protein degeneration and decomposition [46] and continued new protein synthesis, as like Aluminium ions [47]. But from 6 ppm onwards there was significant abiotic stress induction could bring forth oxidative damage of protein synthesis machinery which made soluble protein content

decrease significantly. Excess concentrations of copper widely can affect key cellular processes in plants as a result of which reactive oxygen species (ROS) formation takes place which has been assigned to be a potential cause of damage of the biosynthesis process of biological macromolecules such as DNA, proteins, and lipids [7, 5].

Table 1: Determination of Mitotic inhibition in *Lathyrus sativus* L. root tips with different concentrations of copper treatment

Treat-ment PPM /24hr	Total no of cells counted	Total no of cells in division	Prophase		Metaphase		Anaphase		Telophase		MI ± SD	% of Mitotic inhibition
			No of cells	%	No of cells	%	No of cells	%	No of cells	%		
Control	1800	500	382	76.04	67	13.4	32	6.4	19	3.8	27.09±2.12	0
2	2528	476	369	56.51	82	17.22	49	12.39	66	13.86	19.02±2.52***	29.78
4	2532	415	244	58.79	63	15.18	74	17.83	34	8.19	16.39±2.12***	39.40
6	2432	363	251	69.14	38	10.46	37	10.19	47	12.9	15.33±2.12***	43.41
8	2388	240	199	82.91	14	5.83	6	6.17	12	5	10.11±2.12***	62.67
10	2145	111	102	91.89	3	2.7	3	2.7	3	2.7	5.15±2.12***	80.98

*Determination of Mitotic inhibition in Lathyrus sativus L. root tpis with different concentrations of copper treatment summarizes the cytological effect of different concentrations of copper on mitotic index and chromosome aberrations in root tip cells of. P verses control (***=<0.0001) following ANOVA and Dunnet's multiple comparison test with each treatment with control.*

This present study displayed the mito-depressive action of copper at higher concentrations (4, 6, 8 and 10 ppm) and its possible interruptions in spindle formation (Table 1, Fig. 5, plate: 5). Inhibition of DNA synthesis at S-phase has been directly correlated altered divisional phases and to the lowering of mitotic index (Table 1) [48]. Nuclear lesions have been a "cytological marker" indicating nuclear poisoning (Fig.5, plate: 6) [49] coupled with inhibition of DNA biosynthesis [50]. Inhibition of phragmoplast resulted in binucleate cell formation during cytokinesis [51] by higher doses of copper has been taken into account (Fig.5, plate: 14). Cellular necrosis leading to apoptosis has been regarded as cellular suicide out of abiotic stress within the cell yielding rapid changes, reflected in both in cellular morphology (Giant cell formation and cytoplasmic shrinkage) (Fig.5, plate: 15 and 16) [52] and biochemistry (decrease in total soluble protein) in many treated cells. Cell division becomes affected hugely; especially, in the S phase in giant cells subjected abiotic stress followed by mitotic arrest and induction of cell expansion [53, 54]. Nuclear budding has been assigned to develop from anaphase laggards which again form a nuclear envelope in telophase to get attached to the nucleus or could remain as broken anaphase bridges (Fig.5, plate: 10) [55]. Sticky chromosomes (Fig.5, plate: 5, 6, 11) could arise due to chromatin fibres intermixing forming some subchromatid connections between chromosomes [56] which is irreversibly bring about cellular death thus emerging as one of happening event for reduction of nomal mitotic index (Table 1) [57]. Possibly Cu^{2+} could form ROS related changes which degrade DNA and some resultant changes in chromatin structure [58], which in turn might inhibit DNA-polymerases, altering the biosynthesis of some key proteins directly involved in cell cycle and spindle assembly or orientation resulting in mitodepression [59]. Also Cu^{2+} could prevents the cells to enter into prophase by blocking interphase whereas higher level of abnormal metaphases and ana-telophases could be due to the resultant interaction of Cu^{2+} spindle proteins, with blocking of mitotic cells to complete respective divisions. Laggards and bridges are the result of aneugenic effect of copper action because they lost the ability to attach by spindle fibres; being a potential source of aneuploidy; failed to participate in normal division and cause genetic disequilibrium in daughter cells which are more likely irreversible [59] thus altering the normal function of mitotic spindle disturbing the chromosome movements to the cell poles. Chromosome gaps/chromatid separations (Fig.5, Plate: 9), which represent losses of chromatin material, might represent damage of the protein part of the chromosome rather than the whole chromosome. Studies on copper induced anaphase bridges, micronuclei, laggards, chromosome stickiness and broken nuclei are available on *Zea mays, Helianthus annuus, Vicia faba* and *Allium sativum,* [58, 60-62].

Micronuclei, an "End point cytogenotoxic biomarker" indicating genome loss, happened to form either as a result of elimination of amplified genetic material [62] or from acentric fragments (clastogenic action), chromosomal losses from malformation of the mitotic spindle (aneugenic action) [48] failing to enter into either of the daughter nuclei during telophase. Micronucleus size can be an effective biomarker parameter to assess the clastogenic and aneugenic effects [63]. So, large micronuclei would indicate an aneugenic effect resulting from a chromosome loss, whereas small micronuclei may prove a clastogenic action resulting from chromosome break. Our results on copper-induced aberrations are in agreement with previous literature data. In this study the maximum frequency of micronuclei was observed at 6 ppm, but their presence was also noticed in 8 ppm (Fig.5, plate 15). Many authors are of thought that this high incidence of ana-telophase chromosome aberrations could be the consequence of copper-induced oxidative stress and generation of reactive oxygen species (ROS) [16]. Reactive oxygen species (ROS) and their intermediary products easily hamper in a negative fashion the chemical configuration of genetic material, physical state and alter its normal condensations after replication or repair process, thereby altering the biological state of nucleic acids. Formation of C-mitoses after copper treatment was also confirmed (Figure 1, Plate: 8) in our study just like other plant systems, such as *Allium sativum* L [61]. Prominent anaphase bridges were observed (Fig.5, plate: 5, 10) as a major aberration throughout the study and this could be due to chromosomal breaks and reunion of broken ends of chromosomes [64] yielding different outcomes, including chromosome breakage, polyploidy, aneuploidy, and possibly cell cycle arrest [65]. So it may be inferred that copper at higher doses (6, 8 and 10 ppm) is a clastrogen that can induce chromosome breaks by the formation of ROS and it can also act as an aneugen that induces micronuclei and lagging chromosomes [66].

The TTC is a colourless stain generally used as an indicator to check the indirect and partial measure of the functionality of mitochondrial electron transport chains [67]. Electrons for the reduction of colourless TTC to coloured formazan [68] were produced from the mitochondrial electron transport system by the dehydrogenase enzyme activity. The insoluble formazan production (red color) in TTC staining was inversely proportional to the concentration of interfering mitochondrial poisoning as a means of screening for cells that have a dysfunctional respiratory chain [69] (Fig. 4). Mitochondria have several functions in living cells [70] and been found to play a major role in including energy production, calcium buffering, and regulation of cellular apoptosis owing to disruption of the membrane potential of mitochondria and subsequent release cytochrome *c* and triggering activation of caspases. [71]. Mitochondria have many proteins and activation of these proteins could promulgate programmed cell death pathways that result in the demise of the cell. In many of these pathways, permeabilization of mitochondrial membranes is a critical event that results in release of various molecules from the mitochondrial inter-membrane space that are crucial for apoptosis [72]. Thus, a variety of key events in apoptosis focus on mitochondria, including the release of caspase activators, changes in electron transport, loss of mitochondrial transmembrane potential, altered cellular oxidation-reduction, and participation of pro- and antiapoptotic Bcl-2 family proteins, all of which later leads to mitochondrial dysfunction [68]. In the present study, decrease in TTC staining and corresponding decrease in absorbance measured, manifested the toxic effect of copper on root cell mitochondrial activity. In this present assay in *Lathyrus sativus* L. root tips the highest concentration copper treatment in roots have a lesser capacity to produce the colour which might be due to the lack of electrons which indirectly show the inhibition of mitochondrial activity and subsequent cellular death brought about by toxic doses of copper in the cellular environment. In the same way, rate of necrosis and apoptosis were hiked in higher (6, 8, and 10 ppm) concentrations. It can be deciphered that in *Lathyrus sativus* L. root tips enhanced apoptosis could be be due to the release of cytochrome *c* through copper induced ROS which lowered the mitochondrial activities and subsequent necrosis and apoptosis (Fig 5, Plate 15, 16).

Similar reports had been reported from experiments with intact lichens and cultured symbionts where with this rapid and inexpensive quantitative method was used as an "Enzymatic biomarker" (dehydrogenase activity) and formation of the coloured product triphenyl formazan (TPF) being produced after reduction of triphenyl tetrazolium chloride (TTC). Exposure to copper significantly reduced the capacity of cultured lichens and bionts to produce formazan, suggesting that copper brought about cellular damage at higher does and could be regarded as respiratory chain poison for mitochondria leading to cellular death [73].

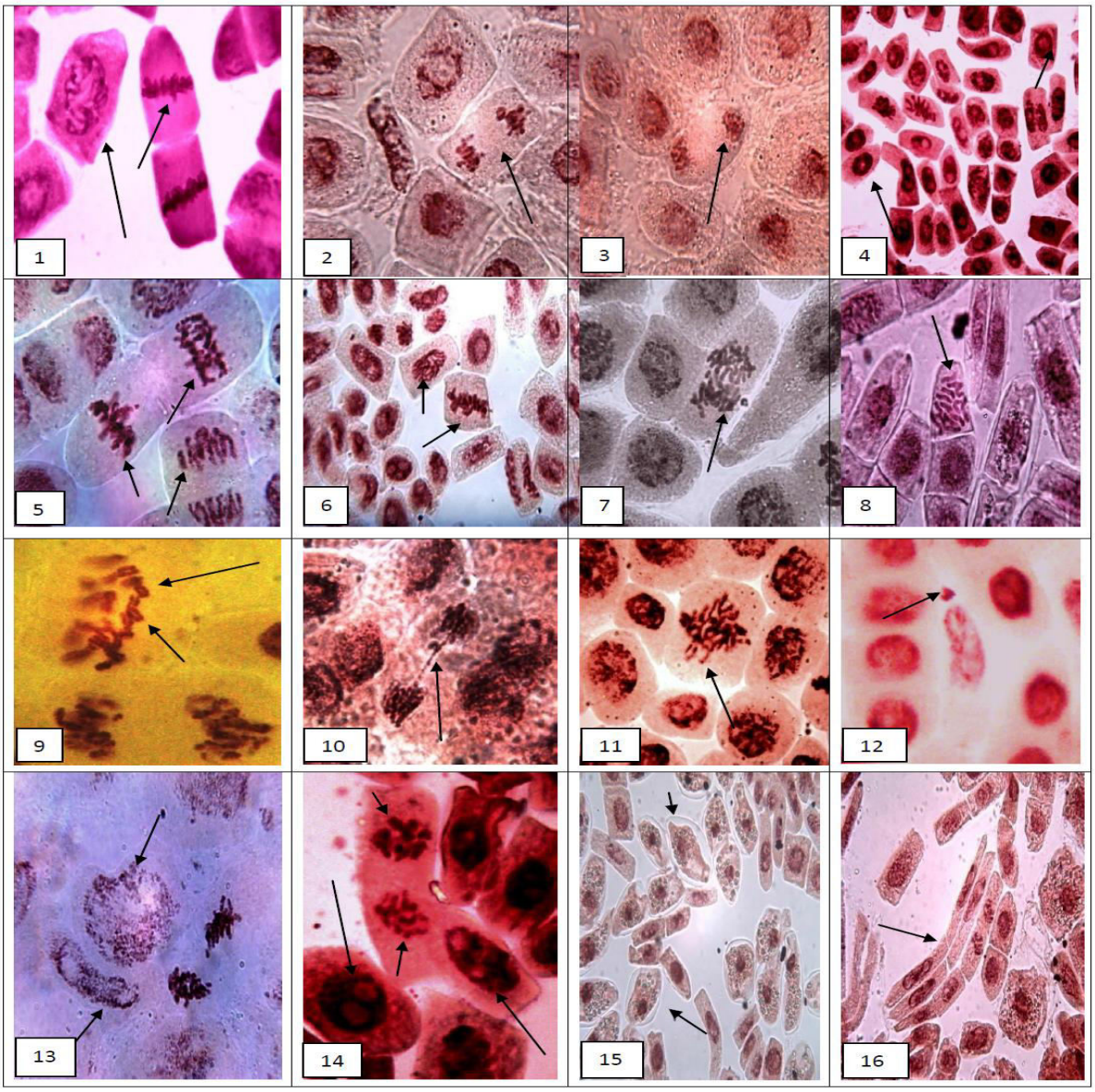


Fig. 5: Abnormal Cytology Pictures after treatment with different concentrations of copper salt at germinating root tips of *L. sativus* L after 72 hrs

1= Control (Prophase and Metaphase), 2= Control (Anaphase), 3= Control (Telophase), 4= nuclear blebbing and Nuclear lesion at 4 ppm, 5= Metaphasic clumping, acentric fragmentation and trophokinesis at 4 ppm, 6=Nuclear lesions, metaphasic stickiness, trophokinesis, and early condensation at 4 ppm treatments, 7= C-Mitosis and Polyploidization at 4 ppm treatment, 8= C-Mitosis at 6 ppm treatment, 9= Heteropycnosis and chromatid swelling (puffiness) at metaphase forming centric fission showing chromosome gaps at 6 ppm treatment, 10= Telophasic stickiness with early fragmentation at 6 ppm treatment, 11= Multipolarity and early separation at metaphase after 6 ppm treatment, 12= Micronuclei formation at 8 ppm treatment, 13= Pulverized nucleus with chromatin erosion at 8 ppm treatment in early apoptotic cells, 14= Binucleate condition with nuclear erosions and disrupted telophase without formation of nuclear membrane at 8 ppm treatment. 15= Necrotic cells with autophagy and degrading nucleus, cells are highly vacuolated with degrading protoplams and nuclear fragmentations at 8 and 10 ppm, 16= Giant Dead cells with nuclear lesion and erosion at 10 ppm treatment.

5. CONCLUSION

In this work, the manifestation of mitodepressive, clastogenic and aneugenic activity together with loss of mitochondrial dysfunction depicted by TTC staining, reiterated the toxic effects of copper in germinating *Lathyrus sativus* L. root tip cells. The present study suggests that employment and use of more than one population biomarker, such as seed germination percentage, root length inhibition with total soluble protein, frequency of micronuclei as cytological

biomarker and a biochemical biomarker *i.e.* root metabolic activity (TTC to formazan formation by mitochondrial dehydrogenase activity) could eke out a simple, straightforward method for identifying the impact of heavy-metal contamination on the genetic material of higher plants. Also, these biomarkers could help detect the effects of the xenobiotics easily, since the responses of various biomarkers have been differential at various levels of the organism's health status. The use of population and cytological biomarkers could be fundamental for accumulating more informative results and to understand clearly the effect of any contaminants on model organisms in ecotoxicological studies. In the present study copper possibly involved in the interactions with DNA, either directly or indirectly *via* oxidative stress via disruption of mitochondrial functions. The amplitude of the responses to copper action in *Lathyrus sativus* L. is enough to conclude that it is important to employ more thorough studies to evaluate the genetic risks of excess copper and its damage at genetic level and of phenotypic repercussions of this injury in other cash crops too. We consider that these results proved the clastogenic and aneugenic potential of copper and they evoke an alarming signal about the increasing presence of copper into environment and its toxicity in the living beings as a result of indiscriminate use of this heavy metal in agriculture as pesticides and fungicides. Excess of copper could bring possible unwanted repercussions on the economically important phenotypic traits in cereals, pulses and all varieties of cash crops and decrease their yields thus negatively influencing the human health; therefore, specific measures have to be taken for preventing soil pollution. Thoroughgoing studies inclusively at molecular level are necessary in order to find those plants which can absorb and accumulate the harmful heavy metals and allow their use in the phytoremediation programs. Also it is recommended to grow some hybrid species which require a minimum of spraying with copper-containing compounds and banning of copper-containing chemical fertilizers and Pesticides, in conjunction with their replacement by some potential relatives possessing strong phytoremediation potentialities.

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