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# Studies on Toxicity of Ag (I) on Plants and Microbes

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## ABSTRACT

Agricultural plants viz. chick pea, black gram and green gram were exposed to various concentrations of Ag(I) (50, 100, 150 and 200 mg/L). Significant decrease in germination percentage and growth of the seedlings were noted with the increase in Ag(I) concentration. Toxic effects of Ag(I) on plants were reflected by, an increase in malondealdehyde level (MDA) which is an index of lipid peroxidation and reduction of photosynthetic pigments. Ag(I) toxicity resulted in a significant hyperactivity of antioxidant enzymes viz. superoxide dismutase (SOD) and catalase in different parts of the plant. The activity of ascorbate peroxidase showed a decrease with an increase in concentration ofAg(I). Toxicity of Ag(I) on different microorganisms viz. *Pseudomonas aeruginosa , Azospirilium*, *Saccharomyces cerevisiae* and *Trichoderma viride* were assessed. The colony count (bacteria) and hyphal growth (fungi) of the test organisms were found to decrease with increase in concentration of Ag(I).

**Keywords**: Ag(I) toxicity; photosynthetic pigments; lipid peroxidation; antioxidant enzymes, microbial toxicity.

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## INTRODUCTION

Silver is one of the toxic forms of heavy metal and has been assigned to the highest toxicity class [1, 2]. Silver from industrial and public wastewater is bound to the activated sludge of wastewater treatment plants. The remaining portion of silver enters the aquatic environment and adsorbed to the sediments at the discharged site and thus immobilized. A small amount of silver is kept in solution by colloidal and complexed material and transported to lakes, estuaries or sea [3]. Annual silver released to the environment from industrial wastes and emissions has been estimated at approx. 2,500 tonnes, out of which 80 tonnes is released into the surface waters and 150 tonnes gets into the sludge of wastewater treatment plants [4, 5]. The major amount of silver (> 94 %) released into the environment remain in the soil or wastewater sludge at the emission site [6].

Silver is known to be released to the environment through its various industrial applications, leading to the possible exposure of aquatic organisms [7]. The accumulation of silver ions in living organisms through the food chain causes numerous diseases and disorders [8, 9]. In humans, accidental ingestion of silver nitrate has produced corrosive damage of the gastrointestinal tract, respiratory irritation and abdominal pain, discoloration of skin, diarrhea, vomiting, shock, convulsions and death [10]. Mild allergic responses have also been attributed to dermal contact with silver [11]. Therefore, industrial wastewater containing silver may cause serious human health hazards as they are being disposed off in water bodies which may be used for an agricultural purpose. Thus, it may be of serious concern to assess the toxicity of silver on plants and the microbes grown in the soil.

A better understanding of total silver concentrations in various environmental compartments and in particular, silver speciation has emerged based on enhanced heavy metal analysis using various ultraclean experimental techniques [12, 13, 14, 15, 16]. Toxicity of silver occurs mainly in the aqueous phase and depends on the concentration of active, free Ag(I) ions. Silver sulfide is the least toxic of all tested silver compounds, because of its low solubility and bioavailability. In soil, sewage sludge, and sediments, in which silver sulfide predominates, the toxicity of silver, even at high total concentrations, is very low. Silver thiosulfate, a highly soluble compound and main component of wastewaters of photoprocessors, has a very low toxicity (e.g. it is 15,000- 17,000 times less toxic than silver nitrate). Silver nitrate is the most toxic silver compound [17].

Earlier reports are available on toxic effects of silver to some plants and bacteria [4, 18, 19]. There are still many unresolved issues and challenges concerning the biological effects of silver nitrate on plants and microbes. The aim of this investigation was to provide more information on toxicity of Ag(I) on leguminous plants and various microbes. The impact of Ag(I) toxicity on photosynthetic pigments and antioxidant enzymes involved in protecting the plant from silver toxicity has been studied. Apart from this, the effect of Ag(I) on microbes viz. *Pseudomonas aeruginosa*, *Azospirillum* sp*, Trichoderma viridae* and *Saccharomyces cerevisiae* has also been investigated.

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## MATERIALS AND METHODS

### Phytotoxicity studies

### Effect of Ag(I) on germination and plant growth

Phytotoxicity tests were performed in order to assess the toxicity of Ag(I) on plants. This test was carried out on three kinds of seeds commonly used in the Indian agriculture (*Cicer aeritimum, Vigna mungo* and *Vigna radiate*). Seeds of these plants were sterilized in 10 % hydrogen peroxide for 20 min. The seeds were then thoroughly washed with distilled water and germinated on moistened filter paper at 25°C. The uniform seedlings were then transferred to the pots (six seeding per pot). These pots were irrigated daily with $AgNO_3$ solution of different concentrations (50, 75, 100, 150, 200, 250 mg/L). Experimental conditions that are used for optimal growth of the plants under regular agricultural conditions were maintained in the growth chamber (28 ºC / 70 % relative humidity during the day, 25ºC / 90 % humidity in night). A photoperiod of 16 hours was maintained. Toxic effect was measured in terms of percent germination and root and shoot lengths after two weeks of $AgNO_3$ treatment. In another set of experiment, leaves, stems and roots from 2 weeks old seedlings were collected from three different types of plants for estimation of photosynthetic pigments and antioxidant enzymes.

### Lipid peroxidation

The level of lipid peroxidation in fresh leaves was measured in terms of malonialdehyde (MDA) content which was determined using thiobarbituric acid method [20]. Initially the leaves were homogenized in 5% (w/v) trichloroacetic acid and then centrifuged. A sample of the supernatant was added to 20% trichloroacetic acid containing 0.5% (w/v) thibarbituric acid. The reaction mixture was incubated at 95ºC for 30 min. The concentration of thiobarbituric acid reacting substances was calculated using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

### Assay of antioxidant enzymes

Plant material (0.5 g leaves) was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1 % insoluble polyvinylpyrrolidone (w/v) at 4°C with mortar and pestle ( 0.1 g FW/ml buffer) , filtered through four layers of cheese cloth and centrifuged at 15,000 X g for 10 min. The supernatant obtained was designated as crude enzyme extract and was used for various antioxidant enzyme assays.

### Ascorbate peroxidase assay

The activity of ascorbate peroxidase was measured by estimating the rate of ascorbate oxidation [21]. 3 mL of enzyme mixture consisted of 50 mM phosphate buffer (pH 7.0), 0.1 mM $H_2O_2$, 0.5 mM sodium ascorbate, 0.1 mM EDTA and 100 µl enzyme extract. The activity was expressed in terms of µmol/of ascorbate oxidized $min^{-1}$ $g^{-1}$.

**Catalase assay**

The activity of catalase was measured by monitoring $H_2O_2$ decomposition at 240 nm in 3 ml reaction mixture containing 50 mM phosphate buffer (pH 7.0), 15mM $H_2O_2$, 100 µl homogenate and 0.1% (v/v) Triton X-100 [22]. The enzyme activity was expressed in terms of µmol of $H_2O_2$ reduced $min^{-1}$ $g^{-1}$.

**Superoxide dismutase activity**

Superoxide dismutase activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium [23]. In the absence of the enzyme the reaction mixture developed the maximum colour which decreased with increasing volume of the enzyme extract. The volume of the enzyme extract corresponding to 50% inhibition of the reaction was calculated by plotting a graph between the enzyme concentration in the reaction mixture and its absorbance at 560 nm and was considered as one unit enzyme. The activity of the enzyme was expressed as unit/g FW.

**Estimation of Photosynthetic pigments content**

Chlorophyll *a*, chlorophyll *b* and carotenoids from leaves of the plants of different heights were extracted in 80% acetone for 24 h in darkness, at 4ºC. The suspension thus obtained was centrifuged at 3000 g for 5 min and the absorbance measured at 460, 645 and 663 nm with a UV-visible spectrophotometer. The pigment concentrations were calculated by equations allowing a simultaneous determination of chlorophylll *a*, chlorophyll *b* and carotenoids in the same solution following the method of Lichtenthaler (1987) [24].

**Microbial toxicity studies**

Microbial toxicity studies were carried out for bacteria, fungi and yeast viz. *Pseudomonas aeruginosa*, *Azospirillum* sp*, Trichoderma viridae* and *Saccharomyces cerevisiae* respectively. The nutrient medium containing 1.5% agar was used for the study. Toxicity was measured in terms of presence or absence of growth, as well as CFU/ml for different concentrations of silver after 24 h and 3 days of incubation for bacteria and fungi respectively.

## RESULTS AND DISCUSSION

**Effects of Ag (I) on germination and plant growth**

The toxic effects of Ag(I) on three plant seeds *viz*. chick pea, black gram and green gram are presented in Table 1. For all three plant seeds, the germination % and the root and shoot lengths of the plants were reduced due to toxicity of Ag(I). Seeds germinated in control are significantly different from the seeds germinated in different concentration of silver nitrate.

Seeds of chick pea and black gram failed to germinate at Ag(I) concentration above 200 mg/L whereas green gram seeds showed germination only up to Ag(I) concentration of 150 mg/L. This difference in germination might be attributed to the high susceptibility of green gram seeds to silver compared to the other two types of plant seeds. Similar effects were observed in root and shoot length of the germinated seedlings.

**Table 1. Effect of different concentrations of Ag(I) on germination % and growth of seedlings after 2 weeks of sowing**

| Seeds | Concentration of silver (mg/L) | Germination % | Root length (cm) | Shoot length (cm) |
|---|---|---|---|---|
| Chick pea | 0 | 100±0 | 15.0±0.03 | 17.0±0.01 |
| | 50 | 100±0 | 6.0±0.03 | 10.0±0.02 |
| | 75 | 80±3.3 | 4.0±0.04 | 7.5±0.02 |
| | 100 | 60±5.7 | 3.2±0.03 | 7.2±0.04 |
| | 150 | 40±3.3 | 2.0±0.01 | 3.6±0.01 |
| | 200 | 20±0 | 1.7±0.03 | 2.0±0.02 |
| | 250 | - | - | - |
| Green gram | 0 | 100±0 | 7.0±0.02 | 18.0±0.03 |
| | 50 | 70±5.7 | 4.1±0.01 | 5.2±0.01 |
| | 75 | 50±0 | 2.9±0.02 | 3.4±0.02 |
| | 100 | 30±3.3 | 1.5±0.03 | 2.2±0.04 |
| | 150 | 10±0 | 1.2±0.04 | 1.4±0.02 |
| | 200 | - | - | - |
| | 250 | - | - | - |
| Black gram | 0 | 100±0 | 5.0±0.01 | 18.0±0.01 |
| | 50 | 80±0 | 4.9±0.02 | 5.7±0.02 |
| | 75 | 70±5.7 | 3.1±0.02 | 3.8±0.02 |
| | 100 | 40±3.3 | 2.3±0.01 | 2.6±0.03 |
| | 150 | 20±0 | 1.9±0.02 | 2.1±0.04 |
| | 200 | 10±0 | 1.2±0.02 | 1.6±0.01 |
| | 250 | - | - | - |

**Table 2. Effect of Ag(I) concentration on various microorganisms**

| Microorganisms | Ag(I) concentration | | | | |
|---|---|---|---|---|---|
| | 0 mg/L | 50 mg/L | 100 mg/L | 150 mg/L | 200 mg/L |
| | Cell count (CFU/ml) at different concentrations of Ag(I) | | | | |
| *Pseudomonas* sp. | $11x10^6$ | $9.8x10^5$ | $5.9x10^4$ | - | - |
| *Azospirillum* sp. | $8.5x10^5$ | $7.5x10^4$ | $4.7x10^3$ | $3.8x10^2$ | - |
| *Saccharomyces cerevisiae* | $9.4x10^5$ | $8.7x10^4$ | $5.8x10^3$ | $1.9x10^2$ | - |
| | Colony diameter (mm) | | | | |
| *Trichoderma viridae* | 90 | 27 | 14 | - | - |

## Lipid peroxidation

In the present study, an increase in malondialdehyde content of root, stem and leaves of the test plants was observed with increase in concentration of Ag(I) used in the treatment (Fig. 1). Accumulation of MDA was relatively higher in the leaves than in the roots and stems. Compared to control plants the leaves of plants treated with Ag(I) at a concentration of 200

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mg/L resulted in approximately three-fold increase in MDA content. Membrane destabilization is generally attributed to lipid peroxidation, due to an increased production of reactive oxygen species [25]. Thus higher MDA content in the tissues of the plant might be due lipid peroxidation and membrane damage caused due to high Ag(I) concentration.

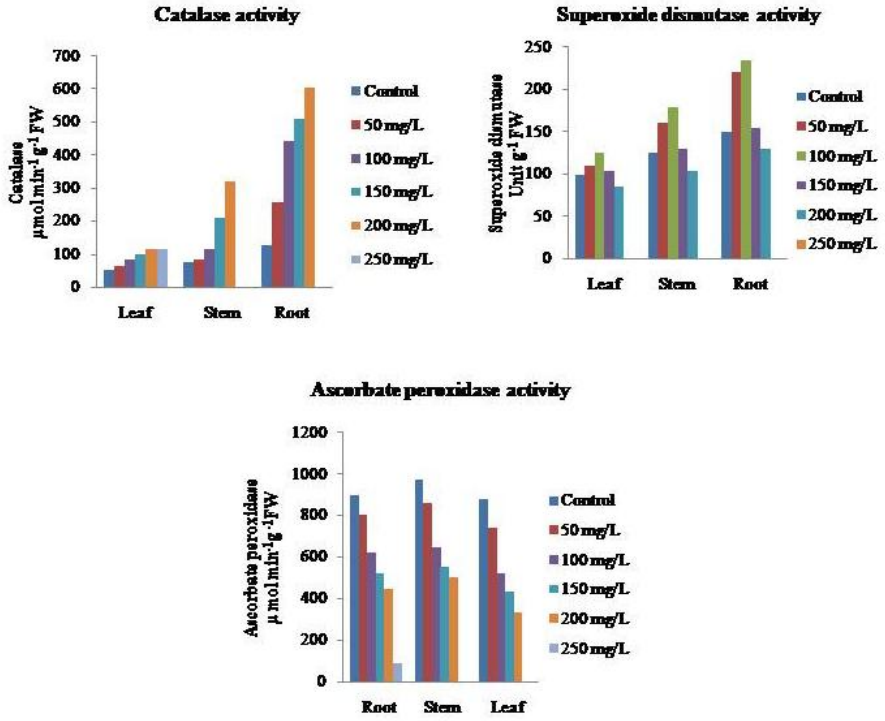


**Fig 1. Antioxidant enzyme activity in chick pea at various concentrations of Ag(I)**

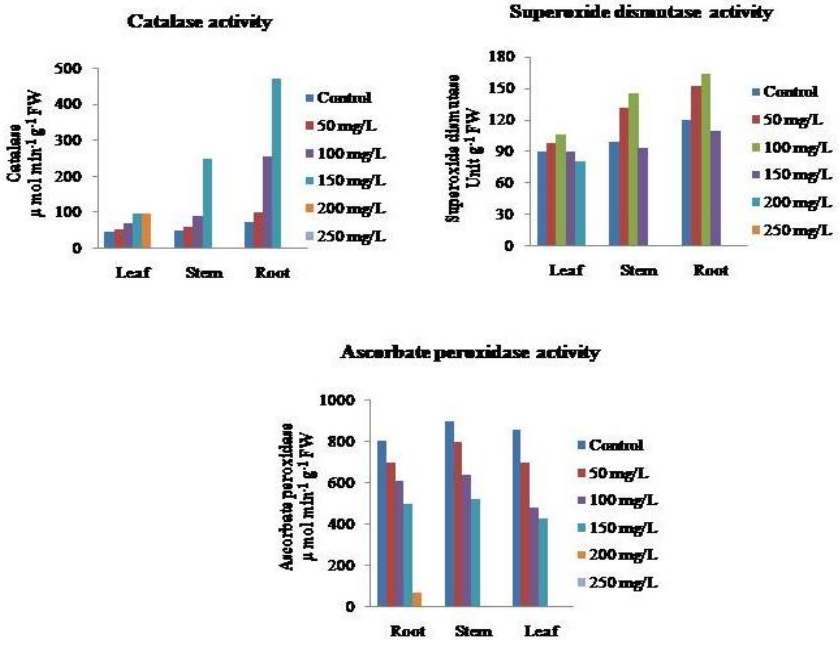


**Fig 2 Antioxidant enzyme activity in black gram at various concentrations of Ag(I)**

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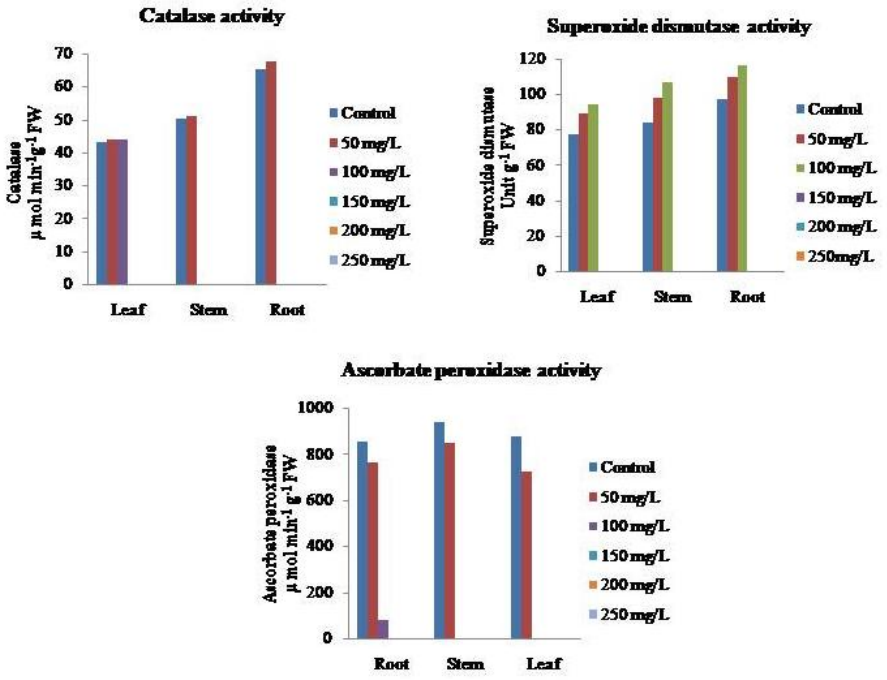


**Fig 3. Antioxidant enzyme activity in green gram at various concentrations of Ag(I)**

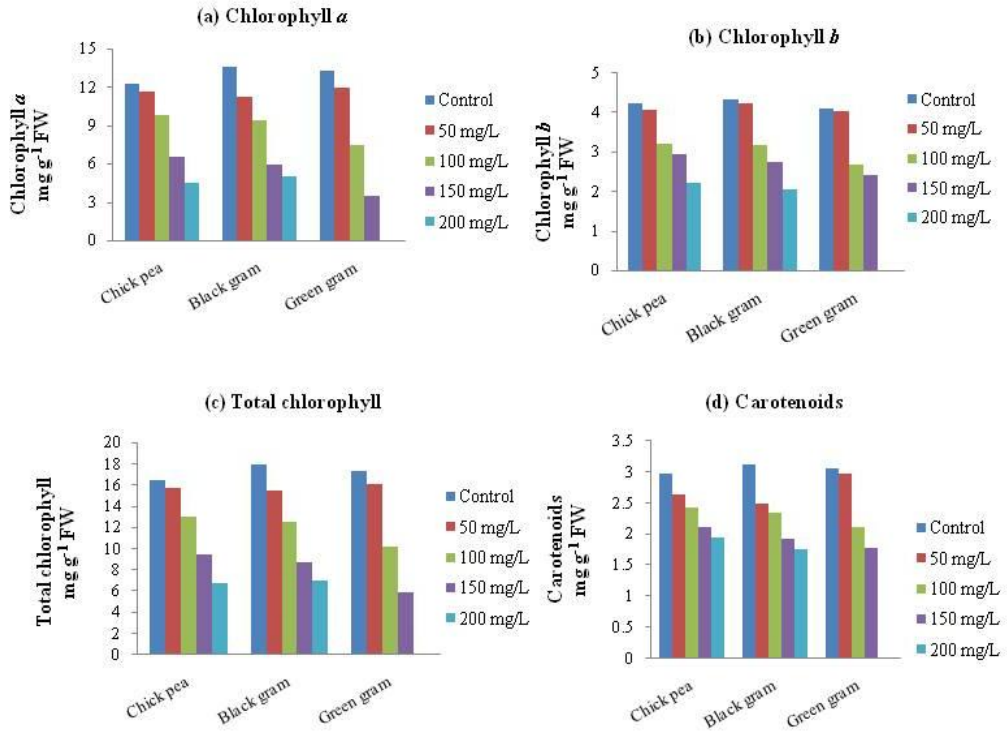


**Fig. 4 Effect of Ag(I) concentrations on (a) Chlorophyll *a* content (b) Chlorophyll *b* (c) Total chlorophyll and (d) Carotenoid content in chicl pea, black gram and green gram**

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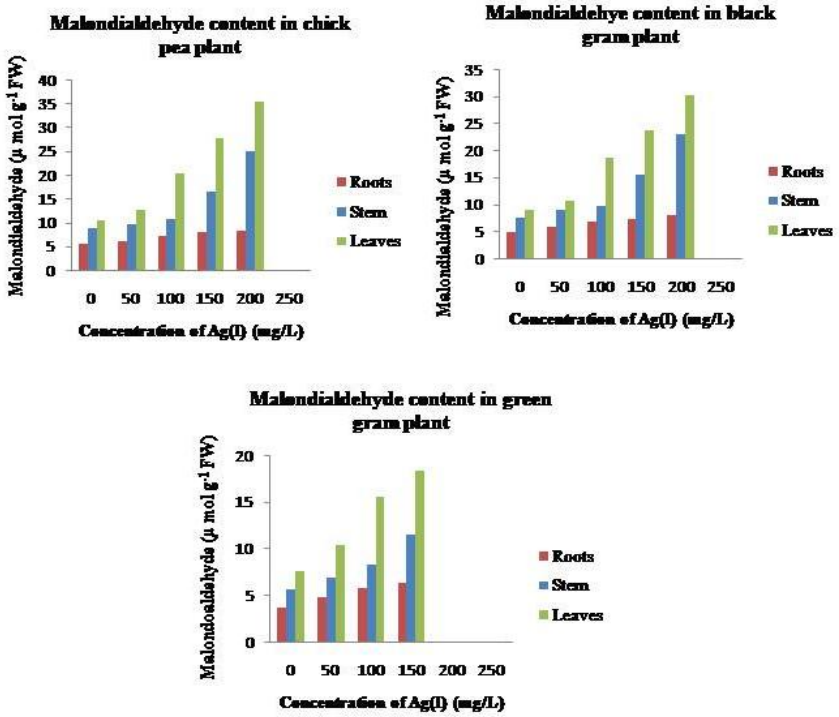


**Fig. 5 Effect of Ag(I) concentration on malondialdehyde content in chick pea, black gram and green gram**

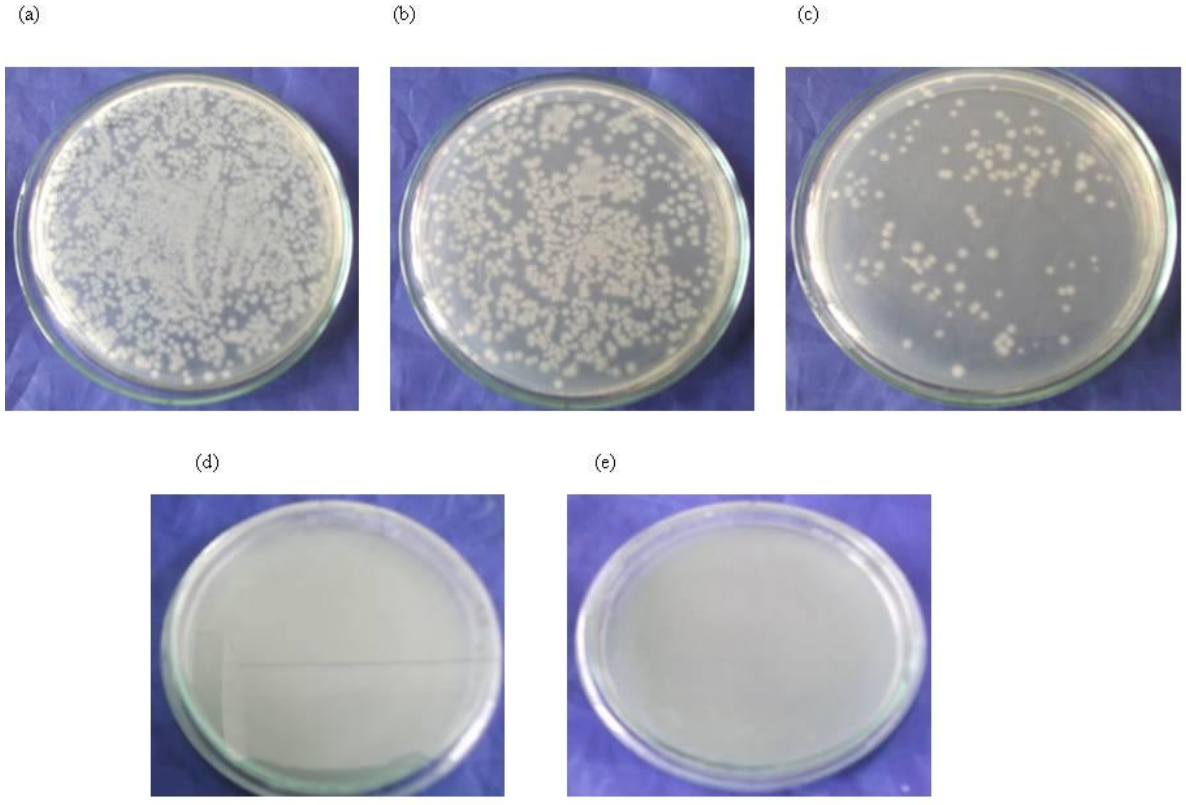


**Fig. 6 Growth of *Pseudomonas* sp. in media containing Ag(I) at concentration of (a) Control (b) 50 mg/L (c) 100 mg/L (d) 150 mg/L(e) 200 mg/L**

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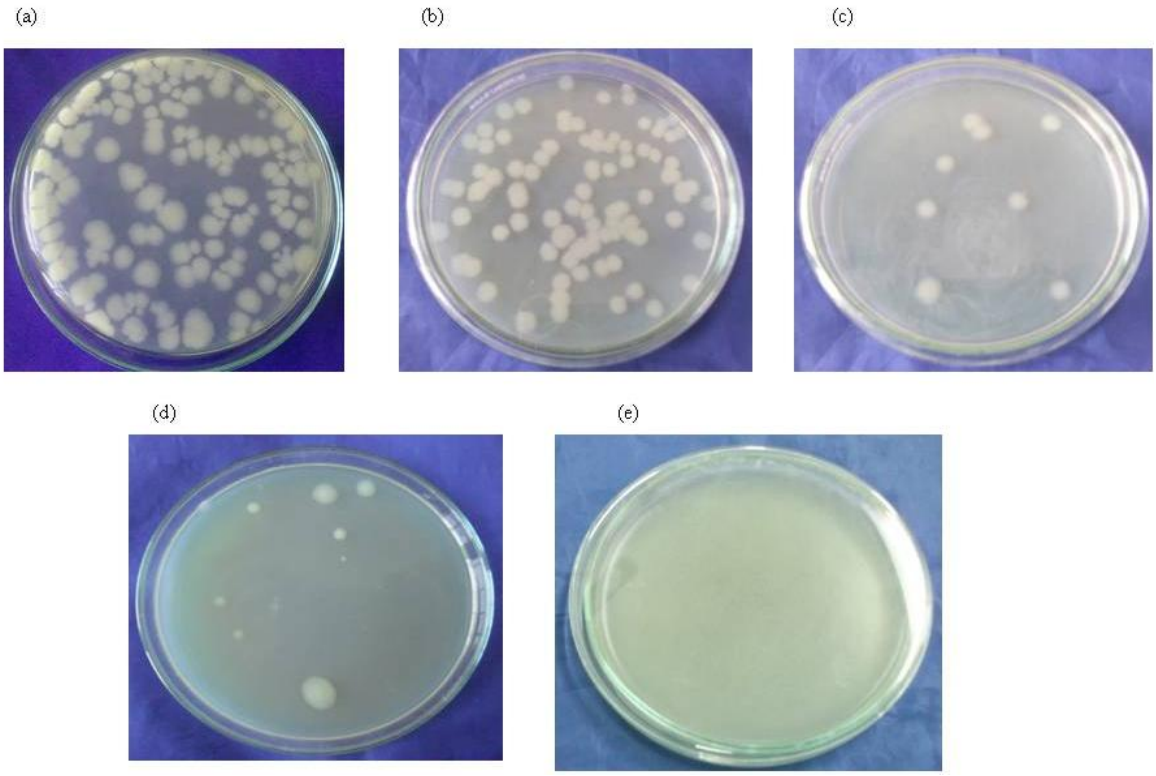


**Fig.7 Growth of *Azospirillum sp*. in media containing Ag(I) at concentration of (a) Control (b) 50 mg/L (c) 100 mg/L (d) 150 mg/L (e) 200 mg/L**

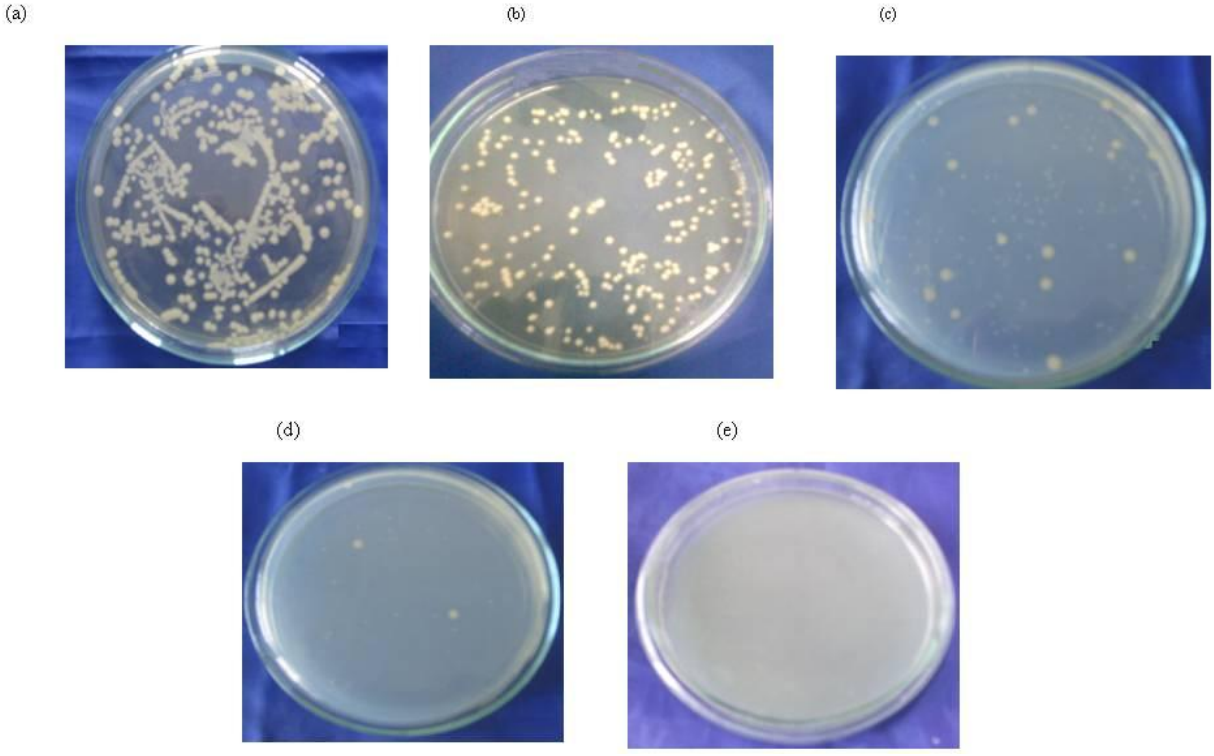


**Fig.8 Growth of *Saccharomyces cerevisiae*. in media containing Ag(I) at concentration of (a) Control (b) 50 mg/L (c) 100 mg/L (d) 150 mg/L(e) 200 mg/L**

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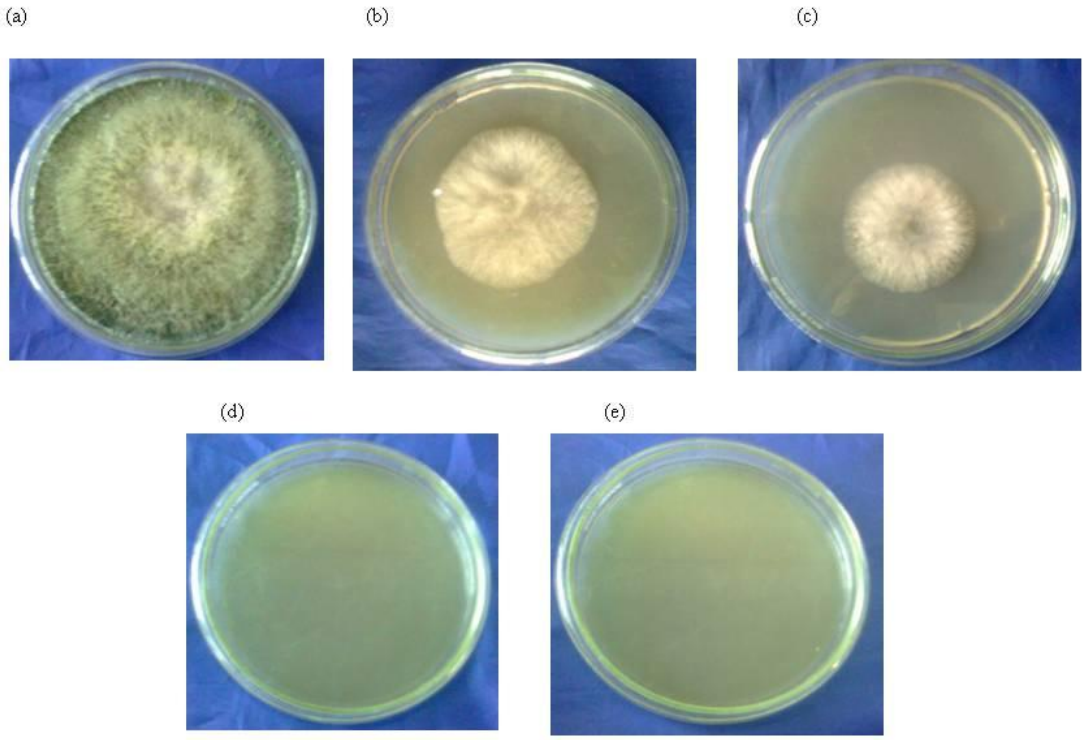


**Fig.9 Growth of *Trichoderma viridae* in media containing Ag(I) at concentration of (a) Control (b) 50 mg/L (c) 100 mg/L (d) 150 mg/L(e) 200 mg/L**

**Antioxidant enzyme assay**

Heavy metals are reported to induce reactive oxygen species in plants [26]. Plants have evolved a complex system involving antioxidant enzymes in order to overcome the damage caused by these reactive oxygen species. In the present study, it was observed that all the three plants showed lower enzyme activities of catalase and superoxide dismutase in leaves compared to roots and stem (Fig. 2, 3 and 4). In chick pea catalase activity showed a steep increase with increasing Ag(I) concentration where as superoxide dismutase activity increased up to a Ag(I) concentration of 100 mg/L and decreased on further increase in Ag(I) concentration. Similar results were observed with catalase and superoxide dismutase activities in black gram. In case of green gram no enzyme activity was noticed beyond 100 mg/L which might be attributed to the high metal stress in these plants. The results of this study also shows that green gram is more susceptible to Ag(I) toxicity because its antioxidant enzyme system could not help the plant to mitigate and repair the damage caused by reactive oxygen species. Ascorbate peroxidase is the member of the ascorbic acid-glutathione cycle, and plays a major role in eliminating poisonous $H_2O_2$ from plant cells. In this study, all concentrations of Ag(I) used in the experiment inhibited ascorbate peroxidase activity which shows that ascorbate peroxidase has the least role in detoxification of $H_2O_2$ due to its sensitivity to Ag(I).

**Estimation of photosynthetic pigments**

The test plants treated with different concentrations of Ag(I) showed variation in photosynthetic pigment content (Fig. 5). In all the three types of plants, treatment with 50 mg/L of Ag(I) exhibited a slight decrease in the values of chlorophyll *a*, chlorophyll *b,* total chlorophyll and carotenoids over the control plants. The deleterious effect of Ag(I) was significant from 100 mg/L and increased with increasing concentrations. The effect was more pronounced in green gram which showed a reduction in chl a, chl b, total chl and carotenoids content by 74%, 41.5%, 70% and 42% respectively on treatment with Ag(I) at concentration of 150 mg/L. At Ag(I) concentrations above 200 mg/L (in chick pea and black gram) and 150 mg/L (in green gram) showed no germination of seed, toxicity test was carried out till 200 and 150 mg/L for the respective plants.

Reduced levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids in the test plants may be attributed to the toxicity of Ag(I) to chlorophyll biosynthesis. Increased lipid peroxidation resulting in high MDA content reported in this study may also cause degradation of photosynthetic pigments.

**Microbial toxicity studies**

Toxicity of Ag(I) was tested on various microorganisms. Initially the toxicity of Ag(I) was studied based on the presence or absence of bacterial and fungal growth in nutrient agar and SDA medium respectively. Four different microbes were grown in culture plate containing different concentrations of Ag(I). Figures 6, 7 and 8 show the toxic effect of Ag(I) on the growth of microbes. Among the four test organisms, *Pseudomonas* sp. showed growth upto 100 ppm concentration. There was no growth in plate containing 150 and 200 mg/L Ag(I). The other two organisms, *Azospirillum* sp. and *Saccharomyces cerevisiae* showed growth upto 150 mg/L. The colony diameter of the tested fungal species (*Trichoderma viridae)* showed a drastic reduction with increasing concentration of Ag(I) and at concentrations of 200 mg/L there was absolutely no growth.

The colony count of the test organisms at different Ag(I) concentration used in this study is shown in table 2. The colony count of *Pseudomonas* sp. in the control plate was $11x10^6$ CFU/mL (Table 2), which was reduced to 5.9 $x10^4$ CFU/mL in the plate containing 100 mg/L Ag(I). The colony count of *Azospirillum* sp. and Saccharomyces *cerevisiae* in the control plate was 8.5 $x10^5$ and 9.4 x $10^5$CFU/mL respectively which also decreased with increase in Ag(I) concentration. Delayed hyphal growth of *T. viridae* was found in the plate containing 100 mg/L caffeine and complete inhibition of growth was observed in the plate containing a Ag(I) concentration of 150 and 200 mg/L.

## CONCLUSION

The results of this investigation show that Ag(I) at a concentration of above 200 mg/L affected seed germination as well as root and shoot length in chick pea and black gram where

as green gram was highly susceptible since the seeds failed to germinate at a concentration above 150 mg/L. Ag(I)- induced oxidative stress was tolerated by these plants through the hyperactivity of antioxidant defense system. The $H_2O_2$ formed by the superoxidation of active oxygen species was quenched by catalase and superoxide dismutase. However, ascorbate peroxidase had a least role in the quenching of $H_2O_2$ due to its high sensitivity to Ag(I). High levels of malondialdehyde accumulation in different parts of the plants might be attributed to lipid peroxidation, due to an increased production of reactive oxygen species. Reduced levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids was observed in all the three test plants due to the toxicity of Ag(I). Apart from phytotoxicity, Ag(I) also showed reduced colony count and hyphal growth with increased concentration of Ag(I) in case of the selected microorganism.

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