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RESEARCH ARTICLE

Glutamic acid assisted phyto-management of silver-contaminated soils through sunflower; physiological and biochemical response

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Abstract

Phytoremediation is a cost-effective and eco-friendly technique for the removal of heavy metal-contaminated soils and water. The less availability and mobility of heavy metals in medium decreased the efficiency of this technique. The mobility and availability of these metals in the medium can be enhanced by the addition of organic chelators. The present study was conducted to investigate the possibility of glutamic acid (GA) in improving silver (Ag) phytoextraction by sunflower (*Helianthus annuus* L.). Different concentrations of Ag and GA were supplied in solution form in different combinations after defined intervals. Results depicted that increasing concentration of Ag significantly reduced the plant biomass, photosynthetic pigments, and antioxidant enzyme activities (like catalase, peroxidase, ascorbate, peroxidase, superoxide dismutase). Furthermore, Ag stress increased the Ag concentration and the production of reactive oxygen species (ROS) in sunflower plants. The addition of GA alleviated the Ag-induced toxicity in plants and enhanced Ag concentration and accumulation in sunflower. The addition of GA enhanced Ag accumulation in sunflower roots by 70, 79, 58, and 66% at 0-, 100-, 250-, and 500-μM Ag treatments, respectively, as compared to control plants. In conclusion, the results showed that Ag significantly reduced the physiological and biochemical attributes in term of reduced growth of sunflower and the addition of GA alleviated the Ag induced toxicity and enhanced Ag uptake. The results suggested that sunflower can be used as hyper-accumulator plant for the removal of Ag under GA. Further studies are required to understand the role of GA at gene and microscopic level in plants.

Keywords Phytoremediation · Photosynthetic pigments · Reactive oxygen species · Concentration

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Introduction

Nanotechnology is an emerging and revolutionary field attracting the researchers around the globe since few years. This interdisciplinary science deals with the synthesis and application of functional nanomaterial produced from metals and carbon at nanoscale (Sökmen et al. 2017; Kannan et el. 2010; Kumari et al. 2017). Many metals like silver (Ag), gold (Au), zinc (Zn), and aluminum (Al) have wide range of applications in nanoparticle structures and templates of the applications; silver has been extensively used against pathogenic microbes especially bacteria having high efficacy against them (Apalangya et al. 2014). Different biochemical and physiochemical methods have been used for the synthesis of silver nanoparticles (AgNPs) including plant-mediated (Prabu et al. 2015), plant terpenoids (Khan et al. 2016), polymers (Zhang et al. 2016), and microwave-assisted (Sökmen et al. 2017). Physicochemical, electromagnetic, antimicrobial, and unique optical properties of

AgNPs made their extensive use in antifungal products, disinfectants, electronic devices, water purification, textile products, and agriculture practices (Ayatollahi et al. 2016; Firdhouse and Lalitha 2015; Korbekandi and Iravani 2012). According to Buric et al. (2015), an amount of 500 t/annum of AgNPs is used to produce products of common use. At the same time on other side of picture, a very serious concern of toxicity and fate of AgNPs arising many questions on human health and environment (Sajid et al. 2015). Inclusion of AgNPs into food and vegetation by using polluted land and contaminated wastewater is posing serious threats to humans and animals health. These AgNPs are directly becoming the part of environment through domestic and industrial effluent and wastewater pouring into rivers and streams (Buric et al. 2015). Application of inorganic and organic fertilizers causing indirect deposition of Ag in soil and water (Nam et al. 2014; Baker et al. 2014). Extensive use of AgNPs in consumable products with transformed physicochemical properties and enhanced activity of AgNPs of size less than 100 nm is threatening the biota (Ma et al., 2015; Farkas et al. 2010). Minute concentrations of AgNPs exert immense toxicity in soil effecting enzymatic activities of soil microbes (Peyrot et al. 2014). Several research studies established negative impacts of AgNPs on edible crops including effects on pepper, reduced seed germination in *Phaseolus radiates* (Mung) and cucumber, reduced biomass of *Cucurbita pepo* (Pumpkin) (Vinkovic et al. 2017; Lee et al. 2012; Barrena et al. 2009; Stampoulis et al. 2009). Moreover, transformation of DNA resulting into abnormal agronomic traits, formation of reactive oxygen species (ROS), and affected transpiration rate is occurring in plants due to Ag (Atha et al. 2012; Dimpka et al. 2013; Sharma et al. 2014). Intake of silver as AgNPs contaminated food is leading exposure to humans resulting into ulcers and blockage of gastrointestinal track (Sajid et al. 2015; Foldbjerg and Autrup 2013).

Many methods, protocols, and techniques like mineralization, extraction, adsorption, microfiltration, and centrifugation have been extensively used but found limited in respect of their efficiency. Recently, an emerging and sustainable approach to remediate or recover silver (AgNPs) getting attention of researchers is phytoremediation. Phytoremediation is a technology that uses plant's natural mechanism for extraction, accumulation, and metabolism of inorganic contaminants from the contaminated soil and water (Evangelou et al. 2015; Padmapriya et al. 2015). Plant (tree, herb or shrub) must be native, hyper-accumulator, efficient in nutrient uptake capacity, network of deep roots, and significant biomass growth (Hanks et al. 2015; Ziarati et al., 2014). A number of studies identified remediation potential of plants in eliminating Ag and AgNP contamination from aquatic or soil media. Fernandes et al. (2017a) reported *Phragmites australis* ability to accumulate AgNPs in roots; Vinkovic et al. (2017) reported AgNP concentrations in roots, stem, and leaves of *Capsicum annuum* L. (pepper plant). Similarly, Lee et al. (2012) identified the bioaccumulations of AgNPs in *Phaseolus radiatus* and *Sorghum bicolor* accordingly.

Sunflower (*Helianthus annuus* L.) is a metal (-oilds) hyperaccumulator plant, capable of tolerating a certain concentrations of different heavy metals without showing toxicity (Atta et al. 2013). The effective phytoremediation of heavy metals using sunflower has been reported by Farid et al. (2017), Dhiman et al. (2017), Ashrafi et al. (2015), and Zalewska and Nogalska (2014). Rizwan et al. (2016) reviewed the toxicity mechanisms of Pb, Cu, Ni Cd, Cr, and Zn in sunflower. Nehnevajova et al. (2007) reported significant increase of metal extraction in sunflower mutant which was 7.5, 8.2, and 9.2 times higher than that in control plants for Cd, Pb, and Zn, respectively. A previous work by Barros-Galvao et al. (2017) proved the supportive role of glutamic acid (GA) for sunflower in growth attributes, biomass, lipids, soluble protein contents, and starch when applied at seedling stage of plant and strengthening the plant in its natural mechanism. Delauney et al. (1993) reported that in higher biomass plants, proline can be synthesized from glutamate or ornithine, which is the main pathway under water stress. The use of GA as mediator to enhance the plant growth for more phytoextration has not been extensively employed before. Keeping in view, the present work is aimed to identify the extraction of silver (Ag) by the sunflower along with the sufficient supply of GA.

Material and methods

Experimental growth conditions

An appropriate clay loam soil (clay 48%, sand 27%, silt 25%) with 0.74 mg kg^{-1} of silver (Ag) concentration was taken from a botanical garden of University of Gujrat, Pakistan. Homogenized soil air dried at room temperature and then ground to particle diameter of 2.0-mm mesh. Soil samples were analyzed for physico-chemical parameters like pH and conductivity. Healthy sunflower seeds were collected from the Oilseed Division of Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan. The seeds were sown into soil settled in plastic pots of 5.0-kg capacity, and whole setup was then placed into wire house. Temperature recorded was relatively low as 22–25 °C at the time of sowing than harvesting as 30–35 °C. Humidity recorded was apposite as 75% during sowing and at harvesting was 80%. After seedling of 15 days, plants were scattered into five plants/pot. A 500-mL solution containing mix fertilizer as 2.19-g L^{-1} N (as $(NH_2)_2CO$), 0.5-g L^{-1} P (as $(NH_4)_2HPO_4$), and 2.14-g L^{-1} K (as K_2SO_4) was given to irrigate plants fortnightly and monthly. All the pots and glassware rinsed with 10% HNO_3 were thoroughly washed using distilled water to neutralize their pH.

Treatments

After the duration of 4 weeks, plants were treated with different concentrations of silver (Ag) as 0, 100, 250, and 500 μM in combination with GA as 0 and 2.5 Mm. The eight treatments were established for the present study as, T_1: CK Ag 0 μM + GA 0 mM, T_2: GA 2.5 mM, T_3:Ag 100 μM, T_4: Ag 250 μM, T_5: Ag 500 μM, T_6: Ag 100 μM + GA 2.5 mM, T_7: Ag 250 μM + GA 2.5 mM, T_8: Ag 500 μM + GA 2.5 mM. Weed growth was prohibited throughout the experimental period. Treatments were applied after every 8 days till further 8 weeks.

Agronomic trait estimation

After a period of 6 weeks of treatment, sampling for growth parameters like plant height, root elongation, number of leaves/plant, leaf area, and biomass determination like root fresh/dry weight, leaf fresh/dry weight, and stem fresh/dry weight was performed. After taking fresh weight, plants were dried at 75 °C/72 h dry mass measurements, while fresh parts as leaf, stem, and root were preserved for physiochemical assessment.

Biochemical assessment

Chlorophyll and carotenoids contents were assessed after 6 weeks of given treatment. Decolorization of leaves was achieved by homogenizing leaves in 85% (*v*/*v*, Sigma) aqueous acetone to extract pigment at 4 °C in the absence of light and continuous shaking. Centrifugation of samples for 4000 rpm for 10 min was performed to get supernatant and analyzed at 663, 644, and 452.5 nm using a UV-Visible spectrophotometer (Halo DB-20/DB-20S, Dynamica Company, London, UK) (Metzner et al. 1965). Equations were solved and extinction coefficient was adjusted to determine the contents of chlorophyll and carotenoids according to (Lichtenthaler, 1987).

Equations for measuring chlorophyll and carotenoid contents:

$$\text{Chlorophyll a } (\mu g/mL) = 10.3 * - E_{663} - 0.98 * E_{644}$$
$$\text{Chlorophyll b } (\mu g/mL) = 19.7 * E_{644} - 3{:}\ 87 * E_{663}$$
$$\text{Total chlorophyll} = \text{chlorophyll a} + \text{chlorophyll b}$$
$$\text{Total carotenoids } (\mu g/mL) = 4.2 * E_{4552.5} - \{(0.0264 * \text{chl a}) + (0{:}\ 426 * \text{chl b})$$

Soluble proteins and antioxidant enzyme estimation

Following Bradford (1976), Coomassie brilliant blue G-250 as dye and bovine serum albumin as standard were used to determine soluble protein after 6 weeks. Frozen plants were grounded with mortar pestle, homogenized using 50-mM sodium phosphate (Na_3PO_4) buffer of pH 7.0 having 1.0-mm EDTA-Na_2 and 2% (*w*/*v*) polyvinyl pyrolidine-40 (PVP-40), and then centrifuged at 11000 rpm for 15 min at 4 °C. Antioxidant enzymatic activities and soluble protein were estimated using supernatant of centrifuged samples. Entire protein was estimated by adding Bradford solution into 100-μL crude extract, and absorbance was determined at 595-nm wavelength.

Protocol described by Aebi (1984) was followed for catalase (CAT) determination. Enzyme extract 100 μL, H_2O_2 100 μL, and 2.9-mL 50-mM phosphate buffer along with citric acid 2.0 mM (pH 7.0) were combined to compose assay mixture. Decrease in the absorbance at 240 nm due to H_2O_2 disappearance (ε = 39.4 mM^{-1} cm^{-1}) was the parameter to assess CAT activity.

Method presented by Nakano and Asada (1981) was adopted to assess ascorbate peroxidase (APX) activity. Enzyme extract 100 μL, ascorbate 100 μL, H_2O_2 100 μL, and 2.7-mL 25-mM potassium phosphate buffer along with citric acid 2.0 mM (pH 7.0) were mixed to compose mixture assay. To determine the oxidation activity of ascorbate, change in wavelength at 209 nm (ε = 2.8 mM^{-1} cm^{-1}) was assessed.

Electrolyte leakage and reactive oxygen species estimation

Fresh leaves and roots of plants after 6 weeks experimentation were placed into test tubes filled with 8.0-mL deionized water. Electrical conductivity (EC) was assessed by putting test tube in water bath having temperature of 32 °C for the duration of 2.0 h. Samples were autoclaved to let them discharge all electrolytes setting temperature till 121 °C for 20 min. Final EC was estimated by dropping the temperature to 25 °C Dionisio-Sese and Tobita (1998).

Formula for EC calculation is,

$$EL = (EC_1/EC_2) \times 100.$$

Hydrogen peroxide (H_2O_2) was estimated by the homogenized plant tissues (50 mg) and phosphate buffer (3 mL) by centrifugation at 6000 ×*g* per 25 min. Mixture of extract solution (3 mL) and titanium sulphate (1 mL 0.1%) was undergo for centrifugation at 6000 ×*g* for 15 min. The yellowish supernatant was measured at 410 nm. The H_2O_2 was calculated by extinction coefficient of 0.28 μ/mol/cm.

Lipid peroxidation was assessed in terms of MDA contents, resultant of lipid peroxidation by thiobarbituric acid (TBA) reaction method described by Heath and Packer (1968) with some modification was used as stated by Dhindsa et al., 1981and Zhang and Kirkham (1994).

Silver (Ag) concentration assessment

Dried plant material at 90 °C was turned into ash using muffle furnace heated at 600 °C for 6.0 h. Nitric acid (HNO_3) and hydrochloric acid (HCl) were mixed in same ratio of each, i.e., 3:0 mL and then ash was dissolved in the solution to convert

the metal nitrates. The samples thus filtered and added deionized water to make volume up to 50 mL in volumetric flask. Total silver (Ag) was estimated by the method of Perkin Elmer Analyst 100, USA. Data analysis was performed using Analytical Software, Tallahassee, USA.

Formula for the calculation of Ag concentration in plant is metal ($\mu g\ g^{-1}$) in plant = metal reading of digested sample ($mg\ L^{-1}$) × dilution factor

where,

$$\text{Dilution factor} = \frac{\text{Total volume of sample (ml)}}{\text{Weight of plant material (g)}}$$

Formula for metal uptake by the plant is,

Metal uptake = metal concentration in plant ($\mu g\ g^{-1}$) × plant's whole dry weight (g) (Zayed et al. 1998) method used to calculate bio-concentration factor.

Statistical analysis

All values are mean of triplicate samples documented in this study. Analysis of variance (ANOVA) was performed by using the statistical package, SPSS version 16.0 (SPSS, Chicago, IL) followed by Tukey's test to identify significant differences among the treatment means.

Results

Figure 1 demonstrates plant height, root length, leaf area, and number of leaves and flowers of sunflower grown under increasing concentration of Ag individually and in combination with GA. Reduction in all growth attributes has been found when Ag stress was applied as compared to control system plants. The metal reduction in increasing trend (Fig. 1) was found in sunflower as the concentration of Ag was increased from 100 to 500 μM. The lowest growth was estimated for the plants applied with the highest level, i.e., 500 μM of Ag.

Instead of the Ag, GA (2.5 mM) addition in control system improved plants growth and reduced the effects of Ag stress in plants. GA improved and maintained the growth of all parts of plant even at highest level of Ag stress (500 μM).

The biomass alterations of sunflower supplied with Ag concentration, and those also treated with GA amendment are demonstrated in Table 1. A reduction trend in root fresh/dry weight, shoot fresh/dry weight, and leaf fresh/dry weight has been found with increasing concentration of Ag. Highest reduction was observed in plants that were applied with 500 μM of Ag in the absence of GA amendment.

At concentration of 2.5 mM of GA, significant increase in biomass of plants in controls was observed in the absence of Ag. Addition of GA to the soil system amended with AgNPs also showed significant increase in the biomass of sunflower. Plants supplied with highest level of Ag (500 μM) with GA showed higher improvements as compared to the only GA-treated plants.

Chlorophyll a, b, total chlorophyll, and carotenoid contents were also measured under Ag and GA as shown in Fig. 2. Sunflowers were found quite responsive towards initial concentrations of Ag (100 μM), and highest response was noticed at 500 μM followed by 250 μM in terms of reduced chlorophyll and carotenoid content. Results ensured the improved chlorophyll and carotenoids content by the addition of GA in control and Ag-treated plants. Plants supplied with Ag along with GA showed tolerance towards Ag stress by maintaining their chlorophylls and carotenoids contents.

The effects of GA amendment under Ag stress for plant soluble proteins and SPAD value are presented in Fig. 3. Control plants mediated with GA without any Ag stress possessed highest levels of soluble protein and SPAD value in leaves and root. Reduced content of soluble protein and SPAD value was identified in plants supplied with Ag concentration without GA. The highest reduction was estimated in plants exposed to highest level of Ag, i.e., 500 μM followed by 250 and 100 μM, whereas the sunflower plants supplied with combine Ag and GA possessed higher levels of soluble protein and SPAD value.

Results of MDA, H_2O_2, and EC under different concentrations of Ag and GA are presented in Fig. 4. An increasing trend in oxidative stress has been resulted by enhancement of MDA and H_2O_2 due to up leveling of Ag concentrations in the solution applied to soil media grown with sunflower. Highest oxidative stress was observed in plants exposed to 500 μM of Ag in the absence of GA amendment. Increase in EC was observed which might be due to the leakage of electrolytes in the solution by plant organ under Ag stress. The electrolyte leakage went higher along with increasing concentrations of Ag, i.e., 100 to 250 μM till 500 μM.

While the plants dealt with GA amendment during Ag stress showed improved resistibility against oxidative stress and production of electrolytes in the plants. The addition of GA (2.5 mM) minimized the production of MDA, H_2O_2, and controlled EL as compared to the only Ag-treated plants. Even the addition of GA to the plants exposed to Ag also showed suppressed oxidative stress production in the plants. Plants exposed to highest concentration of Ag (500 μM) showed maximum production of ROS, but in case of combined application of GA and Ag, the production of ROS was constrained.

Antioxidant enzymatic activities in sunflower under Ag stress alone and with GA are presented in Fig. 5. At the initial level of Ag, sunflower plants responded in terms of high activities of antioxidant enzymes boosting the defense system of plant to resist change in normal functioning. The highest activities were observed in plants evaluated with 250 μM of Ag followed by 100 μM. While the activities decreased at the highest concentration of Ag stress, i.e., 500 μM to the plant which weakens the defense system to resist the change.

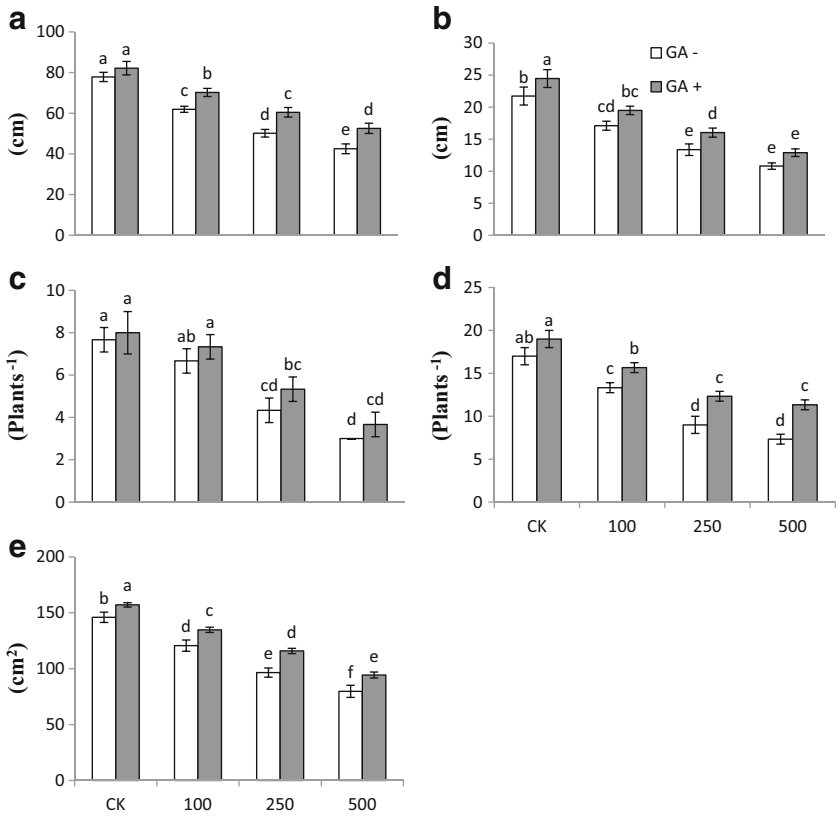

Fig. 1 Effect on plant height (**a**), root length (**b**), number of flowers (**c**), number of leaves (**d**), and leaf area (**e**) in sunflower treated with increasing concentration of Ag (0, 100, 250, and 500 μM) and glutamic acid (0, 2.5 mM). Values are demonstrated as means of three replicates along with standard deviation. Different letters indicate that values are significantly different at $P < 0.05$

Upward arrow shows for enhanced enzymatic activities and downward arrow for suppressed activities.

100μM of Ag↑ < 250 μM of Ag↑ > 500 μM of Ag↓

Mediation of GA with Ag stress boosted up the activities of SOD, POD, CAT, and APX in leaves and roots of plant. Plants resisted to highest Ag stress level by improved defensive mechanism in terms of enhanced enzymatic activities in plants.

500μM of Ag↓ < 500μM of Ag + 2.5mM of GA↑

The concentration and accumulation of Ag in sunflower plants estimated with and without GA are depicted in Table 2. Plants provided with increasing dose of Ag, concentrated, and accumulated Ag in their leaf, stem, and root. The

Table 1 Effect of Ag and GA on fresh and dry biomass of roots, stems, and leaves (g plant^{-1}) of sunflower

Treatment	Fresh weight (g)			Dry weight (g)		
	Leaf	Stem	Root	Leaf	Stem	Root
CK	19.4 ± 0.67b	40.5 ± 1.52a	18.2 ± 1.10b	5.86 ± 0.15b	13.5 ± 1.00a	6.90 ± 0.50a
G.A 2.5	23.3 ± 0.90a	43.6 ± 1.46a	21.3 ± 1.20a	6.73 ± 0.30a	14.3 ± 0.70a	7.26 ± 0.25a
Ag 100	15.8 ± 0.45c	28.9 ± 1.40c	15.9 ± 0.30 cd	4.26 ± 0.25c	9.56 ± 0.60c	5.40 ± 0.36b
Ag 100 + G.A 2.5	17.9 ± 0.55b	35.1 ± 0.88b	17.5 ± 0.55bc	5.23 ± 0.20b	11.7 ± 0.55b	6.36 ± 0.40ab
Ag 250	11.3 ± 1.05d	18.6 ± 1.37de	12.5 ± 0.45ef	3.23 ± 0.25d	7.06 ± 0.40d	4.16 ± 0.35c
Ag 250 + G.A 2.5	14.0 ± 0.50c	26.0 ± 1.73c	14.4 ± 0.51de	4.46 ± 0.26c	9.07 ± 0.40c	5.40 ± 0.46b
Ag 500	8.60 ± 0.65e	15.4 ± 1.20e	9.06 ± 0.40 g	2.23 ± 0.25e	5.00 ± 0.50e	2.76 ± 0.25d
Ag 500 + G.A 2.5	12.0 ± 0.50d	20.6 ± 1.60d	11.5 ± 0.55f	3.26 ± 0.24d	6.43 ± 0.40de	3.93 ± 0.30c

Values are the means of three replications ± SD. Variants possessing the different letters are statistically significant at $P > 0.05$

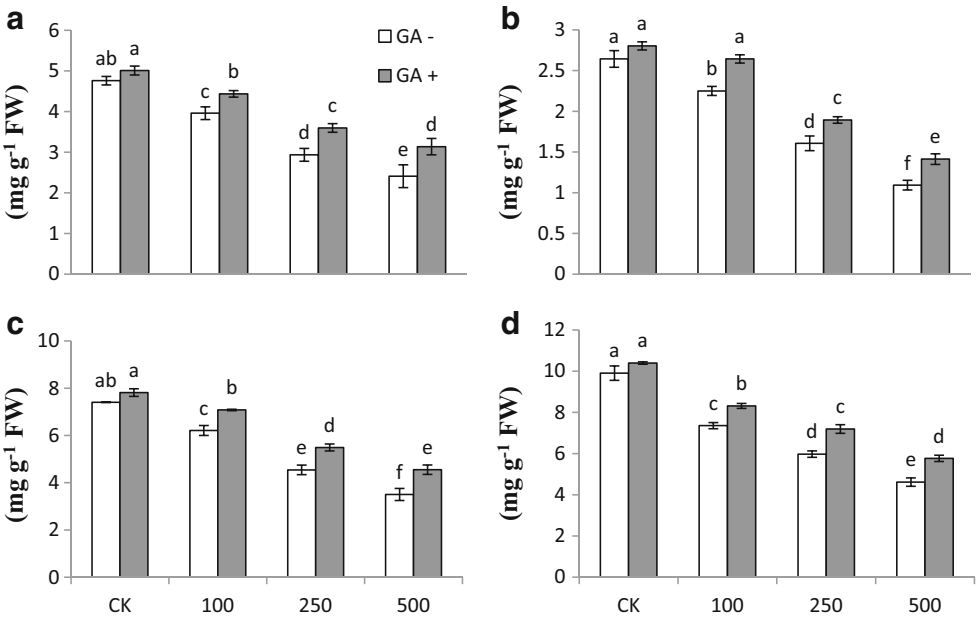

Fig. 2 Effect on chlorophyll a (**a**), chlorophyll b (**b**), total chlorophyll (**c**), and carotenoids (**d**) in sunflower treated with increasing concentration of Ag (0, 100, 250, and 500 μM) and glutamic acid (0, 2.5 mM). Values are demonstrated as means of three replicates along with standard deviation. Different letters indicate that values are significantly different at $P < 0.05$

concentration went higher along with the rising levels of treatment applied, i.e., 100, 250, and 500 μM, while the same trend was observed in all three parts of plant under GA application. The concentration and accumulation were increased by 61, 52, 21, 17, 70, 79, 58, and 66% in the root of sunflower at 0, 100, 250, and 500-μM Ag treatments along with GA, respectively. The Ag concentration and accumulation significantly increased by the addition of GA when applied with all Ag levels. The correlation among Ag concentration and growth attributes and physiological and biochemical attributes are given in Supplementary Tables 1 and 2.

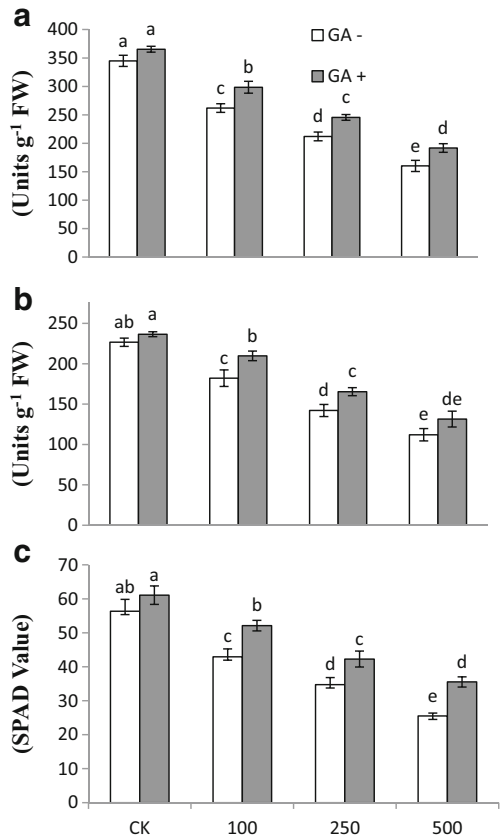

Fig. 3 Effect on soluble protein in leaf (**a**), soluble protein in root (**b**), and spad value (**c**) in sunflower treated with increasing concentration of Ag (0, 100, 250, and 500 μM) and glutamic acid (0, 2.5 mM). Values are demonstrated as means of three replicates along with standard deviation. Different letters indicate that values are significantly different at $P < 0.05$

Discussion

The agronomic traits of sunflower, i.e., fresh/dry weights of leaf, stem, and root and growth attributes including plant height, root length, number of leaves, number of roots, and leaf area are depicted in Table 1 and Fig. 1, respectively. Plants exposed to the higher concentrations of Ag in the solution of $AgNO_3$ possessed suppressed biomass of leaf, stem, and root either freshly weighted or dried. Biomass reduction was also identified in pepper plant (*C. annuum* L.), and the reason investigated by Vinkovic et al. (2017) is the abnormal cytokinin response (hormone growth) due to Ag stress applied in the form of $AgNO_3$ and also in the form of AgNPs. The reduction trend went higher along with increasing levels of Ag exposure to sunflower in this study supporting the findings of Tomacheski et al. (2017) performed experiment for oat and lettuce had reduced biomass when exposed to the Ag levels as compared to the control system plants. Fresh and dry weights reduced due to the Ag treatment applied, but more reduction was noticed with $AgNO_3$ as compare to Ag NPs applied to *Spirodela polyrhiza* (Jiang et al. 2014). *S. polyrhiza* appeared to be green and healthy in control system as

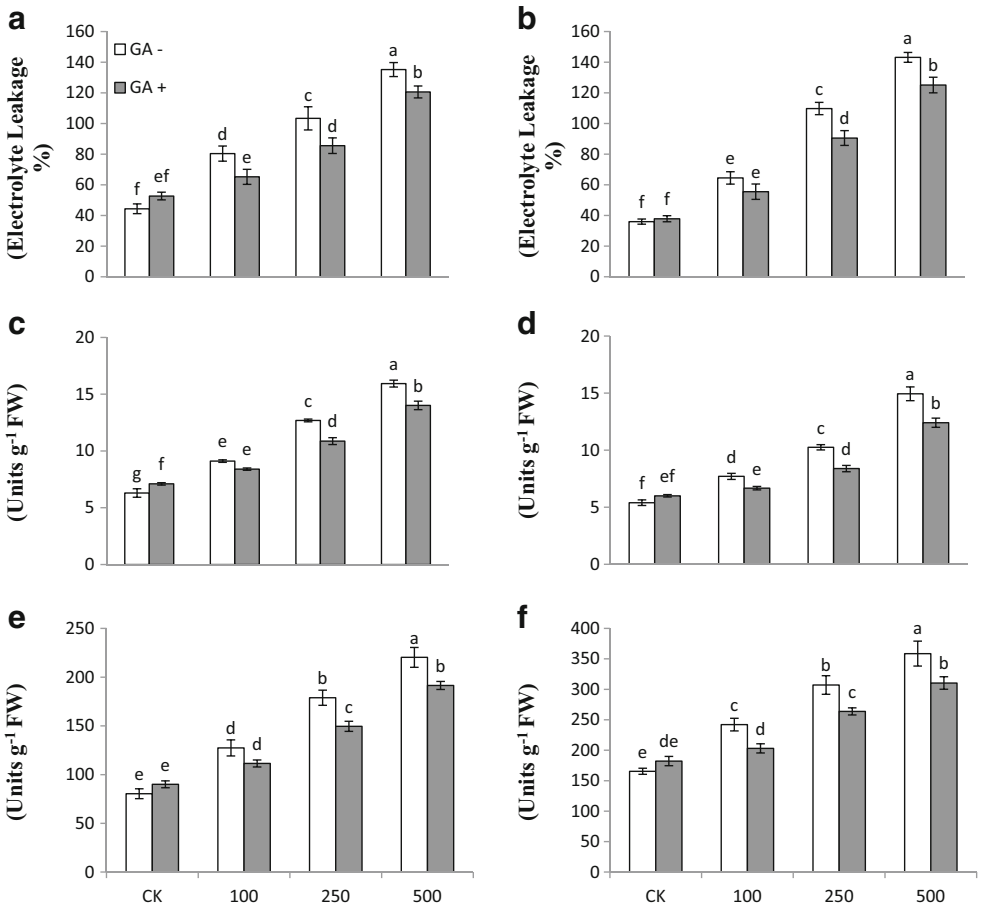

Fig. 4 Effect on EL in leaf (**a**), el in roots (**b**), MDA in leaves (**c**), MDA in roots (**d**), H_2O_2 in leaves (**e**), and H_2O_2 in roots (**f**) in sunflower treated with increasing concentration of Ag (0, 100, 250, and 500 μM) and glutamic acid (0, 2.5 mM). Values are demonstrated as means of three replicates along with standard deviation. Different letters indicate that values are significantly different at $P < 0.05$

compared to Ag applied, which appeared to be injured and morphologically damaged. The reduced biomass of sunflower can be the result of Ag accumulation as stated by Vinkovic et al. (2017) for pepper plant. Plants with GA amendment came up with improved biomass of sunflower even at higher level of Ag content and stress. Jia et al. (2017) investigated the promoting role of GA in terms of plants growth could be the possible reason for improved biomass of sunflower in our study. Addition of GA strengthen the plants to combat any stress applied to plant and maintaining the biomass is the confirmation given by Liu et al. (2011) that can support our results.

Root enlargement majorly effected by the Ag content applied to the medium grown with mung bean plant because Ag accumulated more in root section as compared to the above ground parts of plants stated by Kumari et al. (2017). The findings of Kumari et al. (2017) also found significant reduction in sunflower root length along with plant height, number of leaves/flowers, and leaf area. The highest reduction of root length noticed in sunflower applied with the highest level of Ag content, i.e., 500 μM followed by 250, 100, and 0 μM. Tomacheski et al. (2017) depicted negative effects of Ag on plant growth in terms of reduced activities of soil microorganisms. *C. pepo* (zucchini) also showed reducing trend for growth attributes under increasing Ag exposure and toxicity (Stampoulis et al. 2009) and similar trend noticed in present study for different levels of Ag applied to sunflower. Growth promoter GA investigated by Jia et al. (2017), and Barros-Galvao et al., 2017enhanced the plant height, root length, number of leaf/flower, and leaf area of sunflower even at stress bearing condition due to Ag content. Sunflower even at the highest stress level (Ag 500 μM) maintained its growth in terms of all parameters when supplied with GA amendment.

A significant reduction in chlorophyll and carotenoid content was observed in present study (Fig. 2). Lower chlorophyll and carotenoid content induced by Ag application has previously been reported for tomato plants (*Solanum lycopersicum* L.) by Cekic et al. (2017). Plants dealt with Ag content at any level showed suppressed chlorophyll and carotenoid contents as compared to the plants under control condition. Qian et al. (2013) reported reduced phytosynthesis rate in terrestrial plants along with reduced water content under Ag stress. In present study, lowest chlorophyll and carotenoid contents were noticed with 500 μM of Ag treated plants followed by 250 and 100 μM. According to Forde and Lea (2007), GA strengthens the plants in all aspects that include synthesis of chlorophyll and carotenoid contents, which is the supportive finding for our results. Plants supplied with GA during all

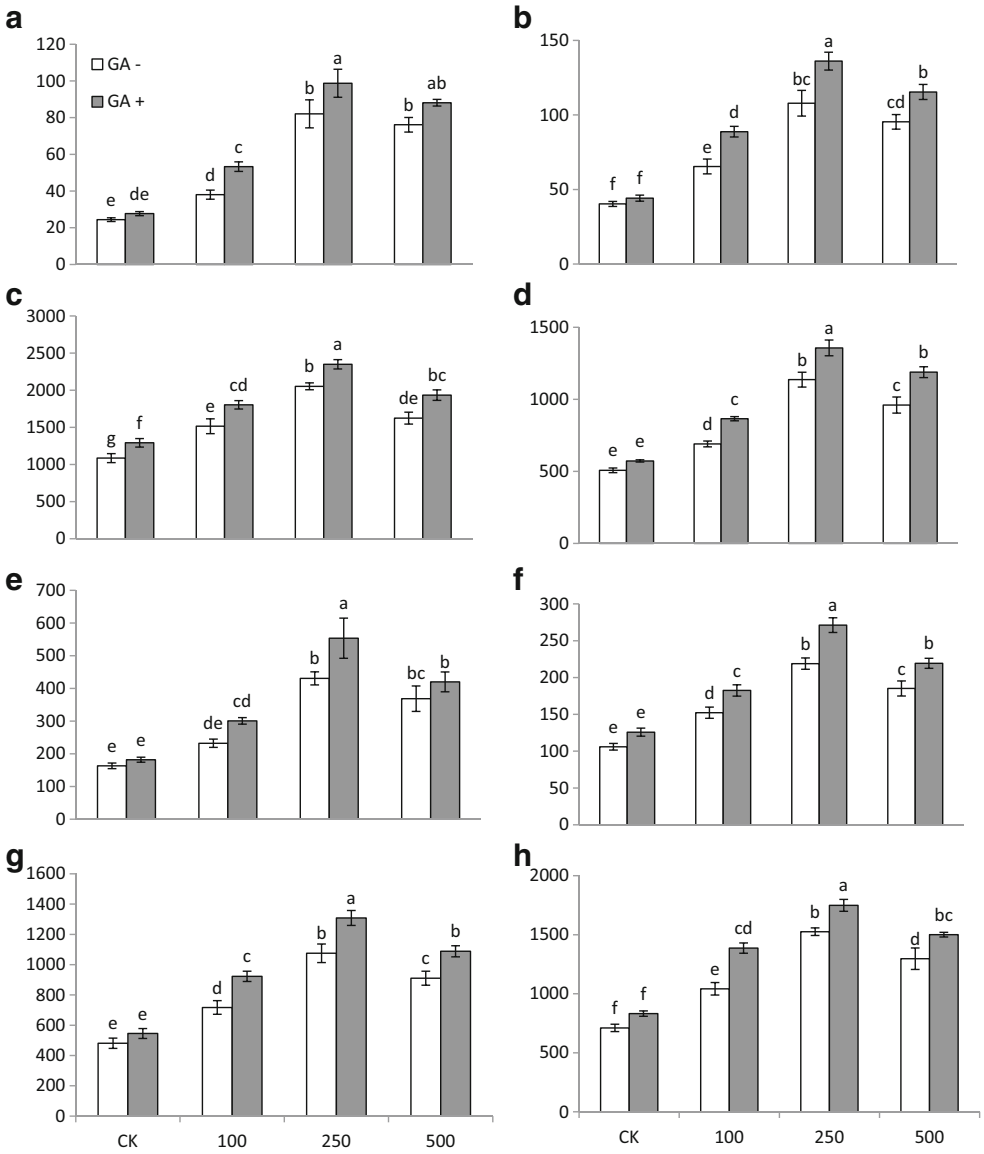

Fig. 5 Antioxidant enzyme activities, SOD in leaf (**a**), SOD in root (**b**), POD in leaf (**c**), POD in root (**d**), APX in leaf (**e**), APX in root (**f**), CAT in leaf (**g**), and CAT in root (**h**) in sunflower treated with increasing concentration of Ag (0, 100, 250, and 500 μM) and glutamic acid (0, 2.5 mM). Values are demonstrated as means of three replicates along with standard deviation. Different letters indicate that values are significantly different at $P < 0.05$

three levels of Ag stress showed enhanced contents of chlorophyll and carotenoids as compared to non-GA-treated plants.

Reducing trend of soluble protein (SP) and SPAD value in sunflower under increasing level of Ag is depicted in Fig. 3. Aquilina and Blundell (2016) reported that Ag interaction with protein's normal organized structure leading to its disruption causing SP deficiency. The finding of present study is also showing SP content deficiency in sunflower relation with Ag and also variations along with the varying level of Ag applied. Highest concentration of Ag affected SP level in mungbean plant (*Vigna radiate* L.) identified by Kumari et al. (2017). Further evidence to our study is sunflower treated with highest level of Ag, i.e., 500 μM possessed lowest level of SP followed by 250 and 100 μM as compared to the control system plants. Sunflower treated with GA in combination with Ag concentration, which showed improved levels of SP contents, is supporting the statement of Jia et al. (2017) about amending the role of GA for the plants bearing any environmental stress. Forde and Lea (2007) stated the boosting role of GA for enzymatic activities in plants in response to the damage leading to SP depletion. The addition of GA improved SP level of sunflower treated even with the highest level of Ag, i.e., 500 μM is verifying the study of Doumett et al. (2010) and Aquilina and Blundell (2016).

Enhanced ROS has been observed in plants under heavy metal stress reported by Farid et al. (2017) and Shakoor et al. (2014), and the similar expression was noticed in present study as shown in Fig. 4. The plants showed tolerance towards initial concentrations of Ag (100 μM) due to enhanced activities of antioxidant enzymes than at the highest level of Ag, i.e., 500 μM. Li et al. (2017) reported enhanced MDA production in leaves of soybean and upgraded contents of H_2O_2 in root of rice plants when exposed to Ag. Our findings are further supported by Li et al. (2017) and Cekic et al. (2017)

Table 2 Effect of GA on Ag concentration (mg kg^{-1}) and Ag accumulation (μg $plant^{-1}$) in roots, stems, and leaves of sunflower

Treatment	Ag concentration (mg kg^{-1})			Ag accumulation (μg $plant^{-1}$)		
	Leaf	Stem	Root	Leaf	Stem	Root
CK	1.06 ± 0.15 g	1.163 ± 0.04f	1.53 ± 0.51 g	6.27 ± 1.01e	15.80 ± 1.61e	10.54 ± 3.29d
G.A 2.5	1.16 ± 0.04 g	1.45 ± 0.08f	2.48 ± 0.50 g	7.83 ± 0.53e	20.85 ± 1.03e	18.00 ± 3.16d
Ag 100	67.13 ± 6.50f	95.00 ± 5.01e	122.15 ± 13.00f	287.11 ± 39.58d	906.83 ± 9.75d	660.81 ± 98.41c
Ag 100 + G.A 2.5	95.50 ± 5.05e	145.46 ± 15.01de	186.00 ± 5.10e	499.12 ± 10.36c	1717.16 ± 256.22bc	1183.89 ± 75.43b
Ag 250	141.24 ± 3.11d	186.28 ± 6.29 cd	230.40 ± 10.0d	456.36 ± 30.85c	1314.76 ± 33.54 cd	959.00 ± 72.84bc
Ag 250 + G.A 2.5	193.83 ± 7.51c	242.06 ± 10.31bc	280.50 ± 10.0c	867.04 ± 81.65b	2194.47 ± 129.22a	1516.36 ± 141.91a
Ag 500	261.76 ± 7.60b	295.43 ± 10.00ab	361.96 ± 17.85b	584.03 ± 59.08c	1478.81 ± 180.63c	1004.38 ± 139.70b
Ag 500 + G.A 2.5	328.95 ± 7.85a	327.26 ± 53.85a	425.56 ± 15.05a	1073.85 ± 71.74a	2095.38 ± 284.89ab	1676.91 ± 187.71a

Values are the means of three replications ± SD. Variants possessing the different letters are statistically significant at $P > 0.05$

who found, enhanced MDA content in tomato plants under Ag stress. Organic acids proved for their assisted role to overcome ROS production in *Brassica napus* (Shakoor et al. 2014), sunflower (Farid et al. 2016), and *Lemna minor* (Sallah-Ud-Din et al., 2017) under different metal stress. The application of GA along with all three levels of Ag showed for sunflower inhibiting ROS production in leaves and roots.

Plant defense system that activates to overcome the production of ROS produced under heavy metal stress has been reported by many scientists. McShan et al. (2014) reported enhanced antioxidant enzymatic activities at initial level of Ag stress and reduction at higher stress level. Similar trend of enzymatic activities was reported in sunflower exposed to Cr stress by Farid et al. (2017). Enhanced antioxidant defense mechanism triggered by ROS production to repair DNA damage and to overcome oxidative stress due to Ag toxicity is investigated by Saed-Moucheshi et al. (2014) and Cekic et al. (2017). Plants been reported for restoring, improving, and strengthening their defense system even in stressful conditions when supplied with amendments of organic acids (Ehsan et al. 2014; Farid et al. 2017, 2016, 2015). Similarly, in the present study, sunflower supplied with amino acid, i.e., GA showed improved enzymatic activities to combat even the highest stress of 500 μM of Ag. The sudden reduction in enzymatic activities at highest Ag stress further restored or improved by the application of GA. As mentioned above, GA noticed for reducing ROS production in plants due to Ag stress is related to modified defense system of the sunflower.

Sunflower extensively reported for concentrating and accumulating heavy metals in leaf root and stem parts of plant (Farid et al. 2017). The Ag concentration and accumulation in sunflower are depicted in Table 2. Concentration and accumulation increased along with the level of Ag exposure to sunflower. Hanks et al. (2015) reported roots of *Pistia stratiotes* with highest concentration of Ag as compared to shoots. Ag accumulated in belowground parts (roots) of salt marshes plant revealed in the study of Fernandes et al. (2017). The path of Ag exposure also matters either by roots or by foliar stated by Li et al. (2017), who reported more accumulation is leaves as compared to roots. In the present study, exposure path was via roots; plants accumulated more Ag. Aquilina and Blundell (2016) stated that the similar trend of Ag concentration in Kingdom Fungi is a further verification of our findings. Organic acid amendment that enhanced metal solubility and bioavailability to the plants has been widely reported. The GA also investigated for its potential to enhance bioavailability of metals in media by detaching them from organic fraction in the same medium (Doumett et al. 2010). In present study, sunflower supplied with Ag concentration in combination with GA possessed more concentration and accumulation in plant as compared to the only Ag-treated plants. The relation among Ag concentration in different parts of plant with morpho-physiological and biochemical attributes is clearly shown in Supplementary Tables 1 and 2, where the increase and decease can be found for better understanding of the response of sunflower. The highest Ag was possessed in plants treated with the combination of GA and 500 μM that can relate to enhanced growth of sunflower due to GA application, which is a growth regulator too as described by Jia et al. (2017).

Conclusions

In conclusion, our results showed that plant growth and photosynthetic pigments decreased, while ROS increased under Ag stress. GA significantly enhanced plant growth and chlorophyll content by lowering the production of ROS and enhanced antioxidant enzyme activities. GA also enhanced Ag concentration and accumulation in sunflower plants. Thus, data indicated that GA application can increase Ag uptake by sunflower and reduced its toxic effects in plants as depicted by increase in plant growth and biomass. However, studies based on DNA and gene changes are required to better understand the response of sunflower under Ag and GA.

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