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Effects of $CuSO_4$ and $AgNO_3$ on artemisinin and phenolic compound in shoot cultures of *Artemisia annua* L.

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Abstract

In the present study, the effect of exogenous silver nitrate (Ag^+) and copper sulfate (Cu^{2+}) applications on production of artemisinin and phenolic compounds as well as oxidative stress in the shoot cultures of *Artemisia annua* was investigated. A significant decrease in the shoot biomass and the total chlorophyll was observed in the shoots exposed to increased Ag concentration. Additionally, a significant increase in the carotenoid content was evident in the shoots (1.5 fold). The shoots exposed to Cu exhibited a marked increment in the biomass (1.5 fold), the total chlorophyll (2.2 fold) and the carotenoid content (1.4 fold). Ag and Cu induced generation of H_2O_2, LOX activity and increased production of MDA via both enzymatic and non-enzymatic peroxidation of membrane lipids one week after elicitation. In this study, we found that Ag and Cu markedly induced GST activity at the concentrations as low as 100 µM Ag and 25 µM Cu. Furthermore, Ag and Cu elicited accumulation of phenolic compounds (maximum value: 6.5 mg/gfw) and phenylalanine ammonia-lyase activity (2.5 folds). Artemisinin content was increased in the shoot cultures supplemented only with 5 µM of $CuSO_4$ (0.65%). It was revealed that both Cu and Ag affected the phenolic compound biosynthesis in the shoot cultures of *Artemisia*. However, artemisinin production was only affected by Cu.

Key words: *Artemisia annua*, Artemisinin, Copper sulfate, Lipoxygenase, Silver nitrate

Introduction

Signal molecules in plants are subjected to stresses and elicitors, often resulting in accumulation of secondary metabolites. The mechanisms include production of reactive oxygen species (ROS), the hypersensitive reaction, the lipid oxidation, and the activation of enzymes involved in the secondary metabolism or detoxification such as phenylalanine ammonia lyase (PAL) and glutathione S-transferase (GST) (Edwards and Dixon, 1991; Garcia-Brugger *et al.*, 2006; Roco *et al*., 1993). Elicitors are also well-known as research tools to investigate elements of the complex pathways and signaling interactions in the plant secondary metabolism. Despite the previously mentioned signals relating to ROS signaling, there is increasing evidence of a close connection between ROS and other various signaling systems in plant cells such as lipid-derived compounds dependent signaling pathways (Glyan'ko, 2011). The oxylipins play a crucial role as an origin of signaling compounds in abiotic and biotic stress reactions (Wasternack, 2007). The formation of oxilipins is commonly observed in ROS-mediated or enzyme-catalyzed of reactions. Interestingly, it has been observed that the presence of the heavy metals and wounding can induce oxylipins levels (Imbusch and Mueller, 2000). No doubt, many oxylipins biologically take part in induction of plant secondary metabolism.

Metal ions (such as Al, Ag and Cu) function as abiotic elicitors and induce both de novo synthesis and rapid accumulation of defense-related secondary metabolites (Aftab *et al*., 2012; Khalili *et al*., 2009; Narula *et al*., 2005). The promotion mechanisms of accumulation of the plant secondary metabolites by the metals have not been clarified yet. However, ROS signaling, lipid peroxidation, and formation of oxylipins are observed in most cases (Mithofer *et al*., 2004).

Artemisia annua L., a memebr of Asteraceae, contains artemisinin, a sesquiterpene endoperoxide, which is the only pharmaceutical compound utilized for malaria curing. However, the level of artemisinin in *A. annua* is relatively low (0.01-0.8% dry weight) and restricting commercialization of the product (Ferreira *et al*., 1997; Bhakuni *et al*., 2001). Therefore, many groups have attempted to increase production of artemisinin in

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tissue culture of *A. annua*. Elicitation has been considered as the most effective strategy to enhance production of the secondary metabolites, especially in cultured cells and organs. Some elicitors such as chitosan (Putalun *et al*., 2007), acetyl salicylic acid (O'Donnell *et al*., 1996), methyl jasmonate (Walker *et al*., 2002), gibberlic acid (Woerdenbag *et al*., 1993) and yeast extract (Baldi and Dixit, 2008) have been demonstrated to improve the artemisinin production. They have been investigated with respect to single elicitor on a particular cell line in most of these studies. But considering the complexicity of artemisinin biosynthesis, it is reasonable to examine the combined effect of these yield enhancement strategies.

Heavy metals enhance secondary metabolism in plants or plant cells in vitro. Since the existence of heavy metals in medium leads to oxidative stress and production of reactive oxygen as a secondary stress, the antioxidant response of shoot cultures of *A. annua*is is investigated in this study in cultures with different concentrations of silver nitrate ($AgNO_3$) and copper sulfate ($CuSO_4$).

Experimental design and culture conditions

Artemisia shoot culture: *Artemisia annua* L. seeds were obtained from Iranian Biological Resource Center (IBRC), Tehran, Iran. The seeds were surface sterilized using 70% ethanol solution for 30 s and 3% solution of sodium hypochlorite for 20 mins. and then rinsed with distilled water for three times. The sterilized seeds were cultured on Murashige and Skoog (MS) medium containing 30 g/l sucrose and 10 g/l agar. Cultures were incubated in a growth chamber at 25±2 °C with 16-hrs. photoperiod under cool-white light (35 mmol m^{-2} s^{-1}). After one month the seedlings were sub-cultured 3 times (with intervals of 20 days) on the same medium conditions. 3 months later the shoots, those contain 3 internode, were transferred to liquid MS medium and then were exposed to elicitors ($AgNO_3$: 1, 10 and 100 µM, $CuSO_4$ 5, 10 and 25 µM, the control treatment had no elicitor. The shoots were grown for seven days, harvested, rinsed and were used for following analysis.

Shoot dry weight: The shoot dry weight of samples was determined after over drying at 75°C for 48 hrs.

Photosynthetic pigments: The concentrations of the total chlorophyll and carotenoids of the shoots were assayed using the method of Arnon (1949) and Lichtenthaler (1987). Extracts were made from a 100 mg fresh sample in 10 ml 80% (v/v) acetone and measured at the wavelengths of 645, 663, and 470 nm with a UV/VIS spectrophotometer (JENWAY 6300).

Measurement of H_2O_2: Hydrogen peroxide content of the shoots was measured spectrophotometrically according to Alexieva *et al*. (2001). The fresh shoot samples of 0.05 g were grinded and homogenized with 1.5 mL extraction buffer containing 0.1% trichloroacetic acid (TCA) in a mortar and pestle on the ice. The homogenate was centrifuged at 12000 × g for 15 minutes to produce supernatant. The reaction mixture consisted of 0.1% TCA, shoot extract supernatant (0.5 mL), 10 mM potassium phosphate buffer (0.5 mL) and reagent (1.0 mL, 1 M KI). After 1 hour of reaction in a dark place, the absorbance of the solution was measured at 390 nm. A standard diagram prepared with known concentrations of H_2O_2 was utilized to calculate the amount of hydrogen peroxide.

Malondialdehyde content: Malondialdehyde (MDA) was measured according to the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). 100 mg of shoots was homogenized with 0.1% trichloracetic acid and centrifuged at 15000 ×g for 10 mins. The supernatant (1.0 mL) was mixed with 20% TCA (2.5 mL) containing 0.5% TBA and heated in a boiling water bath for 30 mins. and allowed to be quickly cooled in an ice bath. The supernatant was centrifuged at 9000 rpm for 10 mins., and absorbance was read at 532 nm and adjusted for nonspecific absorbance at 600 nm. The concentration of MDA was estimated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Lipoxygenase (LOX), Phenylalanine ammonia lyase (PAL) and Glutathione S-transferase (GST) assays: One hundred milligrams of the fresh stems was homogenized in 1.5 mL of 0.1 M Tris-HCl buffer (pH 8.5) containing 1% PVP (w/v), 1 mM $CaCl_2$, and 10% (v/v) glycerol. After centrifuging at 11,000 × g for 20 min at 4 °C, the supernatant was used as a source for the LOX enzyme activity test. LOX activity was examined according to Axelrod *et al*. (1981). The reaction mixture, in which LOX activity was measured, is composed of 1.95 mL of potassium phosphate buffer (50 mM, pH 7.0) containing linoleate (50 mM) and 50 µL of enzyme extract. The absorbance change per minute at 25 °C was measured at 234 nm. The reported results were based on ΔOD g fresh $weight^{-1}$ min^{-1}. 100 milligrams of fresh stems was homogenized in 1.5 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. After centrifuging at 12000 × g for 20 min at 4 °C, the supernatant was used as a source for PAL and GST activity assessments. PAL activity was assayed in accordance with Zucker (1965). Increase in the absorbance of shoots extract due to PAL activity was recorded spectrophotometrically at the wavelength of 290 nm. PAL activity was expressed as unit/g fresh weight. Glutathione-S-transferase activity was measured using the method described by Carmagnol *et al*. (1981) which lead to a reduction in absorbance at 340 nm due to glutathione (GSH) oxidation.

Assays of phenolic compounds: Ground shoots (100 mg) were extracted in 80% methanol and measured with Foline-Ciocalteu method (Singleton and Rossi, 1965). The total phenolics were calculated according to gallic acid standard curve and were expressed as mg g^{-1} fresh weight.

Artemisinin quantification: An accurate and repeatable High-performance thin-layer chromatography (HPTLC) method with the help of a TLC scanner was done on the shoot culture extracts of

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A. annua for artemisinin quantification (Agarwal *et al*. 2007; Quennoz *et al*. 2010). Briefly, concentrated ethyl acetate extract of different shoot culture extracts (100 mg) were dissolved in 3 mL diethyl ether (repeated three times), Then they were filtered to 10 mL. One µL of the sample was on a prewashed silica gel TLC aluminium foil 60 (20×10 cm^2 with the thickness of 0.2 mm; E. Merck, Darmstadt, Germany). The application rate was fixed at 150 nL/s, the slit dimension was kept at 4×0.1 mm^2, and a scanning speed of 20 mm/s was employed. The mobile phase was consisted of hexane:acetone (80:20). The migration distance was 60 mm. For derivatization of artemisinin spots, the developed HPTLC plate was dried with a hair drier, immersed for 1s in a solution of vanillin in ethanol (1 %), and then sulphuric acid in ethanol (5%), and finally heated at 110 °C for 5 mins. Determination was done at 596 nm using a TLC Scanner 3 (CAMAG, Muttenz, Switzerland). For the quantitative analysis, a standard calibration curve was prepared in the range of 200 to 800 µg/mL using different concentrations of artemisinin (200, 400, 600, and 800 µg/mL) as the standard material (Sigma Aldrich, USA). The relationship between the concentrations and the height peak of the diagrams was determined using the minimum square method (R^2 value). Validation of the HPTLC method was calculated as the percent recovery of spiked extract sample with standard artemisinin at 200 µg/mL concentration. Limit of detection (LOD) and limit of quantification (LOQ) were calculated using the formulae based on the signal-to-noise ratio, LOD = 3 × S/N' and LOQ = 10 × S/N', where S = signal height and N'= noise height.

Statistical analysis: An *in vitro* experiment was conducted completely randomized design with 3 replications. Statistical differences between the mean values were compared using one-way multivariate analysis of variance (MANOVA) and least significant different. Results were reported as mean ± standard deviation (SD) and P<0.05 was considered as statistical significant.

Results

Higher dry weight of shoots value of *A. annua* was found in shoots grown in culture medium with Ag 1 µM and Cu 5 and 10 µM. In contrast, lower dry weight of shoots was obtained with shoots exposed to 100 µM of Ag. Shoot growth was not affected by the application of Ag 10 µM and Cu 25 µM in the medium, compared to the control (Fig. 1).

The Ag treated shoots showed an increase (P< 0.05) of 39.5% in chlorophyll content for the treatment of Ag 1 µM and a decrease of 16% and 26% for the treatments of Ag 10 and 100 µM, respectively. In Cu^{2+} treated shoots, an increase in Cu concentration of the culture medium resulted in a significant (P< 0.05) increase in total chlorophyll content of the shoots (Fig. 2A). Regardless of heavy metal concentrations, carotenoid content in shoots of *A. anuua* was similar in all treatments (except Cu 25 µM). The highest content of carotenoid was at Ag 10 µM; while the lowest content was at Cu 25 µM (Fig. 2B).

The production of H_2O_2 and MDA was influenced by Ag and Cu stress (Fig 3A, B). The contents of H_2O_2 and MDA were increased in shoots of *Artemisia* plants in both stresses under all treatments. It was observed that the production of MDA was higher in Cu^{2+} treated shoots than the content in the Ag^{+} treated shoots.

Silver nitrate and copper toxicity increased LOX, GST and PAL activities (Fig. 4A, B and C). Plants exposed to Ag and Cu exhibited a marked increment in LOX activity. The highest activity (11.46 U/gfw/min) was recorded in shoots grown in Cu treatment. PAL activity also increased in all concentrations of Ag, reaching 2.7 folds greater than the control shoots after 7 days. A significant decrease was observed in PAL activity in plants exposed to increased concentrations of Cu. According to the results (Fig. 4C), GST activity was significantly (P<0.05) increased up to 2.7 and 1.8 times in shoots exposed to Ag (1 and 10 µM) and Cu (5 and 10 µM) respectively, but not in Ag 100 µM and Cu 25 µM treatments, in comparison with the control treatment.

The total phenolic contents of the shoots were drastically (P<0.05) influenced by Ag concentrations in the culture medium. A significant increase was observed in the phenolic compounds at all concentrations of the Ag treatments. However, a significant (P<0.05) increase was observed in the phenolic compounds of *Artemisia* shoots at 5 and 10 µM of Cu treatments, but not under Cu 25 µM stress condition (Fig. 5A).

The current study attempted to determine the effects of Ag and Cu, on the synthesis and accumulation of bioactive compounds in *A. annua*. The highest artemisinin content (0.65%) was obtained in Cu 5 (Fig. 5B).

Discussion

It is generally shown that plants under heavy metal stresses undergo growth reduction, especially under the Ag and Cu stress (Ali *et al*., 2006; Khalili *et al*., 2009). Growth of the shoots of *A. annua* was reduced by Ag 100 µM which has observed in other plant species (Suh *et al*., 2013; Xiao *et al*., 2010). Higher growth (dry weight of shoots) value of *A. annua* was found in shoots grown in culture medium with Ag 1µM. Thus, in the concentrations above 10 µM, silver nitrate was a limiting factor for the growth of *A. annua*. A similar trend was observed for Cu, where growth was inhibited at Cu concentrations above 10 µM.

Exposure of plants to high levels of metals inhibits photosynthesis as well as problems in distribution of water and nutrients (Mohanpuria *et al*., 2007; Wojcik and Tukiendorf, 2004). The negative effects of heavy metals on chlorophyll are in agreement with those previously reported for other plants (Aftab *et al*., 2012; Yusuf *et al*., 2011). With increasing Ag concentration in the medium, there was trend in the decreased total chlorophyll. The reduction observed in the shoot biomass at Ag 10-100 is similar to the reduction

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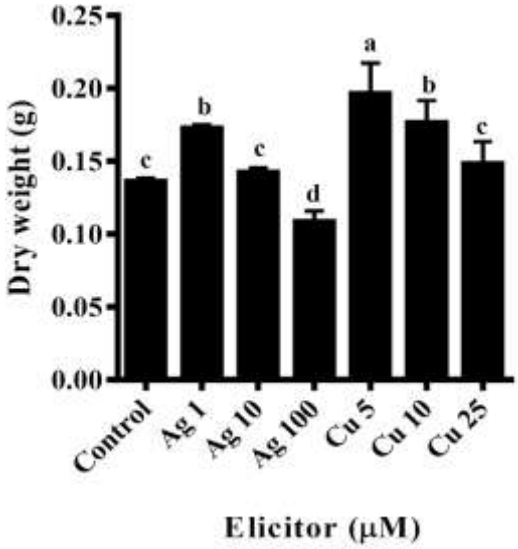

Fig 1: Effects of $AgNO_3$ (Ag) and $CuSO_4$ (Cu) concentrations on shoot dry weight in shoot cultures of *Artemisia anuua*. Values are means of triplicate results.

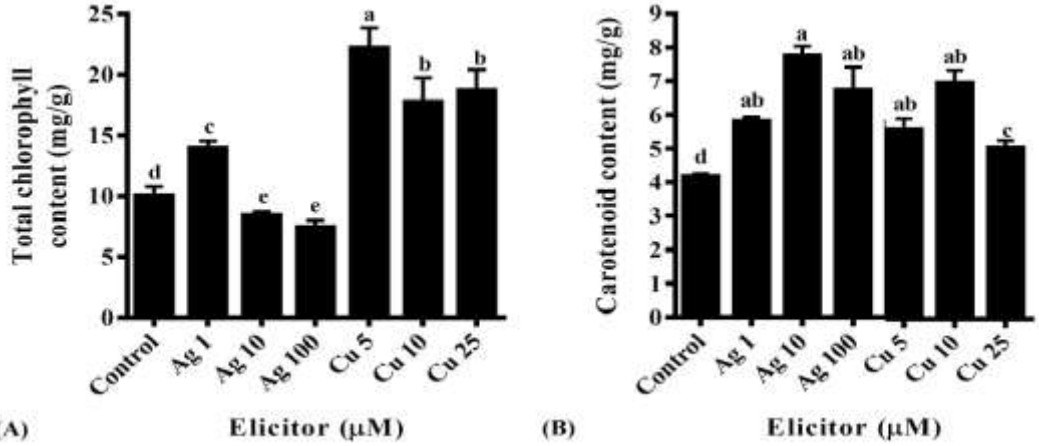

Fig 2: Effects of $AgNO_3$ (Ag) and $CuSO_4$ (Cu) concentrations on total chlorophyll (A) and carotenoid content (B) in shoot cultures of *Artemisia anuua* one week after elicitation. Values are means of triplicate results.

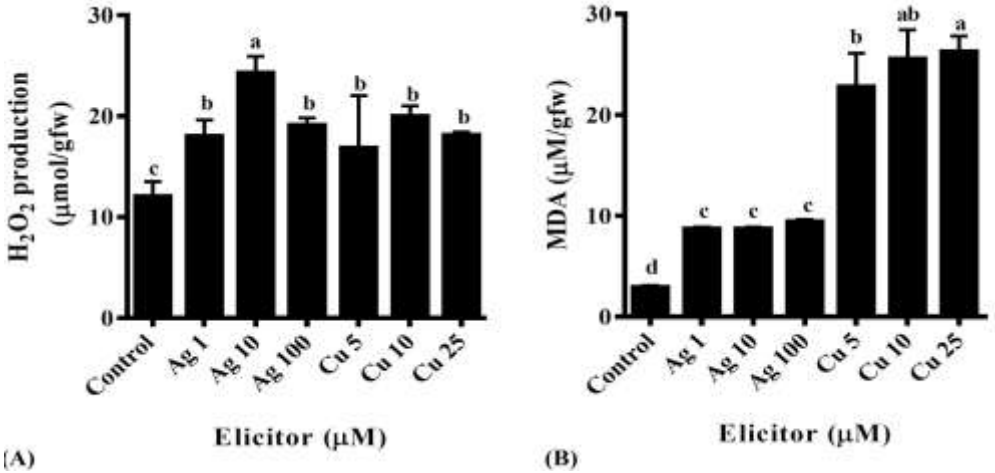

Fig 3: Effects of $AgNO_3$ (Ag) and $CuSO_4$ (Cu) concentrations on H_2O_2 (A) and MDA production (B) in shoot cultures of *Artemisia anuua* one week after elicitation. Values are means of triplicate results.

observed in the pigment content. However, total chlorophyll was significantly ($P<0.05$) increased with increasing Cu concentration in the medium. Carotenoids are pigments with multiple functions in the metabolism

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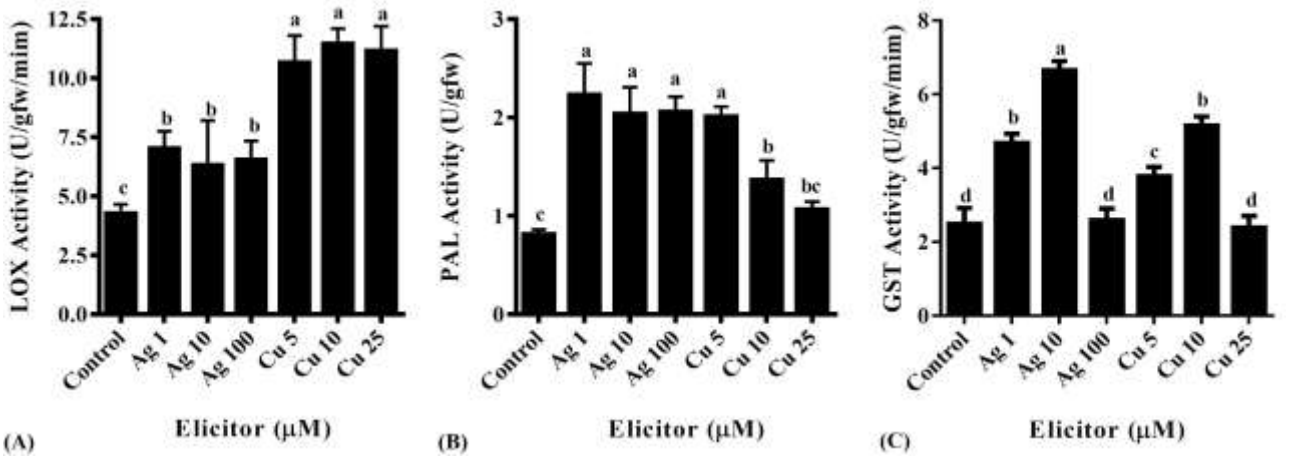

Fig 4: Effects of $AgNO_3$ (Ag) and $CuSO_4$ (Cu) concentrations on LOX (A), PAL (B) and GST (C) activities in shoot cultures of *Artemisia anuua* one week after elicitation. Values are means of triplicate results.

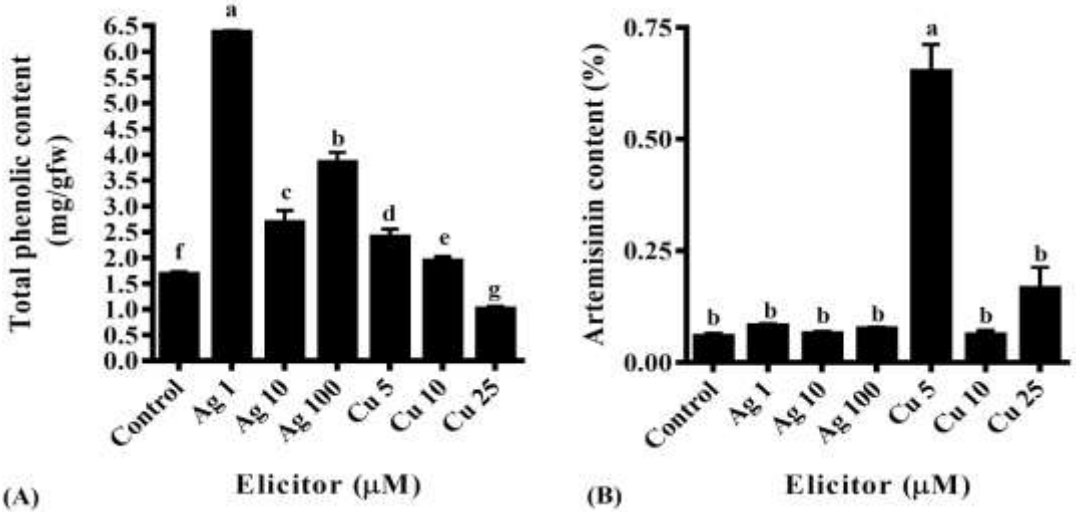

Fig 5: Effects of $AgNO_3$ (Ag) and $CuSO_4$ (Cu) concentrations on total phenolic compound (A) and artemisinin content (B) in shoot cultures of *Artemisia anuua* one week after elicitation. Values are means of triplicate.

of the plants, including tolerance to oxidative stress. The antioxidant actions of carotenoids are based on their singlet oxygen quenching properties, and their ability to trap peroxyl radicals (Holzwarth *et al*., 2006). Ahmed *et al*. (2013), showed that Cu-induced increment in carotenoids contents of safflower leaves was demonstrative of high potential of non-enzymatic antioxidant defense.

Heavy metal stress in plants results in oxidative stress and overproduction of reactive oxygen species as a secondary stress. When plants are exposed to heavy metals, the balance of free radical metabolism is shifted toward accumulation of H_2O_2. In this study, treatment of *A. annua* shoot cultures with Ag and Cu increased H_2O_2 production. H_2O_2 can induce both non-enzymatic and enzymatic metabolic processes of peroxidation of polyunsaturated fatty acids (PUFA) in membrane lipids. Malondialdehyde, which is a non-enzymatic peroxidation product of PUFA in the membrane, is considered an indicator of free radical stress. Lipoxygenase (LOX) enzyme, involved in production of important signaling molecules and defense metabolites in plants, catalyze enzymatic lipid peroxidation in octadecanoid pathway (Andreou *et al*., 2009). However, involvement of the octadecanoid pathway in heavy metal-inducible production of secondary metabolite is unclear. Oxylipins (oxidized metabolites of unsaturated fatty acids) are products of two, non-enzymatic and enzymatic, peroxidation of PUFA in the membrane (Wasternack, 2007). In this study, we examined both non-enzymatic and enzymatic ~~of~~ peroxidation of PUFA. Treatment of shoot cultures of *A. annua* by Ag and Cu led to induction of the LOX activity, and an increase in the MDA production, one week after elicitation. It has been demonstrated that oxylipins, as signaling molecules in plants, participate in different defense reactions against pathogen and herbivore attack, and are probably responsible for the heavy metal-induced defense responses (Mithofer *et al*., 2004).

The generation of ROS is commonly observed in plants under stress, playing a dual role: as plant toxic compounds and as regulators of biological processes

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(Fujita *et al*., 2006; Mittler, 2006; Parida and Das, 2005). ROS levels are controlled by both enzymatic and non-enzymatic (such as phenolic and carotenoids) antioxidants (Jaleel *et al*., 2009). Glutathione S-transferases (GSTs) are considered detoxification enzymes, and play vital roles in the detoxification of xenobiotics, protective activities against oxidative tissue damage and may act as stress signaling proteins (Loyall *et al*., 2000). The addition of Ag^{+} and Cu^{2+} induces accumulation of GST mRNA in suspension-cultured tobacco (Boot *et al*., 1993). It has been suggested that glutathione transferase and glutathione peroxidase plays a regulatory role in maintaining the redox state of the cell in *Arabidopsis* when they are exposed to Cu (Smith *et al*., 2004). In this study, we found that Ag and Cu markedly induced GST activity at concentrations as low as 100 µM Ag and 25 µM Cu. Plants respond to heavy metals with various detoxification pathways as well as responses to secondary stress effects. The observed stress response of GST activity demonstrates its involvement in detoxification of Ag and Cu in shoots of *A. annua*.

It has also been reported that heavy metal stress increases phenolic metabolites synthesis in plants, supporting the protective role of these phenolic compounds (Michalak, 2006; Sakihama *et al*., 2002). All phenylpropanoids are extracted from cinnamic acid. Cinnamic acid itself is generated from phenylalanine due to function of phenylalanine ammonia-lyase (PAL). It has been observed that phenolic compounds are especially increased in Ag^{+} treated shoots, while these compounds are increased only in shoots under Cu^{+5} µM treatment. On the other hand, increase in the PAL activity in both treatments, particularly Ag treatment, was well evident.

Diosgenin was enhanced at low concentrations of Cu stress in in vitro-cultivated plants of *Dioscorea bulbifera* (Narula *et al*., 2005). Cu also induced production of betalains in *Beta vulgaris* (Trejo-Tapia *et al*., 2001). Similarly, Khalili *et al*. (2009) has shown the stimulatory effects of Ag on accumulation of silymarin in hairy roots of *Silybum marianum*. The increase in the artemisinin production has been reported in *A. annua* under heavy metal stress (Aftab *et al*., 2012; Li *et al*., 2012).

In the present study, a noticible increase in artemisinin content was observed when the shoots were treated with 5 µM Cu. A direct connection between ROS production and artemisinin content has been reported by several groups (Guo *et al*., 2010; Pu *et al*., 2009). Aftab *et al*. (2012) have proposed that heavy metal-induced artemisinin biosynthesis is ROS-dependent, suggesting that ROS acts in converting dihydro-artemisinic acid to artemisinin during artemisinin biosynthesis.

Hence, our study verified that Cu might lead to improve the artemisinin content in shoot cultures of *A. annua*, being in agreement with numerous researches, which shows that the plants respond to abiotic elicitors by activating an array of defense mechanisms, including induction of biosynthesis of secondary metabolites.

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