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PRETREATMENT WITH SALICYLIC ACID AND NITRIC OXIDE MITIGATED SILVER NANOPARTICLES TOXICITY AND ENHANCED THEIR REMOVAL IN MEDICINAL *PHLOMIS TUBEROSA* PLANTS

ELHAM GHASEMIFAR, GHADER HABIBI* and GOLAMREZA BAKHSHI-KHANIKI

Department of Biology, Payame Noor University (PNU), PO BOX 19395-3697 Tehran, Iran

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Since silver nanoparticles (AgNPs) are used as nanofungicides and nanopesticides in agriculture, the toxicity of AgNPs as well as $AgNO_3$ must be determined. Besides this, we evaluated the combined effects of salicylic acid (SA) and nitric oxide (NO) on responses of *Phlomis tuberosa* plants to Ag-induced stress. The results of growth parameters together with measurement of malondialdehyde (MDA) indicated that exposure to 1000 mg L^{-1} of AgNPs or $AgNO_3$ exerted more toxicity, which was closely associated with the over–accumulation of ROS and the reduction of photochemical functioning. However, SNP (NO) and SA addition successfully alleviated adverse impact of AgNPs on *Phlomis* seedlings. Maximum amelioration of Ag-induced stress was found by combined treatments of SA+NO. *Phlomis* plants primed with SA+NO exhibited higher synthesis of chlorophyll *b* and carotenoid pigments to ameliorate AgNP-induced adverse effects on chlorophyll fluorescence parameters. SA+NO led to high levels of proline under both AgNPs and $AgNO_3$ treatments. A further increase in antioxidants (phenolic compounds) was observed in NO-primed plants under AgNPs-induced stress, which was attendant with the high level of CAT and APX activities. Increase in total Ag translocation into shoot organs and cell survival were also enhanced by SA+NO under AgNPs stress. We concluded that SA+NO mitigated the inhibitory effects of AgNPs stress on the photosynthetic apparatus by increasing the phenolic compounds and carotenoids as well as by regulating accumulation of Ag, ROS and antioxidants. The present findings provide important knowledge to design strategies that minimize the negative impact of AgNPs and $AgNO_3$ on crops.

Keywords: antioxidant status, exogenous nitric oxide, phenolic compounds, photochemical activity, salicylic acid, silver nanoparticle.

INTRODUCTION

Today nanotechnology and nanoparticles (NPs) are used to promote plant growth and productivity, which play important roles in sustainable development (Zhand et al., 2018; Szőllősi et al., 2021). However, they have positive and negative aspects and pose environmental challenges as well. Due to increased accumulation of NPs in soil and aquatic ecosystems, research on toxic effects of NPs on microbes, invertebrates, and aquatic organisms, including algae and plants, is needed to ensure sustainable use in food production efforts. Additionally, it has been reported that the use of NPs leads to alterations in growth, biological function, gene expression, and development of plants (Szőllősi et al., 2021). In this regard, specific research on NPs toxicity in plants is urgently needed to use nanotechnology safely (Zulfiqar et al., 2019). However, natural silver concentrations are generally low in the environment but silver concentrations are continuously increasing at higher levels in Ag contaminated soil or water (Tripathi et al., 2017; Saleeb et al., 2019). It is

* Corresponding author, e-mail: ghabibi@pnu.ac.ir

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reported that AgNP has no significant effect on soil biota at concentrations as low as 0.14 mg Ag kg^{-1} (Colman et al., 2013). As a result, we used higher silver concentrations to assess toxic range of silver in soil.

Silver nanoparticles (AgNPs) have been used as novel nanopesticides and nanofungicides (Zhand et al., 2018). Importantly, in recent years, the toxicity of AgNPs to various species has been studied in a number of reports (Lee et al., 2012; Wang et al., 2013; Noori et al., 2020). In many plant species, AgNPs cause adverse effects on gene expression (Kaveh et al., 2013; Rizwan et al., 2021), cell division and/or cell elongation (Vishwakarma et al., 2017), growth and development (Zulfiqar et al., 2019; Wang et al., 2020) and photosynthetic electron transport (Biba et al., 2021). They also cause significant increase in ROS formation and lipid peroxidation in *Oryza sativa* L. (Nair and Chung, 2014), potato (Bagherzadeh Homaee and Ehsanpour, 2016), soybean (Li et al., 2017) and *Cucumis sativus* (Zhang et al., 2018).

In this regard, plant priming by free radical nitric oxide (NO) and phenolic phytohormone salicylic acid (SA) can alleviate the negative effects of abiotic stress in plants (Habibi, 2019; Sharma et al., 2020; Prakash et al., 2021). Later studies suggest that initial exposure to NO (SNP) protects pea seedlings against AgNPs by counteracting accumulation of Ag and ROS, and antioxidants (Tripathi et al., 2017). In another study, SNP increased the activities of antioxidant enzymes and proline accumulation, which was linked with increasing NO levels in the roots (Amooaghaie et al., 2018). Numerous studies have revealed that silicon further increased the NO level in the AgNPs-treated *Brassica juncea* plants, which diminished oxidative stress-effects of silver nanoparticles (Vishwakarma et al., 2020; Kolbert and Ördög, 2021). Additionally, exogenous application of SA enhances heavy metal stress tolerance by bettering the antioxidative defense system, osmolyte accumulation and ionic homeostasis (Sharma et al., 2020). Moreover, application of SA in rice plants has also been shown to reduce the toxicity of arsenic when exogenously applied at appropriate concentrations (Faizan et al., 2021), and increases in the endogenous levels of this hormone were found to play an important role in the response to Ag-induced stress in cucumber plants (Zhang et al., 2018).

Although others have reported how individual NO alleviates AgNPs-induced oxidative stress (Tripathi et al., 2017; Amooaghaie et al., 2018), to our knowledge, no study has analyzed the role of SA or SA+NO in the mitigation of AgNPs-induced stress in plants. Here, we presented the first evidence showing that combined priming was more effective than SA or NO alone for *Phlomis tuberosa* plants exposed to AgNPs stress, however, the crosstalk between NO and SA needs further investigation. In addition, to better understand the effects of AgNPs, we investigated plant physiological and molecular responses upon exposure to AgNPs in comparison to silver nitrate ($AgNO_3$). This study aims to highlight alleviating aspects of SA, NO and SA+NO and critically discusses how these two molecules reduce the negative impact of AgNPs and $AgNO_3$ on *Phlomis tuberosa* plants, as well as improve the removal efficiency of these toxicants from soil. The findings of this work provide important knowledge, which may be considered for safe application of nanomaterials in agriculture.

MATERIALS AND METHODS

PLANT MATERIAL AND TREATMENTS

Seeds of *Phlomis tuberosa* (commonly known as Jerusalem Sage) were collected from Mishoudagh, near the city of Marand, 65 km south of Tabriz (45°38' E, 38°22' N) at an elevation of 1800 m, East-Azarbaijan Province (NW Iran). The plant material was identified by one of us and the voucher specimens have been deposited at two herbaria located at the Faculty of Pharmacy, Tehran University of Medical Sciences, and the National Botanical Garden of Iran, northwest of Tehran. The seeds were sown at the top of cylindrical plastic pots filled with perlite and then watered with 500 ml of half-strength Hoagland solution. For priming treatments, *Phlomis tuberosa* seeds were soaked in aerated solutions of sodium nitroprusside (SNP, as a NO donor) and salicylic acid (SA) for 12 h. The concentrations of NO and SA were chosen according to the effect of different NO (0.05, 0.1, 0.5, 1, 5 and 10 mM SNP) and SA (0.05, 0.1, 0.5, 1, 5 and 10 mM SA) concentrations on *Phlomis* seed germination as well as on seedling growth in a preliminary study, which showed that 0.1 mM NO and SA priming significantly promoted seed germination and seedling growth (Fig. 1). Sixteen weeks after sowing, when the plants were about 10 cm (3 inches) tall, the

Fig. 1. Effect of NO and SA on the germination of *Phlomis tuberosa* seeds. *Phlomis* seeds were exposed to 0, 0.05, 0.1, 0.5, 1, 5 and 10 mM SNP or SA for 12 h.

pots were irrigated with 1000 ppm AgNPs or $AgNO_3$ solution dissolved in tap water for 21 days (Ag treatment). The Ag nanoparticle product (AgNPs) was procured from a reliable company called "Iranian Nanomaterials Pioneers, Mashhad, Iran". The physicochemical traits of this nano-product are as follows: Purity: 99.99%; APS: 5-8 nm; SSA: 25-42 m^2/g; Color: black; Morphology: spherical; True Density: 10.9 g/cm^3. Equivalent silver salt ($AgNO_3$) was purchased from Sigma–Aldrich and utilized as a bulk control. The concentration of silver nanoparticle was chosen according to the effect of different AgNPs (5, 50, 100, 1000 and 2000 ppm) concentrations on seed germination as well as on seedling growth in a preliminary study (Fig. 2). At 1000 ppm AgNPs, the germination percentage halved as compared with that of the control and it was chosen as

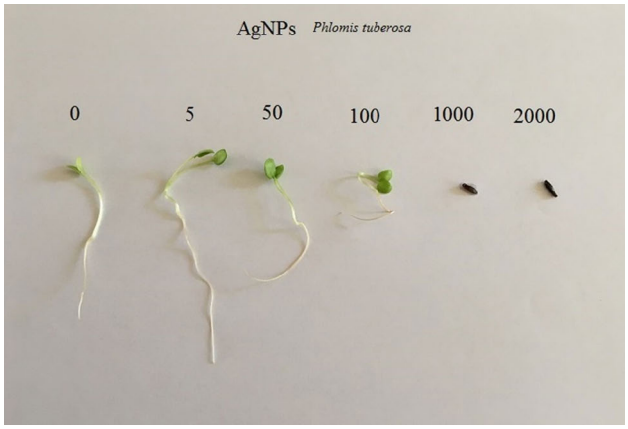

Fig. 2. Effect of AgNPs on the germination of *Phlomis tuberosa* seeds. *Phlomis* seeds were exposed to 0, 5, 50, 100, 1000 and 2000 ppm AgNPs for 48 h.

a semi-lethal concentration (Zhang et al., 2008) of AgNPs for further experiments (data not shown). Control plants were treated with the nutrient solution without Ag, NO and SA. The plants were grown in a greenhouse under day/night temperature of 25-30/19-21°C, relative humidity of 60-65% and daily photon flux density of about 350-400 µmol m^{-2} s^{-1} throughout the experimental period.

HARVEST

After 21 days of Ag treatment, the plants were harvested for morphological and physiological analyses. After determination of the fresh weight (FW), the leaves were dried for 48 h at 70°C for determination of the dry weight (DW). For latter physiological analysis, the samples were stored immediately in liquid N_2 until assay.

ASSAY OF CHLOROPHYLLS AND CAROTENOIDS

For determination of the leaf concentration of chlorophyll and carotenoids, the samples were homogenized in methanol as described by Lichtenthaler and Wellburn (1983). The homogenate was filtered, and after centrifugation at 1000 rpm for one minute, the absorbance of supernatants was determined at 400–700 nm.

CHLOROPHYLL A FLUORESCENCE MEASUREMENTS

Chlorophyll *a* fluorescence transients (OJIP transients) were recorded daily using a Pocket–PEA chlorophyll fluorimeter (Plant Efficiency Analyser,

Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 32 1JL, England) between 9:00–11:00 a.m. in dark-adapted leaves for at least 30 minutes. We determined some groups of chlorophyll *a* fluorescence parameters using the JIP–test (Strasser et al., 2004), which were shown in the following section:

- F_v/F_o, the efficiency of the water–splitting complex on the donor side of PSII
- F_v/F_m, the maximal photochemical efficiency
- PI_{abs}, the performance index
- φ_{Eo}, the quantum yield of electron transport

DETERMINATION OF SOLUBLE SUGARS, STARCH AND PROLINE

The extraction and quantification of total soluble proteins were measured using the method of Bradford (1976). Proline concentrations were determined as described by Bates et al. (1973). Leaf samples from each group were homogenized with 3% (w/v) sulphosalycylic acid at 4°C and the homogenates were centrifuged at 3,000g for 20 min. The supernatant was boiled with acetic acid and acid ninhydrin, and then the absorbance was read at 520 nm.

The standard curve was created using proline (Sigma).

Soluble sugars concentrations were measured by the method of Quentin et al. (2015). Leaf tissues were extracted with 2.5 mL 80% ethanol in a water bath for 2h at 30°C. After centrifugation at 3,000 g for 10 min, the supernatants were treated with anthrone-sulfuric reagent, and then the absorbance at 630 nm was determined. Glucose (Sigma) was used for the standard curve. The pellets were used for starch analysis by the method of Magné et al. (2006). Starch was dissolved after resuspendation in a 4 : 1 (v/v) mixture of 8 N HCl/dimethylsulfoxide, and the supernatant was mixed with iodine–HCl solution and the absorbance at 600 nm was determined. Starch (Merck) was used as the standard curve.

ASSAY OF PHENYLALANINE AMMONIA-LYASE (PAL) ACTIVITY AND RELATED METABOLITES

To estimate PAL activity, a leaf sample was homogenized with 50 mM sodium phosphate buffer (pH 7.0) containing 2% (w/v) polyvinylpolypyrrolidon (PVPP), 2 mM EDTA, 18 mM β-mercaptoetanol and 0.1% (v/v) Triton X-100. Formation of cinnamic acid was estimated by spectrophotometry at 290 nm by the method of Zucker (1965). One unit (U) of PAL activity was expressed as the amount of the enzyme that produced 1 nmol cinnamic acid per h. Total phenolic content was evaluated by the method of Velioglu et al. (1998). Gallic acid was used for constructing the standard curve. The results were defined as mg gallic acid (GA) per gram of the fresh weight. The total flavonoid content was measured using a standard curve of quercetin and expressed as mg quercetin equivalent (QE)/100 g extract. For determination of the anthocyanin content the leaves were homogenized in an ice bath with 3 ml HCl-methanol solvent (1: 99, v/v) and the supernatant was filtered after allowing the samples to stay in darkness at 5°C for 24 h. The amount of anthocyanin was calculated from the absorbance at 550 nm.

ASSAY OF ANTIOXIDANT ENZYMES ACTIVITIES AND RELATED METABOLITES

Determination of the activity of antioxidant enzymes and concentration of related metabolites were done by the method of Habibi and Hajiboland (2012). Fresh samples were ground in the presence of liquid nitrogen and measurements were undertaken using a spectrophotometer. Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to the method of Giannopolitis and Ries (1977). The enzyme was extracted in 25 mM HEPES (pH 7.8) with 0.1 mM EDTA and the supernatant was added together with the reaction mixture containing 0.1 mM EDTA, 50 mM Na_2CO_3 pH 10.2, 13 mM methionine, 63 µM nitroblue tetrazolium chloride (NBT), 13 µM riboflavin. For the determination of catalase (CAT, EC 1.11.1.6) activity samples were harvested and freshly homogenized in cold extraction buffer containing 50 mM phosphate (pH 7.0) and assayed spectrophotometrically by following the degradation of H_2O_2 at 240 nm by the method of Simon et al. (1974). The reaction medium contained 50 mM phosphate buffer, 10 mM H_2O_2 and one unit was defined as 1 µmol H_2O_2 decomposed min^{-1}. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was extracted and determined by the method of Boominathan and Doran (2002). The activity was assayed using 50 mM phosphate buffer (pH 7), 0.2 mM EDTA, 0.5 mM ascorbic acid and 50 µg BSA. Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobar-

bituric acid according to the procedure of Habibi and Hajiboland (2012). The soluble protein extraction and assay were carried out as described by Bradford (1976).

QUANTIFICATION OF NO CONCENTRATION

Nitric oxide concentrations were determined spectrophotometrically according to the method of Wu et al. (2016). The leaves were homogenized in 50 mM cool acetic acid buffer (pH 3.6, containing 4% zinc diacetate). The homogenate was centrifuged at 10,000 g for 15 min at 4°C, and the supernatant was collected. The obtained samples after the last centrifugation were mixed with cool acetic acid buffer, and then centrifuged. The two supernatants were combined and 0.1 g of charcoal was added. Absorbance was read at 540 nm, and the NO concentration was determined using a standard curve plotted with known concentrations of $NaNO_2$.

DETERMINATION OF SILVER CONTENT

Plant materials were ground in the extraction buffer containing 5 ml of a mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$) (v/v, 4:1) at 130°C for 1 h. After cooling, 5 ml of concentrated hydrochloric acid (HCl) was added and incubated at 115°C for 20 min. Determination of Ag content was carried out using an Inductively-Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, INTEGRA XL2, GBC; Australia).

HPLC ANALYSIS

The leaf extract (0.5 g) was homogenized in methanol (5 ml) and centrifuged at 3000 g prior to HPLC analysis. The HPLC analysis was done using an Agilent 1290 high-performance-liquid-chromatography (HPLC) system (Santa Clara, CA, USA) with an Agilent 1290 diode-array detector (DAD) according to Sinrod et al. (2019). Separation was achieved on a 25 cm × 4.6 mm Eurospher 100-5 C18 analytical column with pre-column provided by Knauer (Berlin, Germany). Data acquisition and integration were carried out with EZchrom Elite software. A 20 µl sample of the methanol extract in leaves was injected into an HPLC column through a 3900 Smartline Autosampler injector equipped with a 100 µL loop. Separation was performed using 0.02% trifluoroacetic acid in water (elution A) and methanol (elution D). The total running time was 55 minutes at a flow rate of 0.5 ml/min, and the column temperature was maintained at 20°C.

STATISTICAL ANALYSIS

The experiments were done in a complete randomized block design with 4 replications. Statistical analysis was done using Sigma Stat (3.5) with the Tukey test ($p < 0.05$).

RESULTS

The growth of *P. tuberosa* was measured and compared with the control plants. The dry mass of the shoot declined under both $AgNO_3$ and AgNPs treatments. In the present study, reduction of the shoot dry mass was significantly mitigated by NO and combined treatments of NO and SA. The dry mass of the root decreased in $AgNO_3$ treatments (Fig. 3) while in AgNPs treated seedlings the dry mass was not negatively affected. Interestingly, priming seeds with SA, NO or SA +NO alleviated the deleterious effects of AgNPs stress on the root growth. Under $AgNO_3$ stress, while the root dry mass was improved by SA prim-

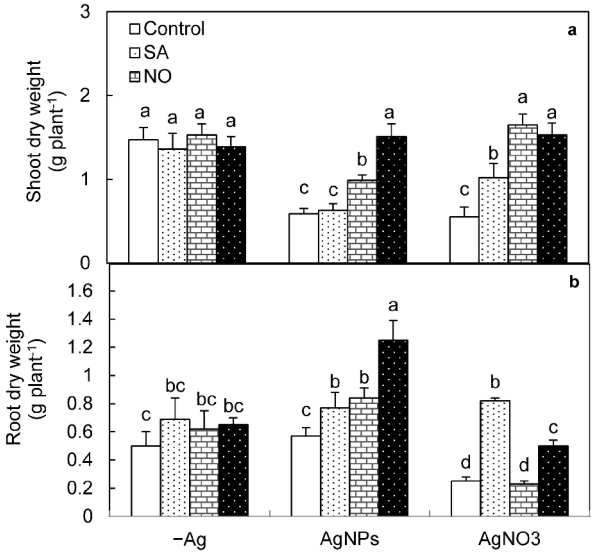

Fig. 3. Effect of SA and NO addition on the shoot and root dry weight of *Phlomis tuberosa* under $AgNO_3$ or AgNPs-stressed conditions. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

ing, it was not affected by exposure to other priming factors, compared with the control.

Both shoot and root Ag contents were significantly increased by both $AgNO_3$ and AgNPs treatments, and further increased by $AgNO_3$ treatment (Table 1). The highest accumulation of Ag was observed in SA+NO pretreated *Phlomis* leaves under AgNPs stress, however, this increase in Ag concentration was significantly prevented in roots by SA+NO priming.

In the control plants, NO application caused an increase in carotenoids levels. Under $AgNO_3$ and AgNPs treatments, the contents of total chlorophyll and carotenoids were not affected (Fig. 4). Under AgNPs treatments, while Chl*b*/Chl*a* ratio and carotenoids content were not affected by the exposure to SA or NO alone, they were significantly increased by the combination of SA and NO.

According to the results, a clear decrease in the relative amplitude of the IP (F_m) phase from the OJIP curve was observed in plants treated with AgNPs (Fig. 5). However, this decrease in IP

Table 1. Effect of SA and NO addition on Ag concentration (µg g^{-1}) in *P. tuberosa* plants under $AgNO_3$ or AgNPs-stressed conditions. Data of each row within each parameter indicated by the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

Treatment	Shoot Ag^+ concentration	Root Ag^+ concentration
Control	0.07 ± 0.002^e	0.10 ± 0.003^d
$AgNO_3$	1.79 ± 0.06^c	2.64 ± 0.13^a
$AgNO_3$+SA+NO	2.53 ± 0.09^b	0.63 ± 0.05^c
AgNPs	1.37 ± 0.04^d	1.21 ± 0.04^b
AgNPs+SA+NO	3.92 ± 0.10^a	0.72 ± 0.01^c

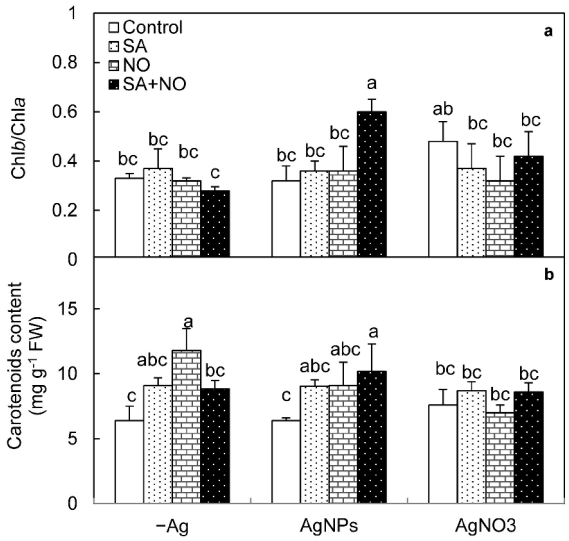

Fig. 4. Effect of SA and NO addition on the chlorophyll and carotenoids contents in *Phlomis tuberosa* leaves under $AgNO_3$ or AgNPs-stressed conditions. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

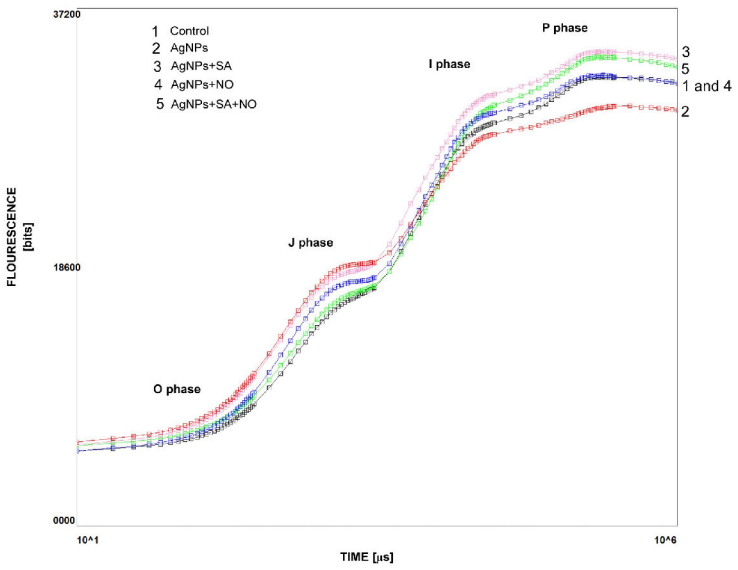

Fig. 5. Effect of SA and NO addition on the chlorophyll *a* fluorescence induction curve of *Phlomis tuberosa* leaves under AgNPs-stressed conditions.

phase was prevented in AgNPs treatment by SA or NO alone and their combination priming. In addition, we observed a slight increase in the OJ phase of the fluorescence rise only in the AgNPs-treated leaves, as compared to the control plants (Fig. 5). Although, the maximum quantum yield (F_v/F_m) of leaves was not affected by $AgNO_3$ and AgNPs treatments (Fig. 6), we detected that the performance index of photosystems (PI_{abs}), oxygen–evolving complex efficiency of PSII (F_v/F_o), quantum yield of electron transport (φ_{Eo}) were reduced after exposure to both $AgNO_3$ and AgNPs stresses (Fig. 6). However, this decrease in PI_{abs}, F_v/F_o and φ_{Eo} was significantly prevented in AgNPs treatment by H_2O_2+NO priming.

The results showed that proline contents were significantly increased by AgNPs and $AgNO_3$ treatments, and further increases were observed in plants primed with SA+NO (Fig. 7). While soluble sugars concentration was improved by AgNPs treatment, it was not affected by exposure to SA or NO priming, compared with the control. Under $AgNO_3$ treatment, while the starch content was not affected by exposure to SA or NO alone, it was significantly increased by the combination of SA and NO.

Under non-stress conditions, phenolic and flavonoids contents were not influenced by exposure to SA and NO priming (Fig. 8). Phenolic and flavonoids contents were significantly enhanced by $AgNO_3$ application, which was associated with a marked increase in PAL activity. Under AgNPs

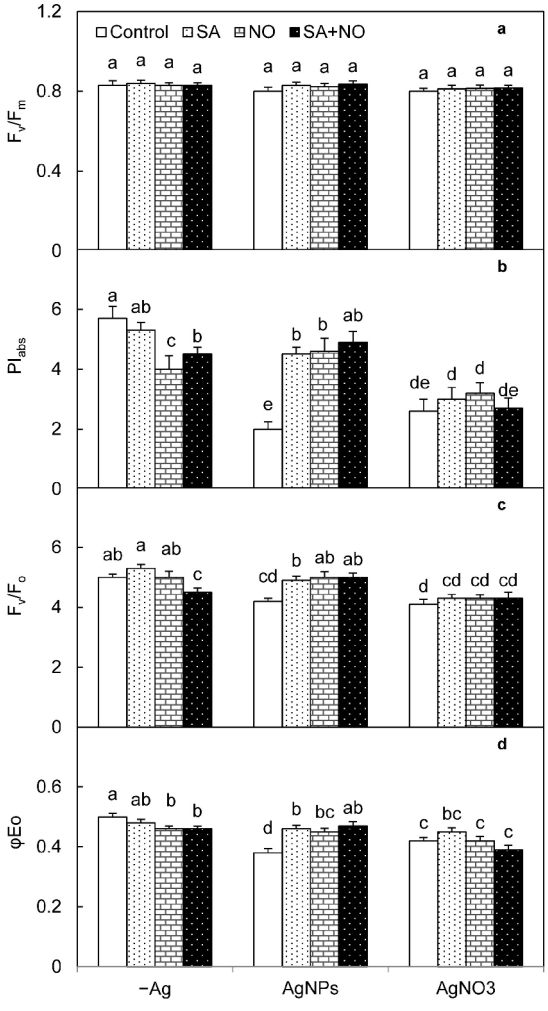

Fig. 6. Effect of SA and NO addition on the maximum quantum yield (F_v/F_m), oxygen-evolving complex efficiency of PSII (F_v/F_o), quantum yield of electron transport (φ_{Eo}) and performance index (PI_{abs}) of *Phlomis tuberosa* leaves under $AgNO_3$ or AgNPs-stressed conditions. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

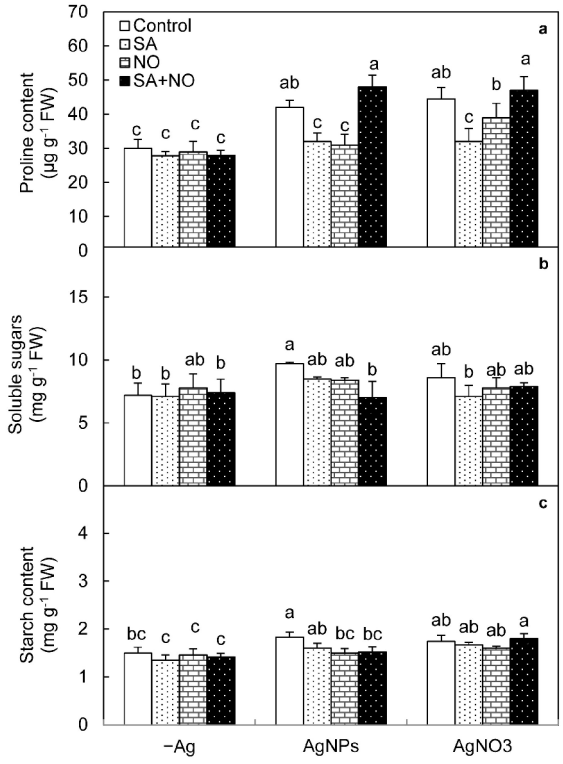

Fig. 7. Effect of SA and NO addition on the soluble sugars (mg g^{-1} FW), starch (mg g^{-1} FW) and proline (μg g^{-1} DW) contents in *Phlomis tuberosa* leaves under $AgNO_3$ or AgNPs-stressed conditions. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

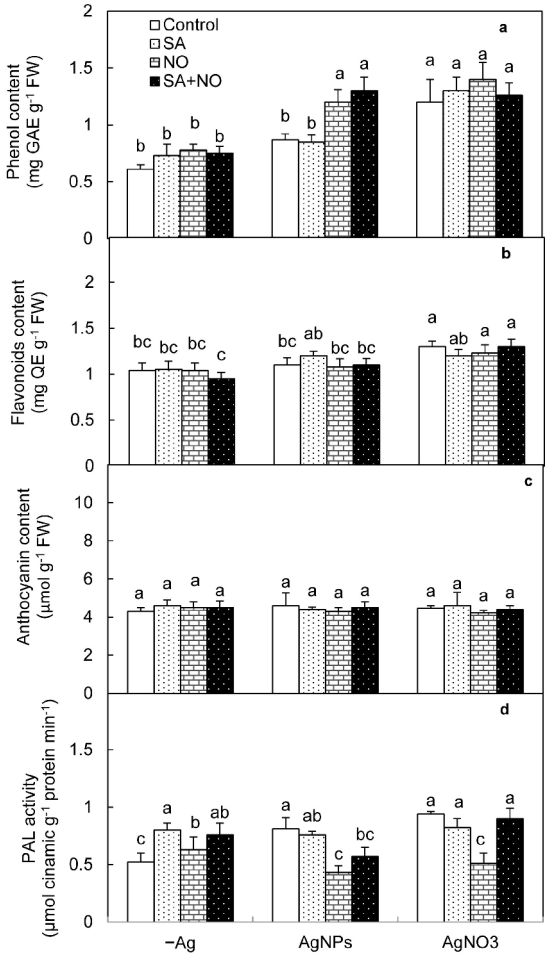

Fig. 8. Effect of SA and NO addition on the total phenol, anthocyanin and flavonoids contents, and the activity of phenylalanine ammonia-lyase (PAL) in *Phlomis tuberosa* leaves under $AgNO_3$ or AgNPs-stressed conditions. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

treatments, while the content of phenolic and flavonoids was increased by NO and SA+NO priming, it was not affected by exposure to SA priming alone, compared with the control. In the present study, the content of anthocyanin was not significantly affected by single SA and NO or by combination of SA+NO under both $AgNO_3$ and AgNPs treatments. We assessed the main phenolic acids in the methanol extract of *Phlomis* leaves using HPLC analysis (Table 2). With exogenous application of SA, sinapyl alcohol, chlorogenic acid and ascorbic acid contents were significantly increased, as compared with the control (Table 2). AgNPs also considerably enhanced the contents of sinapyl alcohol, ascorbic acid and cinnamic acid. On the other hand, addition of SNP or SA further increased the level of these phenolic compounds under AgNPs-induced stress. The highest chlorogenic acid was estimated in AgNPs+SA+NO treatment (Table 2).

The superoxide dismutase (SOD) activity in the primed AgNPs-stressed plants remained unchanged, but decreased in response to priming under $AgNO_3$ application (Fig. 9). The CAT activity was significantly increased by AgNPs stress, and further increase was observed under combined AgNPs and priming treatments (Fig. 9). A similar observation was recorded for the ascorbate peroxidase (APX) activity pattern in plants exposed to AgNPs and combined priming. Compared with $AgNO_3$ treatment alone, the $AgNO_3$ and priming combination caused higher CAT activity. The malondialdehyde (MDA) content in *Phlomis* leaves exposed to $AgNO_3$ or AgNPs significantly increased (49.1% and 45.5%, respectively; $p \leq 0.05$), as compared to the control (Fig. 10). However, the priming with NO or SA+NO depressed the content of MDA under $AgNO_3$ treatment. Furthermore, under AgNPs treatment, SA+NO-pretreated plants showed more dramatic decreases in MDA contents than the plants that were primed with SA or NO alone. The results indicated that NO contents remained unchanged in the non-primed Ag-stressed plants, but increased in response to SA +NO priming under both $AgNO_3$ and AgNPs treatments (Fig. 10).

DISCUSSION

ACCUMULATION OF SILVER NANOPARTICLES AND SILVER IONS AND EFFECT ON GROWTH PARAMETERS

The result of this study showed that AgNPs significantly reduced the shoot dry mass of *P. tuberosa*. These results were in agreement with previous studies, which confirmed that AgNPs can reduce the growth and development of many plant species (Zuverza-Mena et al., 2016; Zulfiqar et al., 2019; Wang et al., 2020). Measuring the growth of roots revealed that the significant decreases were only recorded under $AgNO_3$ stress, which could be correlated with increased accumulation of Ag in the roots and shoots of $AgNO_3$–treated seedlings. The above findings suggest that Ag in both forms, i.e., bulk silver and nano silver

Table 2. Effect of SA and NO addition on the content of phenolics (mg ml^{-1}) in leaves of *Phlomis tuberosa* under $AgNO_3$ or AgNPs-stressed conditions. Data of each row within each parameter indicated by the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

Treatment	Sinapyl alcohol	Chlorogenic acid	Ascorbic acid	Cinnamic acid
Control	0.17 ± 0.02^{c}	0.001 ± 0.00^{d}	1.64 ± 0.06^{d}	0.024 ± 0.00^{b}
SA	0.38 ± 0.06^{b}	0.022 ± 0.003^{b}	2.62 ± 0.16^{b}	0.033 ± 0.002^{ab}
NO	0.16 ± 0.01^{c}	0.023 ± 0.004^{b}	1.75 ± 0.09^{d}	0.023 ± 0.003^{b}
SA+NO	0.17 ± 0.04^{c}	0.014 ± 0.003^{c}	2.26 ± 0.04^{c}	0.042 ± 0.005^{a}
AgNPs	0.37 ± 0.02^{b}	0.001 ± 0.00^{d}	3.03 ± 0.07^{a}	0.037 ± 0.002^{a}
AgNPs+SA	0.32 ± 0.06^{b}	0.016 ± 0.001^{c}	3.02 ± 0.16^{a}	0.044 ± 0.003^{a}
AgNPs+NO	0.53 ± 0.09^{a}	0.015 ± 0.001^{c}	2.92 ± 0.09^{a}	0.042 ± 0.007^{a}
AgNPs+SA+NO	0.12 ± 0.04^{c}	0.087 ± 0.003^{a}	1.61 ± 0.04^{d}	0.031 ± 0.009^{b}

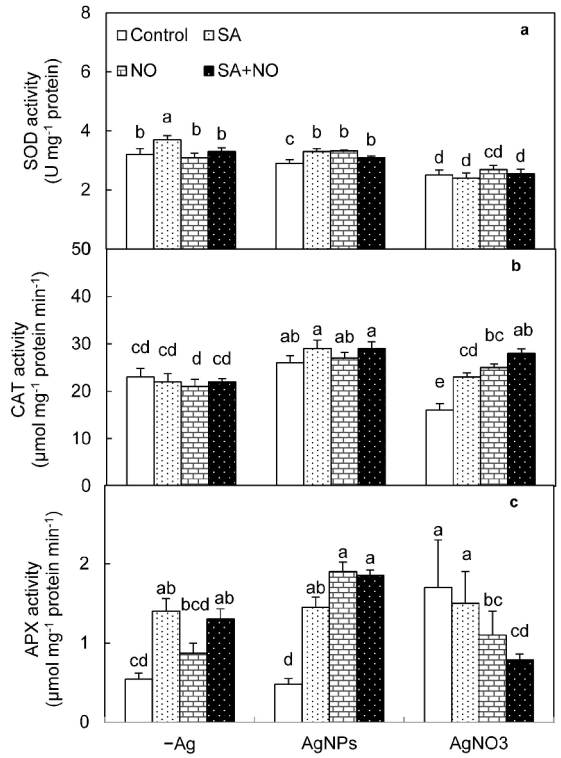

Fig. 9. Effect of SA and NO addition on the activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) in *Phlomis tuberosa* leaves under $AgNO_3$ or AgNPs-stressed conditions. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

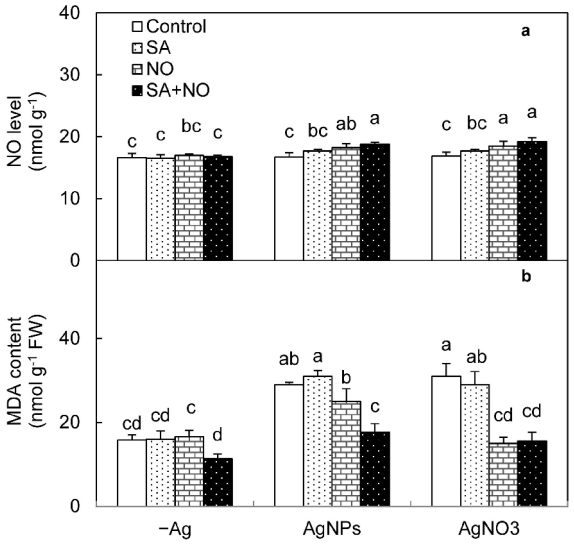

Fig. 10. Effect of SA and NO addition on the concentration of nitric oxide (NO) and malondialdehyde (MDA) in *Phlomis tuberosa* leaves under $AgNO_3$ or AgNPs-stressed conditions. Bars indicated with the same letter are not significantly different ($p < 0.05$, Tukey test). Values are the mean ± SD (n = 4).

imparted toxicity on cell division and/or cell elongation (Singh et al., 2014; Vishwakarma et al., 2017). However, the growth inhibition under AgNPs was mitigated by NO and SA+NO priming. Similar results were obtained by Tripathi et al. (2017), who reported that the addition of SNP (a donor of NO) alleviated AgNPs-induced adverse effects on growth parameters in *Pisum sativum* seedlings. Additionally, our results are consistent with the findings of Amooaghaie et al. (2018) for *Brassica nigra*, who found that the application of NO improved plant growth under AgNPs and Ag^{+} ions stresses. Furthermore, plants pretreated with SA+NO exhibited better growth under AgNPs conditions than plants that were pretreated with

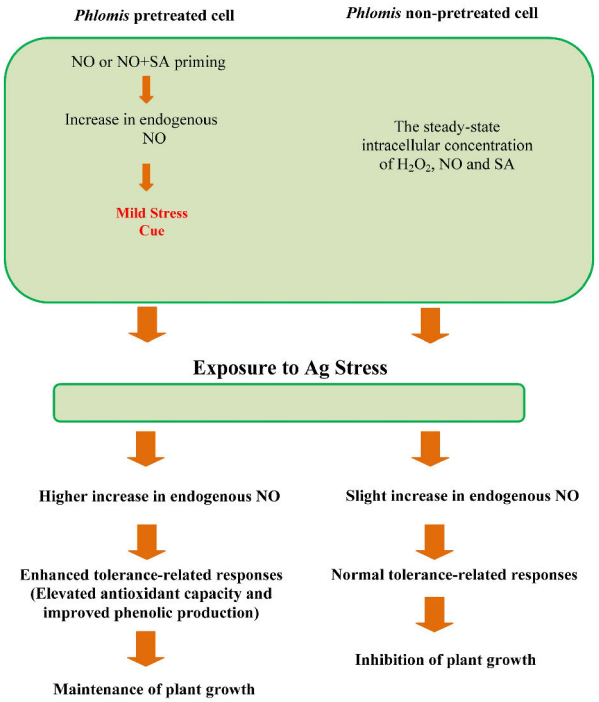

Fig. 11. Priming with exogenous SA or NO induces AgNPs-stress tolerance of *Phlomis tuberosa* plants and improves physiological homeostasis and plant growth.

SA or NO alone, suggesting that SA+NO application was more effective than SA or NO alone for *Phlomis* plants exposed to AgNPs stress. As a result, pretreatment with SA+NO tended to increase Ag translocation from roots into photosynthetic organs under AgNPs stress while saving proper growth.

EFFECT OF SILVER NITRATE AND SILVER NANOPARTICLES ON TOTAL CHLOROPHYLL, CAROTENOIDS AND CHLOROPHYLL FLUORESCENCE TRANSIENT MEASUREMENTS

The results showed that both AgNPs and $AgNO_3$ decreased photochemical activity of *Phlomis* plants, which was attendant with the largest decline in F_v/F_o, quantum yield of electron transport (φ_{Eo}) and performance index of photosystems (PI_{abs}) (Fig. 4). Because of the lower F_v/F_o under AgNPs or $AgNO_3$ stress, it can be assumed that the reduction in the water–splitting complex activity at the donor site of PSII was due to the transient toxicity induced by Ag^+ in our study (Habibi and Ajori, 2015). The decrease in φ_{Eo} may be associated with replacement of Ag^+ ions with Cu^+ ions in plastocyanin as a copper-containing protein that mediates electron-transfer. This replacement of Ag^+ ions with Cu^+ ions causes disturbance or inactivation of the photosynthetic electron transport (Jansson and Hansson, 2008; Biba et al., 2021). Our results showed that AgNPs negatively affected F_v/F_o, (φ_{Eo}) and PI_{abs}, however, seed priming with the combination of SA and NO significantly improved these parameters under AgNPs stress. This improvement of photochemical activity was dependent on significant accumulation of chlorophyll *b* and carotenoids. In fact, *Phlomis* leaves accumulate carotenoids for light harvesting and photoprotection (Aragón–Gastélum et al., 2020; Habibi, 2020; Habibi, 2021). Here, we could correlate carotenoid accumulation with electron transfer enhancement in the *Phlomis* plants primed with SA+NO.

We also estimated the changes in the OJIP curve in response to AgNPs stress to determine the stress-induced effects on photosynthesis (Tripathi et al., 2016; Liang et al., 2019). After exposure of *Phlomis* plants to AgNPs, there was a clear decline in the relative amplitude of the IP (F_m) phase in parallel with the increase in the OJ (F_0) phase of the fluorescence rise, suggesting that the silver nanoparticle treatments affected all the components of electron transport chain from PSII towards PSI. This higher OJ phase was dependent on the dissociation of LHCII and PSII complex or the reduced electron flux from Q_A to Q_B (Kalaji et al., 2014; Hamdani et al., 2015). The same impacts on PS-II under silver nanoparticle treatments were previously reported by Dewez and Oukarroum (2012). However, when SA and NO-pretreated plants were subjected to AgNPs, the increased IP phase revealed that the priming with SA and NO enhanced the improvement of PSII photochemical efficiency. Our results are consistent with the findings of Tripathi et al. (2017) for *Pisum sativum* seedlings, who reported that the seed priming with NO ameliorated AgNp-induced adverse effects on chlorophyll fluorescence parameters. To conclude, further analysis will be needed to understand the effect of SA+NO priming on the photochemical processes as well as fast chlorophyll *a* fluorescence kinetics.

CHANGES IN COMPATIBLE SOLUTES BY BOTH SILVER IONS AND SILVER NANOPARTICLES

Previous studies showed that a severe reduction of sugar contents as well as increase in proline contents were found in rice (*Oryza sativa* L.) seed-

lings treated with AgNPs (Nair and Chung, 2014). To the contrary, in this study, a significant increase of soluble sugars concentration was obtained under AgNPs treatment. Sugars, as important signaling molecules, are known to accumulate during stress in leaves (Wingler et al., 2000; Zhang et al., 2018). In this study, a significant increase in the accumulation of soluble sugars was recorded upon exposure to AgNPs, which possibly function in the stress tolerance of *Phlomis* plants. Many plants, when exposed to salinity stress, accumulate proline in large quantities, as a ROS scavenger and osmotic regulator (Chun et al., 2018). In this study, we showed that priming with SA+NO led to high levels of proline under both AgNPs and $AgNO_3$ treatments. This result was in line with the findings of Ali et al. (2017) who showed a large increase in total proline contents when wheat cultivars were subjected to salinity in the presence of 0.1 mM SNP. In addition, our results are in agreement with many previous reports that SA is involved in enhancing the proline level under heavy metal stress (Faraz et al., 2019).

CHANGES IN ANTIOXIDANT ACTIVITY, PHENOLIC AND NO LEVELS

Phenolic compounds are one kind of secondary metabolites, which can inhibit lipid peroxidation (Oh et al., 2009) and subsequently enhance the plant resistance to environmental stresses (Blasco et al., 2013; Su et al., 2018). Keeping this in view, when AgNPs-exposed plants were primed with NO or SA+NO, increases in the accumulation of these compounds are likely related to the quenching of oxidative stress and to general defense. In this study, with exogenous application of SA or NO alone under AgNPs-induced stress, increases in the accumulation of sinapyl alcohol, chlorogenic acid, cinnamic acid and ascorbic acid compounds may be involved in the protective mechanism in *Phlomis* upon AgNPs stress (Ford et al., 2010; Zhang et al., 2018; Noori et al., 2020).

It was reported that the accumulation of nonenzymatic antioxidants such as phenolic compounds and flavonoids is closely associated with marked increases in the activity of antioxidant enzymes such as SOD, CAT and APX in order to inhibit overproduction of ROS (Laxa et al., 2019; Hasanuzzaman et al., 2020). Similarly, in the present study, the accumulation of phenolic compounds correlated with a high level of CAT and APX activities in NO-primed plants under AgNPs stress, and phenolic compounds and flavonoids appear to be major players in ROS scavenging.

Here, Ag exposure caused lipid peroxidation as indicated by the higher MDA levels. Similar results were reported by Mo et al. (2021), who showed that Ag at high concentration (100 mg/L) caused membrane damages. However, the use of SA+NO greatly reduced MDA content and mitigated oxidative damages, by upregulating of antioxidative enzymes activities in response to AgNPs stress. The present results are in agreement with the previous ones, which revealed that the use of NO reduced salt-induced inhibitory effects in *Pisum sativum* seedlings (Tripathi et al., 2017) and *Brassica nigra* (Amooaghaie et al., 2018) via enhancement of antioxidant components involved in removing ROS. In addiotion, our results are consistent with the findings of Zhang et al. (2018) for *Cucumis sativus*. They reported that SA alleviated Ag-induced stress by increasing the activities of defense-related enzymes. Interestingly, combined priming was more effective than SA or NO alone in alleviating AgNPs-induced stress in *Phlomis* plants.

CONCLUSION

This study suggested that both $AgNO_3$ and AgNPs at 1000 ppm adversely decreased the growth, pigments and photosynthesis due to the enhanced level of Ag and significant lipid peroxidation. However, SA and SNP addition successfully reduced the levels of oxidative stress marker (MDA) and consequently ameliorated the adverse impact of Ag on *Phlomis* seedlings. Strong amelioration of Ag-induced stress under SA+NO priming was achieved by accumulation of antioxidant compounds (such as carotenoids, chlorogenic acid, cinnamic acid and ascorbic acid) which consequently preserved photochemical functioning. The increased OJ part of the OJIP curve and the decreased IP phase of the curve in parallel with the reduction of the water–splitting complex activity and PI_{abs} showed that the AgNPs stress impaired the whole photosynthetic electron transport flux in the *Phlomis* leaves, however, combined priming mitigated AgNPs-induced adverse effects on these chlorophyll fluorescence parameters. The obtained results suggest that SA +NO can improve the removal efficiency of AgNPs

toxicants from the environment. These findings provide valuable information for development of sustainable strategies in order to reduce the negative impact of AgNPs and $AgNO_3$ on crops, which will be important knowledge to support the sustainable use of AgNPs in agriculture.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Elham Ghasemifar, Ghader Habibi and Golamreza Bakhshi-Khaniki. The first draft of the manuscript was written by Ghader Habibi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. The authors have no relevant financial or non-financial interests to disclose.

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