STUDY OF SILVER NANOPARTICLES UPTAKE BY *Helianthus annuus* CROP IN SALINITY CONDITIONS

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Abstract

Engineered nanoparticles (NPs) are used in different industrial products, including cosmetic, pharmaceutics, clothes, electronic and agriculture products. In the past years the use of silver nanoparticles (AgNPs) expanded significantly, especially due to their antibacterial and antifungal properties. Despite the benefits in using AgNPs for different purposes, they enter in the environment can be problematic and have different mechanisms of accumulation, internalization and toxicity in plants. Moreover, plant growth and development is limited by salinity conditions, an abiotic stress parameter, especially in arid, semi-arid and irrigated areas in tropical and sub-tropical places. The germination, seedling and plant growth and, consequently, the productivity of the plants decrease, causing economic and social impacts. In this context, the aim of the present study was to track the uptake of AgNPs by sunflower (*Helianthus annuus*), a metal hyperaccumulator plant. For this, experiments were conducted in soil and hydroponic mediums where plants were treated with salinity and two different concentrations of AgNPs. Four groups were studied: control (without exposure to AgNPs and NaCl), salinity (group exposed to 100 mM of NaCl), AgNPs (group exposed to 5 or 100 $mg.kg^{-1}$ of AgNPs (the lowest concentration was used only in hydroponic experiment) and AgNPs plus salinity (group exposed to 5 or 100 $mg.kg^{-1}$ of AgNPs and 100 mM of NaCl). At the end of the experiments, plants were harvested; roots, shoots and leaves were separated and samples were prepared for elemental analysis, lipid peroxidation and pigment analysis. Results showed the internalization of Ag in the cortex of roots from sunflower crop in hydroponic medium. K content in roots was negatively affected by nanoparticles and salinity treatments. The exposure of sunflower to AgNPs and salinity seems to intensify silver accumulation in leaf and root tissues.

1. INTRODUCTION

Silver (Ag) nanoparticles (NPs) are engineered nanomaterials with widespread application in the industry of everyday products because their antimicrobian and antifungical properties. These metal based nanoparticles received attention in the last years not only due their specific and useful characteristics but also because the risks

that they could cause to the environment, through their uptake by plants and small animals, affecting the food chain and, consequently, the whole ecosystem. Benefits and risks of toxicity for live organisms caused by different types of engineered NPs are not complete understood since they depend on the format, size and composition of the nanomaterials. To know the dynamic of NPs in different mediums like soil, sludge or water, and after they be absorbed by live organisms, detailed studies are required, including the whole cycle of the nanomaterials in the medium and in the respective biological organism which uptake them. Depend on the medium and characteristics of the NPs, they could became dissolved or in agglomerate form and their chemical state could be modified, changing the toxic potential of silver [1]. Since AgNPs have been an important component of a variety of consumer products, it is probable they exist in the environment in consequence of the industrial activity and possible discharge routes. Thus, it is necessary to evaluate their interaction with different live organisms, especially plants because they are producers and, consequently, the base of the food chain [2]. By other side, the use of AgNPs in agriculture increased due the application of nano-encapsulated agrochemicals aiming to optimize the outcomes through soil and culture practices, since AgNPs present antimicrobial and nutritional properties. Moreover, benefits in the use of AgNPs in plant biotechnology have been reported [3]. However, when in high concentrations, AgNPs could accumulated in plant cells, became toxic and inhibit the growth of the plants when applied in high concentrations [3].

Plant growth and development is limited by salinity conditions, an abiotic stress parameter, especially in arid, semi-arid and irrigated areas in tropical and sub-tropical places. In general, the germination, seedling and plant growth and, consequently, the productivity of the plants decrease, causing economic and social impacts [4]. Due the high concentration of sodium (Na) and chlorine (Cl) ions and the inhibition of nutrient uptake from the soil, the plant nutrition is negatively affected. For example, salinity stress disturbs the uptake and accumulation of essential nutrients such as potassium (K) and calcium (Ca) [5]. Thus, field practices have been studied aiming to improve crop development under salinity conditions, such as K application to overcome the effects of salinity in pearl millet [5]. In addition, Hurtado and collaborators [6] reported the benefit in the application of silicon (Si) to sorghum and sunflower crop in salinity environment.

Besides that, the role of nanoparticles in plant development under salinity stress has been studied, especially because the application of nutrients in such nano structures make them better absorbed by the plant cells. The interaction between AgNPs and salinity was evaluated in grass pea germination [7]. The germination speed index was calculated for the different studied treatments, showing a positive result in the application of AgNPs to seed germination when exposed to different salinity levels [7]. Mozafari and collaborators [8] reported the benefits of iron nanoparticles and potassium silicate treatments for grape (Khoshnaw cultivar) exposed to salinity environment.

In the present work, the main aim was to evaluate the uptake of silver nanoparticles by sunflower (*Helianthus annuus*), a metal hyperaccumulator plant [9], exposed to salinity stress, using accelerator's based techniques. Sunflower is an important economic crop, especially in Ukraine, Russian Federation, European Union, Argentine and Turkey, the main worldwide producers [10], used for oil seed and other commodity production, to feed animals and for bioenergy proposes. Moreover, this plant is known to be a metal accumulator, making it an important green option to remove metals from the soil (phytoextraction technology) [11]. Thus, it is essential to understand the AgNPs effect on this plant because i) it could be a source of silver toxicity to other live organisms; ii) it could be used for phytoextraction of silver in contaminated soil; iii) the evaluation of AgNPs role in the plant development under salinity stress is a valuable data for producers from arid, semi-arid and irrigated regions, since it may support new tools and technologies for field practices.

2. MATERIAL AND METHODS

2.1 Samples' experiment design

All experiments regarding the plant crop and sample preparation were conducted at the Department of Biology - Biotechnical Faculty of the University of Ljubljana. Seeds of sunflower (*Helianthus annuus*) were germinated in verniculite at the growth chamber under controlled conditions (photoperiod: 16/8h day/night; cool white fluorescent illumination: 550 μmol m^{-2} s^{-1}; constant temperature: 20 °C; humidity: 50%). When the plants presented two leaves (about 1 cm of length), they were carefully transferred to the crop medium (hydroponic or soil) and kept in the growth chamber during the experiment time. Fig. 1 shows a scheme of the plant crop experiments.

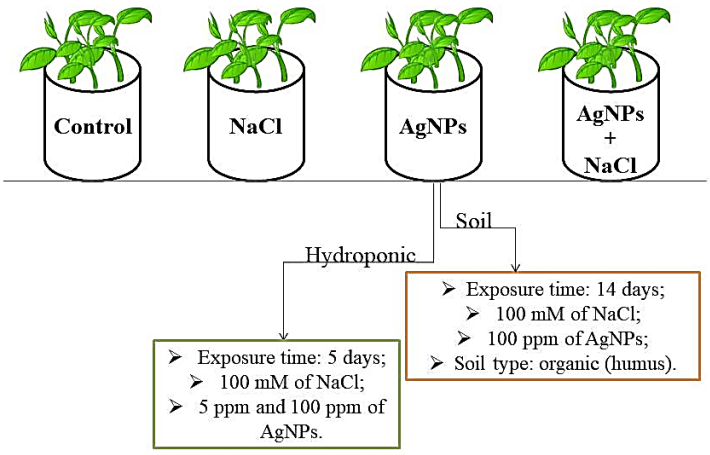

FIG. 1. Scheme of the plant experiments performed in hydroponic and soil crop mediums. In both cases, the plants were kept in the grow chamber under controlled conditions of light, temperature and humidity. The concentrations of AgNPs and salinity as well as the time of exposure is shown.

2.2 Hydroponic experiment

Hydroponic experiments were conducted using both 5 $mg.kg^{-1}$ or 100 $mg.kg^{-1}$ concentrations of AgNPs. For each concentration, four experimental groups were considered: control group, group exposed to 100 mM of salinity solution (NaCl), group exposed to AgNPs (AgNps) and the group exposed to a combination of salinity (100 mM) and AgNPs (NaCl+ AgNPs). For each group, one pot filled with 800 mL of hoagland solution was prepared and four plants were placed in each pot. 100 ml of AgNPs solution (5 or 100 $mg.kg^{-1}$ of AgNPs nanopowder (Sigma Alderich) dissolved in 100 ml of bidestilated water) was added in the pots of groups treated with nanoparticles. Groups treated with salinity received a total solution of 100 mM of NaCl dissolved in bidestilated water, which was applied during three consecutive days to achieve a gradual increase in salinity and therefore reduce the osmotic shock for the plants. Finally, 100 ml of bidestilated water was added to the control group to make equal the volume of solution in all groups. The experiment was repeated twice, totalizing eight plants per group. The exposure time of each repetition was five days.

At the end of the experiment, plants were harvested. Roots, stems and leaves were separated, washed in bidestiled water and properly prepared for elemental micro analysis. Sample preparation for elemental mapping and composition is a crucial step to obtain reliable information about the chemical element's localization in the biological tissues [12, 13]. The procedure followed in this work is described in details by Vogel-Mikus and collaborators [12] and consists in the cryo-fixation of the plant tissue, specimen sectioning at low temperature (about -25 °C) and freeze-drying. The sectioned specimens were 25 µm and 60 µm thicker. After freeze-dried, the integrity of the samples was verified by optical microscopy. Samples were accommodated in specific holders according the analytical technique setup requirements. Additionally, dried and pulverized root material was pressed into pellets for silver chemical speciation analysis.

2.3 Soil experiment

Humus soil (3.5 kg) was contaminated with 100 $mg.kg^{-1}$ of AgNPs nanopowder (Sigma Alderich) dissolved in bidestilated water. The nanoparticle solution was very well mixed with the soil and the mixture was placed inside a black plastic bag to get stabilization for two weeks. After that, sunflower plants were transferred to the appropriate pots filled with the contaminated soil. Again, four plant groups were considered for the experiment: control group, group exposed to 100 mM of salinity solution (NaCl), group exposed to AgNPs (AgNps) and the group exposed to NaCl+ AgNPs. The total solution of salinity was applied during three consecutive days in the plants. Since the experiment took place during two weeks and it was expected plants get bigger than in the hydroponic experiment, four pots per group were prepared, and two plants were accommodated in each pot. In total, eight plants per group were crop. In the end of the period, the plants were carefully harvested and washed with bidestiled water. Roots and leaves were separated and weighted. One fraction of the plant material was

prepared for lipid peroxidation, as an indication of oxidative stress in roots and leaves, following the protocol described by Hodges and collaborators [14]. Lipid peroxidation content was expressed as malonaldehyde (MDA) equivalent production in these plant organs; the other part was freeze-dried during 72 h, approximately. Photosynthetic pigment content was evaluated in dried and pulverized sunflower leaves according Lichtenthaler and Buschmann (2005) [15] methodology. Additionally, elemental analysis was carried out in leaves and roots. For this, samples were prepared in duplicate by pressing the dried and pulverized root and leaves material in pellets. The concentration of Ag was also determined in soil samples. In this case, samples were prepared in triplicate by pressing the soil into pellets.

2.4 Elemental analysis

The uptake of AgNPs by sunflower and the elemental composition of all plant samples were evaluated by X-ray spectroscopies, namely Particle Induced X-ray Emission (PIXE) [16] and X-ray Fluorescence (XRF) [17]. Both techniques provide the elemental composition of the material with good resolution and limit of detection for trace and major elements. Moreover, these methods can be setup for micro analysis, giving a map of the chemical element distribution in the material with resolution about 1 µm or less [18, 19].

In this work, PIXE was carried out by using a focused ion beam combined with a scanning system – in this case known as microPIXE. MicroPIXE measurements were performed at the Low and Medium Energy Departament, of the Josef Stefan Institute (JSI – Ljubljana, SL) [20, 12]. The incident proton beam (3 MeV) on the samples was delivered by a 2 MV Tandetron accelerator. The current was maintained in 150 pA approximately and the time of each scan varied between 3 and 14 h. The emitted X-rays were collected by a silicon drift detector (SDD) and a high-purity germanium (HPGe) detector. Sunflower samples were placed between two thin foils of Pioloform film on the aluminum holder, which was accommodated in a specific metallic support inside the reaction chamber. The pressure inside this chamber was kept about 10^{-6} mbar. PIXE spectra were fitted by GeoPIXE [21] and GUPIXWIN [22] softwares. The incident charge measurements were performed using a chopper.

XRF measurements of plant bulk samples were performed at JSI using a PeduzoT02 system with an americium (Am) excitation source. Elemental concentrations were calculated using AXIL X-ray analysis package [23]. MicroXRF was also carried out to complement results of Ag distribution in root samples. In this case, 25 µm thick root samples were irradiated at the ID-21 XRF beam line of ESRF synchrotron source (Grenoble, FR). The XRF spectra were fitted by PYMCA [24].

Additional analyses of root samples were carried aiming to check the chemical speciation of silver in the plants through X-ray Absorption Near Edge Structure (XANES). The measurements were performed at the P64 beamline of PETRA III (DESY laboratory, Hamburg – Germany) [25]. XANES spectra were fitted by Athena software [26].

2.5 Data analysis

Data of different parameters (lipid peroxidation, pigments, elemental concentration) were taken to perform one way ANOVA followed by Tukey. For each individual experiment every parameter was compared among the treated groups (statistically significant difference: $p<0.05$).

3. RESULTS AND DISCUSSION

3.1 Ag uptake – Hydroponic experiment

Root samples of sunflower exposed to silver nanoparticles (100 $mg.kg^{-1}$) were analyzed by XANES in order to check the chemical state of silver after the interaction with the medium and plant tissues. The analysis of the spectra (data no showed) revealed that the major part of silver was retained as nanoparticles. Some small deviations in the spectra were observed, probably because the introduction of organic ligands, such as sulfur (S) and oxygen (O) due the incorporation of the metal into the plant.

Considering this result, the uptake of AgNPs by sunflower was verified in the plant tissues. Figure 2 shows the localization and concentration of silver in root samples (60 µm thick) of sunflower treated with AgNPs and AgNPs + NaCl, for both concentrations of nanoparticles, obtained by microPIXE. The average concentrations of

Ag in the samples exposed to the higher concentration of AgNPs were (10600 ± 54) mg.kg^{-1} and (2800 ± 50) mg.kg^{-1} in samples of these groups, respectively. Plants treated with 5 mg.kg^{-1} of AgNPs and 5 mg.kg^{-1} + 100 mM of NaCl also uptake silver nanoparticles in the concentrations of (1300 ± 24) mg.kg^{-1} and (358 ± 26) mg.kg^{-1}, respectively. In all elemental maps was observed that Ag is concentrated in the root cortex, with some hot spots in the proximities of the epidermis and less distributed around the xylem. This same pattern of Ag distribution in root samples was also observed using microXRF facility (Figure 3). As expected, silver was not found in roots from control and salinity groups. In root from control group, the fitting of the PIXE spectrum resulted in a concentration value below the minimum detection limit (MDL) (MDL = 117 mg.kg^{-1}). Similar result was found for root samples treated with salinity (MDL = 33 mg.kg^{-1}).

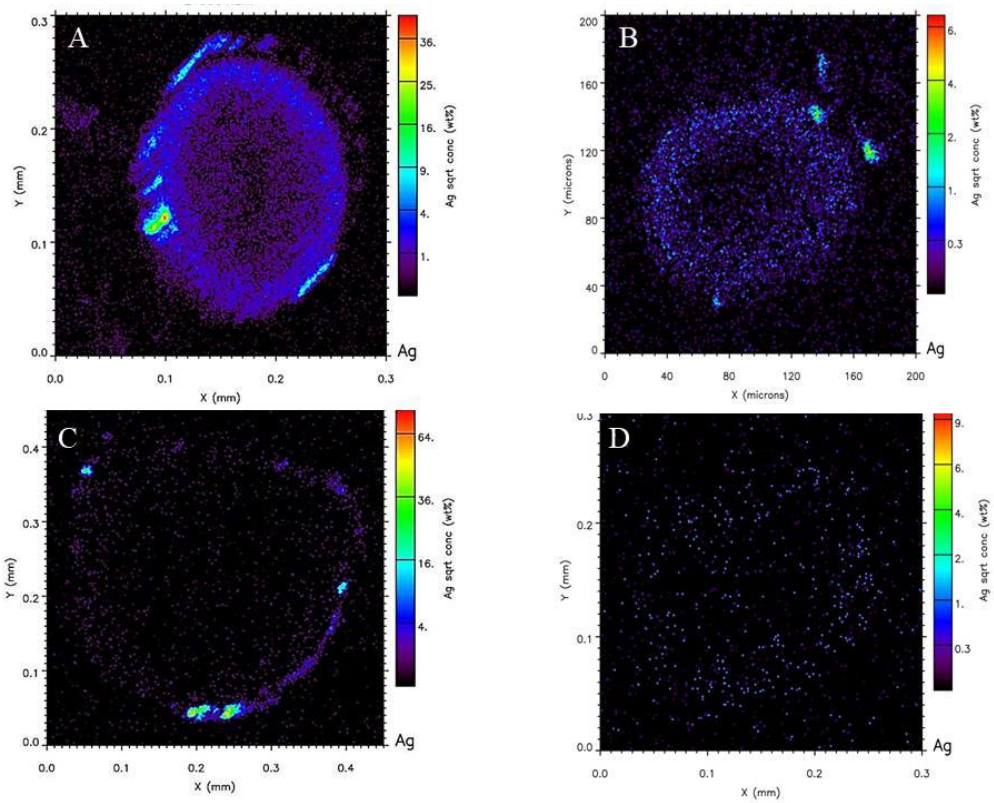

FIG. 2. Silver distribution in root samples of sunflowers exposed to 100 mg.kg^{-1} (A) and 5 mg.kg^{-1} (B) of AgNPs, 100 mg.kg^{-1} AgNPs plus 100 mM of NaCl (C) and 5 mg.kg^{-1} AgNPs plus 100 mM of NaCl (D). The elemental maps were obtained through GeoPIXE fitting of the PIXE spectra..

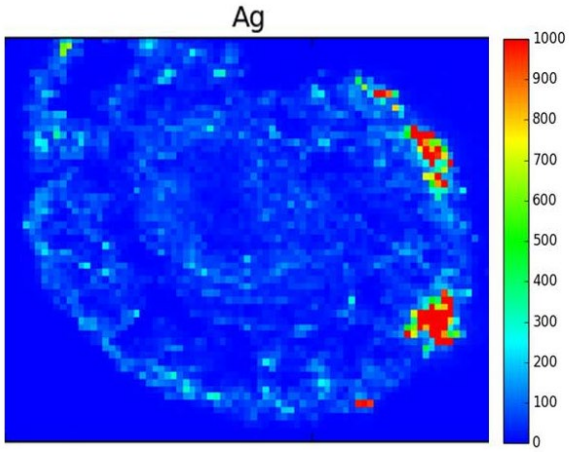

FIG. 3. Silver distribution in root of sunflower exposed to 100 mg.kg^{-1}. The scale bar indicates the silver content qualitatively: less intense Ag signal (blue color) to higher intense Ag signal (red color). Result obtained using microXRF.

Potassium (K) was quantified in root bulk samples by XRF. Figure 4 shows its behavior in function of the treatments. It is notable that the stress caused by salinity and AgNPs have an influence in the absorption of K by the plants and their nutrition. The combination of both treatments increased the concentration of K in the plant tissue in comparison with AgNPs treated group. Positive effect of lower concentrations of AgNPs than 100 mg.kg^{-1} applied to seed germination or plants cultivated in salinity environment was observed for different crops [7, 27]. In the present work, even the lowest concentration of AgNPs (5 mg.kg^{-1}), decreased the potassium absorption by sunflower, corroborating the effect dependence of type and concentration of nanoparticle on the plant development.

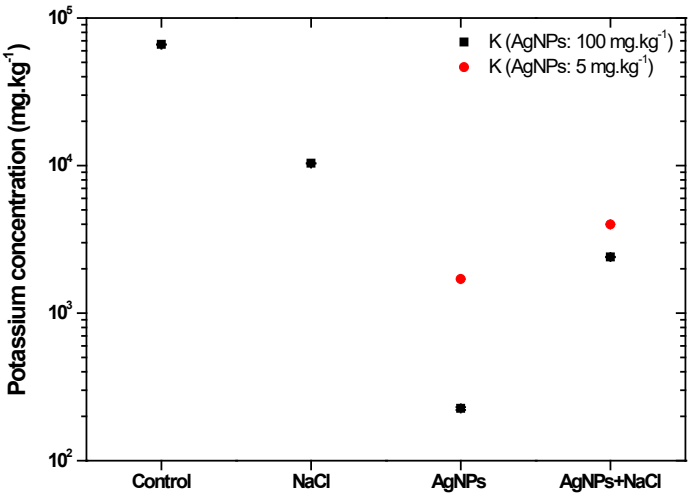

FIG. 4. Potassium concentration in roots of sunflower crop in hydroponic medium. Red circles and black squares correspond to samples from groups treated with 5 mg.kg^{-1} and 100 mg.kg^{-1} of AgNPs, respectively. Uncertain bars are not visible in the plot since they range between 1% to 5% of the concentration value.

In order to study a possible translocation of Ag in the plant tissues, stems and leaves were analyzed by microPIXE. Stems from plants treated 100 mg.kg^{-1} of AgNPs showed a silver concentration of 72 mg.kg^{-1}, which was approximately the MDL value. However, in leaves of the same group was not detected silver above the MDL. Although the low content of Ag in stems, it seems that the stress caused by the treatments with nanoparticles and salinity changed the uptake of nutrients such as calcium (Figure 5). The content of this element decreased in fifty per cent, approximately, in plants treated with AgNPs+NaCl in comparison with the control group.

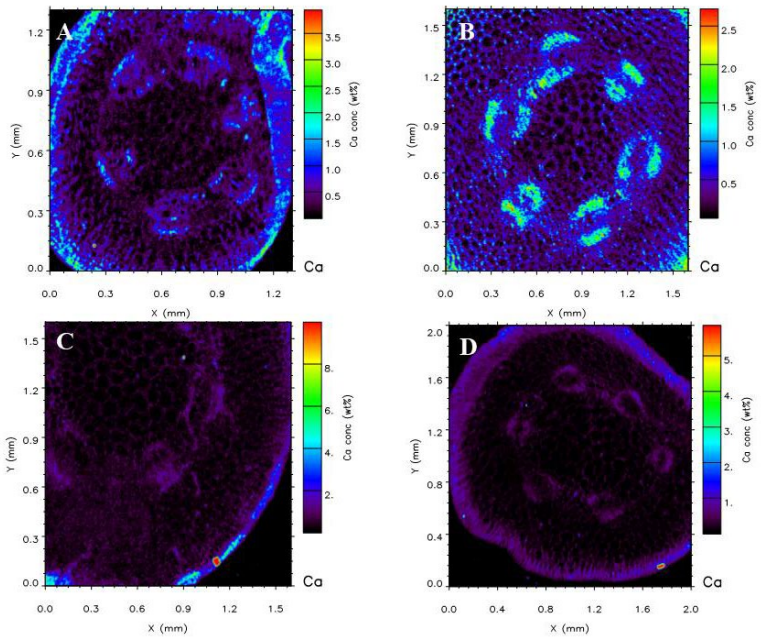

FIG. 5. Calcium distribution in stems from control (A), 100 mM of salinity (NaCl) (B), 100 mg.kg^{-1}of AgNPs (AgNPs) (C) and 100 mg.kg^{-1} AgNPs plus 100 mM of salinity (AgNPs+NaCl) (D) groups. Ca distribution maps were obtained by microPIXE and quantified using GeoPIXE software.

3.2 Ag uptake – Soil experiment

Figure 6 shows the concentration of Ag in soil, root and leaves of sunflower, obtained by XRF. Ag content in samples from Control and Salinity groups was bellow of the LOD. Salinity increased the AgNP uptake in sunflower plants (roots and leaves). Therefore, the concentration of Ag in the soil treated with NaCl+AgNPs is lower than in NaCl-free soil since the AgNPs concentration was the same in the begin of the experiment for both soil groups. (the soil Ag concentrations at the beginning of the experiment were the same).

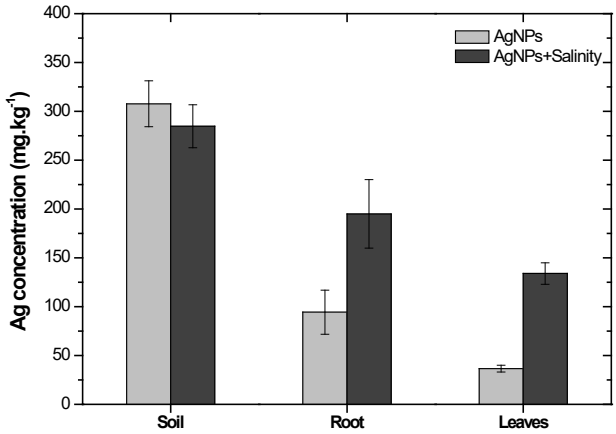

FIG. 6. Ag concentration of soil, roots and leaves of sunflower samples. Data obtained by XRF in triplicate (duplicate for leaves). Uncertain bars correspond to the standard deviation of the mean..

The stress caused in sunflower plants due the esposure to AgNPs and salinity is demonstrated by the results of lipid peroxidation (Figure 7) and pigment analysis (Table 1) The level of chlorophyll A decreased in function of all treatments while chlorophyll B content decreased in the presence of the salinity and with the combination of AgNPs and salinity. However, only salinity did not change carotenoids content in sunflower leaves. In this case, nanoparticles were responsible to decrease carotenoids in the plants and this effect was intensified by the addition of salinity. A decrease in chlorophyll concentration shows a deteriorating effect of the stressors present (either salinity, NP or the combination of both) on the basic plant function, that is photosynthesis. Figure 8 shows how the plants were affected by the treatments. This means that the primary function of the plant is threatened. As already reported, salinity is an important stress parameter to seed germination and plant grown [5]. In the present work, its effect was observed mainly in chlorophyll content. Larue and collaborators [28] reported that AgNPs did not change the content of photosynthetic pigments in lettuce submitted to 1, 10 and 100 µg/g of AgNPs treatment through foliar exposure. These founds corroborate that the interaction of NPs with plants is different for each crop as well as the effect caused by the nanomaterial, which is dependent of the exposure (foliar or radial), crop conditions and nanoparticle characteristics [29].

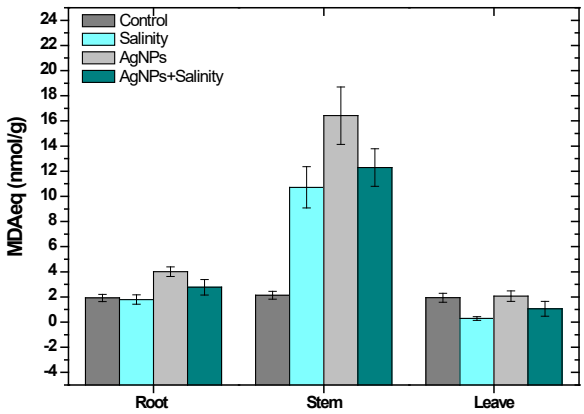

FIG. 7. Lipid peroxidation content in root, stem and leave samples of sunflower crop in soil. Analyses were performed in triplicate. Uncertain bars correspond to the standard deviation of the mean..

TABLE 1. Chlorophyll and carotenoids concentration in leave samples of sunflower crop in soil. Analyses were performed in triplicate. Uncertain bars correspond to the standard deviation of the mean. Different letters in the same colunn mean statistical difference (ANOVA One Way + Tukey: $p<0.05$).

Experimental groups	Chlorophyll A (mg/g)	Chlorophyll B (mg/g)	Carotenoids (mg/g)
Control	5.3556 ± 0.03288[a]	2.8188 ± 0.10875[a]	0.9031 ± 0.01256[a]
Salinity	4.6184 ±0.18446[b]	1.8466 ± 0.14983[b]	0.8411 ± 0.03530[a]
AgNPs	3.2784 ± 0.395[c]	1.4520 ± 0.08681b,[c]	0.6093 ± 0.1964[b]
AgNPs+Salinity	2.3101 ± 0.09836[d]	1.1297 ± 0.11816[c]	0.4210 ± 0.02657[c]

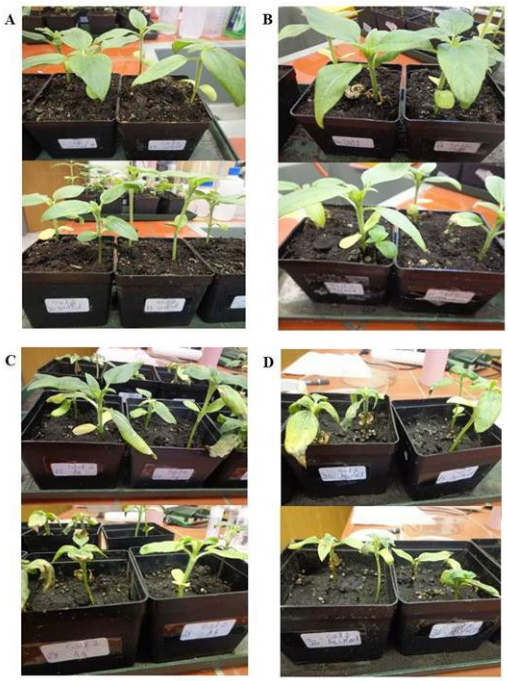

FIG. 8. Sunflower crop in soil at the moment of harvest – 14 days of treatment. A) control group; B) Salinity group; C) AgNPs group and D) AgNPs+Salinity group. The pictures shos the appearance of the plants in the end of the experiment.

4 CONCLUSIONS

It was evaluated the uptake of AgNPs by sunflower crop in soil and hydroponic medium under salinity stress. NPs crossed the root barrier and were relocated in to the upper parts of plants. The exposure to AgNPs and salinity affected the plant growth and development as shown by lipid peroxidation and pigment results. Such effects showed to be dependent on the treatment condition and crop medium. The application of nanoparticles in the agriculture still demanded studies about the uptake and translocation of these nanomaterials by different plant cultures, using different NPs concentration and composition and crop conditions. Results obtained will contribute for the development of a sustainable application of nanomaterials to the agro systems, preserving the environment and ensuring the agri food production safety

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