CHAPTER SIX

Phytotoxicity of silver nanoparticles and defence mechanisms

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1. Introduction

Continuous formation, production and utilization of nanoparticles (NPs) and their unregulated release into aquatic as well as terrestrial systems via number of pathways, have resulted in a growing concern over their impending environmental effects [1,2]. Among different available NPs, silver nanoparticles (AgNPs) are of particular interest because of their well-known antibacterial and antifungal properties due to which they have been implemented in a wide range of commercial products such as medical devices, textiles, food packaging, and healthcare and household products [3,4]. AgNPs are known to induce toxicity in prokaryotic [5], eukaryotic [3] and aquatic [6] as well as in *in vitro* systems [7]. The (cyto)toxicity of

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AgNPs has been attributed to several possible mechanisms, including disruption of cell-membrane integrity [5], protein or DNA binding and damage [8], reactive oxygen species (ROS) generation [9] as well as apoptotic cell death [10]. However, it is still not clear to which degree the toxicity of AgNPs results from AgNPs *per se* and how much toxicity is related to the released Ag^+ [11–13]. Since AgNPs are prone to various environmental (bio)transformation, which modifies their properties influencing their transport, fate and possible toxicity [14], proper characterisation of AgNPs as well as direct detection and localisation within plant tissues are indispensable to reveal direct interaction of silver nanoparticles with plants.

Plants are the vital part of ecosystems as primary producers and they play a significant role in accumulation and biodistribution of many environmentally released substances. They are very likely to be influenced by AgNPs, serving as a potential pathway for AgNP-transport and bioaccumulation into food chains [15]. Therefore, any negative effects of NPs upon plant growth could cause significant changes in the ecosystem, potentially causing irreversible damage. Plants can be adversely affected by AgNPs indirectly, via AgNP-containing products for human usage that are being released to the environment [16]. Moreover, they can also be directly exposed to AgNPs through application of the commercially available products that are being implemented in agriculture, since nanotechnology has been applied in plant production to increase plant growth [17] and to improve pest and disease management [18]. Toxicological studies of AgNPs conducted on plants marked positive, negative or neutral impacts [19,20,20a], which depend on various factors regulating the uptake and accumulation in plants such as plant species and age, the nanoparticle size and concentration, as well as on the test conditions i.e. temperature, duration and method of exposure [21–24].

In this chapter, recently published studies on plant uptake and accumulation of AgNPs and their effects on different aspects of plant physiology (Fig. 1), including germination, growth and morphological and ultrastructural changes as well as photosynthesis have been reviewed. Moreover, we present here a discussion about AgNP roles in promotion of oxidative stress, followed by responsive plant antioxidant machinery, as well as in changes in protein expression, which require further research to understand the molecular response triggered by AgNPs in plants.

2. Uptake and accumulation

There is growing evidence that AgNPs are released into the different types of environment including soil, water and air from where they could be

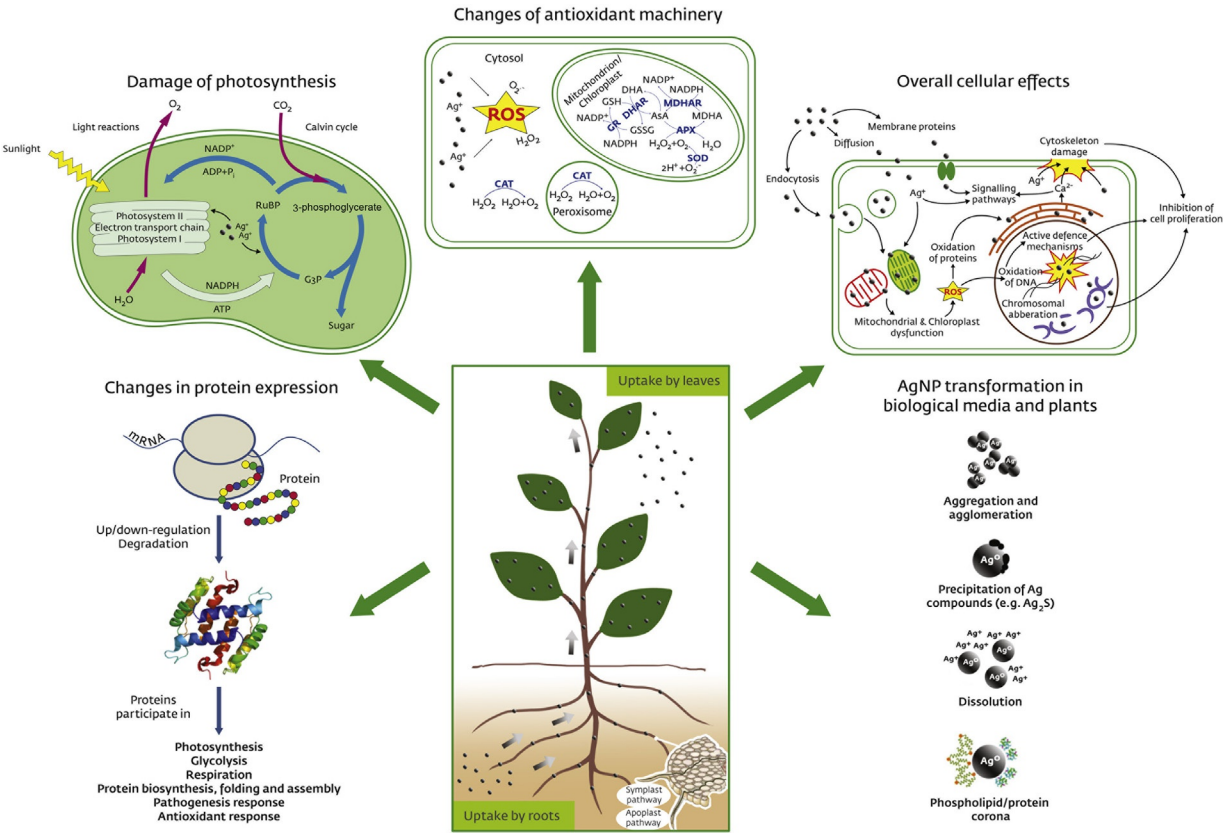

Fig. 1 Schematic illustration of silver nanoparticles (AgNPs) interactions with terrestrial plants.

taken and accumulated in plants. Studies of AgNP-induced phytotoxic effects conducted on different plant species reported contradictory results, showing that impacts of AgNPs on plants largely depend on the type and concentration of nanoparticles, plants species, tissue exposed, and the experimental conditions [reviewed in [25–31]]. Various types of AgNPs can exhibit distinct characteristics depending on their size, charge and surface properties, which can influence their behaviour, uptake as well as toxicity [32,33]. For example, exposure to smaller particles with larger surface areas resulted in higher cellular uptake and toxic responses [34,35]. Moreover, different characteristics of the media used for plant growth (pH, ionic strength, redox conditions, etc.) could modify initial properties of synthetized AgNPs and consequently influence their bioavailability, bioaccumulation as well as biological effects. Most data regarding the fate of AgNPs and their behaviour come from investigations in aquatic systems and several reports pointed out differences in AgNPs behaviour in deionized water used for preparation of stock solution and various media used for exposure [reviewed in [14]]. Studies focused on the environmental transformations of AgNPs show that they undergo slow oxidative dissolution by molecular oxygen and protons, reactions with reduced sulphur species or chloride, adsorption of polymers, natural organic matter or proteins, and aggregation that depends on media and coatings [reviewed in [26,33,36]]. The most important processes for the bioavailability of AgNPs and their biological effects include agglomeration or aggregation of NPs to form larger particles, oxidation of elemental silver (Ag^0) to silver ion (Ag^+) and subsequent dissolution to dissolved Ag^+ species, speciation and solubility of Ag^+ in solution and reactions modifying the reactivity of AgNPs [26]. For example, the presence of ions in the medium will effectively destabilize the AgNPs, leading to aggregation [33,37]. Agglomeration can reduce the mobility and modify the initial concentration of AgNPs, thus influencing their toxic effects [38,39]. To stabilize AgNPs against aggregation, different coating are applied including carboxylic acids (citrate), polymers (polyvinylpyrrolidone, PVP), polysaccharides (gum arabic, GA), and surfactants (cetyltrimethylammonium bromide, CTAB and sodium dodecyl sulfate, SDS), which change the surfaces of AgNPs and thus affect their behaviour and transformations in the medium [33] as well as their toxicity to plants [32,40,41]. Additionally, the aerobic conditions result in the oxidation of AgNPs and release of Ag^+, which can then have a major role in toxicity. Dissolution is very much influenced by the pH and the presence of strong binding ligands, which can be very different in the various types of media

used for plant growth [26]. For example, presence of Cl^- in exposure solution would result in formation of AgCl, thus removing free Ag^+ [42]. Sulfidation i.e. formation of Ag_2S is also possible, which can limit AgNPs bioavailability and toxicity [43]. On the other hand, AgNP transformation in the soil and other solid media has been poorly analysed [44,45], due to the lack of adequate techniques. Several studies have shown that surface reactive particles, such as clays and organic matter-coated particles present in soil, can affect the behaviour of AgNPs, favouring their aggregation and thus decreasing the risk of toxicity [22,32,42,45]. Moreover, it has been reported that biotransformation of AgNPs such as dissolution, sulfidation, aggregation, and adsorption of macromolecules such as proteins can occur during interaction with biological environments, for example at root surface or even after AgNP internalization within the plant tissue [11,43]. Despite the importance of AgNP transformation for their toxicity, in a lot of studies investigating AgNP toxic effects on plants, the size and the state of AgNP aggregation were often measured only before experiments began, in order to characterize synthetized AgNPs [20,23,24,46–50]. For example, it was found that AgNPs with different morphologies and sizes (triangular, 47 nm; spherical, 8 ± 2 nm and decahedral, 45 nm) induced a differential response regarding plant growth and gene expression in Arabidopsis (*Arabidopsis thaliana*) seedlings, but no characterization of the particles in the Murashige and Skoog medium used for plant cultivation and/or during the time course of experiment was done. Still, in many studies more thorough characterization of AgNPs was performed immediately after addition to exposure media [32,51–53] as well as during and after the experiment [32,34,35,38,54–57]. Substantial aggregation of AgNPs was observed in nutrient solution used for duckweed *Lemna minor* cultivation due to the high ionic strength and change in pH, so dilution of the original medium was used for exposure [58]. In the work of Barrena et al. [38], besides biological effects such as germination of cucumber (*Cucumis sativus*) and lettuce (*Lactuca sativa*), the stability of the 2 nm AgNPs was investigated in different media by dynamic light scattering (DLS) analysis and transmission electron microscopy (TEM) images, which showed that there were no relevant changes in the size distribution and stability of AgNPs before and after the experiments. However, zeta potential measurements indicated that AgNPs had less negative charge after addition to media, probably due to the interaction of particle surface coating with molecules from the media, thus forming biomolecular or protein corona [59]. Moreover, a small increase of about 1 nm in particle size has been observed by DLS, which corresponds to this

coating phenomenon. Similarly, Jiang et al. [53] investigated toxicity of 7.8 nm AgNP-GA (0, 0.5, 1, 5, and 10 mg L^{-1}) on duckweed *Spirodela polyrhiza* and found that the size of AgNPs was slightly increased after addition to the 10% Hoagland medium. In a study on AgNP effects on Arabidopsis, TEM analysis showed aggregation of AgNPs during the exposure in the 1/4 Hoagland medium [60], which is in agreement with other studies [34,35,56]. The percentage of AgNP dissolution has been also examined as an important factor influencing the AgNP toxicity and mostly low amounts of free Ag^+ have been found [32,53,61,62]. However in some cases the dissolution was measured in stock solutions of AgNPs, which however does not have to correspond to what happens in exposure media. For example, dissolution of 20 nm AgNPs was observed in the 1/4 Hoagland medium after 24 h, but it decreased after 14 days of exposure [60], possibly because of the precipitation of Ag^+ with electrolytes in the exposure medium or by adsorbing with other particles in the solution [43]. Similar initial increase and then decrease of free Ag^+ released from AgNPs with time in exposure solution was also reported by Jiang et al. [55]. In investigations of AgNP toxicity on algae, Miao et al. [39,63] showed that AgNPs aggregated with time in the exposure medium (seawater) and that a considerable amount of Ag^+ was released from AgNPs. Mirzajani et al. [64] found that 20 nm AgNPs had a lower impact on rice (*Oryza sativa*) after prolonged incubation in a medium compared to fresh mixture, possible due to aggregation and agglomeration of AgNPs. Das et al. [65] investigated AgNP toxicity on tomato (*Lycopersicon esculentum*) in soil-plant system through 72 weeks long experiment and found that agglomeration of AgNPs was high during early phase of exposure, while the dissolution rate increased with time in soil. Moreover, 40 nm AgNP-PVP was found to be more phytotoxic to thickspike wheatgrass (*Elymus lanceolatus*) and red clover (*Trifolium pratense*) than $AgNO_3$, probably because transformation products induced by aging in biosolids resulted in larger pools of extractable Ag^+ than those from $AgNO_3$-biosolids exposures [66]. As pointed out by Reidy et al. [14], sufficient characterization of nanoparticles in the same solution and under the same exposure conditions as used in the experiment should be performed in toxicological studies, since this is crucial for understanding what actually interacts with cells/organisms. The limitations in knowledge about AgNPs (bio)transformation can be attributed to the lack of adapted techniques for the characterization of nanomaterials in complex matrices, such as soil or plants. At present, many methods are used for the characterization of AgNP different physicochemical properties

(e.g., size, distribution), such as DLS, TEM, atomic force microscopy (AFM), flow field flow fractionation (FlFFF), fluorescence correlation spectroscopy (FCS), nanoparticle tracking analysis (NTA), etc. However, all these methods have some shortcomings and the characterization of AgNPs often requires the combination of different analytical methods [67].

Various studies show both positive and negative impacts of AgNPs on plants, depending on factors affecting the uptake and accumulation in plants [20,30]. Uptake of AgNPs depends on size and shape of AgNPs (negatively correlated) as well as on exposure concentration (positively correlated) [27]. In most investigations regarding the AgNP phytotoxicity, AgNP uptake and accumulation by plants was confirmed by measuring total Ag content by inductively coupled plasma—mass or atomic emission spectrometry (ICP-MS, ICP-AES) or atomic absorption spectroscopy (AAS) without further specification of silver forms [48,49,52,53,68–70]. These methods do not allow sources of Ag to be distinguished and might also include Ag that has not been taken up in cells. Therefore, uptake and toxicity studies require appropriate controls with an Ag salt (most often $AgNO_3$ has been used) as well as repeated washing of plant material in presence of a strong Ag ligand, such as cysteine, to remove not internalized particles and Ag^+ before analysis of Ag content [26]. Most studies have shown that Ag mostly remains associated with the roots and the translocation to the shoots is usually very low [25,32,35,47]. In several studies, a higher Ag content in root and shoot was observed after exposure to AgNPs compared to treatment with $AgNO_3$, suggesting an accumulation of the nanoparticulate form of silver [35,68,71,72]. Presence of particles as dark depositions detected by TEM has been considered as confirmation of AgNPs uptake in the form of nanoparticles in various plant species [23,46,47,53,54,68], while in some studies the elemental composition analysis of depositions, confirming Ag presence, was also performed [22,73–75]. However, most of the presented results are from studies performed in aqueous media, although soil is more relevant substrate for the plant growth and it could be polluted by AgNPs through sewage sludge and/or wastewater [76]. Moreover, as already mentioned the bioavailability and toxicity of AgNPs is different in the soil. For example, the bioaccumulation and effect of AgNPs on mung bean (*Phaseolus radiatus*) and sorghum (*Sorghum bicolor*) growth was lower in soil than in agar [22]. The amounts of Ag^+ released from AgNPs during the exposure period showed that Ag^+ was largely responsible for the apparent toxicity in agar, while the influence of the Ag^+ was negligible in soil medium, which may be attributed to their binding to organic substances

in soil and lower bioavailability [44]. In an experiment investigating the fate of AgNP-PVP in a sludge-amended soil cultivated with wheat (*Triticum aestivum*) and rape (*Brassica napus*), it was shown that Ag transfer into plants was very low and no translocation of Ag to the shoots was found, because Ag was associated with soil S-rich particles (as Ag heteroaggregates and adsorbed/complexed Ag species) and thus unavailable [77].

More recent studies and development of new techniques such as laser scanning confocal microscopy, scanning and transmission electron microscopy (SEM, TEM) coupled with an energy dispersive X-ray spectrometer (EDX) and single particle-inductively coupled plasma-mass spectrometry (SP-ICP-MS) allowed precise localization and identification of AgNPs not only in plant tissues, but also inside the cell. Moreover, synchrotron radiation (SR) based techniques has been recently successfully used in the analysis of NPs in plants, such as micro-X-ray fluorescence (μ-XRF) for elemental distribution and/or X-ray absorption spectroscopy (XAS) and X-ray absorption near edge structure spectroscopy using a microfocused beam (μXANES) for chemical speciation (reviewed in Castillo- Michel et al. [78]. Micro-particle induced X-ray emission (μPIXE) and Rutherford backscattering spectroscopy (μRBS) are also promising new tools for AgNPs distribution and *in situ* quantification [79] as well as X-ray micro and nano-computed tomography (μ-and nano-CT) for 3D distribution of NPs [72]. Various studies showed that plant roots are the main route of plant's exposure to AgNPs. AgNPs have to cross cell walls and plasma membranes of epidermal layers in roots to enter vascular tissues (xylem) and to be translocated to the leaves. Investigating effects of 6 nm AgNP-GA on the common grass (*Lolium multiflorum*), Yin et al. [32] found that many AgNPs adsorbed to the surface of roots, although TEM analysis also revealed the presence of particulates inside the root cells. Similarly, AgNPs were observed to be adhered onto the surface of mung bean and mustard root cells [48], while they were detected in *Allium cepa* root cells after exposure to AgNPs coated with PVP (9.4 nm), citrate (61.2 nm) and CTAB (5.6 nm) [40]. Moreover, uptake of 40 nm AgNPs has been demonstrated in columella of Arabidopsis roots by a confocal/multiphoton microscope [25]. Based on the 3D resolution data from the laser scanning confocal microscope Geisler-Lee et al. [34] suggested that a possible route for AgNP transport in Arabidopsis root tips grown in hydroponic growth media was from border cells to root cap, epidermis and columella and then to the initial cells of the root meristem. Moreover, Geisler-Lee et al. [80] reported that AgNPs gradually accumulated in a time sequence in Arabidopsis root tip

and elongation region during 4 weeks of hydroponic growth; on the 14th day AgNPs entered root hair cells, while by the 17th day they were transported in vascular tissue, both phloem and xylem. Similarly, in alfalfa (*Medicago sativa*) exposed to 11 nm AgNP-PVP synchrotron-based μ-XRF analysis showed that silver nanoparticles mainly accumulated in the columella border cells and elongation zone [81]. In duckweed *Landoltia punctata* exposed to 13 nm AgNP-PVP, silver was distributed throughout the root tip and had the highest concentrations near the apical meristem [56]. At subcellular level, TEM analysis showed that 20 and 40 nm AgNPs could be found in cell walls and at plasmodesmata, while for the 80 nm AgNPs accumulation was observed, but the sizes of AgNPs detected in the cell wall were smaller, indicating that partial dissolution and size reduction may be necessary for plant uptake [34]. Moreover, aggregated AgNPs were also present in cell walls, especially in the middle lamella as well as in the plasmodesmata, demonstrating that symplastic transport could be physically blocked and that AgNPs might pass intercellular space via apoplastic transport, which is also in agreement with results of Stegemeier et al. [81]. Massive accumulation and internalization of 10 nm AgNPs in Arabidopsis root was also confirmed by Bao et al. [82], who observed individual particles and aggregated clumps all over the cell wall, middle lamella plasma, intercellular space, and in the vacuole. Cell internalization of AgNPs of much larger size than is the size of the cell wall pores (3–8 nm) [83,84], raise the question how these AgNPs can pass through the cell wall. Previously, it has been proposed that direct uptake of nanoparticles by the plants is possible for AgNPs smaller than 5 nm, which can cross the cell wall [85]. The underlying mechanisms for plant uptake of large AgNPs are still poorly understood, but it is possible that AgNPs may induce the formation of new and larger size pores or cause ruptures in root structure, which allows the passage of large AgNPs through the cell wall [11,31]. Further, to get inside the cell, AgNPs needs to pass the plasma membrane. The potential entries through the lipid bilayer are not yet clarified, but it has been suggested for other nanoparticles that they can cross the cell membranes either by the endocytic processes or using embedded transport carrier proteins or ion channels [11]. The combination of μ-CT and μ-XRF analyses [72] showed the presence of localized AgNP accumulation regions adhering on the epidermis of wheat roots while nano-CT technique revealed that AgNPs accumulated preferentially in discontinuities between root epidermal cells as well as attached on root hairs. Pradas del Real et al. [72] consider that these results support two possible ways of AgNP entry: (i) accumulation of AgNPs in the epidermis,

which can cause rupture of tissue and facilitate the transfer of AgNPs inside roots [86] and/or (ii) root hairs, which have a thin cell wall and take up nutrients by transport, diffusion and endocytosis [87]. It has already been suggested that translocation of AgNPs is aided by endocytosis [26,88], which includes the creation of vesicle that enfold the material and finally transport AgNPs from plasma membrane to the cells [20]. However, in root tissues of wheat [47] and rocket (*Eruca sativa*) [46] AgNPs were not observed by TEM analysis, which suggests that they remain on the root surface and that the effects of AgNPs are mediated primarily by Ag^+ released by oxidative dissolution of NPs at the root interface. Since the root tip cell walls of wheat seedlings were rich in matrix components, Vannini et al. [47] suggested that wheat seedlings exposed to toxic concentrations of AgNPs attempt to prevent or reduce their uptake into root cells by restricting metal ions to the apoplast by binding them to the cell wall or to cellular exudates. Different plant species are differently rich in matrix components and thus differently protected from the AgNPs uptake which could partly explain inconsistencies in uptake results. Moreover, in *L. multiflorum*, μXRF/μXANES analysis showed spots of oxidized silver within root tissues, indicating AgNP oxidation and dissolution within or on biological surfaces [32]. Therefore, Yin et al. [32] proposed two mechanisms of internalization of silver: (i) direct AgNPs uptake by the roots followed by the release of oxidized silver species within the root tissues and (ii) dissolution of the AgNPs on the root surface followed by internalization of the ionic species by the roots. Ag^+, which are isoelectric to Cu^+, can enter the cell by the high-affinity Cu transporter [26]. Furthermore, the accumulation of Ag in the alfalfa root apoplast, determined by XRF analysis, and the presence of small NPs in root cell walls, revealed by TEM, suggest uptake of partially dissolved NPs and translocation along the apoplast [81]. Bao et al. [82] used the macerozyme R-10 tissue extraction method followed by SP-ICP-MS to study the uptake and size distribution of AgNPs in Arabidopsis. Both SP-ICP-MS and TEM measurements indicated that AgNPs accumulated predominantly in the apoplast and that the size of AgNPs in plant tissue was two to three times larger than the originally dosed AgNPs, indicating the AgNP biotransformation processes were involved. Similarly, the combination of μXRF/XAS and nanoXRF revealed the presence of single AgNPs as well as AgNP aggregates in the vascular region of sunflower (*Helianthus annuus*) roots, and identified Ag in coordination to sulphur ligands at the Casparian strip [89]. In wheat, μ-XANES analysis revealed that Ag was mostly present as AgNP in the epidermis, but inside the roots Ag was distributed in the cell walls of the cortex

as a mixture of Ag-thiol species and other ionic Ag species, evidencing the biotransformation of Ag [72]. Moreover, no Ag^0 was observed inside roots, which implies that AgNPs were completely dissolved and complexed by organic ligands, which is contrary to results obtained on Arabidopsis [34,82]. Since Ag was detected inside the cortex cells, in the endodermis and in the central cylinder, these results suggest both apoplastic and symplastic transfer of Ag in monovalent form. Based on the results of silver distribution analysed by μXRF/XAS after 24 and 60 h of exposure, Stegemeier et al. [56] proposed that the primary route of AgNPs uptake into duckweed *L. punctata* roots appears to be through attachment onto the root surface (root cap), dissolution assisted by the acidic local environment in root cap mucilage, and internalization (and possibly reprecipitation) of dissolved silver. Direct uptake of AgNPs or sulfidized AgNPs is likely occurring in parallel as Ag^0 was also present beside a mixture of Ag_2S and Ag-thiol. However, due to the capabilities of plants to reduce metal ions to elemental NPs inside plant tissues [54,90], it is possible that AgNPs in plant cells come either from the direct uptake of AgNPs or *in vivo* reduction of Ag^+ dissolved from AgNPs at root surface and taken in cell [25,56]. In Arabidopsis root cells [34] as well as in *L. punctata* [56] treated with $AgNO_3$, Ag^0 was detected, implying the conversion of Ag^+ to Ag^0; so it can be concluded that either AgNPs or Ag^+, in ionic form or converted to Ag^0 was transported through intercellular space in cell walls. Results obtained with μPIXE/RBS and XANES analyses confirmed that Ag inside root tissue of uncoated 40 nm AgNP-treated lettuce was mainly present as Ag^+, with a small contribution from Ag^0 (as AgNPs), but these methods also do not distinguish if only ions are internalized or AgNPs are internalized and dissolved inside the cells [79]. A combination of high resolution imaging techniques including nano secondary ion mass spectrometry (NanoSIMS), a nanoscopic scale resolution chemical imaging mass was used to quantitatively investigate elemental distribution in freshwater green alga *Chlamydomonas reinhardtii* exposed to AgNPs [91]. Results confirmed the entrance of AgNPs into the cells of alga since silver present in the periplasmic space was identified as AgNPs. However, silver present in the cytoplasm was identified as Ag_2S particles and Ag-thiol. Moreover, when algae were exposed to Ag^+, Ag was also found as particles in the periplasmic space, but their crystal structure was completely different from that of silver crystals arising from exposure to AgNPs, which excluded the possibility of the formation of AgNPs from Ag^+ in the periplasmic space of cells. By contrast, Ag_2S particles were detected in the cytoplasm after exposure to both Ag^+ and AgNPs,

implicating that the Ag_2S particles in the cytoplasm were formed from Ag^+ ion released from AgNPs. Furthermore, Wang et al. [91] did not observe AgNPs inside the vesicle or the endosome around the cell membrane or on the way to the endomembrane system, suggesting that there was no endocytosis or passive diffusion of AgNPs into the cytoplasm. In conclusion, there is currently no definite evidence of whether the AgNPs enter the cell intact, or Ag^+ released from the AgNPs at root surface are taken in cell with subsequent formation of AgNPs in plants.

After root uptake, translocation of AgNPs to the shoots requires transport across the root, including Casparian strip to the xylem. Previous studies suggested that AgNPs can move mostly through apoplast, reaching the cortex and vascular tissue [34,89]; once AgNPs are in the vascular tissues, they could be transported to the leaves via the long-distance transport [80]. Presence of AgNPs in leaves of different species has been documented mostly by TEM and confocal microscopy [54,68,80]. Bao et al. [82] showed that after the uptake and internalization by Arabidopsis root, AgNPs could be translocated toward the plant shoot since AgNP aggregates were observed (by TEM) around the cell wall and in the vacuole of the leaf. Detection of AgNPs in stem, leaves and fruit of tomato was confirmed by environmental scanning electron microscope which can differentiate AgNPs from the other present nanoparticles [70]. Several investigations revealed that the distribution of silver accumulation in shoot organs is species-specific. For example, in investigation where poplars (*Populus deltoides* × *nigra*) and Arabidopsis were exposed hydroponically to AgNPs of different sizes (5 and 10 nm AgNPs coated with polyethylene glycol (PEG) and 25 nm AgNP-carbon), Arabidopsis accumulated much more Ag (measured by ICP-MS) in leaves than in the stem or flower tissues, whereas poplars accumulated Ag at similar concentrations in leaves and stems [35]. Mustard (*Brassica campestris*) and mung bean (*Vigna radiata*) exposed to AgNPs in Hoagland medium accumulated more Ag in stem than in leaves [48]. In tobacco exposed to AgNPs low amount of silver were determined in leaf tissue, but presence of AgNPs was not detected by TEM [74], indicating that different Ag^+ species could be transported to shoot. Moreover, μPIXE analysis was used to identify a possible translocation of Ag towards the leaves of lettuce, but Ag was not detected in the stem [79]. Therefore, Larue et al. [79] suggested that Ag mostly dissolved inside plants, resulting with formation of Ag^+, which are more mobile and can distribute homogeneously in plant tissue leading to a low local Ag concentration, below detection limit. However, the more sensitive

μXRF analysis detected AgNP as well as other Ag species in the vascular bundles of the stem [79], thus confirming that AgNPs can be transferred through the vascular system to the shoot. Although most studies investigated root uptake of AgNPs, it has been demonstrated that in cotyledons, which emerged in direct contact with growth medium amended with AgNPs, AgNPs could enter through the pores of stomata and then be accumulated in stomata and in cell wall grooves between pavement cells [80]. Moreover, in lettuce leaves foliar uptake of AgNPs (uncoated, 40 nm) through stomata has also been demonstrated [92]. In that study, Ag agglomerates were detected by both, μXRF and SEM analyses all over the surface of leaves, between the guard cells and in the sub-stomatal chamber. Ag was also detected inside the leaf parenchyma either inside cells or associated to the cell walls. Also, AgNPs were observed in the main vein, inside the bundles and in the cell wall thickenings. In the majority of the analyzed regions, μXANES spectra identified a mixture of AgNPs and secondary species, including Ag-thiol, and other Ag^+ species such as AgCl. The mechanisms of foliar transfer of NPs are mostly unknown. Results of Larue et al. [92], showing that NPs were embedded in the leaf cuticle and present in the sub-stomatal chamber, suggest that AgNPs might follow both the cuticular and stomatal pathways to penetrate inside lettuce leaves.

Although a great progress has been made in understanding the uptake of AgNPs in plants, many questions still remain. Factors affecting AgNPs bioavailability and uptake have been identified, but it is necessary to reveal the mechanisms of AgNPs internalization in roots as well as upward transport to shoots. The mechanisms of foliar uptake and distribution of AgNPs in leaves are still insufficiently explored.

3. Effects on germination, growth, morphology and ultrastructure

3.1 Effects on seed germination and plant growth

Positive, negative or neutral effects of AgNPs on plant development were investigated in many studies via seed germination, when the germination rate and root length are measured and via growth of seedlings and plants, in which root/shoot elongation and dry weight are used to assess acute effects of NP form of silver on plant physiology. In the study of Tripathi et al. [73], biosynthesized 22 nm AgNPs (1000, 3000 and 5000 μM in Hoagland medium) significantly decreased pea (*Pisum sativum*)

seed germination. Similarly, Barrena et al. [38] observed that 100 $\mu g\,mL^{-1}$ of laboratory-synthesized AgNPs (29 nm) significantly reduced germination index in cucumber and lettuce. In the study of El-Temsah and Joner [21] the effects of three types of AgNPs with different particle sizes (2, 5 and 20 nm), applied in 0–100 $mg\,L^{-1}$ concentrations, were evaluated using seed germination tests with ryegrass *(Lolium perenne)*, barley (*Hordeum vulgare*) and flax (*Linum usitatissimum*). The results showed that AgNPs at the concentration of 10 $mg\,L^{-1}$ had a certain inhibitory effect on germination, but there were no trends indicating that smaller particles were more toxic than the larger ones and it seems that effect depended on plant species. The absence of concentration- and particle size-dependant effects observed in this study may be due to saturation or equilibrium being reached, possibly involving an ionic component that was not distinguished in this study [21]. Toxicity of two types of spherical AgNPs, 6 nm AgNP-GA and 21 nm AgNP-PVP, as well as of $AgNO_3$ (1, 10 or 40 $mg\,L^{-1}$) to the seeds of 11 species of wetland plants on the filter paper and in soil was investigated [24]. It was observed that the exposure to 40 $mg\,L^{-1}$ AgNP-GA significantly inhibited the germination of *Scirpus cyperinus*, *Juncus effusus* and *Phytolacca americana*. Moreover, AgNP-GA had more negative effects compared to $AgNO_3$. Authors Yin et al. [24] suggested that the high toxicity of AgNP-GA was not only due to the presence of Ag^+, but it is more likely a result of the combination of size, coating and perhaps even surface charge. Moreover, the only significant effect of AgNPs on germination in the soil experiment was inhibition of *P. americana* germination after exposure to 40 $mg\,L^{-1}$ AgNP-GA [24], which indicates that the exposure medium also has an influence on AgNPs toxicity. After Arabidopsis seeds exposure to 75 and 300 $\mu g\,L^{-1}$ of 20 nm AgNP-citrate, it was found that plants suffered gradual degenerative seed viability with a decreasing germination rate in successive generations [80]; there was no difference in seed germination among E0 generation; however, seed germination rates decreased from the initially exposed E0 generation through the first (E1) to third (E3) generations exposed to AgNP-citrate. Furthermore, a stronger toxicity of AgNP-citrate compared to equivalent dosage of $AgNO_3$ was observed [80]. Similarly, a 40 nm AgNPs and the same concentration of $AgNO_3$, ranging from 200 to 1600 $mg\,L^{-1}$, were also found to inhibit seed germination of black mustard (*Brassica nigra*) in a dose-dependent manner and the AgNPs also had stronger inhibitory effect than Ag^+ [93]. These results implicate that AgNPs inhibitory effects observed in germinating seeds are not only due to the dissolved Ag^+ released from AgNPs, but also

can be partially attributed to the nanoparticles themselves. However, the results showed that both AgNPs and $AgNO_3$ suppressed lipase activity and reduced the conversion of lipids into soluble and reducing sugars, which led to the decline in oilferous seed germination.

Although AgNPs mainly had negative effects on seed germination, there are also some opposite results. Namely, treatment with spherical 30–40 nm AgNPs (10, 20 and 30 $\mu g\,mL^{-1}$) accelerated *Boswellia ovalifoliolata* germination; on the solid medium with AgNPs, germination completed within 7–10 days, while for control seeds it took 10–20 days [94]. Similar results were also found after exposure of pearl millet (*Pennisetum glaucum*) seeds to 20 and 50 $mg\,L^{-1}$ of 13 nm AgNPs [95]. Study of Almutairi and Alharbi [96] on seeds of watermelon (*Citrullus lanatus*), zucchini (*Cucurbita pepo*) and corn (*Zea mays*) treated with 20 nm AgNPs showed that the AgNPs concentrations at which the highest germination rate was observed differed among investigated species; for zucchini it was 0.5 $mg\,mL^{-1}$, for corn 1.5 $mg\,mL^{-1}$ and for watermelon 2 $mg\,mL^{-1}$, which indicates that three different crop species had different dose responses to AgNPs in terms of germination. Fayez et al. [97] reported that although 25 nm AgNPs significantly decreased grain germination of barley (*Hordeum vulgare*) when applied in high concentrations (0.5 and 1 mM), the lower concentration of 0.1 mM positively affected the seed germination. On the other hand, in several studies, exposure to AgNPs did not result with any effects on germination. No effect on germination percentage of flax was observed after exposure to 2, 5 and 20 nm AgNPs [21]. Moreover, AgNPs of different sizes (20, 50 and 65 nm) applied in 50 ppm concentration showed no significant effect on seed germination of broad bean (*Vicia faba*) [98]. The lack of effect on germination was also recorded after exposure of 11 wetland species to AgNP-PVP [24], in castor bean (*Ricinus communis*) seeds exposed to AgNP-PVP [69], in radish (*Raphanus sativus*) seeds treated with colloidal AgNPs [99], Arabidopsis seeds after treatment with 10 nm AgNPs [68] and after treatment of rocket seeds with 10 nm AgNP-PVP [46]. From the results of all presented studies it can be concluded that the susceptibility of plant seeds to AgNPs exposure is more dependent on the plant species than on the physico-chemical characteristics of the particles themselves.

Beside germination, the effects of AgNPs on plant growth were also observed through measurements of root and shoot elongation as well as of fresh and dry weight. AgNPs (20 nm) had a retarding effect on root growth of corn seedlings and significant decrease was observed at all tested concentrations, but particularly at the highest ones, 1.5 and 2 $mg\,mL^{-1}$ [96].

Exposure of broad bean to 50 ppm of AgNPs of different sizes (20, 50 and 65 nm), significantly decreased the root length with the most reduced root growth recorded after exposure to the AgNPs of the smallest size [98]. Furthermore, 100 $\mu g\,mL^{-1}$ of 29 nm AgNPs had negative influence on cucumber and lettuce root growth [38]. Similarly, the exposure to 10 $mg\,L^{-1}$ of 10 nm AgNP-PVP inhibited the length of roots and shoots of wheat seedlings [47]. In the study of Tripathi et al. [73], root and shoot length as well as fresh and dry weight of pea seedlings were significantly declined due to the addition of 1000 and 3000 μM of 22 nm AgNPs, although in roots the toxic effects of AgNPs were stronger than in shoots. Furthermore, the 20 nm AgNPs applied in 0.5 and 1 $mg\,L^{-1}$ concentrations significantly reduced root elongation and root and shoot weight as well as leaf area of rice seedlings [100]. Similar effects were observed for seedlings of radish exposed to 2 nm AgNPs [99], pearl millet treated with 13 nm AgNPs [95], barley exposed to 25 nm AgNPs [97] and mustard treated with 47 nm AgNPs [50]. In all these abovementioned studies concentration-dependent reduction in root and shoot elongation was recorded, although the effects were more pronounced in root growth, which is in accordance with the fact that the roots come in direct contact with the exposure medium containing AgNPs. Moreover, these observations are in correlation with the fact that most silver remains associated within the roots [32,74]. In the study of El-Temsah and Joner [21], it was found that the length of shoots of germinating seeds of three plant species responded differently to AgNPs of different sizes; 2 nm colloidal AgNPs inhibited shoot growth of flax and ryegrass at 10 $mg\,L^{-1}$, while the inhibitory effect was observed for all threes species at 20 $mg\,L^{-1}$. 10 mg L^{-1} of 5 nm AgNPs reduced shoot growth of flax and barley, but this effect did not occur at higher concentrations, while for ryegrass inhibition was observed in all tested concentrations (10, 20 and 100 $mg\,L^{-1}$). Exposure to 20 $mg\,L^{-1}$ of 20 nm AgNPs resulted with an inhibitory effect on shoot growth of all three species. Presented results indicate that effects of AgNP-exposure on root and shoot growth are also very much dependent on the plants species, although AgNPs size and concentration also contribute to observed inhibitory effects. To determine if toxic effects on plant growth are the result of the nanoparticulate form of AgNPs or a consequence of the dissolution of Ag^{+}, effects of both types of silver were investigated in several studies. Tobacco seedlings grown in the nutrient medium, supplemented with 100 μM of 50 nm AgNP-citrate or $AgNO_3$ at the same concentration, exhibited significantly reduced roots [75]. Furthermore, the 6 nm AgNP-GA induced significantly higher growth reduction in common grass

than $AgNO_3$ [32]. Moreover, overall growth effects were more pronounced after exposure to 6 nm AgNP-GA than to 25 nm AgNP-GA, suggesting the particle size-mediated toxicity of AgNPs [32]. In Arabidopsis, 10 nm AgNPs reduced fresh weight and root length in a concentration-dependent manner and the observed effects were more pronounced after treatments with AgNPs than with $AgNO_3$ [68]. Similar observation was also reported for the growth of roots and shoots of black mustard after exposure to 40 nm AgNPs [93]. Results obtained in these studies demonstrate that AgNP inhibitory effects on plant growth can be ascribed not only to dissolved Ag^+ released from AgNPs, but also can be partially attributed to the nanoparticles themselves. However, there are studies which reported that $AgNO_3$ can induce more toxic effects on plant growth than AgNPs; Gubbins et al. [58] reported concentration-dependent growth inhibition of duckweed *L. minor* after exposure to 20 and 100 nm AgNP-citrate; however, the greater effects of Ag^+ compared to AgNPs were observed. Stronger effects of $AgNO_3$ on root and shoot elongation compared to AgNPs were also reported for seedlings of mustard [50] and barley [97]. Cvjetko et al. [40] investigated the toxicity of $AgNO_3$ and AgNPs with different coatings and sizes: 61.2 nm AgNP-citrate, 9.4 nm AgNP-PVP and 5.6 nm AgNP-CTAB on *A. cepa* roots. Among tested AgNPs, the strongest inhibitory effect on root growth was recorded for AgNP-CTAB, probably due to the highest uptake of these positively charged NPs of the smallest size, although ionic form of Ag was confirmed to be more toxic than any of the AgNPs applied. In the study of Yasur and Rani [69], <100 nm AgNP-PVP (100–4000 mg L^{-1}), had no significant effects on the root or shoot length of castor bean seeds, while $AgNO_3$ showed drastic effects on shoot and root growth percentage in a concentration-dependent manner. In the study of Yin et al. [24], it was observed that exposure of 11 wetland plants to 40 mg L^{-1} of 6 nm AgNP-GA, 21 nm AgNP-PVP and $AgNO_3$ in culture conditions, significantly reduced root length of 9, 6 and 8 species, respectively, while leaf length was less affected. In the soil study, exposure to AgNP-GA negatively affected seedling growth of only three tested plant species, while AgNP-PVP and $AgNO_3$ reduced the growth of only one species [24], evidencing that the exposure media also might influence toxicity of AgNPs. Moreover, Lee et al. [22] reported that growth of mung bean and sorghum seedlings was adversely affected by exposure to 10 nm AgNPs-citrate (0, 5, 10, 20, and 40 mg L^{-1}) in the agar medium, with mung bean being more sensitive than sorghum. However, plant growth in the soil was not inhibited, despite exposure to substantially higher concentrations

of AgNPs relative to the agar test. The reasons for this involve changes in the physicochemical properties of nanoparticles in soil as discussed in section uptake and accumulation.

Contrary to all presented findings, some positive effects of AgNPs on plant growth were also recorded. In the study of Savithramma et al. [94], 30–40 nm AgNPs applied in 10, 20 and 30 $\mu g\,mL^{-1}$ concentrations showed positive effects on growth of *Boswellia ovalifoliolata* seedlings in the concentration-dependent manner. This positive effect was ascribed to AgNPs ability to generate new pores on the seed coat during penetration, which may help to influx the nutrients inside the seed or NPs may carry the nutrients along with them, leading to rapid germination and growth rate [94]. Also, positive effects of 20 nm AgNPs on the growth of watermelon, zucchini and corn seedlings was observed [96]. Similarly, AgNP-PVP had positive effects on root and leaf elongation of *Carex lurida* in culture conditions and AgNP-GA on biomass of *L. multiflorum* in soil experiment [24]. We can conclude that the impact of AgNPs on seed germination, plant growth and development on plants largely depends on various factors such as plant species, growth stage of plant, composition and concentration of the nanoparticles, and the experimental setup (treatment period, media composition, and method of exposure). Negative, positive or neutral effects could be observed on seed germination, while the effect of AgNP on plant growth was more negatively affected with more impact on the plant roots.

3.2 Effects on morphology and ultrastructure

As roots are the major target of AgNPs toxic effects, several authors investigated root morphology of various plant species treated with AgNPs of different sizes, concentrations and coatings. In the study of Tripathi et al. [73], anatomical structures of pea root seedlings grown in Hoagland medium for 15 days revealed that due to the addition of 1000 and 3000 µM of biosynthesized 22 nm AgNPs, the number and length of root hairs was drastically decreased compared to control. Furthermore, the superficial root cap cells of germinating wheat seedlings, treated for 5 days with 10 $mg\,L^{-1}$ of 10 nm AgNP-PVP, undergo degradation and plasmolysis occurs in a greater extent in AgNP- than $AgNO_3$-treated cells; in these cells large vacuoles and periplasmic space was observed [47]. Similarly, Yin et al. [32] found that root tip cells of common grass seedlings failed to develop root hairs, had highly vacuolated and collapsed cortical cells and broken epidermis and root cap after exposure to 40 $mg\,L^{-1}$ of 6 nm AgNP-GA for 5 days; in contrast,

seedlings exposed to identical concentrations of $AgNO_3$ showed no such abnormalities. Moreover, Vannini et al. [46] reported that in rocket seedlings root tip cells were also more vacuolated after exposure to AgNPs than in the root cells after treatment with $AgNO_3$. Furthermore, in the study of Pokhrel and Dubey [52], 73.4 $\mu g\,mL^{-1}$ of AgNP-citrate (56 nm) and 200 $\mu g\,mL^{-1}$ $AgNO_3$ both caused changes in primary root cells at the zone of elongation in 7 days old maize seedlings; cells were consistently elongated after AgNP-treatment, while they appeared thinner and irregular after exposure to $AgNO_3$. On the contrary, light microscopy did not reveal any significant changes in the organization of root apical meristem and elongation zone in the study of tobacco seedlings after 30 days-exposure to 100 μM of 50 nm AgNP-citrate, while the roots of seedlings treated with the same concentration of $AgNO_3$ were thicker with reduced root cap [75].

In several studies the impact of AgNPs on the seedlings roots was analysed in the organelles at the ultrastructural level by transmission electron microscopy (TEM). Major changes were observed in plastids, vacuole and endoplasmic reticulum (ER) of root cap, meristems and differentiating cells. The number of the amyloplasts and the size of the smooth endoplasmic reticulum (sER) in the root cap columella cells of rocket seedlings were reduced after both AgNP-PVP- and $AgNO_3$-treatments [46]. However, only AgNPs induced morphological modifications of sER in the region of cell elongation and differentiation of root samples; in particular, an extensive swelling was observed. In wheat seedlings exposed to AgNP-PVP [47], no starch grains were noticed in the root cap columella amyloplasts and in the plastids of meristematic cells. Moreover, the vacuolization increased in the differentiating cells and an extensive swelling was observed in ER, whose tubules appeared packaged in regular structures which occupy a large area of the cell. In this root area the production of a large number of lateral roots primordia was observed, similarly as in wheat roots exposed to AgNPs [54]. Lateral root primordium originated very early in the AgNP-PVP-exposed root apex and it initiated immediately under the meristematic root apex area in the inner root region. This suggests that AgNPs affect mechanisms controlling lateral root production by the pericycle [47], probably by binding of Ag^+ to ethylene receptor [101], thus blocking ethylene induced inhibition of lateral root production usual for dark-grown seedlings. Since in both studies by Vannini et al. [46,47] it was demonstrated by TEM that AgNPs did not enter the root cells, while some electron-dense spots were associated with the cell walls of outer root tip cells, it has been suggested that the effects of AgNPs are mediated primarily by Ag^+ released from NPs at the

root interface. However, there are some investigations showing that observed morphological effects of AgNP exposure can be a result of the immediate uptake of AgNPs by root cells as discussed in part uptake and accumulation.

Beside changes in root cells, AgNPs also induce morphological modifications in the leaves of seedlings of various plant species. Tripathi et al. [73] reported that leaf chloroplasts in mesophyll cells, proto-xylem, meta-xylem and phloem of pea seedlings exposed to AgNPs (1000 and 3000 μM) were adversely affected. After exposure to 3000 μM AgNPs mesophyll tissue was not differentiated into palisade and spongy parenchyma cells, while lower epidermal cells of the leaf midrib were more elongated compared to treatment with 1000 μM AgNPs. On the contrary, in the study of Peharec Štefanić et al. [75] leaf anatomy showed no significant changes in the cell organization of tobacco seedlings exposed to 100 μM AgNP-citrate compared to the control; however, leaves of seedlings treated with 100 μM $AgNO_3$ were thinner with bigger chloroplasts. In several studies the impact of AgNPs on leaves of exposed seedlings was also analysed at the ultrastructural level and TEM studies revealed changes primarily in the chloroplasts; disturbances in their shape, thylakoid system, plastoglobules and the starch content were observed. Chloroplasts in the needles of old Scots pine (*Pinus sylvestris*) seedlings, treated by spraying seedlings' aerial parts with 50 ppm of AgNPs, have been modified from lenticular to round [102], while chloroplasts of English oak (*Quercus robur*) seedlings, also treated by spraying with the same AgNPs, contained large starch granules [103]. In the leaves of 100 μM AgNP-citrate treated tobacco seedlings, chloroplasts were swollen with dilated thylakoid systems and bigger plastoglobules than the control; chloroplasts in seedlings exposed to 100 μM $AgNO_3$ showed similar changes as those found after AgNP-exposure [75]. These results are partially in agreement with those reported by Qian et al. [68], where 3 mg L^{-1} of both 10 nm AgNPs and Ag^+ reduced cell size and disrupted the thylakoid membrane structure in Arabidopsis seedlings; however, the impact on chloroplast ultrastructure was less pronounced in $AgNO_3$-treated seedlings. In general, chloroplasts were shown to be the most sensitive organelles in the leaves of seedlings of different plant species exposed to AgNP-induced stress.

Morphological modifications and ultrastructural changes of roots, stems and leaves were also observed in the fully developed stage of various plant species. In *A. cepa*, the highest applied AgNP concentration of 100 ppm induced complete disintegration of cell walls for most of the cells [104]. Anatomical investigations of the transverse sections of *Bacopa monnieri* roots

exposed to AgNPs in 10 ppm concentration showed disappearance of the characteristic air chambers and partition filaments in root cortex, although the same observations were obtained after exposure to 10 ppm $AgNO_3$ [105]. In the same study light microscopy analysis also revealed structural aberrations in the stem anatomy including alterations of shape, size and distribution of xylem elements, after both silver treatments. In the study of Cvjetko et al. [74] microscopic analyses showed that the root tip cells of tobacco adult plants were highly vacuolated after 7 days of exposure to both 100 μM AgNP-citrate and $AgNO_3$. Furthermore, TEM study showed that after both types of treatments only nuclei could be observed within the root cells, due to large vacuoles; however, exposure to $AgNO_3$ also resulted with partly destroyed root cells, while nuclei were highly damaged [74]. In the same study, AgNPs were detected in the intermembrane space of the root cells, which proves their direct uptake and accumulation in the root cells [74]. In the study of tobacco plants exposed to AgNPs, leaf semithin sections showed no significant changes in the cell organization, except for the difference in the leaf thickness were the leaves of AgNP-treated plants were thinner than the control ones [74]. Moreover, Fayez et al. [97] reported that the leaf chlorosis of barley plants exposed to 25 nm AgNP or $AgNO_3$ appeared after 7–12 days, which was dependent on the dose and type of treatments, although leaf chlorosis was more evident after treatments with $AgNO_3$ than with AgNPs. Furthermore, analysis of leaf ultrastructure showed destruction of chloroplasts, mitochondria and nucleus after both types of treatments. Namely, in response to 1 mM AgNPs, chloroplasts had few small plastoglobuli and well defined grana thylakoids and stroma lamellae, although a reduction in size of grana thylakoids to stroma lamellae was observed; additionally, nuclear envelope was ruptured. After exposure to 1 mM $AgNO_3$ somewhat opposite effects were recorded; chloroplasts were characterized with big plastoglobuli, condensed stroma matrix, formation of vesicles and dilated grana thylakoids, while stroma lamellae lost their organization. Moreover, the mitochondrial envelope and cristae were partially or totally degenerated and in the nucleus clumping of nuclear chromatin into more densely packed material was observed [97]. In duckweed *S. polyrhiza*, ultrastructural changes in chloroplasts were observed after 72 h of exposure to 6 nm AgNP-GA and 20 nm AgNP-PVP (10 mg L^{-1}); chloroplasts were characterized with accumulated starch and large starch grains as well as with fewer intergranal thylakoids [106]. In leaf cells of tobacco plants, chloroplasts were smaller and somewhat swollen and ruptured, although with well-developed thylakoid system after exposure to

AgNP-citrate, while those found in cells of $AgNO_3$-treated plants were bigger than in the control [74]. Ultrastructure damage of chloroplasts, mitochondria and nucleus found in abovementioned studies confirms that these organelles are the main targets affected by AgNPs, although chloroplasts were shown to be the most sensitive organelles.

In conclusion, the impact of AgNPs on seed germination and growth, morphology and ultrastructure of plants depends on various AgNP characteristic such as size, chemical composition, surface structure and oxidative dissolution. Their influence also seems to depend on the plant species and growth stage of plant, the type of substrate (soil or different nutrient media) the concentrations of nanoparticles involved and the manner of the application (foliar or soil). After AgNP accumulation in plants, they generally start degrading the quality of plants with generally negative impact on the root growth of germinating seedlings and the fresh biomass of the plant through the reduction in root elongation and biomass. Since roots are the first target tissue to confront with AgNP solution, toxic symptoms appear more in roots rather than in shoots, but AgNPs also induce morphological modifications in the stem and leaves. The main cell targets affected by AgNP toxicity are chloroplasts, mitochondria and nucleus, although there is still an open debate is the toxicity caused by AgNPs themselves or released Ag^+.

4. Induction of oxidative stress

Studies published so far suggest that exposure to metal NPs can induce increased generation of reactive oxygen species (ROS) [107], which can react with proteins, lipids and DNA molecules, resulting in a number of metabolic disorders, destruction of cell membranes and, in consequence, cell death. Production of superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), common ROS, was studied by Panda et al. [108], who reported their increased levels in *A. cepa* roots after 2 h-exposure to AgNPs (37 nm, 0, 5, 10, 20, 40, 80 mg L^{-1}). Exposure of rice seedlings to 0.5 and 1 mg L^{-1} of 20 nm AgNPs also resulted in a dose-dependent increase of ($O_2^{\bullet -}$) and H_2O_2 in roots and shoots after 1 week [100]. Moreover, in the study of Galazzi et al. [109] elevated H_2O_2 production in transgenic soybean (*Glycine max*) plants was found after 14-days-treatment with 50 mg kg^{-1} of 60 nm AgNP-citrate, while increase in ROS production was also observed in rice seedlings exposed to uncoated AgNPs (18.34 nm and concentration 30 and 60 μg mL^{-1}) for 7, 14 and 21 days [64]. A significant dose-dependent increase of ROS was also found after

exposure of aquatic plants *Lemna gibba* to 1 and 10 mg L^{-1} of 50 nm AgNPs [62] and *S. polyrhiza* to 0.5, 1.0, 5.0 and 10 mg L^{-1} of 6 nm AgNPs [106]. In the study of Cvjetko et al. [40], exposure of *A. cepa* roots to AgNPs stabilized with three different surface coatings, citrate (AgNP-citrate; 61.2 nm), polyvinylpyrrolidone (AgNP-PVP; 9.4 nm) and cetyltrimethylammonium bromide (AgNP-CTAB; 5.6 nm), resulted with significant increase in ROS content compared to the control at concentrations of 50, 75 and 100 μM, with AgNP-PVP and AgNP-CTAB exhibiting concentration-dependent increase. Moreover, in the same study AgNP-treatments exhibited lower toxicity compared to exposure with $AgNO_3$ indicating AgNP toxicity is not necessarily associated with dissociation of Ag^+ ions. This result can be, at least partially, attributed to AgNPs coatings, since addition of coating can stabilize nanoparticles, reducing dissociation of Ag^+ ions and thus diminish its toxicity [69]. Nair and Chung [60] also reported stronger concentration-dependent increase of ROS formation in $AgNO_3$-treated Arabidopsis seedlings compared to AgNP-treated ones. Moreover, in the study of tobacco seedlings exposed to citrate-coated AgNPs for 30 days it was found that higher concentrations of 50 nm AgNPs (100 μM) induced elevated production of ROS; however, despite the higher accumulation of Ag in seedlings exposed to AgNPs than in those treated with $AgNO_3$, effects of $AgNO_3$ were found to be more toxic than those of nanoparticles [75]. On the contrary, higher accumulation of total ROS and ($O_2^{\bullet -}$) in potato (*Solanum tuberosum*) plantlets exposed to 20 nm uncoated AgNPs was recorded in comparison to $AgNO_3$-exposed ones after application of 10 and 20 mg L^{-1} concentrations of both silver forms [110], which suggests that the effects of AgNPs on ROS production are not unambiguous. The study in which adult tobacco plants were treated with 61 nm AgNP-citrate (25, 50, 75, 100 and 500 μM) no changes in ROS accumulation were recorded in comparison to the control [74], thus suggesting that the response to AgNP-imposed stress might also be dependent on the plant developmental stage. Still, in the majority of presented studies, AgNPs of different sizes, concentrations and coatings induced ROS formation, regardless of the duration of the exposure and investigated plant species. Therefore, toxicity of AgNPs in plant cells in majority of the cases can be attributed to the generation of ROS, whose production might explain the effects of nanoparticles on plants. However, at this point it cannot be stated with certainty if AgNPs induce generation of ROS directly or indirectly through Ag^+ ions.

When generation of ROS exceeds the capacity of the cellular antioxidant defence system, oxidative stress occurs, which may lead to inactivation

and damage of membrane lipids, proteins and DNA molecule. Malondialdehyde (MDA) is one of the final products of oxidative modification of lipids, and changes of its concentration indicate membrane lipid peroxidation under ROS action as the effect of cellular injury of membrane lipids. It is widely used as an indicator of oxidative stress in plant cells and tissues [111]. Beside lipid peroxidation, protein oxidation, one of the covalent modifications of proteins induced by ROS or other products of oxidative stress, is also often analysed to confirm the presence of oxidative stress. Increased level of lipid peroxidation was reported for rice [100], Arabidopsis [60] and mung bean [112] seedlings exposed to AgNPs. In the study of Cvjetko et al. [40], in which effects of AgNPs stabilized with different coatings were analysed, it was found that treatments of *A. cepa* roots with 5.6 nm AgNP-CTAB exhibited concentration-dependent increase in MDA and carbonyl content, while exposure to 60 nm AgNP-citrate and 9.4 nm AgNP-PVP resulted with significantly lower values, which was partially attributed to the smaller size of AgNP-CTAB. The very small size of NPs is believed to cause higher toxicity in plants and uptake of AgNPs has been already associated with particle size and concentration [113]. Moreover, the strong effect of CTAB-coated AgNPs can also be attributed to the coating itself because the cell membrane is negatively charged and may enter into electrostatic interactions with the AgNP-CTAB, due to positively charged CTAB. The weakest impacts were recorded for citrate-coated AgNPs, which were of the biggest size; namely, due to the formation of the aggregates as well as to negative charge, the uptake of AgNP-citrate by *A. cepa* root cells was probably somewhat difficult and the AgNPs surface available for interaction with organic molecules decreased [114], thus lowering their toxic effects. In the study of Barbasz et al. [115] two wheat callus cultures, one sensitive to oxidative stress and the tolerant one, were exposed to 20, 40 and 60 ppm of 17 nm AgNPs and it was found that a stronger increase of MDA content was observed in sensitive cultivar than in the cells of tolerant callus. Peharec Štefanić et al. [75] reported that in tobacco seedlings increased contents of MDA and protein carbonyls were recorded only after the highest concentration (100 μM) of the 50 nm AgNP-citrate was applied. However, after exposure of adult tobacco plants to the same concentrations of AgNP-citrate, none of the applied AgNP-concentrations induced a significant increase in MDA and protein carbonyl content neither in roots nor in leaves [74], thus suggesting that plant response to AgNPs might be also dependent on plant age and/or developmental stage. In several studies, comparison between plants exposed to AgNPs with those exposed to the same concentration of

$AgNO_3$ was performed. In some of them $AgNO_3$-exposure imposed greater stress than AgNP-exposure [40,74,75,115], which confirms that Ag^+ is generally more toxic for plants than Ag nanoparticles and that the AgNP-toxicity probably does not depend solely on the Ag^+ ions dissociated from nanoparticles. However in some cases the results are not unambiguous. For example, Galazzi et al. [109] reported that after exposure of non-transformed (NT) and transformed (T; which after transformation gained tolerance to herbicide) soybean plants to AgNPs and $AgNO_3$ for 14 days, increase in MDA content was recorded for both soybean genotypes. However, in NT plants increase was much higher after exposure to $AgNO_3$ compared to AgNPs, while in T plants MDA content was of similarly elevated values after both types of treatments. This suggests that various factors might influence plant's response to AgNP-imposed stress.

Considering the impact on the DNA molecule, AgNPs were found to induce DNA damage [40,74] and influence gene expression [51,68,116]. Kumari et al. [104] reported that uncoated AgNPs (100 nm, 25, 20, 75, and 100 ppm) may have a genotoxic effect in *A. cepa* roots, while in the same plant species it was found that biologically synthesized AgNPs of 20 nm applied in 5, 10 and 20 $\mu g\,mL^{-1}$ concentrations caused severe mitotic and meiotic abnormalities in the root tips and flower bud cells [116]. Moreover, Patlolla et al. [51] demonstrated that 25, 50 and 100 $mg\,L^{-1}$ concentrations of 60 nm uncoated AgNPs significantly increased the number of chromosomal aberrations, micronuclei, and decreased the mitotic index in exposed *V. faba* roots compared to control. Decrease in mitotic index was also found after exposure of *A. cepa* roots to 100 μM AgNP-PVP and AgNP-CTAB [40]. In several studies an alkaline protocol of the plant comet assay, a reliable and simple method which is widely used for estimation of the extent of induced DNA damage, was applied to investigate the possible damaging effects of AgNPs on plant DNA. In *A. cepa* roots exposure to 20, 40 and 80 $mg\,L^{-1}$ of 37 nm AgNPs induced DNA damage in a dose-dependent manner [108]. In the study of Cvjetko et al. [40], in which the effects of AgNPs coated with citrate, PVP and CTAB as well as of $AgNO_3$ were investigated, a significant increase in tDNA was recorded after exposure to 75 μM AgNP-CTAB and 100 μM AgNP-PVP, while treatments with $AgNO_3$ induced even stronger effects. These results indicate that AgNP effect on DNA molecule is a result of particulate form, but also suggest that DNA sensitivity to AgNPs is dependent on the nanoparticle coating, size as well as on concentration. After exposure of tobacco seedlings to AgNP-citrate, comet assay revealed that $AgNO_3$ induced an increase in tail

DNA from even the lowest applied concentration (25 μM), while the exposure to AgNPs resulted in significantly increased values of oxidative stress parameters, including tail DNA, only after the highest applied concentration (100 μM) [75]. Vannini et al. [47] found no changes at the DNA level in wheat seedlings treated with 1 and 10 mg L^{-1} of 10 nm AgNP-PVP. Moreover, Cvjetko et al. [74] reported that none of the applied AgNP-citrate concentrations (25, 50, 75, 100 and 500 μM) induced increased DNA tail in roots or leaves of the adult tobacco plants compared to the control, while $AgNO_3$-treatments induced significant DNA damage in root tissue at all of the applied concentrations and in leaves after two highest investigated concentrations (100 and 500 μM). The overall results indicate that ROS formation and oxidative stress can play a critical role in phytotoxicity mechanism but AgNP size, overall surface charge and/or surface coating as well as on the plant species and developmental stage may influence plant response to AgNPs.

Plants have developed very efficient ROS scavenging system, which depends on the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and stimulate the production of antioxidant molecules such as ascorbic acid (AsA) and glutathione (GSH). Most of the studies dealing with AgNPs impact on plants reported changes in activities of antioxidant enzymes, although results are not unambiguous. Within a cell, the SOD constitutes the first line of defence against ROS since its neutralization of superoxide radical to H_2O_2 is very effective in preventing damage to biologically important molecules. Vannini et al. [46] reported increased SOD activity and expression after exposure of rocket seedlings to treatments with 10 mg L^{-1} of 10 nm AgNP-PVP. Peharec Štefanić et al. [75] found enhanced SOD activity in tobacco seedlings exposed to AgNPs, which was in agreement with the low level of oxidative damage found in that tissue. The induction of SOD activity was additionally confirmed in the same study by proteomic analysis, where up-regulation of Fe-SOD was found. Significant increase in SOD activity was also reported for potato plantlets exposed to AgNPs [110]. In callus cultures of two wheat varieties, treatments with AgNPs did not cause a significant change in SODs activity in the variety tolerant to oxidative stress, suggesting that in the tolerant callus, other antioxidants, such as GSH, whose increased content was measured, may be involved in cell protection, and/or, applied nanoparticle concentrations were not stressful for this variety [115]. On the other hand, in the sensitive wheat callus, changes in SOD activity were concentration-dependent, thus indicating a

mobilization of enzymatic antioxidant systems. A slight decrease in SOD activity was observed in non-transformed soybean plants after treatment with AgNPs, while exposure of transformed, herbicide tolerant plants resulted with much higher increase in activity of this enzyme [109]. However, a strong increase in the CAT activity was detected in both soybean genotypes AgNPs, which suggests that this antioxidant enzyme has a key role in the protection against the toxic effects of ROS generated by AgNPs. These findings suggest that AgNPs phytotoxicity might be dependent on the sensitivity of the particular plant species or even on the cultivar or a variety of the same species.

To protect cells against ROS completely, antioxidant enzymes such as CAT, PPX and APX have to remove H_2O_2 generated by SOD dismutation of $(O_2^{\bullet -})$. Moreover, GR and GSH are two major components of AsA-GSH pathway, which also plays a significant role in protecting cells against ROS. Significant increase in the activities of CAT, APX and GR was found after exposure of potato plantlets to AgNPs, and $AgNO_3$ [110]; however, in AgNP-treated plantlets, GR activity was significantly decreased at higher concentration, which was accompanied with higher reduction in GSH and AsA compared to plants exposed to $AgNO_3$, according to which authors suggested that AgNPs had higher toxicity than the equivalent mass of Ag ions. Barbasz et al. [115] reported an increase of GSH levels in callus cells of both wheat genotypes exposed to AgNPs at all used concentrations (20, 40 and 60 ppm); however, the greater change of GSH content was recorded in the tolerant variety in comparison to the sensitive one, which indicated that this nonenzymatic compound was the main antioxidant, the synthesis of which was activated in the tolerant variety in conditions of oxidative stress induced by AgNPs. In the sensitive variety, a smaller increase in GSH level was accompanied with simultaneous activation of SOD and PPX; therefore, authors suggested that in the defence mechanism against AgNP-imposed stress, both enzymatic and nonenzymatic antioxidants are operating [115]. In the study performed on *A. cepa* roots, AgNP treatments induced the PPX activity, while significant concentration-dependent decrease in CAT and APX activities was noticed, suggesting that PPX was the enzyme involved in lowering the ROS level [40]. Elevation of PPX and inhibition of CAT activity has also been reported in *B. monnieri* [105] and sensitive wheat calli [115] after exposure to AgNPs. The highest reduction of CAT activity observed in both sensitive and tolerant wheat calli at the highest applied concentrations of AgNPs corresponded with the highest amount of generated ROS, which indicates that CAT synthesis

was probably inhibited by strong oxidative stress [115]. After exposure of castor bean seedlings to AgNPs, increase in PPX activity was not concentration-dependent, while CAT activity was inhibited [69]. In the study of Peharec Štefanić et al. [75] elevated APX activity, along with the decreased PPX and unchanged CAT activity, suggested that APX was a key enzyme responsible for catalysing the conversion of H_2O_2 into H_2O after exposure of tobacco seedlings to AgNPs. On the other hand, increased CAT activity was recorded in *S. polyrhiza* [106] and water hyacinth (*Eichhornia crassipes*) [117] exposed to AgNPs. In roots of adult tobacco plants, exposure to AgNPs did not induce significant changes in activity of PPX, while lower AgNPs concentrations induced higher CAT activity, which was is in good correlation with no measurable changes in oxidative stress parameters, indicating that AgNPs induced mild oxidative stress, which could be efficiently alleviated by antioxidant enzymes [74]. The same study showed that in leaf tissue, no changes were recorded in APX activity, while PPX activity increased at lower concentrations and decreased at higher concentration [74]. Moreover, AgNPs did not induce any significant oxidative stress in tobacco leaves. Since the Ag concentration in leaves was much lower than in roots, it is possible that some of the changes observed in the leaves were just a consequence of the stressful events that took place in the roots. A decrease in PPX activity at higher AgNP concentrations, after an initial increase at lower concentrations, was also recorded in leaves of *Pelargonium zonale* plants [118]. Several reports in which AgNP toxicity was compared with those of $AgNO_3$ [74,75] suggested that exposure to $AgNO_3$ induced severe oxidative stress accompanied with stronger response of antioxidant enzymes; in some cases even severe inhibition of activity was observed [69], possible due to Ag^+ binding to SH groups in enzymes. However, similar effects between AgNP- and $AgNO_3$-exposure were also observed [115]. Moreover, it has been demonstrated that AgNPs were even more toxic than Ag^+ [68] and could alter the transcription of genes involved in antioxidant synthesis and change the balance between the oxidant and antioxidant systems. Presented results suggest that different antioxidant enzymes could play key roles in eliminating ROS accumulated as a result of nanosilver exposure but the exact mechanism of AgNP action on antioxidant system in plants is yet to be proven.

Results obtained on oxidative damage to lipids, proteins, and DNA molecule as well as on changes in the activity of plant antioxidant enzymes in plants treated with AgNPs suggest that oxidative stress could have an important role in the phytotoxicity of AgNPs.

5. Effects on photosynthesis

Photosynthesis is a key process for life on Earth in which plants and other photosynthetic organisms, like algae and cyanobacteria, change light energy into chemical energy. In these organisms photosynthesis represents a major physiological process for the maintenance of cellular viability and growth. Photosynthetic process is known to be very sensitive to stress caused by adverse environmental conditions, such as high light, drought, salinity, heat and heavy metals [119]. Chlorophyll as a major photosynthetic pigment is responsible for capturing light energy and its content can be used as an indicator of plant photosynthetic capacity [120]. A decrease in chlorophyll content as a parameter of AgNP phytotoxicity has been noticed in various organisms including marine and freshwater microalgae [63,121,122], aquatic plants [41,53,55], crop plants [20,30,50,65,100,123] as well as a model plant Arabidopsis [57,60,68,124]. Beside chlorophyll content, chlorophyll *a* fluorescence has been proposed as a sensitive, non-destructive, rapid, and efficient method for detecting the impacts of environmental stress on photosynthetic efficiency [125,126]. Light energy absorbed by chlorophyll molecules can be either used for photochemistry, be re-emitted as heat or be re-emitted as fluorescence. Since these three processes are in competition with each other, the yield of chlorophyll fluorescence emission gives us valuable information about the quantum efficiency of photochemistry, which is responsible for providing energy and reducing power for CO_2 assimilation. The analysis of changes in chlorophyll fluorescence kinetics provides detailed information on the structure and function of the photosynthetic apparatus and various parameters derived from fluorescence measurement data have been widely used as sensitive biomarkers of phytotoxic effects [126]. Several authors evaluated AgNP phytotoxicity by measuring different fluorescence parameters, most often maximum quantum yield (F_v/F_m) of photosystem II (PSII), which corresponds to the efficiency by which an absorbed photon will be trapped by PSII reaction centres [125], and found that PSII efficiency in various algae and plants was significantly reduced after AgNP exposure [20,30,53,55,63,68]. The change of fluorescence parameters under a light-adapted state, which can provide information about the efficiency of photochemical reactions and/or heat dissipation of chlorophyll excitation energy, has also been analysed [50,53,127–129]. More recently, a measurement of rapid fluorescence induction curves with high resolution and the parameters of the so-called JIP-test have been used to assess the behaviour of various

components of photosynthetic apparatus in plants and algae exposed to AgNPs [121,123,127,129,130].

Significant amount of data on AgNP-induced toxic effect on photosynthesis come from investigations on photosynthetic algae. In alga *C. reinhardtii* short-term (1 − 2 h) exposure to 801–879 nM of uncoated 40 nm AgNPs inhibited the photosynthetic yield of the PSII [61]. Dewez and Oukarroum [127] found the evidence of a structural deterioration of PSII reaction centre, the alteration of the oxygen evolving complex and the inhibition of electron transport activity at the in cells of *C. reinhardtii* exposed to 5 and 10 $\mu mol\,L^{-1}$ of 50 nm AgNPs for 6 h. Moreover, operational PSII quantum yield in the light and the non-photochemical quenching value decreased, indicating that there was no activation of photoprotective mechanisms. Analysis of fluorescence induction curves in *C. reinhardtii* cells after 24 h incubation with 80 nm AgNPs at concentrations of 2 and 20 $\mu mol\,L^{-1}$ confirmed the inhibition of electron transport in PSII but showed the absence of direct effect on reactions of oxidation of the photosystem I (PSI) pigment P700 [130]. Moreover, analysis of induction curves for delayed fluorescence revealed a decrease in the energization of the photosynthetic membranes in presence of AgNP. Navarro et al. [130] investigated effect of nine differently coated AgNPs (chitosan, lactate, PVP, PEG, gelatin, sodium-dodecylbenzenesulfonate, citrate, dexpanthenol and carbonate) with average diameters ranging from 17 to 456 nm, on photosynthetic yield of *C. reinhardtii*. After 1 h exposure all differently coated AgNPs proved to be toxic and toxicity was neither related to particle size nor to the coatings. In marine cell wall-lacking microalga *Dunaliella tertiolecta* and freshwater microalga *Chlorella vulgaris* treatment with uncoated 50 nm AgNPs (0.1, 1, and 10 $mg\,L^{-1}$) for 24 h decreased chlorophyll content and induced a decrease in the photosynthetic performance mostly due to a change in the maximum quantum yield for primary photochemistry and electron transport activity [121,122]. However, AgNPs had a more negative effect on *D. tertiolecta* compared to *C. vulgaris*, which could be due to the absence of cell-wall in *D. tertiolecta* and/or biotransformation of AgNPs in seawater growth medium. In marine macroalga *Ulva lactuca*, suppression of the quantum efficiency of PSII electron transport in the light was found at AgNP concentrations higher than 15 $\mu g\,L^{-1}$ [128]. In marine diatoms, photosynthetic activity and chlorophyll content were severely suppressed by 10 nm AgNP-PVP in *Thalassiosira weissflogii* [63] as well as by 10 nm oleylamine-coated AgNPs in *Skeletonema costatum* [131]. In aquatic plant *Wolffia globosa* exposed to AgNPs for 3 days (20 nm diameter, 0.1, 1, and 10 $mg\,L^{-1}$), the

decrease in chlorophyll content and photosynthetic ability after AgNP-citrate treatments was much smaller when compared to those obtained after treatments with AgNPs coated with adenosine triphosphate disodium (ATP) [41]. In another duckweed, *S. polyrhiza*, 72 h-exposure to 10 nm AgNP-GA as well as to 20 nm AgNP-PVP applied in 0.5, 1, 5, and 10 mg Ag L^{-1} concentrations inhibited maximum quantum yield and effective quantum yield in light and reduced chlorophyll content [53,55]. Moreover, AgNP-PVP inhibited the photoprotective capacity of PSII, decreased carotenoid content and induced inhibition of Rubisco activity [55]. Contrary, Shabnam et al. [129] revealed that in *S. polyrhiza* fronds exposed to uncoated AgNPs (10–30 nm, 1, 10, 50, and 100 mg L^{-1}, for 24 h) F_v/fm and electron transport rate remained mostly unaltered, while parameters of chlorophyll fluorescence showed marginal decline. Regarding investigations on crop plants, in seedlings of wheat and sunflower treatments with AgNPs (uncoated, 20–30 nm, 10–100 ppm) for 24 h, did not cause any significant alteration in PSII efficiency [123], while in lettuce 7-day foliar exposure to uncoated 40 nm AgNPs (1, 10 or 100 μg AgNPs g^{-1} FW) did not exhibit toxic effects on photosynthetic pigment content [92]. Contrary, in rice seedlings after 1-week exposure to AgNPs, a significant reduction in total chlorophyll and carotenoids contents was observed [100]. After 15 days the treatment with 1 and 3 mM of uncoated AgNPs significantly declined photosynthetic pigments and chlorophyll fluorescence in pea [73] and mustard seedlings [50]. Similarly, photosynthesis and CO_2 assimilating efficiency were severely disrupted in tomato after a long-term exposure (56 days) to AgNP-PEG (10 nm, 10 mg kg^{-1}) as a significant decrease in chlorophyll content as well as in Hill activity and the rate of photosynthesis were observed [65]. The 7-day exposure of tobacco adult plants to differently coated AgNPs (citrate, PVP and CTAB) revealed that positively charged AgNP-CTAB had more adverse influence on chlorophyll content and fluorescence parameters than AgNPs stabilized with other two coatings (M. Tkalec et al., unpublished results). Contrary, in leaves of brown mustard (*Brassica juncea*) seedlings exposed to AgNPs (30 nm) higher chlorophyll contents and improved photosynthetic quantum efficiency were recorded, which indicates that more number of reaction centres are in an "open state" to carry out light reaction [23]. On the other hand, in Arabidopsis treatments with AgNPs of different concentrations (0.5–3000 mg L^{-1}), sizes (10, 20 and 40 nm), coatings (uncoated, citrate-coated) and of different exposure periods (3–4 days, 14 days) decreased total chlorophyll content as well as the photosynthesis efficiency [57,60,68,124,132]. Moreover Li et al. [132] found

that AgNPs caused an adverse effect on transcript levels of psbA, rbcL as well as the cyclic electron transport chain-related gene transcripts, thereby reducing ATP synthesis. Regardless of one study with positive effects [23], the vast majority of investigations showed that different AgNPs, regardless of their characteristics, the duration of the exposure and investigated plant species, can have a detrimental impact on the structure and function of the photosynthetic apparatus.

Majority of literature data on AgNP toxicity in various species showed that AgNP-induced decrease in the chlorophyll content was in correlation with the reduced chlorophyll fluorescence yield [53,55,73,121,132]. Several studies showed that the negative effect of AgNP-exposure on photosynthetic parameters can be correlated with the increase in ROS generation [55,60,65,73,122,124,132]. Increased ROS formation can be responsible for chlorophyll deterioration, which then affects the conversion of light energy into photosynthetic electron transport and consequently reduces PSII photochemistry [121]. For example, in *C. vulgaris* efficient detoxification of AgNPs-induced ROS species by antioxidant enzymes activity allowed continuation of photosynthesis at growth-inhibitory AgNPs concentration. On the other hand, analysis of the rapid rise of chlorophyll fluorescence revealed that AgNP-induced structural deterioration of PSII reaction centre, the alteration of the oxygen evolving complex, and the inhibition of electron transport activity, which could result in formation of excess ROS [127]. Moreover, this effect was stronger during light exposure compared to dark condition, which may indicate that AgNPs can be photosensitized and capable of producing ROS that do additional damages to PSII functional integrity. Zou et al. [41] found that AgNPs can induce a decline in activity of Hill reaction, a first step of photosynthesis, which also indicates the negative effects on functional integrity of PSII and electron transfer efficiency. Using chlorophyll extract, it was found that AgNP-induced decrease in photosynthetic capacity may be related to a loss of excited electrons from chlorophyll molecules through an effective interaction between chlorophyll molecule and AgNPs [133,134]. Furthermore, it was also shown that AgNPs induced inhibition of Rubisco activity [55], which can lead to slowing down of CO_2 assimilation, as also observed by Das et al. [65]. As a consequence of decreased energy consumption excess, excitation energy is generated and this can promote ROS formation in the chloroplast [55]. This is consistent with changes in chloroplast structure observed after AgNP treatment in several investigations [74,97,106]. Alternatively, it is possible that the decrease in chlorophyll content as well

as in the levels of *psbA* gene [132], which encodes PSII reaction centre protein D1, in plants exposed to AgNPs represent a photoprotective mechanism to reduce light absorption and photochemical reactions in leaf, consequently decreasing toxic ROS. Although there are growing evidences that production of ROS is one the mechanisms through which AgNPs induce their toxic effects, the underlying mechanisms and the cause-and-effect relationship are still not entirely clarified.

As in the studies of AgNPs' effects on growth, germination, oxidation stress and other physiological processes, there is still no clear answer to whether the toxic effect on the photosynthesis is caused directly by nanoparticulate form or Ag^+ released from AgNPs. It has been established that AgNPs are prone to oxidation in aqueous solutions depending on pH, ionic strength, and the presence of ligands [26]. In most of the studies considering phytotoxic effects of AgNPs, the contribution of Ag^+ coming from AgNPs dissolution has been accounted by exposing plants to $AgNO_3$. In some studies no considerable difference in photosynthesis-related parameters was observed between effects of silver in the form of nanoparticles and Ag^+ ions [41,130] while in others it was demonstrated that nanoparticulate form of silver has less toxic effects on photosynthesis than ionic form [50,53,123,128,129]. Most of these investigations showed significantly higher Ag content in the plants exposed to Ag^+ than those exposed to AgNPs [53,123,128,129], which could explain the more negative effect of Ag^+ than that of AgNPs. In the experiments where Ag^+ dissociated from AgNPs in exposure solution were removed either by diafiltration or free Ag^+ were complexed with ligands such as thiols, no toxicity of AgNPs on PSII quantum yield and chlorophyll content was observed, indicating indirect toxicity of AgNPs [63,135]. These findings are in agreement with abundant literature data showing that AgNPs toxicity is caused mainly by the release of Ag^+ from nanoparticles [22,46,47,55,56,63]. It is well known that Ag ions can block the electron flow in PSII, which then causes production of ROS [136]. Analysis of chlorophyll fluorescence measurements in *S. polyrhiza* revealed that exposure to Ag^+ decreased electron transport rate and increased heat dissipation [129]. Moreover, it was reported that silver can interfere with chlorophyll biosynthesis and/or breakdown [136], by interaction of Ag^+ with thiol group of enzymes of chlorophyll biosynthesis. However, in some studies amount of Ag^+ present in media was very low (1–3%), so it was assumed that it cannot explain the inhibitory effect on photosynthesis [53,61,135]. In a few studies on Arabidopsis, a more pronounced decrease of the various photosynthetic parameters induced by

AgNPs compared to $AgNO_3$ was, at least partially, explained by the high level of Ag accumulation in plants treated with AgNPs [57,60,68,124]. Several investigations showed presence of AgNPs not just in roots but also in the leaves [68,79,80,82], confirming that Ag in plant tissues can arise from direct uptake of AgNPs and contribute to the AgNP toxicity on photosynthetic processes. However, as already mentioned, only a small fraction of AgNPs has been transported from the roots to the shoots [32,35,47,82], which implies that the negative effects of AgNPs on roots, which provide nutrition for physiological activities including photosynthesis and chlorophyll synthesis, can significantly reflect on photosynthetic processes, which take place in leaves [132]. In investigation on algae, the photosynthetic yield in *C. reinhardtii* after exposure to AgNPs with different coatings was comparatively lower when calculated as a function of measured Ag^+ concentrations than that of algae which were exposed to Ag^+ [135]. However, since excess cysteine completely blocked toxic effects, authors suggested that interaction between the particles and the algae can increase Ag^+ release from AgNPs, thus causing toxic effect [61,135]. It has been suggested that the production and secretion of ROS by algae might lead to increased Ag dissolution [137,138]. Several authors proposed that toxic effects of AgNPs on plants can be attributed to free Ag^+, originating from absorbed nanoparticles in plant cells [55,62]. As previously mentioned, Larue et al. [92] revealed AgNP agglomerates in various regions of the lettuce leaves, but also detected significant changes of internalized AgNPs, including partial or total oxidation of AgNPs, and formation of secondary Ag^+ species such as Ag^+ bound to thiols and AgCl. The results show that the negative effects of AgNPs on photosynthesis are associated with Ag accumulation in the root and/or in the shoot, but it is not yet fully explained whether the effects come from nanoparticles, Ag^+ or both.

Based on presented literature data we can conclude that exposure to AgNPs has a detrimental impact on the structure and function of the photosynthetic apparatus. Certain divergence in the AgNP-imposed effect observed can be attributed to differences in the type of coating, size, time of exposure or plant species used in experiment as has already been established for toxic effects on other parameters like growth, germination, etc. The question whether the toxic effect on photosynthesis is specific for nanoparticles or it is the result of the action of Ag^+ released from AgNPs in exposure solution and/or after biotransformation in the cellular structures remains unsolved.

6. Changes in protein expression

The area of nanomaterial phytotoxicity has attracted attention in recent years, but still the mechanisms involved in changes of plant protein expression as a result of exposure to nanoparticles are unknown. Therefore, it is necessary to develop a more standardized approach in order to understand the plant-NPs interactions. Proteomics techniques, which detect quantitative and qualitative changes in protein expression profiles, are powerful tool for the identification of proteins related to specific developmental and/or environmental signal [139]. Therefore, to examine the molecular bases of AgNPs phytotoxicity, proteome analysis can be applied. Information obtained from proteomic studies can improve the knowledge of interactions between plants and nanoparticles, since these studies reflect the nanoparticles effects on gene expression. So far proteomic-based approach for studying plant responses to AgNP-induced stress has been employed in only a few studies [46,47,75,140,141]. This chapter throws light on the current literature since results of these studies offer new insight into plant response to AgNP exposure and provide important information to support the sustainable use of AgNPs.

From the obtained data it is difficult to draw unambiguous conclusions since in different studies different sizes of nanoparticles were applied and AgNPs were stabilized with different coatings or were uncoated (Table 1). Namely, Mirzajani et al. [140] exposed rice seedlings to 18.34 nm uncoated AgNPs; 15 nm AgNPs without coating were also applied in treatments of soybean seedlings [141]. In the proteomic studies of exposure of rocket and wheat seedlings, 10 nm AgNP-PVP were used [46,47]. AgNP-citrate was examined in tobacco seedlings (50 nm, [75] and soybean plants (60 nm, [109]), while in the study conducted on the green algae *C. reinhardtii* the 20 nm AgNP-PEG was applied [142]. Moreover, the exposure times also differed among published studies and extended from short exposures of two [141], three [142] or 5 days [46,47] to much longer treatments which lasted for 14 [109], 20 [140] and 30 days [75]. Exposure media used for AgNP-treatments included soaked filter paper [46,47], silica sand [141], liquid [140,142] or solid nutrient medium [75] and deionized water [109]. It is also important to emphasize that proteomic methodology applied in these studied mostly involved protein separation by means of two-dimensional electrophoresis followed by either nanoLC-ESI- [46,47,109,142] and nanoLC/FTICR- [140] or

Table 1 Proteomic studies of plant and green algae response to AgNP-imposed stress.

Species	AgNP size (nm)	AgNP coating	AgNP concentration	Exposure time (days)	Exposure medium	Analysed tissue	Proteomic methodology	Reference
Eruca sativa (rocket)	10	Polyvinyl-pyrrolidone	$10\,mg\,L^{-1}$	5	Filter paper soaked in deionized water	Roots	2-DE, nanoLC-ESI-MS/MS	[46]
Chlamydomonas reinhardtii	20	Polyethylene glycol	$215\,\mu g\,L^{-1}$	3	MBL medium	Whole cell	2-DE, nanoLC-ESI-MS/MS	[142]
Triticum aestivum (wheat)	10	Polyvinyl-pyrrolidone	$10\,mg\,L^{-1}$	5	Filter paper soaked in deionized water	Roots and shoots	2-DE, nanoLC-ESI-MS/MS	[47]
Oryza sativa (rice)	18.34	Uncoated	30, $60\,\mu g\,mL^{-1}$	20	Hydroponic medium	Whole seedlings	2-DE, nanoLC/FTICR MS	[140]
Glycine max (soybean)	15	Uncoated	2 ppm	2	Silica sand	Roots and cotyledons	Gel-free, nanoLC-ESI-MS/MS	[141]
Nicotiana tabacum (tobacco)	50	Citrate	$100\,\mu M$	30	Solid MS medium	Whole seedlings	2-DE, MALDI TOF/TOF MS	[75]
Glycine max (soybean)	60	Citrate	$50\,mg\,Kg^{-1}$	14	Deionized water	Leaves	2-D DIGE, nanoLC-ESI-MS/MS	[109]

MALDI-TOF/TOF-MS/MS [75]. Only the study of Mustafa et al. [141] employed gel-free nanoLC-ESI mass spectrometry.

In the studies of Mirzajani et al. [140] and Peharec Štefanić et al. [75] the proteomes of whole rice and tobacco seedlings, respectively, were analysed upon AgNP-exposure. In rice, 28 protein spots showed significant differences in response to treatment with uncoated AgNPs and the majority of AgNP-induced changes was associated with decreased abundance [140], contrary to tobacco, in which 39 responsive proteins were detected upon exposure to AgNP-PVP, majority of which was up-regulated [75]. Given the prolonged exposure of the seedlings to silver treatments (from seeds to fully developed seedlings) in these two studies, observed changes in the proteomes reflect the adjustment of plant metabolism to AgNP-induced stress. Contrary, in the following studies, where proteome changes were analysed after a few days of exposure, differences in protein expression might be a result of instant up-/down-regulation in response to AgNPs. After five-day exposure of rocket seedlings to AgNP-PVP, only the proteome of root tissue was analysed and 22 AgNP-responsive proteins were found, among which 15 proteins exhibited enhanced expression [46]. The same treatment applied to wheat seedlings resulted with 27 responsive proteins in roots and 12 in shoots, majority of which was up-regulated; interestingly, no common proteins in roots and shoots were found [47]. Mustafa et al. [141] reported that in the differential analysis of soybean seedlings exposed to uncoated AgNPs, the abundances of 107 root proteins were significantly changed, while in cotyledons only 9 proteins were found to be responsive; majority of the proteins were found to be down-regulated. Moreover, only one protein, glyoxalase II 3, was found to be common for roots and cotyledons, although its response was opposite; in the root tissue, glyoxalase II 3 expression was down-regulated, while in cotyledons it revealed enhanced expression. In our study on adult tobacco plants, only several common proteins (osmotin, basic beta-1,3-glucanase, CBP20, Fe-SOD, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triose phosphate isomerase (TPI) and malate dehydrogenase (MDH)) were found to be regulated by AgNPs in both roots and shoots (P. Peharec Štefanić et al., unpublished). This tissue-dependent response probably results from differences in metal content, as silver accumulation in roots was several times higher than in leaves, after both types of treatments. Tissue-specific response has not been extensively studied so far, although reports on metal-induced stress show that proteins in roots and leaves are differentially regulated, generating different detoxification mechanisms [143]. Abovementioned proteomic analyses

[47,141], P. Peharec Štefanić et al., unpublished], in which roots and shots were separately analysed, show that roots are the major site of proteome changes thus confirming that roots are the principal targets of the toxic effects of AgNPs. Additionally, studies in which coated AgNPs (PVP [46,47]; PEG, [142]; and citrate, [75]) were applied, resulted with mostly up-regulated responsive proteins, while in two studies performed with uncoated AgNPs [140,141] majority of the proteins exhibited decreased expression. These findings could be related with higher stability of coated AgNPs, which are less prone to release Ag^+ ions compared to uncoated ones. Despite abovementioned differences, some common features of identified responsive proteins in these published studies can be found; namely, performed studies have indicated that AgNPs predominantly affected proteins related to cell metabolism, stress response and signalling.

Majority of the proteomic analysis performed so far showed that many of the identified proteins with differential expression after plant treatments with AgNPs were those involved in the processes of primary metabolism. In the studies of the AgNP effect on wheat [47], soybean [141] and tobacco seedlings [75] as well as on green alga *C. reinhardtii* [142] it was revealed that proteins involved in photosynthesis were impacted. The study on tobacco seedlings exposed to AgNPs reported the up-regulation of proteins involved in primary reactions of photosynthesis i.e. protein PsaD as important part of PSI, proteins of the oxygen-evolving complex of PSII and plastid ATP synthase CF1 epsilon subunit, as well as enzymes involved in carbon reactions i.e. Rubisco, Rubisco activase 2, the enzyme that is required for the activation of Rubisco and beta-carbonic anhydrase, which ensures a sufficient amount of CO_2 for fixation by Rubisco [75]. These findings suggest that the up-regulation of enzymes involved in photosynthesis might help seedlings exposed to AgNPs to accelerate their energy production and produce additional reducing power, which is necessary to overcome AgNP-induced stress. However, the opposite effects on photosynthesis-involved proteins after AgNP-exposure were also recorded. Namely, several proteins of PSII complex were down-regulated after exposure of flooded soybean seedlings to AgNPs [141]. Moreover, Galazzi et al. [109] reported decreased expression of Rubisco small and large subunits and oxygen-evolving enhancer protein 2 of PSII after exposure of soybean plants to AgNPs. The later was also found after exposure of *C. reinhardtii* to AgNPs [142], although chlorophyll a–b binding protein of PSII and Rubisco activase were found to be up-regulated. The negative impact of AgNP-treatment on photosynthesis of wheat seedlings was also indicated by the down-regulation of

HCF136 protein, which is essential for photosystem biogenesis [47]; however, in the study conducted on *C. reinhardtii* this protein was up-regulated [142].

The enhanced expression of proteins involved in glycolysis i.e. plastidic aldolase, TPI, GAPDH and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (iPGAM) was recorded after exposure of tobacco seedlings to AgNPs [75]. Moreover, GAPDH was found to be a responsive protein in AgNP-exposed rice seedlings [140]. These proteins improve a plant's ability to maintain glycolysis in order to ensure sufficient energy production. Moreover, TPI and GAPDH are multifunctional proteins, which exhibit activities distinct from their classical functions, such as involvement in oxidative stress response [144]. In the study on soybean seedlings in response to AgNPs treatment under flooding stress, among proteins that revealed enhanced expression were G-proteins, which are regulatory proteins that function as essential signal transducers in hypoxia signalling pathway, which can help plants to alter their carbohydrate metabolic pathways to support ATP generation through glycolysis and fermentation under oxygen-deprived conditions [141]. In general, the enhanced expression of proteins involved in photosynthesis and glycolysis may serve to ensure sufficient energy to cope with stress in AgNP-exposed seedlings. Moreover, up-regulation was also found for MDH [46,47,75] and NAD-dependent isocitrate dehydrogenase [142], proteins involved in tricarboxylic acid (TCA) cycle, which links glycolysis to the mitochondrial electron transport chain. Moreover, expression of mitochondrial and chloroplast ATP synthase subunit was also enhanced after exposure of plants to AgNPs [75], which indicates increased ATP production. However, an exposure of transformed, herbicide tolerant soybean plants to AgNPs resulted with down-regulation of TPI and mitochondrial ATP synthase subunit [109]. Still, enhancement of the protein expression, found in the majority of the studies, suggests that metabolic adaptations of plants might play an important role in mitigating the stress imposed by AgNPs. Alterations in energy metabolism are aimed to enhance energy production in an immediately available form, such as ATP, needed for increased biosynthesis of stress-related compounds [145].

Studies have shown that plant exposure to AgNPs often results with activation of different proteins responsible for biosynthesis, folding, assembly, translocation and degradation of proteins in many cellular processes. For example, the expression of eukaryotic initiation and elongation factors, which have important roles in protein synthesis regulation, translation, elongation and mRNA turnover, were found to be up-regulated after AgNP

exposure in several studies [46,75,142]. Moreover, among proteins involved in protein folding, peptidyl-prolyl cis-trans isomerases (PPIases), which catalyse a rate-limiting step in protein folding, were found to be up-regulated in *C. reinhardtii* [142] and tobacco seedlings [75] upon exposure to AgNPs, although their down-regulation was reported in soybean plants [109]. Up-regulation of special class of proteins, heat shock proteins (Hsps), whose enhanced expression has an important role in maintenance of protein homeostasis in plants under a wide range of environmental stressors [146], was also found in several studies. Peharec Štefanić et al. [75] reported the up-regulation of chloroplastic-like 20 kDa chaperonin and Hsp70 protein after exposure of tobacco seedlings to AgNPs. Moreover, Vannini et al. [46] found enhanced expression of the 17.4 kDa class heat shock protein in rocket seedlings exposed to AgNPs, although the expression of the heat shock protein 70–2, involved in ER-associate degradation, was decreased. Exposure of *C. reinhardtii* to AgNPs resulted with up-regulation of ClpB chaperon, a member of Hsp 100 family [142]. Moreover, Kunitz family trypsin and protease inhibitor proteins increased in soybean seedlings exposed to AgNPs [141], probably to degrade irreversibly damaged proteins since stress conditions result in the aggregation of misfolded proteins [147]. In compliance with that, in rice seedlings exposed to AgNPs some proteins involved in protein synthesis/degradation processes, including proteasome subunit α-10 and subunit β-11, were identified [140]. Since it is known that Ag^+ acts on protein structure [148] and that AgNPs can interact with proteins [149] enhanced abundance of proteins involved in degradation of damaged proteins as well as in protein synthesis and folding may have an important role in adaptation of plants to silver-imposed stress.

It was shown that AgNP-treatments can also alter the expression of some pathogenesis related (PR) proteins, such as β-1,3-glucanase and chitinase, that are usually induced in response to wounding or infection [150], but are also involved in metal stress response [47,151]. In the study of Peharec Štefanić et al. [75], the up-regulation of basic beta-1,3-glucanase and acidic chitinase was found in tobacco seedlings exposed to AgNPs. Since AgNPs were observed in plasmodesmata, cell wall, and middle lamella [34], it is possible that the accumulation of beta-1,3-glucanase, which has a roll in the regulation of callose at plasmodesmata [152], might increase their permeability in response to AgNPs as has been observed for some metals [153]. Moreover, jacalin lectin family protein was found to be up-regulated in rocket seedlings after exposure to AgNPs [46], while in the study of wheat seedlings exposed to nanosilver, the enhanced expression of several PR proteins was found [47].

Most of the proteomic research on plant response to various stress factors revealed positive correlation between stress tolerance and increased abundance of antioxidant proteins [154]. It has been shown that AgNP-induced stress can also accelerate the production of cellular antioxidant enzymes in order to reduce ROS. In the rocket seedlings exposed to nanosilver an increased abundance of SOD and Type2 peroxiredoxin (PRX), a thiol peroxidase, was found [46], while Mirzajani et al. [140] reported the up-regulation of SOD, APX and glutathione-*S*-transferase (GST) in rice seedlings exposed to AgNPs. Enhanced expression of CAT, APX and Fe-SOD, proteins involved in defence against oxidative stress, was also found after exposure of tobacco seedlings to AgNPs [75]. However, there are opposite results; SOD isoforms were found to be down-regulated in soybean [109] and *C. reinhardtii* [142] exposed to AgNPs. Still, majority of the findings confirm that enhanced expression of proteins involved in defence against oxidative stress in seedlings exposed to AgNPs could be one of the protective strategies against nanosilver-induced ROS formation. It is well-known that accumulation of detoxification-related and ROS scavenging enzymes mitigates harmful effects of oxidative stress as well as increased amounts of toxic byproducts of cellular metabolism as a consequence of imbalances in cellular homeostasis [154,155].

The up-regulation of calcium-binding messenger proteins (CaM), such as calmodulin, one of the most extensively studied Ca^{2+}-sensing proteins involved in cell signalling, was also recorded in plant tissue after AgNP-treatment [140]. Intracellular changes in Ca^{2+} ions in response to different stimuli detected by calmodulin influence the activities of CaM-binding proteins, which have been implicated in plant adaptation to adverse environmental conditions [156].

Proteomic studies published so far investigated impact of AgNPs mostly on plant seedlings, while studies comparing the effects on young (seedlings) and fully developed (adult) plants are completely lacking, except for our studies on tobacco. Comparing changes in protein expression in tobacco seedlings [75] and adult plants (Peharec Štefanić et al., unpublished) exposed to AgNP-citrate, we found correlation in 37 proteins (25 in leaves, 5 in roots, and 6 in both roots and leaves), majority of which are proteins of carbohydrate and energy metabolism. These results indicate that there are proteins responsive to AgNP-induced toxicity, regardless of the age of the plant age and that exposure to AgNP represents stress factor which induces imbalances in cellular metabolic pathways, especially aerobic respiration and photosynthesis, leading to enhanced ROS formation [145]. However, it is

important to emphasize that the majority of these common proteins were up-regulated in seedlings, but down-regulated in roots and leaves of adult plants, which suggests that the mode of their expression is dependent on plant developmental stage as well as on duration of the exposure (7 days for adult plants vs. 30 days for seedlings from the seed stage). Moreover, more prominent oxidative stress and activation of antioxidant enzymes was found in tobacco seedlings after 30 days of exposure [75] compared to adult plants [74] exposed to AgNPs for 7 days. In the recent study of Galazzi et al. [109] it was reported that after exposure of soybean plants to AgNPs, leaf proteome analysis showed that the majority of the responsive proteins were down-regulated, which corresponded with low values of oxidative stress parameters and activities of antioxidant enzymes. These results indicate that adult plant, which have fully developed root system and leaves, exhibit different response to AgNP-induced stress than the young plants, exposed to the nanosilver from the seed stage. Similar observations were reported for tobacco seedlings and adult plants exposed to heavy metal stress [157,158].

Only three studies published so far have compared changes in plant proteomes after simultaneous exposure of the same plant species to AgNPs and $AgNO_3$ [46,75,109], while one study was conducted on green alga *C. reinhardtii* [142]. Proteomic studies performed on seedlings [46,75] revealed significantly higher number of $AgNO_3$-responsive proteins compared to AgNPs. Vannini et al. [46] found only four proteins in rocket seedlings, exposed to AgNPs and $AgNO_3$ for 5 days, which were common to both treatments, while 18 and 39 proteins were specifically expressed after AgNP- or $AgNO_3$-exposure, respectively and were all mainly up-regulated. Low level of overlap of identified proteins as well as much higher number of responsive proteins found in $AgNO_3$- compared to AgNP-exposed seedlings indicates that AgNPs and $AgNO_3$ cause distinct changes in the proteome of the root cells. However, there is evidence that they share some common mechanisms of action. Namely, both treatments induced the accumulation of proteins related to metabolism of sulphur, an important constituent of many stress-related compounds, such as GSH, cysteine, methionine and thioredoxin. Moreover, both Ag treatments consistently induced two key enzymes in cysteine biosynthesis: *O*-acetylserine(thiol)lyase in $AgNO_3$-treated roots, and cysteine synthase in AgNPs-treated roots. Cysteine can chelate dissolved Ag^+ and alter the surface chemistry, aggregation, and dissolution of zero-valent AgNPs [159]. Moreover, cysteine is a direct coupling step between sulphur and its incorporation into GSH, important in plant stress tolerance to

ROS. In addition, AgNP exposure caused the accumulation of a vitamin-B12-independent methionine synthase isozyme, which is involved in the biosynthesis of the methionine, the sulphur-containing amino acid. These results indicate that in both Ag treatments the metabolism of sulphur not only plays an important role in growth and development of roots, but it is also involved in Ag tolerance. Moreover, both Ag treatments activated some common enzymatic and nonenzymatic pathways of ROS detoxification machinery, including SOD and PRX [46] in order to cope with ROS generation induced by AgNPs and/or Ag^+. On the other hand, in tobacco seedlings treated with the AgNPs and $AgNO_3$ for 30 days, it was found that among 49 identified proteins, only 12 of them exhibited different expression depending on the type of exposure (AgNPs or $AgNO_3$), thus indicating that dissociated Ag could be in large part involved in AgNPs toxicity [75]. The majority of the proteins were up-regulated by both treatments and were those involved in the processes of primary metabolism, indicating enhanced energy requirements to cope with Ag^+ stress. This can be correlated with up-regulation of Hsps and PR proteins, as well as enhanced expression of several proteins involved in defence against oxidative stress (CAT, APX and Fe-SOD) [75], which were activated against silver-induced toxicity. The study conducted on the green alga *C. reinhardtii* revealed that majority of the proteins were up-regulated by both AgNPs and $AgNO_3$, among which the proteins with the highest score were those involved in thiamine biosynthesis, Calvin cycle and photosynthesis [142]. These results also suggest that AgNPs and $AgNO_3$ both tend to regulate metabolism in similar ways, thus suggesting that the toxicity is mainly due to release to free Ag^+. However, no increase in regulation of typical antioxidant enzyme was found in algae; the only identified protein involved in antioxidant system was SOD, which was found to be down-regulated by both treatments compared to the control [142], indicating species-specific differences in response to (nano)silver. In our study on adult tobacco plants treated with AgNPs and $AgNO_3$ for 7 days, AgNP-exposure resulted with somewhat higher number of differentially expressed proteins in roots and leaves compared to treatment with $AgNO_3$, which was more pronounced in leaf compared to root (Peharec Štefanić et al., unpublished). Moreover, a high overlap of differentially expressed proteins between AgNPs and $AgNO_3$ treatments was found in roots; namely, only four out of 29 proteins had different expression level depending on the type of the treatment. On the contrary, in leaf tissue half of the proteins (18 out of 35) exhibited different expression level between AgNPs and $AgNO_3$ exposure, which is more in accordance with findings obtained in rocket [46] and soybean

[109]. In the study by Galazzi et al. [109], in which leaf proteomes of non-transformed (NT) and transformed (T, herbicide tolerant) soybean plants were compared after exposure to AgNPs and $AgNO_3$ for 14 days, it was found that 22 proteins were differentially abundant for NT-AgNP vs. NT-$AgNO_3$ group, while 9 differentially abundant proteins were found for T-AgNP vs. T-$AgNO_3$, which suggested that beside silver form, the difference in plant genome may also affect the interaction between plant and (nano)silver-exposure. Namely, in NT-soybean treatment with $AgNO_3$ had a more negative effect on expression on photosynthesis-related proteins compared to AgNP, although the expression of the majority out of 10 overlapping proteins was more down-regulated by AgNPs; on the contrary, in T-soybean all five overlapping proteins were more repressed by $AgNO_3$ treatment. These data add further evidences that the AgNP effects are not simply due to the release of Ag ions, which is in accordance with results obtained on AgNPs stability analyses.

7. Conclusion

In conclusion, the uptake of AgNPs by plants depends on the size and shape of AgNPs as well as on the exposure concentration, but mechanisms of AgNPs internalization and distribution in plants are not fully understood. Their impact on morphological and physiological features of plants depends on AgNP characteristics, transformation possibilities as well as on the plant species and developmental stage and the way of exposure. Since roots are the first tissue to be in contact with AgNP solution, toxic symptoms appear more frequently in roots than in shoots, although AgNPs also induce morphological modifications in the stem and leaves. The main subcellular targets affected by AgNPs are mitochondria, nucleus and in particular chloroplasts, which is in line with detrimental impact of AgNPs on the structure and function of the photosynthetic apparatus. Moreover, damaged chloroplasts contribute to ROS generation and oxidative stress has an important role in the phytotoxicity of AgNPs. However, the underlying mechanisms of AgNP-mediated ROS production need further investigations. Proteomic analyses indicate that AgNPs predominantly affect proteins related to cell metabolism, stress response and signalling. The question whether phytotoxic effect is specific for nanoparticles or it is the result of the action of Ag^+ released from AgNPs in exposure solution and/or after biotransformation in the cellular structures remains unresolved.

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References

[1] P.C. Ray, H. Yu, P.P. Fu, Toxicity and environmental risks of nanomaterials: challenges and future needs, J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev. 27 (2009) 1–35.

[2] S. Smita, S.K. Gupta, A. Bartonova, M. Dusinska, A.C. Gutleb, Q. Rahman, Nanoparticles in the environment: assessment using the causal diagram approach, Environ. Health 11 (2012) S13.

[3] M. Ahamed, R. Posgai, T.J. Gorey, M. Nielsen, S.M. Hussain, J.J. Rowe, Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila melanogaster*, Toxicol. Appl. Pharmacol. 242 (2010) 263–269.

[4] T.M. Tolaymat, A.M. El Badawy, A. Genaidy, K.G. Scheckel, T.P. Luxton, M. Suidan, An evidence-based environmental perspective of manufactured silver nanoparticle in syntheses and applications: a systematic review and critical appraisal of peer-reviewed scientific papers, Sci. Total Environ. 408 (2010) 999–1006.

[5] A.K. Suresh, D.A. Pelletier, W. Wang, J.W. Moon, B. Gu, N.P. Mortensen, D.P. Allison, D.C. Joy, T.J. Phelps, M.J. Doktycz, Silver nanocrystallites: biofabrication using *Shewanella oneidensis*, and an evaluation of their comparative toxicity on Gram-negative and Gram-positive bacteria, Environ. Sci. Technol. 44 (2010) 5210–5215.

[6] J. Fabrega, J.C. Renshaw, J.R. Lead, Interactions of silver nanoparticles with *Pseudomonas putida* biofilms, Environ. Sci. Technol. 43 (2009) 9004–9009.

[7] R. Foldbjerg, Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549, Arch. Toxicol. 85 (2011) 743–750.

[8] S. Arora, J. Jain, J.M. Rajwade, K.M. Paknikar, Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells, Toxicol. Appl. Pharmacol. 236 (2009) 310–318.

[9] Y.H. Hsin, C.F. Chen, S. Huang, T.S. Shih, P.S. Lai, P.J. Chueh, The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells, Toxicol. Lett. 179 (2008) 130–139.

[10] P. Gopinath, S.K. Gogoi, P. Sanpui, A. Paul, A. Chattopadhyay, S.S. Ghosh, Signaling gene cascade in silver nanoparticle induced apoptosis, Colloids Surf. B. Biointerfaces 77 (2010) 240–245.

[11] E. Navarro, A. Baun, R. Behra, N.B. Hartmann, J. Filser, A.J. Miao, A. Quigg, P.H. Santschi, L. Sigg, Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi, Ecotoxicology 17 (2008) 372–386.

[12] S. Kim, J.E. Choi, J. Choi, K.H. Chung, K. Park, J. Yi, D.Y. Ryu, Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells, Toxicol. In Vitro 23 (2009) 1076–1084.

[13] K. Kawata, M. Osawa, S. Okabe, In vitro toxicity of silver nanoparticles at non-cytotoxic doses to HepG2 human hepatoma cells, Environ. Sci. Technol. 43 (2009) 6046–6051.

[14] B. Reidy, A. Haase, A. Luch, K.A. Dawson, I. Lynch, Mechanisms of silver nanoparticle release, transformation and toxicity: a critical review of current knowledge and recommendations for future studies and applications, Materials (Basel) 6 (2013) 2295–2350.

[15] C.M. Rico, S. Majumdar, M. Duarte-Gardea, J.R. Peralta-Videa, J.L. Gardea-Torresdey, Interaction of nanoparticles with edible plants and their possible implications in the food chain, J. Agric. Food Chem. 59 (2011) 3485–3498.
[16] S.N. Luoma, Silver nanotechnologies and the environment: old problems or new challenges, in: The Project on Emerging Nanotechnologies Report, Woodrow Wilson International Center for Scholars, Washington, D.C., 2008.
[17] B.S. Sekhon, Nanotechnology in agri-food production: an overview, Nanotechnol. Sci. Appl. 20 (2014) 31–53.
[18] A. Servin, W. Elmer, A. Mukherjee, R. De la Torre-Roche, H. Hamidi, J.C. White, P. Bindraban, C. Dimkpa, A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield, J. Nanopart. Res. 17 (2015) 92.
[19] Pallavi, C.M. Mehta, R. Srivastava, S. Arora, A.K. Sharma, Impact assessment of silver nanoparticles on plant growth and soil bacterial diversity, 3 Biotech 6 (2016) 254.
[20] D.K. Tripathi, A. Tripathi, S. Singh, Y. Singh, K. Vishwakarma, G. Yadav, S. Sharma, V.K. Singh, R.K. Mishra, R.G. Upadhyay, N.K. Dubey, Uptake, accumulation and toxicity of silver nanoparticle in autotrophic plants, and heterotrophic microbes: a concentric review, Front. Microbiol. 8 (2017) 7.
[20a] S.K. Verma, A.K. Das, M.K. Patel, A. Shah, V. Kumar, S. Gantait, Engineered nanomaterials for plant growth and development: a prospective analysis, Sci. Total Environ. 630C (2018) 1413–1435.
[21] Y.S. El-Temsah, E.J. Joner, Impact of Fe and Ag nanoparticles on seed germination and differences in bioavailability during exposure in aqueous suspension and soil, Environ. Toxicol. 27 (2012) 42–49.
[22] W.-M. Lee, J.I. Kwak, Y.-J. An, Effect of silver nanoparticles in crop plants *Phaseolus radiatus* and *Sorghum bicolor*: media effect on phytotoxicity, Chemosphere 86 (2012) 491–499.
[23] P. Sharma, D. Bhatt, M.G. Zaidi, P.P. Saradhi, P.K. Khanna, S. Arora, Silver nanoparticle-mediated enhancement in growth and antioxidant status of *Brassica juncea*, Appl. Biochem. Biotechnol. 167 (2012) 2225–2233.
[24] L. Yin, B.P. Colman, B.M. McGill, J.P. Wright, E.S. Bernhardt, Effects of silver nanoparticle exposure on germination and early growth of eleven wetland plants, PLoS One 7 (2012) e47674.
[25] X. Ma, J. Geiser-Lee, Y. Deng, A. Kolmakov, Interactions between engineered nanoparticles (ENPs) and plants: phytotoxicity, uptake and accumulation, Sci. Total Environ. 408 (2010) 3053–3061.
[26] R. Behra, L. Sigg, M.J.D. Clift, F. Herzog, M. Minghetti, B. Johnston, A. Petri-Fink, B. Rothen-Rutishauser, Bioavailability of silver nanoparticles and ions: from a chemical and biochemical perspective, J. R. Soc. Interface 10 (2013) 20130396.
[27] M. Thwala, S.J. Klaine, N. Musee, Interactions of metal-based engineered nanoparticles with aquatic higher plants: a review of the state of current knowledge, Environ. Toxicol. Chem. 35 (2016) 1677–1694.
[28] W. Du, W. Tan, J.R. Peralta-Videa, J.L. Gardea-Torresdey, R. Ji, Y. Yin, H. Guo, Interaction of metal oxide nanoparticles with higher terrestrial plants: physiological and biochemical aspects, Plant Physiol. Biochem. 110 (2017) 210–225.
[29] M. Rizwan, S. Ali, M.F. Qayyum, Y.S. Ok, M. Adrees, M. Ibrahim, M. Zia-ur-Rehman, M. Farid, F. Abbas, Effect of metal and metal oxide nanoparticles on growth and physiology of globally important food crops: a critical review, J. Hazard. Mater. 322 (2017) 2–16.
[30] D.K. Tripathi, S. Singh, S. Singh, R. Pandey, V.P. Singh, N.C. Sharma, S.M. Prasad, N.K. Dubey, D.K. Chauhan, An overview on manufactured nanoparticles in plants: uptake, translocation, accumulation and phytotoxicity, Plant Physiol. Biochem. 110 (2017) 2–12.

[31] X. Ma, J. Yan, Plant uptake and accumulation of engineered metallic nanoparticles from lab to field conditions, Curr. Opin. Environ. Sci. Health 6 (2018) 16–20.
[32] L. Yin, Y. Cheng, B. Espinasse, B.P. Colman, M. Auffan, M. Wiesner, J. Rose, J. Liu, E.S. Bernhardt, More than the ions: the effects of silver nanoparticles on *Lolium multiflorum*, Environ. Sci. Technol. 45 (2011) 2360–2367.
[33] C. Levard, E.M. Hotze, G.V. Lowry, G.E. Brown Jr., Environmental transformations of silver nanoparticles: impact on stability and toxicity, Environ. Sci. Technol. 46 (2012) 6900–6914.
[34] J. Geisler-Lee, Q. Wang, Y. Yao, W. Zhang, M. Geisler, K. Li, Y. Huang, Y. Chen, A. Kolmakov, X. Ma, Phytotoxicity, accumulation and transport of silver nanoparticles by *Arabidopsis thaliana*, Nanotoxicology 7 (2013) 323–337.
[35] J. Wang, Y. Koo, A. Alexander, Y. Yang, S. Westerhof, Q. Zhang, J.L. Schnoor, V.L. Colvin, J. Braam, P.J. Alvarez, Phytostimulation of poplars and Arabidopsis exposed to silver nanoparticles and Ag^{+} at sublethal concentrations, Environ. Sci. Technol. 47 (2013) 5442–5449.
[36] V.K. Sharma, K.M. Siskova, R. Zboril, J.L. Gardea-Torresdey, Organic-coated silver nanoparticles in biological and environmental conditions: fate, stability and toxicity, Adv. Colloid Interface Sci. 204 (2014) 15–34.
[37] M. Thwala, N. Musee, L. Sikhwivhilu, V. Wepener, The oxidative toxicity of Ag and ZnO nanoparticles towards the aquatic plant *Spirodela punctuta* and the role of testing media parameters, Environ. Sci. Process. Impacts 15 (2013) 1830–1843.
[38] R. Barrena, E. Casals, J. Colón, X. Font, A. Sánchez, V. Puntes, Evaluation of the ecotoxicity of model nanoparticles, Chemosphere 75 (2009) 850–857.
[39] A.J. Miao, Z. Luo, C.S. Chen, W.C. Chin, P.H. Santschi, A. Quigg, Intracellular uptake: a possible mechanism for silver engineered nanoparticle toxicity to a freshwater alga *Ochromonas danica*, PLoS One 5 (2010) e15196.
[40] P. Cvjetko, A. Milošić, A.M. Domijan, I. Vinković Vrček, S. Tolić, P. Peharec Štefanić, I. Letofsky-Papst, M. Tkalec, B. Balen, Toxicity of silver ions and differently coated silver nanoparticles in *Allium cepa* roots, Ecotoxicol. Environ. Saf. 137 (2017) 18–28.
[41] X. Zou, P. Li, J. Lou, H. Zhang, Surface coating-modulated toxic responses to silver nanoparticles in *Wolffia globose*, Aquat. Toxicol. 189 (2017) 150–158.
[42] C.O. Dimkpa, J.E. McLean, D.W. Britt, A.J. Anderson, Bioactivity and biomodification of Ag, ZnO, and CuO nanoparticles with relevance to plant performance in agriculture, Ind. Biotechnol. 8 (2012) 344–357.
[43] G.V. Lowry, K.B. Gregory, S.C. Apte, J.R. Lead, Transformations of nanomaterials in the environment, Environ. Sci. Technol. 46 (2012) 6893–6899.
[44] C. Coutris, E.J. Joner, D.H. Oughton, Aging and soil organic matter content affect the fate of silver nanoparticles in soil, Sci. Total Environ. 420 (2012) 327–333.
[45] N.A. Anjum, S.S. Gill, A.C. Duarte, E. Pereira, I. Ahmad, Silver nanoparticles in soil–plant systems, J. Nanopart. Res. 15 (2013) 1896.
[46] C. Vannini, G. Domingo, E. Onelli, B. Prinsi, M. Marsoni, L. Espen, M. Bracale, Morphological and proteomic responses of *Eruca sativa* exposed to silver nanoparticles or silver nitrate, PLoS One 8 (2013) e68752.
[47] C. Vannini, G. Domingo, E. Onelli, F. De Mattia, I. Bruni, M. Marsoni, M. Bracale, Phytotoxic and genotoxic effects of silver nanoparticles exposure on germinating wheat seedlings, J. Plant Physiol. 171 (2014) 1142–1148.
[48] H. Mazumdar, Comparative assessment of the adverse effect of silver nanoparticles to *Vigna radiata* and *Brassica campestris* crop plants, Int. J. Eng. Res. Appl. 4 (2014) 118–124.
[49] A. Gorczyca, E. Pociecha, M. Kasprowicz, M. Niemiec, Effect of nanosilver in wheat seedlings and *Fusarium culmorum* culture systems, Eur. J. Plant Pathol. 142 (2015) 251–261.

[50] K. Vishwakarma, N. Upadhyay, J. Singh, S. Liu, V.P. Singh, S.M. Prasad, D.K. Chauhan, D.K. Tripathi, S. Sharma, Differential phytotoxic impact of plant mediated silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) on *Brassica* sp, Front. Plant Sci. 8 (2017) 1501.
[51] A.K. Patlolla, A. Berry, L. May, P.B. Tchounwou, Genotoxicity of silver nanoparticles in *Vicia faba*: a pilot study on the environmental monitoring of nanoparticles, Int. J. Environ. Res. Public Health 9 (2012) 1649–1662.
[52] L.R. Pokhrel, B. Dubey, Evaluation of developmental responses of two crop plants exposed to silver and zinc oxide nanoparticles, Sci. Total Environ. 452 (2013) 321–332.
[53] H.S. Jiang, M. Li, F.Y. Chang, W. Li, L.Y. Yin, Physiological analysis of silver nanoparticles and $AgNO_3$ toxicity to *Spirodela polyrhiza*, Environ. Toxicol. Chem. 31 (2012) 1880–1886.
[54] C.O. Dimkpa, J.E. McLean, N. Martineau, D.W. Britt, R. Haverkamp, A.J. Anderson, Silver nanoparticles disrupt wheat (*Triticum aestivum* L.) growth in a sand matrix, Environ. Sci. Technol. 47 (2013) 1082–1090.
[55] H.S. Jiang, L.Y. Yin, N.N. Ren, S.T. Zhao, Z. Li, Y. Zhi, H. Shao, W. Li, B. Gontero, Silver nanoparticles induced reactive oxygen species via photosynthetic energy transport imbalance in an aquatic plant, Nanotoxicology 11 (2017) 157–167.
[56] J.P. Stegemeier, B.P. Colman, F. Schwab, M.R. Wiesner, G.V. Lowry, Uptake and distribution of silver in the aquatic plant *Landoltia punctata* (duckweed) exposed to silver and silver sulfide nanoparticles, Environ. Sci. Technol. 51 (2017) 4936–4943.
[57] M. Ke, Q. Qu, W.J.G.M. Peijnenburg, X. Li, M. Zhang, Z. Zhang, T. Lu, X. Pan, H. Qian, Phytotoxic effects of silver nanoparticles and silver ions to *Arabidopsis thaliana* as revealed by analysis of molecular responses and of metabolic pathways, Sci. Total Environ. 644 (2018) 1070–1079.
[58] E.J. Gubbins, L.C. Batty, J.R. Lead, Phytotoxicity of silver nanoparticles to *Lemna minor* L, Environ. Pollut. 159 (2011) 1551–1559.
[59] M. Lundqvist, J. Stigler, T. Cedervall, T. Berggård, M.B. Flanagan, I. Lynch, G. Elia, K. Dawson, The evolution of the protein corona around nanoparticles: a test study, ACS Nano 5 (2011) 7503–7509.
[60] P.M.G. Nair, I.M. Chung, Assessment of silver nanoparticle-induced physiological and molecular changes in *Arabidopsis thaliana*, Environ. Sci. Pollut. Res. 21 (2014) 8858–8869.
[61] E. Navarro, F. Piccapietra, B. Wagner, F. Marconi, R. Kaegi, N. Odzak, L. Sigg, R. Behra, Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*, Environ. Sci. Technol. 42 (2008) 8959–8964.
[62] A. Oukarroum, L. Barhoumi, L. Pirastru, D. Dewez, Silver nanoparticle toxicity effect on growth and cellular viability of the aquatic plant *Lemna gibba*, Environ. Toxicol. Chem. 32 (2013) 902–907.
[63] A.J. Miao, K.A. Schwehr, C. Xu, S.J. Zhang, Z. Luo, A. Quigg, P.H. Santschi, The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances, Environ. Pollut. 157 (2009) 3034–3041.
[64] F. Mirzajani, H. Askari, S. Hamzelou, M. Farzaneh, A. Ghassempour, Effect of silver nanoparticles on *Oryza sativa* L. and its rhizosphere bacteria, Ecotoxicol. Environ. Saf. 88 (2013) 48–54.
[65] P. Das, S. Barua, S. Sarkar, S.K. Chatterjee, S. Mukherjee, L. Goswami, S. Das, S. Bhattacharya, N. Karak, S.S. Bhattacharya, Mechanism of toxicity and transformation of silver nanoparticles: inclusive assessment in earthworm-microbe-soil-plant system, Geoderma 314 (2018) 73–84.
[66] A.H. Jesmer, J.R. Velicogna, D.M. Schwertfeger, R.P. Scroggins, J.I. Princz, The toxicity of silver to soil organisms exposed to silver nanoparticles and silver nitrate in biosolids-amended field soil, Environ. Toxicol. Chem. 36 (2017) 2756–2765.

[67] A. Yan, Z. Chen, Detection methods of nanoparticles in plant tissues, in: Ö. Çelik (Ed.), New Visions in Plant Science, IntechOpen, London, 2018, pp. 84–113.
[68] H. Qian, X. Peng, X. Han, J. Ren, L. Sun, Z. Fu, Comparison of the toxicity of silver nanoparticles and silver ions on the growth of terrestrial plant model *Arabidopsis thaliana*, J. Environ. Sci. 25 (2013) 1947–1956.
[69] J. Yasur, P.U. Rani, Environmental effects of nanosilver: impact on castor seed germination, seedling growth, and plant physiology, Environ. Sci. Pollut. Res. 20 (2013) 8636–8648.
[70] L.V. Antisari, S. Carbone, A. Gatti, G. Vianello, P. Nannipieri, Uptake and translocation of metals and nutrients in tomato grown in soil polluted with metal oxide (CeO_2, Fe_3O_4, SnO_2, TiO_2) or metallic (Ag, Co, Ni) engineered nanoparticles, Environ. Sci. Pollut. Res. 22 (2015) 1841–1853.
[71] D. Stampoulis, S.K. Sinha, J.C. White, Assay-dependent phytotoxicity of nanoparticles to plants, Environ. Sci. Technol. 43 (2009) 9473–9479.
[72] A.E. Pradas del Real, V. Vidal, M. Carrière, H. Castillo-Michel, C. Levard, P. -Chaurand, G. Sarret, Silver nanoparticles and wheat roots: a complex interplay, Environ. Sci. Technol. 51 (2017) 5774–5782.
[73] D.K. Tripathi, S. Singh, S. Singh, P.K. Srivastava, V.P. Singh, S. Singh, S.M. Prasad, P.K. Singh, N.K. Dubey, A.C. Pandey, D.K. Chauhan, Nitric oxide alleviates silver nanoparticles (AgNps)-induced phytotoxicity in *Pisum sativum* seedlings, Plant Physiol. Biochem. 110 (2017) 167–177.
[74] P. Cvjetko, M. Zovko, P. Peharec Štefanić, R. Biba, M. Tkalec, A.M. Domijan, I. -Vinković Vrček, I. Letofsky-Papst, S. Šikić, B. Balen, Phytotoxic effects of silver nanoparticles in tobacco plants, Environ. Sci. Pollut. Res. 25 (2018) 5590–5602.
[75] P. Peharec Štefanić, P. Cvjetko, R. Biba, A.M. Domijan, I. Letofsky-Papst, M. Tkalec, S. Šikić, M. Cindrić, B. Balen, Physiological, ultrastructural and proteomic responses of tobacco seedlings exposed to silver nanoparticles and silver nitrate, Chemosphere 209 (2018) 640–653.
[76] S.A. Blaser, M. Scheringer, M. MacLeod, K. Hungerbuhler, Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles, Sci. Total Environ. 390 (2008) 396–409.
[77] A.E. Pradas del Real, H. Castillo-Michel, R. Kaegi, B. Sinnet, V. Magnin, N. Findling, J. Villanova, M. Carrière, C. Santaella, A. Fernández-Martínez, C. Levard, Fate of Ag-NPs in sewage sludge after application on agricultural soils, Environ. Sci. Technol. 50 (2016) 1759–1768.
[78] H.A. Castillo-Michel, C. Larue, A.E. Pradas del Real, M. Cotte, G. Sarret, Practical review on the use of synchrotron based micro-and nano-X-ray fluorescence mapping and X-ray absorption spectroscopy to investigate the interactions between plants and engineered nanomaterials, Plant Physiol. Biochem. 110 (2017) 13–32.
[79] C. Larue, H. Castillo-Michel, R.J. Stein, B. Fayard, E. Pouyet, J. Villanova, V. -Magnin, A.E.P. del Real, N. Trcera, S. Legros, S. Sorieul, Innovative combination of spectroscopic techniques to reveal nanoparticle fate in a crop plant, Spectrochim. Acta B At. Spectrosc. 119 (2016) 17–24.
[80] J. Geisler-Lee, M. Brooks, J. Gerfen, O. Wang, C. Fotis, A. Sparer, M. Xingmao, B.V. Howard, M. Geisler, Reproductive toxicity and life history study of silver nanoparticle effect uptake and transport in *Arabidopsis thaliana*, Nanomaterials (Basel) 4 (2014) 301–318.
[81] J.P. Stegemeier, F. Schwab, B.P. Colman, S.M. Webb, M. Newville, A. Lanzirotti, C. Winkler, M.R. Wiesner, G.V. Lowry, Speciation matters: bioavailability of silver and silver sulfide nanoparticles to alfalfa (*Medicago sativa*), Environ. Sci. Technol. 49 (2015) 8451–8460.
[82] D. Bao, Z.G. Oh, Z. Chen, Characterization of silver nanoparticles internalized by Arabidopsis plants using single particle ICP-MS analysis, Front. Plant Sci. 7 (2016) 32.

[83] N.C. Carpita, D.M. Gibeaut, Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth, Plant J. 3 (1993) 1–30.
[84] A. Chesson, P.T. Gardner, T.J. Wood, Cell wall porosity and available surface area of wheat straw and wheat grain fractions, J. Sci. Food Agric. 75 (1997) 289–295.
[85] K.J. Dietz, S. Herth, Plant nanotoxicology, Trends Plant Sci. 16 (2011) 582–589.
[86] P. Wang, N.W. Menzies, E. Lombi, B.A. McKenna, M.D. de Jonge, E. Donner, F.P.C. Blamey, C.G. Ryan, D.J. Paterson, D.L. Howard, Quantitative determination of metal and metalloid spatial distribution in hydrated and fresh roots of cowpea using synchrotron-based X-ray fluorescence microscopy, Sci. Total Environ. 463 (2013) 131–139.
[87] F. Schwab, G. Zhai, M. Kern, A. Turner, J.L. Schnoor, M.R. Wiesner, Barriers, pathways and processes for uptake, translocation and accumulation of nanomaterials in plants—critical review, Nanotoxicology 10 (2016) 257–278.
[88] J. Fabrega, S.R. Fawcett, J.C. Renshaw, J.R. Lead, Silver nanoparticle impact on bacterial growth: effect of pH, concentration, and organic matter, Environ. Sci. Technol. 43 (2010) 7285–7290.
[89] G. Martinez-Criado, J. Villanova, R. Tucoulou, D. Salomon, J.P. Suuronen, S. Labouré, C. Guilloud, V. Valls, R. Barrett, E. Gagliardini, Y. Dabin, ID16B: a hard X-ray nanoprobe beamline at the ESRF for nano-analysis, J. Synchrotron Radiat. 23 (2016) 344–352.
[90] A.T. Harris, R. Bali, On the formation and extent of uptake of silver nanoparticles by live plants, J. Nanopart. Res. 10 (2008) 691–695.
[91] S. Wang, J. Lv, J. Ma, S. Zhang, Cellular internalization and intracellular biotransformation of silver nanoparticles in *Chlamydomonas reinhardtii*, Nanotoxicology 10 (2016) 1129–1135.
[92] C. Larue, H. Castillo-Michel, S. Sobanska, L. Cécillon, S. Bureau, V. Barthès, L. Ouerdane, M. Carrière, G. Sarret, Foliar exposure of the crop *Lactuca sativa* to silver nanoparticles: evidence for internalization and changes in Ag speciation, J. Hazard. Mater. 264 (2014) 98–106.
[93] R. Amooaghaie, F. Tabatabaei, A.-M. Ahadi, Role of hematin and sodium nitroprusside in regulating *Brassica nigra* seed germination under nanosilver and silver nitrate stresses, Ecotoxicol. Environ. Saf. 113 (2015) 259–270.
[94] N. Savithramma, S. Ankanna, G. Bhumi, Effect of nanoparticles on seed germination and seedling growth of *Boswellia ovalifoliolata* an endemic and endangered medicinal tree taxon, Nano Vis. 2 (2012) 61–68.
[95] A. Parveen, S. Rao, Effect of nanosilver on seed germination and seedling growth in *Pennisetum glaucum*, J. Clust. Sci. 26 (2015) 693–701.
[96] Z.M. Almutairi, A. Alharbi, Effect of silver nanoparticles on seed germination of crop plants, J. Adv. Agric. 4 (2015) 280–285.
[97] K.A. Fayez, B.A. El-Deeb, N.Y. Mostafa, Toxicity of biosynthetic silver nanoparticles on the growth, cell ultrastructure and physiological activities of barley plant, Acta Physiol. Plant. 39 (2017) 155.
[98] E.A. Abdel-Azeem, B.A. Elsayed, Phytotoxicity of silver nanoparticles on *Vicia faba* seedlings, N. Y. Sci. J. 6 (2013) 148–156.
[99] N. Zuverza-Mena, R. Armendariz, J.R. Peralta-Videa, J.L. Gardea-Torresdey, Effects of silver nanoparticles on radish sprouts: root growth reduction and modifications in the nutritional value, Front. Plant Sci. 7 (2016) 90.
[100] P.M. Nair, I.M. Chung, Physiological and molecular level effects of silver nanoparticles exposure in rice (*Oryza sativa* L.) seedlings, Chemosphere 112 (2014) 105–113.

[101] B.K. McDaniel, B.M. Binder, Ethylene receptor 1 (ETR1) is sufficient and has the predominant role in mediating inhibition of ethylene responses by silver in *Arabidopsis thaliana*, J. Biol. Chem. 287 (2012) 26094–26103.
[102] M. Aleksandrowicz-Trzcinska, A. Szaniawski, M. Studnicki, M. Bederska-Blaszczyk, A. Urban, The effect of silver and copper nanoparticles on the growth and mycorrhizal colonization of Scots pine (*Pinus sylvestris* L.) in a container nursery experiment, iForest 11 (2018) 690–697.
[103] J. Olchowik, R. Bzdyk, M. Studnicki, M. Bederska-Blaszczyk, A. Urban, M. - Aleksandrowicz-Trzcinska, The effects of silver and copper nanoparticles on the condition of English oak (*Quercus robur* L.) seedlings in a container nursery experiment, Forests 8 (2017) 310.
[104] M. Kumari, A. Mukherjee, N. Chandrasekaran, Genotoxicity of silver nanoparticles in *Allium cepa*, Sci. Total Environ. 407 (2009) 5243–5246.
[105] C. Krishnaraj, E.G. Jagan, R. Ramachandran, S.M. Abirami, N. Mohan, P.T. Kalaichelvan, Effect of biologically synthesized silver nanoparticles on *Bacopa monnieri* (Linn.) Wettst. plant growth metabolism, Process Biochem. 47 (2012) 651–658.
[106] H.S. Jiang, X.N. Qiu, G.B. Li, W. Li, L.Y. Yin, Silver nanoparticles induced accumulation of reactive oxygen species and alteration of antioxidant systems in the aquatic plant *Spirodela polyrhiza*, Environ. Toxicol. Chem. 33 (2014) 1398–1405.
[107] J. Yang, W. Cao, Y. Rui, Interactions between nanoparticles and plants: phytotoxicity and defense mechanisms, J. Plant Interact. 12 (2017) 158–169.
[108] K.K. Panda, V.M. Achary, R. Krishnaveni, B.K. Padhi, S.N. Sarangi, S.N. Sahu, B.B. Panda, In vitro biosynthesis and genotoxicity bioassay of silver nanoparticles using plants, Toxicol. In Vitro 25 (5) (2011) 1097–1105.
[109] R.M. Galazzi, C.A. Lopes Júnior, B. de Lima, F.C. Gozzo, M.A.Z. Arruda, Evaluation of some effects on plant metabolism through proteins and enzymes in transgenic and non-transgenic soybeans after cultivation with silver nanoparticles, J. Proteomics 191 (2019) 88–106.
[110] M.B. Homaee, A.A. Ehsanpour, Silver nanoparticles and silver ions: oxidative stress responses and toxicity in potato (*Solanum tuberosum* L.) grown in vitro, Hortic. Environ. Biotechnol. 57 (2016) 544–553.
[111] P. Sharma, A.B. Jha, R.S. Dubey, M. Pessarakli, Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions, J. Bot. 2012 (2012) 217037.
[112] P.M.G. Nair, I.M. Chung, Physiological and molecular level studies on the toxicity of silver nanoparticles in germinating seedlings of mung bean (*Vigna radiata* L.), Acta Physiol. Plant. 37 (2015) 1719.
[113] T. Silva, L.R. Pokhrel, B. Dubey, T.M. Tolaymat, K.J. Maier, X. Liu, Particle size, surface charge and concentration dependent ecotoxicity of three organo-coated silver nanoparticles: comparison between general linear model-predicted and observed toxicity, Sci. Total Environ. 468–469 (2014) 968–976.
[114] G. Šinko, I. Vinković Vrček, W. Goessler, G. Leitinger, A. Dijanošić, S. Miljanić, Alteration of cholinesterase activity as possible mechanism of silver nanoparticle toxicity, Environ. Sci. Pollut. Res. Int. 21 (2014) 1391–1400.
[115] A. Barbasz, B. Kreczmer, M. Oćwieja, Effects of exposure of callus cells of two wheat varieties to silver nanoparticles and silver salt ($AgNO_3$), Acta Physiol. Plant. 38 (2016) 76.
[116] N. Saha, S.D. Gupta, A glimpse on silver nanoparticles genotoxicity in higher plants, Glob. J. Nanomedicine 2 (2017) 555583GJO.MS.ID.555583.
[117] P.U. Rani, J. Yasur, K.S. Loke, D. Duta, Effect of synthetic and biosynthesized silver nanoparticles on growth, physiology and oxidative stress of water hyacinth: *Eichhornia crassipes* (Mart) Solms, Acta Physiol. Plant. 38 (2016) 58.

[118] M. Hatami, M. Ghorbanpour, Effect of nanosilver on physiological performance of *Pelargonium* plants exposed to dark storage, J. Hortic. Res. 21 (2013) 15e20.
[119] E.H. Murchie, T. Lawson, Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications, J. Bot. 64 (2013) 3983–3998.
[120] A.D. Richardson, S.P. Duigan, G.P. Berlyn, An evaluation of noninvasive methods to estimate foliar chlorophyll content, New Phytol. 153 (2002) 185–194.
[121] A. Oukarroum, S. Polchtchikov, F. Perreault, R. Popovic, Temperature influence on silver nanoparticles inhibitory effect on photosystem II photochemistry in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*, Environ. Sci. Pollut. Res. Int. 19 (2012) 1755–1762.
[122] A. Oukarroum, S. Bras, F. Perreault, R. Popovic, Inhibitory effects of silver nanoparticles in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*, Ecotoxicol. Environ. Saf. 78 (2012) 80–85.
[123] P. Pardha-Saradhi, N. Shabnam, P. Sharmila, A.K. Ganguli, H. Kim, Differential sensitivity of light-harnessing photosynthetic events in wheat and sunflower to exogenously applied ionic and nanoparticulate silver, Chemosphere 194 (2018) 340–351.
[124] A. Sosan, D. Svistunenko, D. Straltsova, K. Tsiurkina, I. Smolich, T. Lawson, S. Subramaniam, V. Golovko, D. Anderson, A. Sokolik, I. Colbeck, Engineered silver nanoparticles are sensed at the plasma membrane and dramatically modify the physiology of *Arabidopsis thaliana* plants, Plant J. 85 (2016) 245–257.
[125] G.H. Krause, E. Weis, Chlorophyll fluorescence and photosynthesis: the basics, Annu. Rev. Plant Biol. 42 (1991) 313–349.
[126] R.J. Strasser, M. Tsimilli-Michael, A. Srivastava, Analysis of the chlorophyll a fluorescence transient, in: G.C. Papageorgiou, Govindjee (Eds.), Chlorophyll A Fluorescence, Springer, Dordrecht, 2004, pp. 321–362.
[127] D. Dewez, A. Oukarroum, Silver nanoparticles toxicity effect on photosystem II photochemistry of the green alga *Chlamydomonas reinhardtii* treated in light and dark conditions, Toxicol. Environ. Chem. 94 (2012) 1536–1546.
[128] A. Turner, D. Brice, M.T. Brown, Interactions of silver nanoparticles with the marine macroalga, *Ulva lactuca*, Ecotoxicology 21 (2012) 148–154.
[129] N. Shabnam, P. Sharmila, P. Pardha-Saradhi, Impact of ionic and nanoparticle speciation states of silver on light harnessing photosynthetic events in *Spirodela polyrhiza*, Int. J. Phytoremediation 19 (2017) 80–86.
[130] D.N. Matorin, D.A. Todorenko, N.K. Seifullina, B.K. Zayadan, A.B. Rubin, Effect of silver nanoparticles on the parameters of chlorophyll fluorescence and P 700 reaction in the green alga *Chlamydomonas reinhardtii*, Microbiology 82 (2013) 809–814.
[131] J. Huang, J. Cheng, J. Yi, Impact of silver nanoparticles on marine diatom *Skeletonema costatum*, J. Appl. Toxicol. 36 (2016) 1343–1354.
[132] X. Li, M. Ke, M. Zhang, W.J.G.M. Peijnenburg, X. Fan, J. Xu, Z. Zhang, T. Lu, Z. Fu, H. Qian, The interactive effects of diclofop-methyl and silver nanoparticles on *Arabidopsis thaliana*: growth, photosynthesis and antioxidant system, Environ. Pollut. 232 (2018) 212–219.
[133] W.F. Falco, A.M. Queiroz, J. Fernandes, E.R. Botero, E.A. Falcão, F.E.G. Guimarães, J.C. M'Peko, S.L. Oliveira, I. Colbeck, A.R.L. Caires, Interaction between chlorophyll and silver nanoparticles: a close analysis of chlorophyll fluorescence quenching, J. Photochem. Photobiol. A Chem. 299 (2015) 203–209.
[134] A.M. Queiroz, A.V. Mezacasa, D.E. Graciano, W.F. Falco, J.C. M'Peko, F.E.G. Guimarães, T. Lawson, I. Colbeck, S.L. Oliveira, A.R.L. Caires, Quenching of chlorophyll fluorescence induced by silver nanoparticles, Spectrochim. Acta A Mol. Biomol. Spectrosc. 168 (2016) 73–77.
[135] E. Navarro, B. Wagner, N. Odzak, L. Sigg, R. Behra, Effects of differently coated silver nanoparticles on the photosynthesis of *Chlamydomonas reinhardtii*, Environ. Sci. Technol. 49 (2015) 8041–8047.

[136] Q.S. Xu, J.Z. Hu, K.B. Xie, H.Y. Yang, K.H. Du, G.X. Shi, Accumulation and acute toxicity of silver in *Potamogeton crispus* L, J. Hazard. Mater. 173 (2010) 186–193.
[137] D. He, J.J. Dorantes-Aranda, T.D. Waite, Silver nanoparticle-algae interactions: oxidative dissolution, reactive oxygen species generation and synergistic toxic effects, Environ. Sci. Technol. 46 (2012) 8731–8738.
[138] H. Qian, K. Zhu, H. Lu, M. Lavoie, S. Chen, Z. Zhou, Z. Deng, J. Chen, Z. Fu, Contrasting silver nanoparticle toxicity and detoxification strategies in *Microcystis aeruginosa* and *Chlorella vulgaris*: new insights from proteomic and physiological analyses, Sci. Total Environ. 572 (2016) 1213–1221.
[139] S.P. Gygi, R. Aebersold, Mass spectrometry and proteomics, Curr. Opin. Chem. Biol. 4 (2000) 489–494.
[140] F. Mirzajani, H. Askari, S. Hamzelou, Y. Schober, A. Römpp, A. Ghassempour, B. Spengler, Proteomics study of silver nanoparticles toxicity on *Oryza sativa* L, Ecotoxicol. Environ. Saf. 108 (2014) 335–339.
[141] G. Mustafa, K. Sakata, Z. Hossain, S. Komatsu, Proteomic study on the effects of silver nanoparticles on soybean under flooding stress, J. Proteomics 122 (2015) 100–118.
[142] A.L. Lindgren, The Effects of Silver Nitrate and Silver Nanoparticles on *Chlamydomonas reinhardtii*: A Proteomic Approach, Degree Project for Master of Science in Ecotoxicology, University of Gothenburg, 2014.
[143] K. Lee, D.W. Bae, S.H. Kim, H.J. Han, X. Liu, H.C. Park, C.O. Lim, S.Y. Lee, W.S. Chung, Comparative proteomic analysis of the short-term responses of rice roots and leaves to cadmium, J. Plant Physiol. 167 (2010) 161–168.
[144] M.A. Sirover, On the functional diversity of glyceraldehyde-3-phosphate dehydrogenase: biochemical mechanisms and regulatory control, Biochim. Biophys. Acta 1810 (2011) 741–751.
[145] K. Kosová, P. Vítámvás, M.O. Urban, I.T. Prášil, J. Renaut, Plant abiotic stress proteomics: the major factors determining alterations in cellular proteome, Front. Plant Sci. 9 (2018) 122.
[146] M.H. Al-Whaibi, Plant heat-shock proteins: a mini review, J. King Saud. Univ. Sci. 23 (2011) 139–150.
[147] J.X. Liu, S.H. Howell, bZIP28 and NF-Y transcription factors are activated by ER stress and assemble into a transcriptional complex to regulate stress response genes in Arabidopsis, Plant Cell 22 (2010) 782–796.
[148] W. Ghandour, J.A. Hubbard, J. Deistung, M.N. Hughes, R.K. Poole, The uptake of silver ions by *Escherichia coli* K12: toxic effects and interaction with copper ions, Appl. Microbiol. Biotechnol. 28 (1988) 559–565.
[149] V. Banerjee, K.P. Das, Interaction of silver nanoparticles with proteins: a characteristic protein concentration dependent profile of SPR signal, Colloids Surf. B Biointerfaces 111 (2013) 71–79.
[150] C.T. Wu, G. Leubner-Metzger, F. Meins, K.J. Bradford, Class I beta-1,3-glucanase and chitinase are expressed in the micropylar endosperm of tomato seeds prior to radicle emergence, Plant Physiol. 126 (2001) 1299–1313.
[151] P. Beáta, M. Ildikó, Plant defense against heavy metals: the involvement of pathogenesis-related (PR) proteins. Recent progress in medicinal plants, in: A.S. Awaad, G. Kaushik, J.N. Govil (Eds.), Mechanism and Action of Phytoconstituents, vol. 31, Studium Press (India) Pvt. Ltd., New Delhi, 2011, pp. 179–205.
[152] R. Zavaliev, A. Levy, A. Gera, B.L. Epel, Subcellular dynamics and role of Arabidopsis beta-1,3-glucanases in cell-to-cell movement of tobamoviruses, Mol. Plant Microbe Interact. 26 (2013) 1016–1030.
[153] I. Corrales, C. Poschenrieder, J. Barcelo, Boron-induced amelioration of aluminium toxicity in a monocot and a dicot species, J. Plant Physiol. 165 (2008) 504–513.

[154] K. Kosová, P. Vítámvás, M.O. Urban, M. Klíma, A. Roy, I.T. Prášil, Biological networks underlying abiotic stress tolerance in temperate crops—a proteomic perspective, Int. J. Mol. Sci. 16 (2015) 20913–20942.
[155] K. Kosová, P. Vítámvás, I.T. Prášil, J. Renaut, Plant proteome changes under abiotic stress—contribution of proteomics studies to understanding plant stress response, J. Proteomics 74 (2011) 1301–1322.
[156] K.A. Wilkins, E. Matthus, S.M. Swarbreck, J.M. Davies, Calcium-mediated abiotic stress signaling in roots, Front. Plant Sci. 7 (2016) 1296.
[157] M. Tkalec, P. Peharec Štefanić, P. Cvjetko, S. Šikić, M. Pavlica, B. Balen, The effects of cadmium-zinc interactions on biochemical responses in tobacco seedlings and adult plants, PLoS One 9 (2014) e87582.
[158] K. Majsec, P. Cvjetko, S. Tolić, M. Tkalec, B. Balen, M. Pavlica, Integrative approach gives new insights into combined Cd/Cu exposure in tobacco, Acta Physiol. Plant. 38 (2016) 142.
[159] A.P. Gondikas, A. Morris, B.C. Reinsch, S.M. Marinakos, G.V. Lowry, H. Hsu-Kim, Cysteine-induced modifications of zero-valent silver nanomaterials: implications for particle surface chemistry, aggregation, dissolution, and silver speciation, Environ. Sci. Technol. 46 (2012) 7037–7045.