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ORIGINAL ARTICLE

Endoreduplication changes in five in-vitro-grown crops upon treatment with silver nanoparticles

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Abstract

Silver nanoparticles (AgNPs) are now often utilized in agriculture and horticulture. One of the common applications is to use AgNPs as antimicrobial agents in tissue cultures instead of using standard sterilization procedures. However, in addition to beneficial effects they have also cyto- and genotoxic ones, inducing DNA damage and changing cell cycle dynamics. The aim of this study was to determine the effect of AgNPs at different concentrations (50–100 ppm) on endoreduplication, DNA content, and growth of seedlings of five crops: rapeseed, white mustard, sugar beet, red clover, and alfalfa, cultivated in vitro. Flow cytometry was used to establish genome size and DNA synthesis patterns in the roots, hypocotyls, and leaves of first-leaf-pair seedlings. AgNP-treatment did not influence germination or genome size, but increased root length and endoreduplication intensity. The increases were especially pronounced in species/organs with high polysomaty. We suggest that enhanced endopolyploidization is a defense mechanism against the stress induced by AgNPs causing mitotic division disruption.

Key message

Application of silver nanoparticles at 50–100 ppm in vitro promotes root elongation and endoreduplication, especially in highly polysomatic species. Such treatment does not affect germination or genome size.

Keywords Cell cycle · Cytotoxicity · Endoreduplication · Flow cytometry · Nanosilver · Seedling growth

Introduction

Nanotechnology, commonly used during the last decade in medicine, cosmetic industry, and the production of different household goods, has lately gained attention also in agriculture and horticulture. In crop production, nanoparticles (NPs) are applied to deliver pesticides, fertilizers, and nutrients (for review see Ranjan et al. 2021). They also support sensors used for real-time monitoring of crops, soil, and the environment, enabling precision farming. Moreover, due to the antimicrobial activity of NPs, such as those of silver, gold, platinum, zinc or copper, they are successfully used for controlling bacterial and fungal contamination in tissue cultures (e.g., Mahna et al. 2013; Taghizadeh et al. 2014; Álvarez et al. 2019; Moradpour et al. 2016; Parzymies et al. 2019). NPs can also be used as carriers in plant transformation as well as for enhancing seed germination and plant bioactive compound production (Álvarez et al. 2019).

Nevertheless, NPs must be used with caution because of their genotoxic and cytotoxic activity, especially at high concentrations (> 50–100 mg L^{-1}). Genotoxicity of NPs can result from physical interactions with DNA, the reduction of cell DNA repair ability or from increased production of reactive oxygen species (ROS), causing antioxidant depletion and gene expression alterations (Wang et al. 2013). NPs can also be toxic to plants due to their chemical and physical characteristics, e.g., they can associate with cell structures,

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including proteins, or fill pores, making them unavailable for transport (Dietz and Herth 2011). There are numerous papers reporting the effect of NPs on the mitotic index (MI), the formation of chromosomal and nuclear aberrations, and cell death (Ghosh et al. 2019; Ranjan et al. 2021).

The effect of NPs on plants is largely dependent on the physicochemical properties, including chemical composition, concentration, and size. The concentration of nanoparticles affects the response of plants to the presence of NPs; with increasing concentration the toxicity enhances, which is due to the increased accumulation of NPs in plant tissues and oxidative stress. The concentration-dependent effect is also connected to the treatment procedure and plant growth stage (Wang et al. 2022). On the other hand, the toxicity of NPs depends on their size; the toxicity of small-sized AgNPs is much higher than that of large-sized particles. For example, cytotoxic and genotoxic effects in *Allium cepa* increases with the reduction of the particle diameter (Scherer et al. 2019). This is because small particles have a larger specific surface, and also they can easier pass through the cell membrane (Nie et al. 2023).

Silver NPs (AgNPs) constitute about 25% of the total nanomaterial-based products, including those used in agriculture (Ranjan et al. 2021). They can enhance seed germination, promote root and shoot growth as well as increase metabolite production (for review see Mahajan et al. 2022). Due to the antibacterial and antifungal properties of silver, AgNPs at the concentration 10–250 mg L^{-1}, mostly spherical in shape, are commonly used for sterilization in tissue cultures (Mahna et al. 2013; Taghizadeh et al. 2014; Moradpour et al. 2016; Parzymies et al. 2019). However, as with other NPs, AgNPs have both beneficial and phytotoxic effects. Studies using *A. cepa* as a model system (Allium test) for studying genotoxicity in plants revealed that AgNPs of 24–100 nm at 50–100 mg L^{-1} cause DNA damage, cell death, an increased frequency of cells with micronuclei, a decrease in MI, disturbed metaphase, and chromosomal breaks and bridges (Kumari et al. 2009; Panda et al. 2011; Sobieh et al. 2016). Similar effects were observed after AgNP-treatment (particles of 20–65 nm) at 25–100 mg L^{-1} of *Vicia faba* roots (Patlolla et al. 2012; Abdel-Azeem and Elsayed 2013). Such cytotoxic effects of AgNPs of 30 nm could lead to perturbances in cell cycle dynamics. Although analyses have yet to be reported using AgNPs, the application of titanium dioxide NPs at 5–150 mg L^{-1} to wheat seedlings induces cell cycle arrest at the G_0/G_1 phase (an accumulation of 2 C cells; Silva et al. 2016). But, when ZnO NPs of 75–85 nm (mostly cuboidal to hexagonal–cuboidal in shape) at 200–800 mg L^{-1} are applied to *A. cepa* roots they cause the arrest of cells at the G_2/M checkpoint (an increased proportion of 4C cells), which may be a plant strategy to cope with DNA damage by providing time for its repair (Ghosh et al. 2016).

Disturbances in mitosis in response to NPs treatments suggest that they may have an effect on a modified cell cycle called endoreduplication. This process, common in the angiosperms, involves DNA amplification that is not followed by mitosis, resulting in endopolyploid cells (4C → 8C → 16C → 32C → 64C, and so on; Nagl 1976a, b). Because enhanced cell ploidy usually coincides with an increase in the size of a cell, endoreduplication is responsible for cell expansion, and as such can be a compensation mechanism for the lack of growth by cell division. It is tissue- and species-specific and typically occurs in differentiating cells no longer associated with the mitotic cycle. Endoreduplication can also be induced by biotic or abiotic stresses (Lang and Schnittger 2020; Qi and Zang 2020). In endopolyploid cells gene expression is enhanced, and metabolic activity increases. Molecular components of the mitotic cycle and endoreduplication are closely connected, e.g., CDKB1 and A2-type cyclins, which are responsible for mitosis, are repressed in endoreduplicating cells, and the inhibition of mitosis often affects endoreduplication intensity. Thus, our research is on the effect of NPs on endoreduplication in plants which, to the best of our knowledge, has not been studied previously.

Flow cytometry (FCM) is a unique and convenient method to study endoreduplication. It allows an accurate detection of nuclei with different DNA contents (ploidy) within a plant/plant organ (Sliwinska et al. 2022). There are numerous reports on the determination of the intensity of endoreduplication in different organs of wild plant species as well as crops using FCM (e.g., Galbraith et al. 1991; Lukaszewska and Sliwinska 2007; Bainard et al. 2012; Sliwinska et al. 2015; Ducár et al. 2018), therefore the method was applied to the present study.

In this research, five polysomatic (possessing mitotic as well as endopolyploid cells) crop species were selected and the endoreduplication intensity in the roots, hypocotyls, and leaves of seedlings exposed to AgNPs and $AgNO_3$ in vitro was analyzed by FCM. Additionally, the nuclear DNA content in the leaves and the length of the roots and hypocotyls of these seedlings was established. The aim of the study is to determine changes in the DNA synthesis pattern (cell cycle and endoreduplication) and seedling growth upon treatment with AgNPs at different concentrations. This information can be used in in vitro cultures and can be a basis for optimizing crop production in which NPs are utilized.

Materials and methods

Plant material

Seeds of rapeseed (*Brassica napus* L. var. *napus*) cv. Osorno, white mustard (*Sinapis alba* L.) cv. Rodena, sugar beet (*Beta*

vulgaris L.) cv. Elvira, red clover (*Trifolium pratense* L.) cv. Viola, and alfalfa (*Medicago sativa* L.) cv. Radius were used as plant material. Seeds were sterilized 1 min in 70% (v/v) ethanol, followed by 12 min in 1.5% (v/v) commercial sodium hypochlorite solution (Chemia, Bydgoszcz, Poland), and then washed three times in double-distilled sterile water. Sterilized seeds were placed in 350 mL jars (10 seeds per jar) containing 50 mL of half-strength MS medium (Murashige and Skoog 1962). The medium contained 2.2 g L^{-1} MS basal medium (Sigma Aldrich, Saint Louis, USA) supplemented with 15 g L^{-1} sucrose (Chempur, Piekary Śląskie, Poland), and was solidified with 7 g L^{-1} agar (Vitro LAB-AGAR, Biocorp, Warsaw, Poland). The pH of the media was adjusted to 5.8 prior to autoclaving. Cultures were maintained at 24 ± 1°C in a growth chamber with light intensity 40 µmol photons m^{-2} s^{-1} (L36W/77 Fluora lamps, OSRAM, Munich, Germany) and 16/8 h light/darkness photoperiod.

Treatments

The treatment solutions were applied to the surface of the medium (1 mL per jar) using a sterile pipette before sowing the seeds in the following conditions: (i) no treatment (MS); (ii) solution used by the producer (Nanoparticles Innovation NPIN s.c., Łódź, Poland) for nanosilver suspension preparation (S); it did not contain AgNPs; this treatment was used to enable detecting the possible effect of the solution itself on germination, seedling growth, and DNA content and synthesis pattern; (iii) silver nitrate ($AgNO_3$; Sigma Aldrich, Saint Louis, USA) at 50, 75, and 100 ppm; (iv) AgNPs solution (Nanoparticles Innovation NPIN s.c., Łódź, Poland) at 50, 75, and 100 ppm. Spherical AgNPs of 20 ± 3 nm diameter (as measured by scanning transmission electron microscopy, Nova NanoSEM 450, FEI, accelerating voltage of 30 kV) stabilized with citric acid were used. According to the manufacturer, the AgNPs were produced by the seeded-mediated growth method. The synthesis was set to obtain the final concentration of AgNPs at 100 ppm. Before application, a nanoparticle solution was exposed to sonication for 5 min using an ultrasonic cleaner ("Ultron" Zakład Urządzeń Elektronicznych, Dywity near Olsztyn, Poland) to prevent AgNPs aggregation. Treatments with S and $AgNO_3$ were considered as additional controls to MS, aiming to eliminate the possible effect of the solution used to suspend the AgNPs and of the silver ions, respectively, to allow capturing the specific effect of AgNPs on studied seed and seedlings parameters.

Seed germination and morphological characteristics of the seedlings

Germination percentage for each species was established 7 days after sowing the seeds on the medium and incubating them at the conditions described above for growing seedlings; seeds with a visible radicle were counted. For each species and treatment 100 seeds (germinated in ten jars) were tested.

When the seedlings reached the first-leaf-pair stage they were removed from the medium and before exposing them to flow cytometric analysis the length of the roots and the hypocotyls was measured. For each species and treatment ten seedlings were analyzed.

Flow cytometry (FCM)

Estimation of genome size

Leaves of first-leaf-pair seedlings were used for cytometric estimation of nuclear DNA content. The following internal standards were applied: for rapeseed and white mustard, *Vicia villosa* cv. Minikowska (2C = 3.32 pg; Dzialuk et al. 2007); for sugar beet, *Raphanus sativus* cv. Saxa (2C = 1.11 pg; Doležel et al. 1994); for red clover, *Petunia hybrida* PxPc6 (2C = 2.85 pg; Marie and Brown 1993); and for alfalfa, *Zea mays* CE-777 (2C = 5.43 pg; Lysak et al. 1998). Samples were prepared as previously described (Sliwinska and Thiem 2007) using nuclei isolation buffer (0.1 M Tris; 2.5 mM $MgCl_2 \cdot 6H_2O$; 85 mM NaCl; 0.1% v/v Triton X-100; pH 7.0) supplemented with propidium iodide (PI; 50 µg mL^{-1}) and ribonuclease A (50 µg mL^{-1}). For each sample, about 5000 nuclei were analyzed, using a CyFlow Ploidy Analyzer flow cytometer (Sysmex Partec GmbH, Görlitz, Germany); a linear signal amplification was applied. Analyses were performed on ten biological replicates. Histograms were analyzed using a CyView 1.6 computer program (Sysmex Partec GmbH, Görlitz, Germany). The coefficient of variation (CV) of the G_0/G_1 peak of a sample species ranged between 3.50 and 5.73%. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of a sample species/internal standard on a histogram of fluorescence intensities.

Estimation of endoreduplication intensity

Endoreduplication was established in the roots, hypocotyls, and leaves of first-leaf-pair seedlings using the same sample preparation procedure and cytometer as for genome size estimation. Instead of an internal standardization, an external standardization was applied to establish the position of 2C peak on an FCM histogram; for each species, the young leaf of this species was used as an external standard. To fit peaks with high DNA content within the histogram, logarithmic signal amplification was used and the number of analyzed nuclei was enhanced to about 10,000. Analyses were performed on ten biological replicates. After evaluation of the histograms using a CyView 1.6 computer program,

the following measures were applied: the number of endocycles, the percentage of nuclei with different DNA contents, the mean C-value, the (Σ > 2C)/2C ratio, and the super cycle value (SCV); for details of the measures see Sliwinska et al. (2022).

In this study, nuclei having at least 8C DNA were considered to be endopolyploid, since it is not possible to distinguish by FCM the 4C nuclei originated from the cells that have just entered endoreduplication (i.e., in the G_1 phase of the first endocycle) from those originated from the cells in the G_2 phase of the mitotic cycle.

Statistical analysis

The results were analyzed statistically using a one-way analysis of variance (ANOVA) and Duncan's test ($P = 0.05$). Analyses were conducted employing STATISTICA v.13.3 (StatSoft, Poland). The percentage data from the germination tests were subjected to ANOVA after angular transformation, although actual percentages are presented in Fig. S1.

Results

Seed germination and morphological characteristics of the seedlings

Germination of the seeds varied between 84.5 and 98%, depending on the species (Fig. S1). There were no significant effects on germination of any of the treatments in any of the studied species.

AgNPs, especially when applied at high concentrations, increased the length of the roots of all species except alfalfa (Fig. 1). This increase was particularly apparent in white mustard seedlings after application of AgNPs at 100 ppm; their roots were longer by over 1.5-fold than those of the seedlings grown on MS medium alone (5.8 cm v. 9.1 cm). AgNPs already at 50 ppm caused significant elongation of the roots of this species (by 40%).

AgNPs did not have an impact on the length of the hypocotyls of four out of the five species; only the hypocotyls of white mustard seedlings exposed to AgNPs at 100 ppm were shorter (by about 30%) than in seedlings grown on MS medium.

Genome size and endoreduplication intensity

The 2C DNA content (genome size) in the seedlings grown on MS medium alone was: rapeseed 2.256 pg, white mustard 1.061 pg, sugar beet 1.473 pg, red clover 1.181 pg, and alfalfa 3.646 pg (Table S1). The values estimated here fall within the range of genome sizes for these species previously published in the Kew Plant DNA C-values Database (Leitch et al. 2019). None of the treatments significantly changed the genome size of any of the investigated species.

All studied species were polysomatic, i.e., endoreduplicated nuclei (8C, 16C, 32C) occurred in their organs in addition to mitotic ones (2C and 4C) (Figs. S2–S7). However, the endopolyploidy level varied, depending on the species and organ. The nuclei with the highest endopolyploidy, 32C (three endocycles), occurred in the roots of white mustard and in the hypocotyls of rapeseed, white mustard, and sugar beet. No more than two endocycles (endopolyploid nuclei with 8C and 16C DNA) occurred in the roots of rapeseed and sugar beet and in the hypocotyls of alfalfa. No more than one endocycle (endopolyploid nuclei with 8C DNA) took place in the roots and the hypocotyls of red clover and roots of alfalfa. No endopolyploid nuclei were detected in the leaves of red clover and alfalfa; only mitotic nuclei, possessing 2C and 4C DNA, were present. In the leaves of the remaining species one endocycle occurred, resulting in the presence of 0.3–2.9% 8C nuclei.

Although the number of endocycles did not change in the treated plants, the intensity of endoreduplication was affected by AgNPs; however, this effect was negligible in the organs of alfalfa (Tables 1, 2, 3, 4, 5). Generally, as revealed by the three measures, the mean C-value, the (Σ > 2C)/2C ratio, and the SCV, AgNPs increased endoreduplication. Nevertheless, the extent of this effect varied depending on the species and organ. In most cases (except those mentioned below) $AgNO_3$ did not affect endoreduplication intensity. Also, no impact of the solution for AgNPs suspension without AgNPs (S) on endoreduplication was found.

In the roots and the hypocotyls of rapeseed seedlings, all three of the above measures indicated enhanced endoreduplication intensity, regardless of the AgNPs concentration (Table 1). In the leaves of this species, although the same tendency occurred for the first two measures, only the highest dose of AgNPs increased their values significantly as compared to all controls. As revealed by SCV, the application of $AgNO_3$ caused a decrease in endoreduplication intensity in the leaves in comparison with the MS control and all other treatments.

In white mustard, AgNPs increased endoreduplication intensity in the roots and the hypocotyls even at the lowest concentration (except for SCV in the hypocotyls), while in the leaves the application of AgNPs at 75 and 100 ppm increased the measures significantly (Table 2). In the roots and the hypocotyls of the seedlings treated with $AgNO_3$ at 75 and 100 ppm the (Σ > 2C)/2C ratio was lower than in the non-treated seedlings (MS).

In sugar beet the positive relationship between endoreduplication and the application of AgNPs was obvious in the roots and the hypocotyls (Table 3); however, not in the leaves. In this organ, only the mean C-value and the (Σ > 2C)/2C ratio in the seedlings treated with the highest

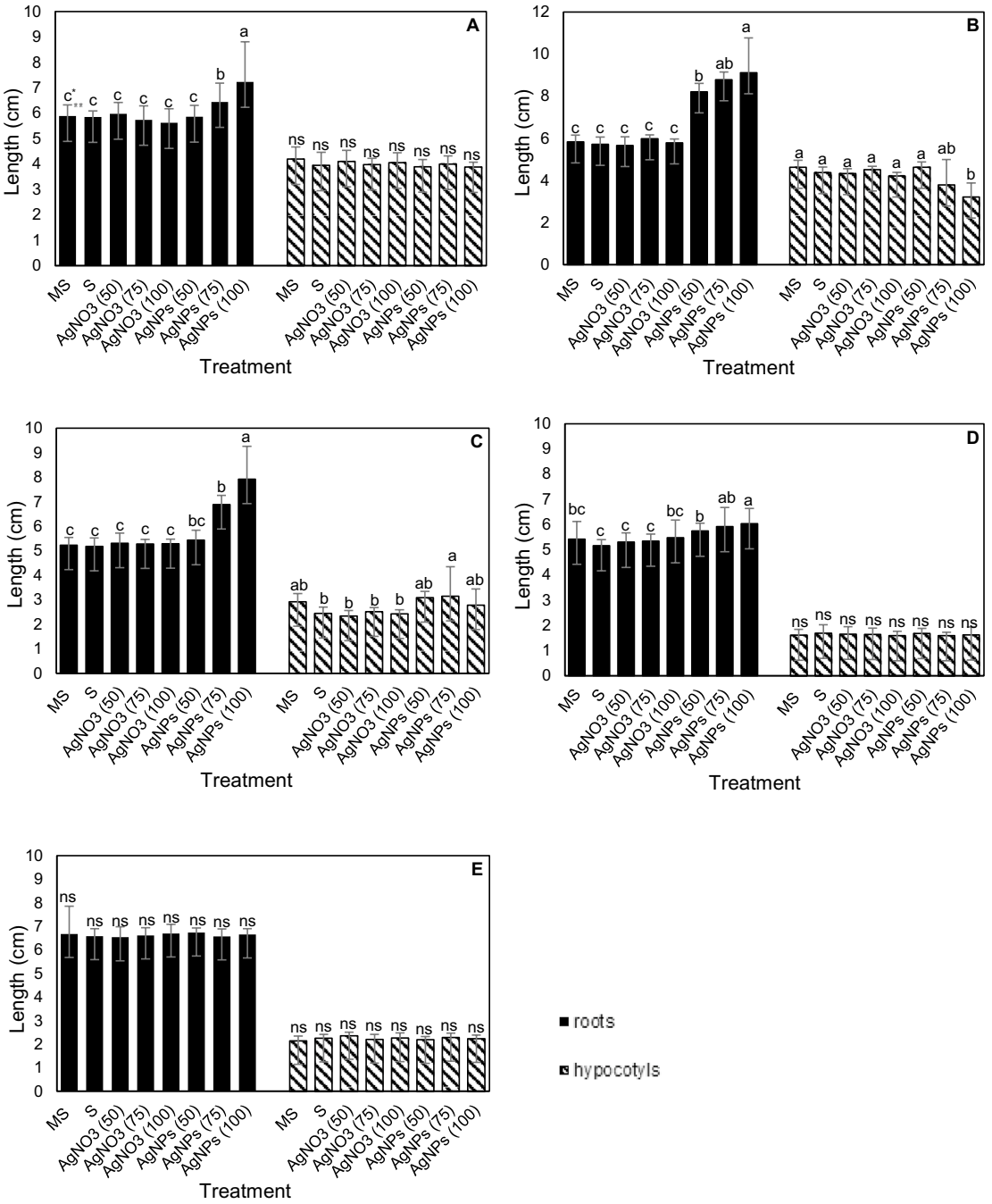

Fig. 1 Length of the roots and hypocotyls of seedlings of five crop species grown in vitro and treated with $AgNO_3$ and silver nanoparticles (AgNPs) at 50, 75, and 100 ppm. **A** rapeseed; **B** white mustard; **C** sugar beet; **D** red clover; **E** alfalfa. *MS* Murashige and Skoog medium, *S* the solution used for AgNPs suspension preparation. ∗Values for particular species and organ corresponding to the bars marked with the same letter do not differ significantly at $P = 0.05$ (Duncan's test); *ns* values corresponding to the bars do not differ significantly

dose of AgNPs were higher than in the seedlings grown on MS alone, but at the same time they did not differ from those in the seedlings treated with $AgNO_3$ at 50 ppm.

A different pattern of the effect of AgNPs on endoreduplication was observed in the seedlings of red clover, where endoreduplication intensity was lower than in the other three species (Table 4). Although the mean C-value (and in the case of the roots also SCV) confirmed the positive effect of AgNPs on endoreduplication intensity in the roots and the leaves at all concentrations, in the hypocotyls this value was not significantly different for all applied treatments. The same tendency was indicated by the $(\Sigma > 2C)/2C$ ratio;

Table 1 Measures of endoreduplication intensity in different organs of the seedlings of rapeseed grown in vitro and treated with $AgNO_3$ and silver nanoparticles (AgNPs) at different concentrations (50, 75, and 100 ppm)

Treatment	Organ	No. of endocycles	Mean C-value		$(\sum > 2C)/2C$		SCV	
MS	Root	2	5.076 ± 0.174	b*	2.569 ± 0.319	c	0.355 ± 0.030	b
S		2	5.099 ± 0.216	b	2.650 ± 0.328	c	0.377 ± 0.048	b
$AgNO_{3\,(50)}$		2	5.094 ± 0.196	b	2.488 ± 0.285	c	0.369 ± 0.043	b
$AgNO_{3\,(75)}$		2	5.045 ± 0.311	b	2.467 ± 0.480	c	0.355 ± 0.062	b
$AgNO_{3\,(100)}$		2	5.135 ± 0.137	b	2.678 ± 0.161	c	0.376 ± 0.031	b
$AgNPs_{\,(50)}$		2	5.602 ± 0.250	a	3.171 ± 0.416	b	0.453 ± 0.040	a
$AgNPs_{\,(75)}$		2	5.488 ± 0.340	a	3.272 ± 0.229	b	0.427 ± 0.056	a
$AgNPs_{\,(100)}$		2	5.706 ± 0.251	a	3.659 ± 0.334	a	0.451 ± 0.049	a
MS	Hypocotyl	3	5.334 ± 0.364	b	1.834 ± 0.391	b	0.405 ± 0.064	b
S		3	5.332 ± 0.387	b	1.713 ± 0.335	b	0.407 ± 0.064	b
$AgNO_{3\,(50)}$		3	5.251 ± 0.371	b	1.624 ± 0.243	b	0.391 ± 0.055	b
$AgNO_{3\,(75)}$		3	5.171 ± 0.371	b	1.799 ± 0.332	b	0.382 ± 0.063	b
$AgNO_{3\,(100)}$		3	5.340 ± 0.247	b	1.739 ± 0.188	b	0.400 ± 0.037	b
$AgNPs_{\,(50)}$		3	5.827 ± 0.248	a	2.349 ± 0.394	a	0.467 ± 0.037	a
$AgNPs_{\,(75)}$		3	5.931 ± 0.257	a	2.390 ± 0.382	a	0.487 ± 0.036	a
$AgNPs_{\,(100)}$		3	6.023 ± 0.450	a	2.502 ± 0.367	a	0.504 ± 0.067	a
MS	Leaf	1	2.316 ± 0.103	bc	0.167 ± 0.078	bcd	0.009 ± 0.002	ab
S		1	2.276 ± 0.084	c	0.136 ± 0.045	d	0.010 ± 0.005	ab
$AgNO_{3\,(50)}$		1	2.279 ± 0.061	c	0.148 ± 0.035	bcd	0.006 ± 0.002	c
$AgNO_{3\,(75)}$		1	2.280 ± 0.041	c	0.149 ± 0.024	bcd	0.006 ± 0.002	c
$AgNO_{3\,(100)}$		1	2.259 ± 0.076	c	0.139 ± 0.051	cd	0.005 ± 0.001	c
$AgNPs_{\,(50)}$		1	2.370 ± 0.066	ab	0.204 ± 0.049	abc	0.008 ± 0.002	b
$AgNPs_{\,(75)}$		1	2.385 ± 0.115	ab	0.210 ± 0.082	ab	0.011 ± 0.003	a
$AgNPs_{\,(100)}$		1	2.436 ± 0.141	a	0.255 ± 0.121	a	0.010 ± 0.002	ab

MS Murashige and Skoog medium, *S* the solution for AgNPs suspension preparation, *SCV* super cycle value

*Values for the particular organ (in columns) followed by the same letter do not differ significantly at $P = 0.05$ (Duncan's test)

however, its increase was statistically proved only for the highest dose of AgNPs.

Applied treatments did not affect endoreduplication in the hypocotyls and the leaves of alfalfa, the species also characterized by low endoreduplication (Table 5). Most of the measures revealed no significant differences between the treatments. Only in the roots of this species did the SCV value indicate higher endoreduplication intensity in the seedlings treated with AgNPs at 50 ppm.

Discussion

AgNPs are currently often used to agriculture and horticulture; however, their influence on germination, growth, and plant development is not always clear. Both beneficial and toxic effects have been reported, especially related to the phytotoxicity of AgNPs at different doses in various crops. Because of the ambiguous results of the previous studies on plant response to AgNP-treatment, in the present research five important for agriculture plant species, rapeseed, mustard, sugar beet, red clover, and alfalfa, were selected and changes in their DNA synthesis patterns (cell cycle and endoreduplication intensity) and growth of cultured in vitro seedlings upon treatment with AgNPs at three concentrations were studied. In addition, an impact of AgNPs on seed germination and genome size was established.

Seed germination is an essential prelude for successful crop establishment. Therefore, a huge effort is made by seed companies to enhance it; its decrease upon any treatment is highly undesirable. The use of AgNPs during germination is beneficial, neutral, or inhibitory, dependent mostly on their dose. For example, AgNPs at 50 mg L^{-1} improve germination percentage of *Brassica juncea* and *B. nigra*, while at higher dosages (100–1600 mg L^{-1}) there is reduced germination (Sharma et al. 2012; Amooaghaie et al. 2015). AgNPs increase germination of *Boswellia ovalifoliolata* seeds, but even at low concentrations they negatively affect germination of fenugreek and tobacco (Savithramma et al. 2012; Hojjat and Hojjat 2015; Biba et al. 2020). However,

Table 2 Measures of endoreduplication intensity in different organs of the seedlings of white mustard grown in vitro and treated with $AgNO_3$ and silver nanoparticles (AgNPs) at different concentrations (50, 75, and 100 ppm)

Treatment	Organ	No. of endocycles	Mean C-value		(∑ > 2C)/2C		SCV	
MS	Root	3	7.038 ± 0.353	c*	4.387 ± 0.244	b	0.625 ± 0.056	b
S		3	6.901 ± 0.436	c	4.240 ± 0.452	bc	0.622 ± 0.078	b
$AgNO_{3\ (50)}$		3	7.043 ± 0.258	c	4.061 ± 0.283	bc	0.634 ± 0.040	b
$AgNO_{3\ (75)}$		3	7.074 ± 0.346	c	3.961 ± 0.293	c	0.636 ± 0.055	b
$AgNO_{3\ (100)}$		3	6.998 ± 0.293	c	4.025 ± 0.166	c	0.617 ± 0.045	b
$AgNPs_{\ (50)}$		3	7.485 ± 0.228	b	5.376 ± 0.372	a	0.700 ± 0.042	a
$AgNPs_{\ (75)}$		3	7.714 ± 0.323	ab	5.359 ± 0.357	a	0.729 ± 0.054	a
$AgNPs_{\ (100)}$		3	7.792 ± 0.288	a	5.344 ± 0.550	a	0.741 ± 0.046	a
MS	Hypocotyl	3	5.993 ± 0.185	c	4.545 ± 0.506	c	0.456 ± 0.038	ab
S		3	5.947 ± 0.300	c	4.479 ± 0.128	cd	0.448 ± 0.052	ab
$AgNO_{3\ (50)}$		3	6.005 ± 0.303	bc	4.132 ± 0.192	cd	0.470 ± 0.045	ab
$AgNO_{3\ (75)}$		3	5.987 ± 0.282	c	4.031 ± 0.453	d	0.464 ± 0.044	ab
$AgNO_{3\ (100)}$		3	5.889 ± 0.456	c	4.020 ± 0.570	d	0.428 ± 0.077	b
$AgNPs_{\ (50)}$		3	6.273 ± 0.227	ab	5.509 ± 0.503	b	0.490 ± 0.038	a
$AgNPs_{\ (75)}$		3	6.280 ± 0.243	ab	5.512 ± 0.574	b	0.496 ± 0.050	a
$AgNPs_{\ (100)}$		3	6.353 ± 0.207	a	6.247 ± 0.594	a	0.498 ± 0.041	a
MS	Leaf	1	2.318 ± 0.055	d	0.166 ± 0.042	d	0.009 ± 0.004	e
S		1	2.317 ± 0.044	d	0.182 ± 0.026	cd	0.003 ± 0.004	de
$AgNO_{3\ (50)}$		1	2.319 ± 0.031	d	0.164 ± 0.018	d	0.010 ± 0.003	cde
$AgNO_{3\ (75)}$		1	2.358 ± 0.071	cd	0.189 ± 0.048	cd	0.011 ± 0.002	bcd
$AgNO_{3\ (100)}$		1	2.347 ± 0.085	d	0.183 ± 0.053	cd	0.010 ± 0.003	bcde
$AgNPs_{\ (50)}$		1	2.440 ± 0.036	bc	0.230 ± 0.021	bc	0.017 ± 0.003	bc
$AgNPs_{\ (75)}$		1	2.493 ± 0.077	ab	0.269 ± 0.050	ab	0.018 ± 0.006	ab
$AgNPs_{\ (100)}$		1	2.568 ± 0.211	a	0.315 ± 0.134	a	0.025 ± 0.020	a

MS Murashige and Skoog medium, *S* the solution for AgNPs suspension preparation, *SCV* super cycle value

*Values for the particular organ (in columns) followed by the same letter do not differ significantly at $P = 0.05$ (Duncan's test)

no changes in germination percentage occur when seeds of faba bean, tomato, kale, and radish are treated with AgNPs at different concentrations (Abdel-Azeem and Elsayed 2013; Zuverza-Mena et al. 2016; Tymoszuk 2021). In our studies germination percentage remained the same for all species, regardless of the treatment, which confirmed the lack of impact on these of Ag^+ or AgNPs at 50–100 ppm. Since subsequent growth of the seedling in the present and previous studies was nonetheless affected by AgNPs it can be assumed that either the seed coat constituted an effective barrier to them or, even if they penetrated the seed, they require a longer time than germination per se to have been effective.

It is known that metal NPs in the soil/substrate can enter root tip tissues through the rhizodermis or apoplast and be transported through the symplast to the other plant organs, affecting their development (Szöllösi et al. 2020). As in germination, AgNPs promote or inhibit plant growth, which is species- and dose-dependent, but also is determined by phytohormonal regulation (Sharma et al. 2012). AgNPs at high doses inhibit root growth of such species as radish, tobacco, *Arabidopsis thaliana*, and *Physalis peruviana* (Qian et al. 2013; Zuverza-Mena et al. 2016; Timoteo et al. 2019; Biba et al. 2020). Growth of both roots and shoots of *B. juncea* is inhibited by 200 mg L^{-1} AgNPs, but at lower concentrations (25–100 mg L^{-1}) those organs increase in length (Sharma et al. 2012); there is a similar effect of AgNPs on root and shoot length of fenugreek (Hojjat and Hojjat 2015). The present study confirms the beneficial effects of AgNPs on root length of four out of five species; only alfalfa did not respond to the treatment. In contrast, the growth of hypocotyls of most of the species was not affected, probably because AgNPs enter this organ later than into the roots, i.e., when they have already grown. Nevertheless, the highest dose of the AgNPs inhibited growth of white mustard hypocotyls.

Plant growth occurs as a result of the mitotic cycle (an increase in cell number) and endoreduplication (an increase in cell volume; cell expansion). Therefore, to understand NPs effect on plant growth studying of those processes is necessary. The impact of AgNPs on the mitotic cycle is well

Table 3 Measures of endoreduplication intensity in different organs of the seedlings of sugar beet grown in vitro and treated with $AgNO_3$ and silver nanoparticles (AgNPs) at different concentrations (50, 75, and 100 ppm)

Treatment	Organ	No. of endocycles	Mean C-value		$(\sum > 2C)/2C$		SCV	
MS	Root	2	4.667 ± 0.179	c*	3.210 ± 0.155	c	0.274 ± 0.038	b
S		2	4.657 ± 0.251	c	3.267 ± 0.448	c	0.262 ± 0.048	b
$AgNO_{3\ (50)}$		2	4.668 ± 0.229	c	3.288 ± 0.383	c	0.278 ± 0.049	b
$AgNO_{3\ (75)}$		2	4.727 ± 0.188	c	3.389 ± 0.182	bc	0.281 ± 0.045	b
$AgNO_{3\ (100)}$		2	4.681 ± 0.165	c	3.318 ± 0.114	c	0.272 ± 0.036	b
$AgNPs_{\ (50)}$		2	4.937 ± 0.154	b	3.623 ± 0.342	ab	0.323 ± 0.031	a
$AgNPs_{\ (75)}$		2	4.965 ± 0.151	b	3.737 ± 0.404	b	0.325 ± 0.035	a
$AgNPs_{\ (100)}$		2	5.149 ± 0.267	a	3.833 ± 0.300	a	0.360 ± 0.058	a
MS	Hypocotyl	3	5.945 ± 0.195	b	3.185 ± 0.449	b	0.458 ± 0.029	b
S		3	5.971 ± 0.244	b	3.120 ± 0.421	b	0.457 ± 0.033	b
$AgNO_{3\ (50)}$		3	6.098 ± 0.356	b	3.093 ± 0.300	b	0.475 ± 0.053	b
$AgNO_{3\ (75)}$		3	6.108 ± 0.068	b	3.051 ± 0.222	b	0.483 ± 0.010	b
$AgNO_{3\ (100)}$		3	6.000 ± 0.364	b	2.967 ± 0.213	b	0.463 ± 0.058	b
$AgNPs_{\ (50)}$		3	6.625 ± 0.174	a	3.701 ± 0.300	a	0.563 ± 0.037	a
$AgNPs_{\ (75)}$		3	6.672 ± 0.289	a	3.767 ± 0.400	a	0.562 ± 0.050	a
$AgNPs_{\ (100)}$		3	6.703 ± 0.189	a	4.015 ± 0.361	a	0.574 ± 0.033	a
MS	Leaf	1	2.567 ± 0.175	b	0.344 ± 0.158	bc	0.018 ± 0.010	ab
S		1	2.581 ± 0.231	ab	0.344 ± 0.176	bc	0.023 ± 0.013	ab
$AgNO_{3\ (50)}$		1	2.589 ± 0.039	ab	0.367 ± 0.044	abc	0.013 ± 0.005	b
$AgNO_{3\ (75)}$		1	2.570 ± 0.089	b	0.346 ± 0.073	bc	0.015 ± 0.004	b
$AgNO_{3\ (100)}$		1	2.556 ± 0.064	b	0.328 ± 0.059	c	0.016 ± 0.003	b
$AgNPs_{\ (50)}$		1	2.685 ± 0.196	ab	0.406 ± 0.117	abc	0.029 ± 0.028	a
$AgNPs_{\ (75)}$		1	2.700 ± 0.141	ab	0.446 ± 0.117	ab	0.023 ± 0.011	ab
$AgNPs_{\ (100)}$		1	2.718 ± 0.065	a	0.463 ± 0.081	a	0.022 ± 0.008	ab

MS Murashige and Skoog medium, *S* the solution for AgNPs suspension preparation, *SCV* super cycle value

*Values for the particular organ (in columns) followed by the same letter do not differ significantly at $P = 0.05$ (Duncan's test)

recognized. Only their application at low concentrations (5 and 50 mg L^{-1}) in Allium test promoted mitosis (increased MI; Prokhorova et al. 2013), while in other studies they disrupted the normal dynamics of the cell cycle by causing chromosomal aberrations, arresting cells in the G_1 or G_2 phase or slowing the progression from DNA synthesis to nuclei/cell division, resulting in the decrease of MI or even DNA degradation and cell death (Kumari et al. 2009; Panda et al. 2011; Sobieh et al. 2016; Patlolla et al. 2012; Abdel-Azeem and Elsayed 2013; Ghosh et al. 2019). AgNP-treatment can be regarded as a stress factor, leading to the question as to what defense mechanism a plant can activate in response. Since AgNPs seem to disturb mitotic cycle regulation at the molecular level, it can be hypothesized that they trigger DNA synthesis without mitosis, i.e., endoreduplication. Although the role of endoreduplication in stress responses is not quite clear, an increase in its intensity occurs under stresses such as exposure to UV irradiation, salinity, or heavy metals (Adachi et al. 2011; Barkla et al. 2018; Hendrix et al. 2018; Matsuda et al. 2018). Endoreduplication is induced by DNA double-strand breaks caused by irradiation, suggesting that plants have evolved a distinct strategy to sustain growth under genotoxic stress (Adachi et al. 2011; Matsuda et al. 2018). Such a strategy could be also activated as a response to DNA damage by NPs. Moreover, studies by Bhosale et al. (2018) suggest that endoreduplication triggers in particular the expression of cell-wall-related genes, and fortified walls could be a stronger barrier to NPs than those of cells of lower ploidy. Present results confirm that endoreduplication is an AgNP-stress response. In four out of five species studied here, endoreduplication was enhanced in seedlings treated with AgNPs. This was especially evident in the roots and hypocotyls of the highly polysomatic species rapeseed and sugar beet, where the application of AgNPs at the highest concentrations increased the proportion of endopolyploid cells by 7–8%. This increase was also substantial in all organs of white mustard: 6% in the roots, 3% in the hypocotyls, and 2% in leaves. Red clover, which is a species characterized by low endopolyploidy (only 8C nuclei with a low proportion 1–3% in the roots and hypocotyls)

Table 4 Measures of endoreduplication intensity in different organs of the seedlings of red clover grown in vitro and treated with $AgNO_3$ and silver nanoparticles (AgNPs) at different concentrations (50, 75, and 100 ppm)

Treatment	Organ	No. of endocycles	Mean C-value		$(\sum > 2C)/2C$		SCV	
MS	Root	1	2.588 ± 0.079	b*	0.381 ± 0.077	bc	0.010 ± 0.004	b
S		1	2.586 ± 0.078	b	0.370 ± 0.072	bc	0.012 ± 0.002	b
$AgNO_{3\ (50)}$		1	2.587 ± 0.049	b	0.369 ± 0.042	bc	0.012 ± 0.003	b
$AgNO_{3\ (75)}$		1	2.588 ± 0.077	b	0.382 ± 0.065	bc	0.010 ± 0.004	b
$AgNO_{3\ (100)}$		1	2.548 ± 0.120	b	0.342 ± 0.106	c	0.011 ± 0.006	b
$AgNPs_{\ (50)}$		1	2.695 ± 0.044	a	0.428 ± 0.046	ab	0.024 ± 0.005	a
$AgNPs_{\ (75)}$		1	2.697 ± 0.098	a	0.427 ± 0.077	ab	0.026 ± 0.010	a
$AgNPs_{\ (100)}$		1	2.737 ± 0.125	a	0.481 ± 0.122	a	0.024 ± 0.009	a
MS	Hypocotyl	1	2.922 ± 0.136	ns	0.722 ± 0.173	ns	0.024 ± 0.007	bc
S		1	2.918 ± 0.077		0.721 ± 0.078		0.021 ± 0.007	bc
$AgNO_{3\ (50)}$		1	2.923 ± 0.147		0.723 ± 0.146		0.023 ± 0.011	bc
$AgNO_{3\ (75)}$		1	2.907 ± 0.114		0.718 ± 0.156		0.020 ± 0.006	c
$AgNO_{3\ (100)}$		1	2.931 ± 0.126		0.708 ± 0.138		0.027 ± 0.009	abc
$AgNPs_{\ (50)}$		1	2.936 ± 0.070		0.709 ± 0.091		0.027 ± 0.007	abc
$AgNPs_{\ (75)}$		1	2.946 ± 0.108		0.704 ± 0.131		0.032 ± 0.009	ab
$AgNPs_{\ (100)}$		1	2.969 ± 0.186		0.704 ± 0.160		0.038 ± 0.022	a
MS	Leaf	0	2.080 ± 0.016	b	0.042 ± 0.009	c	0.000 ± 0.000	ns
S		0	2.080 ± 0.012	b	0.042 ± 0.006	c	0.000 ± 0.000	
$AgNO_{3\ (50)}$		0	2.082 ± 0.015	b	0.043 ± 0.008	c	0.000 ± 0.000	
$AgNO_{3\ (75)}$		0	2.081 ± 0.017	b	0.042 ± 0.010	c	0.000 ± 0.000	
$AgNO_{3\ (100)}$		0	2.084 ± 0.012	b	0.044 ± 0.006	bc	0.000 ± 0.000	
$AgNPs_{\ (50)}$		0	2.110 ± 0.036	a	0.059 ± 0.020	ab	0.000 ± 0.000	
$AgNPs_{\ (75)}$		0	2.112 ± 0.024	a	0.059 ± 0.013	ab	0.000 ± 0.000	
$AgNPs_{\ (100)}$		0	2.126 ± 0.058	a	0.068 ± 0.035	a	0.000 ± 0.000	

MS Murashige and Skoog medium, *S* the solution for AgNPs suspension preparation, *SCV* super cycle value

ns—Values for the particular organ (in columns) do not differ significantly (Duncan's test, $P = 0.05$)

*Values for the particular organ (in columns) followed by the same letter do not differ significantly at $P = 0.05$ (Duncan's test)

responded with only a slight increase of endoreplication intensity, while there was no response in any of the organs of alfalfa. This corresponds with the lack of the response in the growth of the roots and hypocotyls upon AgNP-treatment of these two perennial species (only red clover roots slightly elongated when high doses of AgNPs were applied). Moreover, alfalfa cv. Radius used here is, according to the breeder's information, highly resistant to abiotic stresses, which can explain its undisturbed growth.

The only organs studied here where endoreduplication did not occur were leaves of red clover and alfalfa, which made possible to conclude on the influence of AgNPs on the cell cycle. As FCM analyses revealed, in the leaves of red clover AgNPs application increased the proportion of 4C nuclei from 4% in the control seedlings to 5.5–6%, depending on dose, in the treated ones. This confirms the arrest of the cell cycle in the G_2 phase caused by AgNPs in some cells. In the leaves of alfalfa cell cycle activity was stable regardless of the treatment, which again confirmed its tolerance to stress.

In the present research treatment with $AgNO_3$ at the same concentrations as AgNPs was used to check if the Ag^+ ions (which are also present in the suspension of AgNPs) applied alone affect studied processes. Previous research has shown that Ag^+ ions are released from the AgNPs and cause a toxicity (for review see Navarro et al. 2008). However, in some studies Ag^+ ion concentration after AgNPs treatment is low, thus additional toxicity of AgNPs-cell interaction is suggested. In the Allium test, where different forms of silver were used, AgNPs application caused less cytotoxicity and greater genotoxicity than Ag + ions alone (Panda et al. 2011). In *Arabidopsis* AgNPs are more toxic than Ag^+ ions alone (Qian et al. 2013). Here, no significant effect of the application of $AgNO_3$ on plant growth and endoreduplication occurred, which confirms that observed changes in growth and endoreduplication upon AgNP-treatment are the effects of AgNPs themselves.

Some studies suggest that environmental stress can induce changes in nuclear DNA content. For example,

Table 5 Measures of endoreduplication intensity in different organs of the seedlings of alfalfa grown in vitro and treated with $AgNO_3$ and silver nanoparticles (AgNPs) at different concentrations (50, 75, and 100 ppm)

Treatment	Organ	No. of endocycles	Mean C-value		$(\sum > 2C)/2C$		SCV	
MS	Root	1	3.491 ± 0.219	ab*	1.430 ± 0.349	ns	0.082 ± 0.032	b
S		1	3.496 ± 0.114	ab	1.479 ± 0.108		0.076 ± 0.031	b
$AgNO_{3\ (50)}$		1	3.451 ± 0.116	b	1.460 ± 0.257		0.068 ± 0.030	b
$AgNO_{3\ (75)}$		1	3.503 ± 0.124	ab	1.371 ± 0.168		0.088 ± 0.026	b
$AgNO_{3\ (100)}$		1	3.524 ± 0.229	ab	1.421 ± 0.277		0.090 ± 0.040	b
$AgNPs_{\ (50)}$		1	3.641 ± 0.250	a	1.365 ± 0.339		0.122 ± 0.046	a
$AgNPs_{\ (75)}$		1	3.521 ± 0.119	ab	1.361 ± 0.158		0.090 ± 0.019	b
$AgNPs_{\ (100)}$		1	3.524 ± 0.116	ab	1.464 ± 0.329		0.087 ± 0.014	b
MS	Hypocotyl	2	3.697 ± 0.058	ns	0.719 ± 0.068	ns	0.184 ± 0.012	ns
S		2	3.669 ± 0.204		0.726 ± 0.063		0.181 ± 0.033	
$AgNO_{3\ (50)}$		2	3.592 ± 0.222		0.772 ± 0.115		0.164 ± 0.034	
$AgNO_{3\ (75)}$		2	3.567 ± 0.197		0.725 ± 0.073		0.158 ± 0.033	
$AgNO_{3\ (100)}$		2	3.592 ± 0.364		0.725 ± 0.185		0.167 ± 0.051	
$AgNPs_{\ (50)}$		2	3.648 ± 0.080		0.775 ± 0.059		0.175 ± 0.015	
$AgNPs_{\ (75)}$		2	3.685 ± 0.096		0.771 ± 0.058		0.179 ± 0.012	
$AgNPs_{\ (100)}$		2	3.600 ± 0.038		0.747 ± 0.063		0.168 ± 0.005	
MS	Leaf	0	2.180 ± 0.071	ns	0.101 ± 0.043	ns	0.000 ± 0.000	ns
S		0	2.166 ± 0.070		0.092 ± 0.043		0.000 ± 0.000	
$AgNO_{3\ (50)}$		0	2.172 ± 0.087		0.097 ± 0.054		0.000 ± 0.000	
$AgNO_{3\ (75)}$		0	2.162 ± 0.055		0.089 ± 0.033		0.000 ± 0.000	
$AgNO_{3\ (100)}$		0	2.168 ± 0.074		0.094 ± 0.046		0.000 ± 0.000	
$AgNPs_{\ (50)}$		0	2.168 ± 0.053		0.092 ± 0.031		0.000 ± 0.000	
$AgNPs_{\ (75)}$		0	2.185 ± 0.028		0.102 ± 0.017		0.000 ± 0.000	
$AgNPs_{\ (100)}$		0	2.190 ± 0.032		0.105 ± 0.019		0.000 ± 0.000	

MS Murashige and Skoog medium, *S* the solution for AgNPs suspension preparation. *SCV* super cycle value

ns—Values for the particular organ (in columns) do not differ significantly (Duncan's test, $P = 0.05$)

*Values for the particular organ (in columns) followed by the same letter do not differ significantly at $P = 0.05$ (Duncan's test)

the application in vitro of the heavy metal, zinc, causes a decrease in 2C DNA content in *Viola arvensis* regenerants (Sychta et al. 2020). Small variations in genome size were also found in wheat seedlings upon the treatment with titanium dioxide NPs; however, these did not exceed the range of normal intraspecific variation (Silva et al. 2016). In the present research AgNPs did not change the genome size of any of the five crops, which confirms no genotoxic effect of AgNPs at 50–100 ppm.

Conclusions

AgNP-treatment in vitro increases endoreduplication in seedlings of rapeseed, white mustard, sugar beet, red clover, and alfalfa, although this effect is more evident in the species/organs with high endopolyploidy. This increase coincides with the longer roots, but in most of the species AgNPs do not affect the length of the hypocotyls at the first-leaf-pair stage of seedling development. At the concentrations used here (50–100 ppm) AgNPs do not affect seed germination or genome size. Thus, they not only have no phytotoxic effect but also promote cell expansion in roots, which can be beneficial for in vitro culture. However, the possibility of negative effect of AgNPs on plants when applied at higher concentrations cannot be excluded. Therefore, the application of AgNPs in tissue cultures can be recommended, but the treatment should be optimized for each plant material.

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to writing of the manuscript; KB: performing the experiments and evaluation of FCM histograms; ES: contribution to establishing the experimental design, supervising the experiments, writing drafts and final version of the manuscript.

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