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The effects of silver nanoparticles synthesized with an aqueous extract of *Agrimonia eupatoria* L. on winter wheat and barley varieties

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Abstract: Silver nanoparticles represent a potential solution for mitigating the negative effects of temperature stress on cereals. This study investigates the impact of silver nanoparticles on winter varieties of wheat and barley during the tillering phase, focusing on proline concentration, antioxidant activity and extract yield under winter field conditions. Silver nanoparticles (AgNPs) were synthesized using a green method with an aqueous extract of the plant *Agrimonia eupatoria* L. (fam. Rosaceae). Two winter cereal varieties, Simonida (*Triticum aestivum* L.) and Nonius (*Hordeum vulgare* L.), were foliar treated with 5 and 10 mg/mL AgNPs–H_2O. The experiment lasted for 10 days, during which the minimum recorded temperature was –7 °C under field conditions. The proline concentration was increased in both varieties treated with nanoparticles compared to the controls. Antioxidant activity was assessed using the DPPH method for both treated and untreated samples, with ascorbic acid used as a positive control. Antioxidant activity has increased in all treated samples compared to the untreated samples. Only specific concentrations of AgNPs–H_2O increased the extract yield. Based on these results, our study emphasizes the potential of AgNPs–H_2O to improve the tolerance of winter cereals to low temperatures.

Keywords: green synthesis; cereals; proline; antioxidant activity; low temperatures.

INTRODUCTION

Temperature changes, whether in the form of increases or decreases, affect the rate of plant development, with temperature stress disrupting cellular

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metabolism and functionality. Such stress induces morphological, physiological and biochemical changes, depending on the plant species and the duration of stress exposure.[1] Low temperatures (< 0 °C) represent temperature stress, while the acclimatization of winter cereal varieties begins at temperatures below 10 °C. Low temperatures, as stress factors, cause yield loss through a reduction in the number of productive tillers, spikes and grains, resulting in shorter stems, smaller leaf area and decreased photosynthesis. Tillering in cereals is a growth and development phenophase during which secondary shoots are formed at the tillering node. The rate and intensity of tillering largely depend on climatic conditions. The optimal temperature for the tillering phase is 15–17 °C. When temperatures are lower, tillering slows down, and it ceases below 6 °C. During the tillering phase, winter cereal varieties can be sensitive to low temperatures, depending on their intensity and duration. Under stressful conditions, one of the most detrimental effects on plants is oxidative stress, which leads to the formation of reactive oxygen species, causing protein and nucleic acid damage, lipid oxidation, cell membrane damage and ultimately the inhibition of plant growth and development, which may result in the plants' inability to survive.[2–4]

Silver nanoparticles (AgNPs) are a type of metal nanoparticles with unique biological, chemical and physical characteristics, such as: catalytic activity, chemical stability, high electrical conductivity, specific optical and thermal properties.[5] The application of silver nanoparticles in agriculture holds multiple significances, including their use as nanofertilizers,[6] nanopesticides,[7] nanobiosensors and nanometeorological instruments,[8] for improving soil properties,[9] as growth stimulators and as agents for fruit ripening.[10] Silver nanoparticles enhance yield, antioxidant activity and proline content in cereals under cold stress conditions.[11,12]

The synthesis of AgNPs, as well as other nanomaterials, can be physical, chemical or biological. Biological synthesis is also referred to as green synthesis.[13] The green synthesis of silver nanoparticles offers ecological advantages over chemical and physical methods. These methods are simple, environmentally friendly and suitable for commercial applications, as they do not require high energy consumption, high temperatures, pressures or toxic chemicals.[14] The synthesis consists of three steps: extraction, the use of reducing agents and the application of nontoxic materials. Biological methods can yield nanoparticles of specific sizes and shapes, which is one of the most important requirements in synthesis. Green synthesis of silver nanoparticles utilizes molecules derived from biological systems such as plants, microorganisms, fungi and algae.[15] Molecules obtained from extraction from biological systems, such as phenols, terpenoids, amino acids, vitamins, polysaccharides, proteins, enzymes, tannins, alkaloids and alcohol compounds, are important as reducing and stabilizing agents.[16] Silver

nanoparticles obtained through green synthesis, in contrast to chemical synthesis, exhibit long-lasting antibacterial effects and lower phytotoxicity.[17]

In our study, an aqueous extract of the plant *Agrimonia eupatoria* L. (fam. Rosaceae) was used to synthesize nanoparticles. In addition to its antioxidant and antibacterial properties, this plant possesses anti-inflammatory, neuroprotective, antidiabetic, hepatoprotective and anticancer properties.[18] Due to its high content of bioactive compounds, *A. eupatoria* has exceptional reducing ability, which is a crucial step in the synthesis of metallic nanoparticles. Furthermore, Marković *et al*.[19] identified optimal conditions for the synthesis of silver nanoparticles using this plant in their study, which further supports its selection.

Various strategies have been employed to overcome the negative effects of stress: selection of tolerant genotypes, application of different plant growth regulators and use of organic fertilizers. Species and varieties that can tolerate stress, combined with nanotechnology in agriculture, could be an effective strategy for achieving sustainable production and increasing yields under stress conditions.[20,21]

The aim of this research is to investigate the effect of silver nanoparticles on winter varieties of wheat and barley during the tillering phase, focusing on increasing resistance to low temperatures by analysing proline content, antioxidant activity and extract yield.

EXPERIMENTAL

Chemicals

Silver nitrate ($AgNO_3$, Sigma Aldrich). 1,1-Diphenyl-2-picrylhydrazyl (DPPH, Tokyo Chemical Industry, Tokyo). L-Ascorbic acid ($C_6H_7O_6Na$, Carl Roth GmbH, Germany). Methanol (CH_3OH, Zorka, Serbia). Ninhydrin and orthophosphoric acid ($C_9H_6O_4$ and H_3PO_4, Centrohem, Serbia). Glacial acetic acid, toluene and sulfosalicylic acid (CH_3COOH, C_7H_8 and $C_7H_6O_6S$, Hemos, Serbia). The solutions and chemicals were of analytical grade.

Preparation of plant aqueous extract for the synthesis of silver nanoparticles

The aqueous extract of the plant *A. eupatoria* was prepared using Muruzović *et al*[18] method. Dried and powdered plant material weighing 60 g was immersed in 800 mL of distilled water and left at room temperature for 24 h. The plant material was soaked with the same amount of distilled water and filtered every 24 h, three times. The obtained filtrate was collected and then dried using a rotary evaporator (DLAB, RE 100 S) at 40 °C. The dried extracts were subsequently stored in a refrigerator at 4 °C.

Green synthesis of silver nanoparticles

The synthesis of silver nanoparticles was carried out according to the Marković *et al*.[19] method $AgNO_3$ was used as the silver source to produce AgNPs, while the aqueous extract of *A. eupatoria* served for the reduction and stabilization of silver ions (the color change from light yellow to dark brown confirmed the synthesis). $AgNO_3$ was dissolved at a concentration of 5 mM, and the reaction was performed at 25 °C, pH 4, using 1 % plant extract and stirred for 3 h on a magnetic stirrer (magnetic stirrer MSH 300). After synthesis, the suspension was centrifuged at 4500 rpm for 20 min (Centric 150). After centrifugation, the supernatant was

removed and the precipitated nanoparticles were dried at 40 °C and stored at 4 °C. The synthesis of silver nanoparticles was monitored spectrophotometrically at wavelengths ranging from 200 to 800 nm. The characterization of AgNPs–H_2O, including transmission electron microscopy (TEM), UV–Vis spectrophotometry and FTIR spectroscopy was described previously by Marković *et al*.[19]

Growing conditions for cereals, treatment and sampling

Two varieties of winter cereals were analyzed: wheat (*Triticum aestivum* L.), variety Simonida and barley (*Hordeum vulgare* L.) variety Nonius. These varieties are known for their resistance to low temperatures and considering the variability of these factors, it is important to investigate their additional resistance through the application of silver nanoparticles. The experiment was conducted over a period of 10 days in an experimental field of the Agricultural Advisory Service in Kragujevac (44º10′00″N, 20º58′00″E) during the 2023/2024 growing season. Meteorological data on minimum and maximum temperatures were collected through daily measurements. Each variety was sown on an experimental plot of 9 m^2, with a sowing density of 500 seeds per m^2. The experiment was conducted in three replications. One replication was 3 m^2, with each square meter representing different growth conditions for the plants. Within each variety, one square was a control, the second was treated with 5 mg/mL and the third square was treated with 10 mg/mL. The treatment was applied foliarly at concentrations of 5 and 10 mg/mL AgNPs–H_2O. After ten days of the treatment, the aboveground parts of the cereals were collected and transported to the laboratory in liquid nitrogen. In the laboratory, the plant material was macerated using the Muruzović *et al*. method, employing a methanolic solvent. The obtained filtrate was collected and dried in a rotary evaporator at 40 °C (DLAB, RE 100 S). The extracts were then stored in a refrigerator at 4 °C. This procedure was used to prepare extracts that were analyzed for proline content, antioxidant activity and extract yield.

Preparation of extracts for determining the extract yield from wheat and barley samples

The extract yield was calculated for all samples collected. Methanol was used in the maceration process to determine the extract yield. For each sample, 5 g of dried and ground aboveground plant material stems and leaves (LSA) was used.

Determination of proline

The proline content was determined spectrophotometrically using Bates *et al*[22] method. The plant extract was homogenized in a porcelain mortar with a 3 % solution of sulfosalicylic acid, after which the homogenate was filtered. Ninhydrin reagent and glacial acetic acid were added to the filtrate. The mixture was then incubated at 100 °C for 1 h. The reaction was interrupted by transferring the test tubes to ice and then toluene was added while stirring. After separating the toluene phase from the aqueous layer, the toluene phase containing proline was taken for absorbance measurement spectrophotometrically at a wavelength of 520 nm (UV-5100B spectrophotometer). Pure toluene was used as a blank. The proline concentration was determined from a standard curve prepared with known concentrations of proline, using the same method as for the samples. The proline concentration was expressed in μmol/g of extract. Each sample was measured in three replicates.

Determination of antioxidant activity

The determination of antioxidant activity was conducted using the DPPH method according to the description by Kumarasamy *et al*.[23] The dry extract was dissolved in methanol (1000 μg/mL), after which a series of double dilutions was prepared. To each diluted sample

of 2 mL, 2 mL of 40 mM DPPH solution was added and allowed to stand in the dark for 30 min at room temperature. After that, the absorbance was measured at 517 nm using a UV--5100B spectrophotometer. A control with methanol, instead of the sample, was prepared in parallel. Ascorbic acid was used as the standard. All samples and controls were tested in three replicates. Based on the obtained results, the percentage inhibition of DPPH radicals and the IC_{50} value were determined. The inhibition assessment was calculated using the following equation:

$$\text{Inhibition (\%)} = 100((A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}) \quad (1)$$

where A_{control} is the absorbance of the control sample and A_{sample} the absorbance of the extract. The IC_{50} value is the effective concentration at which 50 % of the DPPH radicals are neutralized. This value was obtained from the graph of neutralization activity (%) versus sample concentration, as described in detail in the work by Comic *et al*.[24]

Statistical analysis

Statistical analysis of the data was performed using Excel software (build 16.0.17328.20124, version 2402) and SPSS software (IBM SPSS Statistics, version 26). The obtained mean values of proline concentration and mean values of antioxidant activity expressed as IC_{50} for DPPH were analyzed using two-way analysis of variance (ANOVA) with a significance threshold of $p \leq 0.05$. This test evaluate whether there are statistically significant differences between the analyzed groups.

RESULTS AND DISCUSSION

Based on the characterization of the nanoparticles at the Institute of Nuclear Sciences Vinča, the nanoparticles exhibit isometric morphology and a uniform size distribution (average diameter of 35±1 nm), as confirmed by transmission electron microscopy (TEM) and high-resolution TEM (HRTEM) analyses. The use of scanning transmission electron microscopy (STEM) with high-angle annular dark-field (HAADF) imaging and energy-dispersive spectroscopy (EDS) confirms the crystalline nature of AgNPs. Fourier transform infrared spectroscopy (FTIR) analysis identifies identical functional groups in the plant extracts and corresponding AgNPs, indicating the role of phytochemicals in the reduction of silver ions. Spectrophotometric monitoring of the synthesis process, influenced by various parameters, provides insights into the kinetics and optimal conditions for the formation of AgNP–H_2O Marković *et al*.[19]

During the ten-day experiment, daily temperatures ranged from –7 to 12 °C, Fig 1. Temperature, as a negative abiotic factor affecting cereals, has primarily been studied in the context of high temperatures and heat stress. There is a substantial body of research addressing the various effects of silver nanoparticles under heat stress conditions.[25,26] However, it is interesting to note that there is limited research examining the impact of silver nanoparticles on cereals in the early developmental stages exposed to low temperatures.

The highest proline concentration was recorded in the barley samples treated with a concentration of 5 mg/mL AgNPs–H_2O (1.213 μmol/g), followed by the wheat samples treated with a concentration of 5 mg/mL AgNPs–H_2O (1.17

μmol/g). The lowest concentrations were recorded in the control samples of wheat (1.074 μmol/g) and barley (1.031 μmol/g), as shown in Fig. 2. The wheat and barley samples treated with the concentration of 5 mg/mL AgNPs–H_2O had higher proline concentrations than those treated with the concentration of 10 mg/mL AgNPs–H_2O. Control samples of wheat and barley exhibited the lowest proline concentrations compared to both treatments with 5 and 10 mg/mL AgNPs–H_2O. Overall, the proline concentration was higher in the treated samples compared to the control samples, indicating that treatment with AgNPs––H_2O positively affects proline accumulation in wheat and barley during exposure to low temperatures, Fig. 2.

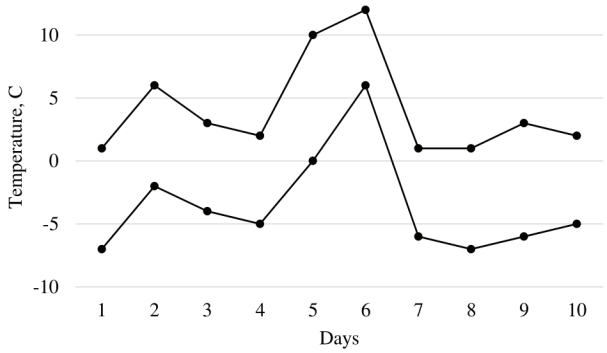

Fig. 1. Temperature fluctuations during the ten-day duration of the experiment.

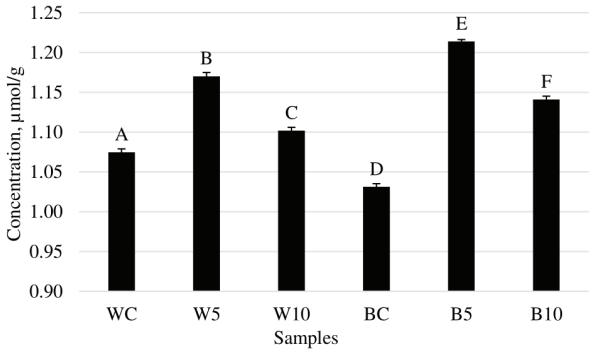

Fig. 2. The average proline content in wheat and barley samples, expressed in μmol/g of extract with standard error. The tested samples are labeled as follows: control – WC, treatment with 5 mg/mL – W5 and with 10 mg/mL – W10. Barley samples are labeled as follows: control – BC, treatment with 5 mg/mL – B5 and with 10 mg/mL – B10.

The obtained average values of proline concentration were subjected to a two-way analysis of variance (ANOVA test) with a significance threshold of $p \leq 0.05$, which showed statistically significant differences among the groups. The student Newman–Keuls test for multiple comparisons confirmed significant

differences between all groups. The mean values of the samples are labeled with different letters. Based on these analyses, it was concluded that there are significant statistical differences in proline concentration among the examined groups, Fig. 2.

Our results on proline accumulation are consistent with the previous studies.[30,31] These studies have shown that gold nanoparticles stimulate proline accumulation in plants exposed to low temperatures. Similarly, in our study, silver nanoparticles also increased proline concentration during exposure to low temperatures, suggesting their potential as cryoprotectants in plants under stress conditions.

According to Li *et al*.[3] repeated exposure to low temperatures over a certain period can also affect cereal productivity. While lower concentrations of nanoparticles may stimulate antioxidant mechanisms in plants, higher concentrations can lead to oxidative stress that the plants cannot overcome.[27] The highest antioxidant activity was recorded in the wheat samples treated with a concentration of 10 mg/mL AgNPs-H_2O (IC_{50} = 80.73 μg/mL) and in the wheat samples treated with a concentration of 5 mg/mL AgNPs-H_2O (IC_{50} = 118.51 μg/mL). Meanwhile, the barley samples treated with 5 mg/mL AgNPs–H_2O had the highest level of antioxidant activity compared to all other barley samples. However, compared to the positive control (ascorbic acid IC_{50} = 54.01 μg/mL), all tested samples, both treated and untreated, showed limited antioxidant activity. The antioxidant activity of AgNPs–H_2O was low, Fig. 3.

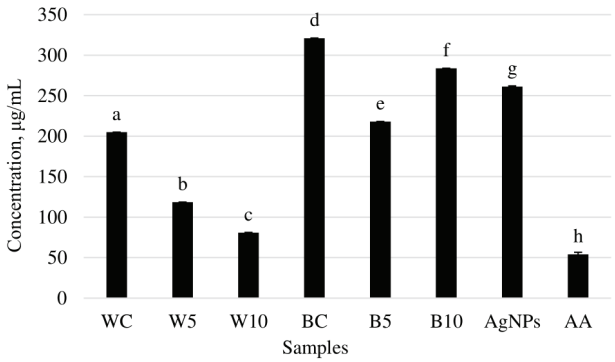

Fig. 3. The antioxidant activity, expressed as average IC_{50} values for DPPH, measured in wheat and barley samples, silver nanoparticles and ascorbic acid with standard error. The tested samples are labeled as follows: control – WC, treatment with 5 mg/mL – W5 and with 10 mg/mL – W10. Barley samples are labeled as follows: control – BC, treatment with 5 mg/mL – B5 and with 10 mg/mL – B10. AA and AgNPs refer to ascorbic acid and silver nanoparticles, respectively.

The obtained antioxidant activity values, expressed as average IC_{50} values for DPPH, were also subjected to an ANOVA test with a significance threshold

of $p \leq 0.05$, which showed statistically significant differences among the groups. Subsequently, the student Newman–Keuls test for multiple comparisons was used, confirming that all groups differ statistically significantly from each other. The mean values of the samples are labeled with different letters. Based on these tests, it was concluded that there are statistically significant differences in antioxidant activity among the examined groups.

Similar to our study, Islam *et al.*[27] used silver nanoparticles and examined their effect on antioxidant activity in wheat and barley grass exposed to temperatures of 5–10 °C. The obtained values showed that wheat grass had higher antioxidant activity than barley grass. Gorczyca *et al.*[28] investigated the antioxidant activity in the roots and leaves of wheat, with and without the presence of nanoparticles. Antioxidant activity was assessed using the enzymes catalase and superoxide dismutase, revealing that the values in treated plants did not differ from the control group. In the study by Budhani *et al.*[29] five different commercial silver nanoparticles negatively affected germination, root growth and shoot length in wheat. Venzhik *et al.*[30] demonstrated that gold nanoparticles at a concentration of 20 μg/mL had the most favorable effect on wheat seedling survival at –3 °C, increasing leaf length, chlorophyll content and carotenoid levels without MDA accumulation. Their subsequent research confirmed that gold nanoparticles can enhance wheat seedling tolerance to low temperatures, with higher survival rates at –3 °C compared to the control, although damage increased at –5 and –7 °C.[31]

Extraction of the aerial parts of plants, stems and leaves (LSA) was performed for 3 wheat samples and 3 barley samples to determine the extract yield for each sample. After complete solvent removal, the extract yield was obtained in g and %, as shown in Table I. The extract yield varied depending on the cereal species and treatment. The wheat samples showed an extract yield of 1.39 g (27.8 mass %) for samples treated with a concentration of 5 mg/mL AgNPs-H_2O (W5), while the samples treated with a concentration of 10 mg/mL AgNPs-H_2O (W10) had a slightly higher yield of 1.62 g (32.4 mass %). The control wheat sample (WC) had a yield of 1.42 g (28.4 mass %). For the barley samples, the control sample (BC) recorded the highest extract yield of 1.67 g (33.4 mass %). The sample treated with 5 mg/mL AgNPs-H_2O (B5) had a yield of 1.65 g (33 mass %), while the sample treated with 10 mg/mL AgNPs-H_2O (B10) showed a slightly lower yield of 1.59 g (31.8 mass %), as shown in Table I.

Numerous studies highlight the importance of silver nanoparticles with effects depending on the genotype of the studied organism, particle size, concentration, nanoparticle coating agents, application method, degree of dispersion and phytochemical properties.[32–35] In our research, silver nanoparticles with an average diameter of 35±1 nm were used, which contributed to increased antioxidant activity and proline content in both wheat and barley varieties compared

to the control groups. Accordingly, the application of silver nanoparticles in agriculture could be an effective approach to enhance resilience and mitigate stress caused by low temperatures during the tillering stage.

TABLE I. Extract yield for wheat and barley samples

Sample	Yield, g	Yield, mass %
WC	1.42	28.4
W5	1.39	27.8
W10	1.62	32.4
BC	1.67	33.4
B5	1.65	33
B10	1.59	31.8

CONCLUSION

Foliar application of silver nanoparticles at concentrations of 5 and 10 mg/mL affected antioxidant activity and proline concentration in both winter cereal varieties: Simonida (*T. aestivum* L.) and Nonius (*H. vulgare* L.) at the tillering stage during low temperatures. Treated plants exhibited better antioxidant activity compared to untreated plants. The highest antioxidant activity was recorded in the wheat samples treated with 10 mg/mL AgNPs–H_2O and the barley samples with 5 mg/mL AgNPs-–H_2O, suggesting that lower nanoparticle concentrations more effectively stimulate antioxidant mechanisms in barley. Overall, the wheat samples demonstrated better antioxidant activity than the barley samples. The highest proline concentrations were recorded in the plants treated with 5 mg/mL, both in wheat and barley, while the plants treated with 10 mg/mL had lower proline concentrations. These results confirm that a lower concentration of silver nanoparticles more effectively increases proline concentration. The yield of the extracts was highest in the wheat treated with 10 mg/mL and in the control barley sample. These results suggest that silver nanoparticles may potentially mitigate the negative effects of low temperatures on cereals and further enhance their resilience.

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ИЗВОД

ЕФЕКТИ СРЕБРНИХ НАНОЧЕСТИЦА СИНТЕТИСАНИХ ВОДЕНИМ ЕКСТРАКТОМ *Agrimonia eupatoria* L. НА ОЗИМЕ СОРТЕ ПШЕНИЦЕ И ЈЕЧМА

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Сребрне наночестице представљају потенцијално решење за ублажавање негативних ефеката температурног стреса на житарице. Ова студија испитује утицај сребрних наночестица на озиме сорте пшенице и јечма током фазе бокорења, фокусирајући се на концентрацију пролина, антиоксидативну активност и принос екстракта у зимским пољским условима. Сребрне наночестице (AgNPs) синтетисане су коришћењем зелене методе са воденим екстрактом биљке *Agrimonia eupatoria* L. (fam. Rosaceae). Две сорте озимих житарица, Simonida (*Triticum aestivum* L.) и Nonius (*Hordeum vulgare* L.), третиране су фолијарно са 5 и 10 mg/mL AgNPs–H_2O. Експеримент је трајао 10 дана, током ког је минимална забележена температура била –7 °C, у пољским условима. Концентрација пролина била је повећана код сорти третираних наночестицама у поређењу с контролама. Антиоксидативна активност, одређена је DPPH методом за третиране и нетретиране узорке, уз аскорбинску киселину као позитивну контролу. Антиоксидативна активност била је повећана код свих третираних узорака у односу на нетретиране узорке. Принос екстраката су незнатно повећале само одређене концентрације AgNPs–H_2O. Ово наглашава потенцијал AgNPs–H_2O за побољшање толеранције озимих житарица на ниске температуре.

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