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The role of silver nanoparticles in yellow lupine (*Lupinus luteus* L.) defense response to *Fusarium oxysporum* f.sp. *lupini*

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This study presents the influence of silver nanoparticles (AgNPs) on the growth of yellow lupine (*Lupinus luteus* L.cv. Diament and *Lupinus luteus* L.cv. Mister), and some metabolic reactions triggered by AgNPs during the seed germination stage and development of the seedling. Also, the role of AgNPs in defense mechanisms of the above of yellow lupine varieties against hemibiotrofic patogen *Fusarium oxysporum* f.sp. *lupini*. AgNPs enhanced the growth of yellow lupine seedlings, particularly root length and fresh biomass. Furthermore, AgNPs triggered defense-related phytohormones, such as abscisic acid (ABA), jasmonates (JA/MeJA), and salicylic acid (SA), which were involved in defense response of yellow lupine against *F. oxysporum* infection. The application of AgNPs significantly enhanced the growth of yellow lupine seedlings, increasing root length by over 400% and fresh biomass by 183% compared to the control. Moreover, AgNPs also significantly triggered an important defense-related phytohormone ABA, which increased by 103- and 38-times in Diament and Mister varieties, respectively. AgNPs influenced soluble sugar levels, such as sucrose and fructose, in yellow lupine, which may be related to defense mechanisms. The treatment with AgNPs induced a hormetic effect, where the roots of seedlings exhibited increased growth and defense responses at low concentrations. The level of gibberellic acid (GA) increased by 556% and 297% in AgNP-pretreated embryo axes of Diament and Mister varieties, respectively. Sugar levels, such as sucrose and fructose, were also influenced by AgNPs. In Diament variety, sucrose and fructose levels increased by 60% and 146%, respectively. However, *F. oxysporum* infection caused a strong decline in sugar levels. Overall, the study suggests that AgNPs can be used to enhance plant growth and defense against pathogens.

Keywords Silver nanoparticles, *Fusarium oxysporum*, Phytohormones, Superoxide anion, Soluble sugars, Yellow lupine

Nanoparticles (NPs) are defined as nanoobjects with all external dimensions in the nanoscale, where the lengths of nanoobjects do not differ significantly[1,2]. Furthermore, according to the European Commission[3] "nanoscale' is defined as the size range from 1 to 100 nm. Due to NPs small size and large surface to volume ratio, they show increased chemical reactivity and improved mechanical, catalytic, optical and electrical properties[4]. As reported by[5], as particle size decreases, the proportion of atoms located at the surface increases, which raises its tendency to adsorb, interact and react with other atoms, molecules and complexes to achieve charge stabilization. Additionally, their miniscule size allows NPs to be incorporated within aqueous suspensions and behave as a colloid. Such unique properties of NPs have been shown as beneficial for a wide range of applications. As a

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result, manufactured or engineered NPs have been intentionally produced and used in variety of applications[6]. It is worth mentioning that one of the most commonly used types of NPs are metal-based or inorganic NPs. In the present study we focus on silver nanoparticles (AgNPs), which are one of the most commonly investigated NPs[7]. Silver at the nanoscale have been used for antimicrobial sterilization and in biomedical fields because of its intrinsic therapeutic properties[8]. AgNPs have been used not only for medical products and devices but also for food packaging, solar energy absorption and as a catalyst for numerous chemical reactions[9]. Numerous research results indicate that AgNPs have both strong antimicrobial as well as antifungal activity, mostly due to its ionic charges (Ag^+)[10,11]. Furthermore, AgNPs have the ability of to anchor and penetrate bacterial cell walls disturbing therefore vital processes of the bacteria, resulting in the death of the bacteria[12]. It should also be emphasised that AgNPs occurring inside the cells cause induction of oxidative stress, modulation of signal transduction pathways and disruption of intracellular organelles and biomolecules[13]. The AgNPs can putatively modulate cellular signaling and acts by dephosphorylating tyrosine residues on key bacterial peptide substrates, thus inhibiting microbial growth[14]. Therefore, AgNPs possesses multiple modes of inhibitory action against microorganisms and they are more effective compared to synthetic fungicides[15]. Also, as reported by[16], AgNPs are also considered to be the most important NPs with fungicidal and viricidal properties[17]. In addition to their bactericidal activity, small AgNPs (< 10 nm in diameter) affect viruses although the microbicidal effect of silver mass in this case was weak. It was also concluded by[11] that, by applying a specific stabilizing agent, one can tune the selectivity of AgNP toxicity towards desired pathogens. For example, positively charged arginine-stabilized AgNPs were the most biocidal among all studied nanoparticles. In turn, the strongest fungicidal properties were noted for negatively charged AgNPs obtained using (−)-epigallocatechin gallate. Additionally,[18] reported that the use of NPs in the form of nanopesticides may protect crops from fungal and bacterial infections. Their strong pesticidal, antifungal, antiviral, and bactericidal effects were also previously reported by[19]. Current phytopathologists are using NPs and their chemical properties to support the effectiveness of plant defense against biotic stressors[20]. Additionally, bio-control methods of phytopathogens with the use of AgNPs are being often proposed from the past few years[21,22]. It was also demonstrated that biologically produced AgNPs can support seed germination and plant growth, improving chlorophyll content/photosynthetic efficiency, and increasing fertilizer and water efficiency, when they were used at optimum concentration for a given plant species[23].

As reported by[24], AgNPs exhibited antifungal properties against pathogens such as *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida glabrata* and *Aspergillus brasiliensis*[25]. Additionally, AgNPs also inhibited the activity of pathogenic fungi such as *Alternaria alternate*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Botrytis cinerea* and *Curvularia lunata*[26].

In the present experimental work, we first show the influence of AgNPs on yellow lupine growth and metabolic responses during the seed germination stage and development of the seedlings, and during cross-talk of AgNPs and *F. oxysporum* f.sp. *lupini* infection and development of Fusarium wilt. More importantly, we highlight the newly emerging mechanism of action of AgNPs on yellow lupine growth and biochemical responses in the seed germination stage and development of the seedlings, during plant—hemibiotrophic pathogen interactions. The first goal of this study was to investigate the effect of AgNPs as well as cross-talk between AgNPs and fungal pathogen *F. oxysporum* f.sp. *lupini* on the growth of embryo axes of germinating seeds and seedling roots, both *Lupinus luteus* L. cv. Diament as well as *Lupinus luteus* L. cv. Mister variety. In addition, it was important to check whether application the above AgNPs have impact on limiting the development of infection and diseases Fusarium wilt. The second goal was to verify whether in embryo axes of germinating seeds and seedling roots of the above-mentioned varieties of yellow lupine, an enhanced generation of signal molecules, such as defence-related phytohormones, i.e. abscisic acid (ABA), jasmonic acid (JA)/methyl jasmonate (MeJA), 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor) and salicylic acid (SA) in response to pretreatment of AgNPs, *F. oxysporum* and cross-talk of AgNPs and *F. oxysporum* occurs. In turn, the third goal was to verify whether AgNPs and cross-interactions of both stress factors, regulate the level of giberelic acid and indole 3-acetic acid—phytohormones playing important roles in seed germination. The fourth goal was to determine whether application of AgNPs changed concentration of key defence primary metabolites such as soluble sugars that play a central role in plant growth, development and stress responses. We hypothesize that application of AgNPs will regulate the growth and raise the metabolic status of yellow lupine seedlings. At the same time, AgNPs as priming agents, will enhance the defense mechanisms of embryo axes of germinating seeds against *F. oxysporum* f.sp. *lupini*. Therefore, as a result of AgNPs application and cross-talk between AgNPs and *F. oxysporum* levels of SA, ABA, JA and ACC in yellow lupine cells are expected to increase. Besides, the changes in concentrations of phytohormones such as giberelic acid and indole 3-acetic acid, playing important roles in seed germination will observed. Moreover, post-infection changes in sugar levels will be associated with the induction of defense mechanisms of lupine against *F. oxysporum*. It is assumed that a application of AgNPs may lead to limitation of infection development as well as fusariosis.

In this paper, we would like to answer the important questions: what is the role of AgNPs at the hormetic dose in stimulating the growth and metabolism of embryo axes of germinating yellow lupine seeds and what is their effect on the defense responses in germinating yellow lupine seeds against *F. oxysporum* and to what extent AgNPs inhibit the development of infection and disease Fusarium wilt? It should be emphasized that we were used a stable model system, i.e. embryo axes of germinating seeds pretreated with AgNPs or non-pretreated and inoculated or non-inoculated with *F. oxysporum*, cultured hydroponically on mineral medium. These research results provide new scientific knowledge, as the importance of AgNPs is addressed in the regulation of growth and generation of reactive oxygen species (superoxide anion) and signaling molecules as phytohormones and soluble sugars involved in plant defense mechanisms, as well as issues concerning the effect of these molecules on inhibiting infection caused by *F. oxysporum*.

Research on yellow lupine (*Lupinus luteus* L.), a member of the legume family (*Fabaceae* L.), has an enormous practical importance[27]. The cultivation of this leguminous is getting grate attention both economic as well as

scientific experts[28,29]. The lupins are an interesting group of legume crop species produce large seeds containing up to 40% protein[30]. For example, yellow lupine is mainly used as a feed for livestock and poultry[31,32], but it is also cut for silage. Besides, lupine seeds can be an alternative to soybean in all livestock species due to their high content in good quality protein[28]. Additionally, it is a very effective N-fixing legume, accumulating about 330 kg N ha^{-1}[33] and can be used for land reclamation[34]. In turn, *F. oxysporum* has been found to cause disease in a large number of plant species[35]. Besides, *F. oxysporum* f.sp. *lupini* is one of the key fungal pathogen of lupins[36]. This hemibiotrofic pathogen is a facultative parasite, i.e. possessing both biotrophic and necrotropic feeding mechanisms[37,38], and it is responsible for Fusarium wilt as well as pre-emergent sprout root and post-emergent seedling rot. This pathogen is representative of soilborne pathogens inhabiting the soil for a long time in the form of chlamydospores, whose hyphae penetrate the roots, spreading in the tissues, colonizes and metastasizes in xylem vessels, and causes systemic wilting, as well as pre-emergent sprout root and post-emergent seedling rot[35,39]. Colonization and clogging of vessels, in addition of secretion of several toxins by the fungus, play a major role in wilt symptom development and progression[40–45]. Regardless of the above, as reported by[46], AgNPs can also be easily produced by *F. oxysporum* aqueous extracts and may be a feasible, low-cost, environmentally friendly method for generating stable and uniformly sized AgNPs.

Results

The effect of silver nanoparticles and *Fusarium oxysporum* f.sp. *lupini* on the growth of the embryo axes of yellow lupine germinating seeds and the roots of seedlings. Analysis of disease symptoms

The results of measurements of the length and fresh weight of the embryo axes of yellow lupine germinating seeds and the roots of seedlings of Diament variety exposed to AgNPs showed that this NPs cause strong stimulation for the growth, the so-called hormetic effect (Figs. 1 and 2). An increase in the lengths of the roots from AgNPs variant for both varieties versus time of experiment were observed. Similarly to the Diament variety, in the Mister variety the root length of AgNPs variant was significantly higher at all-time points than in the case of the other experimental variants. For example, at 48, 72 and 96 h (h) of experiment, the length of embryo axes of germinating seeds and roots of seedlings from AgNPs variant of Diament variety were greater 44%, 7% and 73% in relation to the control, respectively. In turn, in case of Mister variety for AgNPs variant, at 48, 72 and 96 h the length of embryo axes of germinating seeds and roots of seedlings were greater 17%, 31%, 47% and 63% in relation to the control. Therefore, the highest of the lengths of seedling roots for Diament and Mister varieties exposed to AgNPs were observed at 96 h of experiment. The embryo axes of germinating seeds and seedling roots both Diament and Mister varieties pretreated with AgNPs at the above of hours were longer, not only in comparison to the control, but also in comparison to other experimental variants. Besides, 72 and 96-h roots of seedlings of Mister cultivar from AgNPs + Fusarium variant were longer than the roots of Fusarium variant; the roots from AgNPs + Fusarium variant were greater 9% and 13% in relation to Fusarium variant. In turn, at 48 h fresh weight of the roots from AgNPs + Fusarium variant of Diamond variety was 15% greater than in the control (Fig. 1c, Table 1). At subsequent time points, i.e. at 72 and 96 hpi the length and fresh weight of the roots from AgNPs + Fusarium variant of Diament variety were lowest, significantly lower than the roots from other experimental variants.Therefore, at 72 and 96 hpi the length and fresh weight of the above roots from AgNPs + Fusarium variant were less than 42% and 40%, and 40% and 61% in relation to the control, respectively. In turn, fresh weight of hypocotyl of yellow lupine germinating seedlings showed that up to 48 h it was significantly higher in the case of AgNPs and AgNPs + Fusarium variants of Diament variety in comparison to the control (i.e. it was higher 22% and 11%, respectively) and Fusarium variant (Fig. 1e). Another trend versus time is observed in Mister variety; the highest fresh weight of hypocotyl from AgNPs + Fusarium variant was observed at 72 hpi and it was higher in comparison to the other experimental variants (Fig. 1f).

The effect of silver nanoparticles and *Fusarium oxysporum* f.sp. *lupini* on concentrations of phytohormones

The effect of silver nanoparticles and Fusarium oxysporum f.sp. lupini on concentrations of defence-related phytohormones

The analytical results of the QuEChERS method showed a significant accumulation of ABA in the embryo axes of germinating seeds and the roots of yellow lupine seedlings from AgNPs + Fusarium variant, in the case of both Diament as well as Mister varieties (Fig. 3a,b). Moreover, from 0 to 72 h of the experiment, there was very strong increase in the ABA content in the embryo axes and the roots of yellow lupine seedlings from AgNPs + Fusarium variant. Therefore, at 24, 48 and 72 hpi the concentration of ABA in AgNPs + Fusarium variant of Diament variety was 1230%, 263% and 1770% higher in relation to the control, respectively.

At 72 hpi the highest level of ABA in the roots of *L. luteus* L.cv. Diament was detected; this level was 240 ng g^{-1} FW. In turn, in the case of Mister variety the highest level of ABA in 48 h-roots from AgNPs + Fusarium variant was noted; this level was 105 ng g^{-1} FW and it was two times lower than in Diament variety. At 48 h of experiment, the concentration of ABA in AgNPs and AgNPs + Fusarium variants of Mister variety was 627% and 2224% higher in comparison to the control, respectively. Moreover, attention is drawn to the high concentration of ABA in embryo axes of AgNPs variant and AgNPs + Fusarium variant for Mister variety at the beginning of the experiment, i.e. at 0 h.Besides, an increase in the level of ABA in embro axes of germinating seeds pretreated with AgNPs (AgNPs variant) for Diament cultivar between 48 and 72 h of experiment should be noted In 72-h the roots of seedlings from AgNPs variant of of Diament variety, the level of ABA increased by an average 1477% in relation to the control, while in 48-h embryo axes of germinating seeds from AgNPs variant of Mister variety increased by an average 251% in relation to the control, respectively.Also, between 24 and 48 hpi an increase in ABA level in infected embryo axes of germinating seeds (Fusarium variant) for Mister cultivar was observed; this level was 21 ng g^{-1} FW. The concentration of ABA in Fusarium variant of Mister variety was 521%

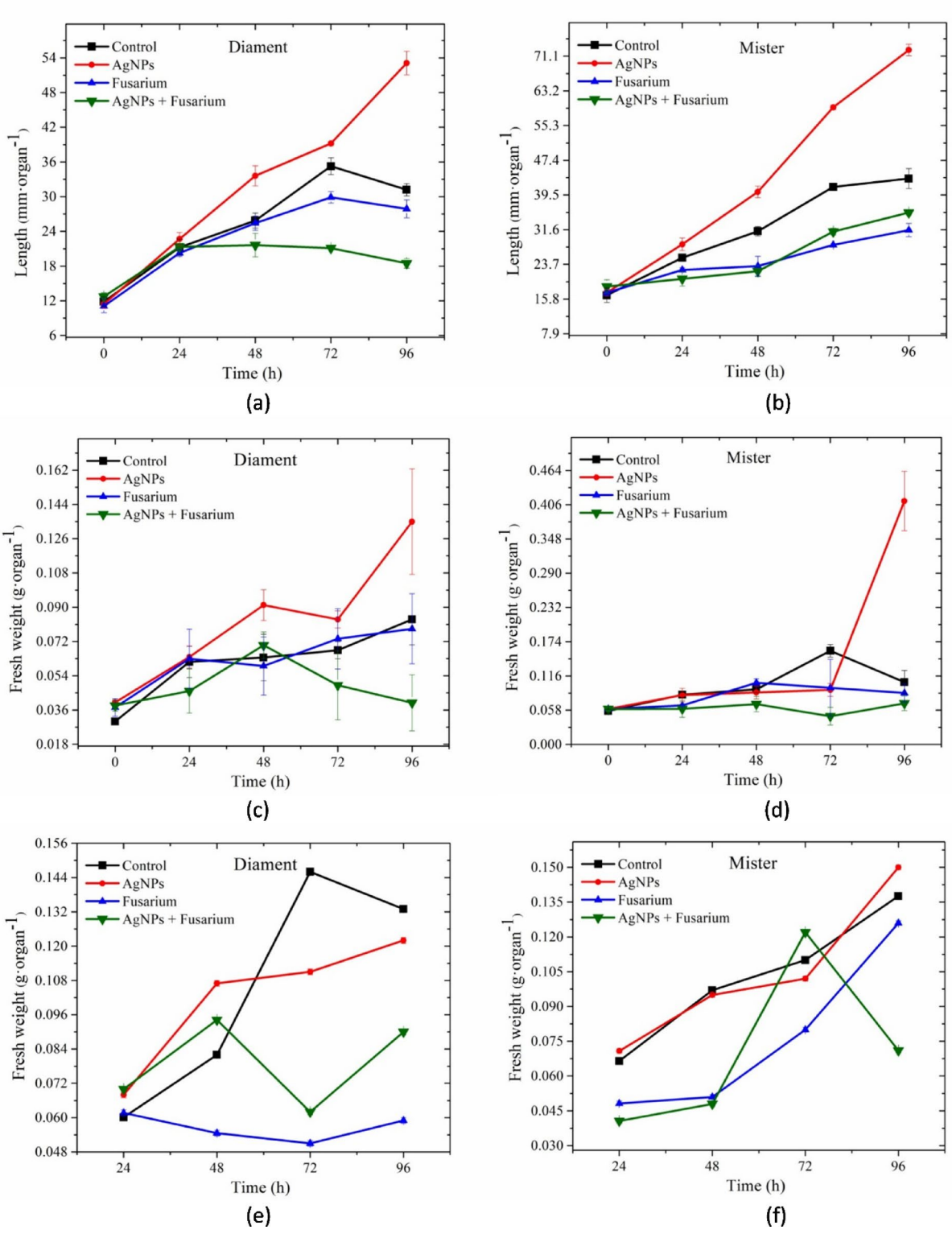

Fig. 1. Morphometric measurements of the length (**a**,**b**) and fresh weight (**c**,**d**) of embryo axes and the roots of yellow lupine germinating seeds seedlings (*Lupinus luteus* L.cv. Diament (**a**, **c**) or *Lupinus luteus* L.cv. Mister (**b**, **d**)), pretreated with silver nanoparticles (AgNPs) or non- pretreated with AgNPs and non-infected or infected with *Fusarium oxysporum* f.sp. *lupini*. Fresh weight of hypocotyl of yellow lupine (*L. luteus* L.cv. Diament and *L. luteus* L.cv. Mister) (**e**, **f**) germinating seedlings. Statistical significance between individual comparisons is presented in Table S2.

Fig. 2. 48-h embryo axes of germinating seeds of yellow lupine (*Lupinus luteus* L.cv. Diament or *Lupinus luteus* L.cv. Mister), pretreated with AgNPs or non-pretreated with AgNPs and non-infected or infected with *Fusarium oxysporum* f.sp. *lupini*.

Time (h)	Variants			
	Control	AgNPs	Fusarium	AgNPs + Fusarium
Lupinus luteus L.cv. Diament				
24	None	None	None	None
48	None	Browning on the entire surface of the roots, the roots stronger, better trurgor than in the control	Single roots show browning, some meristems dried out	Browning on the entire surface of the roots, more firm than in Fusarium variant
72	None	Slight browning of the roots, stronger and longer than the roots from the control	Good turgor, only some roots show browning, smaller symptoms (more resistant) than on the roots of the Mister variety	Browning on the entire surface of the roots
96	none	AgNPs cause root elongation in comparison to the control, lateral root reduction, slightly darker than the control	Less browning and the roots thicker than with AgNPs + Fusarium variant, root apical meristems are necrotic	Browning of the roots, hard, root apical meristems necrotic
Lupinus luteus L.cv. Mister				
24	None	None	None	None
48	None	Good turgor, slightly browning of the roots, from hypocotyl side, more firm than in the control	Slightly blue due to infection, some meristems dried out, slight reduction in turgor	Browning on the entire surface of the roots, more firm than in + Fusarium
72	None	Slightly lower firmness than the control, slight browning of most the roots	The roots with the strongest symptoms, the strongest browning of the roots, blueness of the apical meristems, necrotic changes	Turgor better than in + Fusarium variant, blueness of the root tips
96	None	AgNPs cause root elongation in comparison to the control	The roots look worse than in Fusarium variant of the Diament variety, the roots are more limp, turgor is reduced, root pical meristems are necrotic, more sensitive to Fusarium than the roots of Diament variety	AgNPs cause thicker roots and more surface browning, root apical meristems necrotic, more elongated than in Fusarium variant

Table 1. Disease symptoms on embryo axes of germinating seeds and seedling roots of yellow lupine (*Lupinus luteus* L.cv. Diament or *Lupinus luteus* L.cv. Mister) pretreated with AgNPs or non-pretreated with AgNPs and non-infected or infected with *Fusarium oxysporum* f.sp. *lupini*. Four culture variants were applied: embryo axes of germinating seeds pretreated with AgNPs, not inoculated and cultured on Hoagland medium (AgNPs); embryo axes pretreated with AgNPs, inoculated and cultured on Hoagland medium (AgNPs + Fusarium); embryo axes non-pretreated with AgNPs, not inoculated and cultured on Hoagland medium (control); embryo axes non-pretreated with AgNPs, inoculated with *F. oxysporum* and cultured on Hoagland medium (Fusarium).

higher than in the control.In turn, the analysis of the SA content in the roots of yellow lupine seedlings for the Mister variety, a very high peak at 48 h of the experiment for AgNPs variant demonstrated; it was 360 ng g^{-1} FW (Fig. 3d). This level of SA in the roots of yellow lupine seedlings from AgNPs variant of the Mister variety was significantly higher (365%) than in the control of the Mister variety. Additionally, the level of SA in AgNPs variant of the Mister variety was significantly higher than in experimental variants of Diament variety (Fig. 3c). Besides, already at 24 h increase in SA level in embro axes of germinating seeds pretreated with AgNPs for Diament variety was observed. Then a decrease in SA concentration and an increase again up to 96 h of the experiment. In the Mister variety, between 24 and 72 hpi of the experiment, an increase in content of SA in embryo axes of germinating seeds and seedling roots for Fusarium, AgNPs + Fusarium and control variants was detected. However, these contents were lower than in AgNPs variant. In the case of the Diament variety, significant fluctuations in the SA level were recorded. The level in the SA content between 24 and 72 h of the experiment was lower than in the control. Additionally, the concentration of SA at 96 hpi is noteworthy, where the level of SA in the roots of seedlings of the Diament from AgNPs + Fusarium variant was the highest, higher (13%) than in the control and much higher in other experimental variants. In turn, at 96 h in Mister variety roots

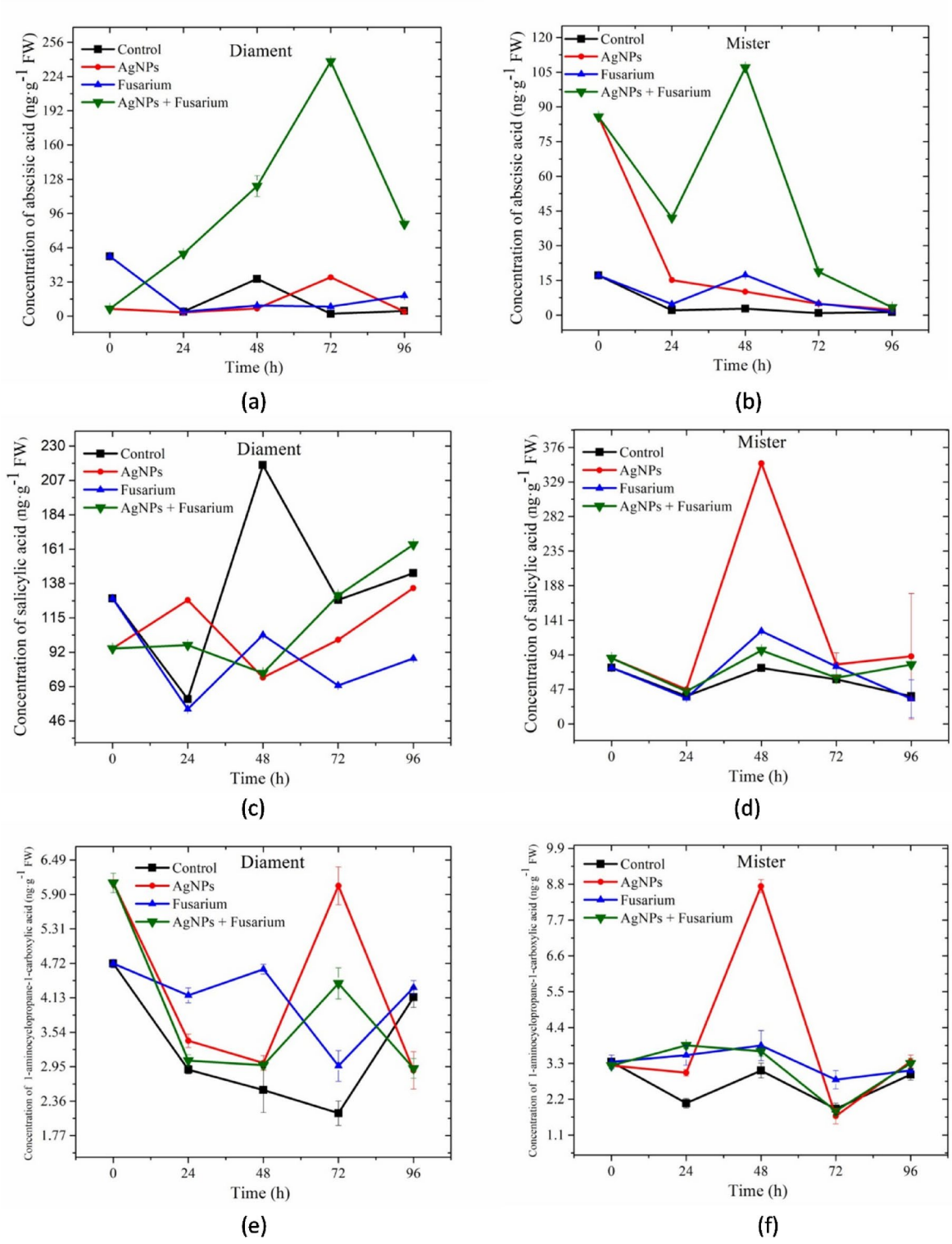

Fig. 3. Concentrations of phytohormones in embryo axes of germinating seeds and the roots of seedlings of yellow lupine *Lupinus luteus* L.cv. Diament (**a**, **c**, **e**, **g**, **i**) and *Lupinus luteus* L.cv. Mister (**b**, **d**, **f**, **h**, **j**) from the control (non-pretreated) and pretreated with AgNPs, and non-infected and infected with *Fusarium oxysporum* SCHLECHT f.sp. *lupini*, and cross-talk of AgNPs and *Fusarium oxysporum* f.sp. *lupini*. Statistical significance between individual comparisons is presented in Table S2.

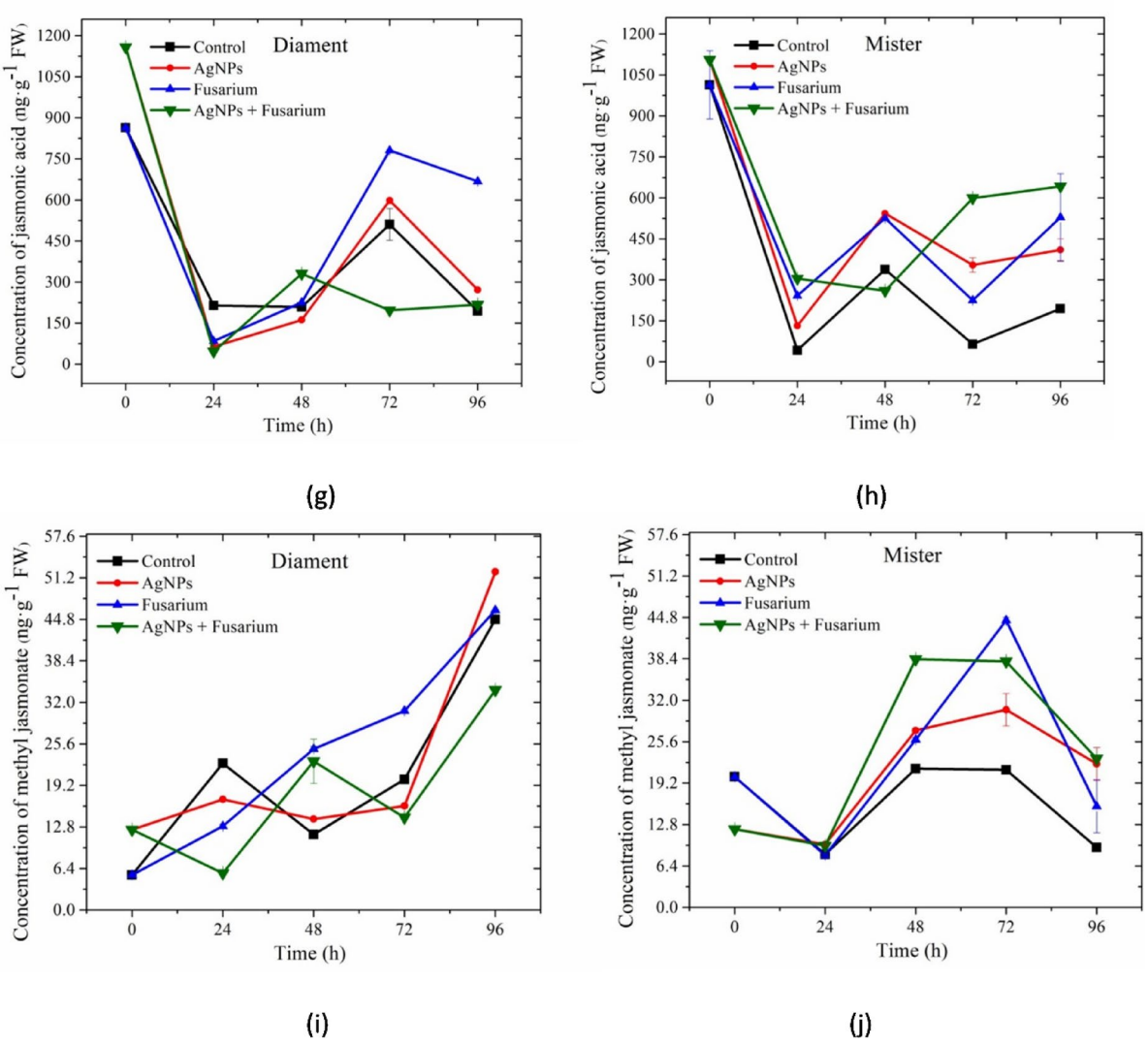

Figure 3. (continued)

from AgNPs and AgNPs + Fusarium high level of SA were observed; these levels were 142% and 113% higher than in the control and higher in Fusarium variant.

Analysis of 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor) contents showed that at 0 h of the experiment high its level (i.e. 6.195 ng g^{-1} FW) in the embryo axes of germinating yellow lupine seeds of the Diament variety from AgNPs and AgNPs + Fusarium variants occurred; this level was significantly higher than in the control and Fusarium variants (4.72 ng g^{-1} FW) (Fig. 3e). In 0-h embryo axes of Diament variety, the level of ACC was 29% and 20% higher in AgNPs and AgNPs + Fusarium variants in relation to the control, respectively. Then, at 24 h a very strong drop in ACC content, both in the embryo axes of germinating yellow lupine seeds of the Diament variety from AgNPs, AgNPs + Fusarium and control variants was observed. This low level in the above variants persisted for up to 48 h. In the case of Fusarium variant, content of ACC in 48-h embryo axes of germinating yellow lupine seeds was 4.70 ng g^{-1} FW. In 48-h embryo axes of Diament variety, the level of ACC was 18%, 17% and 81% higher in AgNPs, AgNPs + Fusarium and Fusarium variants in relation to the control, respectively. Moreover, the main attention is drawn to a significant increase in the level of ACC in 72-h roots of the Diament variety from the variant AgNPs; this level amounted 6.195 ng·g^{-1} FW. Also, an increase in ACC concentration in 72-h roots of AgNPs + Fusarium variant for the Diament variety was noted; this level was 4.425 ng g^{-1} FW. It should be emphasized that in 72-h roots of Diament variety, the concentration of ACC was 181%, 103% and 38% higher in AgNPs, AgNPs + Fusarium and Fusarium variants in relation to the control, respectively. In turn, at 96 h the decrease in the ACC content in the roots of seedlings from AgNPs and AgNPs + Fusarium variants was detected. In 96-h roots of Diament variety, the concentration of ACC was 30% and 29% lower in AgNPs and AgNPs + Fusarium variants in relation to the control, respectively.

The opposite tendency to the above was noted in 96-h roots from Fusarium variantof Diament variety; the level of ACC was higher 4% in the roots from the above of the variant in relation to the control. Moreover, an interesting result is a very high peak indicating the high content of ACC in 48-h axes of germinating lupine seeds of Mister variety pretreated with AgNPs (AgNPs variant); this level amounted 8.9 ng g^{-1} FW. The concentration

of ACC in the above of embryo axes from AgNPs variant of Mister variety was higher 120% in relation to the control. It should be mentioned that a similar trend was detected in the roots of germinating seeds of the Diament variety, but at 72 h of the experiment. At 24 hpi, the highest level of ACC is visible in the embryo axes pretreated with AgNPs and inoculated with *F. oxysporum* of Diament variety; this content was higher in relation to the control and other experimental variants. In the case of 24-h embryo axes from Fusarium variant and AgNPs variant of Mister variety, the ACC level at the above time point was also significantly higherthan in the control. The concentration of ACC in the above of embryo axes from AgNPs, AgNPs + Fusarium and Fusarium variants of Mister variety was higher 45%, 85% and 71% in relation to the control, respectively. Additionally, at 48 hpi contents of ACC in embryo axes of Mister variety from Fusarium and AgNPs + Fusarium variants was higher 25% and 19% than in the control, respectively.. At subsequent time points, a mild decrease in ACC and again a mild increase in control and in these variants were found.

The concentration of jasmonic acid (JA) is very strongly decreasing between 0 and 24 h of the experiment in the embryo axes of two yellow lupine cultivars (Fig. 3g,h). In addition, it should be mentioned that in 0 h experiment the highest level of JA in the embryo axes of germinating seeds two varieties of lupins was found. The concentration of JA in 0-h embryo axes from AgNPs, AgNPs + Fusarium and Fusarium variants of Diament variety was higher 34%, 22% and 20% in relation to the control, respectively. At 48 h, the highest content of JA in embryo axes from AgNPs + Fusarium variant for Diament variety was observed, while in embryo axes from AgNPs + Fusarium variant of Mister variety this level at time point was lowest. The level of JA in 48-h embryo axes from AgNPs + Fusarium variant of Diament variety was higher 58% in relation to the control, while in 48-h embryo axes from the above variant of Mister variety this level was lower 27% than in the control. It should be emphasized that between 48 and 72 hpi and at 96 hpi, opposite trends in JA concentration in embryo axes from AgNPs + Fusarium variant for Diament and Mister cultivars were observed. In turn, between 48 and 72 h strong accumulation of JA in infected embryo axes from Fusarium variant of Diament variety, significantly higher than in other experimental variants was demonstrated, while in Mister variety the opposite trend occurred. The concentration of JA in 72-h embryo axes from Fusarium variant of Diament variety was higher 53% in relation to the control. In turn, the level of JA in 72-h embryo axes from Fusarium variant of Mister variety was higher 247% in relation to the control. Also, the content of JA in 72-h embryo axes from AgNPs and AgNPs + Fusarium variants of Mister variety was higher 446% and 823% in relation to the control, respectively.

Moreover, from 0 to 96 h an increase in methyl jasmonate (MeJA) content in the embryo axes of germinating seeds and seedling roots of Diament variety was detected (Fig. 3i). The highest content (54.4 ng g^{-1} FW) in 96-h roots pretreated with AgNPs of Diament variety was found, and the lowest (32 ng g^{-1} FW) in AgNPs + Fusarium variant. The concentration of MeJA in 96-h embryo axes from AgNPs variant of Diament variety was higher 16% in relation to the control. In turn, strong fluctuations in the MeJA level in the Mister variety was observed (Fig. 3j). Between 0 and 24 h of the experiment, a decrease in the content of MeJA, then between 24 and 48 h a strong increase, generally remaining at a high level in the next time point, and at 96 h a decrease in the concentration of this phytohormone. However, the highest level of MeJA was shown at 48 hpi, in pretreated with AgNPs and infected with *F. oxysporum* embryo axes from AgNPs + Fusarium variant of Mister variety and at 72 hpi in infected embryo axes from Fusarium variant. The content of MeJA in 48-h embryo axes from AgNPs + Fusarium variant of Mister variety was higher 79% in relation to the control, while it was higher 109% in 72-h embryo axes from Fusarium variant.

The effect of silver nanoparticles and Fusarium oxysporum f.sp. lupini on concentrations of giberelic acid and indole 3-acetic acid, phytohormones playing important roles in seed germination.

From 24 to 96 h of experiment, the concentration of giberelic acid (GA) in pretreated with AgNPs and infected with *F. oxysporum* embryo axes of germinating seeds and the roots of seedlings increased very strongly, i.e. from 3.4 to 27 ng g^{-1} FW (Fig. 4a,b). The concentration of GA in 96-h the roots of AgNPs + Fusarium variant of Diament variety was higher 358% in relation to the control, while GA level in 96-h the roots of Fusarium variant was higher 113% in relation to the control. In embryo axes from the AgNPs variant of Diament variety, a very slight decrease in GA level from 0 to 48 h, after which at 72 h strong growth occurred (Fig. 4a). Also, fluctuations in GA content in Fusarium variant were also observed. In turn, at 48 h, reduction in GA level in embro axes from AgNPs, AgNPs + Fusarium and Fusarium variants of Mister variety in relation to control was detected. The concentration of GA in 48-h embryo axes from AgNPs, AgNPs + Fusarium and Fusarium variants of Mister variety was lower 53%, 16% and 55% in relation to the control, respectively. Then, a very strong growth in concentration of GA in 72-h embryo axes from Fusarium variant, as well as in AgNPs and AgNPs + Fusarium variants was noticeable. At 96 h significant reduction in the content of GA in these variants was observed.

At the beginning of the experiment, i.e. at 0 h, concentrations of indole 3-acetic acid (IAA) (26 ng·g-1 FW) in embryo axes of germinating seeds of Diament variety from AgNPs variant was higher than in the control and Fusarium variant. Therefore, the concentration of IAA in 0-h embryo axes from AgNPs, AgNPs + Fusarium and Fusarium variants of Diament variety was higher 52%, 34% and 29% in relation to the control, respectively. Between 0 and 24 h reduction in IAA content in pretreated with AgNPs and pretreated with AgNPs and infected with *F. oxysporem* of embro axes of Diament variety was detected. From 24 to 72 h very strong incresae in GA content in embryo axes from all experimental variants, and at the next time point, a slow decline was observed. However, the concentration of IAA in all experimental variants (AgNPs + Fusarium, Fusarium, AgNPs) was lower than in the control (Fig. 4c,d). Between 24 and 48 h of experiment, increase in IAA level in the embryo axes of germinating seeds and seedling roots of Mister variety was noted. The concentration of IAA, in 48-h embryo axes from AgNPs, AgNPs + Fusarium and Fusarium variants of Mister variety was higher 142%, 256% and 553% in relation to the control, respectively. Moreover, the highest IAA content in Mister variety was noticed in 96-h the roots of AgNPs + Fusarium variant, it was higher 266% in relation to the control. Moreover, attention is also drawn to the significant increase in the level of IAA in the roots of seedlings from the AgNPs variant, it

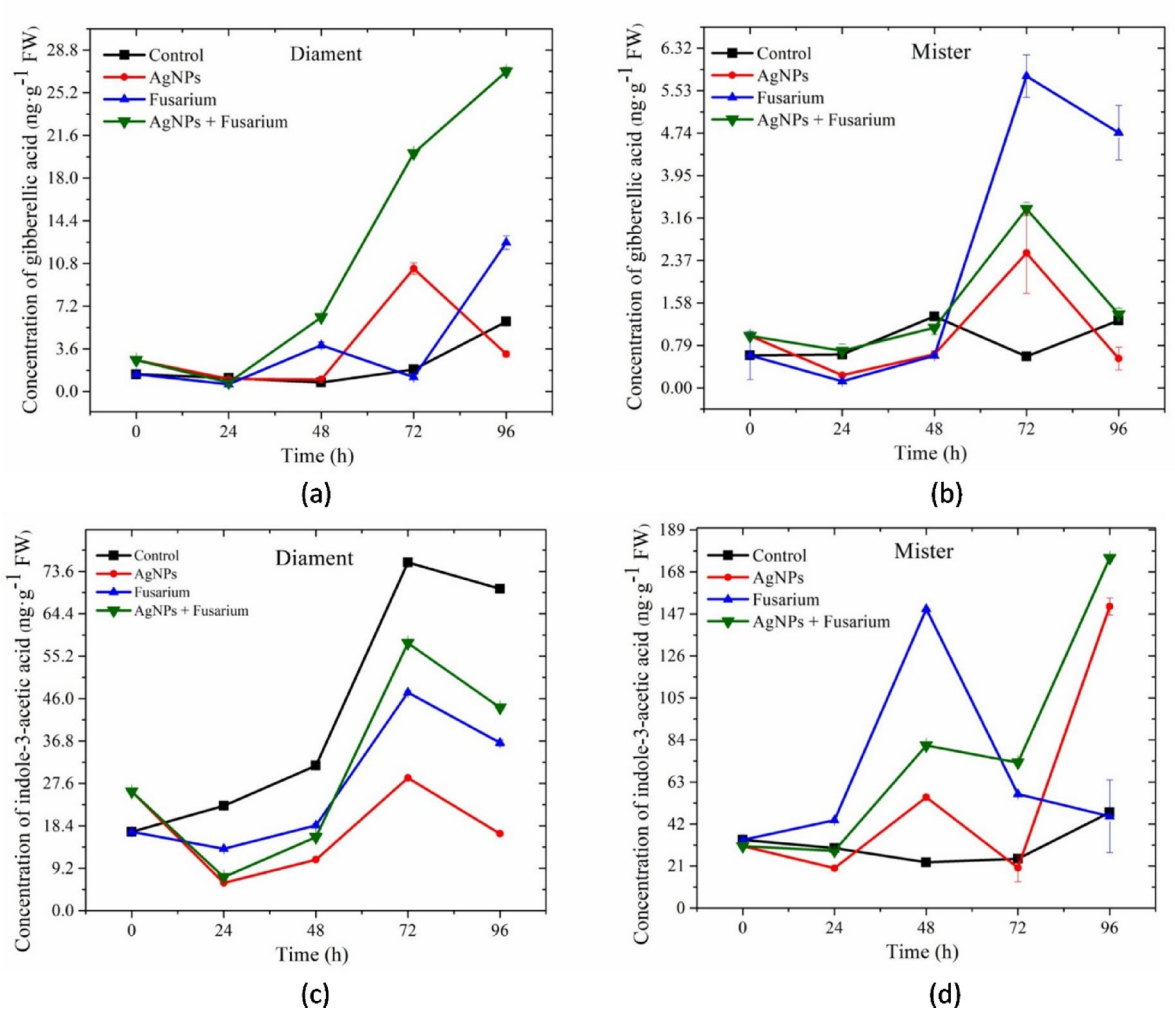

Fig. 4. Concentrations of phytohormones playing important roles in seed germination, i.e. giberelic acid and indole 3-acetic acid in embryo axes of germinating seeds and the roots of seedlings of yellow lupine *Lupinus luteus* L.cv. Diament (**a**, **c**, **e**, **g**, **i**) and *Lupinus luteus* L.cv. Mister (**b**, **d**, **f**, **h**, **j**) from the control (non-pretreated) and pretreated with AgNPs, and non-infected and infected with *Fusarium oxysporum* SCHLECHT f.sp. *lupini*, and cross-talk of AgNPs and *Fusarium oxysporum* f.sp. *lupini*. Statistical significance between individual comparisons is presented in Table S2.

was higher 215% in relation to the control. Moreover, between 72 and 96 h, a significant increase in the level of IAA in the roots of seedlings of Mister variety from the AgNPs and AgNPs + Fusarium variants is noteworthy, a completely different trend.

The effect silver nanoparticles and *Fusarium oxysporum* f.sp. *lupini* on soluble sugar concentrations in yellow lupine

Concentration of sucrose and its monosacharides in embryo axes of germinating seeds and the roots of seedlings of Diament and Mister cultivars showed different tendencies (Fig. 5). Significant fluctuations in sucrose level in the above plant material versus time were observed. From 0 to 24 h, an increase in the level of sucrose in pretreated with AgNPs embryo axes of germinating seeds and the roots of seedlings (AgNPs variant) of the Diament variety was found, this level was 18% higher than in the control. The highest accumulation of sucrose in 96-h infected roots of seedlings from Fusarium variant, both in the case of Diament as well as Mister cultivars was noted. The concentration of sucrose in 96-h roots of Fusarium variant for Diament and Mister variety was higher 63% and 44% than in the control, respectively. Also, at 48 hpi the reduction in sucrose level in pretreated with AgNPs and infected embryo axes (AgNPs + Fusarium variant), and in infected with *F. oxysporum* embryo axes (Fusarium variant) and in pretreated with AgNPs embryo axes (AgNPs variant) in relation to the control was noteworthy.

In turn, it should be emphasized that a strong increase in sucrose level in infected with *F.oxysporum* embryo axes of germinating seeds and the roots of seedlings (Fusarium variant) and in pretreated with AgNPs embryo axes of germinating seeds and the roots of seedlings (AgNPs variant)) was visible in the Mister variety. It should

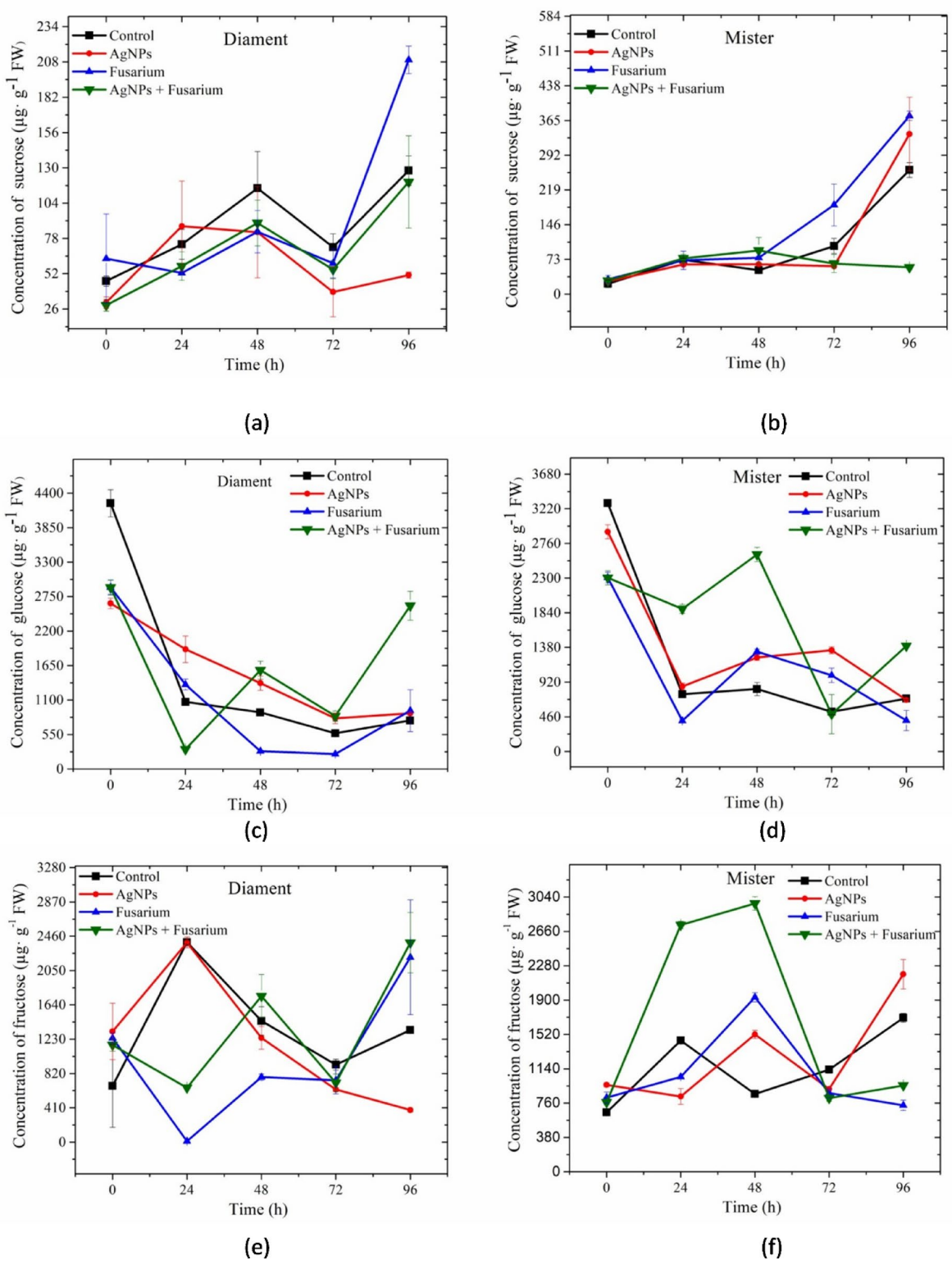

Fig. 5. Concentrations of sucrose (**a**) and monosaccharides (**b**) in yellow lupine *Lupinus luteus* L.cv. Diament (**a**, **c**, **e**) and *Lupinus luteus* L.cv. Mister (**b**, **d**, **f**). Seedling roots control (non-pretreated) and pretreated with silver nanoparticles (AgNPs), and infected with *Fusarium oxysporum* f.sp. *lupini*, and cross-talk of AgNPs and *Fusarium oxysporum* SCHLECHT f.sp. *lupini*. Statistical significance between individual comparisons is presented in Table S2.

be emphasized that the lowest sucrose content was recorded in 96 h pretreated with AgNPs and infected roots of seedlings (AgNPs + Fusarium variant) for Mister variety.

Between 0 and 24 h of the experiment, glucose concentration decreased significantly in all experimental variants for both varieties. Then, the increase in glucose concentration in embryo axes of germinating seeds from AgNPs + Fusarium between 24 and 48 h of the experiment for Diament and Mister cultivars draws attention. The concentration of glucose in 48-h embryo axes of AgNPs + Fusarium variant for Diament and Mister varieties was higher 74% and 215% than in the control, respectively. Moreover, in the above variant at 72 hpi a decrease in the content of glucose occurs and at the next time point, i.e. at 96 h, there is significant increase again. It should also be mentioned that at 48 h of experiment, the glucose content in the AgNPs variant of the Diament variety was higher 51% than in the control. A similar tendency in glucose level at this time point for the Mister variety was observed. Besides, the level of glucose in 48-h embryo axes of Diament variety decreased in relation to the control, while in Mister variety the level of glocose was higher than in the control. Additionally, between 0 and 24 h of the experiment, the content of fructose increased in embryo axes of AgNPs variant of Diament variety and in the control. It should be emphasized that the high level of fructose in 24-h and 48-h embryo axes from AgNPs + Fusarium variant of Mister variety is noteworthy. The concentration of fructose in 24-h and 48-h embryo axes of AgNPs + Fusarium variant of Mister variety was higher 87% and 245% than in the control, respectively.

Correlation coefficients between observed traits in relation to the roots of yellow lupine seedlings in the context the effect of AgNPs and *Fusarium oxysporum* f.sp. *lupini*

The empirical distribution of observations for the 12 observed traits was normal. The MANOVA results indicated that the times (Wilks $\lambda = 0.00000003381$, $F = 479.80$, $p < 0.001$), variants (Wilks $\lambda = 0.00000000141$, $F = 130.76$, $p < 0.001$) and the time × variant interaction (Wilks $\lambda < 0.0000000001$, $F = 51.17$, $p < 0.001$) were significantly different when investigated in terms of all the 12 traits jointly. Furthermore, the ANOVA results indicated that the main effects of times and variants as well as time × variant interaction were statistically significant for all the observed traits (Table 2).

The range of variation in lenght of root increased with increasing time from 0 to 96 h (Fig. 6). For fresh weight of root, there was similar variance at 0 h and 48 h. For ABA, the largest range of observed values was at 72 h, and the smallest at 24 h (Fig. 6). At 48 h, SA and ACC had the highest variability (Fig. 6). At 0 h, IAA and sucrose showed the lowest variability (Fig. 6).

Very high variability for DC was observed for lenght of root, MeJA and glucose (Fig. 7). For control of Mister (Mc), a large range of results was observed for fresh weight of root and JA (Fig. 7). Particularly noteworthy is the stability of fresh weight of root after application of D + Ag, D + Ag + F and D + F (Fig. 7). In contrast, the application of M + Ag, M + Ag + F and M + F strongly differentiated IAA values compared to Mc (Fig. 7).

Significant positive correlations were observed between 16 traits pairs: lenght of root–fresh weight of root (0.23), ACC–JA (0.36), ACC–SA (0.54), ACC–glucose (0.29), lenght of root–MeJA (0.25), lenght of root–IAA (0.29), lenght of root–sucrose (0.42), fresh weight of root–fructose (0.21), GA3–ABA (0.53), GA3–JAMe (0.30), IAA–JAMe (0.24), IAA–fructose (0.19), JA–glucose (0.60), SA–JAMe (0.19), ABA–glucose (0.26), and sucrose–fructose (0.25) (Fig. 8). Significant negative correlation coefficients were observed between nine traits pairs: lenght of root–JA (−0.36), lnght of root–glucose (−0.52), fresh weight of root–ACC (−0.18), fresh weight of root–JA (−0.28), JA–sucrose −0.22), JA–fructose (−0.20), ABA–sucrose (−0.18), JAMe–glucose (−0.22), and glucose–sucrose (−0.31) (Fig. 8).

Figure 9 shows variability of the 12 traits for the combinations of times and variants in terms of the first two canonical variables. In the graph, the coordinates of the point for particular combinations of times and variants are the values for the first and second canonical variables, respectively. The first two canonical variables

Source of variation	Time	Variant	Time × Variant	Residual
The number of degrees of freedom	4	7	28	80
Lenght of root	2344.85***	373.5***	267.18***	1.507
Fresh weight of root	0.047**	0.052***	0.061***	0.012
ABA	4250.26***	16,041.45***	4182.02***	13.2
SA	19,192.3***	12,857.3***	7254.5***	207.5
ACC	8.37438***	5.14861***	5.15479***	0.04122
JA	2,811,034***	122,174***	72,766***	1386
MeJA	1785.338***	147.807***	324.47***	2.489
GA3	192.9632***	173.3629***	52.9441***	0.5665
IAA	10,092.3***	6568***	3010.3***	459.9
Sucrose	84,598***	12,161***	10,591***	2484
Glucose	18,363,562***	833,453***	1,151,693***	20,983
Fructose	2,671,856***	636,513***	1,407,127***	38,552

Table 2. Mean squares from two-way analysis of variance for 12 observed traits. Mean values and standard deviations (s.d.) for 12 observed traits were presented in Table S1. The relationships between times and variants, independently, were presented in Figs. 1 and 2, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$.

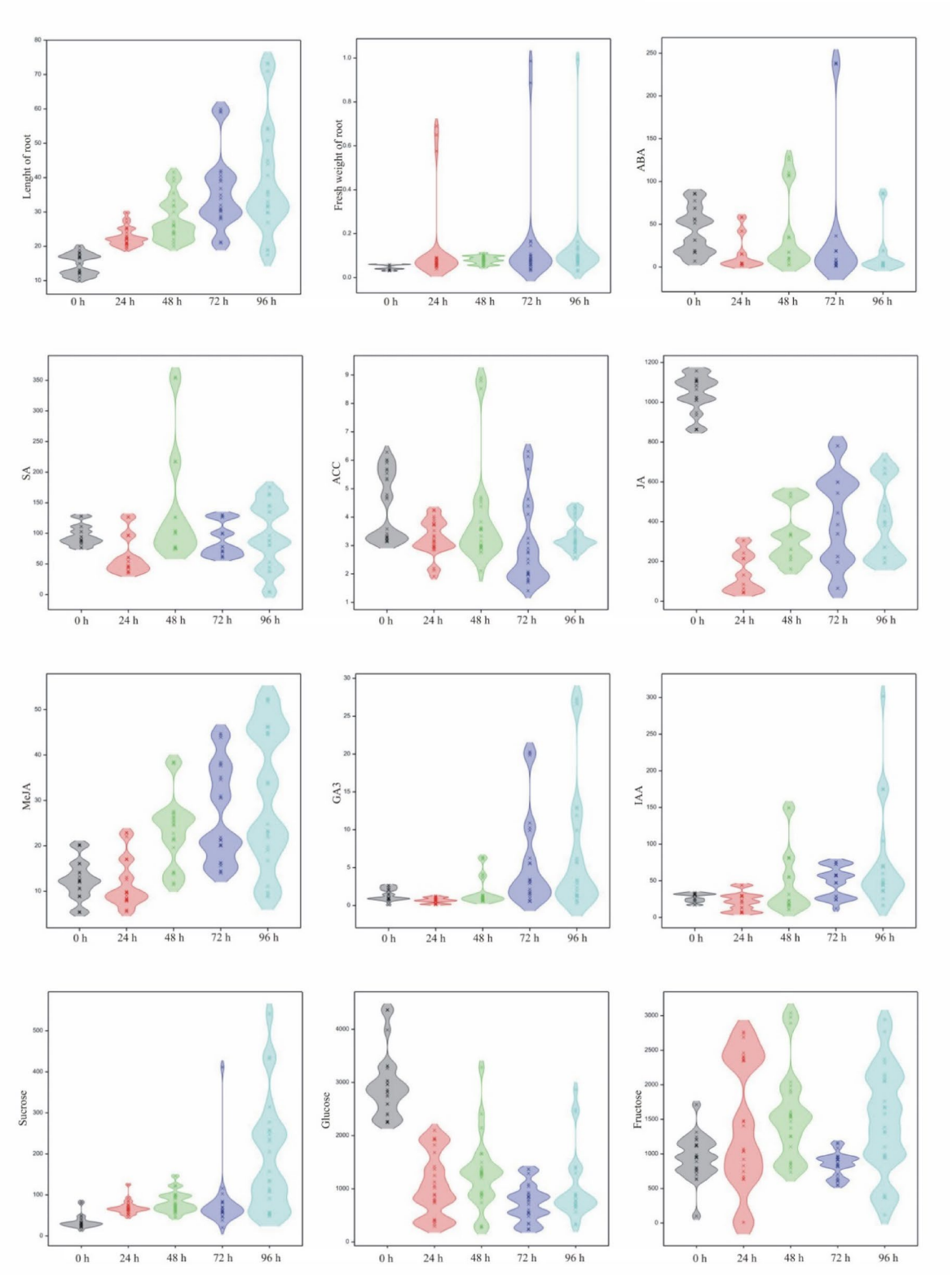

Fig. 6. Density plots for observations of 12 observed traits (lenght of root, fresh weight of root, ABA, SA, ACC, JA, MeJA, GA3, IAA, sucrose, glucose and fructose) for particular times.

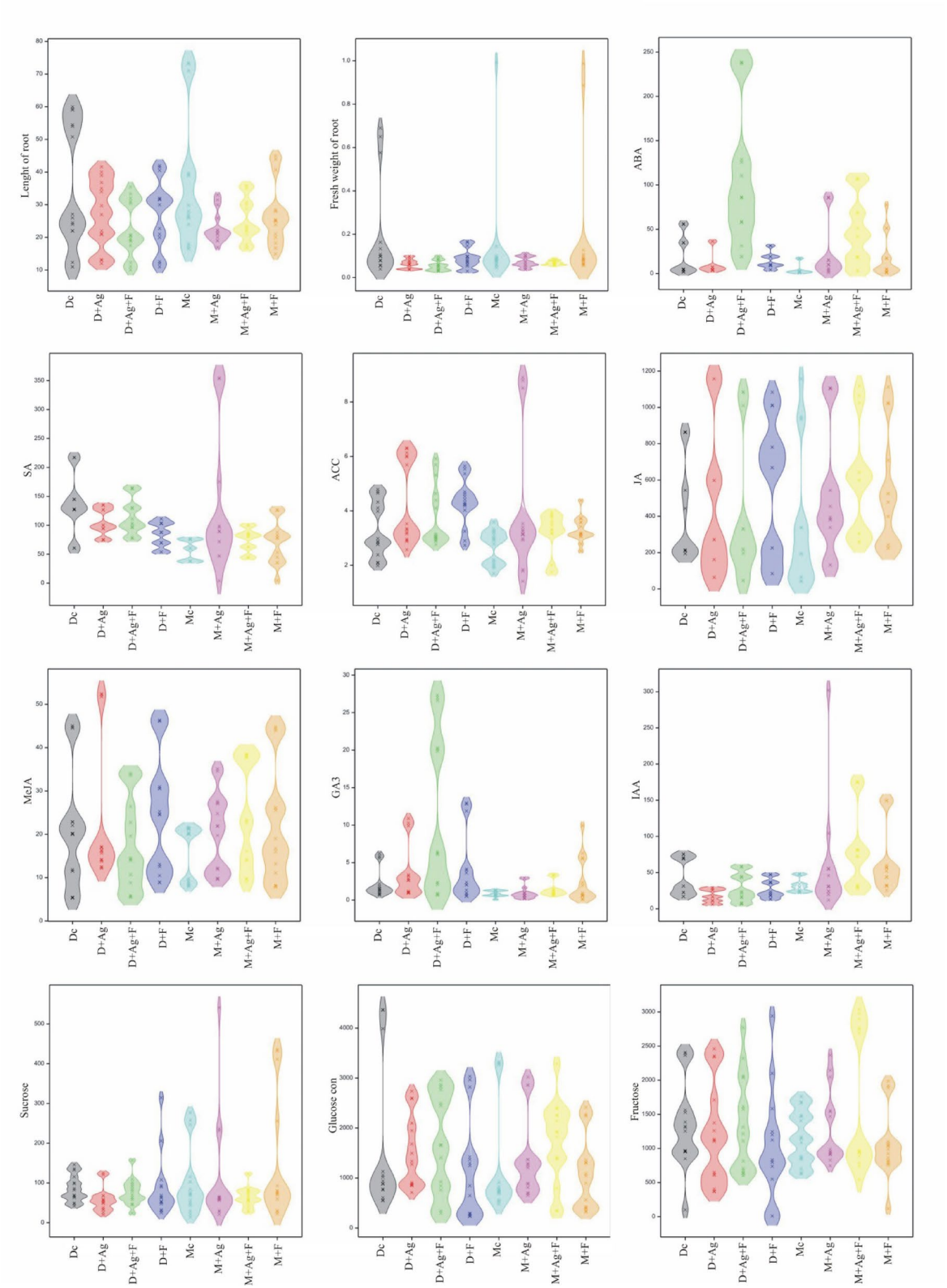

Fig. 7. Density plots for observations of 12 observed traits (lenght of root, fresh weight of root, ABA, SA, ACC, JA, MeJA, GA3, IAA, sucrose, glucose and fructose) for particular variants.Values of elementary contrasts between combinations of times and variants for eleven observed traits were presents in Table S2. The vast majority of contrasts were statistically significant.

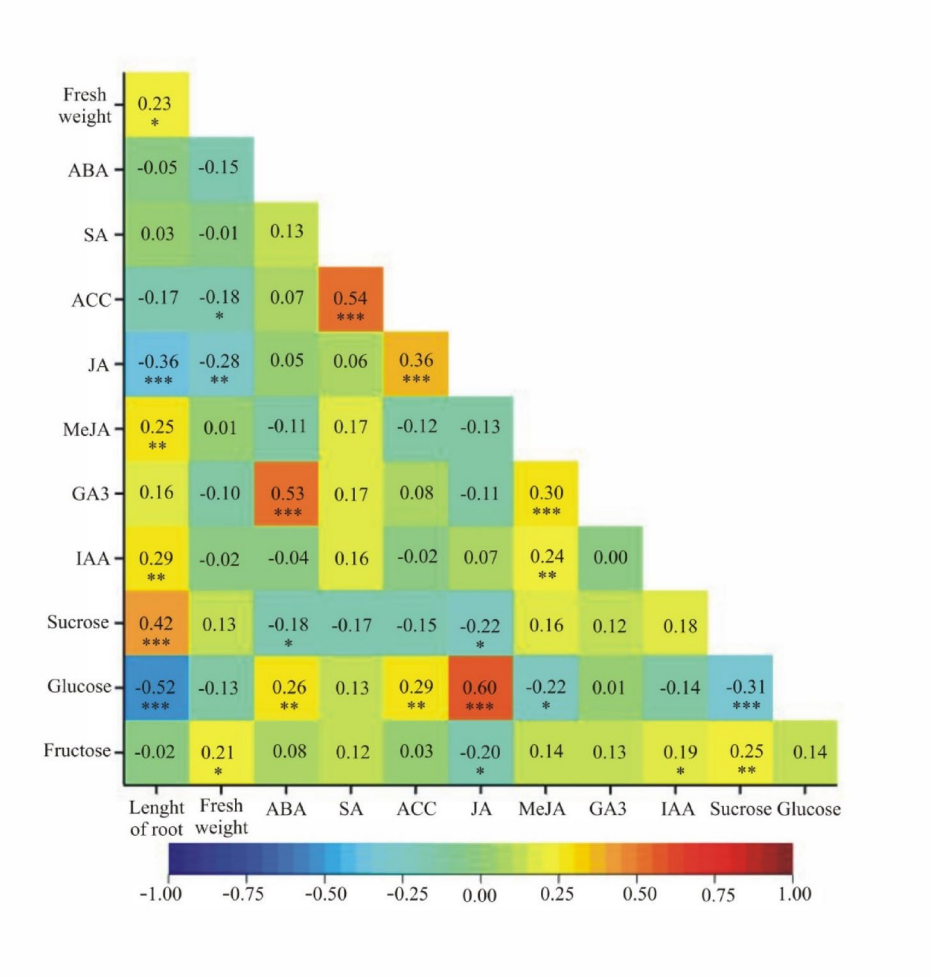

Fig. 8. A heatmap for correlation coefficients calculated and tested between all pairs of observed traits (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The correlation coefficients ranged from − 1 (blue) to 1 (red).

accounted for 65.23% of the total multivariate variability between the individual combinations of times and variants. Significant positive linear relationships with the first canonical variable were found for ACC (0.38), GA3 (0.55), SA (0.75), ABA (0.45) and MeJA (0.59). The second canonical variable was significantly positively correlated with lenght of root (0.47), ABA (0.39), GA3 (0.52) and sucrose (0.34). However, second canonical variable was significantly negatively correlated with ACC (–0.40), JA (–0.75) and glucose (–0.50). The greatest variation in terms of all the 12 traits, based on the measured Mahalanobis distances, was found for Diament + Ag Nanoparticles + Fusarium at 72 h and Mister + Ag Nanoparticles at 48 h (the Mahalanobis distance between them amounted to 111.47). The greatest similarity was found for Mister + Fusarium at 0 h and Mister + Ag Nanoparticles + Fusarium at 0 h (1.82). The values of the Mahalanobis distances for all the pairs of combinations of times and variants are presented in Table S1.

The effect of AgNPs and *F. oxysporum* f.sp. *lupini* on generation of superoxide anion in the embryo axes of germinating seeds and the roots of seedlings of diament and mister varieties

Considerable differences in $O_2^{\cdot-}$ level were detected in the cells of the roots of yellow lupine (*L. luteus* L.cv. Diament and *L. luteus* L.cv. Mister), pretreated with AgNPs or non-pretreated with AgNPs and non-infected or infected with hemibiotrophic pathogen *F. oxysporum* f.sp. *lupini* (Fig. 10). Relative release of the superoxide anion ($O_2^{\bullet-}$) was investigated by staining fragments of the roots with the $O_2^{\bullet-}$—specific indicator, dihydroethidium (DHE). The presence of $O_2^{\bullet-}$ oxidizes DHE to ethidium, whereupon it emits fluorescence. The DHE-derived fluorescence began to appear, in the roots of germinating seeds of Diament and Mister varieties from 0 h. Already at 0 h after inoculation, a very high DHE-derived fluorescence, especially in the roots of Diament variety from Fusarium variant was observed, and covered generally a large area of tissue (third column). In the above cells, the signal came from the entire cytoplasm and from the cell walls. Also, strong fluorescence from the cell walls and cell nuclei in cells of 0-h-old roots from AgNPs + Fusarium variant of Diament variety attracts attention (fourth column). In *F. oxysporum*-inoculated roots of germinating seeds of Diament variety, the fluorescence derived from $O_2^{\cdot-}$ decreased versus time. In turn, in root cells from AgNPs + Fusarium variant of Diament variety, fluorescence increased versus time, especially until 72 hpi, and at the next time point it was lower but

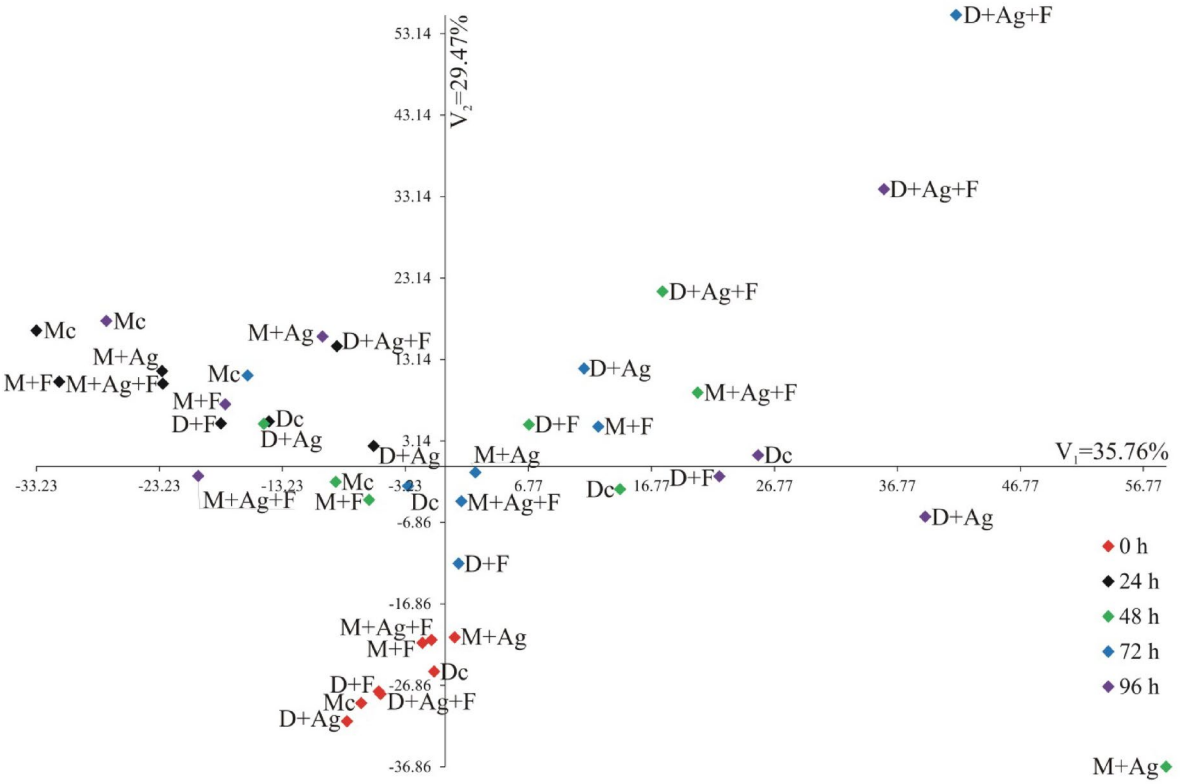

Fig. 9. Distribution of combinations of times and variants in the space of the first canonical variable (V_1) and the second canonical variable (V_2). Dc, Diament control; D + Ag, Diament + Ag Nanoparticles; D + Ag + F, Diament + Ag Nanoparticles + Fusarium; D + F, Diament + Fusarium; Mc, Mister control; M + Ag, Mister + Ag Nanoparticles; M + Ag + F, Mister + Ag Nanoparticles + Fusarium; M + F—Mister + Fusarium.

fluorescence still maintained. At 72 hpi, the strongest fluorescence emission indicating strong generation of $O_2^{\cdot-}$ in the cells from AgNPs + Fusarium variant of Diament variety was observed. Moreover, fluorescence emission was also observed in root cells with AgNPs variant. However, this signal was significantly weaker in comparison to other experimental variants, i.e. Fusarium and AgNPs + Fusarium. It is worth mentioning that from 0 to 48 h, $O_2^{\cdot-}$ generation in the cells of AgNPs variant occurred in the cell walls, while at 72 and 96 h it also occurred in the cytoplasm.

In turn, in the root cells of the tested experimental variants of the Mister variety, significantly weaker fluorescence related to $O_2^{\cdot-}$ generation than in the root cells of the Diament variety was observed. From 0 to 96 h, the signal coming from $O_2^{\cdot-}$ decreased versus time. Moreover, from 24 h of experiment, fluorescence occurred mainly in the cell walls or cell nuclei of root cells of the Mister variety. In the Mister cultivar, similarly to the Diament cultivar, the strongest fluorescence among all analyzed time points was observed at 0 h in root cells inoculated with *F. oxysporum*; the signal comes from the cytoplasm of the cell nuclei. Also, fluorescence appeared in the cytoplasm and cell walls of the AgNPs + Fusarium variant and the AgNPs variant, but in AgNPs variant it was was slightly less than in Fusarium and the AgNPs + Fusarium variant. At 24 h, the generation of $O_2^{\cdot-}$ from cell nuclei was clearly visible.

Materials and methods

Plant material and growth conditions

Yellow lupine (*Lupinus luteus* L.cv. Diament and *Lupinus luteus* L.cv. Mister) seeds of the S-elite class were used in the experiments, these were obtained from the Plant Breeding Company in Tulce near Poznan in Poland. The Diament and Mister varieties are resistant to fusariosis according to the data provided by the Plant Breeding Company in Tulce, Poland. The Diament variety is characterized by very high yield potential, sweetest variety because lowest alkaloid content and the highest resistance to Fusarium wilt of lupines. The seeds of this variety of lupine are characterized by a very high nutritional value, high protein content and low alkaloid content. Moreover, it should be mentioned that Diament is the sweetest variety on the market. In turn, special features of yellow lupine Mister are the highest seed crude fat content on the market, the most uniformly maturing variety, good lodging resistance after flowering, a drought-tolerant variety, which provides secure cultivation and good nutritional parameters, i.e. protein content and low proportion of alkaloids. Surface-sterilization of seeds was performed by Morkunas et al.[36]. Yellow lupine seeds were surface-sterilized, immersed in sterile water and left in an incubator (23 °C). After 6 h of imbibition, the seeds were transferred onto Petri dishes and immersed in a small amount of water to support further absorption. After a subsequent 72 h of seed germination, the

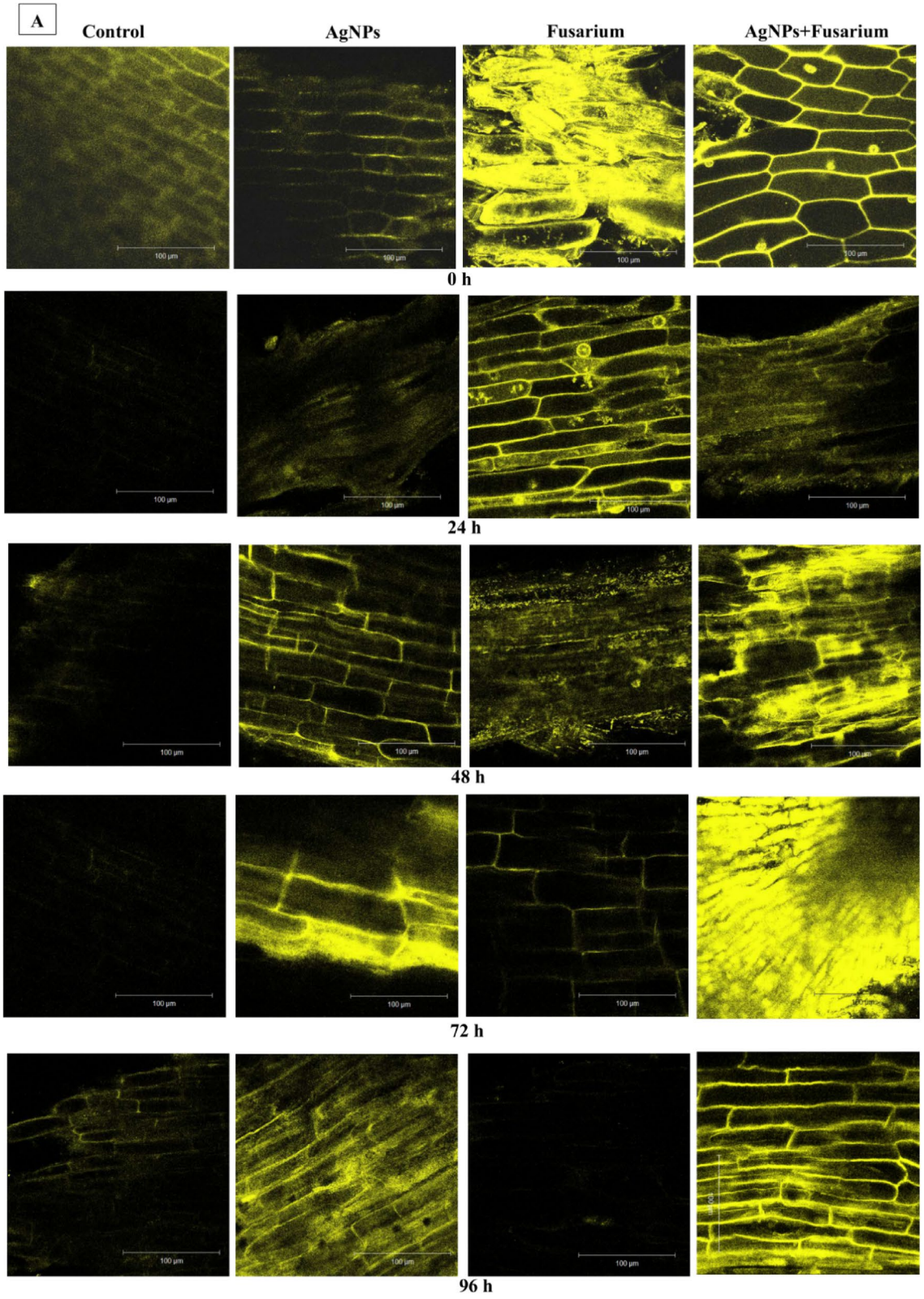

Fig. 10. Relative generation and cytochemical localization of superoxide anion ($O_2^{\cdot-}$) in the embryo axes of germinating seeds and the roots of seedlings of yellow lupine (*Lupinus luteus* L.cv. Diament and *Lupinus luteus* L.cv. Mister), pretreated with AgNPs or non-pretreated and non-inoculated or inoculated with *F.oxysporum*. Yellow fluorescence came from DHE (dihydroethidium), which is observed by a Zeiss LSM 510 confocal microscope, (objective magnification of 20x), scale bar 100 µm.

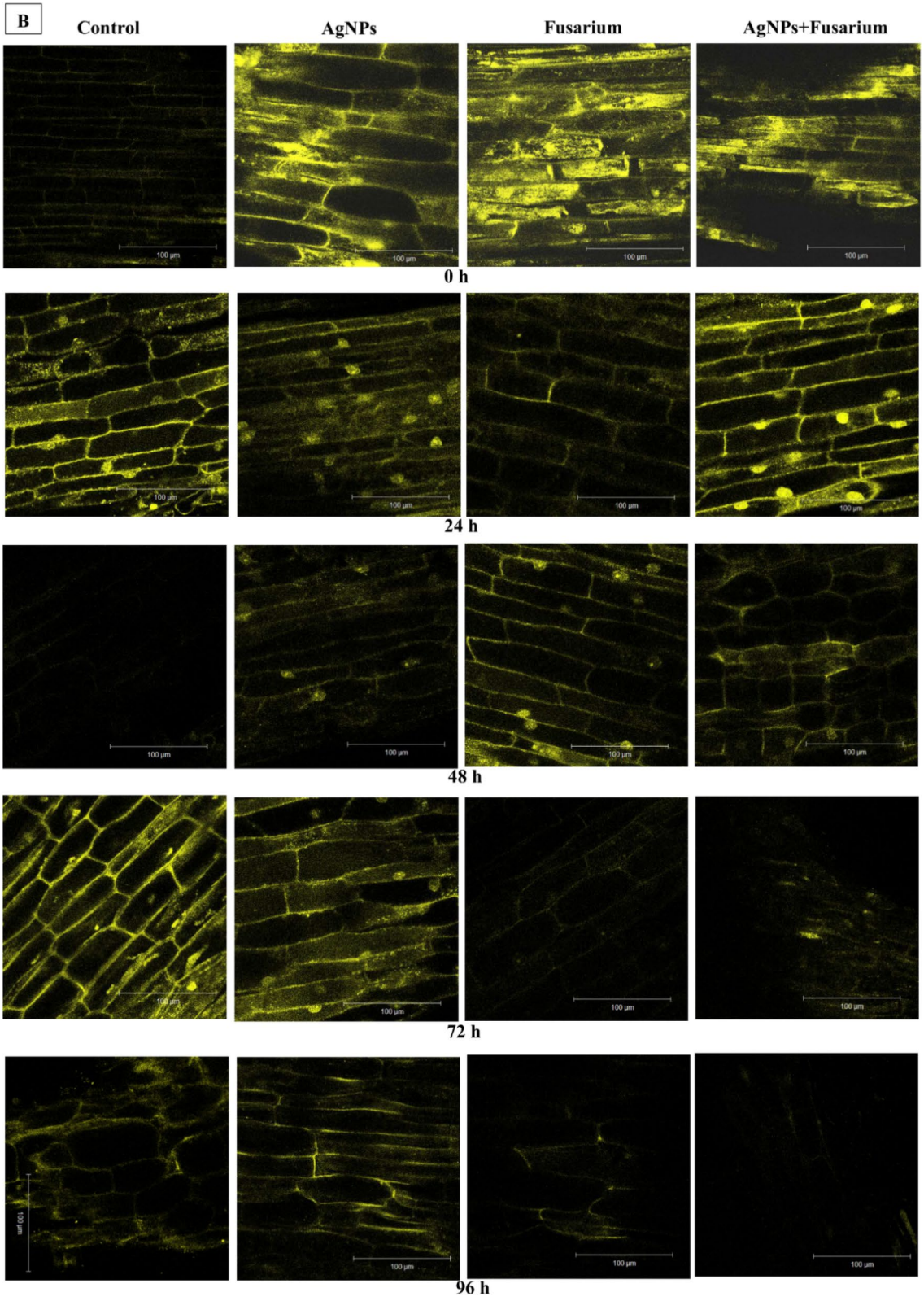

Figure 10. (continued)

germinating seeds were non-treated or pre-treated with an aqueous solution of AgNPs (Sigma Aldrich, product no 798738, 30 wt% dispersion in ethylene glycol, 1 ml AgNPs 30 wt% dispersion in ethylene glycol·L-1water) during 7 h. Next at the beginning of the study (0 h), the germinating seeds pre-treated with AgNPs or non-treated, were inoculated with a *F. oxysporum* f.sp. *lupini* spore suspension or non-inoculated and were transferred to

hydroponic grow boxes containing Hoagland medium. Hydroponic boxes were covered with dark foil to mimic soil conditions. Four culture variants were applied: embryo axes of germinating seeds pretreated with AgNPs, not inoculated and cultured on Hoagland medium (AgNPs); embryo axes pretreated with AgNPs, inoculated and cultured on Hoagland medium (AgNPs + Fusarium); embryo axes non-pretreated with AgNPs, not inoculated and cultured on Hoagland medium (control); embryo axes non-pretreated with AgNPs, inoculated with Fusarium and cultured on Hoagland medium (Fusarium) (Fig. 2). Samples were collected for analyses at 0 h and after 24, 48, 72, and 96 h of culture, following that they were frozen at – 80 °C to determine the contents of phytohormones and soluble sugars concentrations. Furthermore, it should be stressed that the hypocotyls and the roots were used for measurements of length and fresh weight. In turn, embryo axes of germinating seeds and the roots of yellow lupine seedlings were used as objects for all other studies carried out in this work.

Preparation of spore suspension and inoculation

Fusarium oxysporum f.sp. *lupini* strain K-1018 (subsequently referred to as *F. oxysporum*) was obtained from the Collection of Plant Pathogenic Fungi, Laboratory of the Plant Diseases Clinic and Pathogen Bank, Institute of Plant Protection—National Research Institute in Poznań. The pathogen was incubated in the dark at 23 °C in Petri dishes (diameter 9 cm) on a potato dextrose agar (PDA) medium (pH 5.5, Sigma-Aldrich, Poznań, Poland). After 3 weeks of growth an *F. oxysporum* spore suspension was prepared. The spore suspension was obtained by washing the mycelium with sterile water and shaking with glass pearls. Then the number of spores was determined using a hemocytometer chamber (Bürker, Labart, Gdańsk, Poland). Embryo axes of germinating seeds were inoculated with the spore suspension at a concentration of 5×10^6 spores per 1 mL. Inoculation was performed by injecting 10 µL of spore suspension into the upper part of the embryo axis of germinating seeds and additionally also by spraying the embryo axes of germinating with the inoculum.

Morphometric measurements of the embryo axes and the roots of yellow lupine seedlings

Morphometric measurements, i.e. the length and fresh weight of the embryo axes of germinating seeds and the roots of yellow lupine seedlings, both Diament and Mister varieties were done. Plant material were collected at 0, 24, 48, 72 and 96 h of culture. Then the results were the average of the measurements of at least 10 organs and were expressed as per 1 organ, i.e. in cm·organ^{-1} and fresh weight in g·organ^{-1}.

Detection of phytohormones concentration

Plant hormones salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA/MeJA), indole-3-acetic acid (IAA) were determined by modified QuEChERS method of[47] using internal deuterated standards and followed by liquid chromatography tandem mass spectrometry (LC–MS/MS). Approximately 100 mg of plant tissue was ground in liquid nitrogen and further homogenized with 1.6 mL of 80% (v/v) acetonitrile containing 1 mM 2,6-di-tert-butyl-4-methylphenol and 5% (w/v) formic acid. The homogenates were agitated overnight at 4 °C then about 300 mg of solid $MgSO_4$ and NaCl (3:1) were added. Samples were subsequently vortexed and agitated for 30 min at room temperature. The next homogenates were centrifuged at 10 000 × *g* for 10 min at 20 °C to separate water and acetonitrile phases and the upper acetonitrile phase was transferred to new tubes and evaporated to dryness under nitrogen stream at 45 °C. The remaining residue was suspended in 1 mL of 1 M formic acid and loaded onto Bakerbond SPE column pre-conditioned with 1 mL of methanol and 2 mL of 1 M formic acid. After sample application, the columns were washed with 2 mL of 1 M formic acid and the retained analytes were eluted with 0.5 mL of 80% (v/v) methanol. Elutes were then lyophilized and suspended in 100 µL of 35% (v/v) methanol with 0.1% formic acid (w/v) and analysed with LC–MS/MS.

Analysis was performed on a Shimadzu Nexera XR UHPLC system (Shimadzu, Kyoto, Japan) with Ascentis Express C-18 column (100 × 2.1 mm, 2.7 µm, Supelco, Bellefonte, PA, USA) as a stationary phase that was kept at 35 °C. The mobile phase consisted of (A) 0.1% formic acid and (B) methanol with 0.1% formic acid delivered as a binary gradient at a flow rate of 0.4 mL min^{-1}. The gradient started at 35% B, then was raised linearly to 90% B during the next 4 min, and then to 100% B during the next 2 min. The LC was interfaced with a triple quadrupole mass spectrometer (LCMS-8045, Shimadzu) operated in the positive and negative modes, with the electrospray as the ionization source. Multiple reaction monitoring (MRM) acquisition was done by monitoring the 211.3/133.30 m/z and 214.10/134.30 m/z transition for JA and d_5-JA, respectively, 137.30/93.00 and 141.40/97.10 for SA and d_4-SA, 263.00/153.00 and 269.20/159.05 for ABA and d_6-ABA, 176.20/130.30 and 178.20/132.30 for IAA and d_2-IAA. The peak area of the diagnostic product ions under optimized conditions was used for the phytohormones quantification. Data acquisition and processing was performed with LabSolutions software 5.8 (Shimadzu, Kyoto, Japan).

Detection of superoxide anion radical generation

The generation of $O_2^{\cdot-}$ was detected according to the method using the dye dihydroethidium (DHE, Sigma–Aldrich) described by[39] and[48]. DHE is non-fluorescent, can passively cross the membrane of live cells, and can be oxidized by $O_2^{\cdot-}$ to a fluorescent dye detected by laser confocal microscopy at Laboratory of Electron and Confocal Microscopy, Faculty of Biology, Adam Mickiewicz University. The fluorescence is proportional to the intracellular $O_2^{\cdot-}$ level. The production of $O_2^{\cdot-}$ in embryo axes of germinating seeds and the roots of seedlings was observed following the staining of surfaces with 100 µM DHE in 3 mM DMSO after immersing for 12 h at room temperature in darkness. After rinsing with 100 µM $CaCl_2$ solution, at pH 4.75, root sections were observed using a Zeiss LSM 510 confocal microscope, (objective magnification of 20x). An argon laser was used for excitation at 488 nm, with emission at 565–615 nm following background subtraction. All images were obtained at the same depth, and were analyzed by the LSM Image Browser software, version 4.2. It should be stressed that images presented in the study for all assays included fragments of embryo axes near the half-length. Fluorescence and confocal microscopy experiments were repeated three times with similar results.

Determination of soluble sugar contents

For determination of sucrose, glucose and fructose contents in samples, a mass spectrometer coupled to a liquid chromatograph (UPLC-MS/MS) method was used[49]. About 100 mg of embryo axes and the roots of yellow lupine (samples) were homogenized in liquid nitrogen and next with 1.5 mL of 50% (v/v) of ethanol. The samples were then agitated for 2 h at 10 °C, centrifuged at 10 000 × g for 10 min and 0.5 µl of supernatants were injected into the system. The concentration range used for sucrose and glucose quantification was from 1 to 100 µg ml^{-1}. Results were expressed as the mean and standard deviation of three replicates in mg g^{-1} of fresh weight. The LC–MS/MS analysis were performed with a Shimadzu Nexera XR UHPLC/LCMS-8045 system (Kyoto, Japan) with an Agilent Poroshell 120 HILIC-Z (Agilent) 100 × 2.1 mm, 2.7 µm column, maintained at 35 °C. The mobile phase was a mixture of (A) 0.1% ammonium hydroxide in water and (B) 0.1% ammonium hydroxide in acetonitrile applied at a flow rate of 0.35 ml min^{-1} in a 11 min gradient as follows: 0–4 min, 85–60% B; 4–6 min, 60% B; 6–11 min, 85% B. Analytes were ionized in negative mode as $[M–H]^-$. The mass spectrometer was operated under multiple-reaction monitoring (MRM) mode. The MRM transitions used for sugars quantitation were as follow: glucose and fructose 179.10/89.00; sucrose 341.00/88.90 m/z. The MS parameters for each analyte and analog were optimized separately by direct infusion of individual standard solution.

Statistical analysis

The normality of the distribution of the 11 observed traits was assessed using the Shapiro–Wilk's normality test[50]. A two-way (time, variant) multivariate analysis of variance (MANOVA) was performed to determine the overall effects of the time and variant as well as time variant interaction on the variability of the 11 traits. Two-way analysis of variances (ANOVA) were conducted to determine the main effects of both factors and their interaction on each individual trait. Mean values and standard deviation (s.d.) were calculated for each combination of time and variant. Fisher's least significant differences (LSDs) were estimated and homogeneous groups were designated based on these LSD values. Pearson's linear correlation coefficients were calculated to evaluate the relationships between the 11 observed traits and visualized using heatmap[51]. To compare the average values of individual characteristics between selected combinations of times and variants, appropriate contrasts were calculated using the two-sample *t*-test for all the traits studied. Canonical variate analysis was used for a multitrait assessment of the similarity between the combination of times and variants studied[52]. The Mahalanobis distances were calculated as a measure of "polytrait" variation across the combination of times and variants[53]. The statistical analysis was performed using GenStat 23rd edition[54]

Discussion

The results of this study for the first time show the mechanism of action of AgNPs on the growth of yellow lupine, in association with the changes in plant growth regulator concentration, i.e. GA_3 and IAA, and soluble sugars during the seed germination stage and development of the seedling. As a result of AgNPs application, very strong stimulation of the growth was observed both in the case of seedling roots of Diament as well as Mister variety (Fig. 1). The longest roots of lupine seedlings and the highest increase in fresh biomass were observed at 96 h of the experiment (Fig. 1). The melanization process progressing over time was also observed (Table 1). It is also noteworthy that at an earlier time point 72 h, the high level of GA3 in the the roots of seedlings of both tested yellow lupine varieties from AgNPs variant was detected (Fig. 4). GA3 is an important plant growth regulator that is actively involved in cell elongation, control various aspects of seed germination process, and other important physiological functions in plant growth and development[55]. In light of new literature data on the effect of metal-based NPs on plants as well as on the basis of our previous research[56]. It is suggested in the current study that AgNPs induced the phenomenon of hormesis in the case of yellow lupine embryo axes and seedling roots.

As reported Calabrese and Baldwin[57], the phenomenon of hormesis is very well know in biomedical literature, but becoming more broadly discussed also for biological research. Moreover, the concept of hormesis has generated considerable attention also in the area of nano-toxicology[58]. As reported[59]. AgNPs display unique biological activities and can be use as novel biostimulators. Stimulation of length and number of tomato (*Solanum lycopersicum*) the roots at a hormetic dose was observed. The published literature contains research results indicating that different types of ENMs, including AgNPs may induce a hormetic response in in vitro[60–62] and in vivo[63–69] models concerning both biomedical, biological and ecological studies[70]. It has been proven that hormetic effect was observed in the case of plants such as poplars (*Populus deltoides* × nigra) and *Arabidopsis thaliana* growing hydroponically on nutrient media, exposed to PEG-coated 5 and 10 nm AgNPs, and carbon-coated 25 nm AgNPs at a wide range of concentrations[71]. Moreover, the hormetic response caused by AgNPs was also demonstrated for common bean (*Phaseolus vulgaris*), corn (*Zea mays*) plants, vanilla (*Vanilla planifolia*) shoots, sugarcan (*Saccharum* spp.) shoots, seedlings of wheat[72–76]. On the other hand, the colloidal AgNPs induced also hormesis in the cells of human organism, e.g. in A549 human epithelial cells. The study of Sthijns et al.[62], provided also evidences for hormesis caused by AgNPs demonstrating that exposure to a low non-toxic dose of AgNPs might protect against a second toxic dose.

In view of the above, the regulation of growth and metabolism by AgNPs involves a hormetic effect. However, from literature reports it is also known that NPs may cause an increase in the uptake of mineral elements from the medium. The results obtained by[75] has proved that AgNPs are hypothesized also to enhance water and nutrient use efficiency in plants. These research revealed that AgNPs used in 25 ppm concentration caused significant increase leaves and highest grain yield.

In this study, quantitative analysis of phytohormones such as GA3 and IAA—playing important role in the germination process and the regulation of seedling growth and development showed fluctuations. However, after the application of AgNPs the highest level of the GA3 and IAA generally was found in 72-h roots of

seedlings both varieties, except for IAA in Mister variety. High contents of these growth regulators at 72 h may be associated with significant stimulation of the growth of seedling roots at 96 h.

The research performed by[77] showed that the application of NPs caused increase the synthesis of indole acetic acid (IAA) and gibberellins (GA), which in turn improved plant growth under drought stress. It has been proven that the application of NPs protected the membranes, enhanced water uptake, and maintained water relationship. Several reports suggest that proper concentrations of AgNPs are vital in increasing the efficiency of seed germination process, plant growth, improving chlorophyll content and photosynthetic efficiency, and increasing fertilizer and water efficiency[77–80].

Assuming the occurrence of hormesis based on our results of studies, apart of root growth stimulation by AgNPs, also stimulation of the cellular response was demonstrated (Figs. 1 and 3). Our the results revealed significantly enhanced the generation of phytohormones such as SA, ACC and JA/MeJA in the roots of seedlings both Diament and Mister cultivars at specific time points (Fig. 3). The increased concentration of these phytohormones may indicate that the applied dose of AgNPs in our experimental model system have the ability to induction of priming. In germinating seeds, endogenous hormones interact with each other, rather than working independently, to regulate the processes of plant development and defense response[81,82]. It should also be taken into account that the seed germination phase is the metabolically active phase. Additionally, our results from confocal microscopy showed that AgNPs caused increase in generation of $O_2^{\cdot-}$ in root cells of yellow lupine both Diament and Mister varieties (Fig. 10). In the control material, fluorescence indicating the presence of $O_2^{\cdot-}$ was reported only sporadically in the cells. Besides, analysis of confocal microscopy images revealed that $O_2^{\cdot-}$ was also involved in the defense mechanism of yellow lupine root cells infected with *F. oxysporum* (Fusarium variant) or pretreated with AgNPs and infected with *F. oxysporum* (AgNPs + Fusarium variant) of Diament variety. In the *F. oxysporum* variant, a strong signal indicating the generation of $O_2^{\cdot-}$ came from the entire cytoplasm and from the cell walls. This strong generation of $O_2^{\cdot-}$ was visible already at the beginning of inoculation in the Fusarium variant, the intensity of which decreased versus time and was limited to fluorescence originating only from the cell walls and nuclei. In the case of the AgNPs + Fusarium variant, a high level of $O_2^{\cdot-}$ generation was observed not only immediately after inoculation, but also at 72 hpi, with the signal coming mainly from the cell walls and nuclei at 0 h, and mainly from the protoplast at 72 h. In the case of the Mister variety, the signal was significantly weaker and limited to the early phase after inoculation. It was found that the strong postinfection generation of oxidative stress in the Diament variety may be related to the induction of the autophagy process in root cells inoculated with *F. oxysporum* from Fusarium variant (data prepared for publication).

As mentioned above, in this work it was also important understanding the role of AgNPs in the defense response of two yellow lupine varieties.Therefore, based on the analysis of disease symptoms (Table 1) and electronograms from transmission electron microscope (unpublished data, the results prepared for publication), it was found that the Diament lupine variety was more resistant than the Mister variety. Embryo axes and the roots of Diament variety pretreated with the AgNPs and infected with *F. oxysporum* (AgNPs + Fusarium variant), especially in early phase infection were more firm than infected with *F. oxysporum* (Fusarium variant). Data published by the Plant Breeding Company in Tulce show that the Diament variety is known as the sweetest variety because it contains the lowest alkaloid content. Analyses of sucrose and monosaccharides concentration showed that the content of these saccharides in seedling roots of Diament variety was the higher in relation to other experimental variants in 24-h embryo axes pretreated with AgNPs (AgNPs variant) (Fig. 5). Sugars are not only the primary substrate providing energy and structural material, but may also act as signal molecules interacting with the hormonal signaling network[83–85]. Moreover, sugars regulate cellular activity at multiple levels, from transcription and translation to protein stability and activity[86]. Also, sugars have multiple roles as nutrients and signalling compounds for immunity[36,87,88]. As reported by[89], many plant responses to the attack of a fungal pathogen are closely connected with the pathways regulating the level of sugar in the plant cell and ensuring energy homeostasis. Our research results has shown that between 0 and 24 hpi, a significant decrease in monosaccharide levels was observed in embryo axes pretreated with AgNPs and infected with *F. oxysporum* (AgNPs + Fusarium variant) of Diament variety (Fig. 5). A similar trend in glucose content in the 24-h embryo axes of the Mister variety from AgNPs + Fusarium variant occured. These results indicates that sugars were not only used in the plant's defense mechanism, but were also consumed by *F. oxysporum*. It is also worth noting that at 24 hpi in the roots of yellow lupine seedlings of the Diament variety from AgNPs + Fusarium and Fusarium variants, the level of sucrose and monosaccharides was lower than in the control, which confirms our conclusion. However, at subsequent time points an, alternating increase and decrease in these monosaccharides was observed, and again an increase at the last time point of the experiment. Concentration of the above sugars was significantly higher than in other experimental variants. In turn, the Mister variety showed very high monosaccharide accumulation between 0 and 72 hpi. Therefore, the reaction of these two lupine varieties is different, which is related to their different sensitivity to *F. oxysporum* infection and functioning of defense mechanisms. Mister variety has the highest seed crude fat content, protein content and low proportion of alkaloids. The increased resistance of Diament variety to *F. oxysporum* may be related to the sugar level. Our previous multi-year research revealed that sugar signals may contribute to immune responses against pathogens, including *F.oxysporum* and probably function as priming molecules leading to pathogen-associated molecular patterns (PAMP)-triggered immunity and effector-triggered immunity in plants. These putative roles also depend greatly on coordinated relationships with hormones and the light status in an intricate network. Additionally, our previous research results indicate that high sugar levels may lead to a reduced virulence of *F. oxysporum* f.sp. *lupini* through the limitation of mycotoxin production, infection and fusariosis development[85]. Also, according to the concept of Van den Ende[90] soluble carbohydrates can replace water (e.g. under drought stress) causing keep membrane surfaces "hydrated" and prevent membrane fusion by maintaining the space between phospholipid molecules[91]. Besides, small soluble sugars such as glucose, fructose, sucrose can also act as signalling molecules that change gene expression regulating plant metabolism, defense reaction, plant growth and development[87,92]. Additionally,

soluble vacuolar sugars may participate in vacuolar antioxidant processes, intimately linked to the well-known cytosolic antioxidant processes functioning during stress, which may contribute to increase plant tolerance to stress[90,91]. Our research results also showed that AgNP application caused increase in fresh weight of 48-h embryo axes and hypocotyls of Diament variety, both in the case of AgNPs and AgNPs + Fusarium varieties. Also, the application of *Trichoderma longibrachiatum*, similarly as AgNPs stimulated growth of seedlings (i.e. both shoot and root length) infected *F. pseudograminearum*[93].

Besides, there are evidences both from in vitro as well as in vivo studies, that AgNPs exhibit a significant inhibitory effect on the growth and development of *F. oxysporum*[94]. It was observed in the case of different pathosystem but this effect depended on the used concentration of AgNPs. For example, experimental results of[95] revealed that AgNPs at concentration of 80 µg/mL inhibited mycelial growth of *F. oxysporum* by 50% in relation to the control. Besides, electron microscopy observations demonstrated that AgNPs had a damaging effect on fungal hyphae, causing deformation of cell membranes. Also, the research by Huang et al.[96] showed that AgNPs at a dose of 400 µg/ml caused the inhibition rate of mycelium growth by 61% in relation to the control, while complete inhibition of conidia germination occurred at 100 µg/ml of AgNPs. Furthermore, concentrations of 75–150 mg/L AgNPs significantly limited the development of *F. oxysporum* f.sp. *lycopersici* in vitro on PDA plates[97] (Sánchez et al. 2023). Moreover, the mechanisms of AgNPs action on fungal pathogens, including *F. oxysporum* can be diverse. Frequently, it involves damage to the cell wall and the cell membrane of the pathogen. Electron microscopy observations revealed that AgNPs cause accumulation of vacuoles and vesicles in fungal cells, leading to the leakage of cellular contents[98]. AgNPs can also induce the production of reactive oxygen species (ROS) in plant host, which are toxic to fungal cells, leading to damage of proteins, lipids, and DNA, ultimately causing pathogen cell death [[99] and literature cited in this review]. AgNPs may interfere with fungal metabolic pathways, e.g. AgNPs can disrupt cellular respiration processes and energy production in fungal cells[94,98]. The precise mechanism of AgNPs action on *F. oxysporum* cells remains the subject of detailed research. However, the multidirectional mechanism of action allows for avoiding pathogen resistance development, making AgNPs a promising agent in combating fusariosis. Their efficacy and the difficulty in developing resistance by pathogens suggest that AgNPs may serve as an alternative or complement to conventional fungicides in plant protection.

Conclusions

The results of this study indicate that the applied dose of AgNPs caused significant stimulation of the growth in comparison to the control and other experimental variants; it indicates hormesis phenomenon. Moreover, application of AgNPs enhanced plant metabolism, i.e. induced generation of $O_2^{\cdot-}$ and caused accumulation of selected defense-related phytohormones at specific time points. Besides, the results of our research demonstrated that AgNP alone induced better trurgor, lateral root reduction and root melanization in comparison to the control. Additionally, AgNPs regulated yellow lupine defense responses to hemibiotrophic pathogen, *F. oxysporum*. The differences were found in the response of Diament and Mister varieties to the application of AgNPs and in the defense response to fungal pathogen. The results obtained showed that ABA plays an important role in defense response of the roots both varieties of yellow lupine, pretreated with AgNPs and infected with *F. oxysporum* (AgNPs + Fusarium variant). It has been demonstrated that application of the AgNPs caused the changes in soluble sugar levels, metabolites playing key roles in the defense mechanisms of host plant. The response of yellow lupine both Diament and Mister varieties to AgNPs or fungal pathogen *F. oxysporum* or cross-talk of AgNPs and *F.oxysporum* was dependent on the time period after AgNPs pretreatment and the time after inoculation. Besides, analyzing defense responses of the above lupin varieties, similar trends were found over time in the case of biochemical indicators tested, as well as significantly different ones. The results of our the research will improve understanding of the plant–hemibiotrophic pathogen interactions under AgNPs and provide novel information on host plant, yellow lupine and *F. oxysporum* pathosystem.

Data availability

All data included in this study are available upon request by contact with the corresponding author.

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Author contributions

Anielkis Batista: conceptualization and designed the experiments, performed experiments, analyzed the data, wrote the paper and contributed to the discussion on the data, editing and performed literature analysis. Jacek Kęsy: contributed to the research methodology and measurements of phytohormone and soluble sugar concentration. Katarzyna Sadowska: performed inoculation; culture of Fusarium oxysporum f.sp. lupini and preparation of spore suspension and inoculation, participation in the preparation of hydroponic culture, collection plant material for analyses and preparation of references section. Zbigniew Karolewski: performed inoculation, participation in the preparation of hydroponic culture and collection plant material for analyses. Jan Bocianowski: performed statistical analysis of the data and contributed to performing elementary comparisons between particular levels of analyzed factors, prepared Tables, figures and the table S1 to supplementary, performed the formal analysis and proofread the manuscript. Agnieszka Woźniak: prepared figures. Iwona Morkunas: conceptualization and designed the experiments, performed experiments, research management, analyzed the data, wrote the paper, especially significant contribution to the result and discussion section, performed the formal analysis, edited the whole manuscript, and proofread the manuscript.

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Declarations

Competing interests

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