

Article

Nano-Silver Particles Reduce Contaminations in Tissue Culture but Decrease Regeneration Rate and Slows Down Growth and Development of *Aldrovanda vesiculosa* Explants

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Abstract: *Aldrovanda vesiculosa* is a carnivorous water plant which is endangered by extinction worldwide. The number of natural stands and populations has decreased; therefore, there is a need for its active protection. The best method would be an in vitro culture. One of the main problems is disinfection of the explants. Therefore, it was decided that we should treat the explants with nano-silver particles. The explants were shoot fragments which were disinfected with sodium hypochlorite and then placed in a liquid 1/5 MS medium, supplemented with silver nanoparticles (AgNPs) at a concentration of 5 mg·dm⁻³. It was observed that AgNPs reduced the number of contaminations but also led to necrosis of the shoots. The shoots, which undertook regeneration in presence of AgNPs, were smaller and did not form traps; however, after being moved to fresh media twice, they started to develop normal leaves. Taking into consideration both disinfection and regeneration rates, it might be advisable to disinfect *aldrovanda* shoots in sodium hypochlorite only, without AgNPs. The results of the research might indicate a toxic activity of AgNPs towards water plants, which seems a big problem, as nanoparticles are commonly used in all the fields of life. However, the matter should be studied further.

Keywords: AgNPs; disinfection; initiation; in vitro; micropropagation; nanomaterials; nanoparticles; waterwheel plant



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1. Introduction

Aldrovanda vesiculosa L. (Droseraceae), a waterwheel, is a carnivorous water species endangered by extinction all over the world, and it is under protection in all European countries. One of the biggest populations of the species is located in East Poland. Unfortunately, the number of natural stands and populations of *Aldrovanda* has decreased, both in Poland and worldwide [1]. According to Kamiński [2], in 1987, there were 14 confirmed localities of the waterwheel and, nowadays, there are only six left. The author investigated the factors affecting the survival of *aldrovanda* in the temperate climate of Poland, and suggested that the species should be reintroduced into new, better, and substitute localities. *Aldrovanda vesiculosa* does not form mature seeds in the Polish climate; therefore, it may be propagated only vegetatively. As the number of existing donor plants is low, it is important to use the methods which are the least harmful to the plants. Propagation in vitro allows for the production of the critically endangered plant species, as it allows us to obtain a lot of good quality and healthy plantlets, with no harm to the mother plants. Tissue culture has been commonly used for the propagation of many rare and endangered plant species [3–5].

There is little information on the micropropagation of *A. vesiculosa*. Kondo et al. [6] described the disinfection method and initiation of the *aldrovanda* cultures from seeds of the Japanese race of the species. The authors disinfected seeds with 1% benzalkonium chloride, sodium hypochlorite, and ethanol. The seeds were placed in B5 Gamborg liquid medium with 2% sucrose, the addition of 20–50 mg·dm⁻³ of gibberellic acid, and a few

drops of antibiotic. After a day, the seeds were individually placed in the fresh medium of the same composition and kept at 22 °C under a light. After a week, 60% of the seeds germinated and formed shoots and proliferated. The authors managed to obtain regeneration from turions as well, but, at the same time, they stated that the growth of the Polish race is different.

Adamec et al. [7] studied the *in vitro* cultivation of aldrovanda, and they proposed the media for micropropagation of the species. The authors used the plants obtained from the Kondo cultivation. The authors studied the medium for aldrovanda micropropagation and suggested that the half strength of B5 with only 500 mg·dm⁻³ KNO₃ was the best. They attempted to initiate the cultures from seeds but stated that, due to a high contamination rate (52.3%) and low germination (44.4%), the method was not reliable. Therefore, the authors advised using defoliated shoots and mild surface sterilization. However, the method itself was not provided.

Plant tissue cultures are commonly applied not only for the conservation of endangered plant species, but for wider use in the propagation of crop species, the production of bioactive compounds, or as models for scientific studies. However, to make all that possible, the plant material has to be disinfected before tissue culture initiation. That stage might decide the success of micropropagation, as only contamination-free cultures are used for further regeneration of plant material, which would lead to obtaining healthy and good quality plants.

To obtain *in vitro* cultures free of contaminations, the plant material has to be surface disinfected. It is extremely difficult to find a balance between the use of disinfection substances strong enough to get rid of any microorganisms, and mild enough to not kill the plants. The disinfection part is also highly important because remaining microorganisms usually grow fast in tissue culture. There are many sterilizing substances used for the surface disinfection of plant material. The most often used are calcium hypochlorite (CaOCl), sodium hypochlorite (NaOCl), ethanol (EtOH), hydrogen peroxide (H₂O₂), mercuric chloride (HgCl₂), silver nitrate (AgNO₃), fungicides, and antibiotics. Some disinfecting substances are harmful to plant tissues and, therefore, they negatively affect organogenesis, or even lead to necrosis due to their phytotoxicity. As such, the type, concentration and exposition time of disinfectants are important. Sometimes, it seems almost impossible to obtain pathogen-free cultures. Bacteria seem especially difficult to eliminate [8]. Therefore, researchers and plant producers look for new methods and substances.

One of the latest ideas is the use of nanomaterials, which are substances of very small size (1–100 nm) and unique properties [9]. There are different nano-substances, metals, and metal oxides, including silver (Ag), gold (Au), zinc (ZnO), copper (Cu), titanium dioxide (TiO₂), magnesium oxide (MgO), nickel (Ni), iron oxide (Fe₃O₄), and silicon (Si) [10,11]. At present, nanotechnology is present in every aspect of life—medicine, food, or the environment—and nanomaterials are available in different forms, such as powder, particles, clusters, tubes or films, and sizes. The best-known activity of nanoparticles (NPs) is its ability to eliminate different microorganisms [12]. Silver nanoparticles are the most often used nanomaterials in different sectors, especially in agriculture [13].

In the plant production sector, silver nanoparticles (AgNPs) were developed as plant growth stimulators [14] or components of new fertilizers [15], as well as plant protection products [16,17] and herbicides [18]. Recently, nanoparticles have been suggested as biostimulators that might improve plant propagation [19,20] or enhance fruit ripening [21,22]. NPs have also been used in plant tissue culture to improve plant growth, increase yield, improve seed germination [23], enhance bioactive compounds production, and enable genetic transformations [10,24,25]. AgNPs have been proven to reduce microbial contaminations in various plants and may be used for surface disinfection of explants. The antimicrobial property of silver NPs seems well studied. Its effectiveness depends on size, dimension, and type of nanoparticles [25]. According to Sarmast et al. [26], AgNPs show a high ability to eliminate microorganisms without negative effects on plant growth and development, but many researchers have studied the influence of AgNPs on explant disinfection and

plant regeneration, and they indicated both positive and negative effects in the case of plants. Shokri et al. [27] suggested that substances used for sterilization are not neutral for the environment, humans or animals. Kim et al. [25] indicated that the influence of NPs on explants excised from different plant species should be studied to determine a proper method for AgNPs' application in plant material disinfection and cultivation, especially given that plants are a basic element of the world and a source of food. Knowledge of AgNPs' influence on plants is therefore important, especially because the common use of AgNPs increases the chance that they would be released into the environment [28–30]. Silver is the second most toxic metal to aquatic organisms after mercury [31], and, therefore, further studies are needed to clarify these contradictory observations concerning growth stimulation or inhibition of AgNP-treated plants [32]. Recently, there has been a different, green approach proposed. There are three possible means of nanoparticles synthesis: physical, chemical, and biological. The last one, concerning the biogenic synthesis of NPs, is described as nontoxic [33]. The “green biotechnology” methods include obtaining nanoparticles from plants' organs, algae, or even microbes. It was possible to obtain AgNPs from the *Daucus carota* extract [34] or wheat straw extracted lignin [35]. The “green” methods are promising, but there is still little information on the subject.

The presented study aimed to evaluate the influence of the addition of nano-silver particles into the media on the survival rate of explants, tissue culture initiation, and regeneration ability, and then to evaluate the morphological features of regenerating shoots of *Aldrovanda vesiculosa* in tissue culture.

The manuscript describes two important matters. The described study is, to my knowledge, the first research concerning the influence of AgNPs on water plant growth and development in the presence of AgNPs. The toxicity of NPs towards such plants is of great importance, as the extensive use of these substances creates a risk that their residues might infiltrate natural water tanks and threaten plants, and, in turn, jeopardize biodiversity and the environment.

The other achievement described concerns the micropropagation of *Aldrovanda vesiculosa*, a carnivorous water plant species which is critically endangered by extinction. Because only a little amount of plant material is available, it is difficult to conduct experiments. Due to the hydration of tissues and content of different aquatic microorganisms and lake water in the traps, it is extremely difficult to obtain contamination-free in vitro cultures.

2. Materials and Methods

The plant material was *Aldrovanda vesiculosa* shoots with apex, collected from the Orchowe lake in East Poland, in June. The shoots were transported immersed in lake water in plastic containers. In the laboratory, the shoots were defoliated and the tip parts of 2–3 cm in length were used for the experiment. The explants were disinfected in the following steps: 1—rinse in water with a few drops of Ludwik (GRUPA INCO S.A., Warszawa, Poland), twice for 10 min; 2—dip in 70% ethanol (Chempur, Piekary Śląskie, Poland) for a few seconds; 3—disinfect with a 0.5% of sodium hypochlorite (NaOCl), (Chempur); 4—rinse 3 times in sterile distilled water and then place in a liquid media, described below. After 24 h, in the laminar flow chamber, the explants were taken out from the media and immersed for a few seconds in 0.5% NaOCl, 70% ethanol, or 0.5% NaOCl, followed by 70% ethanol. Then the explants were placed in the fresh liquid media of the same content, individually in the Erlenmeyer flasks of the 100 mL volume. Half of the media was supplemented with silver nanoparticles (AgNPs) solution (Sigma-Aldrich, Saint Louis, MO, USA, catalog no. 576832), at a concentration of $5 \text{ mg} \cdot \text{dm}^{-3}$.

The media used in the experiment (1/5 MS) consisted of 5-times diluted Murashige and Skoog (MS) [36] macro- and micronutrients composition, which is the most popular media and is recognized as suitable for most plant species. The media was diluted because *Aldrovanda vesiculosa* grows in peat bogs and lake water poor in nutrients, and, as a carnivorous plant, it is sensitive to high amounts of nitrogen.

The medium was supplemented with thiamine (vit. B₁)—0.1 mg·dm⁻³ (Sigma-Aldrich, Saint Louis, MO, USA), pyridoxine (vit. B₆)—0.5 mg·dm⁻³ (Sigma-Aldrich, Saint Louis, MO, USA), niacine (vit. PP)—0.5 mg·dm⁻³ (Sigma-Aldrich, Saint Louis, MO, USA), glycine—2.0 mg·dm⁻³ (Chempur, Poland), myo-inositol—100 mg·dm⁻³ (Sigma-Aldrich, Saint Louis, MO, USA), and sucrose—20 g·dm⁻³ (Chempur, Piekary Śląskie, Poland). The media pH was established at 5.5 and steam sterilized for 21 min at the temperature of 121 °C and under 1 hPa pressure. The flasks with explants were placed in a growing room at the temperature of 25 °C with 16-h photoperiod. The sources of light were fluorescent day-light lamps with 30 μmol·m⁻²·s⁻¹ light intensity (Philips TLC 58W/84).

The experiment lasted for 8 weeks. The observations concerning the contamination rate and regeneration of the explants were performed once a week. At the end of the experiment, the following features were evaluated: the number of explants with the symptoms of contamination, the number of explants with the symptoms of necrosis, and the number of regenerating shoots. Morphological observations regarding the length of shoots, length of internodes, and length of petioles and traps, were also performed with the use of a Keyence VHX-950F stereoscopic microscope.

The regenerated and contamination-free shoots were placed in the fresh liquid media of the same macro- and micronutrient content, but without the addition of AgNPs, for further growth. Observations were performed after the next 8 weeks and the regenerated shoots were again placed in the fresh media. Passages were completed every 8 weeks, as it was the time when the regenerating shoots were long enough to be divided. Each time, only the green parts were used.

3. Results

It was noted that the sterilizing substance type and the presence of silver nanoparticles (AgNPs) in the media influenced disinfection efficiency (Table 1).

Table 1. The contamination rate and regeneration of *Aldrovanda vesiculosa* in vitro, depending on the use of sodium hypochlorite (NaOCl) or ethanol (EtOH) for explants disinfection, and the addition of silver nanoparticles (AgNPs) to the 1/5 Murashige and Skoog (MS) culture media.

Disinfecting Substance	AgNPs	No. of Contamination-Free Explants (%)	No. of Necrotic Explants (%)	No. of Regenerating Explants (%)
NaOCl	–	61	36	25
	+	77	58	19
EtOH	–	12	0	12
	+	24	24	0
EtOH-NaOCl	–	62	50	12
	+	100	100	0

Based on visual observations, it was noted that the highest number of explants without the symptoms of disinfection was obtained when they had been disinfected with both ethanol and sodium hypochlorite and placed in the media supplemented with silver nanoparticles (100%). Unfortunately, that mixture caused necrosis in all the explants. Comparing the number of contamination-free explants, it might have been observed that sodium hypochlorite allowed us to obtain more disinfected stem pieces than did ethanol (138 in comparison to 36). Ethanol was the least efficient in the disinfecting of explants, as only 12% were free of fungus or bacteria. The addition of AgNPs increased the number of disinfected explants up to 24%. Looking at the data obtained, it was observed that supplementation of the media with AgNPs allowed for the obtaining of a higher number of disinfected explants in all treatments, but, at the same time, it decreased the number of regenerating shoots.

The highest number of regenerating explants was obtained when sodium hypochlorite was used for disinfection. Based on visual observations, it was noted that the addition of silver nanoparticles to the media influenced the morphological development of the shoots (Table 2, Figures 1 and 2).

Table 2. Morphological features of the regenerating explants of *Aldrovanda vesiculosa*, depending on the addition of AgNPs to the 1/5 MS culture media.

Media	Morphological Features of <i>Aldrovanda</i> Shoots
1/5 MS	Shoots are properly developed with visible traps The average stem length (green part): 112 mm Visible lateral shoots The average length of internodes: 2.48 mm The average length of petioles: 1.79 mm The average length of traps: 1.52 mm
1/5 MS + AgNPs	Shoots are smaller, no traps formed The average stem length (green part): 8.24 mm The average length of internodes: 0.5 mm

Shoots cultivated in the media supplemented with AgNPs regenerated much slower. They were smaller, with an average stem length of 8.24 mm and short internodes, less than 1 mm, while those cultivated in the media without AgNPs were much longer (over 10 cm), with longer internodes (2.48 mm on average). The biggest disadvantage of the use of silver ions was the lack of traps, which, in the case of a carnivorous plant species, allow to obtain nutrients. Shoots cultivated in the media without AgNPs formed traps on most of the shoots, and they were 1.52 mm long.



Figure 1. Morphological features of the regenerating explants of *Aldrovanda vesiculosa*, depending on the addition of silver nanoparticles (AgNPs) to the 1/5 MS culture media.

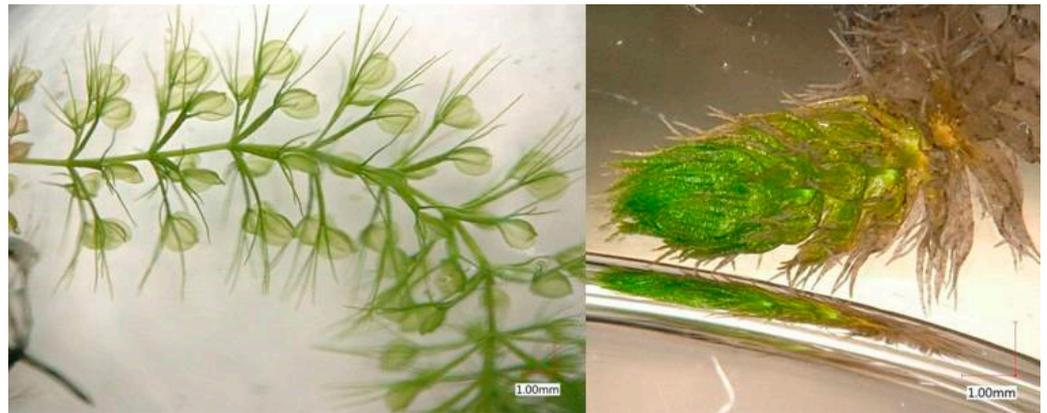


Figure 2. The influence of the AgNPs 1/5 MS media supplementation on the *Aldrovanda vesiculosa* trap formation in tissue culture.

The shoots which regenerated after disinfection were placed in the fresh media and undertook further growth. Shoots that had been cultivated without the addition of AgNPs in the media grew normally, with a production of lateral shoots, while those that had been cultured in the presence of AgNPs were much smaller, with shorter internodes and no traps (Figure 3). However, after the second passage, the shoots started to produce longer petioles and traps (Figure 4).



Figure 3. Subsequent influence of the AgNPs 1/5 MS media supplementation on the *Aldrovanda vesiculosa* morphological features of shoots after the first 8-week passage in the AgNPs-free media.



Figure 4. Subsequent influence of the AgNPs 1/5 MS media supplementation on the *Aldrovanda vesiculosa* morphological features of shoots after the third 8-week passage in the AgNPs-free media.

4. Discussion

Disinfection of plant material is a crucial step in plants' micropropagation, as only contamination-free explants may be used for tissue culture initiation. It is especially important in the case of plants endangered by extinction, for which only a limited amount of source explants are available. A reliable method of surface sterilization guarantees the success of tissue culture initiation, with no harm to donor specimens.

The presented paper concerns a disinfection method for *Aldrovanda vesiculosa* tissue culture. The species is critically endangered by extinction and present in only a few stands worldwide; therefore, it is important not to reduce existing populations. There is little information regarding surface sterilization methods for *A. vesiculosa*, and none of them concern the European races. Kondo et al. [6] initiated a tissue culture of the Japanese Hanyu race of aldrovanda from seeds, which were collected and surface sterilized with 1% benzalkonium chloride for 5 min, then 1–2% of sodium hypochlorite solution for 5 min, and then 70% ethanol for a few seconds. Next, the seeds were rinsed in distilled water 3–4 times and placed in a B5 Gamborg liquid medium supplemented with 2% sucrose, 20–50 mg·dm⁻³ of gibberellic acid, and some drops of penicillin and streptomycin, with the pH set up to 5.5 for a day. The next day, each seed was placed individually in the same medium in tubes and shaken under the light. After a week, 60% of the seeds germinated without any symptoms of contamination. The authors tried to initiate the cultures from shoot tips, but they failed, and stated that it was too difficult, as they contained many aquatic microorganisms in the traps. The authors managed to initiate the cultures from winter buds with the same method as seeds. At the same time, the authors stated that the growth of the Polish race is different.

The studies on the Polish race of *A. vesiculosa* were conducted by Adamec et al. [7]. However, the authors used the plant material obtained from Kondo. They tried to obtain a disinfected in vitro culture of aldrovanda from seeds, and they obtained over 44% germinated seedlings and over 52% contaminations. The authors stated that it was not sufficient, and recommended the use of defoliated shoot pieces and mild surface disinfection. However, the method was not described.

As the disinfection of aldrovanda is difficult and there is little plant material available, we decided to use silver nanoparticles to surface-disinfect the explants. Unfortunately, the use of AgNPs was disadvantageous, as it led to the obtaining of a high number of necrotic

explants and inhibited regeneration of shoots. One of the latest ideas is the application of nanomaterials for the surface sterilization of plants.

There have been many experiments carried out to study the influence of AgNPs as a disinfection agent for plant material, and the harmful effects of nano-silver were proved on more than 600 microorganisms. Abdi et al. [37] applied AgNPs for the first time to control bacterial contaminations in *Valeriana officinalis*. They obtained single node explants from plants grown in a greenhouse and surface disinfected with 70% EtOH for 1 min, 10% Clorox for 1 min, and then applied AgNPs at a concentration of $100 \text{ mg}\cdot\text{dm}^{-3}$ for 180 min. They obtained 89% contamination-free cultures and nano-silver did not affect either regeneration or rooting of plants. Similarly, Taghizadeh and Solgi [38] noted a positive effect of AgNPs application in *Cynodon dactylon* "Teefgreen". They treated explants with 30% Clorox for 15 min, then rinsed them in distilled water and soaked for 30, 60, or 120 min in 100 or $200 \text{ mg}\cdot\text{dm}^{-3}$ AgNPs. The application of $200 \text{ mg}\cdot\text{dm}^{-3}$ of the solution decreased the total number of contaminations (11.1%), especially fungi ones (0%), which are the most common contaminants during tissue culture initiation. Moradpour et al. [39] obtained the highest percentage of disinfected and regenerating explants of *Hevea brasiliensis* when AgNPs were used at a concentration of $10 \text{ mg}\cdot\text{dm}^{-3}$ for 20 min, in comparison to mercuric chloride or sodium hypochlorite. The effect of AgNPs application was studied by Mahna et al. [40] in *Arabidopsis thaliana* seeds, potato leaves, and tomato cotyledons. The authors noted that the use of $100 \text{ mg}\cdot\text{dm}^{-3}$ for 1 or 5 min was ideal for disinfection, and the treatment had no negative effect on explants.

AgNPs are also applied in media supplementation. Sarmast et al. [26] observed that disinfection of *Araucaria excelsa*, combined with the immersion of explants in $200 \text{ mg}\cdot\text{dm}^{-3}$ AgNPs for 180 min, reduced the contamination rate from 61.5% to 11.3%, while the addition of $400 \text{ mg}\cdot\text{dm}^{-3}$ in the media decreased the contamination rate from 81.25% to 18.75%. Both methods were also used by Arab et al. [41], and the authors noted that both methods—explants immersion and media supplementation—reduced contamination rate in almond \times peach rootstock. Parzymies et al. [42] used AgNPs both as immersion and media supplementation in *Pennisetum alopecuroides* tissue culture initiation. They obtained the highest number of contamination-free explants when the explants were soaked in $100\text{--}250 \text{ mg}\cdot\text{dm}^{-3}$ AgNPs solution, or when the AgNPs were added to the media at a concentration of $4 \text{ mg}\cdot\text{dm}^{-3}$ for nodes and $16 \text{ mg}\cdot\text{dm}^{-3}$ for tillers. Spinoso-Castillo et al. [43] proved that the addition of 50 to $200 \text{ mg}\cdot\text{dm}^{-3}$ AgNPs to the medium completely decontaminated cultures of *Vanilla planifolia*. Supplementation of media with AgNPs was also used in the presented experiment, and it was observed that the treatment reduced the contamination rate, which confirms the results of other researchers.

The addition of AgNPs to the media, apart from reducing the contamination rate, may influence the growth and development of explants. Shokri et al. [44] noted that bacterial contamination was significantly reduced in the case of *Rosa* hybrid explants when they had been soaked in a solution of $200 \text{ mg}\cdot\text{dm}^{-3}$ of AgNPs for 20 min. However, supplementation of the medium with $100 \text{ mg}\cdot\text{dm}^{-3}$ AgNPs not only reduced the contamination rate but inhibited the phenolic compounds' exudation as well.

The positive effect of the AgNPs media supplementation was observed in many plant species cultivated in vitro. In the case of *Tecomella undulata*, the number of regenerating shoots and their number increased when the MS medium contained $10 \text{ mg}\cdot\text{dm}^{-3}$ AgNPs [45], while the use of a higher concentration, $60 \text{ mg}\cdot\text{dm}^{-3}$, enhanced the number of shoots and their length [46]. The positive effect of the AgNPs use was also noted in the case of cowpea and brassica [47].

However, the use of nanomaterials gives rise to many doubts and questions, and they might be deposited in tissues [48]. The phytotoxicity of AgNPs is assessed with such features as growth potential, seed germination rate, biomass, or leaf surface area [49–51].

In the presented experiment, the use of AgNPs reduced the contamination rate of explants but noticeably inhibited the regeneration capacity and growth of shoots. A similar effect was proved by many researchers. Spinoso-Castillo et al. [43] studied the influence of

AgNPs on *Vanilla planifolia* shoot regeneration and they obtained the highest shoot number of shoots, over 14, when the media contained 25 or 50 mg·dm⁻³ AgNPs, but the use of nano-silver at a higher concentration of 200 mg·dm⁻³ decreased the number of shoots to 4.55. In the case of *Lupinus termis*, the use of 0.1 to 0.5 mg·dm⁻³ of AgNPs reduced shoot and root elongation and fresh weight [51]. AgNPs reduced plant height and biomass in *Capsicum annuum* exposed to AgNPs 0.01 to 1 mg·dm⁻³ [22]. AgNPs inhibited the growth of many other plant species, such as *Cucumis sativus* [52], *Phaseolus radiates*, *Sorghum bicolor* [53], and *Lemna minor* [54].

5. Practical Applications and Future Research Perspectives

The results obtained in the presented experiment may be used for the tissue culture initiation of *Aldrovanda vesiculosa* worldwide, which may lead to active conservation of the species through in vitro propagation and reintroduction of the obtained plants into their natural habitat.

The conducted experiment has shown that AgNPs added to tissue culture media inhibited the growth and development of waterwheel plants. In the case of that carnivorous species, the lack of traps might prevent the plants from catching their prey. Therefore, further research should be conducted concerning the effect of AgNPs and other nanoparticles used at different concentrations on the development of both *aldrovanda* and other plant species in order to find a safe way to use these novel substances.

6. Conclusions

Nanomaterials have great potential to be used for plant tissue culture to disinfect the plant material, but their effect has to be tested on each plant species. Undoubtedly, AgNPs reduce microbial contaminations during tissue culture initiation, making it possible to establish a healthy culture but, on the other hand, they may have a negative effect on further regeneration of explants. In the case of *Aldrovanda vesiculosa*, it might be concluded that the inhibition of the regeneration capacity of explants outweighs the disinfection ability of the AgNPs used as a media supplementation. Therefore, it is advisable to surface-sterilize explants with sodium hypochlorite only.

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