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Studying the effect of ionic form and nano silver and lead on micropropagation stages of ornamental plant Brassica oleracea var.acephala

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ABSTRACT

Introduction: The technology of in vitro plant culture by supplementing classical breeding methods has been widely accepted for genetic modification and plant improvement. The ornamental Kale (Brassica oleracea var. acephala) has considerable attention due to its properties such as colorful aerial parts, durability, and good resistance to adverse environmental conditions. The role of different forms of heavy metals in in vitro culture is still unclear. Therefore, there are conflicting reports including positive and negative effects of these compounds on plant in vitro culture. The purpose of this research was to investigate the effects of lead nitrate [$Pb(NO_3)_2$], silver nitrate ($AgNO_3$), and silver nanoparticles (AgNPs) on in vitro cultureof Brassica oleracea var. acephala.

Materials and Methods: This study was conducted in two separate experiments comprising: applying treatments on callus induction and applying treatments on shoot regeneration. $Pb(NO_3)_2$, $AgNO_3$ and AgNPs were added at differentiation concentrations (0, 10, 25, and 50 mg L-1) in MS media. The traits such as browning index, pathogen contamination, rooting percentage, callus diameter, callus growth, fresh and dry weight, callus induction, shoot and root regeneration were evaluated after 30 days from the culture.

Results: The results indicated the positive effect of heavy metals in both nano and ionic metals forms. It seems that the concentration of silver nanoparticles should be considered lower due to its greater efficiency in stimulating shoot regeneration.

Conclusion: However, the achievements of these experiments can be the initial basis for other research in the field of the application of nano forms in plant in vitro culture.

1. Introduction

One of the important aspects of plant biotechnology techniques is the culture of cells, tissues, and plant organs in a controlled, in vitro conditions. The technology of in vitro plant culture, by complementing classical breeding methods, has been widely accepted for genetic modification and plant improvement [1]. Plant tissue culture or in vitro culturinvolves all methods of growing cells, tissues, or plant organs in a specific way within a controlled environment for the mass production of uniform plant materials [2]. The in vitro culturtechnique has commercial importance because it enables the rapid production of many new plant varieties

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in a limited and controlled laboratory space and/or the production of pathogen-free plants. Besides, saving time and production costs are among the advantages of tissue culture [3]. Utilizing of in vitro culture creates a key difference between plant propagation in the field and in the laboratory environments [2]. Almost all transgenic plants are generated through in vitro culture processes, making the optimization of micropropagation in different plant species a vital and important role in the success of gene transfer methods and cell studies [4, 5].

Nanotechnology has created a wide range of new applications in biotechnology and agricultural industries because of its possess unique physicochemical properties, including a high surface area, high reactivity, variable pore size, and particle morphology. The size of nanoparticles ranges from 1 to 100 nanometers and exists in various forms such as organic compounds, metals, and metal oxides. Nowadays, silver nanoparticles (AgNPs) play a significant role in industrial applications [6]. The mechanism of AgNPs is not yet fully understood, but it has been suggested that these particles gradually release silver ions (Ag+), which can disrupt microbial cell structures. Therefore, AgNPs is used to control microbial contamination. Metallic silver is valuable, but under normal conditions, its concentration poses no toxicity risk and does not inhibit plant growth [7-9].

Among the major species of Brassicaceae, six species including (Brassica rapa, B. nigra, B. oleracea, B. carinata, B. juncea and B. napus) are widely used worldwide as fodder, oilseed, spices and ornamental plants. Three of them are diploid including (B. nigra, B. rapa, and B. oleracea), while the other are allopolyploid. However, many researches have confirmed their genetic relationships. The kale or mustard family includes many economically important plant species. An ornamental variety of this genus, known as ornamental kale, is part of this group [10, 11]. Brassica oleracea var. acephala belongs to the family Brassicaceae and is a high-potential plant known as ornamental kale [12].

In vitro culture has many applications in plant breeding, including the Brassicacea family. Researchers have used microspores or stamen as explants in somatic embryogenesis in Brassica species [13-16]. There are limited reports on the using of other explants such as hypocotyls [17], protoplasts [18], and immature cotyledons [19] in B. napus. The somatic embryogenesis was performed from cotyledon explants in ornamental kale [20]. Somatic embryos were also obtained using hypocotyl explants in cauliflower [21, 22]. Krzyżanowska et al. (2006) indicated an efficient method for plant regeneration in cabbage through embryogenesis from stamen explants by B5 medium [23]. In another study, different genotypes of ornamental kale were investigated by application of stamen culture process. In this experiment, six genotypes with the highest embryo production rate were identified [24]. The seed explants were used in Brassica juncea L. in in vitro culture. Results showed that this protocol is effective in genetic improvement with a transgenic approach because the viral infections are controlled in this method [25]. Brassica oleracea var. capitata has also been used to introduce an efficient regeneration protocols [26], including: meristematic apex [27], roots [28, 29], lateral buds [28], androgenic embryos [23], immature zygotic embryos [30], leaf and petiole [31]. Some researchers studied the effect of medium, developmental stage, embryo age, cold treatment and medium supplements on the regeneration of plants derived from microspore in four F1 hybrids of ornamental kale. For instance, solid B5 medium containing 1% agar showed the best response in all genotypes. Also, cold treatment at 4°C increased the regeneration rate of plants by 73% [32]. The microspore culture was studied in 63 ornamental kale genotypes and the results showed that embryogenesis was induced in NLN culture medium in only six genotypes [33]. Taghizadeh and Solgi (2015) showed that the application of 0.1 mgL-1 benzyladenine (BA) in combination with 1 mgL-1 2,4-D provided acceptable regeneration in ornamental kale. Moreover, the enrichment culture medium supplemented with 0.5 mg L-1 benzyladenine and indolebutyric acid (IBA) caused the development of shoots in ornamental kale [34]. An efficient protocol for shoot regeneration of Brassica juncea L. was developed using organic medium components and growth promoters from vermicompost and coelomic fluid. Formulated organic plant tissue culture media (vermicompost extract (30%) supplemented with 20 mgL-1 coelomic fluid showed maximum shoot regeneration compared to MS medium supplemented with 1 mg L-1 6-benzyladenine and 0.1 mg L-1 naphthalene acetic acid (NAA). It was also shown that vermicompost extract containing humic acids along with coelomic fluid affected shoot regeneration from cotyledons [25].

In recent years, ornamental kale has considerable attention in Iran due to its colorful aerial parts, durability, and good resistance to adverse environmental conditions, especially cold weather, making it a popular ornamental plant in urban landscapes [34]. Today, various varieties of ornamental kale are used as one of the common autumn and winter plants in landscapes. Considering the economic value of this species and to accelerating the breeding programs, an easy and rapid method is needed to provide new varieties according to market demand. Ornamental kale is currently an imported plant, and its hybrid seeds are expensive. Therefore, optimizing in vitro culture conditions offers an excellent platform for the mass production, uniformity, and self-sufficiency of this climate-adapted ornamental in Iran. Unlike other Brassica species, fewer studies have been conducted on in vitro culture of ornamental kale [34].

The role of different forms of heavy metals in in vitro culture is still unclear, and there are conflicting reports of the combination of positive and negative effects of these compounds on ornamental kale micropropagation. Due to its ornamental value of ornamental kale, the purpose of this research was to investigate the effects of different concentrations of lead nitrate [$Pb(NO_3)_2$], silver nitrate ($AgNO_3$), and silver nanoparticles (AgNPs) on micropropagation stages of Brassica oleracea var. acephala under in vitro conditions.

2. Materials and Methods

The ornamental kale (Brassica oleracea var. acephala) with the commercial names of “Fringed white” (with variegated, crinkled green leaves) was used in this research. To prepare the culture medium, commercial MS powder (Sigma Company) and sucrose were dissolved in distilled water. Various concentrations of plant growth regulators were incorporated into the culture medium. After adjusting the solution to a total volume of one liter, the pH of the culture medium was set to 7.0 using 1N KOH and NaOH. Subsequently, 7 gL-1 of agar was added to solidify the culture medium. The culture media were sterilized in an autoclave at 121°C and pressure of 2.1 atmospheres for 20 minutes. The n, the culture medium was transferred into 10 cm diameter petri dishes that had been thoroughly washed and sterilized earlier. Various explants, including leaves, the main peduncle, and secondary peduncles from both the white and purple ornamental kale varieties, were surface sterilized by soaked in a beaker containing a few drops of Tween 80 under a gentle stream of tap water for 15 minutes. To disinfecting the explants, they were initially treated with 70% alcohol for 30 seconds, followed by three rinses with distilled water. Subsequently, the explants were disinfected using 20% sodium hypochlorite for 5 minutes, followed by three rinses with distilled water. Then explants with uniform size, were cultured in the medium, and the lids were sealed with parafilm tape. Finally, the petri dishes were placed in a growth chamber and maintained at a temperature of 23 ± 0.1 °C, under a light cycle of 16 hours of light and 8 hours of darkness. This study involved two separate experimental stages: treatment was applied during the callus induction stage, followed by a second treatment during the shoot regeneration stage.

1- Application of treatment in callus induction stage: The cross-sections of the main peduncles of the ornamental kale varieties “Fringed white” were used as explants. The sterilized explants were cultured on a MS medium containing 0.1 mg L-1 2,4-D for callus induction. In order to investigate the effect of heavy metals on in vitro callus induction, two forms of ionic and AgNO3 and ionic lead was added in culture medium. Silver nitrate ($AgNO_3$), and Silver nanoparticles (AgNPs) salts (Merck Company) were added at different concentrations (0, 10, 25, and 50 mgL-1) to MS medium. This experiment was conducted using a factorial arrangemant in a completely randomized block design with three replications.

2-Treatment in shoot regeneration stage: For the shoot regeneration stage, the main peduncles of the ‘Fringed White’ ornamental kale variety were utilized as explants. The base culture medium was MS, complemented with 0.01 mg L^{-1} 2,4-D and 1 mg L^{-1} BA. To investigate the effect of heavy metals on callus development, the derived callus was treated in the culture medium using three components: ionic silver, nano silver, and ionic lead. These heavy metals were incorporated into the MS media at varying concentrations (0, 25, and 50 mg L^{-1}). This experiment was carried out using a Completely Randomized Design (CRD) with three replicates.

Measurement of traits and data analysis

After 30 days from the culture, traits such as browning, pathogen contamination (%??), rooting (%??), callus diameter??), callus growth (??), fresh and dry weight (??), callus induction (%??), shoot and root regeneration (%??) were evaluated. Explants displaying intense brown discoloration and releasing phenolic into the medium were noted as browning. The index for the callus and shoot induction, shoot regeneration, rooting, ???? parameters were percentage (%). and for callus diameter was cm , callus apparent growth The callus induction percentage and plant regeneration for each treatment was calculated as following equation:

$$CI=(n/N)x100$$

Where: N = the total number of explant, n = the number of explant that are induced of the callus, and CI - callus induction percentage or percentage of regeneration, respectively.

Callus quality was ranked with the following scale: no callogenesis=1, low callogenesis=2, medium callogenesis=3 and high callogenesis=4 for each explant.

Analysis of variance (ANOVA) will be used to analyze the data. Data analysis was performed using SAS software. Duncan's Multiple Range Test (DMRT) was used to compare the mean and to determine the significance of statistical differences in treatments at 1 % and 5% level.

3. Results

Experiment 1: Treatment in callus induction stage

The interaction effects of heavy metal type and their concentrations on the evaluated traits are shown in Table 1., It was found that the concentration of 50 mgL-1 silver element, 25 and 50 mgL-1 onano silver element, and 10 mgL-1 lead element showed a significantly increase compared to the control treatment on browning percentage. While other concentrations did not show a significant difference from the control treatment. In relation to the contamination trait (%), the Pb(NO3)2 with a concentration of 50 mgL-1 showed a significant difference from the control treatment, while other elements and other concentrations did not show a significant difference from the control treatment. In the rooting trait, (%) the lead element at a concentration of 25 mgL-1 was the best treatment and became significant compared to the control treatment, and other treatments and concentrations did not show a significant difference on the traits. Based on results, the callus diameter trait, But, the AgNPs at 50 was the best treatment. Also, this treatment was significant with control of AgNo3 and AgNPs and [$Pb(NO_3)_2$]. The silver element was significant in all concentrations compared to the control treatment in the callus growth trait, but the comparison of the results of its different concentrations did not show a significant difference. The concentration of 25 mgL-1 of nanosilver element and in lead nitrate at concentrations of 10 mgL-1 and 25 mgL-1 were significant compared to the control treatment, but they were not significantly different from each other. In the study of the dry weight trait, it was also determined that none of the elements and concentrations showed a significant difference compared to the control treatment (Table 1).

Table 1: Comparison of interaction effects of metal types and metal concentrations on traits evaluated under in vitro conditions in ornamental kale.

Treatments	Concentration (mg L-1)	Dry Weight (mg Kg-1)	Callus Growth (code)	Callus Diameter (cm)	Rooting (%)	Contamination (%)	Browning (%)
AgNO3	0	0.07b	1d	1.2b	0 b	50ab	0 b
	10	0.11ab	3.2a	1.4ab	0 b	25ab	3.5a
	25	0.07b	2.5abc	1.2b	0 b	100a	2ab
	50	0.1ab	3ab	1.5ab	0 b	50ab	3a
AgNPs	0	0.07b	1d	1.2b	0 b	50ab	0 b
	10	0.01ab	1.7cd	1.5ab	0 b	75ab	1.5ab
	25	0.1ab	2.5abc	1.3ab	0 b	75ab	3a
	50	0.1ab	2bcd	1.7a	0 b	25ab	3a
Pb(NO3)2	0	0.07b	1d	1.2b	0 b	50ab	0b
	10	0.1ab	2.7abc	1.4ab	0.2b	25ab	3a
	25	0.12ab	1.7cd	1.5ab	1.2a	50ab	2.2ab
	50	0.16ab	2.5abc	1.5ab	0b	0b	2ab

* Means within each column followed by the same letter are not significantly different at the 5% level ($p > 0.05$).

Table 2 presents the interaction effects between the various metal types and their concentrations on the evaluated traits.

Overall, the results showed that lead was the element most responsible for inducing browning; specifically, both the 25 mg L^{-1} and 50 mg L^{-1} lead concentrations resulted in a significant increase in browning compared to the control treatment, while the other heavy metals did not show a significant effect.

In the study of the rooting trait, only the 25 mg L^{-1} nano silver treatment showed a significant difference compared to the control. In contrast, treatments using other concentrations of silver, nano silver, and lead did not demonstrate a significant effect on rooting.

Regarding callus traits, significant differences in callus growth compared to the control were found across several treatments: 25 mg L^{-1} ionic silver, 25 mg L^{-1} and 50 mg L^{-1} nano silver, and 50 mg L^{-1} lead. Furthermore, for callus diameter, the 25 mg L^{-1} and 50 mg L^{-1} lead treatments were significantly different from each other. However, neither these lead concentrations nor the two silver elements showed a significant difference when compared directly to the control group in terms of diameter. Finally, when evaluating fresh weight, only the 50 mg L^{-1} lead treatment was significant compared to the control, whereas the 25 mg L^{-1} concentration of lead and all concentrations of silver and nano silver treatments showed no significant difference.

Table 2- Comparison of the average interaction effects of metal type on metal concentration on the traits evaluated under in vitro conditions in ornamental kale.

Treatments	Concentrations	fresh weight (mg Kg-1)	callus diameter (cm)	growth of callus (code)	Rooting (%)	Browning (%)
AgNO3	0	1.1b	1.1ab	1b	100a	5a
	25	1.5ab	1.2ab	4a	75a	2.33ab
	50	1.5ab	1.2ab	3ab	66.6a	2.33ab
AgNPs	0	1.1b	1.1ab	1b	100a	5a
	25	1b	1.2ab	4a	33.3b	2.3b
	50	1.5ab	1.2ab	3.3a	66.6a	3ab
Pb(NO3)2	0	1.1b	1.1ab	1b	100a	5a
	25	1.1b	1b	2.3ab	91.6a	1.6b
	50	1.8a	1.2a	4.3a	83.3a	2.6b

* Means within each column followed by the same letter are not significantly different at the 5% level ($p > 0.05$).

The simple effect of lead concentration on shoot length indicated that only the 50 mg L^{-1} concentration resulted in a significant difference compared to the control group; this effect was not observed at lower concentrations. Conversely, when examining browning and fresh weight traits, both lead concentrations (25 mg L^{-1} and 50 mg L^{-1}) showed a significant difference not only from the control treatment but also from each other.

Table 3: effect of lead concentration on the evaluated traits under in vitro cultivation conditions with 1/2MS medium and perlite in ornamental kale

Pb treatments (mg L-1)	Fresh weight	Browning	Shoot length
0	0.39c	6.3a	2.5b
25	0.68b	4.3b	2.2b
50	0.96a	1.3c	4.1a

* Means within each column followed by the same letter are not significantly different at the 5% level ($p > 0.05$).

4. Discussion

The interaction effects of metal type on metal concentration on the evaluated traits showed that the highest percentage of browning was related to nano silver. In terms of contamination, low concentration of silver and in rooting, Pb nitrate at a concentration of 50 mgL-1. The highest callus formation was related to silver element, but the other traits and most treatments did not show a significant difference. According to the results stated, the highest percentage of browning was related to lead element. The highest percentage of rooting was observed with the application of 25 mgL-1 concentration of nano silver, while the other treatments were not significant. The highest callus formation and fresh weight were also related to lead element, while the other traits and treatments did not show a significant difference. A simple study of the effect of lead concentration on the evaluated traits showed that the highest shoot length was obtained with the 50 mgL-1 treatment, while the browning and fresh weight traits were not significant in this experiment.

Based on findings from other researchers, it has been observed that low levels of lead nitrate promote stem elongation and growth in turfgrasse. This observation aligns with the previously noted stimulating influence of lead on the growth of ornamental kale in in vitro condition [35].

In our results, AgSNPs showed antimicrobial activity against in vitro pathogens that was similar to that found by Taghizadeh and solgi, 2015 [34]**.** Recent studies have highlighted the potential of antimicrobial nanoparticles, particularly nano-silver, as effective bactericidal agents in plant tissue culture. Abdi et al. (2008) demonstrated that a concentration of 120 mgL-1 of nano-silver significantly improved explant disinfection in valerian tissue culture without harming the explants [40]. Similarly, Rostami and Shahsavar (2009) found that a lower concentration of 4 mgL-1 nano-silver effectively controlled contamination in olive explants while promoting growth [41]. Gharati et al. (2010) identified 75 ppm of nano-silver as optimal for reducing bacterial contamination in Persian walnut in vitro cultures [42]. Safavi et al. (2011) reported that 50 mgL-1 of nano-silver was fully effective in eliminating bacterial contaminants in tobacco tissue culture [44]. Gholamhoseinpour Anvari et al. (2012) confirmed that adding 10 to 20 mg m^{-3} of nano-silver to culture media effectively controlled bacterial growth in peach × almond hybrids [43]. Fakhrfeshani et al. (2012) explored the antimicrobial properties of a different nano-silver solution on gerbera tissue culture, finding that a concentration of 200 mgL-1 successfully managed bacterial and fungal contamination without negatively impacting plantlet regeneration. The mechanism of action for silver nanoparticles involves the alteration of microbial cell membranes and interference with vital enzymatic functions, ultimately leading to bacterial cell death. The unique properties of nanoparticles, including their larger specific surface area compared to bulk silver, enhance their antimicrobial activity [45].

The inhibitory effects of silver (Ag) ions on microorganisms are partially understood, with several studies highlighting the importance of the positive charge of Ag ions in their antimicrobial activity. This positive charge facilitates electrostatic attraction to the negatively charged cell membranes of microorganisms. However, research by Sondi and Salopek-Sondi (2004) indicates that the antimicrobial efficacy of silver nanoparticles against Gram-negative bacteria is concentration-dependent and linked to the formation of pits in the bacterial cell wall. The accumulation of Ag nanoparticles in the bacterial membrane leads to increased permeability, ultimately resulting in cell death [8]. Sondi and Salopek-Sondi (2004) also suggest that a similar mechanism could contribute to the degradation of the membrane structure of E. coli when treated with Ag nanoparticles. This indicates a need for further investigation into the specific mechanisms by which positively charged Ag nanoparticles exert their antimicrobial effects [8]. Overall, these findings suggest that silver nanoparticles could serve as a viable alternative to traditional chemical disinfectants in the management of microbial populations in plant tissue culture, offering a promising avenue for improving in vitro culture practices.

The supplementation of $AgNO_3$ in callus induction media enhanced significantly dry weight and growth calli in ornamental kale. The research conducted by Rakshit et al. (2010) demonstrated that the addition of silver nitrate ($AgNO_3$) at a concentration of 15 mgL-1 significantly enhanced organogenic callus induction from maize embryos [39]. Similarly, Sridevi and Giridhar (2013) found that hypocotyl explants of Coffea dewevrei produced yellow friable embryogenic callus when cultured on half-strength MS medium supplemented with 6.8 mgL-1 $AgNO_3$ [38]. Fei et al. (2000) reported a notable increase in embryogenic callus induction frequency in buffalograss, achieving a peak of 79.9% at 10 mgL-1 $AgNO_3$ [37]. Lua-Figueiredo et al. (2000) highlighted that callus cultures lacking $AgNO_3$ exhibited reduced fresh weight and a more compact texture, while the inclusion of 17 mgL-1 $AgNO_3$ in media containing 2,4-D or NAA improved callus growth and minimized tissue browning within three to four weeks. The mechanism behind $AgNO_3$'s inhibitory action involves silver ions (Ag^+) interfering with ethylene binding, thereby preventing the accumulation of ethylene in intercellular spaces, which can hinder tissue differentiation. This property of $AgNO_3$ has been shown to enhance in vitro shoot regeneration from callus clumps, as evidenced by various studies. Silver nitrate ($AgNO_3$) serves as a significant ethylene inhibitor, enhancing the regeneration capabilities of various dicots and monocots by suppressing ethylene synthesis [36].

5. Conclusion

The results of this experiment indicate the positive effect of metals in nano and ionic metals forms. The use of certain metals in in vitro plant culture can be considered as a growth stimulant. It seems that the concentration of silver nanoparticles should be considered lower due to its greater efficiency in stimulating shoot regeneration. However, the achievements of these experiments can be the initial basis for other research in the field of the application of nano forms in plant micropropagation.

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