

Enhancing In Vitro Regeneration of Tall Fescue (*Festuca arundinacea*) through Optimized Growth Regulators and Nanoparticle Application

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Abstract

This experiment aimed to optimize the in vitro regeneration of tall fescue (*Festuca arundinacea*) and investigate the effects of ZnO and Ag nanoparticles on its growth. The study evaluated the impact of six combinations of auxin (2,4-D) and cytokinins (BAP and kinetin) on stem and seed explants (Iranian ecotype and Molva foreign genotype) using a completely randomized design with three replications. To assess the effects of nanoparticles on callus induction and regeneration, four concentrations of Ag nanoparticles (0, 20, 40, 60 mg L⁻¹) and ZnO nanoparticles (0, 25, 50, 100 mg L⁻¹) were tested under a completely randomized design with three replications. The results indicated that halved seed explants, the Iranian ecotype, and MS1/2 culture medium produced the best outcomes. The medium containing 1 mg L⁻¹ 2,4-D and 0.1 mg L⁻¹ kinetin was the most effective for callus formation, as well as fresh and dry callus weight, while also reducing the time required for callus induction. Additionally, a medium containing 0.9 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ BAP yielded higher rates of both indirect and direct regeneration. For the rooting phase, a medium with 0.25 mg L⁻¹ NAA and 0.1 mg L⁻¹ 2,4-D resulted in the longest roots and the shortest time to rooting. Analysis of variance revealed that both Ag and ZnO nanoparticles significantly affected the time required for callus induction. Furthermore, Ag nanoparticles significantly influenced the regeneration percentage. Mean comparisons for Ag nanoparticles showed that a concentration of 20 mg L⁻¹ accelerated callus formation, whereas 60 mg L⁻¹ resulted in the lowest callus induction rate. Similarly, ZnO nanoparticles at concentrations of 25, 50, and 100 mg L⁻¹ positively impacted the callus formation rate compared to the control treatment without ZnO nanoparticles.

Introduction

Tall fescue (*Festuca arundinacea*) is a monocotyledonous plant from the Poaceae family, specifically the Pooideae subfamily. This species is an allohexaploid ($2n = 6x = 42$), although diploid ($2n = 28$) and decaploid ($2n = 70$) cytotypes have also been reported (Aminuddin, 1993). *F. arundinacea* is among the most important forage crops in Europe. Beyond its ornamental value, it is cultivated in areas prone to soil erosion (Kasperbauer, 1990). The plant offers several advantages, including tolerance to both warm and cold climates, vertical growth that enhances aesthetic appeal, and high drought tolerance (Barker and Welty, 1997).

The high self-incompatibility of tall fescue significantly slows breeding progress through conventional selection methods (Spangenberg et al., 1995). Biotechnological approaches, however, provide an effective pathway to develop new cultivars of perennial forage crops. Transgenic plants have been generated using techniques such as microprojectile bombardment, *Agrobacterium*-mediated transformation, and silicon carbide fiber-mediated transformation (Dalton et al., 1998). Additionally, micropropagation offers numerous advantages over traditional propagation methods, such as increased reproduction rates and the production of virus- and pathogen-free plant material. However, optimizing tissue culture conditions—considering factors such as genotype, explant type, and culture media composition—is essential for success (Bai and Qu, 2000).

In recent years, nanoparticles (NPs) have been shown to enhance various aspects of plant tissue culture. They effectively eliminate microbial contaminants from explants and positively influence callus induction, organogenesis, somatic embryogenesis, somaclonal variation, and secondary metabolite production (Kim et al., 2017). Nanoparticles are widely utilized to improve seed germination, enhance plant growth and yield, induce genetic modifications, and boost bioactive compound production and plant protection. The effects of NPs depend on their type, concentration, the plant species, and the exposure duration. Recent studies indicate that surface disinfection of explants with NPs significantly reduces microbial contamination. Additionally, incorporating NPs into tissue culture media can eliminate bacterial contamination, enhance the morphogenic potential of explants, and induce somaclonal variation.

Silver nanoparticles (Ag-NPs) have been extensively studied for their antimicrobial properties. Abdi and Salehi (2008) reported the use of Ag-NPs to control bacterial contamination in *Valeriana officinalis*. Similarly, Aghdaei et al. (2012) demonstrated that *Tecomella undulata* explants cultured on MS medium supplemented with 10 mg L⁻¹ Ag-NPs, 2.5 mg L⁻¹ BAP, and 0.1 mg L⁻¹ IAA exhibited higher shoot yield, shoot length, and shootlet formation percentages. However, adverse effects on regeneration were observed at concentrations above 60 mg L⁻¹. Mahna et al. (2013) investigated the effects of Ag-NPs on disinfecting explants of *Arabidopsis* seeds, potato leaves, and tomato cotyledons. Treatment with 100 mg L⁻¹ Ag-NPs for 1–5 minutes effectively reduced microbial contamination without negatively impacting plant vitality. However, higher concentrations adversely affected seed germination and plantlet development.

Zinc oxide nanoparticles (ZnO-NPs) have also demonstrated significant potential in plant tissue culture. They enhance plant regeneration and callus growth (Alharby et al., 2019; Mousavi Kouhi and Lahouti, 2018). ZnO-NPs synthesized using plant extracts have shown strong antibacterial properties in various studies (Pal et al., 2017; Aquisman et al., 2020). This study aimed to optimize the in vitro regeneration of tall fescue and investigate the effects of ZnO-NPs and Ag-NPs on its tissue culture performance.

Materials and Methods

Plant Material, Disinfection, Explant Preparation, Callus Formation, and Regeneration

Based on the findings of an earlier study (Amini et al., 2019), seeds from two genotypes were selected: an Iranian landrace (provided by the Seed Bank of the Faculty of Agricultural Technology, Aburairhan Campus, University of Tehran, Pakdasht, Tehran, Iran) and a Swiss cultivar (Molva) (sourced from the Agroscope Research Station Reckenholz-Tänikon [ART], Zurich, Switzerland). The current study was conducted in three stages: 1) Optimization of somatic embryogenesis, regeneration, and rooting. 2) Assessment of somaclonal variation using ISSR molecular markers. 3) Evaluation of the effects of Ag-NPs and ZnO-NPs on the tissue culture of tall fescue.

In the first stage, seeds were rinsed under running water for 30 minutes and subsequently immersed in a 1 g L⁻¹ solution of Thiram fungicide for 15 minutes. After washing three times with sterile distilled water, seeds were treated with 70% ethanol for 1 minute, followed by a 15-minute soak in a 2.5% sodium hypochlorite solution. Finally, seeds were rinsed three more times with sterile distilled water.

Murashige and Skoog (MS) culture medium was prepared according to the protocol of Murashige and Skoog (1962), containing 30 g L⁻¹ sucrose (0.30%), 8 g L⁻¹ agar (Merck, Germany; Catalog Number: 101347), and adjusted to pH 5.8. Ten seeds were placed in each glass container and incubated in a growth chamber under a photoperiod of 16 hours light and 8 hours dark at 25°C. After approximately 5 days, seeds began to germinate. Explants were collected once the seedlings reached a height of approximately 15 cm and developed key organs.

For callus formation and regeneration experiments, a factorial arrangement was used in a completely randomized design (CRD) with three factors and three replicates per treatment (as Petri dishes). Each Petri dish contained five explant segments placed on 25 ml of medium. The experimental factors included:

Genotypes (Iranian landrace and Swiss cultivar), Explant type (seed and stem), Plant growth regulator (PGR) regimes (MS₁: Control (no PGRs), MS₂: 0.5 mg L⁻¹ 2,4-D + 0.1 mg L⁻¹ kinetin, MS₃: 1 mg L⁻¹ 2,4-D + 0.1 mg L⁻¹ kinetin, MS₄: 2 mg L⁻¹ 2,4-D + 0.1 mg L⁻¹ kinetin, MS₅: 0.5 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ kinetin, MS₆: 0.9 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ BAP).

Measured traits included callus induction percentage, regeneration percentage, direct regeneration percentage, fresh callus weight, dry callus weight, and the number of days to callus formation.

Since the distributions of callus induction percentage, regeneration percentage, and direct regeneration percentage were non-normal and could not be normalized via transformation, the nonparametric Kruskal-Wallis and Mann–Whitney tests were employed. For traits such as fresh and dry callus weights, data were analyzed using analysis of variance (ANOVA), and mean comparisons were performed using Duncan's Multiple Range Test (DMRT). Statistical analyses were conducted using SAS 9.2 and SPSS 16.0.

In Vitro Rooting and Acclimatization

The best response for plant regeneration was observed in MS₃ medium, containing 1 mg L⁻¹ 2,4-D and 0.1 mg L⁻¹ kinetin. Regenerated plantlets measuring 3–4 cm in length were excised from this medium and transferred to Petri dishes containing MS_{1/2} medium. Three treatment combinations were tested for enhanced rooting:

1. 0.25 mg L⁻¹ NAA + 0.1 mg L⁻¹ 2,4-D
2. 0.5 mg L⁻¹ NAA + 0.1 mg L⁻¹ 2,4-D

3. 1 mg L⁻¹ NAA + 0.1 mg L⁻¹ 2,4-D

Petri dishes were monitored every 3–4 days, and rooting characteristics such as the number of days to root formation and root length were recorded for comparison. The rooting experiment followed a completely randomized design (CRD) with three replications. Each replication consisted of ten regenerated plantlets cultured in pots containing 50 ml of the treatment medium. Data collected from this experiment were analyzed using ANOVA and Duncan's Multiple Range Test (DMRT).

Evaluation of Somaclonal Variation Using ISSR Markers

Genetic homogeneity between the regenerated plantlets and the mother plants was assessed using ISSR markers. Total genomic DNA was extracted from young leaves of both in vitro regenerated plants and the mother plant following the CTAB protocol (Murray and Thompson, 1980) with minor modifications. The quality and quantity of the extracted DNA were verified using 1% agarose gel electrophoresis and a spectrophotometer, and DNA samples were diluted to a final concentration of 50 ng µL⁻¹.

Ten ISSR primers were used for DNA amplification. PCR reactions were conducted in a total volume of 10 µL, consisting of: 3 µL double-distilled water, 5 µL PCR amplification mix (10x), 1 µL primer (10 pmol), 1 µL DNA (50 ng µL⁻¹). The PCR program included an initial denaturation step at 94°C for 5 minutes, followed by 34 cycles of: 30 seconds at 94°C, 45 seconds at 56–59°C, 2 minutes at 72°C. A final extension step was performed at 72°C for 5 minutes. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator.

Application of ZnO and Ag Nanoparticles Under In Vitro Conditions

ZnO nanoparticles (ZnO-NPs; Catalog Number: 108849, Merck, Germany) and silver nanoparticles (Ag-NPs; Catalog Number: 119208, Merck, Germany) were purchased in powdered form. A stock solution of silver nitrate (AgNO₃) NPs was prepared at a concentration of 500 ppm in sterile water.

This experiment was conducted as a completely randomized design with three replications, where each Petri dish was considered a replication, and five explants were cultured per dish. Iranian ecotype halved seed explants were used, along with a PGR combination of 1 mg L⁻¹ 2,4-D + 0.1 mg L⁻¹ kinetin, optimized in the tissue culture experiment.

The following nanoparticle treatments were applied to the culture medium before autoclaving:

Ag-NPs at concentrations of 0, 20, 40, and 60 mg L⁻¹

ZnO-NPs at concentrations of 0, 25, 50, and 100 mg L⁻¹

The evaluated traits included: Number of days to callus formation, Callus fresh weight and Regeneration percentage

Normality of data was checked before conducting statistical analysis. Analysis of variance (ANOVA) and treatment mean comparisons were performed using Duncan's Multiple Range Test (DMRT) in SAS software.

Results

Investigating the Effect of Plant Growth Regulator Treatments, Explants, and Ecotypes on Callus Formation and Regeneration

In this study the highest percentages of callus formation and regeneration were observed in MS₃ (1 mg l⁻¹ 2.4-D + 0.1 mg l⁻¹ kinetin) and MS₄ (2 mg l⁻¹ 2.4-D + 0.1 mg l⁻¹ kinetin) (Fig. 1). The effect of MS₆ (0.9 mg l⁻¹ 2.4-D + 0.5 mg l⁻¹ BAP) on the percentage of direct regeneration was higher than that of other treatments (Table 1) (Fig. 1f). According to the results from the mean comparison test for the fresh and dry weight of callus, MS₃ (1 mg l⁻¹ 2.4-D + 0.1 mg l⁻¹ kinetin) and MS₄ (2 mg l⁻¹ 2.4-D + 0.1 mg l⁻¹ kinetin) showed the highest fresh and dry weights of callus (Table 1). The results of present study indicated that seed explants had a higher mean percentage of callus formation and regeneration than stem explants, although no significant difference was observed between them for direct regeneration (Table 2).

Table 1

The effect of plant growth regulators (PGRs) on the means of callus-related traits of tall fescue.

Plant Growth Regulators (PGRs)						
Traits	MS ₁	MS ₂	MS ₃	MS ₄	MS ₅	MS ₆
	Control	0.5 mg l ⁻¹ 2.4-D + 0.1 mg l ⁻¹ kinetin	1 mg l ⁻¹ 2.4-D + 0.1 mg l ⁻¹ kinetin	2 mg l ⁻¹ 2.4-D + 0.1 mg l ⁻¹ kinetin	0.5 mg l ⁻¹ 2.4-D + 0.5 mg l ⁻¹ kinetin	0.9 mg l ⁻¹ 2.4-D + 0.5 mg l ⁻¹ BAP
Callus percentage	24.79 ± 6.59	37.33 ± 6.50	50.29 ± 8.12	44.04 ± 8.10	29.88 ± 7.86	32.67 ± 8.05
Regeneration percentage	56.25 ± 5.27	82.29 ± 4.27	88.08 ± 4.51	69.58 ± 6.41	67.88 ± 6.35	92.92 ± 6.35
Direct regeneration percentage	78.75 ± 7.37	42.00 ± 5.32	42.00 ± 5.32	42.00 ± 5.32	78.75 ± 7.37	80.50 ± 11.47
Number of days to callus formation	10 ± 2.24	11.00 ± 4.21	12.83 ± 5.00	13.00 ± 4.14	8.83 ± 3.21	10.66 ± 5.86
Callus fresh weight,	0.08 ± 0.001	0.04 ± 0.002	0.12 ± 0.03	0.09 ± 0.01	0.04 ± 0.004	0.05 ± 0.004
Callus dry weight	0.02 ± 0.001	0.015 ± 0.001	0.05 ± 0.01	0.05 ± 0.03	0.01 ± 0.001	0.02 ± 0.002
The effect of plant growth regulators (PGRs) on the means of callus-related traits of tall fescue. Values represent the mean value of three replications ± standard deviation						

Table 2

The effect of different explants (stem and seed) on the means of callus-related traits of tall fescue.

explant		
Traits	Stem	Seed
Callus percentage	27.29 ± 4.14	45.71 ± 6.65
Regeneration percentage	30.76 ± 2.36	42.24 ± 6.72
Direct regeneration percentage	16.33 ± 2.35	18.85 ± 3.37
Number of days to callus formation	12.77 ± 3.76	9.33 ± 2.45
Callus fresh weight (g)	0.05 ± 0.006	0.07 ± 0.004
Callus dry weight (g)	0.02 ± 0.002	0.02 ± 0.001
The effect of different explants (stem and seed) on the means of callus-related traits of tall fescue. Values represent the mean value of three replications ± standard deviation		

In Vitro Rooting and Acclimatization

The mean comparison results revealed that the Iranian ecotype required fewer days for root formation (Table 3). Additionally, the root length was significantly greater in the Iranian ecotype compared to other treatment (Table 3). When evaluating the effect of plant growth regulators on root formation and root length, the medium containing 0.25 mg l⁻¹ NAA and 0.1 mg l⁻¹ 2.4-D resulted in the shortest time for root formation and the longest root length (Table 3). Figure 2 shows the roots produced in the Iranian ecotype cultivated in the medium containing 0.25 mg l⁻¹ NAA and 0.1 mg l⁻¹ 2.4-D.

Table 3

The effect of ecotype and plant growth regulator on the number of days to root formation and root length

Variables		Number of days to root formation	Root length (cm)
Ecotype	Iranian landrace	9.00 ^b	5.61 ^a
	Molva cultivar	11.11 ^a	4.40 ^b
Plant growth regulator	0.25 mg l ⁻¹ NAA + 0.1 mg l ⁻¹ 2.4-D	7.50 ^c	7.42 ^a
	0.5 mg l ⁻¹ NAA + 0.1 mg l ⁻¹ 2.4-D	10.33 ^b	4.42 ^b
	1 mg l ⁻¹ NAA + 0.1 mg l ⁻¹ 2.4-D	12.33 ^a	3.25 ^b
In each part values in columns followed by the same letters are not statistically significant based on Duncan's multiple range test ($p \leq 0.05$).			

Investigating Somaclonal Variation through Molecular Markers

In this study, ten ISSR primers were tested, of which three primers produced useful, reproducible bands. Between 5 and 11 bands were amplified per primer, with sizes ranging from approximately 150 to over 800 bp. A total of 24 clear bands were generated, averaging 8 bands per primer (Table 4). The ISSR markers revealed polymorphic bands with a 16% polymorphism rate in the Iranian ecotype (both tissue culture and seed samples) and a 20.83% polymorphism rate in the Molva cultivar (both tissue culture and seed samples) (Fig. 3).

Table 4
Somaclonal variation of seed seedlings and tissue culture of tall fescue based on ISSR primers

Primer number	Primer sequence	Annealing temperature	Total band	Number of polymorph band		Polymorphism (%)	
				Iranian ecotype	Molva cultivar	Iranian ecotype	Molva cultivar
1	(AC) 8 TG	54.8	11	1	2	9.09	18.18
2	(GT) 8 C	52.8	5	1	1	20	20
3	(AC) 8 CT	52.5	8	2	2	25	25
sum		-	24	4	5	16	20.83

Investigating the Effect of ZnO and Ag Nanoparticles on Callus Induction and Regeneration

The mean comparison for Ag-NPs showed that the treatments with 20 mg l⁻¹ and 0 mg l⁻¹ Ag-NPs had the lowest and highest number of days for callus formation, respectively (Table 5). Additionally, increasing the concentration of Ag-NPs negatively affected the regeneration percentage, with a significant decrease in regeneration observed at a concentration of 60 mg l⁻¹ (Table 5). For ZnO-NPs, the concentrations of 25, 50, and 100 mg l⁻¹ resulted in better callus formation compared to the 0 mg l⁻¹ treatment (Table 6). Furthermore, the highest fresh weight of the callus was observed at the concentration of 50 mg l⁻¹ ZnO-NPs (Table 6).

Table 5
The effect of Ag-nanoparticle on callus-related traits of tall fescue.

Concentration(mgl ⁻¹)	Regeneration percentage	Number of days to callus formation
0	0.34 ^{ab}	8.67 ^a
20	0.41 ^a	5.57 ^b
40	0.28 ^{ab}	8.00 ^{ab}
60	0.07 ^b	7.00 ^{ab}
Values in columns followed by the same letters are not statistically significant based on Duncan's multiple range test (p ≤ 0.05).		

Table 6
The effect of ZnO nanoparticle on callus-related traits of tall fescue.

Concentration(mg l ⁻¹)	callus fresh weight	Number of days to callus formation
0	0.10 ^b	8.00 ^a
25	0.16 ^{ab}	7.00 ^b
50	0.19 ^a	7.00 ^{ab}
100	0.11 ^{ab}	7.00 ^{ab}
Values in columns followed by the same letters are not statistically significant based on Duncan's multiple range test (p ≤ 0.05).		

Discussion

The interactions between auxins, cytokinins, and their combinations are generally considered the most crucial factors for regulating growth in tissue culture (Evans et al., 1981; Vasil, 1994). In this experiment, various concentrations of 2,4-D (as an auxin) were tested, with 1 mg l⁻¹ yielding the highest percentage and speed of callus formation. Lee et al. (2012) found that 5 mg l⁻¹ of 2,4-D was the optimal concentration for inducing callus from mature Siberian wild grass seeds. Callus development and regeneration are enhanced when auxin is combined with low concentrations of cytokinins, such as 6-benzylaminopurine (BAP) (Gaba, 2005). In the present study, low concentrations of kinetin and BAP were used, with the highest regeneration and direct regeneration percentages observed at 0.5 mg l⁻¹ of BAP. Cytokinins promote plant differentiation and are commonly used in regeneration media for plant tissue cultures. Additionally, when measuring the dry and fresh weight of callus, culture media containing 1 and 2 mg l⁻¹ of 2,4-D produced the highest dry and fresh weights. Zhang et al. (2006) reported that concentrations of 2 to 5 mg l⁻¹ of 2,4-D promote callus development in tall fescue cultivars. The quality of callus induction and regeneration is influenced by an appropriate auxin-to-cytokinin ratio, although this effect may depend on endogenous cytokinin levels (Bai and Qu, 2001).

The effects of growth regulators on tissue culture responses were examined using immature embryos and mature seeds as explants to optimize tissue culture propagation conditions for tall fescue. Callus formed in media containing BAP significantly increased the explants' capacity for regeneration (Bai and Qu, 2001). In cereal and grass tissue culture, mature seeds, stem meristems, and immature inflorescences are commonly used as explants. Mature seeds are particularly convenient because they are available throughout the year, unlike other explants that must be collected from growing plants (Bai, 2001). In this study, halved seed explants, cut longitudinally from the embryonic portion, were used and showed superior performance across all evaluated traits.

In the present experiment, NAA and 2,4-D auxins were tested for rooting. It was observed that increasing the NAA concentration from 0.25 mg l⁻¹ to 0.5 and 1 mg l⁻¹ resulted in a decrease in both rooting

speed and root length. This suggests that higher concentrations of auxins inhibit rooting-related traits. Poordad et al. (2014) reported that higher NAA concentrations stimulate root differentiation, but excessive amounts have inhibitory effects. Similarly, in a study on *Lathyrus sativus* L., Barpete et al. (2014) found that increasing NAA concentration led to a reduction in root length.

It has been suggested that the genus *Festuca* be screened for somaclonal variation using molecular markers (Valles et al., 1993). In this experiment, regenerated plants and seed plants showed polymorphic bands using ISSR primers, which could be linked to somaclonal variation or the impure characteristics of tall fescue plants. Bahmankar et al. (2015) reported polymorphic bands between regenerated and seed plants in a study using RAPD primers to investigate somaclonal variation in cumin. Similarly, polymorphic bands were found in RAPD markers when studying genetic diversity in thyme plants, which was believed to be related to the thyme pollination system (Bakhtiar et al., 2014).

This study also examined the effects of Ag-NPs and ZnO-NPs on callus induction and regeneration. The application of nanoparticles has been shown to eliminate microbial contaminants from explants and enhance callus formation, organogenesis, somatic embryogenesis, and somaclonal variation (Kim et al., 2017). As disinfection of explants is essential in tissue culture, the results from this study showed that Ag-NPs helped reduce contamination in the culture environment. The incorporation of nanoparticles into the tissue culture media can eradicate bacterial contamination and enhance explant morphogenesis (Kim et al., 2017). Ag-NPs exert their antibacterial effects by adhering to and penetrating bacterial cell walls, causing structural changes in the cell membrane that increase permeability and lead to cell death (Ahmed et al., 2020). The production of free radicals by Ag-NPs further contributes to cell death (Kim et al., 2017).

Various studies have noted that nanoparticles in culture media can induce somaclonal variation by affecting the treatment of plant tissues, cells, or cultivable organs (Wang et al., 2001; Kim and Gopal, 2017). In this experiment, increasing the concentration of Ag-NPs above 20 mg l⁻¹ decreased the evaluated traits. At a concentration of 20 mg l⁻¹, Ag-NPs enhanced regeneration compared to the control, although this increase was not statistically significant. It is suggested that concentrations of Ag-NPs lower than 20 mg l⁻¹ could improve the regeneration process more effectively. Bahmankar et al. (2015) also emphasized the importance of determining the optimal concentration of chemicals, including antibiotics, plant growth regulators, and nanoparticles, for effective plant tissue culture.

The comparison of average effects of ZnO-NPs showed that a concentration of 50 mg l⁻¹ resulted in the highest callus fresh weight. Zinc is a low-consumption but essential element in plants, and nanoparticles like ZnO-NPs exhibit significant antimicrobial activity (Dutta et al., 2023). ZnO-NPs' antimicrobial activity has been attributed to processes such as protein leakage, DNA damage, penetration into cells, and blocking of cell communication channels (Wang et al., 2012). In this study, ZnO-NPs demonstrated beneficial antibacterial properties. At a concentration of 50 mg l⁻¹, ZnO-NPs significantly increased callus fresh weight. However, when the concentration was increased to 100 mg l⁻¹, callus fresh weight decreased. High concentrations of ZnO can hinder plant growth (Paschke et al., 2006). The optimal

concentration of ZnO-NPs and the effects of nanotoxicity may vary depending on factors such as plant species and explant type. For example, increasing the concentration of ZnO-NPs to 30–40 mg l⁻¹ had a detrimental impact on blackberry plantlets' number, length, and fresh weight (Krzepiłko et al., 2024). Nanotoxicity, including free radical production, can interfere with cellular processes and lead to cell death (Shafique et al., 2020). ZnO-NPs may also affect plant cell wall and membrane functions, resulting in oxidative stress and cell death from elevated ROS levels (Shafique et al., 2020).

Moreover, nanoparticles, for the most part, silver (Ag) and zinc oxide (ZnO), being included, have considerably potent molecular effects on both growth and regeneration in plant tissue culture. As an illustration, AgNPs promote the antioxidant activity of plants by the fact that genes related to oxidative stresses are being upregulated, such as SOD and CAT, so thereby protecting the plant cells from the damage robbing by the formation of ROS (Kim et al., 2017). In our experiment, the nanoparticles of Ag and ZnO that were exposed to the tall fescue plants caused the rate of the callus induction and the regeneration of the tall fescue to be different by modulating the antioxidative pathways. Studies have indicated that ZnO nanoparticles are also capable of affecting the plant's metabolic pathways. One research suggests that these nanoparticles can govern the expression of photosynthesis genes, such as those controlling chlorophyll synthesis, in order to deliver a supplementary growth (Alharby et al., 2019). In parallel, our experiments showed that ZnO concentrations exerted a positive effect on the callus formation rate, following the hypothesis that ZnO may exert an effect at the molecular level on growth and regeneration through possible mechanisms of gene expression and changes in metabolic activities related to cellular division and differentiation.

These molecular insights are in support of the hypothesis that nanoparticles, especially Ag and ZnO, may enhance and regulate plant tissue culture processes through effects on genetic and enzymatic activities important for plant development. Our results reveal that Ag and ZnO NPs influence callus formation and regeneration efficiency, possibly by modulating antioxidative enzyme activation and gene regulation in accordance with similar observations in other species (Pal et al., 2017; Aquisman et al., 2020).

Conclusion

This study identified the halved seed micro-sample, the Iranian ecotype, and the MS_{1/2} culture medium as the best conditions for the tissue culture of tall fescue. The culture medium containing 1 mg l⁻¹ 2,4-D and 0.1 mg l⁻¹ kinetin was optimal for callus formation, while the culture medium with 0.9 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ BAP facilitated the highest levels of indirect and direct regeneration. In rooting media with 0.25 mg l⁻¹ NAA and 0.1 mg l⁻¹ 2,4-D, maximum root length and the shortest time to rooting were observed. This study also highlighted the importance of optimizing the concentration of auxins (especially 2,4-D and NAA) for callus formation, callus dimensions, and rooting. Regarding the use of Ag-NPs and ZnO-NPs, 20 mg l⁻¹ Ag-NPs positively affected the evaluated traits, while 60 mg l⁻¹ had a toxic effect and decreased regeneration. ZnO-NPs at 50 mg l⁻¹ promoted callus fresh weight, showing the potential of these nanoparticles for enhancing tissue culture efficiency

Declarations

Authorship contribution statement

Moradiasl M: Writing - original draft, Software, Writing - review & editing, Resources, Perform experiments and collect data, Formal analysis. **Amini F:** Writing - review & editing, Formal analysis, Supervision, Validation, Visualization, Preparation of laboratory materials. **Izadi-Darbandi A:** Writing - review & editing, Supervision, Validation, Visualization.

Data availability

All data generated or analyzed in this study are involved in this manuscript

Competing interests: The authors declared that they have no competing interests.

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Figures

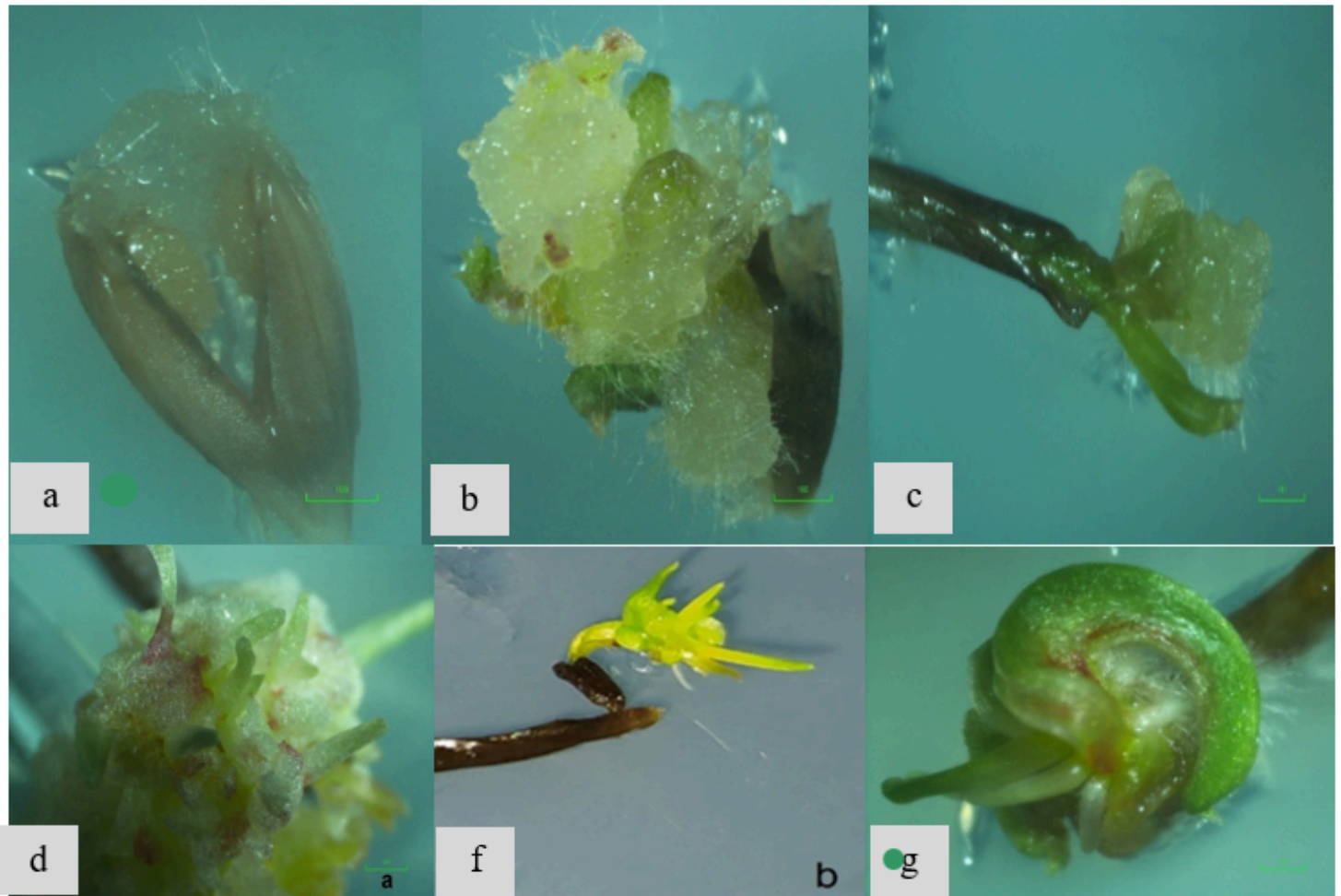


Figure 1

Comparison of callus induction in the culture medium (1 mg l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin), (a, b) callus induction in the halved seed explant, (c) callus induction in the terminal meristem explant of the stem (d) callus regenerated from a halved seed explant, (e) callus regenerated from a stem terminal meristem explant, (f) callus regenerated from a stem terminal meristem explant, (g) direct regeneration from a stem terminal meristem explant.



Figure 2

Roots produced in two samples of the Iranian ecotype cultivated in culture medium (0.25 mg l^{-1} NAA + 0.1 mg l^{-1} 2,4-D)

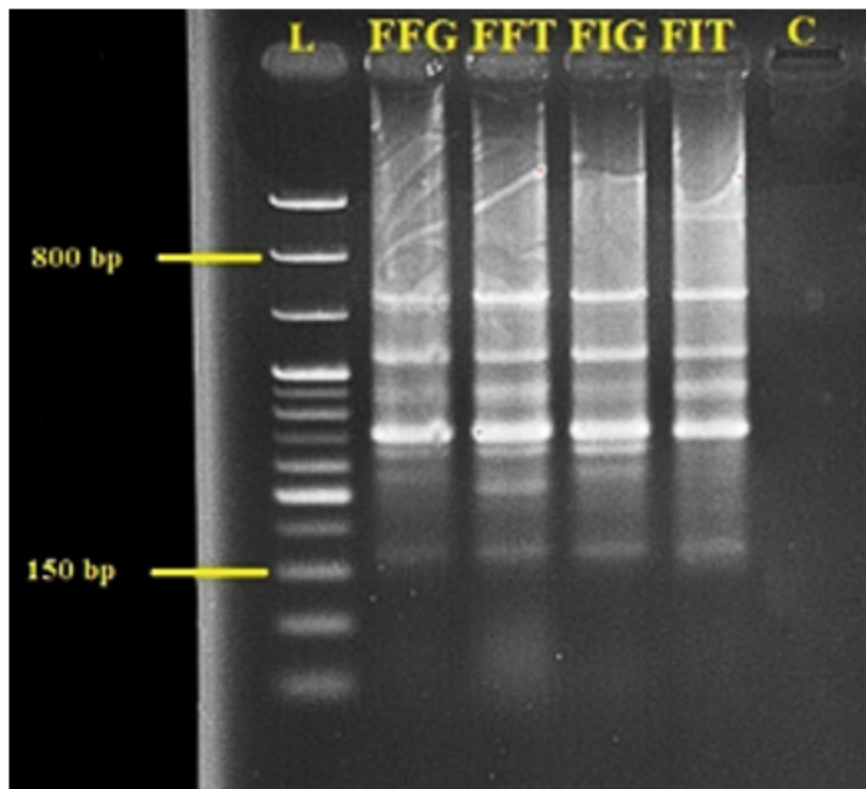


Figure 3

Gel electrophoresis of primer 2 for four treatments, L) Ladder 50 bp (, FFG, FFT, FIG, FIT (Molva genotype by seed, Molva genotype by tissue culture, Iranian ecotype by seed, Iranian ecotype by tissue culture), (C) Control

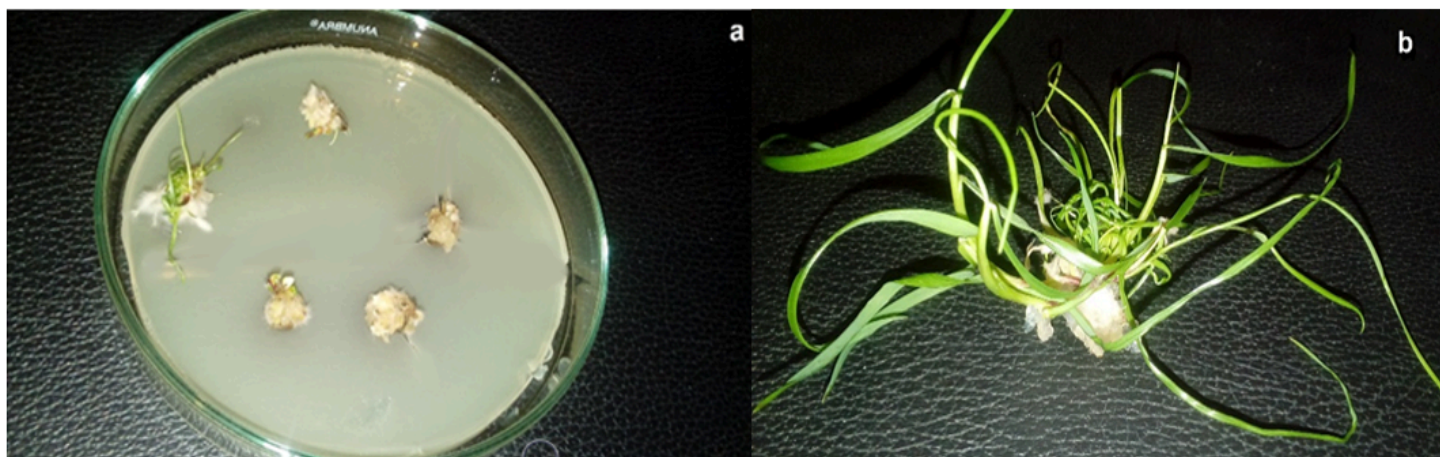


Figure 4

Callus induction(a) and (b) callus regeneration in the medium containing 20 mg l⁻¹ Ag nanoparticles.