

Optimization of tall fescue (*Festuca arundinacea*) callus induction and regeneration in culture medium containing ZnO and Ag nanoparticles

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Abstract

This experiment was conducted to optimize in vitro regeneration of tall fescue (*Festuca arundinacea*) and study of ZnO and Ag nanoparticles effects on it. The effect of six combinations of growth regulators of auxin (2.4-D) and cytokinin (BAP and Kinetin) on stem and seeds halved explant (Iranian and Foreign) was evaluated in a completely randomized design with three replications. To investigate the effect of nanoparticles on callus induction and regeneration, four concentrations of silver nanoparticles (0, 20, 40, 60 mg l⁻¹) and zinc oxide nanoparticles (0, 25, 50, 100 mg l⁻¹) in a completely randomized design with three replications were performed. The results showed that the halved seed micro-sample, the Iranian ecotype, and the MS/2 culture medium had the best results for this part of experiment. The culture medium containing 1 mg l⁻¹ 2.4-D and 0.1 mg l⁻¹ kinetin was the most suitable culture medium for callus formation, number of days to callus formation, callus length and width, callus perimeter and area, fresh and dry weight of callus. Also, the culture medium containing 0.9 mg l⁻¹ 2.4-D and 0.5 mg l⁻¹ BAP had a higher amount than other culture mediums in terms of indirect and direct regeneration. In rooting medium with the concentration of 0.25 mg l⁻¹ NAA and 0.1 mg l⁻¹ 2.4-D, the maximum and minimum root length and days to rooting were observed, respectively. The analysis of variance showed that the effect of silver nanoparticles and zinc oxide nanoparticles on the number of days to callus induction was significant. The effects of silver nanoparticles on the percentage of regeneration also were significant. The results of mean comparisons of silver nanoparticles showed that in the concentration of 20 mg l⁻¹ callus formation was induced more rapidly. However, the treatment of 60 mg l⁻¹ had the lowest average of callus induction. The results of the mean comparison showed the positive effect of zinc oxide nanoparticles on concentration of 25, 50, and 100 mg l⁻¹ on callus formation rate compared to the non-application of zinc oxide nanoparticles.

Introduction

Tall fescue (*Festuca arundinacea*) is a monocotyledonous plant of the Poaceae family under the Pooideae family. This plant is an allohexaploid (2n = 6x = 42), although species with 2n = 28 and 2n = 70 have also been reported (Aminuddin, 1993). *F. arundinacea* is one of the most important forage plants in Europe. In addition to the ornamental aspect, this plant is also cultivated in areas where there is a risk of soil erosion (Kasperbauer 1990). The advantages of the tall fescue plant include tolerance to both warm and cold weather, vertical growth that gives the grass a beautiful appearance, and high tolerance to drought (Barker and Welty, 1997).

High self-infertility of tall fescue causes slow progress in breeding success through conventional selection procedures (Spangenberg et al., 1995). Biotechnological approaches are a significant way to obtain new cultivars of perennial forage. Transgenic plants have been obtained by various methods, such as microprojectile bombardment, Agrobacterium-mediated transformation and silicon carbide fiber-mediated transformation (Dalton et al., 1998). Micropropagation has many advantages over traditional propagation methods. With micropropagation, the reproduction rate can be increased and plant materials free from viruses and other pathogens can be produced. However, it is necessary to optimize tissue culture conditions which involve genotypes, explant tissues and culture media and supplements (Bai and Qu, 2000).

Nanoparticles (NPs) have been shown in recent years to be effective in eliminating microbiological pollutants from explants and to have a beneficial effect on callus induction, organogenesis, somatic embryogenesis, somaclonal variation, and the synthesis of secondary metabolites (Kim et al., 2017). Nanoparticles (NPs) have been widely used to improve seed germination, increase plant growth and yield, induce plant genetic modification, improve the production of bioactive compounds, and increase plant protection. The effects of NPs on different features of plants are depended on NPs type, organism species, exposure period, and NPs dos. Recent studies have shown that surface disinfection of explants with nanoparticles (NPs) significantly reduces microbial contamination in various plants. The addition of nanoparticles (NPs) to tissue culture medium can eliminate bacterial contamination, increase the morphogenic potential of explants, and may lead to somaclonal variation. Abdi and Salehi (2008) used silver nanoparticles (Ag) to control bacterial contamination of *Valeriana officinalis*. The effect of silver nanoparticles (Ag) on the micropropagation of *Tecomella undulata* was also investigated (Devil's Pomegranate). The explants cultured in MS medium with 10 mg l⁻¹ Ag NPs, 2.5 mg l⁻¹ BAP, and 0.1 mg l⁻¹ IAA had higher average shoot yield, shoot length, and percentage of shootlets. However, in higher concentrations (more than 60 mg l⁻¹

¹) adverse effects on stem regeneration were observed (Aghdaei et al., 2012). Mahna et al. (2013) investigated the effect of Ag NPs treatment on the disinfection of Arabidopsis seed surfaces, potato leaves, and tomato cotyledons. The best way to disinfect the explants was to treat them with 100 mg l⁻¹ Ag NPs for one or five minutes. This treatment did not negatively impact the vitality of the plants. However, following treatment with large concentrations of Ag NPs, seed germination as well as leaf and cotyledon residues decreased (Mahna et al., 2013). The combination of Ag nanoparticles at a concentration of 50 mg l⁻¹ in MS medium, according to Sharma, reduces the levels of proline, hydrogen peroxide, and malondialdehyde (MDA) in the seedlings by means of antioxidant enzyme activity. Higher doses of silver nanoparticles (100–400 mg l⁻¹), however, had a detrimental impact on seedling development (Sharma et al., 2012).

ZnO-NPs, in particular, have been reported to enhance plant regeneration and callus growth among other nanoparticles. (Alharby et al., 2019; Mousavi Kouhi and Lahouti, 2018). The antibacterial property of ZnO-NP was also proved in some studies wherein ZnO-NP was synthesized using plant extracts and tested for its anti-microbial properties (Pal et al., 2017; Aquisman et al., 2020).

This experiment was conducted to optimize the in vitro regeneration of tall fescue and study of ZnO and Ag nanoparticle's effects on it.

Materials and Methods

Plant material, disinfection explant preparation, callus formation, and regeneration

Based on the results of the earlier investigation, two sample seeds an Iranian landrace and a foreign genotype (Molva) were chosen (Amini et al., 2019). In the present study, experiments were divided into three steps including 1- optimization of somatic embryogenesis, regeneration, and rooting, 2- evaluation of somaclonal variation of the samples based on ISSR molecular markers, and 3- evaluation of the effect of Ag nanoparticles and ZnO nanoparticles on tissue culture of tall fescue.

In the first step, the seeds were placed in running water for 30 minutes and then, immersed in a Thiram fungicide solution of 1 g l⁻¹ for 15 minutes. The seeds were washed three times with sterile water and in the next step, they were placed in 70% alcohol for one minute. The seeds were placed in two-and-a-half-percent sodium hypochlorite solution for 15 minutes and were washed three times with double sterile distilled water. MS culture medium with pH = 5.8 and 8 g l⁻¹ agar was used for seed culture. Ten seeds were placed inside each glass container and wrapped around them, they were placed in the growth chamber under the conditions of 16 hours of light and 8 hours of darkness at a temperature of 25 C°. After about 5 days, the seeds started to germinate. After sufficient growth and reaching the height of about 15 cm of seedlings and the appearance of the main organs of the plant, explants were taken.

To investigate callus formation and regeneration the experiment was performed in a factorial form using a completely randomized design with three factors and three replications (as Petri dishes). Five explant segments were placed in each Petri dish containing 25ml different medium. Studied factors included two genotypes, two explants (seed and stem) and six different concentrations of plant growth regulations (PGRs): 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). In each samples, callus percentage, regeneration percentage, direct regeneration percentage, callus fresh weight, callus dry weight, callus perimeter, length, width and area were measured.

Since the distribution of the recorded data related to callus percentage, regeneration percentage, direct regeneration percentage was not normal and no transformation was able to induce normality, the Kruskal-Wallis and the Mann-Whitney nonparametric tests were used. The data relating to callus fresh weight, callus dry weight, callus perimeter, length, width and area were subjected to ANOVA and DMRT (Duncan's Multiple Range Test) to evaluate the statistical significance and were expressed as the mean ± SE. SAS 9.2, SPSS 16.0, and Excel software were used for data analysis.

In Vitro rooting and acclimatization

Since the best response of plant regeneration was observed in the MS/2 medium containing 0.1 mg l^{-1} Kinetin and 1 mg l^{-1} 2,4-d, the regenerated 3 to 4 cm long plantlets were excised from the mentioned medium, and then were cultured in Petri dishes containing MS/2 medium. The following three combination treatments were investigated for more rooting: (0.25 mg l^{-1} NAA + 0.1 mg l^{-1} 2,4-D), (0.5 mg l^{-1} NAA + 0.1 mg l^{-1} 2,4-D), and (1 mg l^{-1} NAA + 0.1 mg l^{-1} 2,4-D). Petri dishes were observed every 3–4 days regularly and then characteristics including the number of days to root formation and root length were recorded to compare investigated treatments. The rooting experiment was performed as a completely randomized design with three replications (as pots). Ten regenerated plantlets were placed in each pot containing 50 ml of the investigated medium. The data relating to the rooting experiment were subjected to ANOVA and DMRT (Duncan's Multiple Range Test).

Evaluation of somaclonal variation by ISSR markers

In this experiment, the genetic homogeneity of the regenerated plantlet as compared to mother plants was examined by ISSR markers. Total genomic DNA was extracted from young leaves of the in vitro regenerated plant and mother plant following the CTAB procedure according to Murray and Thompson (Murray and Thompson, 1980) with minor modifications. The quality and quantity of extracted genomic DNA were checked on 1% agarose gel and spectrophotometer and the samples were diluted to a final concentration of $50 \text{ ng } \mu\text{l}$. Five ISSR markers were used for DNA amplification that was performed in a $10 \mu\text{l}$ reaction volume containing $3 \mu\text{l}$ double-distilled water, $5 \mu\text{l}$ PCR amplification kit ($10\times$), $1 \mu\text{l}$ primer (10 pmol), and $1 \mu\text{l}$ DNA ($50 \text{ ng } \mu\text{l}^{-1}$). The amplification program included an initial 5 min denaturation at 94°C , followed by 34 cycles of 30 sec at 94°C , 45 sec at $56\text{--}59^{\circ}\text{C}$, and 2 min at 72°C , with a final extension at 72°C for 5 min. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized on a UV light transilluminator.

Application of ZnO and Ag Nanoparticles in vitro condition

ZnO nanoparticle and Ag nanoparticle powder was purchased from Merck, Germany. A suspension of silver nitrate (Ag) nanoparticles at a concentration of 500 ppm was prepared as a stock solution. In this experiment, in the form of a completely randomized design with three replications where each petri was considered as replication and 5 explants were cultured in each petri, the application of ZnO and Ag nanoparticles was investigated. In this experiment, the Iranian ecotype halved seed explant, and hormone level (1 mg l^{-1} 2,4-d + 0.1 mg l^{-1} Kinetin) which was prepared in the tissue culture experiment were used. Ag nanoparticles were used at levels of 0, 20, 40, and 60 mg l^{-1} and ZnO nanoparticles at levels of 0, 25, 50, and 100 mg l^{-1} . Traits such as the number of days to callus formation and regeneration percentage were evaluated.

Data normality was checked and then. analysis of variance and comparisons of treatment means were performed using Duncan's multi-range test using SAS software.

Results

Investigating the effect of plant growth regulators treatments, explants, and ecotypes on callus formation and regeneration

The highest callus percentage and regeneration percentage were observed in the treatments (1 mg l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin) and (2 mg l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin) (Fig. 1,2). The effect of treatment (0.9 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP) on the direct regeneration percentage was higher than other treatments (Table 1) (Fig. 2, c). The results of this study showed that the seed explant had a higher mean percentage of callus formation and regeneration than the stem explant, but no difference was observed between them for direct regeneration (Table 2). According to the results obtained from the average comparison test on the fresh and dry weight traits of callus, culture medium (1 mg l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin) and (2 mg l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin) accounted for the highest dry weight and fresh dimensions of callus (Table 1).

Table 1
The effect of plant growth regulators (PGRs) on the means of callus related traits of tall fescue.

Plant Growth Regulators (PGRs)						
Traits	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
Callus perimeter	1.94 ± 0.24	1.57 ± 0.22	5.00 ± 0.59	4.83 ± 0.84	1.23 ± 0.74	2.12 ± 0.23
Callus length	0.77 ± 0.02	0.62 ± 0.08	1.86 ± 0.26	1.79 ± 0.09	0.46 ± 0.05	0.76 ± 0.09
Callus width	0.48 ± 0.12	0.37 ± 0.05	1.36 ± 0.13	1.27 ± 0.09	0.37 ± 0.04	0.57 ± 0.09
Callus area	1.06 ± 0.23	0.74 ± 0.05	2.98 ± 0.66	2.87 ± 0.01	0.50 ± 0.07	0.97 ± 0.08

Table 2
The effect of different explant (stem and seed) on the means of callus related traits of tall fescue.

Traits	explant	
	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,	0.05 ± 0.006	0.07 ± 0.004
Callus dry weight	0.02 ± 0.002	0.02 ± 0.001
Callus perimeter	1.69 ± 0.85	3.58 ± 0.45
Callus length	0.63 ± 0.06	1.30 ± 0.08
Callus width	0.45 ± 0.05	0.99 ± 0.09
Callus area	0.96 ± 0.21	1.76 ± 0.29

In Vitro Rooting and Acclimatization

The results of the mean comparison showed that the Iranian ecotype had a lower number of days to root formation (Table 3). Moreover, the means of root length was significantly higher in the Iranian ecotype (Table 3). In the comparison of the average effect of growth regulators on the number of days to root formation and root length, it was observed that culture medium 1 (0.25 mg l^{-1} NAA + 0.1 mg l^{-1} 2,4-D) had the lowest amount for the number of days to root formation and highest amount for root length (Table 3). Figure 3 shows the roots produced in the Iranian ecotype cultivated in a culture medium 1 (0.25 mg l^{-1} NAA + 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
Ecotype	Iranian landrace	9.00 ^b	5.61 ^a
	Molva	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
Values in columns followed by the same letters are not statistically significant based on the Duncan's multiple rang test ($p = 0.05$).			

Investigating somaclonal variation through molecular markers

In this experiment, ten ISSR primers were used, of which 3 primers contained useful information and reproducible bands. 5–10 bands were generated per primer, ranging in size from approximately 150 to over 800 bp. These primers produced a total of 24 clear bands, with an average of 8 bands per primer. In this study, ISSR markers created polymorphic bands, which was 15% in the Iranian ecotype (tissue culture and seed sample) and 21% in the foreign variety (tissue culture and seed sample). Also, the presence of polymorphic bands was 33% in both Iranian ecotypes and foreign cultivars (Table 4).

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

number	Primer sequence	Annealing temperature	Total band	Number of polymorph band	Polymorphism (%)
1	(AC) 8 TG	54.8	11	4	36
2	(GT) 8 C	52.8	5	1	20
3	(AC) 8 CT	52.5	8	3	37
sum	-		24	8	33

Investigating the effect of ZnO Nanoparticles and Ag Nanoparticles on callus induction and regeneration the results of the mean comparison of Ag nanoparticles showed that the treatment of 20 and 0 mg l^{-1} had the lowest and highest number of days to callus formation, respectively (Table 5). In this study, the results of mean comparisons also showed that increasing the concentration of Ag nanoparticles harmed the regeneration percentage, and in the concentration of 60 mg l^{-1} the regeneration was significantly reduced (Table 5). The results of the mean comparing the effect of ZnO nanoparticles showed that the levels of 25, 50, and 100 mg l^{-1} had a better callus formation rate than the level of 0 mg l^{-1} (Fig. 5). It was also observed that callus fresh weight, in the concentration of 50 mg l^{-1} had the highest amount compared to other treatments (Fig. 6).

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

Concentration(mg l ⁻¹)	Regeneration percentage	Number of days to callus formation	Callus						
			percentage	fresh weight	dry weight	perimeter	length	width	area
0	6.00 ^b	10.23 ^b	38.45 ^c	0.07 ^b	0.03 ^b	3.60 ^b	1.36 ^b	1.06 ^b	1.80 ^b
20	24.44 ^a	9.33 ^a	71.11 ^a	0.14 ^a	0.05 ^a	6.20 ^a	2.26 ^a	1.69 ^a	3.38 ^a
40	11.11 ^{ab}	10.88 ^b	51.11 ^{ab}	0.10 ^a	0.04 ^{ab}	4.49 ^{ab}	1.64 ^{ab}	1.27 ^{ab}	2.53 ^{ab}
60	6.67 ^b	10.44 ^b	42.22 ^b	0.08 ^b	0.03 ^b	3.69 ^b	1.34 ^b	1.06 ^b	1.89 ^b
Values in columns followed by the same letters are not statistically significant based on the Duncan's multiple rang test (p = 0.05).									

Discussion

The most crucial elements for controlling growth in tissue culture are typically thought to be the interactions between auxin, cytokinin, and the auxin-cytokinin combination (Evans et al., 1981; Vasil 1994). In this experiment, different levels of 2,4-D were used as auxin, and the level of 1 mg l⁻¹ showed the highest percentage of callus formation and the speed of callus formation. In one experiment, 5 mg l⁻¹ of 2,4-D was the ideal concentration for inducing callus from mature Siberian wild grass seeds (Lee et al., 2012). Callus development and regeneration capacity are enhanced when auxin is combined with low concentrations of cytokinins, such as 6-benzyl amino purine (BAP) (Gaba., 2005). In the present study, low levels of kinetin and BAP were used, and the highest percentage of regeneration and direct regeneration was observed in 0.5 mg l⁻¹ of BAP. Researchers reported that cytokinins increase the differentiation of plants and are mostly used in regeneration medium in plant tissue culture. Also, in the measurement of the dry and fresh weight of the callus, it was observed that the culture medium containing 1 and 2 mg l⁻¹ 2,4-D had the highest dry and fresh weight. These results showed that; The dimensions of the callus are likewise affected by the concentration of 2,4-D; their dimensions grow as the concentration increases from 0.5 to 1 mg l⁻¹. Researchers studying the development of calluses on tall fescue cultivars found that a concentration rise of 2,4-D (2 to 5 mg l⁻¹) results in an increase in callus size (Zhang et al., 2006). In cereals and other plants, the quality of callus induction and the capacity for callus regeneration are enhanced by a certain auxin to cytokinin ratio; however, this effect may depend on the level of endogenous cytokinin (Bai and Qu., 2001). The effects of growth regulators on tissue culture responses were examined using immature embryos and mature seeds as explant tissues in an effort to optimize the tissue culture propagation conditions of tall fescue. Callus produced by a BAP-containing media greatly increased the explants' capacity to regenerate (Bai and Qu, 2001). Mature seeds, stem meristems, and immature inflorescences are often used in the tissue culture of cereals and grasses. Meanwhile, mature seeds are the most convenient explants to use. They have the advantage of being available in all seasons, while other types of explants must be collected from growing plants (Bai, 2001). In this experiment, halved seed explants that were cut longitudinally from the embryonic part were used and had a more significant performance in all the tested traits.

In the present experiment, NAA and 2,4-D auxins were used for rooting. It was observed that by increasing the concentration of NAA from 0.25 mg l⁻¹ to 0.5 and 1 mg l⁻¹, the speed of rooting decreased and the root length also decreased significantly. It can be concluded that the increase of auxin in this experiment had a negative effect on rooting-related traits. Root differentiation can be stimulated by increasing the concentration of NAA, but greater quantities have an inhibitory impact

(Poordad et al., 2014). In a study conducted on the rooting of *Lathyrus sativus* L., it was observed that the root length decreases with increasing NAA concentration (Barpete et al., 2014).

It has been suggested that the genus *Festuca* be screened for somaclonal variation using molecular markers (Valles et al., 1993). The experiment's regenerated plants and seed plants showed polymorphic bands of ISSR primers, which could be linked to somaclonal variation, the impure character of tall fescue plants, or their pollination mechanism. Polymorphic bands were found between regenerated plants and seed plants in a study employing RAPD primers to examine the somaclonal variation of cumin seed and tissue culture plants (Bahmankar et al., 2015). Research on the genetic diversity of mother plant seedlings and *Thymus* regined plant seedlings discovered that polymorphic bands are present in the RAPD marker. They believed the thyme pollination system might have something to do with the alterations they had observed (Bakhtiar et al., 2014).

In this study, for the first time, some aspects of tall fescue tissue culture, including the callus percentage, the characteristics of callus dimensions (callus perimeter and area, callus length and width), fresh and dry weight of callus under the influence of Ag and ZnO nanoparticles were studied. Additional researchers noted that the application of nanoparticles has effectively resulted in the removal of microbial contaminants from explants and highlighted the beneficial impact of nanoparticles in the production of callus, organogenesis, somatic embryogenesis, and somaclonal variation (Kim et al., 2017). Since the main goal of the present study was to investigate the effect of Ag nanoparticles and ZnO nanoparticles on the tissue culture of tall fescue, disinfection of plant explants was considered one of the essential prerequisites in the tissue culture technique. The results of this research showed that Ag nanoparticles were able to reduce the contamination of the culture environment. By incorporating nanoparticles into the tissue culture media, it is possible to eradicate bacterial contamination and enhance the explants' capacity for morphogenesis (Kim et al., 2017). There are numerous accounts of nanoparticles getting into the culture medium and causing somaclonal variation in the way that plant tissues, cells, or cultivable organs are treated (Wang et al., 2001; Kim and Gopal, 2017). In this study, the results of mean comparisons showed that increasing the concentration of Ag nanoparticles more than 20 mg l^{-1} decrease the amount of evaluated traits. The process of regeneration of tall fescue seedlings with the use of Ag nanoparticles in concentration of 20 mg l^{-1} was increased compared to the control, although this increase was not significant. It can be said that if the Ag nanoparticle was used in concentrations less than 20 mg l^{-1} , it would affect the regeneration process more effectively. Researchers suggest that in order to apply chemicals like antibiotics, plant growth regulators, and nanoparticles to plant cells and tissues and get the intended effect, the optimal concentration of each substance should be identified (Bahmankar et al., 2015). In general, the results of this study showed that the effect of Ag nanoparticles on the characteristics of callus formation was significant and callus formation has been induced at a concentration of 20 mg l^{-1} at a faster rate than other treatments. The efficiency of nanoparticles can vary with different genotypes and applications, and this can be due to different reasons such as the difference in nanoscale size, the probability that these structures will lose their properties in the physiological processes of plants, and the properties of nanoscale metals (Lee et al. 2008; Wang et al. 2012).

In the comparison results of the average effect of ZnO nanoparticles, it was observed that in callus fresh weight, the concentration of 50 mg l^{-1} was the highest compared to other treatments. ZnO is a low-consumption but essential element in plants. Many nanoparticles, such as ZnO nanoparticles, show significant antimicrobial activity.

ZnO nanoparticles antimicrobial activity has been attributed to a number of processes, including protein leakage, DNA damage, penetration into cells, and blocking of cell communication channels (Wang et al. 2012). In this study, ZnO was found to have beneficial antibacterial properties. also, ZnO nanoparticles at a 50 mg l^{-1} concentration worked well on callus fresh weight. The callus fresh weight increases when the concentration is increased from 25 to 50 mg l^{-1} , but it decreases when the concentration is increased to 100 mg l^{-1} . High concentrations of ZnO have effects that hinder plant growth (Paschke et al., 2006). The factors that could contribute to the diminished beneficial utilization of nanoparticles by the plant include that, the nanoparticle structures will not dissolve in the medium and that the nanoparticles are of a different grade than the metals in the nutrient medium (Kim et al. 2014). While nanoparticles were tested against the expectation that they could accelerate plant growth and boost regeneration, they might not demonstrate a useful rate of regeneration when compared to standard

procedures carried out in wheat tissue culture. This could be because of the potential for nanotoxicity as well as the inability of plants to utilize nanoparticle structures efficiently (Nalci et al., 2019).

Conclusion

According to the observations of this study the halved seed micro-sample, the Iranian ecotype, and the MS/2 culture medium showed the best results for this experiment. The culture medium containing 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin was the most suitable culture medium for callus formation, number of days to callus formation, callus length and width, callus perimeter and area, fresh and dry weight of callus. Also, the culture medium containing 0.9 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP had a higher amount than other culture mediums in terms of indirect and direct regeneration. In rooting medium with the concentration of 0.25 mg l^{-1} NAA and 0.1 mg l^{-1} 2,4-D, the maximum root length and minimum days to rooting were observed. Considering the observations of optimizing the concentration of auxin growth regulators, especially 2,4-D and NAA in callus formation, the speed of callus formation, callus dimensions, and rooting of tall fescue plant is essential.

In the experiment investigating the use of Ag nanoparticles and ZnO nanoparticles, it was observed that the level of 20 mg l^{-1} of Ag had a positive effect on the evaluated traits, but the concentration of 60 mg l^{-1} had a toxic effect and caused a decrease in the regeneration of the tall fescue plant. Also, different concentrations of ZnO nanoparticles had a positive effect on the fresh weight of callus, and the concentration of 50 mg l^{-1} showed the highest callus fresh weight compared to other treatments.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest in the publication.

Ethical Approval

Not required as no human data or animal samples were used.

Consent to Participate

All authors agreed to participate in the present work.

Consent to Publish

All the authors agreed with the present publication.

Authors Contributions

Fatemeh Amini is the supervisor and suggested the research idea; Meisam Moradi asl collected data, and contributed to interpreting the results. Ali Izadi Darbandi contributed in collecting data and statistical analysis. All authors approved the final manuscript for publication.

Competing Interests

The authors declare that they have no conflict of interests.

Availability of data and materials

All data generated or analyzed during this study are included in the article and its supplementary information.

References

1. Abdi, G., Salehi, H., & Khosh-Khui, M. (2008). NanoAg: a novel nanomaterial for removal of bacterial contaminants in valerian (*Valeriana officinalis* L.) tissue culture. *Acta Physiologiae Plantarum*, 30(5), 709-714.
2. Aghdaei, M., Sarmast, M. K., & Salehi, H. (2012). Effects of Ag nanoparticles on *Tecomella undulata* (Roxb.) Seem. micropropagation. *Effects of Ag Nanoparticles on Tecomella undulata* (Roxb.) Seem. Micropropagation, 21-24.

3. Amini F, Masoumi P, Ramshini H, Norouzian M.A. (2019). Study of genetic diversity in Iranian tall fescue ecotypes using agro-morphological traits, nutritional values and AFLP molecular markers. *Iranian Journal of Genetic and Plant Breeding* 7(2): 54-64.
4. Aminuddin, Johri J.K, Anus M and Balasubramaniam V.R. (1993). Regeneration of Piper betle from callus tissue. *Curr. Sci.* 65, 795-796.
5. Alharby, H. F., Metwali, E. M., Fuller, M. P., Aldhebiani, A. Y. (2016). Impact of application of zinc oxide nanoparticles on callus induction, plant regeneration, element content and antioxidant enzyme activity in tomato (*Solanum lycopersicum* Mill.) under salt stress, *Arch. of Biol. Sci.*, 68(4), 723-735.
6. Aquisman, A. E., Wee, B. S., Chin, S. F., Kwabena, D. E., Michael, K. O., Bakeh, T., Semawi, S., Sylvester, D. S. (2020). Synthesis, Characterization, and Antibacterial Activity of ZnO Nanoparticles from Organic Extract of Cola nitida and Cola acuminata Leaf, *Int. J. Nanosci. Nanotechnol.*, 16(2), 73-89.
7. Bai, Y., & Qu, R. (2001). Factors influencing tissue culture responses of mature seeds and immature embryos in turf-type tall fescue. *Plant Breeding*, 120(3), 239-242.
8. Bai, Y. (2001). Tissue culture and genetic transformation of tall fescue. North Carolina State University
9. Bahmankar, M., Mortazavian, S.M.M., Tohidfar, M. (2015). Determination of Threshold Concentration of Kanamycin to Transfer Gene in Cumin. *Electronic Journal of Biology*, 11:4.
10. Bakhtiar, Z., Mirjalili, M. H., Sonboli, A., Farimani, M. M., & Ayyari, M. (2014). In vitro propagation, genetic and phytochemical assessment of Thymus persicus—a medicinally important source of pentacyclic triterpenoids. *Biologia*, 69(5), 594-603.
11. Barker, R. E., & Welty, R. E. (1997). Registration of ORTFRR-T94 and ORTFRR-F94 tall fescue germplasm with resistance to stem rust. *Crop Science*, 37(3), 134-135.
12. Barpete, S., Khawar, K. M., & Özcan, S. (2014). Differential competence for in vitro adventitious rooting of grass pea (*Lathyrus sativus* L.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, 119(1), 39-50.
13. Dalton, S.J., Bettany A.J.E., Timms, E., Morris, P. (1998) *Plant Sci.*, 132, 31–43
14. Evans, M. J., & Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *nature*, 292(5819), 154-156.
15. Gaba, V. P. (2005). Plant growth regulators in plant tissue culture and development. *Plant development and biotechnology*, 87-99.
16. Kasperbauer, M. J. (1990). *Biotechnology in tall fescue improvement*. CRC Press
17. Kim, D. H., Gopal, J., & Sivanesan, I. (2017). Nanomaterials in plant tissue culture: the disclosed and undisclosed. *RSC advances*, 7(58), 36492-36505.
18. Kim, Y. R., Park, J. I., Lee, E. J., Park, S. H., Seong, N. W., Kim, J. H., ... & Kim, M. K. (2014). Toxicity of 100 nm zinc oxide nanoparticles: a report of 90-day repeated oral administration in Sprague Dawley rats. *International journal of nanomedicine*, 9 (sup2), 109-126.
19. Lee, W. M., An, Y. J., Yoon, H., & Kweon, H. S. (2008). Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): plant agar test for water-insoluble nanoparticles. *Environmental Toxicology and Chemistry: An International Journal*, 27 (9), 1915-1921.
20. Lee, K. W., Chinzorig, O., Choi, G. J., Kim, K. Y., Ji, H. C., Park, H. S., & Lee, S. H. (2012). Callus induction and plant regeneration from mature seeds of Siberian wildrye grass (*Elymus sibiricus* L.). *The Journal of Animal and Plant Sciences*, 22(2), 518-521.
21. Mahna, N., Vahed, S. Z., & Khani, S. (2013). Plant in vitro culture goes nano: nanoAg-mediated decontamination of ex vitro explants. *J. Nanomed Nanotechol*, 4(161), 1.
22. Mousavi Kouhi, S. M., Lahouti, M. (2018). Application of ZnO nanoparticles for inducing callus in tissue culture of rapeseed, *Int. J. Nanosci. Nanotechnol.*, 14(2), 133-141.

23. Murray, M. G., & Thompson, W. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic acids research*, 8(19), 4321-4326.
24. Nalci, O. B., Nadaroglu, H., Pour, A. H., Gungor, A. A., & Haliloglu, K. (2019). Effects of ZnO, CuO, and $\gamma\text{-Fe}_3\text{O}_4$ nanoparticles on mature embryo culture of wheat (*Triticum aestivum* L.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, 136, 269-277.
25. Pal, S., Mondal, S., Maity, J., Mukherjee, R., (2018). Synthesis and characterization of ZnO nanoparticles using *Moringa oleifera* leaf extract: Investigation of photocatalytic and antibacterial activity, *Int. J. Nanosci. Nanotechnol.*, 14(2), 111-119.
26. Poordad, B., Safarnezhad, A., Ebrahimi, M. A., & Bakhshi Khaniki, G. (2014). Investigation of effective factors on sumac In vitro propagation. *Iranian Journal of Rangelands and Forests Plant Breeding and Genetic Research*, 22(1), 25-33.
27. Paschke, M. W., Perry, L. G., and Redente, E. F. (2006). Zinc toxicity thresholds for reclamation forb species. *Water, air, and soil pollution*, 170, 317-330.
28. Sharma, P., Bhatt, D., Zaidi, M. G. H., Saradhi, P. P., Khanna, P. K., & Arora, S. (2012). Ag nanoparticle-mediated enhancement in growth and antioxidant status of *Brassica juncea*. *Applied biochemistry and biotechnology*, 167(8), 2225-2233.
29. Spangenberg, G., Wang, Z.Y., & Wu, X.L. (1995) *Plant Sci.*, 108, 209–217.
30. Valles, M. P., Wang, Z. Y., Montavon, P., Potrykus, I., & Spangenberg, G. (1993). Analysis of genetic stability of plants regenerated from suspension cultures and protoplasts of meadow fescue (*Festuca pratensis* Huds.). *Plant cell reports*, 12(2), 101-106.
31. Vasil, I. K. (1994). Molecular improvement of cereals. *Plant Molecular Biology*, 25(6), 925- 937.
32. Wang, C., Liu, L. L., Zhang, A. T., Xie, P., Lu, J. J., and Zou, X. T. (2012). Antibacterial effects of zinc oxide nanoparticles on *Escherichia coli* K88. *African Journal of Biotechnology*, 11(44), 10248-10254.
33. Wang, Z., Hopkins, A., & Mian, R. (2001). Forage and turf grass biotechnology. *Critical Reviews in Plant Sciences*, 20(6), 573-619.
34. Zhang, W. J., Dong, J. L., Liang, B. G., Jin, Y. S., & Wang, T. (2006). Highly efficient embryogenesis and plant regeneration of tall fescue (*Festuca arundinacea* Schreb.) from mature seed-derived calli. *In Vitro Cellular & Developmental Biology-Plant*, 42(2), 114-118

Figures

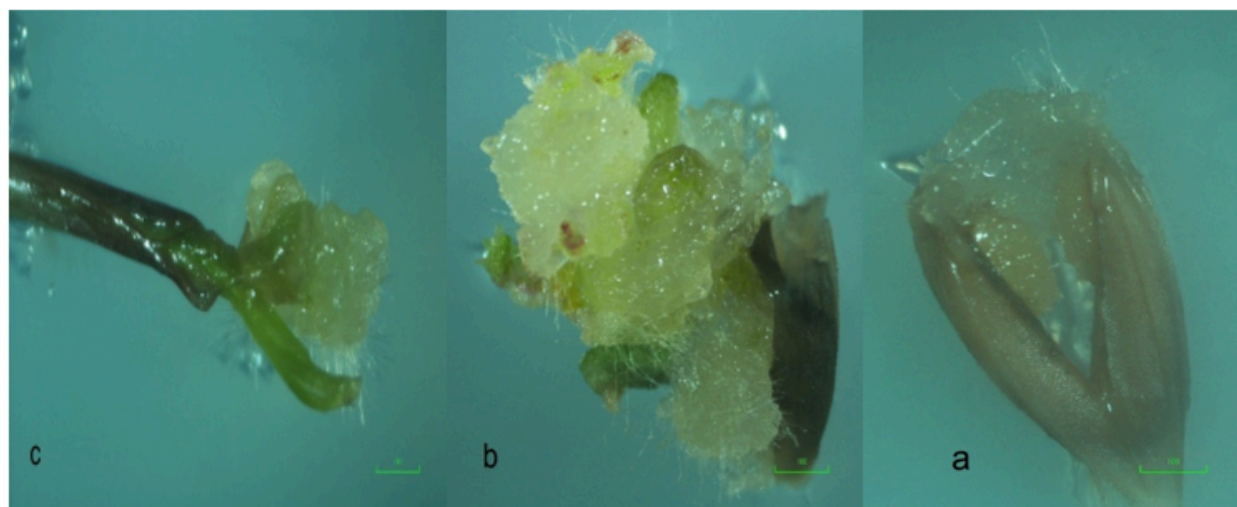


Figure 1

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

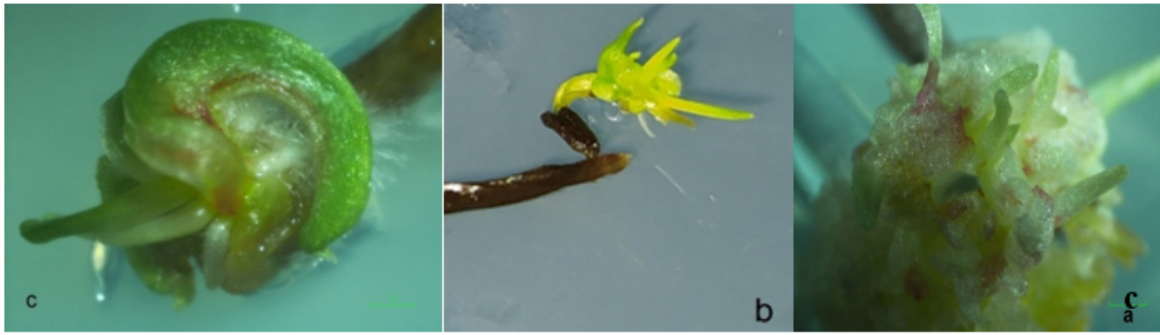


Figure 2

(a) callus regenerated from a halved seed explant, (b) callus regenerated from a stem terminal meristem explant, (c) direct regeneration from a stem terminal meristem explant.



Figure 3

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)

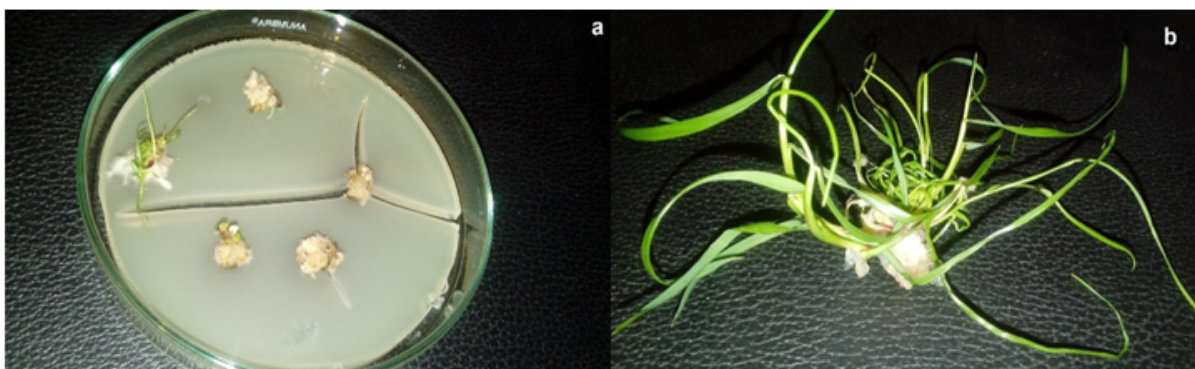


Figure 4

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

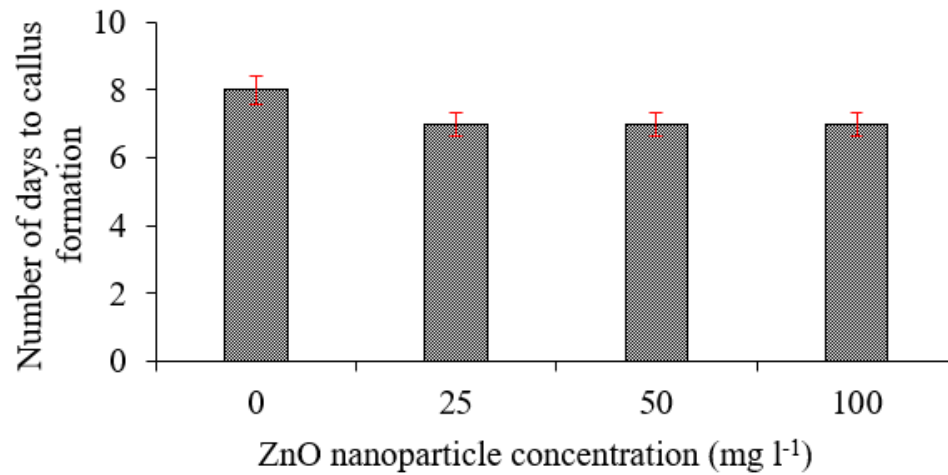


Figure 5

The effect of ZnO nanoparticles on callus formation rate of tall fescue using Duncan's mean comparison test ($P \leq 0.05$).

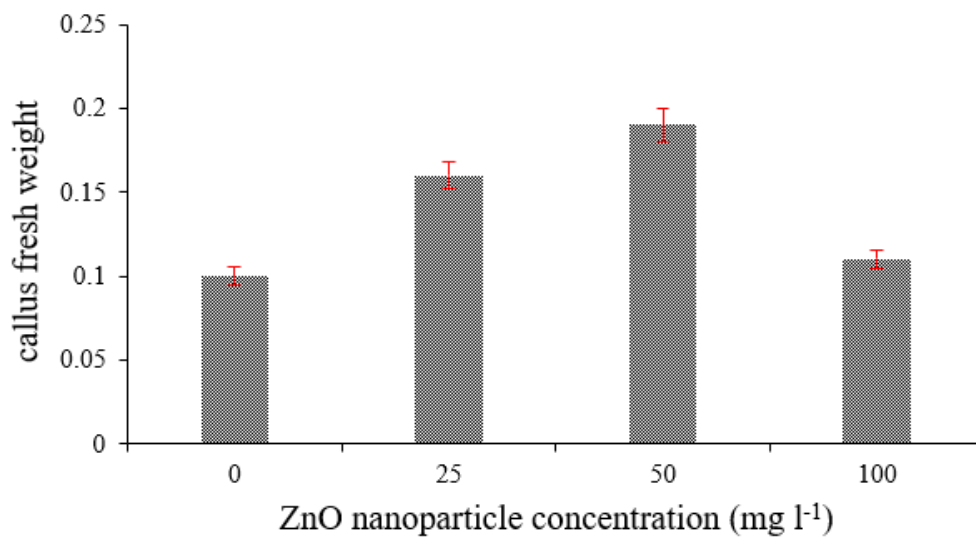


Figure 6

The effect of ZnO nanoparticles on callus fresh weight of tall fescue using Duncan's mean comparison test ($P \leq 0.05$).