

Effect of Nitrate, Ammonium and Mesos on *In Vitro* Multiplication of *Gerbera jamesonii*: New Findings

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Gerbera jamesonii (gerbera) is a commercially cultivated cut flower worldwide. Owing to high levels of heterozygosity, micropropagation is the most suitable approach for the rapid propagation of gerbera cultivars. The optimization of a suitable culture medium is critical for the micropropagation protocol and requires the adjustment of medium components, such as macro- and micronutrients. A full factorial experiment ($2 \times 2 \times 3 \times 3$) was designed using KNO_3 at 0.5X and 1X levels, NH_4NO_3 at 0.5X, 1X, and 1.5X levels, mesos ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and KH_2PO_4) at 0.5X, 1X, and 1.5X levels, and two cultivars (Artist and Brilliance) to analyze the effect of their single and multiple interactions on several *in vitro* growth parameters of gerbera. The current study indicated that the triple interaction among nitrate, ammonium, and mesos affects all growth parameters, and interestingly, $\text{KNO}_3 \times \text{NH}_4\text{NO}_3$ did not affect shoot number. As a result, the highest number of shoots, the lowest number of roots, and the lowest length of the roots were obtained using low concentrations of KNO_3 (0.5X) and NH_4NO_3 (0.5X) and increased levels of mesos (1.5X). This research reports new findings on the interaction effects among macronutrients and suggests how they can be modified to improve the rate of micropropagation as well as the quality of regenerated gerbera plants.

Abstract

Keywords: Ammonium, Gerbera, Mesos, Micropropagation, Nitrate.

INTRODUCTION

Gerbera jamesonii Bolus is an ornamental perennial herb of Asteraceae and native to South Africa and Asia (Kumar *et al.*, 2020). It is favored for its large, colorful flowers, and long-lasting blooming. Flowers can be produced in protected greenhouses under various climatic conditions. Gerberas can propagate both sexually and clonally. However, for commercial purposes, vegetative methods are too slow, and plant tissue culture is an effective method for rapid mass propagation. An efficient and productive *in vitro* culture system depends on the physiological status of the donor plant, genotype, explant type, medium composition, and their interactions. Moreover, the quality of regenerated plants directly affects not only the *in vitro* multiplication process, but also the adaptation and post-harvest stages. MS (Murashige and Skoog, 1962a) is commonly used basal culture medium for gerbera micropropagation (Cardoso and Teixeira da Silva, 2013).

The composition of nutrients in the multiplication medium determines the proliferation rate and quality of *in vitro* plantlets. Supplementation of culture medium with adequate amounts of major essential elements (N, P, K, Ca, Mg, and S) contributes to high efficiency of propagation rate, high-quality plants for post-harvest procedures, and low cost of commercial production. In a current study, $\frac{1}{4}$ or $\frac{1}{2}$ MS was used to improve multiplication rate of gerbera cultivars (da Silva *et al.*, 2020). The same results reported by Shabanour *et al.* (2011) and half strength MS supplemented with 2 mg L⁻¹ BAP (6-benzylaminopurine) induced low number of shoots. Cioc *et al.* (2022) used MS medium supplemented with 1.1 mg L⁻¹ BAP for multiplication of gerbera. However, there is no information regarding the quality of the plants after several subcultures. Additionally, the NH₄⁺ / NO₃ ratio is the main factor affecting the quality of the produced plants. Their excessive application causes vitrification during the first or second subculture in some varieties or after successive subcultures in some tolerant varieties. Therefore, it is crucial to determine the optimum concentration of macronutrients in the culture medium.

Overall, in previous studies, optimization was limited only to reducing all macronutrients together by the same ratio (Cardoso and Teixeira da Silva, 2013), but generally, each macronutrient has a different concentration, and even two of them, including KNO₃ and NH₄NO₃ are present in a higher proportion relative to KH₂PO₄, MgSO₄·7H₂O, and CaCl₂·2H₂O. To date, there have been no reports on the interaction between different concentrations of dominant salts, including KNO₃, NH₄NO₃, and mesos (MgSO₄·7H₂O, CaCl₂·2H₂O, and KH₂PO₄), in the culture medium of gerbera. This study aimed to examine the relationship between different concentrations of these macronutrients and analyze their single effects and interactions. These results provide important clues for improving the micropropagation protocol of gerbera.

MATERIALS AND METHODS

Explant preparation and culture media

Young and immature capitulum explants (with 10 – 15 mm in diameter) of two *Gerbera jamesonii* cultivars (Brilliance and Artist) were collected and used for establishment step. The explants were washed under tap water for 30 min and then immersed in 70% alcohol for one minute followed by thorough washing three times with sterilized distilled water. For the final surface sterilization, explants were immersed in 20% commercial bleach with 0.01% Tween-20 for 20 min, followed by washing three times with sterilized distilled water.

The basal medium used in this research was MS containing 30 g L⁻¹ sucrose, and 0.7% agar. The pH of all media was adjusted to 5.7 before autoclaving at 121 °C and 110 kPa for 30 min.

Establishment step

Sterilized explants were cut into three pieces and cultured in the establishment culture

medium containing 0.5 mg L⁻¹ TDZ (Thidiazuron). Cultures were kept in a growth chamber at 25 °C with a 16 h photoperiod, cool white fluorescent bulbs (PPFD = 35 µmol m⁻² s⁻¹), and 8 h darkness. After two months, regenerated shoots were transferred to the multiplication medium and used for subsequent experiments.

Multiplication step

A full factorial experiment (2 × 2 × 3 × 3) was designed to evaluate the effects of the main macrosalts at various concentrations, including KNO₃ at 0.5X and 1X levels; NH₄NO₃ at 0.5X, 1X, and 1.5X levels; mesos (including KH₂PO₄, MgSO₄ · 7H₂O, and CaCl₂ · 2H₂O) at 0.5X, 1X, and 1.5X levels; and two cultivars (Brilliance and Artist) on *in vitro* propagation parameters. All media contained 0.3 mg L⁻¹ BA (6-benzyladenine) and 0.05 mg L⁻¹ NAA (naphthalene acetic acid). Finally, all possible combinations of factors at all levels produced 36 treatments (Table 1). The concentration of macronutrients was calculated according to the MS medium. In other words, 1X is the corresponding concentration of a mineral in the MS medium. Three replicates (jars) were prepared for each treatment, and each jar included three gerbera axillary shoots. Treatments were transferred to a growth chamber with a 16 h photoperiod, temperature of 25 °C and irradiance of 35 µmol photon m⁻² s⁻¹. The characteristics evaluated after 45 d of culture were the number of shoots, shoot length of the highest shoot (cm), length of the roots (cm), and number of roots.

Table 1. The four factors and their components were used to construct a 2 × 2 × 3 × 3 full factorial design. Concentrations are expressed as MS levels.

Factors		Component	
Cultivar	Artist	Brilliance	-
KNO ₃	0.5X	1X	-
NH ₄ NO ₃	0.5X	1X	1.5x
Mesos	0.5X	1X	1.5x

In vitro rooting

Rooting medium was composed of half-strength MS medium supplemented with 0.5 mg L⁻¹ NAA, 15 g L⁻¹ sucrose, 1.5 g L⁻¹ activated charcoal, and 0.7% agar. Single shoots were cultured on rooting medium and maintained in a growth chamber under the conditions described above for 30 days.

Acclimatization

Rooted plantlets were carefully washed with tap water to remove agar from the roots and transplanted into pots containing peat moss and perlite at a ratio of 1:1. The pots were then transferred to the greenhouse at 24 / 22 ± 2°C (day/night), 80% humidity, and natural light conditions. The pots were kept under plastic sheets, which were then gradually removed.

Statistical analysis

This study used a 2 × 2 × 3 × 3 factorial experiment arranged in a completely randomized design with three replicates. The effects of the treatments were tested for significance using analysis of variance (ANOVA). Duncan's post hoc multiple range test was used to separate significantly different means and to provide homogeneous groups for the means (P < 0.05). All data were subjected to statistical analysis using SAS software (version 7.1; SAS Institute, Cary, NC, USA).

RESULTS

The capitulum explants were successfully established on medium culture (Fig.1). New regenerated shoots were multiplied by subculture on MS containing 0.3 mg L⁻¹ BA and 0.05 mg L⁻¹ NAA. The applied levels of TDZ, BA and NAA in both establishment and multiplication steps are based on our previously unpublished trials in the lab.

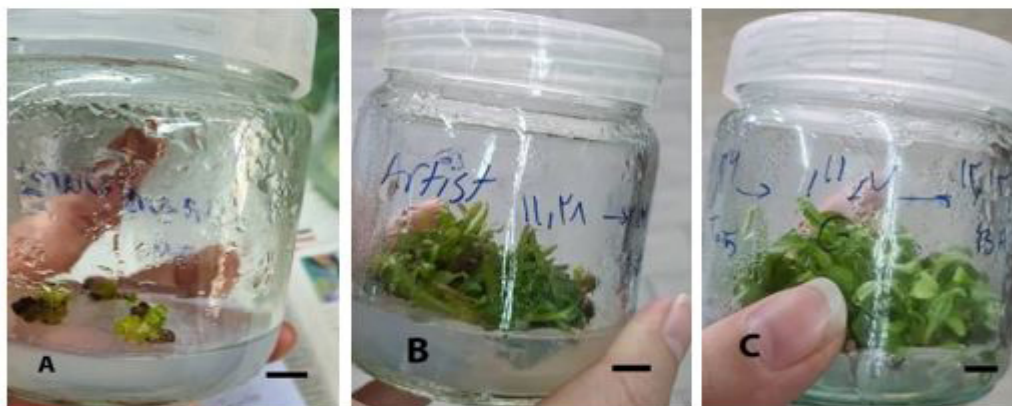


Fig. 1. *In vitro* establishment stage of gerbera capitulum as explants (A); Development of shoots in two gerbera cultivars: Artist (B) and Brilliance (C) (scale bar = 5 mm).

This study was conducted to elucidate the significance of the single effects double, triple, and quadruple interactions of KNO₃, NH₄NO₃, and mesos on multiplication parameters (shoot number, shoot length, root number, and root length) of two gerbera cultivars, Brilliance and Artist. Thus, there were four main factors in analysis of variance. Interaction effects result from the combined effects of the factors on the dependent variable. The interaction effect is significant when the impact of one factor depends on the level of the other factor. Part of the power of ANOVA is the ability to estimate and test interaction effects (Vittoz, 2021). A summary of ANOVA results is presented in table 2.

Table 2. Two-way ANOVA test for shoot number, shoot length, root number, and root length in *Gerbera jamesonii*.

S.o.V	Shoot number	Shoot length	Root number	Root length
Cultivar	0.0004**	0.0032**	<.0001**	0.0074**
KNO ₃	<.0001**	<.0001**	<.0001**	<.0001**
NH ₄ NO ₃	<.0001**	<.0001**	0.04*	0.133 ^{ns}
Mesos	0.5862 ^{ns}	<.0001**	0.3796 ^{ns}	0.2608 ^{ns}
Cultivar × KNO ₃	0.4101 ^{ns}	0.4854 ^{ns}	0.0003**	0.4701 ^{ns}
Cultivar × NH ₄ NO ₃	0.7382 ^{ns}	0.7566 ^{ns}	0.2716 ^{ns}	0.464 ^{ns}
Cultivar × Mesos	0.1177 ^{ns}	0.7538 ^{ns}	0.8051 ^{ns}	0.5493 ^{ns}
KNO ₃ × NH ₄ NO ₃	0.4704 ^{ns}	0.0182*	<.0001**	<.0001**
KNO ₃ × Mesos	0.3144 ^{ns}	0.1327 ^{ns}	0.27 ^{ns}	0.2752 ^{ns}
NH ₄ NO ₃ × Mesos	0.0003**	0.0051**	0.759 ^{ns}	<.0001**
Cultivar × KNO ₃ × NH ₄ NO ₃	0.0784 ^{ns}	0.3214 ^{ns}	0.0682 ^{ns}	0.002**
Cultivar × KNO ₃ × Mesos	0.8497 ^{ns}	0.7629 ^{ns}	0.0410 ^{ns}	0.1480 ^{ns}
KNO ₃ × NH ₄ NO ₃ × Mesos	0.0003**	0.0174**	0.0007**	0.0062**
Cultivar × KNO ₃ × NH ₄ NO ₃ × Mesos	0.2032 ^{ns}	0.2660 ^{ns}	0.2725 ^{ns}	0.0871 ^{ns}

*Significant with p-value < 0.05; **Significant with p-value < 0.01.

Shoot number

As demonstrated by statistical analysis, the effects of $\text{KNO}_3 \times \text{cultivar}$, $\text{NH}_4\text{NO}_3 \times \text{cultivar}$, mesos $\times$ cultivar, $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{cultivar}$, $\text{KNO}_3 \times \text{mesos} \times \text{cultivar}$, $\text{NH}_4\text{NO}_3 \times \text{mesos} \times \text{cultivar}$, and $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos} \times \text{cultivar}$ were not significant at the 0.05 level of probability for shoot number (table 2). However, the effect of the cultivar was statistically significant for this parameter. As shown in table 2, the main effects and interactions are significant. Single effects of KNO_3 and NH_4NO_3 significantly affected shoot number, but mesos did not. In contrast, the triple interaction, $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$, was statistically significant for shoot number (Table 2). This means that each factor independently accounted for variability in the dependent variable in its own right. However, they interact synergistically to explain the variance in the dependent variable. Together, these two factors do something else beyond their separate independent main effects. The significance of the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$ effect versus the insignificance of the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos} \times \text{cultivar}$ effect is considered an advantage for optimizing tissue culture protocols. In fig. 1, the means of this significant triple interaction were compared by using Duncan's multiple range test. Shoot number was highly influenced by low concentrations of KNO_3 (0.5X) and NH_4NO_3 (0.5X), and increased mesos (1.5X) (Fig. 2 and 3). Using this combination, 8.75 shoots per explant were produced. Increasing KNO_3 to 1X led to a sharp decrease in shoot number. At 0.5X KNO_3 and by increasing NH_4NO_3 to 1X or 1.5X, the shoot number was gradually reduced.

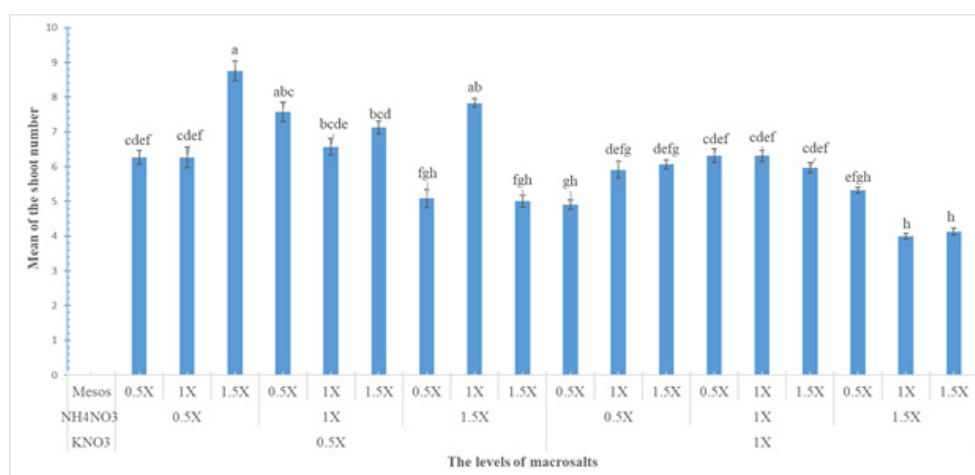


Fig. 2. Comparison of the means of the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$ interaction effect on the number of regenerated shoots in *Gerbera jamesonii* using Duncan's multiple range test. Horizontal axis: KNO_3 at 0.5X and 1X levels; NH_4NO_3 at 0.5X, 1X, and 1.5X levels; mesos at 0.5X, 1X, and 1.5X levels.



Fig. 3. High numbers of regenerated shoots in two *Gerbera* cultivars (left: Brilliance, right: Artist) on multiplication medium containing $0.5 \times \text{KNO}_3$, $0.5 \times \text{NH}_4\text{NO}_3$, and $1.5 \times \text{mesos}$ (scale bar = 5 mm).

Shoot length

Shoot length was also affected by the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$ interaction independent of the cultivar effect (Table 2). According to the results of the mean comparison analysis, higher levels of KNO_3 and NH_4NO_3 produced shorter shoots. However, at low KNO_3 and/or NH_4NO_3 concentrations and low mesos, shorter shoots were produced in both cultivars (Fig. 4).

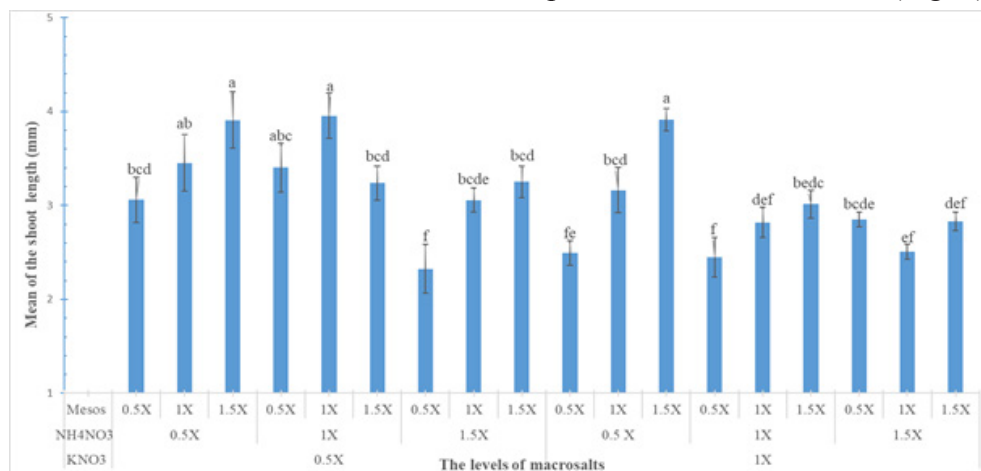


Fig. 4. Comparison of the means of the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$ interaction effect on the length of regenerated shoots in *Gerbera jamesonii* using Duncan's multiple range test. Horizontal axis: KNO_3 at 0.5X and 1X levels; NH_4NO_3 at 0.5X, 1X and 1.5X levels; mesos at 0.5X, 1X and 1.5X levels.

Root number

Root number was also influenced by the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$ interaction instead of the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos} \times \text{cultivar}$ interaction (Table 2). Mean comparison analysis showed that the root number decreased with low KNO_3 (0.5X), low NH_4NO_3 (0.5X), and high mesos (1.5X) (Fig. 5). In contrast, high KNO_3 (1X), low NH_4NO_3 (0.5X) and high mesos (1.5X) concentrations produced a greater number of roots.

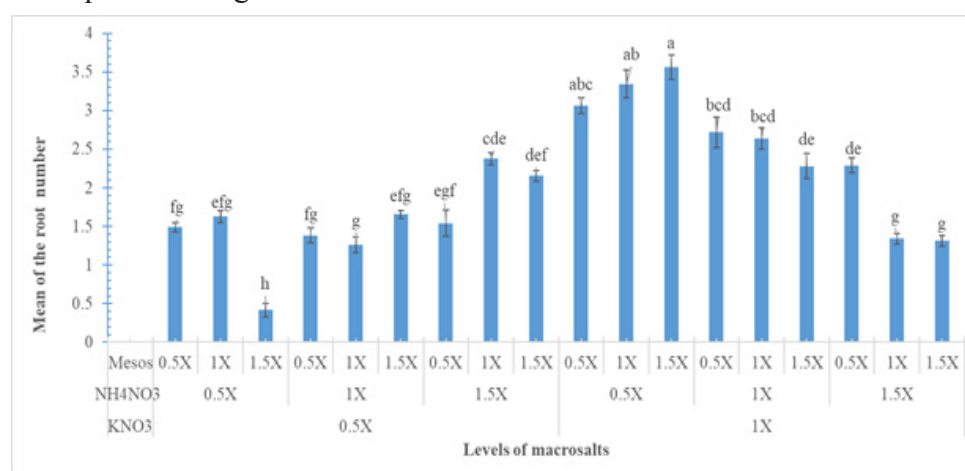


Fig. 5. Comparison of the means of the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$ interaction effect on the number of regenerated roots in *Gerbera jamesonii* using Duncan's multiple range test. Horizontal axis: KNO_3 at 0.5X and 1X levels; NH_4NO_3 at 0.5X, 1X and 1.5X levels; mesos at 0.5X, 1X and 1.5X levels.

Root length

Root length showed the same response as root number, and was influenced by the KNO_3

× NH_4NO_3 × mesos interaction (Table 2). Root length decreased by low KNO_3 (0.5X), low NH_4NO_3 (0.5X) and high mesos (1.5X) as shown by mean comparison test (Fig. 6). In contrast, high KNO_3 (1X), low NH_4NO_3 (0.5X) and low mesos (0.5X) produced greater number of roots.

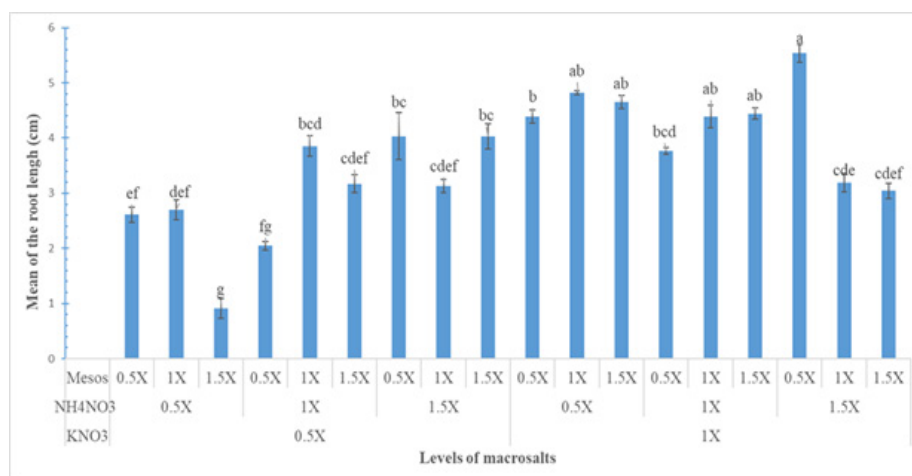


Fig. 6. Comparison of the means of $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$ interaction effect on length of regenerated roots in *Gerbera jamesonii* using Duncan's multiple range test. Horizontal axis: KNO_3 at 0.5X and 1X levels; NH_4NO_3 at 0.5X, 1X and 1.5X levels; mesos at 0.5X, 1X and 1.5X levels.

One of the main results in this research was the insignificance of the $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos} \times \text{cultivar}$ interaction effect on shoot number, shoot length, root number, and root length. This was in contrast to $\text{KNO}_3 \times \text{NH}_4\text{NO}_3 \times \text{mesos}$ interaction effect, which was significant for all four traits.

Acclimatization

In vitro-rooted shoots were transferred to the soil for *ex vitro* establishment. As a result, 80% of plantlets were successfully established and survived (Fig. 7).

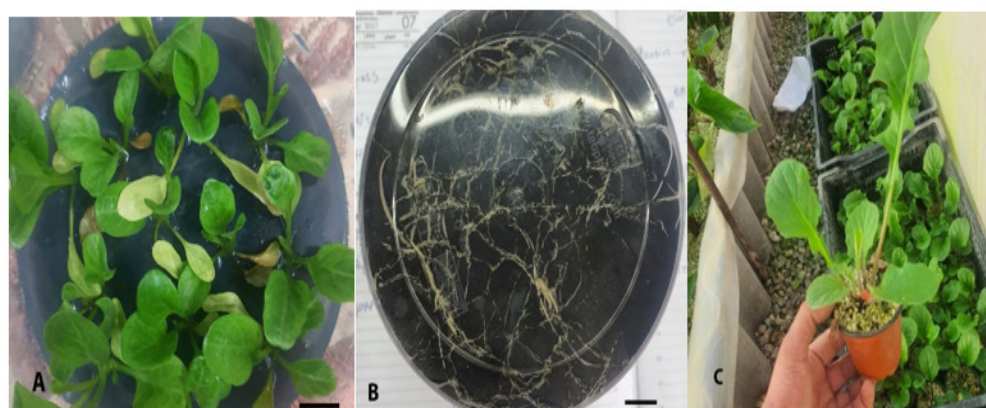


Fig 7. *In vitro* rooted gerbera shoots (A) and (B): Acclimatized plants grown in a greenhouse (C) (scale bar = 10 mm).

DISCUSSION

In gerbera, the effects of mineral nutrients on micropropagation efficiency have mostly been studied based on modification of the MS medium. Mineral availability and uptake in the

culture medium affect the growth, development, and quality of gerbera during *in vitro* culture, and their optimized concentrations in the medium are crucial for improving the proliferation efficiency and plant quality. Despite many reports in the literature, there are no useful universal protocols for commercial laboratories. Additionally, most studies have examined the effects of different strengths of MS medium on the *in vitro* multiplication of gerbera, instead of focusing on the components of the medium. In this regard, we disclosed the alterations in the macronutrient levels of the MS medium and their interaction effects at the multiplication stage of gerbera.

In our investigation, the first remarkable finding was the significance of the interaction among KNO_3 , NH_4NO_3 , and mesos, which is independent of the genotype effect. This result is supported by previous studies that used a half-strength MS medium for different cultivars. Rezende *et al.* (2008) used $\frac{1}{2}$ MS to regenerate 'Jaguar Cream' cultivar. Cardoso and Teixeira da Silva (2012) used $\frac{1}{2}$ MS for proliferation of several gerbera cultivars.

In the current study, we concluded that the combination of low KNO_3 (0.5X), low NH_4NO_3 (0.5X) and high mesos (1.5X) improved the number of shoots produced per explant. According to the statistical analysis, using moderate KNO_3 (1X), shoot number decreased significantly. Moreover, by increasing the NH_4NO_3 concentration, the shoot number began to decrease perceptibly. In a recent study, gerbera shoot culture at MS medium with 50% of salts (MS $\frac{1}{2}$) and 25% of salts (MS $\frac{1}{4}$) resulted in higher shoot formation (3.34 and 3.32 shoots/plants, respectively) (da Silva *et al.*, 2020). Nitrate ions are an essential source of nitrogen for most plant cultures; however, they must first be converted into ammonium inside the cell. Due to its toxicity, ammonium cannot be the sole available source of nitrogen; therefore, nitrate is added to the culture medium. The proportion of NH_4^+ nitrogen is high in MS medium and growth of plant cultures may also be hindered in media due to high concentrations of NH_4^+ even in the presence of high concentrations of NO_3^- at the same time. The free ammonium ion can lead to a rise in ethylene production, at least in whole plants (Ma *et al.*, 2023). High amounts of ammonium ions in the culture medium can cause regeneration of stunted or hyperhydric plants. In *Bryophyllum*, using machine learning based tool, ammonium was identified as a significant factor affecting shoot number, as low concentrations promoted shoot multiplication (Lozano-Milo *et al.*, 2022). In three varieties of *Malus domestica* Borkh, NH_4NO_3 , CaCl_2 , and MgSO_4 at $0.5\times$ MS, was significantly better for two of the three cultivars (Kabybekova *et al.*, 2020).

We also investigated the best concentration of mesos, and in contrast to low amounts of KNO_3 and NH_4NO_3 , high levels of mesos increased the propagation efficiency. Our result was in accordance with da Silva *et al.* (da Silva *et al.*, 2020). They reduced the MS concentration up to one-fourth in the multiplication medium and added 250 mg L^{-1} calcium silicate resulted in 3.32 to 6 shoots per plant. A previous study demonstrated the positive effect of increasing the concentration of mesos salts of MS medium (MgSO_4 , CaCl_2 , KH_2PO_4) to improve the number of shoots (Poonthong and Reed, 2014). Hunková *et al.* (2020) indicated that a treatment of MSx3 mesos components (MgSO_4 , CaCl_2 , KH_2PO_4) increased *in vitro* growth of several berry fruits and *Amelanchier alnifolia* compared to MSx4. Using machine learning to optimize culture medium of *Actinidia arguta*, Hameg *et al.* (2020) demonstrated that low K^+ with high concentration of SO_4^{2-} increased shoot number. In addition, they reduced nitrogen content to 20% but increased mesos up to 200% compared to MS (Hameg *et al.*, 2020). In a previous study, the highest multiplication rate for *Amelanchier alnifolia* was achieved with tripled mesos, whereas *Rubus fruticosus* reacted positively to a lower (1 – 2X) concentration of mesos. Decreasing the concentration of mesos to half led to worse quality in both blackberry and blueberry shoots (Hunková *et al.*, 2020).

Another observation in our study was the growth of longer shoots after the application of higher meso concentrations. A similar effect in micropropagated red raspberries, *Rubus ideaus*, was reported by previous study (Poothong and Reed, 2014). In *Salvia santolinifolia*, MS medium modification with NH_4NO_3 (412 mg L^{-1}), KNO_3 (475 mg L^{-1}) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (880 mg L^{-1}) was found the best medium to increase multiplication rate (Jan *et al.*, 2023).

CONCLUSIONS

This study presents a significant triple interaction effect among macronutrients in the culture medium on the multiplication rate of two gerbera cultivars. We recommend low concentrations of KNO_3 and NH_4NO_3 , in addition to high levels of mesos compared to MS medium, to improve the propagation efficiency of gerbera *in vitro*. Additional detailed analysis could provide new insights into the role of each component of mesos group in the micropropagation of gerbera plants.

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