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Callus Formation in Kochia (*Kochia scopora* L.) Under Different Hormone Concentration and Explant Types

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A B S T R A C T

Objective: Kochia is recently used as a forage and medicinal plant resistant to environmental stresses such as salinity and drought. One of the techniques used in biotechnology and plant breeding is tissue culture.

Methods: Based on this, callus formation and regeneration of Kochia under the influence of hormonal factors and explants types was conducted in a factorial experiment on a completely randomized design with hormonal treatments (9 levels) and explants (3 levels) in 5 replications. After 70 days, the explants were examined for different traits and determined the most suitable explant for callus production.

Results: According to the results regarding changes in the percentage of leaf callus formation, the greatest increase was in MS + 0.5 mg/l Kin + 1 mg/l NAA treatment. For callus formation and percentage of direct rooting, there is a significant difference between leaf explants, cotyledon and hypocotyl. But for the percentage of direct regeneration and the percentage of shoot formation, a significant difference has been observed between the leaf explant with cotyledon and the hypocotyl. The results showed that the highest degree of callus formation occurred in the treatment of MS + 0.5 mg/l Kin + 1 mg/l NAA in the leaf and the axis of the cotyledon, and then in the treatments MS + 1 mg/l NAA and MS + 0.05 mg/l BA + 0.5 mg/l NAA is in the organs of cotyledon and the hypocotyl.

Conclusion: Finally, 2,4-D could lead to more callus formation of the Kochia and it was observed that no callus formation was done in treatments without hormones. Although, to optimize callus formation and regeneration in this plant, it is suggested to also test the effects of environmental variables such as temperature, humidity, and photoperiod, and different levels of growth hormones such as IBA and GA3.

Key words: Auxin, Callus induction, Cytokinin, In vitro, Salinity

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Introduction

Producing salinity-resistant plants with saline water and soil to feed livestock in desert areas is one of the most sustainable ways to protect the environment, and Kochia is one of these resistant plants that can grow in adverse water and soil conditions [1]. Kochia (*Kochia scoparia* L. Schrad) (family *Chenopodiaceae*) is a one-year herbaceous dicotyledonous plant, which is well adapted to dry areas due to its wide and deep root system. Kochia is self-compatible and produces protogynous flowers where the stigmas emerge before anther development [2]. This plant has the ability to penetrate up to a depth of 5 meters in the soil and also grow in very salty soils. Kochia, as a leading plant, is the only species that can grow well in these areas (arid and semi-arid) [3] and it is resistant to saline conditions and germinates well in medium and even high salinity levels [4, 5]. Because of its deep roots, Kochia can receive water from deep in the soil [6, 7, 8]. This plant has a short day and begins to flower during the day less than the threshold, so it can be used for cultivation in the first year in lands with very salty soil.

In the Far East, including China, Korea and Japan, mature seeds is widely used to treat skin diseases and dysuria. Especially, it had been used for breast masses and chest and flank pain [9]. Several types of compounds, such as, triterpenoid glycosides, saponins, and alkaloids have been isolated from *K. scoparia* [10]. Furthermore, *K. scoparia* and components of extracts have been shown to have anti-inflammatory and anti-allergic activities [11].

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Recently, it was reported that *K. scoparia* inhibits angiogenesis in human umbilical vascular endothelial cells as well as proliferation in human prostate cancer cells, which indicates that the extract of this plant may be a useful herbal medicine to prevent prostate cancer progression and angiogenesis [12]. Masses of Kochia exist in the cities of Birjand, Sabzvar, Borujerd, Urmia and Isfahan [13]. In areas that lack fodder, this plant can be used as fodder [14]. Kochia has tasty leaves and branches for livestock [15].

Production of high-quality fodder from saline plants is one of the main goals of agriculture in ecosystems under salinity stress. Kochia is a plant that has the ability to grow in water and soil salinity conditions and has a good capacity to produce fodder [16]. The production and exploitation of fodder from saline plants and the use of soil and saline water resources can be one of the most effective ways to prevent the reduction of cultivated crops. The results of Nabati *et al.*'s study [16] in three cities of Sabzevar, Borujard and Birjand, at three levels of salinity, showed that among the traits studied, only seed yield, harvest index, and 1000 seed weight were affected by salinity stress; However, increasing salinity from 2.5 to 5.10 dS/m did not significantly decrease seed production. In the above research, the researchers found that in a situation where none of the crops have the ability to produce even the minimum biomass, this plant has a high ability to become an oilseed. For this reason, this plant has a lot of work and research capacity. By examining the amount of micro-propagation on Kochia plant through tissue culture, it can be produced, although this method of propagation in the laboratory is common and standard, which will give us the maximum yield in the shortest time [16].

There are many problems in the propagation of plants, both through reproductive and vegetative means. For example, in the reproductive method, seeds may be destroyed or heterogeneous progeny may be obtained. The vegetative method (including planting, transplanting, cuttings, cuttings, etc.) is slow and very expensive. Therefore, cultivation in a glass or laboratory is a more suitable method.

The superiority of the tissue culture method over the conventional culture method in plants is the ability to accurately control the physical and chemical conditions of the environment. In this method, a large number of samples with equal genetic structure can be clearly obtained [17]. Knowledge of tissue culture of many plants, including non-agricultural plants, can help breeders to improve plants and transfer desirable genes and maintain new plants.

The micropropagation method is very attractive in a series of commercial activities due to the high speed in producing new plant species, even with the in-glass method, it is possible to produce plants throughout the year. In a series of species that cannot be propagated by vegetative method, there is a possibility of their propagation by tissue culture method. Today, with the expansion of this cultivation method, many scientific sources have become available about many plant species.

The available references do not provide specific information on callus formation in *Kochia scoparia* L. under varying hormone concentrations and explant types. In *Silene vulgaris*, leaf explants were found to be suitable for callus formation in NAA and BAP media [18]. Similarly, in *Scoparia dulcis* L., leaf explants with different treatment of BAP and IAA or NAA resulted in multiple shoot induction, with the combination of BAP and IAA yielding the highest number of shoots [19].

Contradictory to the general success of leaf explants in the aforementioned studies, the research on mungbean showed that stem, epicotyl, and cotyledon explants also have the potential for plant regeneration [20]. Additionally, the study on *Lycopersicon* species indicated that both stem and hypocotyl explants could form callus, with hypocotyl explants showing superior shoot formation [3].

In many developed countries, including Australia, producers propagate many plant species using established protocols [21]. Spinach is one of the important plants of the spinach family that has been used for callus production. Spinach is one of the two important agricultural vegetables that naturally requires long days, suitable seeds and fertile soil. It also faces many diseases, which is why it is very important to pay attention to spinach tissue culture [22]. In Iran, a study conducted on spinach concluded that regeneration was successful in hypocotyl, cotyledon and root samples [23]. Other plants of this family that have been tissue cultured include quinoa (*Chenopodium Quinoa* L.). The combination of hormones used for each explant included NAA, 2, 4-D hormones. Quinoa is a versatile crop that can be used for fodder, grain and even oil production, but it is a limiting factor such as viral diseases, especially seed-borne diseases, which cause yield reduction and even loss. Therefore, somatic embryogenesis has been carried out for mass production of transgenic plants. Calluses were prepared from hypocotyl samples. Two weeks after cultivation, callus was obtained on modified MS medium. 2, 4-D was used for this medium and the result was declared positive [24]. Considering that there is no report in the literature on tissue culture of kochia plant, the aim of this study is to investigate callus generation and regeneration of this plant in tissue culture medium.

Material and Methods

This research was done in order to investigate the callus formation of Kochia under the influence of hormonal compositions. The seeds required for this research were native seeds collected from farms. For disinfection, first the seeds washed with running water, then immersed them in 70% ethanol alcohol under a laminar hood for 30 seconds, and then were placed them in sodium hypochlorite (three percent active chlorine) and some tween 20 for 10 minutes. All the seeds were washed three times with distilled water for 15 minutes, and then the disinfected seeds were transferred to the MS culture medium. We closed the lid of the containers with parafilm adhesive and kept them in the germinator at 25°C, in the dark for 8 hours. A few days after sowing the seeds, we prepare the hypocotyl and cotyledon explants under the laminar hood. Then, we examined the explants for callus formation in MS culture medium containing different concentrations (NAA, 2, 4-D, Kin, BA) [13]. We putted 4 small samples in each petri dish. The generated callus explants were counted. At first, a preliminary test was conducted to reach the best concentration of the treatments from the concentrations of NAA, 2, 4-D, Kin, BA, and then the appropriate treatment combination (about 9 treatments) be tested in 5 repetitions (Table 1). The experiment was conducted in the form of a completely random design. We prepared leaf explants from seeds grown under a laminar hood. Since they were sealed in the culture medium containers with parafilm adhesive, the leaves were not be contaminated and there is no need for disinfection process. Therefore, under the laminar hood, we transferred the leaves directly to the culture medium. We use different concentrations of NAA, 2, 4-D and Kin, BA for the leaf explant, similar to the hypocotyl and cotyledon explant. After 4 weeks, researchers recorded callus-related traits. The temperature of the samples was set at 25°C ± 0.5°C, and the humidity was set at 70% ± 3% with a light/dark cycle of 16:8. Researchers observed no bacterial or fungal contamination during growth.

Traits such as dry and fresh weight of callus, percent of root and shoot formation, callus formation and mass percentage will be calculated. The calculation of mass moisture percentage was obtained from the following equation:

$$x = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100$$

The data was analyzed by SAS software and the comparison of treatment averages done based on Duncan's test at the 5% probability level. Also, graphs and related figures are drawn using EXCEL software.

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Table 1. The compounds of culture medium used to induce callus production of Kochia scoparia L

	Treatment
1	MS + 0.05 mg/l BA + 0.5 mg/l NAA
2	MS + 1 mg/l NAA
3	MS + 0.5 mg/l Kin + 1 mg/l NAA
4	MS + 0.5 mg/l BA + 1 mg/l NAA
5	MS + 0.05 mg/l BA + 1 mg/l NAA
6	MS + 2 mg/l 2,4-D + 0.5 mg/l BA
7	MS + 2 mg/l 2,4-D + 0.05 mg/l Kin
8	MS; Hormone Free
9	0.5 MS; Hormone free

Results

According to the results of analysis of variance, a significant difference was observed between the treatments in different traits. So, the percentage of shoot formation, the percentage of direct rooting, the percentage of direct regeneration and the degree of callus formation had a significant difference at the 99% confidence level both for the combination of hormones and the type of explant and the interaction effect of the combination of hormones in the explant types (Table 2 and Fig 1.).

Table 2. Analysis of variation in different traits

S. O. V.	df	Days to callus formation	Fresh weight (gr)	Dry weight (gr)	Mass percentage (%)	Callus formation (%)	Regeneration percentage (%)	Root formation (%)	Shoot formation (%)
Hormone	8	237.69**	1.558*	0.0034*	665.20 ^{ns}	11151.3**	2234.152**	2106.5**	2539.1**
Explant types	2	0.415 ^{ns}	2.256*	0.0062*	23.739 ^{ns}	182.13 **	1163.632**	1106.8**	1268.7**
Hormone ×Explant	16	0.872 ^{ns}	1.02 ^{ns}	0.002 ^{ns}	113.80 ^{ns}	1194.85**	673.042**	634.55**	781.93**
Error	72	0.6615	0.722	0.002	121.53	0.1456	2.720	0.3567	10.852
C. V. (%)		10.09	18.7	23.5	17.2	12.5	11.52	2.73	12.72

**,*: significant at 1 % and 5 % probability level, respectively. ns: not significant.

In this research, the callus formation of the Kochia was carried out under the influence of hormonal and explant types. According to the results obtained in the first four weeks, we checked the percentage of callus formation. Regarding the changes in the percentage of leaf callus formation, the highest increase was related to MS + 0.5 mg/l Kin + 1 mg/l NAA treatment and MS + 2 mg/l 2, 4-D + 0.05 mg/l Kin treatment respectively (Table 3). Regarding the changes in the percentage of callus formation on the cotyledon, the highest increase was related to MS + 1 mg/l NAA treatment and MS + 0.5 mg/l Kin + 1 mg/l NAA treatment respectively. Regarding the changes in the percentage of callus formation hypocotyl, the highest increase was observed in MS + 0.5 mg/l Kin + 1 mg/l NAA treatment and MS + 2 mg/l 2,4-D + 0.05 mg/l Kin treatment respectively. While MS treatment without hormone and MS treatment with 0.5 concentration without hormone during four weeks had zero value (Table 3).

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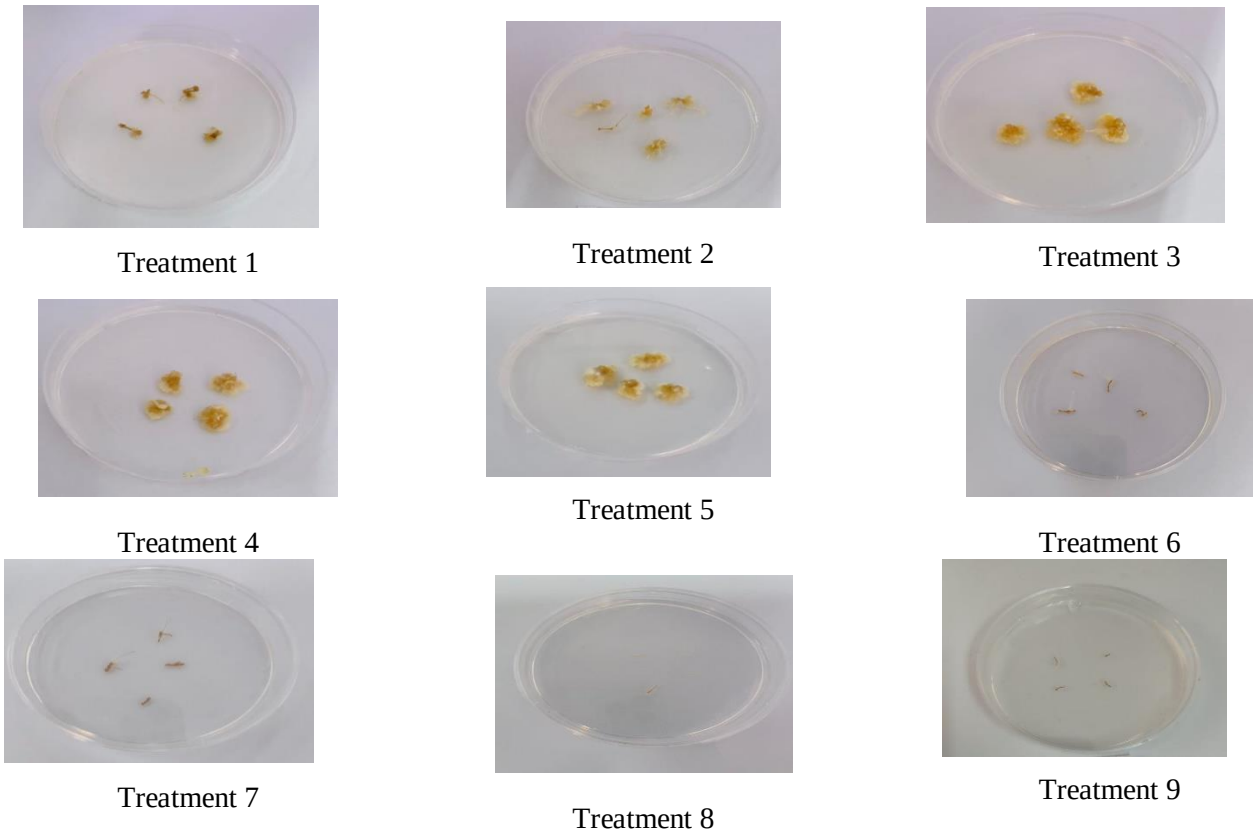


Fig. 1. Callus formation in different treatments of hormonal composition in leaf explant for 4 weeks.

Table 3. Comparison of the mean of different traits of hormone compositions

Hormone composition	Days to callus formation	Fresh weight (g)	Dry weight (g)	Mass percentage (%)	Callus formation (%)	Regeneration percentage (%)	Root formation (%)	Shoot formation (%)
1	11.017a	1.650a	0.072a	20.024a	72.727b	39.335a	43.360a	35.310 a
2	10.250b	0.677ab	0.037ab	17.837a	72.467c	29.827b	32.607b	27.040 b
3	11.080a	0.266b	0.015b	19.695a	92.200a	14.887c	20.810c	8.953 cd
4	9.330c	0.720ab	0.025ab	18.416a	66.700d	15.144c	20.273d	10.010 c
5	9.383c	0.449b	0.015b	22.801a	44.733e	10.00d	16.110e	3.887 de
6	11.120a	0.183b	0.006b	21.579a	42.300f	9.130d	12.967g	5.293 cde
7	10.350b	0.179b	0.007b	22.989a	34.400g	10.450d	14.516f	6.380 cd
8	0.000d	0.000b	0.000b	0.000a	0.000h	0.000e	0.000 h	0.000 e

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9	0.000d	0.000b	0.000b	0.000a	0.000h	0.000e	0.000 h	0.000 e

Means with same letter in each column have not significant difference at 5 % probability level.

The results of Duncan's test showed that there is a significant difference in the degree of callus formation and the percentage of direct root formation between different treatments (except MS treatment without hormone and MS treatment with 0.5 concentration without hormone) in hormonal composition treatments. In percentage of direct regeneration trait, MS + 0.5 mg/l Kin + 1 mg/l NAA treatment and MS + 0.5 mg/l BA + 1 mg/l NAA treatment in one group, MS + 0.05 mg/l BA + 1 mg /l NAA treatment, MS + 2 mg/l 2,4-D + 0.5 mg/l BA treatment and MS + 2 mg/l 2,4-D + 0.05 mg/l Kin treatment in one group and MS treatment without hormone and 0.5 MS treatment without hormones are also in the same group. In percentage of shoot formation traits, MS + 0.05 mg/l BA + 1 mg/l NAA and MS + 2 mg/l 2,4-D + 0.5 mg/l BA treatments in one group and MS treatment without hormone and 0.5 MS treatment without hormones, they are also in the same group, and the rest of the treatments individually have significant differences.

In explant type, percent of callus formation and percentage of direct rooting, there is a significant difference between leaf explants, the cotyledon and hypocotyl. But for the percentage of direct regeneration and the percentage of shoot formation, a significant difference has been observed between the leaf explant with cotyledon and the hypocotyl (Table 4).

Table 4. Comparison of the mean of different traits of explant types

Explant types	Days to callus formation	Fresh weight (g)	Dry weight (g)	Mass percentage (%)	Callus formation (%)	Regeneration percentage (%)	Root formation (%)	Shoot formation (%)
Leaf	8.354 a	0.689a	0.3200a	14.234a	40.046c	8.438b	11.405c	5.466b
Cotyledon	8.050 b	0.289b	0.0133b	16.158a	52.731a	17.065a	20.773d	13.353a
Hypocotyl	7.789 c	0.389b	0.0141b	17.303a	49.794b	17.176a	21.094a	13.252a

Means with same letter in each column have not significant difference at 5 % probability level.

The results showed that the highest degree of callus formation occurred in the treatment of MS + 0.5 mg/l Kin + 1 mg/l NAA in the leaf organs and the axis of the cotyledon, and then in the treatments MS + 1 mg/l NAA and MS + 0.05 mg/l BA + 0.5 mg/l NAA is in the organs of cotyledon and the hypocotyl. According to the results, the highest percentage of direct regeneration, percentage of direct rooting, and percentage of direct stemming were observed in MS + 0.05 mg/l BA + 0.5 mg/l NAA and MS + 1 mg/l NAA treatments in the explant of the cotyledon and the hypocotyl (Table 5).

The results of the correlation test showed that the degree of callus formation, the percentage of shoot formation, the percentage of root formation and the percentage of direct regeneration have a positive and direct correlation with each other at the confidence level of 99% (Table 6).

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Table 5. Comparison of the mean of different traits of explant types× Hormone composition interactions

Hormone composition(a)× explant types(b)	Callus formation (%)	Regeneration percentage (%)	Root formation (%)	Shoot formation (%)
a1 b1	44.00 n	8.00ks	8.00 s	8.00ef
a1 b2	86.68 f	50.00b	53.33 b	46.66 ab
a1 b3	87.50 e	60.00a	68.75a	51.25a
a2 b1	36.40 p	6.82kl	9.09 q	4.54 gf
a2 b2	93.10 c	39.93d	41.37d	34.48 c
a2 b3	87.90 d	44.73c	47.36c	42.10b
a3 b1	95.50 a	18.18f	22.72h	13.63e
a3 b2	86.40 g	9.09lij	13.63n	4.54gf
a3 b3	94.70 b	17.39fg	26.08f	8.69ef
a4 b1	66.70 I	4.17lm	8.33sr	0.00g
a4 b2	76.90 h	26.92e	30.76e	23.07d
a4 b3	56.50 k	13.342h	21.73i	6.95gef
a5 b1	5.00 i	9.17kij	15.00m	3.33gf
a5 b2	62.50 j	12.50hi	25.00g	-0.00 g
a5 b3	66.70 i	8.33kj	8.33r	8.33ef
a6 b1	50.00m	15.00fg	20.00j	10.00ef
a6 b2	41.20 o	8.50 kj	11.76o	5.88gf
a6 b3	35.70q	3.57 m	7.14t	0.00g
a7 b1	52.80 l	12.50hi	16.66k	8.33ef
a7 b2	27.80 s	8.33ks	11.11p	5.55gf
a7 b3	31.60 s	10.52ij	15.78l	5.26gf

Leaf (b1), Cotyledon (b2), Hypocotyl (b3); a1 to a7: composition hormones treatments; Means with same letter in each column have not significant difference at 5 % probability level.

Table 6. Correlation of different traits

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Traits	1	2	3	4	5	6	7	8
1	1.000							
2	0.185**	1.000						
3	0.170**	0.910**	1.000					
4	0.558**	0.254**	0.119**	1.000				
5	0.745**	0.086*	0.081^{ns}	0.383**	1.000			
6	0.486**	0.021^{ns}	0.033^{ns}	0.224**	0.713**	1.000		
7	0.548**	-0.006^{ns}	0.007^{ns}	0.255**	0.754**	0.977**	1.000	
8	0.389**	0.052^{ns}	0.059^{ns}	0.177**	0.627**	0.971**	0.897**	1.000

1- Days to callus formation, 2- Fresh weight, 3- Dry weight, 4- Mass percentage, 5- Callus formation, 6- Regeneration percentage, 7- Root formation, 8- Shoot formation; **,*: significant at 1 % and 5 % probability level, respectively. ns: not significant.

Discussion

According to the results, the highest percentage of direct regeneration, percentage of direct rooting, and percentage of direct stemming were observed in MS + 0.05 mg/l BA + 0.5 mg/l NAA and MS + 1 mg/l NAA treatments in the explant of the cotyledon and the hypocotyl (Table 5). In the research of Blazquez et al. [25], Shahin [13], Morsi et al. [26], Bagheri et al. [27], Asghari Zakaria et al. [28] also found that leaf callus formation increased as a result of MS hormone + 0.5 mg/l Kin + 1 mg/l NAA.

Based on the review of the sources, according to the results of the MS media with 2, 4-D, it could lead to more callus formation of the Kochia. In the research of Eisa *et al.* [24], Hesami *et al.* [29] and Shahin [13], Amirkhani *et al.* [30], Khayatzadeh *et al*. [31] considered effective. In general, the results of the research showed that breeding programs can lead to the strengthening of germination, growth and regeneration of plant organs, which is in accordance with the results of Morsi *et al*.'s research [26].

Therefore, according to the research of Custodio *et al.*, [32] regarding halophyte, it should also be mentioned that in South Khorasan province, the application of plant tissue culture techniques in laboratory conditions in the Kochia species can be used to improve their controlled reproduction. In addition, the germination of the Kochia has a good tolerance to high levels of salinity and it is possible to implement a favorable establishment of this species in the province by applying proper management in the field.

A successful tissue culture technique for this plant is needed. It may also transfer salt-resistant genes. Then, we could use its suspension culture in bioreactors. It could produce active ingredients, like triterpenoid glycosides. Considering the positive results of callus formation in certain conditions, it is suggested to develop operational experiences in the field of callus formation *Kochia scoparia* L. This involves repeated experiments with different hormonal and explant types to obtain more precise improvements in the callus formation process. Considering that the highest degree of callus formation occurred in MS + 0.5 mg/l Kin + 1 mg/l NAA treatment, different combinations of these hormones can be investigated. Better results may be obtained by increasing or decreasing the amounts of these hormones. In addition to the hormones used in this research, the effect of other plant growth hormones can also be investigated. Some hormones may have different effects on callus formation. Also, physical factors such as temperature, humidity and light can have a great effect on callus formation. It is possible to examine the effect of these factors and determine the optimal conditions for callus formation.

Conflict of interest

No potential conflict of interest was reported by the author(s).

Data Availability Statement

The data supporting the findings of this study are available within the article.

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