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Induction of Callus Tissue and Regeneration of Harmala (*Peganum harmala* L.) Influenced by Growth Regulators

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Abstract

There is limited information about the *in-vitro* propagation of harmal. This experiment was conducted as factorial in a completely randomized design with three replications in two separate experiments with different hormonal treatments. In the first experiment, MS medium containing growth regulators with different concentrations of BAP (0 mg/L, 0.5 mg/L, 1 mg/L and 2 mg/L) in combination with NAA at concentrations (0 mg/L, 0.25 mg/L and 5.0 mg/L) and in the second experiment, BAP with concentrations (0 mg/L, 0.5 mg/L, 1 mg/L and 2 mg/L) in combination with 2,4-D with concentrations (0 mg/L, 25 mg/L and 0.5 mg/L). The results showed that the highest callus and regeneration for the harmal plant was the combination of BAP hormone (0.5 mg/L) with NAA (0.5 mg/L). The results of the second experiment showed that callus of explants was carried out in most of the BAP and 2,4-D hormonal treatments and the highest regeneration was observed in the treatment of 1 mg/L BAP with 0.5 mg/L was observed. High BAP concentration in the presence of NAA decreased callus formation and appeared to be better for NAA callus enhancement. The high concentration of BAP in the presence of NAA reduced callus formation and it can be said that NAA was better for increasing callus production.

Keywords:* *Harmala; Callus; Regeneration; BAP; 2,4-D

Introduction

The harmala, scientifically named *Peganum harmala*, is an herbaceous perennial herb of the *Zygophyllaceae* family. Harmala is spread in North Africa, the Mediterranean, Pakistan and Iran. In the importance of the medicinal herb, it is enough that the dried seeds of the herb contain 35% protein, 17% oil and alkaloids harmaline, harmine, harmalol, harman, peganine (Wazinein), isopeganine, dipeganine, deoxyvazine nun. Of this 17%, the proportion of an alkaloid harmaline, harmine, harmalmol and peganine (Wazi Sin) are about 5.8% to 3.8%. The global need for aromatic and medicinal plants is currently high and growing. Most medicinal plants are not easily reproduced using conventional methods and the asexual reproduction method has been invented, which is a useful method of producing such plants and is nowadays widely used for these purposes [1].

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Understanding the mechanism of plant regeneration *in vitro* is useful for genetic manipulation, physiological studies and alkaloid's production. Chemical, mineral, growth hormones and regulators are important factors in plant differentiation and growth. In a study on embryonic callus formation, different concentrations of 2,4-D and NAA were used separately and showed that the highest amount of callus was due to high concentrations of auxin and cytokinin (one to two mg/liter) on *Bunium persicum* plants. In the study of the combination of high concentrations of 2,4-D and low concentration of BAP induced the highest callus of leaves [2].

In a study on *Dracocephalum kotschyi*, two growth regulators of NAA and BAP were found to be more effective in both callus production and regeneration. The combined effect of these two compounds on the induction of undifferentiated callus of *Dracocephalum kotschyi* and the production of root webs has been demonstrated. In another study, it was found that all three leaf specimens, branch and middle meristem had the highest callogenesis in the simultaneous combination of BAP and NAA growth regulators in MS and LS medium. In another study, the highest callus weight was obtained from bud specimens in media containing 0.5 mg/l NAA. The culture medium containing 1 mg/L kinetin in combination with 1 mg/l NAA produced more direct and indirect onions than other media and averaged 5.5 onions sample thumbnail per *Fritillariaim perialis* L.

Fatima et al., an experiment on finger plant (*Digitalis purpurea*) showed that leaves were the best sample thumbnail for callus in relation to hypocotyl, stem and root and were also an effective source of regeneration. See also Kumari and Chandra, showed that the leaves highest levels of secondary metabolites [3].

The present study, by presenting a suitable method for inducing callus and regeneration of harmala, could be used for future studies regarding the use of genetic engineering to increase the production of effective material in this plant as well as the use in suspension culture for effective material production. Therefore, this study aimed to determine the most suitable sample thumbnail and determine the best combination of hormone concentration in the culture of sample thumbnail to achieve the highest number of callus formation and regeneration [4].

Materials and Methods

The seeds of harmala were prepared from Khorasan Razavi agricultural research and natural resource center. The experiment was carried out in 2016 at the tissue culture laboratory of the department of agriculture, Payam Noor university of Mashhad. Seeds were disinfected with 30% alcohol for 1 minute, sodium hypochloride at 2% concentration for 5 minutes. Seeds were rinsed with sterile distilled water three times between disinfection steps. Finally, they were dewatered with sterile filter paper.

Disinfected seeds were cultured in MS germination medium containing 30 g sucrose and 8 g agar at pH 5.7 to 5.8 and 40 seeds per bottle. The bottles were kept in a growth chamber at 25°C for 16 h light and 8 h dark. After germination, germinated seeds were stored in the growth chamber for 1.5 months until, the stem reaches about two centimeters and at this time; sterile seedlings were prepared for leaf and hypocotyl specimens for culture in the target medium [5]. In this study, MS medium containing growth regulators was tested in two experiments. The first experiment with different concentrations of BAP control, 0.5 mg/L, 1 mg/L and 2 mg/L in combination with NAA at concentrations control, 0.25 mg/L and 0.5 mg/L and the second experiment of BAP with concentrations control, 0.5 mg/L, 1 mg/L and 2 mg/L in combination with 2,4-D with concentrations control, 0.25 mg/L and 0.5 mg/L and interaction of NAA with BAP was used [6]. Different samples of leaf, hypocotyl and cut embryos were used for culture. For this purpose, the space of each petri dish was divided into three equal portions, with each sample separately divided into three samples and each petri dish was considered as a replicate. For this purpose, the space of each petri dish was divided into three equal portions, with each sample separately divided into three samples and each petri dish was considered as a replicate. In the first experiment, the effect of different concentrations of BAP and NAA and in the second experiment the effect of different concentrations of 2,4-D and BAP on callus formation and regeneration of three micro-samples were studied statistically [7].

Method of preparation of micro-samples for tissue culture

Leaf micro-samples: Initially, the required tools and the laminar hood environment were sterilized and disinfected (all appliances were autoclaved at 121°C for 20 minutes and used after cooling). Then with a scalpel, seedlings containing leaves of appropriate size were selected and separated, so that the tail of the leaf does not move with the leaf. She scratched the sides of the leaf with a scalpel, it was then transported to the pre-prepared culture medium and it contains different hormones. The micro-samples were cultured in a culture medium that is exactly in contact with it [8].

Hypocotyl micro-samples: The seedlings that had previously cut leaves, its hypocotyls were cut and separated by scalpel, so that the primary bud is not transported with the hypocotyl. It was then transported to the pre-prepared culture medium and it contains different hormones. The micro-samples were cultured in a culture medium that is exactly in contact with it [9].

Embryo micro-samples: Seeds of harmala were first sterilized in 30% alcohol for 1 minute and 2% sodium hypochloride for 5 minutes and washed 3 times with distilled water. After washing, the seeds were cultured in petri dishes containing hormone-free MS medium and kept in the growth chamber for 48 h. During this time the seeds swell and the seed shell softens. Then, with scalpel No. 10, cut the seeds under completely sterile conditions under the laminar hood and with the pressure on the middle of the seed, the embryo came out easily. The embryo was slowly extracted with forceps and placed on a hormone culture medium. After preparing all the micro-samples and transferring them to the culture medium, the petri dish door sealed with par film and transferred to a growth chamber at 25 ± 2°C for 16 h light and 8 h dark. Daily, by observing the slightest contamination in the cultured samples, they removed them from the growth chamber. After four weeks, calluses and regeneration numbers were counted and the data were then analyzed statistically [10].

Statistical analysis

The factorial experiment was conducted in a completely randomized design with three replications. SAS software (9.1) and Excel software were used to draw charts. LSD with 0.01% probability level was used to compare the means [11].

Results and Discussion

First experiment

In the first experiment, the results showed that the effects of BAP, NAA and micro samples (leaf, hypocotyl and cut embryos) and interaction effects of NAA on BAP on callus formation and regeneration were significant at 0.01 levels (Tables 1 and 2).

TABLE 1. **Analysis of variance of BAP, NAA and microbial sample for callus induction and shoot regeneration.**

S.O.V	df	Mean squares	
		Regeneration	**Callus**
BAP	3	2.47^{**}	1.24^{**}
Micro sample	2	2.12^{**}	5.77^{**}
NAA	2	7.67^{**}	16.58^{**}
BAP × Micro sample	6	0.07^{ns}	0.28^{ns}
BAP × NAA	6	4.58^{**}	0.49^{**}
NAA × Micro sample	4	0.09^{ns}	0.36^{ns}
BAP × NAA × Micro sample	12	0.15^{ns}	0.20^{ns}
Error	72	0.21	0.2
Note: *, ** and ns are significant at the level of 0.05 and 0.01 and non-significant, respectively			

TABLE 2. **Interaction effects of BAP and NAA on the mean number of calluses and shoot regeneration in leaf, hypocotyl and embryo micro-sample.**

NAA	BAP	Number of regeneration	Number of callus
0	0	0	0
	0.5	0.24^{g}	0.9^{j}
	1	0.24^{g}	1.25^{i}
	2	1.33^{bc}	2.6^{d}
0.25	0	1.55^{b}	2.01^{e}
	0.5	1.38^{b}	2.75^{c}
	1	0.7^{e}	2.73^{c}
	2	0.41^{f}	1.32^{g}
0.5	0	1^{d}	2.93^{b}
	0.5	2.55^{a}	3^{a}
	1	1.14^{cd}	2.93^{b}
	2	0.8^{e}	1.73^{f}
Lsd	-	0.2	0.06
Note: Numbers with common letters in each column are not significantly different			

Interaction of BAP and NAA on callus formation of all explants showed that the highest mean number of callus formation at 0.01 levels was related to 0.5 mg/l NAA and 0.5 mg/l BAP treatment. The mean comparison of the data of BAP and NAA interaction effects on regeneration showed that the highest regeneration was related to 0.5 mg/l NAA and 0.5 mg/l BAP treatment [12].

Interaction effects of BAP (1 mg/L) and micro-sample in callus formation showed that the highest number of callus formation was observed in leaf micro-sample with 2.41 calluses but in 0.5 mg/L BAP treatment with hypocotyl treatment showed no significant difference (Table 3) [13].

TABLE 3. **Interaction effects of BAP and NAA on the mean number of callus formation and shoot regeneration in leaf, hypocotyl and embryo micro-sample.**

Variables		NAA			BAP			
		0	0.5	1	0	0.5	1	2
Leaf	Number callus	1.44^{a}	2.75^{a}	2.61^{a}	2^{a}	2.37^{a}	2.41^{a}	2.37^{a}
	Number regeneration	0.58^{e}	1.13^{d}	1.63^{c}	1^{d}	1.52^{c}	0.9^{d}	1.1^{d}
Hypocotyl	Number callus	1.25^{b}	2.41^{b}	2.5^{a}	1.85^{b}	2.37^{a}	2.17^{b}	1.85^{b}
	Number regeneration	1.1^{bc}	1.1^{d}	1.4^{c}	1d	1.45^{c}	0.75^{e}	0.75^{e}
Embryo	Number callus	0.78^{d}	1.57^{c}	2.05^{b}	1.23^{c}	2^{b}	1.32^{c}	1.42^{c}
	Number regeneration	0.25^{f}	0.72^{e}	1d	0.53^{e}	1.1^{d}	0.32^{f}	0.64^{f}
Lsd		0.08	0.13	0.11	0.09	0.1	0.09	0.11
Note: Numbers with common letters in each column are not significantly different								

Investigation of interactions of BAP and micro-sample in regeneration showed that the highest interaction of BAP and micro-sample of leaf, hypocotyl and embryo at concentration of 0.5 mg/L and the least of this effect on leaf, hypocotyl and embryo micro-sample at concentration one mg/L was observed (Figures 1 and 2). Cytokinins are among the best growth regulators for regeneration in the culture medium, and among them, BAP has been identified as the most important compound. By examining

the interaction between NAA and micro-sample in callus formation, we observed that the highest leaf callus formation was in 0.25 mg/L and hypocotyl was 0.5 mg/L NAA and the lowest in control treatment [14].

Also, the highest interaction of NAA with a micro-sample was observed at a concentration of 0.5 mg/L and the lowest in control treatment. Examination of the interaction between NAA and micro-sample in regeneration showed that the highest amount of regeneration was observed in leaf, hypocotyl and embryo at a concentration of 0.5 mg/L and the least in control (Figure 3). Interaction effects of NAA, BAP and micro-sample on callus formation showed that the highest amount of callus formation between hormones and a micro embryo was observed at concentrations of 1 mg/L NAA and 0.5 mg/L BAP (Table 4) [15].

FIG. 1. **Calligraphy and regeneration of leaf micro-sample. Interaction between BAP and leaf sample at 0.5 mg/L.**

FIG. 2. **Calligraphy and microenvironment regeneration in hypocotyl of 0.5 mg/l NAA.**

FIG. 3. **Calligraphy and regeneration of fetal explanted related to the interaction between NAA and microbial samples at concentrations of 0.5 mg/L.**

TABLE 4. **Interaction effects of NAA, BAP and type of micro-sample on the mean number of callus formation and regeneration.**

Variables		Number regeneration			Number callus		
NAA	**BAP**	**Embryo**	**Hypocotyl**	**Leaf**	**Embryo**	**Hypocotyl**	**Leaf**
0	0	0	0	0	0	0	0
	0.5	0	0.37^{e}	0.37^{f}	0.65^{f}	^{1e}	1^{e}

	1	0	0.37^{e}	0.37^{f}	0.65^{f}	1.37^{bc}	1.7^{bc}
	2	1c	1^{d}	1.68^{b}	2^{c}	2.75^{a}	3^{a}
0.25	0	1c	1^{d}	1.68^{b}	2^{c}	3^{a}	3^{a}
	0.5	1.37^{b}	1.37^{c}	1.32^{c}	2.32^{b}	3^{a}	3^{a}
	1	0.38^{e}	0.38^{f}	1^{d}	1.32^{e}	2.8^{a}	3^{a}
	2	0.38^{e}	0.37^{f}	0.68^{e}	0.7^{fg}	1.3^{c}	2^{b}
0.5	0	0.68^{d}	1.67^{b}	1.37^{c}	1.66^{d}	2.7^{a}	3^{a}
	0.5	2^{a}	2^{a}	3^{a}	3^{a}	3^{a}	3^{a}
	1	0.68^{d}	1.67^{b}	1.37^{c}	2^{c}	2.7^{a}	2.7^{a}
	2	0.68^{d}	1.67^{b}	1^{d}	1.67^{d}	1.67^{b}	2^{b}
	Lsd	0.3	0.29	0.27	0.3	0.35	0.32
Note: Numbers with common letters in each column are not significantly different							

By studying the interactions of hormones NAA, BAP and micro-sample in regeneration, it was found that the maximum amount of interactions between hormones and micro samples of hypocotyl and embryo was in concentrations of 1 mg/L NAA and 0.5 mg/L BAP was recorded. In tissue culture and cell culture studies, growth and morphogenesis are characterized by the type and concentration of growth regulators and their interactions.

Second experiment

In the second experiment, the effects of BAP, 2,4-D and micro-sample on callus production and direct regeneration in the medicinal plant of *Peganum harmala* L. were investigated. The analysis variance showed that the effect of BAP on callus formation and 2,4-D and micro-sample on callus formation and regeneration was significant at 0.01 level. Interaction of micro-sample in BAP on callus formation and interaction of 2,4-D hormones in BAP on callus formation and regeneration were significant at 0.01 level. No significant differences were observed in the other interactions (Tables 5 and 6) [16].

TABLE 5. **Analysis variance of BAP, 2, 4-D and micro samples for callus formation.**

SOV	**df**	**Mean squares**	
		Regeneration	**Callus**
BAP	3	0.44^{ns}	1.95^{**}
Micro sample	2	2.56^{**}	1.92^{**}
NAA	2	4.7^{**}	19.17^{**}
BAPx × Micro sample	6	0.15^{ns}	0.54^{**}
BAP × NAA	6	2.7^{**}	9.49^{**}
NAA × Micro sample	4	0.06^{ns}	0.078^{ns}
BAP × NAA × Micro sample	12	0.13^{ns}	0.1212
Error	72	0.21	0.15
Note: *, ** and ns are significant at the level of 0.05 and 0.01 and non-significant, respectively			

TABLE 6. **Interaction effects of 2, 4-D and BAP on the mean number of callus formation in the micro-sample.**

2,4-D	0				0.25				0.5				
BAP	0	0.5	1	2	0	0.5	1	2	0	0.5	1	2	Lsd
Callus number	0	1^{d}	1.45^{c}	2.67^{b}	2.83^{a}	3^{a}	2.67^{b}	1.57^{b}	2.83^{a}	3^{a}	3^{a}	1^{d}	0.2
Note: Numbers with common letters in each column are not significantly different													

Mean comparison of interaction effects of BAP and 2,4-D on callus formation showed that the highest mean number of callus formation in treatments of 0.5 mg/L BAP × 0.25 mg/L 2,4-D, 0.5 mg/L BAP × -0.5 mg/L 1 mg/L BAP × 0.5 mg/L D-2,4 were observed and the lowest was related to control treatment. BAP causes improper bud production but with the addition of auxin hormone, the number of buds increased significantly.

In a study on *Passi floraedulis*, it was found that after 8 weeks at the concentrations of 1 mg/L BAP and 2 mg/L 2,4-D the highest callus formation produced. Overall, it can be said that optimizing the concentration of growth regulators in the medium and applying different mediums with a specific concentration of sucrose, can improve regeneration. Overall, it can be said that optimizing the concentration of growth regulators in the medium and applying different mediums with a specific concentration of sucrose, can improve regeneration.

By examining the interactions of 2,4-D, BAP and micro-sample in regeneration, it was found that the highest amount of regeneration was achieved between hormones and a micro-sample of leaf, hypocotyl and embryo in treatment with 1 mg BAP and 0.5 mg. 2,4-D per liter was observed (Table 7).

TABLE 7. **Interaction effects of 2, 4-D and BAP and type of micro-sample on the average number of callus formation and regeneration of micro-sample.**

2,4-D	BAP	Number of regeneration			Number of callus		
		Embryo	Hypocotyl	Leaf	Embryo	Hypocotyl	Leaf
0	0	0	0	0	0	0	0
	0.5	0	0.3^{d}	0.3^{c}	1d	1^{ef}	1^{d}
	1	0	0.3^{d}	0.3^{c}	1d	1.7^{d}	1.7^{c}
	2	0.7^{c}	0.7^{c}	0.25	2.33^{c}	2.7^{ab}	3^{a}
0.25	0	0.65^{c}	1.1^{b}	1.77^{b}	2.7^{ab}	3^{a}	3^{a}
	0.5	0.3^{d}	0.65^{def}	1^{c}	3^{a}	3^{a}	3^{a}
	1	0.3^{d}	0.65^{def}	0.65^{d}	2.33^{c}	2.7^{ab}	3^{a}
	2	0	0.65^{def}	0.5	0.7^{c}	2^{cd}	2^{b}
0.5	0	1b	1b	1c	2.7^{ab}	3^{a}	3^{a}
	0.5	0.65^{c}	1.77^{a}	1.77^{b}	3^{a}	3a	3^{a}
	1	1.77^{a}	2^{a}	2.33^{a}	3^{a}	3a	3^{a}
	2	0	0.3^{d}	0.65^{d}	0.7^{c}	1.3^{e}	2^{b}
	Lsd	0.23	0.28	0.32	0.33	0.33	0.33

Note: Numbers with common letters in each column are not significantly different

Conclusion

Each plant *in vitro* needs different plant growth regulators with certain concentrations. Because of the importance of optimizing the conditions within the culture environmental conditions for micro propagation studies, a suitable protocol for induction and regeneration of callus could be in future studies on the use of genetic engineering. Increase production of the effective substance in medicinal plants as well as use in suspension culture for production of effective substance production. According to experiments on tissue culture of the medicinal plant harmala that were performed in two completely separate experiments, the highest rate of induction of callus formation and regeneration of specimens for tissue culture of the plant in the first experiment, hormonal concentrations of 0.5 mg/l BAP and 0.5 mg/l NAA was observed. High BAP concentration in the presence of NAA decreased callus formation and appeared NAA to be better for callus formation.

The highest induction of callus induction and regeneration in the samples in the second experiment was observed in 1 mg/l BAP and 0.5 mg/l 2,4-D treatment. Increasing BAP concentration in the presence of 2,4-D decreased callus formation and lack of BAP caused good regeneration. The hormone 2,4-D seemed to be necessary to increase regeneration. Generally, 2,4-D and BAP hormones can be used for callus formation and NAA and BAP hormones for regeneration.

Harmala is a valuable herb and medicinal plant. Studying and identifying the regeneration mechanism of this plant is of industrial and medication importance. The use of 2,4-D and BAP growth regulators were the best for callus formation and leaf micro-sample had the best callus production among all micro samples and it was found that the leaf micro-sample was more efficient for regeneration and callus formation than the hypocotyl and embryo samples.

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