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In vitro Plant Regeneration Influence by BAP and IBA in Lentils (*Lens culinaris* Medik)

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Abstract

Lentil is an important food legume crop that is very recalcitrant to shoot regeneration and rooting under *in vitro* conditions. This study aimed to develop efficient and reliable protocol for *in vitro* plant regeneration. The reulsts of this study showed that In this context, 16 Turkish lentil cultivars were shoot tip, stem , hypocotyl, cotyledon and root as explants.

The MS medium containg 4 mg/l BAP induced maximum number (8.25) of shoots per shoot tip explant of cv. Yesil 21. Whereaas, maximum number (28.33) of shoots per cotyledon explant of cv. Ozbek was induced on MS medium containing 1 mg/l IBA.

However,IBA derived shoots were easy to root on MS medium containing 1.87 mg/l NAA. The regenerated explants were transferred to greenhouse for acclimatisation, flowering and seed set. It was concluded that *in vitro* shoot regeneration of lentils (*Lens culinaris* Medikus) was strongly influenced by the type of explant, plant growth regulator concentration and combinations.

Key words: Acclimatisation, auxins, cytokinins, Lentil, micropropagation, rooting

INTRODUCTION

Lentil (Lens culinaris ssp. culinaris) is an important food legume with various uses as food and feed because of its protein-rich grains and straw [1], [2]. The total seed protein content varies from 20 to 35%, with relatively high levels of lysine and sulfur-amino acids [3]. Globally, it is cultivated as a rainfed crop on 3.85 million hectares (m ha) with 3.59 million tonnes (mt) production [1]. It is thought that lentil was brought into cultivation somewher in Southeast Turkey or Northern Syria near the Tigris and Euphrates rivers [4].

Lentil suffers from Narrow genetic base and poor genetic resources, therefore, limiting the application of biotechnological tools for crop improvement. The major problems in Turkey is the narrow genetic base of lentil which must be broadened through introgression of new genes from wild or exotic germplasm because the maintenance of diversity in agriculture is essential to protect plant genetic resources [2]. The genotype–environment interaction and provide greater production stability depends on wide range of genetic diversity and resources of the plant. Therefore, alternative approach for the improvement of this crop is to regenerate plants from single cells and organized tissues and to transfer desirable genes from other sources to complement traditional breeding methods with biotechnology techniques as an alternative. Earlier studies suggeste that *in vitro* culture of lentils is more difficult and the success has been achieved very slowly. First research on lentil tissue culture was reported by Bajaj and Dhanju [5]. It was followed by many researchers like Williams et al [6], Saxena and King [7], Polanco et al. [8], Mallick and Rashid [9], Malik and Saxena [10], [11], Ahmad et al. [12], Polanco and Ruiz [13], Halbach et al. [14], Polanco [15], Ye et al. [16] [17], Khawar and Ozcan [3], Khawar et al. [18], Fratini and Ruiz [19], Sevimay et al. [4], Aasim et al. [20].

Genetic transformation or single gene transfer in lentil has been challenging and more difficult because of its recalcitrant nature to in vitro regeneration [21].

Establishment of an efficient and repeatable *in vitro* regeneration protocol is one of the basic prerequisites for gene transformation and plant breeding [18]. The present study was carried out for the development of reliable and efficient *In vitro* regeneration protocol for 16 of lentil cultivars.

MATERIALS AND METHODS

Material

The present study was carried out using sixteen local turkish lentil varieties namely Sazak 91, Sultan I, Kayı 91, Pul 11, Yeşil 21, Meyveci 2001, Fırat 87, Ozbek, Ciftçi, Erzurum 89, Malazgirt 89, Seyran 96, Kırmızı 51,Yerli Kırmızı, Kafkas and Emre 20. These were obtained from the Department of Field Crops, Faculty of Agriculture, Yuzuncu Yıl University, Van, Turkey.

The seeds were surface-sterilized with 100% commercial bleach (ACE Turkey- 5% NaOCl) for 20 min followed by 3X5 rinses withsterile distilled water. The sterilised seeds were cultured on Petri dishes® (100 x 10 mm diameter) containing MS [22], medium supplemented with 3% sucrose (w/v) 0.7% agar (Sigma agar type A) . The MS regeneration medium contained 4 mg/l BAP with/without 1 mg/l 2,4-D.

The elongated and multiplied shoots derived from their respective explant were obtained and, rooted on MS medium containing 0.19 or 1.90 mg/l NAA. Thereafter, the regenerated plant were hardened and acclimatized under greenhouse condition.

The data were subjected to one way ANOVA statistical analysis and noted on end of each experiment.and compared using Tukey's b test using Minitab statistical software 13.0.

RESULTS

The Table 1 depicts that effects of MS medium conatining cytokinin (4 mg/l BAP) with or without auxin (l mg/l 2,4-D) on shoot regeneration from 5 type of explants of 16 lentil cultivars. The analysis of varience results showed significant diferences ($p < 0.05$) among the cultivars as well as expalnt of the present study. The shoot regeneration reponse on shoot tip explant ranged 2.19 - 8.25 and 0.61-8.15 on MS medium containg 4 mg/l BAP and 4mg/l BAP with 1 mg/l 2,4-D. The maximum shoot regeneration was obtained on shoot tip explant of the cv Yesil-21 cultured on MS medium conatainig 4 mg/l BAP. It was noted that presence of auxin (1 mg/l 2,4-D) with cytokinin (4 mg/l BAP) inhibited the induction of shoots on the shoot tip explant. Due to presence of 2,4-D in medium, the maximum shoot induction on cv Yesil-21 were significantly less compared to shoot induction on shoot tip explant. However, stem node explant ranged 1.45 to 6.67 cv Kirmiz-51 and Sazak-91 respectively on MS medium supplemented with 4 mg/l BAP. Whereas, presence of 1 mg/l 2,4-D in medium again had lower shoot induction ranging 0.61-6.33 on cv.Yesil-21 and Seyran-96 respectively. The cultivars Ciftci, Erzurum and Yerli Kirmizi induced cent percent callus on the stem node explant in the present study. The hypocotyl explant also had variable shoot induction response that ranged 1.07-6.25 and 0.33-6.34 shoots per explant. The shoot induction per explant response on cotyledon was 0.62 – 8.15. It was interestingly to note that presence of cytokinin (4 mg/l BAP) were induced callus on 6 out of 16 lentil cultivar. However, cotyledon explant cultured on MS medium containing 4 mg/l BAP and 1 mg/l 2,4-D induced callus on 15 out of 16 cultivar. Only cv. Kafkas cultivar had shoot induction on aforementioned medium. Root explant produced cent percent callus induction on MS medium containing 4mg/l BAP with or without 1 mg/l 2,4-D.

Effects of 1 or 2 mg/l IBA on shoot regeneration

The Table 1 depicted that shoot regeneration reponse was significantly ($p<0.01$) variable and dependent on explant and variety. As far as 1 mg/l IBA was concerned, maximum and statistically similar shoot induction responce was noted on cv. Ozbek (19.33%) cv. Seyran-96 (21.67%)cv. Ciftci (25.99) and cv Ozbek (28.33) on shoot tip, stem node, hypocotyle and cotyledons repectively. Whereas, it ranged 6.37-19.33, 7.43-21.67, 6.32-25.99 and 6.56- 28.33 on MS medium containing 1 mg/l IBA. However, MS medium containing 2 mg/l IBA showed shoot induction range of 6.22-25.41,6.11-26.99, 4.52-23.33 and 7.62-21.23 on shoot tip, stem node, hypocotyle and cotyledons respectively. The root explant did not show any kind of shoot induction. It was interestangly noted that 1 mg/l and 2 mg/l IBA promoted shoot induction in present study. As far as explant was concerned, highest number (28.33) of shoot induction was noted on 1 mg/l IBA derived cotyledon explants.

Rooting

In vitro rooting is problamatic/complicated in legume specially Fabaceae family. Therefore, extra care should be taken for this aspect. Two concentrations of auxin (NAA) were used in the present study to induce roots. Well developed and healthy shoots from each cultivar were rooted on MS medium containing 0.19 or 1.90 mg/l NAA to evaluate rooting response on the cultivar. Root initiation were observed on all explant along with both the treatment. The analysis of result were significantly different ($p < 0.05$) on the cultivars. The maximum root induction (97.21%) Kayi 91 and (79.85%) Sazak-91 on MS medium containing 0.19 and 1.90 mg/l NAA repectively that ranged 68.15-97.27 and 49.28-79.85% in the present study. Comparasion of two treatment on root induction showed that lower concentration (0.19 mg/l) of NAA was better for root indution. It was observed that all plant induced good rooting that helped in their later growth and development.

All *in vitro* regenerated plantlets were successfully acclimatized under greenhouse condition where they flowered and set seeds. The plantlets showed normal growth and no any sign of abnormality in the greenhouse.

DISCUSSION

Shoot regeneration potential of shoot tip, stem node, hypocotyl, cotyledon and root explants of 16 Turkish lentil cultivars was tested on MS medium containing 4 mg/l BAP with and without 1 mg/l 2,4-D and 1 or 2 mg/l IBA.

Development of effective protocols for lentils micropropagation are very important for pragmatic transformation of the plant. Previous studies on lentil regeneration by Williams et al. [6], Saxena and King [7], Polanco et al. [8], Mallick and Rashid [9], Malik and Saxena [10] [11], Polanco and Ruiz [13], Halbach et al. [14], Polanco [15], Ye et al. [16] [17], Khawar and Ozcan [3], Khawar et al. [18], Fratini and Ruiz [19], Sevimay et al. [4], Aasim et al. [20] point out that irrespective of the explants and culture media repeatable micropropagation including rooting is a tedious job and most often difficult to repeat.

This study showed that initiation and multiplication of shoots was influenced both by concentration and combination of IBA (1 or 2 mg/l) and 4 mg/l BAP with and without 1 mg/l 2, 4-D, explant and the genotype. The results showed that MS medium containing different concentrations of BAP with and without 2,4-D were inhibitory on 5 explants and induced variable amount of callus on cotyledon and root explants. The results emphasise that both concentrations of IBA has high potential for lentil micropropagation; compared to MS medium containing BAP with and without 2,4-D. Whereas, Khawar et al. [18], induced shoots using thidiazuron and rooted them on MS medium containing IBA.

However, no callusing was recorded when either 1 or 2 mg/l IBA was used in regeneration on any of the five explants in this study; which does not confirm the findings of Khawar and Ozcan [3]; they found that IBA promotes callusing at the basal portion of explants along with rare roots on the explants. Contrarily, this study reports that IBA plays an opposite role in lentils. No rooting was noted on the regenerated shoots. There is no previous report on use of auxins for shoot regeneration in lentils; however, IBA based regeneration is reported in another legume cowpea by Aasim et al. [20]. The method provides an easy and alternative mean of regeneration of lentil through tissue culture. The results of this study points out that suitable plant growth regulator, morphological integrity and developmental stage of explants are very important and has key role in the successful regeneration that influence induction of shoots or callusing in agreement with Khawar et al. [23]. Moreover, the regeneration is genotype and plant growth regulators specific. The result also showed multiple shoot regeneration and morphogenetic response that varied significantly depending on the cultivar, explant

Table 1. Effects of 4 mg/l BAP or 4 mg/l BAP-1 mg/l 2,4-D on shoot regeneration from 5 explants of 16 cv of lentil

Cultivar	Shoot tip		stem node		Hypocotyl		Cotyledon		Root	
	4mg/l BAP	4mg/l BAP+1 mg/l 2,4 D	4mg/l BAP	4mg/l BAP+1 mg/l 2,4 D	4mg/l BAP	4mg/l BAP+1 mg/l 2,4 D	4mg/l BAP	4mg/l BAP+1 mg/l 2,4 D	4mg/l BAP	4mg/l BAP+1 mg/l 2,4 D
Sultan1	3.02e	4.81 b	3.62 9d	3.3b	2.11d	2.13d	1.05g	Callus	Callus	Callus
Kayı 91	3..07e	0.61 e	3.42d	1.72d	2.23d	1.16e	1.31g	Callus	Callus	Callus
Pul 11	4.19d	3.10 c	4.16c	1.65d	6.25a	3.19c	4.44d	3.29a	Callus	Callus
YeSil 21	8.25a	1.11 e	2.26e	0.61d	5.63b	0.33f	6.48b	Callus	Callus	Callus
Meyveci 2001	2.19f	2.63d	2.64e	1.1d	4.27c	1.1e	5.27c	Callus	Callus	Callus
Fırat 87	3.17e	3.37c	2.41e	1.6d	5.17b	2.34d	Callus	Callus	Callus	Callus
Ozbek	3.23e	3.43c	2.53e	1.5d	4.64c	Oca.92	8.15a	Callus	Callus	Callus
CiftCi	4.19d	3.21c	2.13e	Callus	4.37c	Şub.18	Callus	Callus	Callus	Callus
Erzurum 89	2.24f	3.15c	2.34e	Callus	2.25d	4.43c	Şub.65	Callus	Callus	Callus
Malazgirt 89	4.17d	5.15b	3.61d	2.41c	1.07e	1.12e	Callus	Callus	Callus	Callus
Seyran 96	3.15e	8.15a	2.92e	6.33a	1.09e	5.16b	Callus	Callus	Callus	Callus
Kırmızı 51	3.23e	2.49d	1.45f	1.16d	2.26d	1.91e	Callus	Callus	Callus	Callus
Yerli Kırmızı	5.31c	5.36b	4.51c	Callus	1.12e	6.34a	0.62	Callus	Callus	Callus
Kafkas	3.03e	4.78b	6.43a	3.44b	2.48d	4.49c	Mar.45	3.19a	Callus	Callus
Emre 20	7.56b	2.87d	5.12b	1.91d	6.11a	3.95c	01.Tem	Callus	Callus	Callus
Sazak 91	5.6c	1.25e	6.67a	1.99d	2.37d	1.99e	Callus	Callus	Callus	Callus

All values in a column shown by different small letters are statistically different at $p<0.05$ using Tukey's b test.

Table 2. Effects of 1 or 2 mg/l IBA on shoot regeneration from various explants of 16 cv of lentil

Cultivar	Shoot tip		Stem node		Hypocotyle		Cotyledon		Roots	
	1 mg/l IBA	2 mg/l IBA	1 mg/l IBA	2 mg/l IBA	1 mg/l IBA	2 mg/l IBA	1 mg/l IBA	2 mg/l IBA	1 mg/l IBA	2 mg/l IBA
Sultan1	9.54f	10.22 i	16.34cd	13.24e	10.13i	6.11j	5.49 f	15.45d	0.00	0.00
Kayı 91	13.52cd	9.25j	7.43g	10.22g	9.62j	5.62jk	12.14c	17.42c	0.00	0.00
Pul 11	14.510c	25.41a	21.23a	15.29d	10.39i	4.52l	16.99b	21.23a	0.00	0.00
YeSil 21	8.671gh	8.25k	9.41f	10.24g	14.38f	23.33a	11.11cd	7.62j	0.00	0.00
Meyveci 2001	17.49b	12.15g	15.31d	13.28e	6.32k	17.51cd	9.23d	21.30a	0.00	0.00
Fırat 87	6.01i	16.11ef	7.34g	26.99a	11.37h	13.49e	5.26f	21.34a	0.00	0.00
Ozbek	19.33a	7.12kl	21.56a	6.11j	24.33b	18.31d	28.33a	8.15j	0.00	0.00
CiftCi	14.33c	19.32cd	13.55e	15.27d	25.99a	9.22g	11.32cd	11.33g	0.00	0.00
Erzurum 89	10.42f	13.23g	8.61f	9.26h	23.33c	7.11li	8.87d	5.44k	0.00	0.00
Malazgirt 89	18.51ab	17.25e	19.18b	13.16e	18.54d	17.24cd	7.23e	19.45b	0.00	0.00
Seyran 96	11.54e	15.34f	21.67a	15.25d	9.09j	10.25f	6.56f	10.11h	0.00	0.00
Kırmızı 51	11.44e	24.24ab	14.19e	21.34b	13.32g	23.22a	17.54b	9.21i	0.00	0.00
Yerli Kırmızı	6.37i	18.95e	9.11f	15.33d	9.35j	13.21e	15.27bc	18.31b	0.00	0.00
Kafkas	9.93g	11.25h	14.13e	8.32i	18.37d	21.34b	10.98cd	14.61e	0.00	0.00
Emre 20	7.24h	6.22m	17.11c	19.33c	15.39e	19.33c	9.55d	4.47 l	0.00	0.00
Sazak 91	10.82f	20.27c	9.23f	12.67f	6.32k	8.12h	12.54c	13.37f	0.00	0.00

All values in a column shown by different small letters are statistically different at $p<0.05$ using Tukey's b test.

and concentration of plant growth regulator in agreement with Ozgen et al. [24].

The shoots that were obtained on MS medium containing IBA could be rooted easily compared to the shoots that were regenerated on MS medium containing BAP with or without 2,4-D. A review of papers published on micropropagation of lentils during last 35 years report either complex and difficult to repeat methodologies with scarce or complete failure of rootings in micropropagated shoots.

All micropropagated plantlets irrespective of the cultivars and explants that were regenerated on IBA were more vigorous and easy to acclimatise in the greenhouse and set seeds compared to those regenerated on BAP with or without 2,4-D. The shoots regenerated on BAP with or without 2,4-D regenrated plantlets died during rooting. All IBA regenerated plantlets showed normal growth and development. The researchers did not find any abnormality in the rooted and acclimatized plantlets in the greenhouse. The researchers meet the objective of establishment of lentil plantletss and are confident that this protocol could help in easy genetic itransformation in future.

Table 3. Differences in rooting percentage (%) of 16 lentil cultivars on MS medium containing 0.19 or 1.90 mg/l NAA

Cultivars	Rooting percentage (%)	
	0.19 mg/l NAA	1.90 mg/l NAA
Sultan1	68.15j	52.76h
Kayı 91	97.21a	73.45c
Pul 11	85.27f	66.17f
YeSil 21	71.35i	49.28i
Meyveci 2001	78.23g	70.36d
Fırat 87	81.25f	63.94g
Ozbek	91.55c	57.42g
CiftCi	88.93b	68.36e
Erzurum 89	76.25h	53.47h
Malazgirt 89	91.24c	62.44g
Seyran 96	93.95b	70.36d
Kırmızı 51	93.07b	79.32a
Yerli Kırmızı	68.44j	74.33b
Kafkas	81.43f	78.84a
Emre 20	82.44f	66.14f
Sazak 91	88.57d	79.85a

All values in a column shown by different small letters are statistically different at $p<0.05$ using Tukey's b test.

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