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PRECONDITIONING EFFECT OF BAP ON *IN VITRO* MULTIPLICATION OF MATURE EMBRYO OF LENTIL (*LENS CULINARIS* MEDIK.)

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ABSTRACT

Lentil (*Lens culinaris* Medik.) is an important legume with high carbohydrate and protein content that is used as main source of food in many countries. Lentil is recalcitrant and difficult to root under *in vitro* conditions. Therefore, present study aimed to determine the preconditioning effect of Murashige and Skoog (MS) medium containing 10 and 20 mg L^{-1} benzylaminopurine (BAP), whereas, postconditioning media were contained of dissimilar levels of BAP combination with indole-3-butyric acid (IBA) for *in vitro* shoot multiplication using mature embryo as explants. Comparing the three culture conditions, the preconditioned embryo explants had significant difference with unconditioned embryo explants in terms of frequency of shoot regeneration (%) and number of shoot per explant. The presence of IBA in culture medium with each increased concentrations of BAP had positively enhanced the number of shoot per preconditioned explant; whereas, shoots length were inhibited variably. The maximum number (16.33) of shoots was recorded on MS medium containing 0.50 mg L^{-1} BAP + 0.10 mg L^{-1} IBA at postconditioning level. Root initiation was observed in all MS media containing IBA after the transfer of shoots. The maximum root induction (75.00%) and mean number of 4.35 roots per shoot was observed on MS medium containing 0.10 mg L^{-1} IBA. The healthy and rooted plants were transferred to glasshouse for acclimatization and exhibited 85% survival. This regeneration protocol may facilitate genetic transformation studies in lentil.

KEYWORDS:
Cytokinin, lentil, mature embryo, preconditioning, regeneration

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is one of the early domesticated and nutritious traditional pulse crops, which has been experiencing a declining area of cultivation and production in Turkey and South-East Asia, due to lower yields [1, 2, 3]. Lentil has originated from the Middle East and spread to the other parts of world. It is being cultivated as a rain-fed crop on 4.32 million hectare area with the global production of 4.97 million tons in 2013. It contains more than 26% protein in seed and considered as a source of amino acids for human and the livestock [4, 5, 6].

Modern and advanced biotechnology techniques including plant tissue culture and genetic transformation has provided new opportunities to improve the germplasm and yield of crop [7, 8]. However, an efficient and reliable shoot regeneration protocol is a prerequisite for lentil genetic transformation strategies [9, 10, 11].

The regeneration frequency can be increased or modified by plant growth regulator combinations, photoperiod, pH, carbohydrates and other growth supplements [12, 13, 14, 15]. Pulse treatment or preconditioning of explants at higher concentrations of cytokinin and auxin is often used for shoot and root multiplication in legume [16, 8]. However, shoot regeneration in lentil has been previously reported through somatic embryogenesis, organogenesis and callus using different explants [17, 10, 18, 11, 2]. But, there is no report on mature embryo explants preconditioning in lentil for shoot multiplication. The present study investigated the effect of BAP preconditioning on lentil mature embryos that may be useful for efficient regeneration leading to genetic transformation.

MATERIAL AND METHODS

Surface sterilization. The seeds of lentil cv. "Sultani Yeşil" were washed with tap water and then surface sterilized using 10% H_2O_2 and few drops of Tween-20 for 15 min followed by 3×5 min rinsing with sterile double distilled water.

The surface sterilized seeds were placed between two pieces of moist filter paper in petri dishes for germination. After two days of germination, mature embryos were excised aseptically and preconditioned on MS medium [19] supplemented with 3% (w/v) sucrose with 10 and 20 mg L^{-1} BAP for 10 days. Mature embryos were placed on gelrite solidified MS medium as the control treatment. The each preconditioning treatment contained 200 embryos with three replications.

Effect of BAP on shoot multiplication. The preconditioned mature embryo explants were cultured on postconditioning regeneration medium containing different concentrations of 0.0, 0.1, 0.25 and 0.5 mg L^{-1}BAP and constant concentration of 0.1 mg L^{-1} IBA. Each treatment was replicated three times with 15 explants in each. At the end of postconditioning treatments, data on shoot induction (%), shoot per explant and average shoot length were recorded for the analysis.

***In vitro* rooting.** Healthy and well developed multiplied shoots (2 to 2.5 cm) were excised and cultured on gelrite solidified ½ strength MS medium with different concentrations of 0.0, 0.1, 0.25 and 0.5 mg L^{-1} IBA supplemented with 0.1% activated charcoal. Ten shoots were inoculated in each magenta box and each treatment consisted of three replications. The experimental data on rooting percentage (%), mean number of root per explant and mean root length were recorded after four weeks of culture.

All media were autoclaved at 121 °C for 20 minutes (1.5 kg cm^{-2} pressure) while their pH was adjusted to 5.6-5.8. All cultures were grown at 16/8 h (light/dark) photoperiod with 25 ± 2 °C. Light intensity of 25 µmol photons m^{-2} s^{-1} was supplied by cool-white fluorescent lamps. However, the plant growth regulators, Gelrite Gellan Gum, and the chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Duchefa Biochemie B.V. (Haarlem, the Netherlands).

***Ex vitro* acclimatization.** Well-developed and rooted plantlets were selected for acclimatization. They were removed from culture media and transferred to the plastic pots containing peat moss and peat moss-perlite (3:1). Each pot were watered once a week with 100 mL tap water and covered with a transparent polyethylene bag to provide a high relative humidity. After ten days, polythene covers were gradually opened and plants were exposed to the light. The adapted plants were transferred to glasshouse and kept under ambient light & temperature between 19-23±2 °C.

Statistical analysis. Data were subjected to one-way analysis of variance (ANOVA; SPSS 17 for Windows, SPSS Inc., Chicago, IL, USA), and post-hoc tests were performed using the least significant difference (LSD) or Duncan's multiple range test (DMRT) at 0.05 level of significance. The treatments were arranged in a completely randomized design. The data given in percentages (%) were subjected to arcsine (√X) transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

The present study investigated preconditioning effect of BAP on mature embryo explants of lentil using gelrite solidified MS medium supplemented with 10 and 20 mg L^{-1} BAP for 10 days (Figure 1a). Therefore, contamination-free seeds were obtained through 10% H_2O_2 treatment and germinated on sterilized moist filter paper for mature embryo explants.

Explants subjected to Murashige and Skoog medium (MS0, control) had significant difference with those embryos treated with 10 and 20 mg L^{-1} BAP in terms of frequency of shoot regeneration (%) and number of shoots per explants (Table 1). Although, there was no significant differences observed on MS medium supplemented with 10 and 20 mg L^{-1} BAP. The frequencies of shoot regeneration were noted 69.67, 83.33 and 100% for 10 and 20 mg L^{-1} BAP and control treatments respectively. Despite of the 100% shoot regeneration frequency, control treatment induced only single shoot per explant; whereas, 3.13 and 2.68 shoots per explants were noted on MS medium containing 10 and 20 mg L^{-1} BAP respectively. Table 2 depicts the effect of preconditioned mature embryo explants when cultured on four postconditioned treatments of

0.00, 0.01, 0.25 or 0.50 mg L^{-1} BAP with 0.10 mg L^{-1} IBA. The explants mainly showed swelling after culture in a postconditioning dose-dependent manner. After one week, postconditioned explants showed fragile callus initiation at the cut ends treated with 0.25 and 0.50 mg L^{-1} BAP-0.10 mg L^{-1} IBA. Thereafter, pale yellow fragile callus followed by shoot bud initiation after two weeks of culture.

Analysis of the results showed that nonconditioning and 10 mg L^{-1} BAP treatment were inferior as compared to 20 mg L^{-1} BAP in terms of shoot regeneration frequency after postconditioning (Figure 1b, c, d). All treatments showed statistically similar shoot regeneration frequencies after postconditioning; however, results varied numerically (Table 2). The explants which were preconditioned with 10 and 20 mg L^{-1} BAP induced 93.33 to 100% shoot regeneration frequency, whereas 96.67 to 100% shoot regeneration frequency was noted on nonconditioned explants after postconditioning levels.

Comparing the results of nonconditioned, 10 and 20 mg L^{-1} BAP preconditioned explants cultured on different concentrations and combinations of BAP-IBA showed that 20 mg L^{-1} BAP treatment had a significant ($P \leq 0.01$) effect on number of shoots per explant. The number of shoots ranged from 1.07 to 11.00, 2.00 to 13.66 and 2.51 to 16.33 and showed significant differences on nonconditioned, 10 mg L^{-1} and 20 mg L^{-1} BAP, respectively (Table 2). The maximum number of shoots were noted with 20 mg L^{-1} BAP preconditioned treatment that postconditioned on MS medium containing 0.50 mg L^{-1} BAP + 0.10 mg L^{-1} IBA (Figure 1e). The number of shoots per explant, induced on 20 mg L^{-1} BAP, were greater compared to 10 mg L^{-1} BAP and nonconditioned explants after each postconditioning treatment. With each increase in BAP concentration accompanied by 0.1 mg L^{-1} IBA at postconditioning step an increment in number of shoots per explant was observed.



FIGURE 1

***In vitro* mature embryo tissue culture of *Lens culinaris*: a- Preconditioned mature embryo explants showing regeneration on control, 10 mg L^{-1} BAP and 20 mg L^{-1} BAP after 10 days of culture; b, c, d, - number of shoots per explants on nonconditioned, 10 mg L^{-1} and 20 mg L^{-1} BAP-preconditioned explants, respectively; e- maximum number of shoots on 20 mg L^{-1} BAP-preconditioned explants after postconditioning on 0.50 mg L^{-1} BAP + 0.10 mg L^{-1} IBA; f- rooting frequency on MS medium containing 0.10 mg L^{-1} IBA; g-acclimatization of *in vitro* regenerated lentil explant growing on peat-moss substrate.**

TABLE 1

Effect of preconditioning treatment of BAP on shoot regeneration of lentil

Treatment (mg L^{-1})	Frequency of shoot regeneration (%) ± S.E*	Number of shoots per explant ± S.E
MS0 (Control)	100^{a}	1.0 ± 0.06^{b}
10 mg L^{-1} BAP	83.33 ± 6.66^{b}	3.13 ± 0.67^{a}
20 mg L^{-1} BAP	69.67 ± 3.33^{b}	2.68 ± 0.55^{a}

* Values shown in column followed by different letters are statistically different using LSD test at 0.01 levels of significances; S.E (standard error)

TABLE 2
Effect of postconditioning with various concentrations of BAP plus 0.1 mg L^{-1} IBA on shoot regeneration of nonconditioned and 10 & 20 mg L^{-1} BAP-preconditioned embryonic node explants of lentil

Post conditioning treatments (mg L^{-1})		Frequency of shoot Regeneration (%) ± S.E*			Number of shoots/explant ± S.E			Shoot length (cm) ± S.E		
BAP	IBA	MS0	10 mg L^{-1} BAP	20 mg L^{-1} BAP	MS0	10 mg L^{-1} BAP	20 mg L^{-1} BAP	MS0	10 mg L^{-1} BAP	20 mg L^{-1} BAP
0.0	0.1	100.00^{a}	100.00^{a}	100.00^{a}	1.07±0.06^{c}	2.00±0.10^{c}	2.51±0.52^{c}	10.58±0.68^{a}	5.75±0.52^{bc}	4.25±0.21^{bc}
0.1	0.1	96.67±3.33^{a}	93.33±3.33^{a}	96.67±6.66^{a}	9.83±0.44^{b}	9.00±1.00^{b}	14.50±0.50^{a}	5.7± 0.46^{bc}	5.04±0.40^{bc}	4.83±0.25^{bc}
0.25	0.1	100.00^{a}	93.33±3.33^{a}	100.00^{a}	10.50±0.28^{b}	9.50±0.57^{b}	14.83±0.33^{a}	4.87±0.31^{bc}	3.96±0.18^{bc}	3.96±0.25^{bc}
0.5	0.1	100.00^{a}	96.67±6.66^{a}	100.00^{a}	11.00±1.25^{b}	13.66±1.09^{a}	16.33±0.33^{a}	5.29±0.61^{bc}	3.91±0.43^{bc}	3.21±0.11^{c}

* Mean values shown by different letters in a block are statistically different using Duncan test at 0.05 levels of significances; S.E (standard error)

TABLE 3
Effect of different concentrations of IBA on rooting of preconditioned explants of lentil

Treatment (mg L^{-1})	Frequency of root induction (%) ± S.E*	Number of roots per plant ± S.E	Root length (cm) ± S.E
MS0 (control)	0.00^{c}	0.00^{d}	0.00^{c}
0.10 IBA	75.00±6.6^{a}	4.35±0.31^{a}	3.99±0.44^{c}
0.25 IBA	50.00±3.3^{b}	2.00±0.21^{b}	4.65±0.35^{b}
0.50 IBA	15.00±1.0^{c}	0.25±0.01^{c}	5.60±0.66^{a}

*Mean values shown by different letters in a column are statistically different using LSD test at 0.05 level of significance; S.E (standard error)

The comparison between nonconditioned explants cultured on postconditioned media; the results showed significant inhibitory effects on shoot length ($P \leq 0.05$). Shoots length of nonconditioned, 10 and 20 mg L^{-1} BAP preconditioned explants when cultured on postconditioned media showed significant differences that ranged from 4.87 to 10.58 cm, 3.91 to 5.75 cm, and 3.21 to 4.83 cm, respectively (Table 2). Maximum shoot length of 10.58 cm was noted on nonconditioned explants cultured on MS medium devoid of BAP with 0.10 mg L^{-1} IBA; whereas, minimum shoot length was noted on 20 mg L^{-1} BAP preconditioned explants cultured on MS medium containing 0.50 mg L^{-1} BAP + 0.10 mg L^{-1} IBA at postconditioned treatment.

***In vitro* rooting.** The regenerated and multiplied shoots were excised and cultured on MS0 (control) and MS medium containing 0.10, 0.25 and 0.5 mg L^{-1} IBA for root induction. The rooting treatments showed significant differences ($P \leq 0.05$) on frequencies of root induction (%), mean number of roots and root length per explants (Table 3). No rooting was observed on MS0 medium (control) devoid of growth regulator. The results revealed that frequency of root induction, number of roots per plantlet and root lengths ranged from 0.00 to 75.00%, 0.00 to 4.35 and 0.00 to 5.6 cm respectively. Comparing four concentrations of IBA, maximum root induction (76.00%) and number of root per plant (4.35) were noted on MS medium supplemented 0.10 mg L^{-1} IBA (Figure 1f). MS medium containing 0.25 and 0.50 mg L^{-1} IBA showed inhibitory effect on root induction and number of root per explant in comparison with 0.1 mg L^{-1} IBA (Table 3). However, increased IBA concentrations in MS media showed positive effect on root length in the present study. Based on results, existence of IBA is necessary for root induction in postconditioned regenerated shoots (Table 3). Interestingly, the regenerated shoots when cultured on MS medium supplemented with any concentration of IBA also induced variable number of shoots per explants.

***Ex vitro* acclimatization.** Healthy plantlets with at least 6-8 cm tall containing 3-4 elongated nodal junctions with well-developed root system were transferred to plastic pot containing peat moss and peat moss-perlite at 3:1 ratio. The results showed the acclimatization of 85% and 60% in peat moss and peat moss-perlite respectively. The best substrate was confirmed as peat moss in the present study (Figure 1g). The peat moss grown plants showed strong roots with about 1.5 – 2 mm thickness at their base.

The grown root easily penetrated into the peat moss after development of number of healthy and fibrous roots that increased surface area of roots to absorb nutrients from the substrate. These roots were profuse and penetration followed by downward

turning in plastic pots containing peat moss. Similar numbers of roots and growth were not observed on peat moss-perlite substrates. After acclimatization in the growth chamber, the plants were transferred to the glasshouse, where they induced flower and set seeds.

DISCUSSION

Successful plant regeneration and multiplication via tissue culture has been widely used for many crops including legumes [20]. Adventitious or direct shoot formation is more important in tissue culture raised plants that provide an opportunity to speed up desired plant multiplication in a short duration. It may be possible reason that *in vitro* high frequency shoot multiplication in tissue culture plants is due to reduced nitrogen content in MS medium; which were also demonstrated in previous studies of chickpea and grasspea [8, 21]. While, Hazarika [22] also reported that nitrogen content involved in many biochemical and physiological processes that regulate plant growth and differentiation.

The present study described preconditioning of embryo explants with BAP followed by culture on different concentrations of BAP with 0.10 mg L^{-1} IBA to find the effect of postconditioning treatments on shoot multiplication. The results clearly showed the superiority of preconditioning and postconditioning over the control irrespective of the applied cytokinin (BAP) concentrations. It is stated that cytokinins as efficient plant cell division and differentiation agents have great influence on shoot formation, quality of the *in vitro* cultured explants, chlorophyll accumulation and leaf senescence [2, 8, 23].

Comparing the preconditioning treatments, 20 mg L^{-1} BAP was superior in terms of shoot regeneration frequency and number of shoots per explants; whereas, nonconditioned explants induced longer shoots. It was established that BAP was the most successful plant growth regulator for axillary shoot initiation in the present study (Table 1). It is well documented that BAP is a potential plant growth regulator that is more effective in enhancing *in vitro* shoot initiation and proliferation [24]. Furthermore, Aasim et al. [24, 16] also emphasized that 10 mg L^{-1} 6-benzylaminopurine (BAP) induced successful *in vitro* shoot regeneration from preconditioned embryonic axis and plumular apices of cowpea. BAP is an early inducer of shoots from preconditioned chickpea embryonic axis and mature embryo reported by Aasim et al. [7]. Similarly, observations were reported by Aasim [11] and Aasim et al. [21]. They emphasized that shoot regeneration improved when immature plumular apices of lentil and plumular apices explants of chickpea were preconditioned with BAP. Contrarily, Barpete et al. [8] reported that preconditioning followed by postconditioning on MS medium with 2-isopentenyl adenine (2iP) or Thidiazuron (TDZ) combined with 0.10 mg L^{-1} IBA had positive effects on shoot multiplication. They emphasized that TDZ-preconditioned explants were more regenerative compared to 2iP-preconditioned explants.

In the present study comparison of nonconditioned and BAP-preconditioned explants suggests that preconditioning enhances the shoot regeneration potential of mature embryo in terms of number of shoots per explant. These results are in agreement with Aasim et al. [24]. They reported shoot regeneration of 100% from plumular apices of cowpea and chickpea. Similarly, Belide et al. [25] and Barpete et al. [20] reported that preconditioning with cytokinin enhanced multiple shoot regeneration in *Pongamia pinnata* and *Lathyrus sativus* respectively. However, cytokinin preconditioning impacts positively on shoot regeneration and multiplication in *Sophoraton kinensis* reported by Jana et al. [26].

According to our results, when preconditioned explants were postconditioned with different concentrations of BAP-IBA in MS regeneration media, they proliferated and induced variable increases in number of shoots per explants which are in agreement with Aasim et al. [7]. They also emphasized the variable effect of cytokinin-auxin (BAP-NAA) concentrations. Furthermore, Barpete et al. [8] also observed variable effects of preconditioned explants when postconditioned with different concentrations of TDZ-IBA and 2iP-IBA.

In the present study, shoot length was decreased by preconditioned and postconditioned treatments. The longest shoot (10.58 cm) was recorded in nonconditioned (control) explants. Although, preconditioned explants also induced variable shoot lengths and decreased shoot length in each increase of BAP-IBA postconditioned treatments. It might be the possible reason that preconditioning treatments induced a reduction in active cell division of regenerating shoots resulting in shoot length reduction. These results are in agreement with Chen et al. [27], Aasim et al. [21] and Barpete et al. [8]; they observed shoots length

reduction in mung bean, chickpea and grasspea respectively. Comparing the nonconditioned explants with preconditioned explants, mean number of shoots per explant was maximum at preconditioned explants on different concentrations of BAP-IBA which is in agreement with Aasim et al. [21] and Barpete et al. [8].

The effectiveness of MS medium containing 0.10 mg L^{-1} IBA to induce maximum rooting frequency and number of roots in lentil is in agreement with Ozdemir et al. [2], who observed MS medium containing 0.19 mg L^{-1} IBA to induce maximum rooting in sixteen lentil cultivars. Similarly, Khawar et al. [10] also found that IBA is helpful in lentil root induction. The present study demonstrated that increased IBA concentrations in rooting media significantly decreased the frequency of root regeneration and number of root per explant. It may be due to cytotoxic effect of growth regulator on meristematic root cells [28]. However, MS medium supplemented with higher concentration of IBA showed an inverse effect on root length. No rooting was noted on shoots cultured in MS medium devoid of growth regulators which is in agreement with Barpete et al. [20] and Ochatt et al. [29] reports.

No negative signs were observed during the acclimatization of plants, and they established in a week during acclimatization condition. Acclimatization in peat moss substrates was superior due to high water-retaining capacity of the substrates. The present study also emphasized the importance of substrate for acclimatization which is in agreement with Kozai [30] and Barpete et al. [31]. The present study showed the effect of BAP preconditioning and postconditioning on lentil mature embryo explants for inducing a high frequency of multiple shoots, acclimatization and seed set. This protocol is a new vista in lentil biotechnology, which will be helpful for easy genetic transformation of this important legume in future.

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