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Direct *in vitro* Regeneration of Lentil (*Lens culinaris* Medik.)

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Abstract: This study surveyed a rapid, efficient and reproducible protocol for *in vitro* shoot regeneration and rooting by different explants and different concentration of BAP. Due to optimization of shoot regeneration, two media including of MS and modified MS (MS salts with double concentration of $CaCl_2$ and B5 vitamins), different explants such as decapitated embryo axes, epicotyls and cotyledonary nodes and different concentrations of BAP hormone (1, 1.5, 2, 2.5, 3 and 4 mg L^{-1}) in two genotypes (Gachsaran and Flip. 92-12 L) were used. The results showed that modified MS is a suitable medium for *in vitro* shoot regeneration of lentil. High levels of BAP caused increasing of shoot regeneration in lentil genotypes. Three milligram per liter of BAP induced the highest level of shoot regeneration. In addition, decapitated embryo explants were the best explants for highest shoot regeneration (5.8) ($p<0.05$). However, increasing of hormone concentration from 2 to 3 and 4 mg L^{-1} caused decreasing in the number of shoots, so 2 mg L^{-1} of BAP was best. For rooting, the *in vitro-in vivo* method of rooting was better than only *in vitro* method. The shoots regenerated in 2 mg L^{-1} BAP had higher rooting percentage than the shoots were regenerated in 3 and 4 mg L^{-1} BAP. These results indicate on the inhibitory effect of high concentration of BAP on root induction. But the genotype didn't have any significant effect on rooting percentage and length of roots.

Key words: Lentil, regeneration, BAP, explants, rooting

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is an important pulse crop which is grown in semiarid regions of the Mediterranean area (Africa and the Middle East), South Asia, South America and western Canada. Lentil seed is a good source of protein (20-36%, compared with 8-12% for cereals) and it's contribution to human nourishment is of vital importance in some areas especially in developing countries (Christou, 1993). It also improves soil fertility by fixing atmospheric nitrogen. *In vitro* culture of lentil (*Lens culinaris* Medik.) is difficult and many techniques have been used for overcoming this problem. Establishing of an efficient and repeatable regeneration protocol is one of the basic prerequisites for gene transformation and plant breeding. Bajaj and Dhanju (1979) have reported the first partial success. They obtained *in vitro* lentil regenerants from meristem tips. Later Williams and McHughen (1986) described a protocol for the regeneration of lentil through callus culture using hypocotyls and epicotyls as explants but the frequency of regeneration was low. Singh and Raghuvansi (1989) reported that plants could be regenerated directly from nodal segments and shoot tips as well as from callus cells without the intervention of callus. Nodal segments and shoot tips produced multiple shoots on a medium supplemented with KIN. Using seed culture, Malik and Rashid (1989) obtained multiple shoots of cotyledonary nodes on a medium with BA. Ye *et al.* (2002) used Murashige (1962) and B5 media containing different concentrations of BAP, TDZ and KIN for *in vitro* regeneration of lentil. Results showed that BAP and TDZ at optimum concentrations (0.2-0.4 and 0.1 mg L^{-1}, respectively) had similar efficiency on induction of shoots from seeds, whereas KIN produced less shoots. They reported that MS salt composition was better than B5 for shoot induction. In their experiment, increasing of calcium concentration was necessary to overcome shoot-tip necrosis. Sarker *et al.* (2003) evaluated the regeneration of different explants on MS media supplemented with different concentrations of various plant hormones. Best multiple shoot regeneration have been achieved in cotyledonary nodes on MS medium supplemented with 0.5 BAP, 0.5 KIN, 0.1 GA3 and 5.5 mg L^{-1} tyrosine. Successful regeneration of legumes has been aided by special determination of critical regeneration parameters such as explant source, genotype, media components and temperature (Parrott *et al.*, 1988). However, the rooting of *in vitro* regenerated shoots present problems in achieving

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whole plant regeneration systems and there are contradictory reports about rooting in this plant. Lentil shoot regeneration using medium containing KIN reported by Williams and McHughen (1986) and Singh and Raghuvanshi (1989), although, in the former case, the regenerated shoots transferred to sand obtaining 11% frequency of rooting, while, in the latter case extremely low frequencies of whole plant regeneration were recovered. In comparison, medium containing BAP showed higher shoot regeneration frequencies. Polanco *et al.* (1988) used MS medium supplemented with NAA for rooting but the frequency of rooting was low. Warkentin and McHughen (1993) reported 50% rooting in shoots, which regenerated from cotyledonary node explants. Polanco and Ruiz (1997) described the inhibitory effect of BAP on *in vitro* and *in vivo* root formation in lentil. They reported 40% rooting in regenerated shoots. Khawar and Ozcan (2002) reported that MS medium containing 0.25 mg L^{-1} IBA was as the best medium for root induction with frequency of 25%. Sarker *et al.* (2003) and Khawar *et al.* (2004) obtained results similar to Khawar and Ozcan (2002) using media supplemented with different concentration of IBA. Polanco and Ruiz (2001) transferred the regenerated shoots into MS medium supplemented with 11.4 μM IAA. They reported that IAA is a better hormone for rooting than NAA and IBA. In experiment of Fratini and Ruiz (2002), among the regenerated shoots from different explants on MS media containing four different cytokinins, the highest rooting percentage obtained from shoots regenerated on media containing 1.25 mM ZEA. TDZ and BA were found to inhibit root development more than KIN and ZEA. Ye *et al.* (2002) believes that NAA is a better than IBA for rooting. Their results showed low frequency of *in vitro* rooting in lentil and inhibitory effect of cytokinins on this procedure. Gulati *et al.* (2001) used micrografting method for overcoming rooting problem. In this method regenerated shoot from cotyledonary nodes on MS medium supplemented with BAP were micrografted on rootstocks. Fratini and Ruiz (2003) evaluated rooting procedure in lentil and other hypogeous legumes based on explant polarity. In this experiment, nodal segments of lentil with an axillary bud were cultured in inverted orientation (apical end in medium). They could obtain higher frequency of rooting than in case of normal orientation. Newell *et al.* (2006) refused the hypothesis of Fratini and Ruiz and reported that aeration is more important than shoot orientation in rooting of lentil. Therefore, in this study, we surveyed a rapid, efficient and reproducible protocol for *in vitro* shoot regeneration and rooting by different explants and different concentrations of BAP.

MATERIALS AND METHODS

Shoot regeneration: This study was conducted in Ferdowsi University of Mashhad, Iran in 2006. We evaluate the effect of different explants and different concentrations of BAP on shoot regeneration of two lentil genotypes such as Gachsaran and Flip. 92-12 L (commonly cultured in Iran). At first, due to determination of best medium for shoot induction, MS medium and modified MS medium with doubled concentration of $CaCl_2$ (750 mg L^{-1}) and B5 vitamins were assayed. Decapitated embryos of two genotypes cultured on these media supplemented with 2 mg L^{-1} BAP. In second experiment, due to determination of best explant and BAP concentration, different explants of Gachsaran genotype (epicotyl, cotyledonary node and decapitated embryos) cultured on modified MS in combinations with four concentrations of BAP (1, 1.5, 2 and 2.5 mg L^{-1}). After that in third experiment, we compared shoot regeneration of two genotypes. In this experiment, we used two genotypes in combination with three concentrations of BAP (2, 3 and 4 mg L^{-1}).

Plant materials: Seeds have been sterilized in 70% ethanol for 30 sec, treated with 2% hypochlorite sodium for 10 min, rinsed 3 times for 10 min with sterilized distilled water. Then, surface sterilized seeds were cultured on 0.4% (w/v) agar-water medium and kept in the dark up to germination in a growth room at 23±2°C. Epicotyls, cotyledonary nodes and decapitated embryos were excised from aseptically three days-old seedlings.

Culture medium and condition: All explants were cultured on MS and modified MS media supplemented with different concentrations of BAP, 3% sucrose, 0.75% agar, pH 5.8 for shoot regeneration in vials. The culture vessels were incubated in the growth room under 16/8 h (light/dark) photoperiod at 24±2°C. Shoots formed after 12 days and then were subcultured on free hormone medium every 12 days to reduce the inhibition effect of BAP on root formation. In third subculture, due to elongation and increasing of shoot numbers, the shoots cut out and transferred with original explants to new medium. The number of regenerated shoots evaluated after 12 days of culture (Fig. 1).

Root induction: Root induction was carried out in two media: *in vitro* and *in vitro-in vivo* rooting media. In first method, 2-4 cm long regenerated shoots were excised and transferred to MS, ½ MS and 1/4 MS+2, 3% sucrose+0.75% agar supplemented with various hormone combinations such as IAA, IBA and

Fig. 1: Shoot regeneration, rooting and acclimatization of lentil: Shoot regeneration of cotyledonary nodes (A) epicotyl, (B) decapitated embryos, (C) on modified MS+2 mg L^{-1} BAP after 12 days, (D) unusual roots formed in *in vitro* rooting medium, (E) Root formation in *in vitro-in vivo* rooting medium and (F) Acclimatization

NAA. The culture vessels were incubated in growth room under 16/8 h (light/dark) photoperiod at 25±2°C.

In second medium, 2-4 cm long regenerated shoots were excised and transferred to modified MS medium supplemented with 10 mg L^{-1} IBA for overnight. Then these shoots transferred to pots containing of perlite:cocopit:sand (1:1:1). In this experiment, the regenerated shoots of decapitated embryos of two genotypes were used on modified MS medium supplemented with 2, 3 and 4 mg L^{-1} BAP. After 3-4 weeks, the number of rooted shoots and the length of roots were recorded (Fig. 1).

Acclimatization: After rooting, plantlets were transferred to small pots containing of perlite: cocopit: sand (1:1:1) in growth room. In order to keep high humidity and better establishment, pots covered by plastic bags and irrigated with 1/2 MS (Fig. 1).

Data analysis: Six replicates with four explants in each replicate were considered. The data from all experiments were statistically analyzed using a completely randomized design. Mean values were evaluated in $p<0.05$ level of significance using Duncan's multiple-range test. Arcsine was applied to normalize data such as percentage data.

RESULTS AND DISCUSSION

First experiment

Effect of culture medium: In all treatment, frequency of shoot regeneration was 100%. Two media were similar in aspect of number and length of regenerated shoots ($p<0.05$). However, calcium concentration and B5 vitamins had a significant effect on quality of regenerated shoots. In MS medium, regenerated shoots had shoot-tip necrosis (STN). Even, subculturing of this shoots on modified MS medium didn't have any positive effect. Nevertheless, shoots in modified MS medium did not show STN symptoms. It was obvious that addition of calcium overcame STN. Researches showed that calcium deficiency, which can be easily promoted by *in vitro* conditions such as low transpiration rate and high humidity, might be the main cause of STN. For example, Parh *et al.* (1998) and Ye *et al.* (2002) reported STN in *in vitro* lentil seedlings. They resolved this problem by doubling of calcium content in MS basal medium. Also Polanco *et al.* (1988) reported that MS salts plus B5 vitamins is better than only MS for *in vitro* culture of lentil.

Second experiment

Effect of explant type and different concentrations of BAP on shoot regeneration: Respecting role of cytokinins on shoot induction and regeneration of lentil, we evaluated

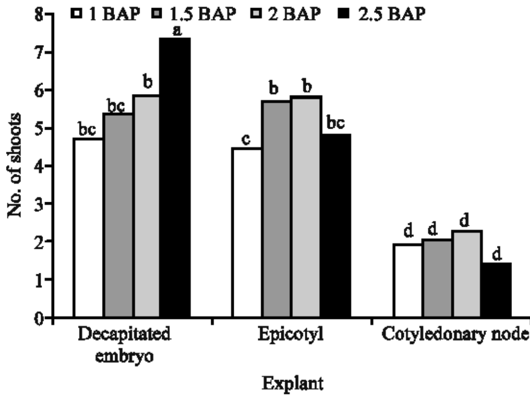

Fig. 2: Effect of explant and different concentrations of BAP (mg L^{-1}) on average number of regenerated shoots in Gachsaran

the effect of different concentrations of BAP on shoot regeneration of different explants (epicotyl, cotyledonary node and decapitated embryos) of Ghachsaran genotype. The response of explants in various concentration of BAP was different ($p<0.05$). Decapitated embryos produced highest number of shoots (5.8) from each explants among other explants. Furthermore, the number of shoots induced in different concentrations of BAP was different and highest number of shoots (4.6) were produced in medium with 2 mg L^{-1} BAP, following on in medium with 2.5, 1.5 and 1 mg L^{-1} BAP, respectively. There was not significant difference between 2.5 and 1.5 mg L^{-1} BAP in terms of the number of regenerated shoots (Fig. 2).

In addition, various explants had different responses in different concentrations of BAP. Decapitated embryos produced the highest number of shoots on modified MS medium supplemented with 2.5 mg L^{-1}. The lowest number of shoots was produced in cotyledonary node in different concentrations of BAP (Fig. 2).

Sarker *et al*. (2003) regenerated 2-6 shoots in cotyledonary node culture and decapitated embryo explants on MS medium supplemented with various concentrations of different hormones. In our experiment, cotyledonary nodes produced lowest number of shoots at 1 mg L^{-1} BAP with no difference in other concentration of BAP. Warkentin and McHughen (1993) and Gulati *et al.* (2001) also, reported multiple shoot regeneration from cotyledonary node explants on MS medium supplemented with 1 mg L^{-1} BAP. Totally, the best explant in respect to the number of shoots was decapitated embryo on MS medium supplemented with 2.5 mg L^{-1} BAP and cotyledonary node regenerated lowest number of shoots in all treatment.

Effect of explant and different concentrations of BAP on average length of regenerated shoots: The length of regenerated shoots among various explants was different. Decapitated embryos and epicotyl produced shoots with similar length (0.4 cm) after almost two weeks whereas shoot length regenerated from cotyledonary nodes was lower than two other explants ($p<0.05$).

There was significant difference among various levels of BAP. In other words, with increasing of BAP concentration, the length of regenerated shoot was decreased. Fratini and Ruiz (2002) have reported similar results. In their study, increasing the concentration of any cytokinin from 1.25 to 10 mM doubled the number of regenerated shoots. They found that the average length of regenerated shoots had adverse relation to the number of regenerated shoots. In other study, Ye *et al.* (2002) reported that with increasing the concentration of BA, higher number of shoots with lower length will regenerate.

Third experiment

Effect of different concentrations of BAP on number of shoot regenerated from two genotypes: The results of third experiment showed that highest number of shoots was obtained at concentrations of 3 and 4 mg L^{-1} and there was not significant difference between them. But, the number of shoot at 2 mg L^{-1} BAP was lower than 3 and 4 mg L^{-1}. There was significant difference between two genotypes in respect of number and height of regenerated shoots. Gechsaran genotype produced lowest number of shoots (4.3) with highest height (0.49 cm) in 2 mg L^{-1} BAP (Table 1). Ahmed *et al.* (1996) reported the different response of genotypes in various concentrations of one hormone. They observed that genotype, GA_3 and BAP levels affected the number of shoots per explant and shoot length. However, in the study of Polanco and Ruiz (2001), genotype did not have any effect on shoot regeneration.

Rooting

Effect of different shoot induction media on rooting: For rooting of regenerated shoots, the *in vitro-in vivo* method has been used. Low rooting percentage (15-20%) obtained by using different *in vitro* rooting media (MS, 1/2MS and 1/4 MS) supplemented with different concentrations of auxins (IAA, IBA and NAA) and different concentrations of sucrose. In other word, mostly, unusual roots and callus structures were formed in the bottom end of shoots. This low frequency of rooting in *in vitro* rooting medium containing auxins also was reported in previous studies. Ye *et al.* (2002) and Khawar *et al.* (2004) observed callus

Table 1: Effect of different concentrations of BAP (mg L^{-1}) on average number and length of regenerated shoots in two genotypes

	BAP					
	NO. of regenerated shoots			Length of regenerated shoots (cm)		
Different level of genotype	2	3	4	2	3	4
Flip. 92-12 L	5.4a	5.6a	5.6a	0.35b	0.26b	0.28b
Gachsaran	4.3b	5.6a	6.1a	0.49a	0.33b	0.27b

Each value is the mean of 6 replications with 4 explants in each replicate. Values within a column followed by different letter(s) are significantly different at the 0.05 probability level using. Duncan's multiple range test ($p<0.05$)

formation on the cut surface of shoots. Williams and McHughen (1986) and Singh and Raghuvanshi (1989) reported 11% of rooting in their study. Khawar and Ozcan (2002) used MS medium containing different concentration of IBA for rooting of 10-days shoots but rooting percentage was 25%. Therefore, we examine the *in vitro-in vivo* method for induction of roots on regenerated shoots from previous experiment.

There was no significant difference between Gachsaran and Flip. 92-12 L genotypes for rooting percentage. Two genotypes had high rooting percentage (60%) in second procedure for rooting. The number (2.3) and length (1.96 cm) of produced roots were similar in two genotypes.

The results showed that rooting percentage in two genotypes have been affected with different concentrations of BAP, which used in shoot induction media. The shoots regenerated in medium containing 2 mg L^{-1}BAP had higher frequency of rooting (70%) than media containing 3 or 4 mg L^{-1} BAP. There was no significant difference between media containing 3 and 4 mg L^{-1} BAP. In addition, there were no differences among different shoot regeneration media for number and length of produced roots.

In earlier studies, inhibitory effect of cytokinins on root induction of shoots has reported. For example, Polanco *et al.* (1988) that used TDZ in shooting medium, reported similar results. They obtained low frequency of rooting by culturing the shoots on MS medium supplemented with NAA. Polanco and Ruiz (1997) that studied the inhibitory effect of BAP on *in vitro* and *in vivo* root formation of lentil, concluded that success in rooting depend on the kind of cytokinin, its concentration and the time elapsed during shoot formation on these media prior transferring to rooting medium. *In vivo* study of root growth of lentil seedlings demonstrated the strong inhibitory effect of BAP on root growth reflected a drastic reduction of the mitotic index of the root meristem.

In current study, due to the time of shoot exposure to cytokinin was short (almost two weeks) and duo to decreasing concentration of this hormone, the inhibitory effect of cytokinin was low. In other hand, duo to using *in vivo* medium instead of *in vitro* medium for root induction of regenerated shoots, higher frequency of rooting have been observed than the previous studies.

This experiment support the aeration hypothesis that first reported by Newell *et al.* (2006) for rooting of microcutting of lentil. They found that medium hypoxia is a limiting factor in *in vitro* rooting process and hypoxic conditions not only limit root initiation but also, limits root elongation. Furthermore, auxin-treated base of the microcutting cannot be dismissed. In addition, a long time placing of the explants in auxins can decrease the frequency of rooting. Then, short period exposure of explants with auxins can be the better way for root induction.

CONCLUSION

Totally, results of these experiments showed that the composition salts and vitamins in medium have clear effect on *in vitro* culture of lentil. In other words, the B5 vitamins and increasing of calcium concentration is necessary to overcome STN. Decapitated embryos produced the highest number of long shoots (5.8) from each explants among other explants. Between two genotypes studied in this article, the Gachsaran produced the higher number of shoots at 2.5 mg L^{-1} so, this treatment can be proper option for shoot regeneration of lentil. Furthermore, *in vitro-in vivo* method was better than *in vitro* method for rooting of regenerated shoots. The shoots regenerated from medium containing 2 mg L^{-1} BAP, produced higher frequency of rooting (70%) than media containing 3 or 4 mg L^{-1} BAP, represents the inhibitory effect of cytokinins on root induction but the type of genotype didn't have any effect on percentage, number and length of produced roots.

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