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Indirect *in vitro* regeneration of lentil (*Lens culinaris* Medik.)

A. Bagheri[1*], V.Ghasemi Omraan[2] and S. Hatefi[3]

1. Collage of Agricultural, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, I.R. Iran,
2. Department of Biotechnology and Plant Breeding, Collage of Agriculture, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, I.R. Iran
3. Department of Agricultural Biotechnology, Paiame noor University of Tehran, P.O. Box: 1599958515 Tehran, I.R. Iran

*corresponding author: abagheri@um.ac.ir

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Abstract

Establishment of an efficient and reproducible regeneration protocol is one of the basic prerequisites for genetic transformation of any crop plant. *In vitro* culture of lentil has proven to be difficult. In spite of a number of reports on the regeneration of this plant, very few satisfying and reproducible protocol has yet been reported. This study carried out for investigation of different hormone treatments and explants in order to establish a reproducible protocol for indirect *in vitro* regeneration of the cultivar Gachsaran (commonly grown in Iran). For this purpose, the effects of 13 different hormone treatments and 4 explants on callus induction and regeneration were studied. Callus with the highest fresh and dry weight was produced on modified Murashige and Skoog (MS) medium containing 1 mg/L α-naphthaleneacetic acid (NAA)and 1 mg/L Zeatin (medium E). Among the explants, decapitated embryos attached to 1/4 of the cotyledon (DEAC) produced callus with the highest fresh and dry weights. In the regeneration stage, calli induced on media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with other hormones did not result in shooting or rooting responses. The highest shooting and rooting responses (75%) were observed for callus induction medium E, using decapitated embryos with a quarter of the cotyledon as the explant.

Keywords: Callus, Phytohormones, Regeneration, Lentil.

Abbreviations: BAP – N6-benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – indole-3-acetic acid; MS – Murashige and Skoog; NAA – α-naphthaleneacetic acid

Introduction

Lentil (*Lens culinaris* Medik.) is an important pulse crop which is grown in semi-arid regions of the world. Lentil seeds are a good source of protein (20–36%, compared with 8–12% for cereals) and their contribution to human nourishment is of vital importance in some areas, especially in developing countries (Christou, 1993). An efficient and reproducible regeneration protocol is the basic prerequisite for genetic engineering and plant breeding practices. *In vitro* culture of lentil has proven to be difficult. Many techniques have been used for overcoming this problem**.** Williams and McHughen (1986) described a protocol for the regeneration of lentil from callus cells using shoot meristem and

epicotyls as explants. However, the frequency of regeneration was less than 50%. When the isolated protoplasts of leaf mesophylic cells were examined for regeneration, single microcallus formation was found to be the sole product (Stiff *et al*., 1986).

The MS media containing different concentrations of 2,4-D have been used to induce callus in the shoot tip and cotyledonary node explants. A better response to callus induction has been observed in cotyledonary node explants (Taleb Bidokhti, 1999). It has been reported that the 2,4-D concentrations in the range of 0.5 - 1 mg/L were most effective in the growth of callus from embryo and embryo axes, and concentrations higher than 5 mg/L have been found to have inhibitory effects (Saxena and King, 1987; Taleb Bidokhti, 1999). The type of explant has been demonstrated to have a definitive role in callus induction and regeneration. For example poorest callus induction is achieved when leaf explants were used and the best response has been observed in node with no organ regeneration (Polanco *et al*., 1988). The success rate of nodal segments, shoot tips and callus have been demonstrated to be higher than other explants (Singh and Raghuvansi, 1989). Furthermore, medium containing 0.5 mg/L 2, 4-D and 1 mg/L kinetin has been shown to have great impact on callus induction with no regeneration (Ghanem *et al*., 1989; Bidokhti, 1999). Regeneration via indirect organogenesis in cotyledonary petiole tissues of lentil has proven to be successful, with a 8-40% regeneration rate, when using MS medium containing 1 mg/L Zeatin and 1 mg/L NAA (Bayrac, 2004). Recently Zaker Tavallaie et at.(2011) have reported an efficient and reproducible lentil regeneration using cotyledon bearing a small part of the embryo axis as explant.

Low rooting frequency has been proven by many researchers to make regeneration a difficult task (Williams and McHughen, 1986; Singh and Raghuvanshi, 1989; Polanco *et al*., 1988; Ye *et al*., 2002; Khawar and Ozcan, 2001; Sarker *et al*., 2003; Khawar *et al*., 2003). Methods such as micro-grafting (Gulati *et al*., 2001), inverted orientation of explants on rooting medium (Fratini and Ruiz, 2003), and the *in vitro* free-soil method (Newell *et al*., 2006) have been developed to overcome rooting problems.

Despite several reports on lentil regeneration, suitable protocols for the efficient use of indirect regeneration for genetic engineering programs have not been developed yet. The objective of this work was the establishment of a reproducible indirect *in vitro* shoot and root regeneration protocol using different explants and different concentrations of hormones in the cultivar Gachsaran of lentil.

Materials and Methods

Plant materials

Identical healthy lentil seeds of the cultivar Gachsaram were collected. These were soaked for 30 sec in 70% (v/v) ethanol and washed with sterilized distilled water, treated with 2% (v/v) sodium hypochlorite for 10 min, and rinsed 3 times for 10 min with sterilized distilled water. Surface sterilized seeds were cultured on 0.4% (w/v) water-agar medium and incubated in the dark at 23 ± 2˚C to germinate. Explants including hypocotyls, decapitated embryos, cotyledonary nodes and DEAC were isolated from 3-day-old seedlings, using a scalpel under sterile conditions.

Culture medium and growth conditions for callus induction

For the purpose of callus induction, explants were cultured on modified MS medium (MS fortified with B_5 vitamins and a two-fold concentrations of $CaCl_2$) containing various hormone concentrations, 3% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8) (Table 1). All media had sucrose as a sugar source, with the exception of medium C, where it was replaced by fructose. Culture vials were kept in the growth chamber at 25 ± 1˚C, under dark conditions for the purpose of callus induction. Generated calli were subcultured into new media was performed every 3-4 weeks. Four weeks following the culture of explants, fresh and dry weights of calli were measured and recorded. For determining the fresh and dry weights of DEAC, the cotyledons were separated from the calli before weighing.

Table 1. Growth regulator composition of culture media used in callus induction and regeneration for different explants.

Callus induction medium		Regeneration medium	
medium	**Hormone** (mg/1)	**medium**	**Hormone** (mg/1)
A_1	0.5 2,4-D	**G**	-
A_2	1 2,4-D	**H**	1 NAA + 1 zeatin
A_3	2.5 2,4-D	**I**	2 kinetin
B_1	0.5 2,4-D + 1 kinetin	**J**	1 kinetin + 1 BAP
B_2	1 2,4-D + 1 kinetin	**K**	0.1 2,4-D + 1 kinetin
B_3	1.5 2,4-D + 1 kinetin	**L**	1 NAA + 1 BAP
B_4	2 2,4-D + 1 kinetin	**M**	1 NAA
B_5	2.5 2,4-D + 1 kinetin	**N**	2 IBA
B_6	3 2,4-D + 1 kinetin	**O**	2 kinetin + 2 IBA
C	5 BAP		
D	10 kinetin+ 1GA		
E	1 NAA + 1 zeatin		
F	1 NAA + 1 BAP		

Media contain MS fortified with B5 vitamins and a two-fold concentration of CaCl2, 3% (w/v) sucrose and 0.8% (w/v) agar.

Organogenesis

Induced calli were transferred to media containing various concentrations of hormones for organogenesis (Table 1). In order to induce shooting, culture vials were kept in the growth chamber at 25 ± 1 ˚C with a 16/8 h light/dark photoperiod, under a cool white fluorescent light ($42\mu molm^{-2}s^{-1}$). The *In vitro-In vivo* method was used to induce roots on regenerated shoots. One week after the last subculture, regenerated shoots were cut into 2-4 cm segments. The segments were cultured on MS medium containing 10 mg/L NAA for 24 h. The treated shoots were transferred to *in vivo* pots consisting of perlite: coco pit: river sand (1:1:1 (v: v: v)) (Figure. 1; A-F).

Acclimatization

For the purpose of further root development and more seedling growth, rooted shoots were transferred into small pots containing *in vivo* medium. In order to adapt the plants to outdoor conditions, the pots were covered with transparent plastics to retain high humidity similar to that of *in vitro* medium.

Statistical analysis of the data was performed as a factorial experiment in a completely randomized design with growth regulator treatments (13 levels) and types of explants (4 levels) as factors in five replicates. Three culture vessels per treatment were used for all experiments. The SPSS for windows (version 16 SPSS Inc. USA) and the MSTAT-C computer programs (Michigan State University, USA) were employed for data analysis. Differences between the means were compared by Duncan's multiple range test at the $p < 0.05$ level of significance. The arcsine transformation was applied to normalize rooting data.

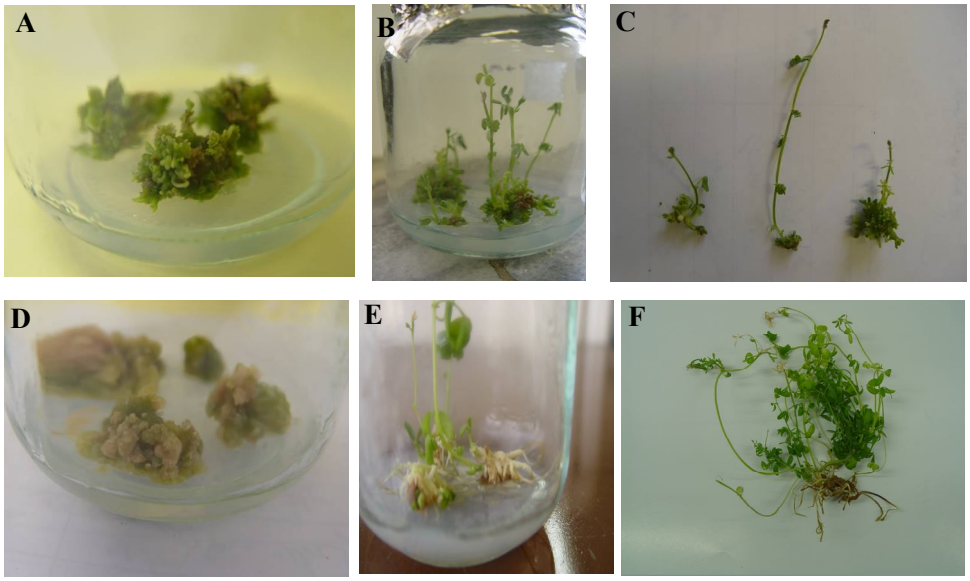

Figure 1. Indirect regeneration of lentil: (A) Calli produced in medium D (10 mg/1 kinetin) a week after transferring to regeneration medium; (B) Regeneration after 3 weeks; (C) Elongation of regenerated shoots; (D) Calli produced in medium E (1 mg/1 NAA + 1 mg/1 zeatin) a week after transferring to MS basal regeneration medium;(E) Regeneration after 3 weeks; (F) Well-developedplantlet.

Results and Discussion

Callus induction

There was no significant difference with respect to the effect of various hormonal treatments and different types of explants and their interactions on the percentage of callus induction. However, significant differences with regard to fresh and dry weights of induced calli were observed. Calli produced on the media containing 2, 4-D were watery and very soft and friable, similar to those observed by Bayrac (2004).

The effect of various hormonal combinations on fresh and dry weights of induced calli

Significant differences among the means of some hormonal treatments for the fresh weight of induced calli were observed. The highest levels of fresh weight were observed in treatments E (1 mg/L NAA + l mg/L Zeatin) and F (1 mg/L NAA + l mg/L N6-benzylaminopurine (BAP)) resulting from rhizogenesis. Finally, the lowest fresh weight was observed in treatment D (10 mg/L Kinetin) (Figure 1**).** In some treatments, there were significant differences among the dry weight means of the calli (Figure 2). Treatment E induced the highest dry weight. In media containing 2, 4-D, by increasing the concentration of this hormone, callus production was reduced. Similar to earlier reports, a larger number of callus was induced on media containing 2, 4-D accompanied by kinetin, as compared to the ones containing only 2,4-D (Saxina and King, 1987; Ghanem, 1995; Taleb Bidokhti, 1999).

The effect of different types of explants on fresh and dry weights of induced calli

Significant difference between the means of fresh and dry weights in various of explants was observed (Figure 3). Amongst explants, DEAC produced calli with the highest fresh and dry weights. Calli produced from cotyledonary nodes and hypocotyls had the lowest dry and fresh weights and the numbers of calli produced by the two explants were approximately equal.

The interaction of hormone treatment, explant type and their subsequent effects on dry and fresh weights of the induced calli

Significant difference with regard to the interaction of hormone treatment and different types of explants on callus fresh weight was observed (Table 2). The highest fresh weight was

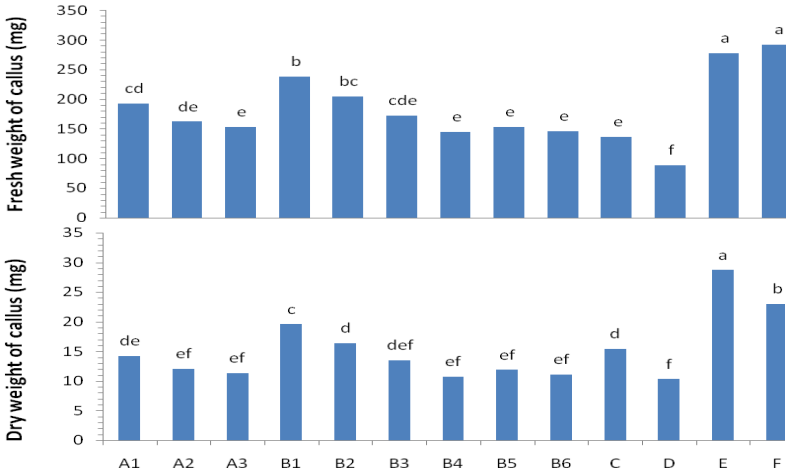

Figure 2. Effects of various hormonal compounds on fresh and dry weights of induced calli. Each bar represents the mean of 20 replications. Bars indicated by similar letters are not significantly different at P = 0.05 (Duncan's new multiple-rangetest).

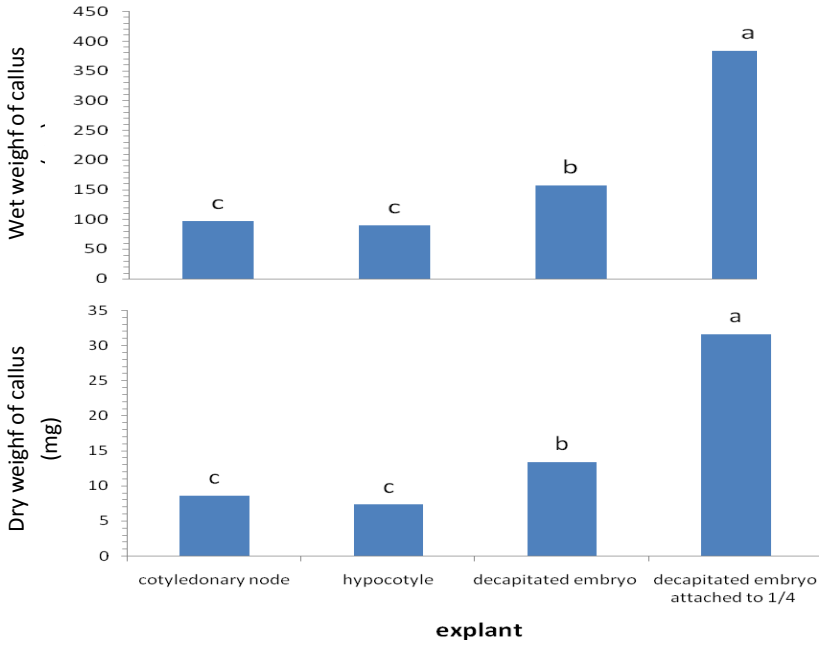

Figure 3. The effects of different explants types on fresh and dry weights of induced calli. Each bar represents the mean of 65 replications. Bars indicated by similar letters are not significantly different at P = 0.05 (Duncan's new multiple-rangetest)

Table 2. Interaction of hormone treatment and explant types and their effects on dry and fresh weights of induced calli (mg).

	Hormonal treatments / Explant	A1	A2	A3	B1	B2	B3	B4	B5	B6	C	D	E	F
Dry weight of induced calli (mg)	Cotyledon node	3.7 ^{jklm}	3.2 ^{klm}	2.7 ^{lm}	9.7 ^{fghijklm}	6.9 ^{hijklm}	6.9 ^{hijklm}	7.5 ^{hijklm}	6.3 ^{hijklm}	6.4 ^{hijklm}	15.5 ^{efg}	11.9 ^{fghi}	20 ^{cde}	10 ^{fghijklm}
	Hypocotyls	2.4 ^{m}	2.7 ^{lm}	2.5 ^{m}	7 ^{hijklm}	6.9 ^{hijklm}	7.3 ^{hijklm}	4 ^{jklm}	5.3 ^{hijklm}	4.6 ^{ijklm}	7.2 ^{hijklm}	3.3 ^{klm}	25.7 ^{bc}	16 ^{efg}
	Decapitated embryo	10.8 ^{fghijk}	12.8 ^{fgh}	11.4 ^{fghij}	15.9 ^{efg}	11.1 ^{fghij}	10.8 ^{fghijk}	7.3 ^{hijklm}	7.9 ^{hijklm}	10.3 ^{fghijk}	15.6 ^{efg}	9.2 ^{ghijklm}	24.9 ^{bc}	26.2 ^{bc}
	Decapitated embryo attached to 1/4	39.9 ^{a}	29.4 ^{b}	28.7 ^{bc}	45.8 ^{a}	40.5 ^{a}	29.9 ^{b}	25 ^{bc}	27.9 ^{bc}	22.8 ^{bcd}	23.5 ^{bcd}	17.3 ^{def}	42.2 ^{a}	39.7 ^{a}
Fresh weight of induced calli (mg)	Cotyledon node	56. 5 ^{lmno}	46.3 ^{mno}	39.4 ^{no}	124 ^{ijklmn}	87 ^{jklmno}	76 ^{jklmno}	101 ^{ijklmno}	81 ^{jklmno}	80 ^{jklmno}	128 ^{hijklm}	96 ^{jklmno}	208.5 ^{fgh}	145.5 ^{fghijk}
	Hypocotyls	37.9 ^{o}	39.7 ^{no}	31.8 ^{o}	85.2 ^{jklmno}	87 ^{jklmno}	95 ^{jklmno}	51.8 ^{lmno}	65.2 ^{klmno}	57.1 ^{lmno}	74 ^{jklmno}	28.5 ^{o}	293.4 ^{de}	215.9 ^{fg}
	Decapitated embryo	155.1 ^{fghij}	195.2 ^{fghi}	134.3 ^{ghijkl}	198.8 ^{fghi}	146 ^{fghijk}	150 ^{fghijk}	94 ^{jklmno}	103 ^{jklmno}	134 ^{ghijkl}	136 ^{ghijkl}	79 ^{jklmno}	225.3 ^{ef}	293.6 ^{de}
	Decapitated embryo attached to 1/4	520.9 ^{a}	368.7 ^{bcd}	409.6 ^{b}	544.6 ^{a}	499.5 ^{a}	371. 4 ^{bcd}	355.5 ^{bcd}	364.7 ^{bcd}	316 ^{cd}	208.7 ^{fgh}	150 ^{fghijk}	384.5 ^{bc}	516.8 ^{a}

Letters A_1-F refers to different media containing different compositions and concentrations of plant hormones used in the callus induction media. Means followed by the same case letters are not significantly different at the 5% probability level by Duncan's new multiple-range test.

obtained using DEAC and the B_1, A_1, F and B_2 media. The lowest fresh weight was gained from hypocotyl explants cultured on A_1, A_3 and D media and explant responses in culture media were different according to the type of hormone treatment.

DEAC cultured on F_1, A_1, B_2, B_1, E media yielded the highest level of dry weight. In fact, this is the first study to use DEAC for callus induction. This explant showed remarkable capacity for callus induction compared to other explants; probably due to the presence of more meristematic centers or nutrient and endogenous hormones available in the cotyledon part of this explant. At the regeneration stage, calli were transferred into nine different media. Calli produced on the media containing 2, 4-D -solely or together with other hormones- showed no response. These calli continued to grow and no shoot or root was regenerated. However, in the medium in which kinetin and 2,4-D were used for callus induction - compared to the media containing only 2,4-D-the induced calli produced more chlorophyll when transferred to the regeneration medium. Similar observations about the inability of calli to induce shoots or roots in the presence of 2,4-D have been reported by other researchers (Williams and McHughen, 1986 ; Saxena and King, 1987; Polanco *et al*., 1988; Ghanem *et al*., 1989; Singh and Raghuvanshi, 1989; Taleb Bidokhti, 1999; Bayrac, 2004). In this study, shoot regeneration only occurred in media D, E and F. Medium D showed lower callus production and shoot regeneration compared to E and F. Altaf (2007). cultured cotyledonary nodes with cotyledons from germinating seedlings in the presence of Benzyladenine (BA) (5 mg/L) and 3% fructose (w/v) (Medium C) and these explants consequently developed into callus and shoot primordia. Also, cotyledonary nodes from germinating seeds were found to produce healthy calli in the presence of kinetin (10 mg/L) and GA (1 mg/L) (Medium D) under dark conditions. In this study, these two media showed low callus production and shoot regeneration in comparison with the E and F media and medium E was chosen for hormonal treatment in order to stimulate regeneration. Callus transferred from this medium to media H, I, K, L, M, N and O did not result in a shooting response. The highest level of shoot and root regeneration was observed in media G (MS without hormone) and J (1 mg/L kinetin + l mg/L BAP). By transferring induced calli into media G and J, similar rate (76%) and number of regenerated shoots (3 shoots) were observed. Healthy shoots were formed after 10 days. However, in medium G, *in vitro* rooting (75%) occurred, while in regeneration medium J, slight rooting was observed, probably as a result of the inhibitory effect of cytokinins on the rooting process. Similarly, in the study carried out Bayrac (2004), when petiole-derived callus were transferred to regeneration medium, a 40% shooting response was observed in the MS medium. The In *vitro-in vivo* method was implemented for root induction in the shoots regenerated in medium J. By means of this method, 65% rooting was obtained. Low degree of rooting, less than 25%, in media containing agar and auxin was also reported by other researchers (Yi *et al*. (2002); Khavar *et al*. (2003); Williams and McHughen (1986); Singh and Raghuvanshi (1989); Khawar and Ozcan (2001). Omran *et al.* (2008) also reported low rooting in agar-based medium. But the results of our current investigation showed that it is possible to obtain high rates of rooting in medium containing agar by implementing the indirect regeneration method. Using DEAC as the explant in this study, high amounts of callus induction and whole-plant regeneration were obtained. This protocol can be used for further investigations including gene transformation or somaclonal variation programs, in regard to this genotype.

In conclusion, the results of our experiment show that among various hormonal treatments and different types of explant, the highest fresh and dry weights of callus and the best rate of shoot and root regeneration (76%) can be obtained by culturing DEAC in MS- callus induction medium containing 1 mg/L NAA plus 1 mg/L zeatin, with subsequent transfer of induced calli into hormone-free MS medium.

Acknowledgments

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