Barisal University Journal Part 1, 4(1):25-34 (2017) ISSN 2411-247X

IN VITRO REGENERATION OF LENTIL (*LENS CULINARIS* MEDIK.) THROUGH CALLUS CULTURE

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Abstract

Fifteen different combinations of plant hormones were tested to establish a suitable regeneration protocol *via* callus culture with four explants namely, shoot tip, hypocotyle, nodal segment and cotyledon of Barimasur-6 (BM-6) variety of lentil (*Lens culinaris* Medik.). Callus with highest fresh weight was produced on MSB_5 (Murashige and Skoog medium with B_5 vitamins) medium supplemented with 1.0 mg/l zeatin and 1.0 mg/l NAA. In the regeneration stage, highest shooting and rooting response was observed on MS medium without any hormonal supplements. It was also observed that callus induced on media supplemented with 2, 4-D alone or in combination with other hormones did not show any positive response toward organogenesis. Among the explants cotyledon explant showed best response toward callus induction as well as shoots regeneration and root formation.

Keywords: Explants, Callus, Hormones, *In vitro* regeneration, Lentil

Introduction

Lentil (*Lens culinaris* Medik.) is the most popular and important source of dietary protein and has been reported to be superior to other pulse crops (Singh et al., 1968; and Gulati et al., 2002). In Bangladesh lentil ranks first in terms of consumption and total area in which different varieties of pulse crop are cultivated. It is evident that the demand of lentil has been steadily increasing for its nutritional quality and acceptability. Thus lentil plays an important role in agricultural system of the South Asian countries with increasing annual demands for human consumption. As lentil has a significant contribution in our economy therefore more attention should be given for improving its quality and production.

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A number of attempts (Erskine et al., 1994; Taylor and Ford, 2008; Gurusamy *et al*., 2012) were made in the past to improve lentil varieties using conventional breeding techniques. However, conventional breeding technique alone could not improve the agronomic qualities of lentil varieties due to lack of resistance sources in the available lentil germplasm. As an adjunct to conventional breeding modern techniques of plant biotechnology can offer some possibilities for improvement of this crop.

One of the basic requirements for the success of crop improvement is to establish a suitable and reliable regeneration system. Plant regeneration from plant cells or callus is necessary if we want to take the benefit from the recent developments in gene transfer techniques. Efforts of regeneration from callus can also provide a possibility of picking somaclonal variations during the culturing process. Reports available regeneration through callus culture has not been studied extensively. Little information is available on *in vitro* cultures of *Lens culinaris* Medik. Williams and Mchughen (1986), Altaf et al. (1999), Bayrc (2004), and Sultana et al. (2012) described a protocol for the regeneration of lentil from callus *via* organogenesis, Saxena and King (1987) *via* embryogenesis, Warkentin and Mchughen (1992) *via* protoplast culture, Sarker et al. (2003), Sarker et al. (2012) *via* direct regeneration technique. However, all of these techniques are genotype specific.

Despite several reports on lentil regeneration, suitable protocols for the efficient use of indirect regeneration for crop improvement programs have not been developed yet. It was this background; 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 BM-6 variety of lentil.

Materials and Methods

Seeds of microsperma type of lentil (*Lens culinaris* Medik.) namely, Barimasur-6 were collected from Bangladesh Agriculture Research Institute (BARI), Joydebpur, Gazipur. Identical healthy seeds were surface sterilized by rinsing them with 70% alcohol for 1 min and then kept in 2% sodium hypochloride supplemented with one drop of Tween 20 for 10 min, which was followed by thorough washing with sterile distilled water to remove the sodium hypochlorite. Surface sterilized seeds were cultured on 0.4% (w/v) water-agar medium and incubated in the dark at 25 ± 2°C.

Shoot tip (ST), hypocotyle (HC), nodal segment (NS) and cotyledon (C) explants (Fig. 1A-F) were used. Explants were isolated from aseptically grown 2 days old seedlings using scalpel and a pair of forceps under sterile condition.

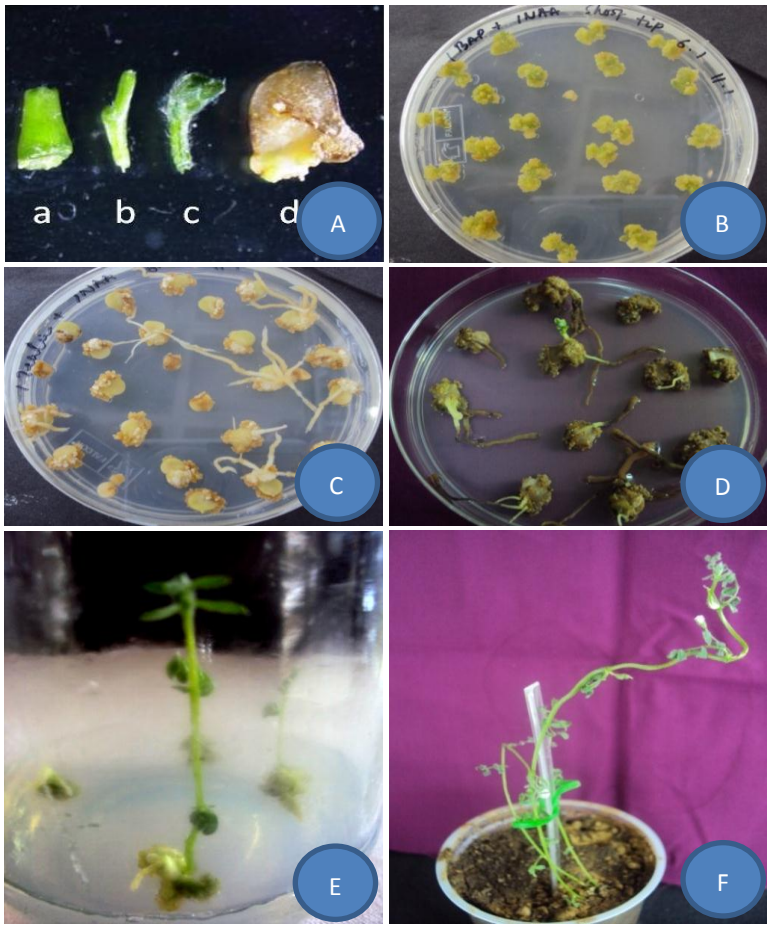

Fig. 1A. Stereomicroscopic view of different explants×9 (a) Hypocotyle (b) Nodal segment (c) Shoot tip (d) Cotyledon. **Fig. 1B.** Callus developed from shoot tip (ST) explant on MSB_5 medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA. **Fig. 1C.** Callus developed from cotyledon (C) explant on MSB_5 medium supplemented with 1.0 mg/l zeatin and 1.0 mg/l NAA. **Fig. 1D.** Initiation of shoots from developed callus on zeatin containing media from cotyledon explants after transfer to MS media without any hormonal supplement. **Fig. 1E.** Adventitious roots developed from the base of the elongated shoots. **Fig. 1F.** Fully developed plantlets growing in pot containing soil, sand and vermiculite (1: 1: 1).

For the purpose of callus induction, explants were cultured on MSB_5 (Murashige and Skoog medium with B_5 vitamins) containing various hormonal supplements, 3% (w/v) sucrose and 0.8% (w/v) agar (Table 1). Culture vials were kept in the growth chamber at 25 ± 2˚C under dark conditions for the purpose of callus induction. Generated calli were sub-cultured into same fresh media every 3 - 4 weeks interval. Fresh weights of calli were measured and recorded after four weeks of the culture. For determining the accurate weights of explants, carefully separated agar from the calli before weighting.

Calli were transferred to media containing various concentrations of hormones, namely, BAP, Kn, NAA, 2, 4-D and zeatin for organogenesis (Table 1). In order to induce shooting, culture vials were kept in the growth chamber at 25 ± 2˚C with a 16/8 hours light/dark photoperiod.

For the purpose of further root development and more seedling growth, the fully developed rooted shoots were carefully taken out of the tube and they were thoroughly washed and transferred to small pots containing soil, sand and vermiculite (1 : 1 : 1) as the potting medium. They were completely covered with transparent cover and were allowed to grow. The inner side of these bags was moistened with water to prevent desiccation. The plants were exposed to the ambient conditions gradually by making holes on cover and removing the cover two days in a week.

Results and Discussion

Attempts were made to regenerate shoots through callus development using four different explants namely, shoot tip (ST), nodal segment (NS), hypocotyle (HC) and cotyledon (C). A total of 15 different media combinations containing different growth regulators were employed. Callus induction from four different explants of BM - 6 variety of lentil was cultured on MSB_5 with and without various concentrations and combinations of BAP, Kn, NAA, zeatin and 2, 4-D. Shoot induction and acclimatization of regenerated shoots was tried using various culture media.

Table 1. Combinations and concentrations of hormonal supplements used in callus induction and shoot regeneration from different explants of BM - 6 variety of lentil.

Callus induction media		Shoot induction media	
Code	Media composition	Code	Media composition
A	MSB_5	J	MS
B	MSB_5 +1.0 mg/l NAA + 1.0 mg/l BAP	K	MS+1.0 mg/l BAP+1.0 mg/l Kn
C	MSB_5 +1.0 mg/l NAA + 1.0 mg/l Kn	L	MS +0.5 mg/l BAP + 0.5 mg/l Kn +0.1 Mg/l GA_3+5.5 mg/l tyrosin
D	MSB_5 +1.0 mg/l NAA + 1.0 mg/l zeatin	M	MS +1.0 mg/l NAA + 1.0 mg/l BAP
E	MSB_5 +0.5 mg/l 2, 4-D	N	MS +1.0 mg/l NAA + 1.0 mg/l Kn
F	MSB_5 +1 mg/l 2, 4-D	O	MS +1.0 mg/l NAA + 1.0 mg/l zeatin
G	MSB_5 +1.0 mg/l 2, 4-D + 1.0 mg/l BAP		
H	MSB_5 +1.0 mg/l 2, 4-D + 1.0 mg/l Kn		
I	MSB_5 +1.0 mg/l 2, 4-D + 1.0 mg/l zeatin		

Induction of callus

Explants were cultured on nine different combinations of hormonal supplements (Table 1). After four weeks of culture differences were observed in case of callus induction among the various combinations of hormonal supplements. The highest amount of callus was observed on MSB_5 medium containing 1.0 mg/l NAA and 1.0 mg/l zeatin. In this media, explants first developed a large number of callus and then rhizogenesis occurred with whitish and thick root from this developed callus. Bagheri et al. (2012) also obtained similar results in lentil when they used cotyledonary petiole as explant for indirect shoot regeneration. Explants inoculated on the medium containing 1.0 mg/l NAA

and 1.0 mg/l BAP produced a small amount of hard compact and smooth callus. Calli produced on media containing 2, 4-D alone and in combination with other hormonal supplements (Media E, F, G, H and I) were very soft, friable and watery, similar to those observed by Bayrac (2004). Amount of callus was very less when the explants were cultured on MSB_5 medium supplemented with NAA and Kn. The response of various explants towards callus induction on MSB_5 medium supplemented with different concentrations and combinations of 2, 4-D, zeatin, BAP, Kn and NAA has been presented in Table 2.

Table 2. Effects of various hormonal supplements and explants towards callus induction in BM - 6 variety of lentil.

Media composition	weight of callus in g (mean ± sd)			
	ST	C	Hypo	NS
MSB_5	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
MSB_5 + 1.0 mg/l NAA + 1.0 mg/l BAP	0.13 ± 0.03	0.16 ± 0.02	0.09 ± 0.01	0.06 ± 0.01
MSB_5 + 1.0 mg/l NAA + 1.0 mg/l Kn	0.06 ± 0.01	0.10 ± 0.03	0.05 ± 0.01	0.02 ± 0.01
MSB_5 + 1.0 mg/l NAA + 1.0 mg/l zeatin	0.10 ± 0.01	0.17 ± 0.02	0.07 ± 0.02	0.06 ± 0.02
MSB_5 + 0.5 mg/l 2, 4-D	0.07 ± 0.01	0.11 ± 0.02	0.06 ± 0.02	0.04 ± 0.02
MSB_5 + 1.0 mg/l 2, 4-D	0.10 ± 0.01	0.18 ± 0.02	0.07 ± 0.01	0.05 ± 0.01
MSB_5 + 1.0 mg/l 2, 4-D + 1.0 mg/l BAP	0.11 ± 0.02	0.14 ± 0.02	0.07 ± 0.03	0.05 ± 0.02
MSB_5 + 1.0 mg/l 2, 4-D + 1.0 mg/l Kn	0.10 ± 0.03	0.15 ± 0.01	0.05 ± 0.02	0.05 ± 0.01
MSB_5 + 1.0 mg/l 2, 4-D + 1.0 mg/l zeatin	0.11 ± 0.03	0.15 ± 0.02	0.07 ± 0.02	0.06±.02

It was observed that cotyledon explants produced highest amount of callus following the application of various combinations of hormonal supplements, while less amount of callus was observed in case of NS explant.

Distinguishable variation was observed in the nature of callus induced from different explants. Callus produced from ST explants was found to be semi hard, friable and pale white on all types of media combination except 2, 4-D containing media after 9-12 days of culture. Considerable number of shoot primordia was also observed from this explant in all types of media composition used in the present investigation (Fig. 1A).

Cotyledon explants responded by hard, compact and green callus with numerous number of roots after 7 -10 days of culture (Fig. 1F). Following 12 - 15 days of culture semi hard and compact callus was observed from NS explant. In most of the cases semi hard, friable, whitish and massive embryo like structures were found when hypocotyle explant was used for callus induction. It may be mentioned here that Vasil and Vasil (1992) reported about the variable nature of callus development in wheat. In this case the colour of the callus was reported to be white, off-white or pale yellow while the nature of the callus was either compact and soft or granular and translucent. Among these calli white as well as soft and compact were found to be embryogenic in nature.

Organogenesis from induced callus

Attempts were made to regenerate shoots from the callus developed from various explants. The induced calli were transferred to MS media supplements with different combinations and concentrations of hormones (as mentioned in Table 1) to observe their effects on shoot regeneration. Among the various explants tested only cotyledon explants responded to shoot regeneration on three media combinations of hormonal supplements, namely, J, K and L (Fig. 1D)**.** ST and Hypo explants formed numerous shoot primordia but these shoot primordia did not show any sign of shoot regeneration and failed to survive. However earlier Williams and McHughen (1986) described a protocol for regeneration of lentil shoots from hypocotyl and epicotyl derived callus. Altaf et al. (1999) also reported regeneration of shoots from shoot apices derived callus.

Time and composition of callus initiation media played an important role on the success of shoot induction. Calli produced on the media contain 2, 4-D alone or in combinations with other hormones showed no response on shoot induction. This calli continued to grow on these media without producing any shoot or root**.** Similar observation 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, 1995; Singh and Raghuvanshi, 1989, Taleb Bidokhti, 1999; and Bayrac, 2004). The overall responses towards shoot regeneration *via* callus derived from different explants are presented in Fig. 2.

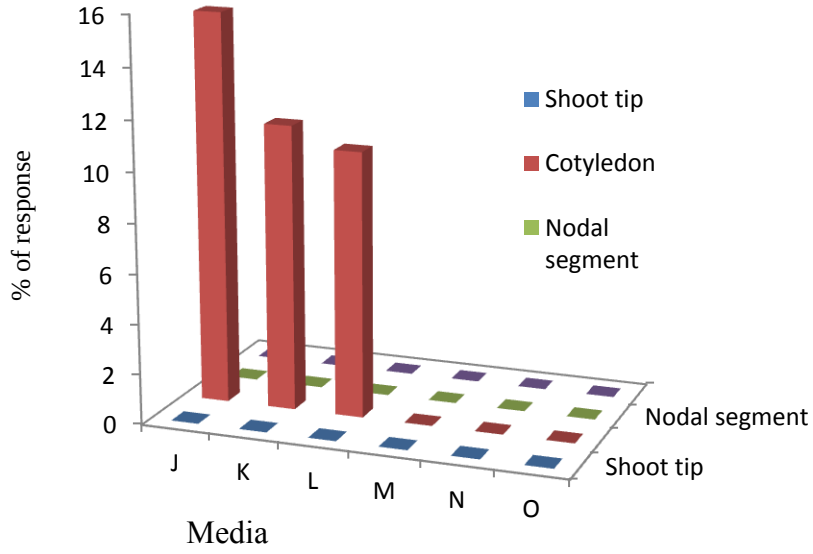

Fig. 2. Responses of induced callus from different explants towards shoot regeneration on MS media with different hormonal supplement.

Regeneration of shoots occurred only in media J, K and L. Among these medium J (MS without any growth regulator) showed better response than media K and L. The percentage of shoot regeneration was found to be 15.6 from cotyledon explant in medium J, whereas, the percentage of response was 11.4 and 10.6 in case of media K and L respectively. Explants cultured on media M, N and O did not exhibit any shoot regeneration capability. In these media newly transferred callus became dark green, increase in volume but did not show any sign of shoot development.

Rooting, transplantation and acclimatization

The ultimate success of organogenesis is depends on the ability of transferred plants in to soil on large scale with high survival rates. Most of the explants produced maximum number of hairy roots in shoot induction medium (MSB_5 media supplemented with zeatin and NAA) and subsequently these hairy roots produced healthy roots when they were transferred to MS basal medium (Fig. 1E). After 3-4 weeks rooted shoots were successfully transplanted to plastic pots containing the equal quantity of soil, sand and vermiculite. About 95% transplanted plantlets survived which bore phenotypically normal flowers and pods (Fig. 1F). Primarily high humid condition was maintained by

covering the plantlets with transparent perforated polythene bag but after 2-4 weeks gradually reduced to ambient level. Similar hardening procedure has been adopted in chickpea by Chaturvedi and Chand (2001).

In conclusion, the results of the present study show that among various hormonal treatments and different explants, the highest amount of callus can be obtained by culturing the cotyledon explants on MSB_5 medium supplimented with 1.0 mg/l NAA and 1.0 mg/l zeatin while the best rate of shoot and root regeneration was obtained by subsquent transfer of induced calli into hormon free MS medium. This protocol can be used for further investigations including genetic transformation or somaclonal variation programs.

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