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# *In vitro* Regeneration in Lentil (*Lens culinaris* Medik.)

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## Abstract

Four varieties of lentil (*Lens culinaris* Medik.), namely BM-1, BM-2, BM-3 and BM-4 of microsperma type were used in *in vitro* regeneration. Among various explants cotyledonary nodes from BM-2 and BM-4 were found to be the best for multiple shoot formation; decapitated embryos being the second best. Best multiple shoot regeneration was achieved on MS medium supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.1 mg/$GA_3$ and 5.5 mg/l tyrosine. Moderate success in root development was achieved on MS supplemented with 25 mg/l IBA.

## Introduction

Grain legumes, commonly known as pulses, are cultivated in the tropics, sub-tropics and temperate regions of the world. In developing countries, grain legumes have gained much importance in view of the acute shortage in the production of animal proteins and the wide prevalence of protein malnutrition. Grain legumes are therefore, considered as the "meat of the poor". Grain legumes are also the main source of protein for livestock feed and inland fish production. Moreover, these crops have the unique ability to fix atmospheric nitrogen symbiotically and thus improve soil fertility.

In Bangladesh, a number of pulses are grown extensively. Most important of these pulses are: lentil (*Lens culinaris* Medik.), chickpea (*Cicer arietinum* L.), blackgram (*Vigna mungo* (L.) Hepper), mungbean (*Vigna radiata* (L.) Wilczek), grasspea (*Lathyrus sativus* L.), field pea (*Pisum* spp.) and cowpea (*Vigna unguiculata* (L.) Walp.). Lentil is most popular in Bangladesh and ranks first in terms of consumption and total area in which different varieties of this crop are cultivated (Gowda and Kaul 1982). Because of its nutritional value, cooking quality and easy digestibility, the demand for this crop has been steadily increasing in the Indian subcontinent, making breeders more and

more conscious about the urgent necessity to step up its production. The foremost problem of lentil is its low yield. The factors that contribute to its low yield may be summed up as follows : (a) narrow genetic base, (b) susceptibility to several diseases and pests and (c) a year to year fluctuation in their productivity.

In addition to conventional breeding procedures, mutation breeding has been attempted in order to evolve high yielding varieties but none of the above methods were successful. The failure was attributed to lack of resistance sources in the available lentil germplasm.

One powerful tool to induce genetic variability available to breeders is to genetically transform the material of choice. One of the basic requirements for success in transformation is to establish a reliable regeneration protocol. Unfortunately, in spite of a number of reports on the regeneration of lentil, no satisfactory and reproducible protocol has been reported.

Regeneration and transformation procedures of lentil are not well developed compared to the success achieved in other grain legumes from Europe and North America. *In vitro* culture of lentil has proved difficult. In the last 20 years, techniques have progressively improved, the first partial success being reported by Bajaj and Dhanju (1979). They obtained *in vitro* lentil regenerants from meristem tips. Later Williams and McHughen (1986) described a protocol for regeneration of lentils from the hypocotyl and epicotyl-derived callus cells. Saxena and King (1987) obtained whole plants from the callus, induced from embryonic axes, while Polanco et al. (1988) reported multiple shoot formation from shoot tips, the first node, and the first pair of leaves in media supplemented with BA or BA and NAA.

Using seed culture, Malik and Rashid (1989) obtained multiple shoots from cotyledonary nodes on a BA-fortified medium. 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 Kn. Malik and Saxena (1992) reported multiple shoot formation by culturing seeds on MS medium supplemented with TDZ. Warkentin and McHughen (1993) reported multiple shoot formation from cotyledonary nodes using BA. These studies indicate that cytokinins induce multiple shoot formation in different types of explants. However, no reliable report on an efficient and stable routine transformation system which is compatible with regeneration of the complete plantlet is available (Chowrira et al. 1995, 1996). It is in this background that the present investigation was

initiated to develop an efficient reproducible and genotype neutral *in vitro* regeneration system of lentil, so that this protocol can be applied dependably to obtain stable transformants.

## Materials and Methods

Four varieties of lentil (*Lens culineris* Medik.) collected from Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh were used in the present investigation. They are : (a) Barimasur-1 (BM-1), (b) Barimasur-2 (BM-2), (c) Barimasur-3 (BM-3) and (d) Barimasur-4 (BM-4).

Different kinds of auxin and cytokinin-supplemented MS medium was used during the present investigation. In certain combinations, some other supplements were added.

Seeds were first soaked in 70% ethanol for one minute and then they were surface sterilized with a 0.1% $HgCl_2$ solution for 15 min and thereafter washed with sterilized distilled water 3 - 4 times. The surface sterilized seeds were then cultured on 0.4% (w/v) water-agar medium and kept in the dark up to their germination in a growth room at 21 ± 2_C in the darkness.

Shoot tip (ST), epicotyl (EC), cotyledonary node (CN), decapitated embryo, immature embryo, leaflet explants were used in this investigation. Shoot tip, epicotyl, cotyledonary nodes, leaflets were excised from aseptically grown three days old seedlings. For the culture of decapitated embryo, surface sterilized seeds were grown on distilled water-agar medium overnight and explants were excised on the following day. In case of embryo explants the overnight-grown sprouted seeds were split open and both root and shoot parts of the mature zygotic embryos were decapitated and subsequently cultured on different media containing various hormonal supplements for shoot regeneration. Immature embryos were collected from field grown material.

For regeneration of shoots all explants were cultured on MS medium with various hormonal supplements, namely, BAP, Kn, NAA, $GA_3$, IAA and tyrosine. The culture vessels were incubated in the growth room under 16/8 hrs light/dark cycle at 25 ± 2_C.

For root induction 2 - 4 cm long regenerated shoots were excised and transferred to MS or half the strength of MS medium supplemented with various hormonal combinations, namely IAA, IBA and NAA. In some rooting experiments liquid MS medium was also used.

## Results and Discussion

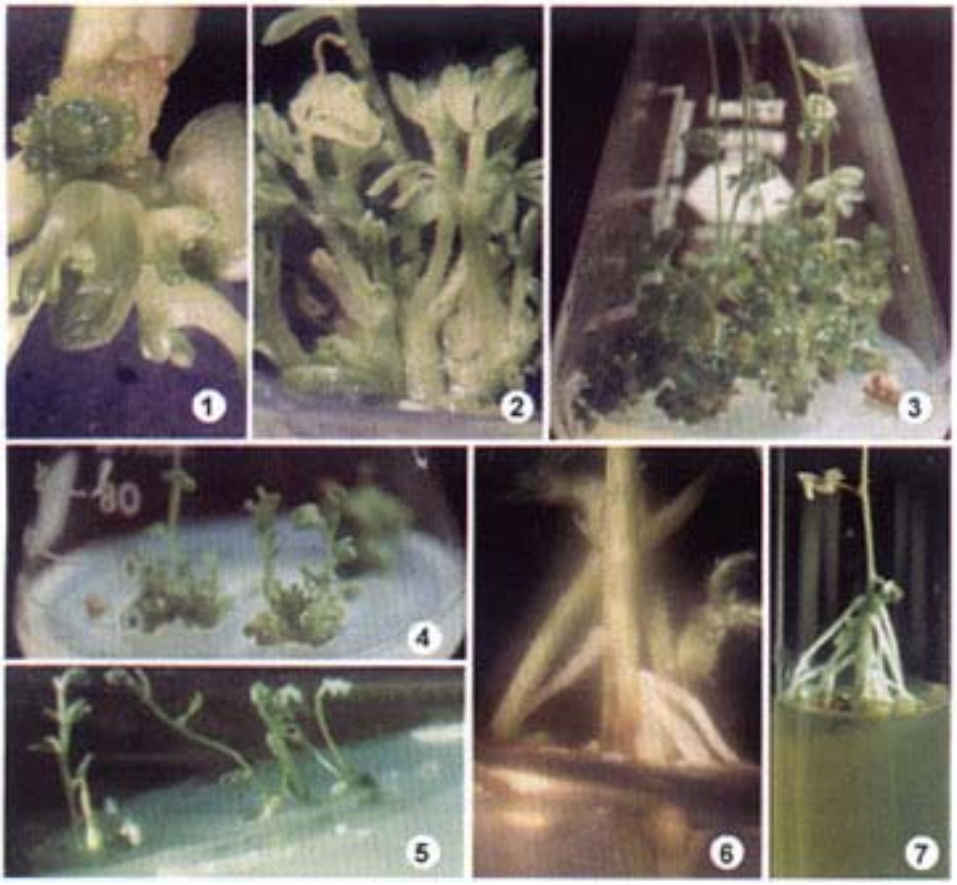
The aim of the present investigation was to develop an efficient transformation compatible regeneration system. Cotyledonary nodes, decapitated and immature embryos, leaflets, hypocotyl, shoot tips, internodal and nodal segments of all the four varieties of lentil (BM-1, BM-2, BM-3 and BM-4) were used as explants for *in vitro* shoot regeneration. Among these explants, only cotyledonary nodes, decapitated embryos, immature embryos, shoot tips and nodal segments initiated multiple shoot formation. Various concentrations and combinations of BAP, Kn, $GA_3$, IAA, NAA and tyrosine were added to MS media as supplements for the above purpose. Experiments were also conducted to induce roots at the base of the *in vitro* grown shoots.

*Shoot regeneration from cotyledonary nodes :* Cotyledonary nodes from BM-2, BM-3 and BM-4 were cultured on MS medium supplemented with different concentrations and combinations of BAP, Kn, $GA_3$, NAA and tyrosine. Shoot formation was observed in all hormonal combinations. However, the best response was obtained when the explants were cultured on MS medium containing 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l $GA_3$ and 5.5 mg/l tyrosine (Table 1). In this combination, 2 - 6 shoots were found to develop from each explant (Fig. 1) and their number was found to increase following the maintenance of these cultures in the same medium (Fig. 2). It has been possible to develop elongated shoots from this type of explants (Fig. 3). These shoots were used for root induction experiments. It was observed that at the base of the regenerated shoots several new shoot buds of different sizes were also found to develop at later stages during the culture (Fig. 2), which finally increased the number of regenerated shoots.

*Shoot regeneration from decapitated embryos:* MS medium with six different combinations and concentrations of BAP, Kn and $GA_3$ as well as tyrosine were used for multiple shoot formation without the formation of callus in four varieties of lentil. However, no remarkable variation was obtained among the varieties regarding shoot formation. The results of this experiment have been presented in Table 1. Among the different hormonal supplements used, MS medium containing 0.5 mg/l BAP, 0.5 mg/l Kn, 0.1 mg/l $GA_3$ and 5.5 mg/l tyrosine was found to be the best for multiple shoot formation in this explant. The number of initial shoots varied from 3 - 5 per explant (Fig. 4). The number of shoots increased upon subculture on the same medium.

*Regeneration of shoots from immature embryos :* The regeneration ability of this explant was also tested on MS medium with four different combinations of BAP, Kn, $GA_3$, IAA and tyrosine. It was possible to regenerate the highest

number of shoots from this explant on MS medium supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.1 mg/l $GA_3$ and 5.5 mg/l tyrosine (Table 1, Fig. 5). In this combination about 88% explants formed shoots.




Figs. **1 - 7** : **1**. Stereomicroscopic view of multiple shoot initiation from cotyledonary nodes of BM-4 on MS medium with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l $GA_3$ and 5.5 mg/l tyrosine. **2**. Developing young shoots from cotyledonary node of BM-2 in the same medium. **3**. Fully developed multiple shoots. **4**. Regeneration of multiple shoots from decapitated embryos of BM-4 on MS medium with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l $GA_3$ and 5.5 mg/l tyrosine. **5.** Shoot formation from immature embryos of BM-1 on MS medium with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l $GA_3$ and 5.5 mg/l tyrosine. **6.** Stereomicroscopic view of root development in half strength of MS medium with 25 mg/l IBA. **7**. Root induction from regenerated shoots in the same medium (note callus formation inhibiting further development of roots).

*Shoot regeneration from nodal segments :* Nodal segments from *in vitro* grown seedlings were used for regeneration of shoots. For this purpose five different combinations of hormonal supplements were used. MS medium supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.1 mg/l $GA_3$, 0.01 NAA and

5.5 mg/l tyrosine showed the best response (Table 1). About 78 % of the cultured explants showed shoot regeneration. The initial shoot buds increased in number upon subculture on the same medium.

*Regeneration of shoots from shoot tips :* Five different combinations of BAP, Kn, $GA_3$ in MS medium were used for the regeneration of shoots from these explants. Among these combinations, 1.0 mg/l BAP, 0.5 mg/l Kn and 0.1 mg/l $GA_3$ showed the best response (Table 1). In this combination about 56% of the cultured explants formed shoots. The number of initial shoots per explant was two to three.

*Effects of tyrosine on the induction of shoots :* It has been proved in case of jute (*Corchorus* spp.) and in wheat (*Triticum aestivum* L.) tissue culture experiments that tyrosine has a positive role towards producing multiple shoots. In the present investigation we also used tyrosine to see its effect on shoot regeneration from cotyledonary nodal explants of different varieties of lentil. Six different concentrations of tyrosine ranging from 1.0 - 25 mg/l were used on MS medium along with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l $GA_3$. Tyrosine at a concentration of 5.5 mg/l was more effective than the other concentrations. The number of shoots were higher than those that developed in other concentrations; and the shoots were healthy and elongated.

*Root induction :* Root induction at the base of the *in vitro* regenerated shoots is still a serious problem. A number of experiments were conducted in the present investigation to induce roots. MS as well as half strength MS medium containing different concentrations and combinations of IBA and IAA were used for this purpose. It was observed that there was no sign of root induction when lower concentrations of auxins were used either in MS or half MS medium in any of the varieties of lentil.

Root induction was observed when shoots were cultured on MS medium supplemented with 25 mg/l IBA (Figs. 6, 7). About 30% of the inoculated shoots were found to produce roots. The number of roots per shoot varied from three to eight. However, roots did not develop from the base of the regenerated shoots but at a level slightly higher than the cut ends (Fig. 6). Furthrmore, it was observed that once inside the auxin-rich medium, the tip of the roots callused (Fig. 7), blocking further growth of the roots (Fig. 7). This abnormal feature of plantlets reduced the survival rate, following their transfer to soil. To avoid callus formation at the tip of developing roots, shoots with the developing roots were transferred to auxin-free medium. It was also possible to induce roots in liquid MS containing 25 mg/l IBA. In the liquid medium the development

**Table 1. Regeneration of multiple shoots from different explants of lentil on MS medium.**

| Explant | MS medium with supplements (mg/l) | No. of explants inoculated | No. of responsive explants | No. of shoots/ explant | % of responsive explants |
|---|---|---|---|---|---|
| Decapitated embrvo | 1.0 BAP + 0.1 $GA_3$ | 240 | 129 | 1 - 2 | 53.75 |
| | 1.0 Kn + 0.1$GA_3$ | 260 | 168 | 1 - 2 | 64.61 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ | 300 | 179 | 1 - 2 | 59.67 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ + 5.5 tyrosine | 450 | 354 | 4 - 5 | 78.67 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ + 0.01 NAA + 5.5 tyrosine | 250 | 184 | 3 - 5 | 73.60 |
| | 0.5 BAP + 0.5 Kn + 0.1 NAA + 0.1 $GA_3$ | 225 | 165 | 2 - 3 | 73.33 |
| Cotyledonary node | 1.0 Kn + 0.1 $GA_3$ | 200 | 124 | 1 - 2 | 62.00 |
| | 1.0 BAP + 0.1 $GA_3$ | 240 | 148 | 1 - 2 | 61.62 |
| | 0.5 BAP + 0.5 Kn | 220 | 152 | | 69.09 |
| | 1.0 BAP + 0.5 Kn | 120 | 79 | 1 - 2 | 65.83 |
| | 1.0 BAP + 1.0 Kn + 0.1 $GA_3$ | 150 | 67 | 2 - 3 | 44.60 |
| | 1.0 BAP + 1.0 Kn + 0.2 $GA_3$ | 130 | 75 | 2 - 3 | 57.69 |
| | 0.5 BAP + 0.5 Kn + 0.25 $GA_3$ | 180 | 112 | 1 - 2 | 62.22 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ | 150 | 108 | 2 - 3 | 72.00 |
| | 0.5 BAP + 0.5 Kn + 0.01 NAA + 0.1 $GA_3$ | 125 | 88 | 1 - 2 | 70.40 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ + 5.5 tyrosine | 350 | 284 | 4-6 | 81.14 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ + 0.01 NAA + 5.5 tyrosine | 210 | 165 | 4-5 | 78.57 |
| Nodal segment | 0.5 BAP + 0.5 Kn + 0.1 NAA + 0.1 $GA_3$ | 60 | 38 | 2 - 3 | 63.33 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ | 45 | 31 | 1 - 2 | 68.88 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ + 5.5 tyrosine | 90 | 69 | 3 - 4 | 76.66 |
| | 1.0 BAP + 0.1 $GA_3$ | 60 | 44 | 1 - 2 | 73.33 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ + 0.01 NAA + 5.5 tyrosine | 96 | 75 | 4 - 5 | 78.12 |
| Shoot tip | 0.5 BAP + 0.5 Kn + 0.25 $GA_3$ | 85 | 42 | 49.41 | 2 |
| | 1.0 BAP + 0.5 Kn | 148 | 82 | 55.40 | 2-3 |
| | 1.0 BAP + 0.5 Kn + 0.1 $GA_3$ | 164 | 92 | 56.09 | 1-2 |
| | 2.0 BAP + 0.5 Kn + 0.3 $GA_3$ | 56 | 31 | 55.36 | 1-2 |
| | 0.5 BAP + 1.0 $GA_3$ | 60 | 32 | 53.33 | 2-3 |
| Immature embryo | 0.5 BAP + 0.5 Kn | 65 | 46 | 1 | 70.76 |
| | 0.5 BAP + 0.5 Kn + 0.1 $GA_3$ + 5.5 tyrosine | 330 | 292 | 1-2 | 88.48 |
| | 1.0 BAP + 0.5 Kn | 130 | 90 | 1-2 | 69.23 |
| | 0.5 BAP + 1.0 IAA | 160 | 120 | 1-2 | 75.00 |

of roots was better and the roots showed no signs of callusing at their tips. Experiments on grafting of differentiated shoots on to the control plant are under way to determine whether this method increases the survival rate of regenerants. Preliminary results indicate that micrografting is more promising for survival than the use of medium containing hormonal supplements.

Several attempts have been made in the past regarding the development of a suitable protocol for *in vitro* shoot regeneration of lentil cultivars growing in Bangladesh (Khanam 1994, Khanam et al. 1995), but, very limited success was achieved. In the present investigation, various explants, namely, decapitated embryos, immature embryos, epicotyl, leaflets and cotyledonary nodes were used for the direct regeneration of plants. It was shown that MS medium containing various hormonal supplements was most effective in generating multiple shoots in the cotyledonary node of the aforesaid explants. They formed healthy shoots with well-developed leaves on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l $GA_3$ and 5.5 mg/l tyrosine. Contrary to these findings, Khanam (1994) and Khanam et al. (1995) obtained best response in multiple shoot regeneration on MS medium containing 0.5 mg/l BAP + 0.5 mg/l Kn + 0.2 mg/l NAA + 100 mg/l CH. Moreover, Polanco et al. (1988) reported the formation of multiple shoots on MS medium with BAP (2.0 mg/l) and NAA (0.2 mg/l) in lentil.

No remarkable variation was observed among the different varieties of lentil in case of multiple shoot regeneration. Using different lentil varieties, Khanam (1994) also found similar response in multiple shoot regeneration. In the present investigation development of roots has been tried using a number of methods. Previous reports indicate that root induction in lentil was achieved on a medium containing NAA or IAA (Polanco et al. 1988) and roots were also induced on hormone free, half strength B5 medium (Warkentin and McHughen 1993). Following the above mentioned reports, several media combinations containing various concentrations of IBA, NAA and IAA were tried in order to induce roots at the base of the regenerated shoots. But the media composition used for root induction by the above workers did not show positive response. Khanam et al. (1994) observed root induction on regenerated shoots after subjecting them to an extremely high concentration of IBA shock (1000 mg/l) for five min. Thereafter, the treated shoots were transferred to 10.0 mg/l IBA containing half MS medium. However, in this investigation the above treatment did not work.

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