IN VITRO PLANT REGENERATION OF LENTIL (*Lens culinaris* Medik.) FROM DIFFERENT EXPLANTS

A

THESIS

BY

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STUDENT NO. : 0905036
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MASTER OF SCIENCE
IN
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DEPARTMENT OF GENETICS AND PLANT BREEDING
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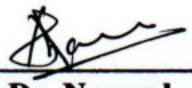

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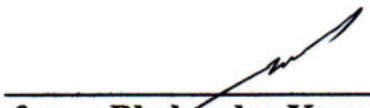

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AUGUST 2010

DEDICATED
TO MY
BELOVED PARENTS

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ABSTRACT

The investigation was carried out in the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur during November, 2009 to April, 2010 with a view to study *in vitro* regeneration of Lentil (*Lens culinaris* Medik) from leaf discs and nodal segments explants. For this purpose, three varieties of Lentil *viz.* BARI Masur -3, BARI Masur -4 and BARI Masur -5 were used to assess their regeneration ability. Leaf disc and nodal segments of the three genotypes of Lentil were cultured on MS medium with different concentrations and combinations of growth regulators. Among the three varieties, BARI Masur -4 showed early callusing (17.67 days) with maximum rate of callus induction (83.33%) incase of leaf disc culture and also showed highest percentage of callus induction (80.55%) with minimum days (17.33 days) with nodal segment as explant. Early and maximum rate of callusing appeared in T_2 (MS +1.5 mg/L 2, 4-D + 0.5 mg/L BAP) and T_3 (MS +2 mg/L 2, 4-D + 0.5 mg/L NAA) from leaf disc and nodal segment respectively. BARI Masur -4 had the highest percentage of shoot regeneration from leaf disc (69.44%) and nodal segment (66.66%) explant. Early and maximum rate of regeneration was found in T_2 (MS+2.0 mg/L Kn + 0.2 mg/L NAA) and T_3 (MS medium +0.5 mg/L BAP+ 0.25 mg/L Kn). The highest number of roots per shoot was counted in BARI Masur -4 (47.23%), in T_3 (MS medium containing 10mg/L IAA) and T_2 (MS medium containing 15mg/L IBA) considering leaf disc and nodal segment as explant. Considering the overall performance, genotype BARI Masur -4 appeared the best for callus formation, shoot regeneration and root formation.

ABBREVIATIONS AND ACRONYMES (Contd.)

Kg	:	Kilogram
Kn	:	Kinetin (6-furfuryl amino purine)
mg/L	:	Milligram per Liter
mm	:	Millimeter
MS	:	Murashige and Skoog
MSE	:	Mean square error
NAA	:	α-Napthelene acetic acid
NaOH	:	Sodium Hydroxide
no.	:	Number
P^H	:	Negative logarithm of hydrogen ion (H^+) concentration
sp.	:	Species
TDZ	:	Thidiazuron
Univ.	:	University
UV	:	Ultra violet
Var.	:	Variety
Viz.	:	Namely

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INTRODUCTION

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INTRODUCTION

Lentil (*Lens culinaris* Medik.) popularly known as masur is an important grain legume of Bangladesh. This commonly grown pulse crop belongs to the sub – family Faboideae (Papilionaceae) under the family Fabaceae (Leguminosae). The generic name of lentil 'Lens' is derived from a Latin word which means disc-shaped seed, and the species name 'culinaris' is derived also from a Latin word meaning a kitchen, signifying its edible seed. The genus Lens comprises five annual species of which only (Lens culinaris Medik) is cultivated (Sindhue and Shinkard, 1983).

According to Ladizinsky (1979) lentil has been originated in Southern Turkey. Cubero (1984) in a detailed review concluded that the region between western Turkey and Kurdis could be its place of origin. It is the oldest and is one of the valuable pulse crops of the world. It is grown in India, Bangladesh, Pakistan, Egypt, Greece, Italy, countries in the Mediterranean basin, Switzerland, U.S.A.

Lentil (*Lens culinaris* Medik) is the main supporting food supplement to rice in Bangladesh. But average lentil yield is poor and it cannot compete with cereals crops. Bangladesh is an agro-based country in the South-East Asia and lentil is one of the major pulses grown in Bangladesh. According to FAO (2009) the area harvested of pulses is 312 thousand ha and production is 258 thousand tons in Bangladesh. The cultivated area of lentil is 179354 acre and yield per acre is 399 kg and production is 71535 M. tons (BBS, 2008).

In developing countries like Bangladesh, pulse can improve the over all nutritional value of cereal based diet. Lentil is considered as the poor man's meat as it is the cheapest source of protein for under privilege people who can not afford to buy animal protein (Gowda and Kaul, 1992). Pulses have there or four times more protein content than rice and ten to fifteen times more than potatoes (Mian, 1976).

In Bangladesh protein nutrition is alarmingly poor, particularly for children and the pregnant and lactating mothers (Mian, 1976; Kaul and Das, 1986). As a result protein deficiency diseases like 'Kwashiorkor' and 'Marasmus' has been reported in Bangladesh. The protein content of Lentil seeds is found to vary from 21.75% to 32.48% (Dimitrove 1973) while the protein contents of rice and wheat are 7.5% and 11.9% respectively. Not only the seeds but also bushy stem and leaf portion of the plant contain considerable amount of protein, which may be used as animal feed. Lentil may become the major source of income for farmers. As lentil has a significant contribution in our economy that is why, more and more attention should be given for improving its quality and higher production.

The factors that contribute to low yield of lentil 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 resistant sources in the available lentil germplasm.

Conventional breeding method alone is insufficient to satisfy the goal as it demands more land, labour and capital. It also takes a long time and infection of several diseases occur. Therefore, it is now evident that to overcome the constraints of lentil production the improvement of this crop is an essential task. The conventional breeding methods are most widely used for crop improvement. But in practical situations, these methods have to be supplemented with plant tissue culture techniques, either to increase their efficiency or to achieve the objectives, which in not possible through the conventional methods

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 establishment 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.

Plant tissue culture techniques provide unique opportunity for overcoming barriers of inter specific cross, asexually gene introgression, period of dormancy etc. and have facilitate rapid development of new verities. Tissue culture techniques have advantages over traditional propagation methods. Cultures are conducted in asceptic environment with the assurance of the production of disease free materials and without risk of re-infection. High frequency regeneration of plants from in vitro cultured tissue is a pre-requisite for successful application of tissue culture technique for crop improvement. The application of biotechnology in combination with the traditional breeding methods will cause the gigantic task of increasing food production.

Tissue culture techniques may also be utilized conveniently to overcome incompatibility barrier through fusion of protoplasts from vegetative cells of interspecific, intergenceric and interfamiliar group (Rao and Chadha, 1986; Rao, 1985).The regeneration of plants from tissue culture is an important and essential component of biotechnological research and sometimes it is required for the genetic manipulation of plant. The techniques of plants tissue culture have been developed as a new and powerful tool for crop improvement (Carlson, 1975; Razdan and Cocking, 1981). So, there is no doubt that in vitro regeneration in lentil has the great potentiality for improving lentil.

Considering the above facts the present investigation was undertaken with the following objectives:

i) To develop efficient protocol for successful regeneration of lentil;
ii) To identify the explants suitable for regeneration and
iii) To study the callus induction and regeneration potentiality of selected varieties of lentil in different growth regulators.

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Lentil (*Lens culinaris* Medik.) is one of the most important pulse crop grown in Bangladesh. Among the legume crops, Lentil is an important food crop in many countries of the world. Researchers have paid much attention on various aspects of improvement, utilization and production of this crop. The improvement of Lentil is possible by both traditional and modern breeding methods. But the traditional method requires long time, high cost and huge labours while the plant biotechnology, now a days, offers many wonderful opportunities for breeders with new chances to solve critical breeding problems at molecular level. There are considerable number of literature regarding callus induction and plant regeneration of Lentil in many countries. However, study with Lentil is very limited in Bangladesh. Related works already conducted by different institutes of the world and have been reviewed and some of the most relevant literatures are cited here in below.

Conventional techniques of crop improvement are lengthy process. The technique of plant tissue culture have been developed as a new and powerful tool for crop improvement (Carlson, 1975; Razdan and Cocking, 1981) and received wide attention of scientific world (D'Amato, 1978; Skirvin, 1979; Larkin and Scowcroft, 1982).

Murashige and Skoog (1962) reported that nutritional requirement for optimal growth of *in vitro* tissue may vary with varieties. The basis of plant tissue culture is the regeneration of different crop plant from different explants (leaf, stem, cotyledon, hypocotyls, root tip, shoot tip etc.) on different nutrient media under sterile conditions. When explants of a plant are grown in a defined medium, an undifferentiated collection of cell arise which is known as callus. The medium can be subsequently manipulated to obtain root, shoot and embryo which then developed into whole plants from this differentiated callus, this process is known as regeneration.

So far it is known that plant regeneration via callus culture is very difficult in grain legumes. In recent years substantial progress has been made in this field (Mascarenhas *et al,* 1975 and Lu *et al.,* 1983). Regeneration has been obtained in a member of grain legumes (Atreya *et al.,* 1984 and Christiansen *et al.,* 1983).

Bajaj and Singh (1980) reported induction of androgenesis in three Indian cultivars of mungbean. Bajaj (1983) also reported the plantlet regeneration from pollen embryos of *Arachis, Brassica* and *Triticum* species. Plantlet regeneration from embryos of *Cajanus sp., Phaseolus aureus* was also reported by Gosal and Bajaj (1979).

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 regenerate from meristem tips. Later Williams and McHughen (1986) described a protocol for regeneration of lentils from the hypocotyl and epicotyl derived callus cells.

A callus is an amorphous mass of loosely arranged thin walled paranchymatous cells arising from the proliferating cells of parent tissue (Dodds and Robetrs, 1990).

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.

In a study Williams and McHughem (1986) conducted with a view to applying cellular and molecular genetic techniques to crop improvement. Shoot, meristem and epicotyl explants of cv. eston were cultured on Murashige and

Skoog (MS) medium containing kinetin and gibberellic acid. The callus produced was able to regenerate shoots in relatively large numbers even after several subcultures. The shoots were rooted in a mist chamber to end whole fertile plants.

Saxena and King (1987) reported that *in vitro* methods to crop improvement, callus was initiated from embryonal axes of cv. Laird cultured on MS medium or on a modified B5 (=B5A) medium supplemented with 2, 4-D. The callus was white friable organised and slow growing. On transfer to B5 a medium without hormones or with benzyladenine and IAA, certain peripheral areas of the callus turned green.Well-organised embryos having cotyledones, shoots and roots which were able to develop into whole plants.

Geetha and Venkatachalam (1997) observed that callus induction from different black gram explants were tested on MS basal medium supplemented with different growth regulators individually and in combination. Explants were produced from hypocotyls, epicotyls, axillary buds, cotyledonary nodes and immature leaves.

Optimum levels for callus induction were 22.8 µM IAA and 16.1 µM NAA and in combination with 2.2 µ M of hypocotyls were most efficient in producing callus.

Mathur and Prakash (1997) reported that, MS medium supplemented with 0.5 mg kinetin and 2.0 mg 2, 4-D/L gave the maximum callus induction.

Callus induction from different black gram explants was studied on MS basal medium supplemented with B-5 vitamins, IAA, NAA, IBA, kinetin and benzyladenine (BA) individually and in combination. For callus induction 22.8 µM IAA or 16.1 µM NAA and in combination with 2.2 µM of BA were optimum levels growth regulators (Geetha and Venkatachalam, 1997).

Malik and Rashid (1989) reported that multiple shoots from cotyledonary nodes of lentil and pea seedling proved possible on medium with cytokinin (10-5 M

Benzylaminopurine [Benzyladenine]). But other parts of the seedlings (root, leaf, hypocotyl) failed to regenerate.

Using seed culture, Malik and Rashid (1989) obtained multiple shoots from cotyledonary nodes on a BA-fortified 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 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.

Singh and Raghuvansi (1989) described a tissue culture procedure for plantlet regeneration directly from nodal segment and shoot tip explants as well as from callus. Nodal segments and shoot tip explants produce a single shoot and roots in 4 weeks on hormone free MS medium only shoot regenerated on a media containing kinetin and multiple shoots formed without intervention by callus or root formation. Best callus formation was on media containing 1.0 mg/1 kinetin and 10.0 mg/l 2,4-D.

Ghanem *et al.* (1990) studied the effect of some growth regulators, 2, 4- D, IAA, IBA, NAA and kinetin on the growth of Lentil (*Lens culinaris* Medik), cultured *in vitro*. They observed the growth of (*Lens culinaris* Medik) from callus derived from different explants. Plant tissue regenerate *in vitro* through two path ways, namely "organogenesis", where shoot buds are organized by concentrated meristematic activity of a number of cells and "embryogenesis", where usually single cell or a small cluster of cells undergo differentiation to produce somatic embryos similar to zygotic embryos. Shoot regeneration is of crucial importance in the realization of the potentiality of cell and tissue culture techniques via plant improvement (Pauk and Purnhauser, 1993).

Khanam *et al.* (1995) observed that multiple shoot regeneration directly from explants and via callus in lentil *(Lens culinaris* Medik) in MS medium containing 0.5 mg/l BAP + 0.5 mg/l Kn + 0.2 mg/l NAA + 100 mg/l CH and MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn, respectively. Root induction in the *in vitro* regenerated shoots was possible only when the base of the shoots were treated with a high concentration of IBA (1.0 mg/1) for a short period and finally transferred to MS medium supplemented with 10.0 mg/l IBA.

Shoot multiplication was induced in cowpea cv. Georgia -21, from shoot tip was reported by Brar *et al.* (1997).They established an experiment in cowpea for shoot multiplication from shoot tip explant. Shoot tips were cultured on MS medium containing BA at 1, 2.5 or 5 mgl^{-1} (4.6, 11.6 or 23.2 μM) combined with 2,4-D at 0.01, 0.1 or 0.5 mg l^{-1} (0.05, 0.5 or 2.3 μM) or NAA at 0.01, 0.1 or 0.5 mgl^{-1} (0.05, 0.5 or 2.7 μM). Treatment with BA induced greater shoot proliferation than those of with kinetin. The highest number of shoots were produced on 5 mg (22.2 μM) BA/L in combination with NAA or 2, 4-D at 0.01 mgl^{-1} (0. 05 μ M).

Nafess *et al.* (1999) showed callus formation and organogenesis of subcultured callus of shoot spices, cotyledonary nodes and stem section of lentils cv. Masoor-85 on media containing various combinations and concentrations of K [kinetin], BA [benzynenzyladenine], IAA, NAA and GA [gibberellic acid].

Teli and Maheshwari (2001) studied that shoot bud formation in *Vigna radiata* occurred when leaf derived callus cultured on MS medium supplemented with kinetin and NAA *in vitro* regeneration of *V. radiata* and *V. mungo* plantlets from shoot tips (apical meristem) cultures was observed within 4 weeks on MS medium supplemented with cytokinin.

Ye *et al.* (2000) reported a system was developed for improving an earlier protocol for the noncallogenic production of shoots from axillary buds on single-node explant. A medium containing MS salts, 3% sucrose and either 1.0 mg/l gibberellic acid or 0.17-0.18 mg/1 indole-3-butyric acid optimise axillary shoot

development and node production for further culture. Root formation was optimal in a medium with 2/3 the standard concentrations of MS salts, 2.0 mg/l alpha-naphthaleneacetic acid and 5% sucrose.

Sarker *et al.* (2003) conducted an experiment with 4 varieties of lentil (*Lens culinaris* Medik.), namely BM-1, BM-2, BM-3 and BM-4 of microsperma type for *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/l GA_3 and 5.5 mg/l tyrosine. Moderate success in root development was achieved on MS supplemented with 25 mg/l IBA.

Shoots were induced from callus cultures of hypocotyls, epicotyls, axillary buds, cotyledonary nodes and immature leaves with varying frequencies in the medium containing kinetin or BA and in combination with IAA or NAA. Multiple shoots were obtained from cotyledonary node segments (Geetha *et al., 1997).*

David J. *et al.* (2004) reported that culturing portions of lentil (*Lens culinaris*) shoot meristems and epicotyls on a medium containing kinetin and gibberellic acid, callus tissue was produced which could be induced to regenerate shoots in relatively large numbers, even after several subcultures. The shoots could be rooted in a mist chamber to yield whole, fertile plants.

Gulati *et al.* (2001) found a protocol for regeneration of shoots of lentil *(Lens culinaris)* was developed multiple shoots (4-5) were regenerated from cotyledonary node explants on MS medium containing 8.8 µM 6-Benzylaminopurine. The protocol was successful with several cultivars of lentil. Polanco and Ruiz (2001) found an efficient and simple method for plant regeneration from immature lentil *(Lens culinaris)* seed. Immature seeds from 1-6 mm of 4 lentil cultivars (Verdina, Pardina, Alpo and Luoa) were cultured *in vitro* on different media include different concentration of N6-Benzylaminopurine

(BAP), alone or in combination with other phytohormones. The highest rooting percentage (88.9%) was obtained from regenerated shoots of Verdina on a medium with 1μM alpha Naphthalene acetic acid.

Amutha *et al.* (2003) observed that regenerated shoots of *V. radiuta* developed prominent roots when transferred to half strength MS medium supplemented with 4.90 μM IBA.

The major constraints to lentil improvement were lack of genetic variability in traits of importance in local germplasm (Sarker *et al,* 1991).

The shoot bud could be rooted on MS medium supplemented with 0.1 mg/ ml indol butyric acid was reported by Tivarekar and Eapen (2001).

Savita *et al.* (2001) found that *in vitro* produced shoots rooted in 0.5 mgl^{-1} IAA resulted in the formation of complete plantlets with an average height of 15 cm in 20 days.

Ahmad, M. *et al.*(1997) reported that an initial step in establishing interspecific hybridization to broaden the genetic basis of lentils (Lens culinaris subsp. culinaris), a set of experiments was carried out to produce an efficient in vitro protocol for propagation of lentil and two of its wild relatives (L. ervoides and L. culinaris subsp. orientalis). The objective of the experiments was to optimize the media (MS) to regenerate shoots in vitro from nodal segments without a callogenic phase. The number of shoots per explant, number of nodes per shoot and shoot length showed that species differences, and gibberellic acid (GA3) and benzyladenine (BA) levels had the largest effects, with only minor interaction effects. The experiments identified a standard protocol based on the optimum levels of growth regulators, MS salts and sucrose concentrations for maximum plant regeneration from the nodal segment of these species. The medium recommended for optimal shoot regeneration without a callogenic stage was MS medium containing 2.89 micro M GA3 and 1.11 micro M BA, and lacking sucrose. Root induction from these shoots was best on MS medium supplemented

with 5.37 micro M NAA. Final successful establishment of regenerated plants was completed by the transfer to a third medium containing half-strength MS salts. The in vitro clonal protocol devised was used for multiplication of Lens interspecific hybrids.

Fratini and Ruiz (2002) studied the first comperative analysis of the effect of the 4 different cytokinins, apply to different lentil *(Lens culinaris* cv. Verdina) explants in 4 different concentration (1.25, 2.5, 5.0 and 10 μM), with regard to the regeneration of shoots and roots of the pulse crop the variable explant, phytohormone and concentration were all highly significant. Mature seed explants showed the highest shoot regeneration overall the phytohormones and concentrations tested. Thidiazuron (TDZ) and Benzyladenine (BA) showed a higher number of regenerated shoots than kinetin (KIN) and zeatin (ZEA) and increase from 1.25 μ M to 10 μM of any cytokinin in general doubled the number of shoot regenerated. The average length of regenerated shoots was inversely proportional to the number of the shoots that regenerated. TDZ and BA inhabited root development more than KIN and ZEA the highest root regeneration frequency was obtained from shoots regenerated on media containing 1.25 μM ZEA. They concluded that in order to obtain the whole plants it is best to regenerate shoots on media containing the cytokinin KIN or ZEA at low concentration to be able to subsequently regenerate roots.

Nag *et al.* (2000) conducted an experiment to observe the effect of different types of auxins (IBA and IAA at 10^{-7} -10^{-5} M; and NAA, 2, 4-D and napthoxyacetic acid (NAA) at 10^{-9}-10^{-7} M), polyamines (Putrescine and spermine at 10^{-6}-10^{-4}; and spermidine at 10^{-9}-10^{-7}) and water stress on rooting of mungbean cv. 105 stem cutting was determined. IBA was the most effective for improving the development of root primordial and second order root, while IAA was the most efficient for establishing root primordial and for increasing total root length per cutting.Singh and Ranu (1998) reported that direct rooting occurred in excised shoot apices with BA (4.0 mgl^{-1}) + NAA (1.0 mgl^{-1}).

Polanco, M.C.; Ruiz, M.L. (1997) studied that the effect of benzylaminopurine [benzyladenine] (BAP) on the formation of roots from lentil shoots regenerated on media containing BAP. Seedling shoot tips, first nodes and bractlets, and immature seeds cultured on the initiation media containing 2.25 or 0.225 mg/litre of BAP regenerated multiple bud shoots. The regenerated shoots formed roots in percentages ranging from 4.6 to 39.9% on a rooting medium (R medium) containing 2 mg/litre of indole-acetic acid. Rooting success on R medium depended upon the cytokinin used in the initiation media, its concentration, and the time elapsed during shoot formation on these media prior to transplanting regenerated shoots to R medium. An in vivo study of root growth of lentil seedlings demonstrated the strong inhibitory effect of BAP on root growth reflected in a drastic reduction of the mitotic index of the root meristem.

Khawar and Ozcan (2002) observed that shoots of lentil cultiver Ali Dayi, after culturing seeds, for 10 days on MS medium, were isolated and rooted on MS medium containing indole-3- butyric acid (IBA) at concentrations of 0.25, 0.50, 1.0 and 2.0 mg/1. The primary response was obtained after 4 weeks, 0.25 mg/L IBA gave the best results with a rooting percentage of 25%. Mean number of 7.87 roots along with mean root length of 7.13 cm. However, all other concentration of IBA failed to induce roots.

Ye *et al.* (2002) developed a protocol based on seed culture for efficient *in vitro* propagation of lentil and best rooting was achieved using MS medium supplemented with 1.5 mg/l NAA.

IBA was effective for rooting of black gram reported by Das *et al.* (2002). Geetha *et al.* (1998) observed that the regenerated shoots were rooted on MS medium supplemented with different concentrations of IBA. Some 75% of the shoots produced roots.

Khawar, K.M.; Sancak,C.; Uranbey, S. and Ozcan, S. (2004) reported that Thidiazuron (TDZ) is among the most active cytokinin like substances and

induces greater in vitro shoot proliferation than many other cytokinins in many plant species. Leaf, stem, stem node and cotyledonary node explants of 2 extensively cultivated Turkish lentil cultivars, Ali Day and Kay 91, were cultured on Murashige and Skoog (MS) media supplemented with various concentrations of TDZ. The present study was conducted to develop a rapid and efficient shoot regeneration system suitable for the transformation of lentil (Lens culinaris Medik.) using TDZ. Cotyledonary nodes and stem nodes after the initial callus stage regenerated prolific adventitious shoots via organogenesis. Shoot or callus formation was not achieved from leaf or stem explants. DMSO as a solvent for TDZ was necrotic on plant tissues and therefore TDZ was dissolved in 50% ethanol to carry out the studies. Cotyledonary nodes showed a higher shoot formation capacity than stem nodes. MS medium supplemented with 0.25 mg/l TDZ produced the highest frequency of shoot formation from cotyledonary nodes in both genotypes. Regenerated shoots (10-20 mm long) rooted in MS medium containing 0.25 mg/l indole-3-butyric acid (IBA). Rooted plantlets were finally transferred to sand in pots..

Regenerated shoots rooted best on MS basal medium containing 9.8, µM IBA (Geetha *et al.* ,1997).To produce whole plants, the shoots were separated and rooted on 0.1 mg (0.5 µM) NAA per litre (Brar *et al.*, 1997).

Fratini and Ruiz (2003) assessed the rooting response of lentil nodal segments in relation to explant polarity. Nodal segments of lentil with an axillary bud cultured in an inverted orientation showed higher rooting frequencies than explants cultured in a percentage (95.35%) and average number of shoots regenerated per explant (2.4) were obtained from explants placed in inverted orientation on MS medium salts with 3% sucrose, supplemented with 5µM indol acetic acid (IAA) and 1µM Kinetin (Kn).

Anju and Pawan (1992) reported that regenerated shoots were rooted on MS basal medium supplemented with either IAA or indolebutyric acid.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 Location, time and duration of the experiment

The present study was conducted in the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding, HSTU, Dinajpur during November, 2009 to April, 2010.

3.2 Experimental materials

The experimental material used in the present investigation is the seed of three Lentil (*Lens culinaris* Medik) varieties viz. BARI Masur-3, BARI Masur-4 and BARI Masur -5 were used to study different parameters associated with plant regeneration.

3.3 Sources of the experimental materials

The seed materials of three Lentil (*Lens culinaris* Medik.) varieties were collected from the Bangladesh Agricultural Development Corporation (BADC), Dinajpur.

3.4 Methods

Various culture media were used in the present investigation depending on specific purposes. A list of them is given below:

A. For Seed Germination

Half strength MS medium (Murashige and Skoog, 1962) medium was used for seed germination.

B. For callus induction

I. T_1= MS medium containing 1 mg/L 2, 4-D

1. T_2= MS medium containing 1.5 mg/L 2, 4-D + 0.5 mg/L BAP

II. T_3= MS medium containing 2 mg/L 2, 4-D + 0.5 mg/L NAA

C. For Shoot regeneration

I. T_1= MS medium containing 1.5 mg/L Kn

II. T_2= MS medium containing 2.0 mg/L Kn + 0.2 mg/L NAA

III. T_3= MS medium containing 0.5 mg/L BAP+ 0.25 mg/L Kn

D. For root formation

I. T_1 =Hormone free 0.5 strength MS medium (Evans *et al.* 1981)

II. T_2 = MS medium containing 15 mg/L IBA

III. T_3= MS medium containing 10 mg/L IAA

E. For watering the plantlets after their transplantation from culture vessel to soil

I. Soil containing 25% garden soil + 50% sand + 25% cow dung

II. Hogland's solution

3.4.1 Preparation of culture media

For the induction of callus and generation of callus of Lentil (*Lens culinaris* Medik) usually MS (Murashing and Skoog, 1962) medium was used, which has been suggested by several scientists. In the present investigation MS medium along with different concentrations of hormones and vitamins were used. A nutrient medium usually consists of organic and inorganic salts; a carbon source and some vitamins and growth regulators were used. Based on the nature of culture and type of explants, the composition of the medium and type of media differ considerably, A composition of MS (Murashige and Skoog, 1962) is given in Table 1.

Different steps of the media preparation

3.4.2 Preparation of stock solution

The preparation of stock solution was the first requisite for the preparation of medium. Stock solution of growth regulators were prepared separately by dissolving the desired quantity of ingredients in appropriate solvent and made the required final volume with distilled water for ready use to expedite the preparation of the medium wherever needed. Separate stock solutions for macronutrients, micronutrients, irons, vitamins, growth regulators etc. were prepared and stored appropriately for use.

Table 1. Composition of stock solution for the preparation of MS (Murashige and Skoog, 1962) medium

Constituents	Concentrations (mg/L)
a) Macronutrients (10X)	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.\ 2H_2O$	440.00
$MgSO_4.\ 7H_2O$	370.00
b) Micronutrients (100X)	
$MnSO_4.\ 4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.\ 7H_2O$	8.60
KI	0.83
$Na_2MoO_4.\ 2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
c) Iron sources (10X)	27.80
$FeSO_4.\ 7H_2O$	37.30
$Na_2EDTA.\ 2H_2O$	
d) Vitamins/Organic nutrients(100X)	2.00
Glycine	0.50
Nicotinic acid	0.50
Pyrodoxine HCl	0.10
Thiamine HCl	100mg
e) Myoinositol	30g
f) Sugar	9g
g) Agar	

Stock solutions were prepared by measuring and dissolving macronutrients in 10 fold (10X) concentration, micro nutrients with 100 folds (100X) concentration, vitamins 100 folds (100X) and minerals with 10 folds (10X). Each of the needed hormones (Auxin and Cytokinin) was dissolved separately and kept in bottle. All the prepared stock solutions were labeled properly and kept in refrigerator at 4° C.

Depending on the type of the medium, the required amount of sucrose was taken in 100 ml beaker half filled with sterilized water and agitated with a magnetic stirrer. Necessary stock solution was added to the beaker one by one. The vitamins and myoinisitol were measured and added to the medium as required.

Before autoclave, regeneration and root inducing media were heated at 150° C to mix the agar and then dispensed to 250 ml conical flasks. Flasks, containing media were stored in refrigerator at a 4°C until needed.

The medium used for leaf callus induction was a modified MS (Murashige and Skoog, 1962) medium containing 2 mg and 1 mg kinetin, 2 mg and 1 mg of BAP and 0.5 mg IAA per liter medium (Dodds and Roberts, 1990). The stock solutions were made for preparation of these media. They were macro nutrients, micro nutrients, iron sources and vitamins. The iron sources and macro nutrients were made in 10 folds. Concentration of other two stock solutions as prepared in 100 fold (100X).

The IAA stock solution was not prepared because there was a possibility of oxidation degradation of the hormone during prolonged storage. The procedure for the preparation of stock solution and media preparation were same as described in case of leaf culture medium. The amount of IAA was dissolved in a few crop of 1N NaOH and transferred it to the medium mixture.

Hormonal stock solutions used in the media

The following growth regulators were used for callus induction media in the present investigation:

A. Auxin:

2, 4-Dichlorophenoxy acetic acid (2, 4-D)

α- Naphthalene acetic acid (NAA)

Indole acetic acid (IAA)

Indole butyric acid (IBA)

B. Cytokinins:

Benzyl amino purine (BAP)

Kinetin (Kn)

The above mentioned hormonal supplements were dissolved in appropriate solvent, against each of them as shown below:

Growth regulators (Phytohormones)	**Solvents**
2, 4-D	70% Ethyl alcohol
NAA	0.1% N NaOH
BAP	0.1% N NaOH
IBA	70% ethyl alcohol
IAA	70% ethyl alcohol
Kinetin	0.1 N HCl

For the preparation of stock solution of any of these hormone 25 mg of each of the hormone powder was taken on a clean watch glass and dissolved in one ml of the particular solvent. The mixture was then taken in a 250 ml measuring cylinder and

volume was made up to 250 ml by further addition of distilled water. The solution was then poured into a clean plastic or glass container and stored at 4^0C and for maximum period of two weeks.

3.4.2.1Steps for the preparation of culture media from stock solution

To prepare one litre (1000 ml) MS (Murashinge and Skoog, 1962) medium the following steps to be following :

I. About four hundred ml distilled water was taken in a two litre Erlenmeyer flask.

II. One hundred ml of macronutrients, 10 ml of micronutrients, 100 ml of irons and 10 ml of vitamins were taken from each of these stock solutions into the flask on a heater cum magnetic stirrer.

III. One hundred mg of myoinositol was added directly to the solution and dissolved properly.

IV. Thirty gm of sucrose was added to this solution and gently agitated to dissolve it completely.

V. Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

VI. Then added more water and made the solution to 1000 ml.

VII. Then with a digital pH meter, the pH of the medium was adjusted to 5.8, with the help of 0.1 N NaOH or 0.1 N HCl whichever it was necessary.

VIII. By adjusting the pH at 5.8, agar was added @ 9 g/L in the solution, to solidify the medium. The mixture was then heated and gently agitated with microwave oven till complete dissolution of agar took place.

IX. Required volume of hot medium was dispensed into culture vessels or vials or conical flasks after proper cooling of the medium the culture vessels were plugged with cork and/or non absorbent cotton and marked with different codes with the help of a glass marker to indicate

specific hormonal combinations, and then autoclaved at 121 ^{0}C and 15 psi for 15 minutes.

3.4.3 Sterilization

It is the prerequisite to ensure aseptic condition for *in vitro* culture technique. So, all the instruments including glasswares and culture media were properly sterilized by autoclaving.

3.4.3.1 Sterilization of culture media

The conical flasks or culture vessels (vials) containing the prepared media were autoclaved at 1.16 kg/cm^2 pressure and 121^{0}C temperature for 22 minutes. After autoclaving the culture vessels were allowed to cool down under normal condition. All the petridishes and vials were marked with permanent marker or sticker to indicate specific phytohormonal combinations.

3.4.3.2 Sterilization of glasswares and instruments

Conical flasks, beakers, test tubes, petridishes, pipettes, metallic instruments like forceps, scalpels, needles and spatula were wrapped with aluminum foils, then were sterilized in an autoclave at a temperature of 121^{0}C for 30 minutes at 1.16 kg/cm^2 pressure.

3.4.3.3 Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing with detergent followed by wiping with 70% ethyl alcohol and savlon. The process of sterilization was repeated at regular intervals. Generally, laminar airflow cabinet was sterilized by wiping the working surface with absolute ethyl alcohol and with the help of UV light.

3.4.3.4 Precautions to ensure aseptic condition

All inoculation and aseptic manipulations were carried out in a laminar airflow cabinet. It was necessary to switch on for at least half an hour before use the Laminar Air flow Cabinet. Then it was cleaned with absolute ethyl alcohol to overcome the surface contamination. Before use, the instruments like scalpels, needles surgical blades etc. were sterilized by dipping in alcohol and then burned in flame inside the Laminar Air flow Cabinet to eliminate contamination. At the time of inoculation, dipping in 70% alcohol and flaming method inside the cabinet for again sterilized these. Both hands were rinsed with 70% alcohol and done repeatedly. Aseptic conditions were maintained during each and very operation for less chances of contamination.

3.4.3.5 Sterilization of experimental materials (Seed)

Matured seeds of three varieties of lentil were washed in running tap water for 3-5 minutes for two or three times to remove the surface organism. The floating seeds were discarded. Later the seeds were dipped in 70% ethyl alcohol for 3-5 minutes with gentle shaking followed by washing with sterile distilled water. Surface disinfections was done by the use of sodium hypochlorite solutions (1% active chlorine) containing 1-2 drops of tween-20 for ten minutes with gentle shaking and then rinsed five times in sterile distilled water. These sterilized seeds were then ready for keeping in the MS (Murashige and Skoog, 1962) media.

3.4.4 Culture techniques

The following culture techniques were employed in the present study-

1. Axenic culture
2. Explants cutlure
3. Subculture or transfer
4. Rooting

3.4.4.1 Axenic culture

Sterilized seeds were placed onto seed germinating MS (Murashige and Skoog, 1962) medium in culture vials. In each vial 8-12 seeds were incubated. The culture was incubated in incubation room till the germination of seed. Six to seven days old leaves were used as the source of contamination free explants.

3.4.4.2 Explants culture

The seedlings raised in axenic culture were used as the source of explants. From the germinated seedling each leaf was cut into 12-15 pieces with 1-15 mm in length by using sterilized surgical blades. Then in each vial four leaf segments and four nodal segments were placed gently, which contained sterilized culture medium with various combinations and concentrations of growth regulators like 2, 4-D, BAP and NAA.

The culture vessels (vials) containing explants were placed under fluorescent light in a room with controlled temperature (22°C±2) using 16 hours photoperiod. The vials were checked daily to note the response and eliminate the development of contamination. Callus was initiated 6-8 days after inoculation and after 16-21 days inoculated explants was transferred onto fresh medium.

3.4.4.3 Sub-culture or transfer

After 16-21 days of inoculation of explants, the calli attained desirable size for transfer to regeneration medium. Then, they were removed aseptically from the vials to new vials containing sterilized induction medium using forceps. The transfer was done inside the laminar airflow cabinet. The sub-cultured media used in the present investigation was MS (Murashige and Skoog, 1962) medium containing different combinations and concentrations of Kn, NAA and BAP.The sub-cultured vials were again incubated at (22°C±2) in 16 hrs Photoperiod. Repeated subcultures were also done at an interval of 15-20 days and incubated under the same temperature as mentioned previously for maintenance of calli and organogenesis. After shoot initiation, more light intensity was used for shoot

elongation. The culture vessels (vials) showing signs of contamination were discarded.

3.4.4.4 Rooting

The sub-cultured calli continued to proliferate and differentiated into shoots. When these shoot grew about 2-3 cm in length were separated from each other and again cultured individually on vials containing sterilized root induction medium to induce root. The media used for root induction were MS medium containing IBA and IAA. The vials containing plantlets were incubated as mentioned previously. Day to day observations was carried out to note the response.

3.4.4.5 *Preparation of pot and transplantation*

Potting mixture containing garden soil, sand and cow dung in the ratio 1:2:1 was mixed properly and autoclaved one hour in 121°C for 30 minutes at 1.16 kg / cm^2. After cooling, the soil mixture was taken into 10 cm plastic pots for growing the plantlets at *in vivo* condition.

When the plantlets became 5-8 cm in height with sufficient root system, they were taken out from the vials. Medium attached to the roots was gently washed out with running tap water. The plantlets were then transplanted to pot containing potting mixture mentioned above. Immediately after transplantation, the plants along with the pots were covered with moist polythene bag to prevent desiccation. To reduce sudden shock, the pots were kept in a growth room for 7-15 days under controlled environments. The interior of the polythene bags was sprayed with distilled water at every 24 hrs to maintain high humidity around the plantlets. At the same time, plantlets were also nourished with Hogland's solution. After two to three days the polythene bags were gradually perforated to expose the plants to natural environment. The polythene bags were completely removed after ten to fifteen days when the plantlets appeared to be self- sustainable. At this stage,

the plantlets were placed in natural environment for 3-10 hours daily. Finally, after 15-20 days they were transferred to the field condition

3.5 Recording of Data

To investigate the effects of different treatments and response of different varieties to callus induction and plant regeneration, data were recorded under the following parameters.

3.5.1 Callus induction

3.5.1.1 Days of callus initiation

Generally callus initiation was started after 16-21 days of incubation of explants. The number of callus initiated over a number of days were recorded. The mean value of the data provided the days required for callus initiation.

3.5.1.2 Number of explants with callus (Percent callus induction)

The number of explants producing in each vial was recorded. The percentage of callus induction was calculated on the basis of the number of explants placed and total number of callus induced.

$$Percent\ callus\ induction = \frac{No.\exp lants\ induced\ Calli}{Total\ no.of\ \exp lant\ \ incubated} \times 100$$

3.5.2 Plantlet regeneration

3.5.2.1 Days to shoot initiation

Shoot initiation started after 25-29 days of incubation of explants. The number of shoots proliferated over a number of days were recorded. The mean value of the data provided the days required for shoot initiation.

3.5.2.2 Number of callus with shoot (percent shoot regeneration)

Number of callus with shoot was recorded and the percentage of shoot regeneration was calculated by following formula.

$$Percent\ \ shoot\ regeneration = \frac{No.of\ calli\ with\ \ shoot}{Total\ no.of\ \ incubated\ calli} \times 100$$

3.5.2.3 Number of shoots with roots

Average number of shoots with roots calculated by using the following formula:

$$\overline{X} = \frac{\sum Xi}{n}$$

Where, $\overline{X}$ = mean of shoots with roots

Σ= Summation

Xi= number of shoots with roots

n= number of observations

3.5.2.4 Number of regenerated plantlets

Total established plants were calculated based on the number of plantlets place in the pots and the number of plants finally established or survived:

$$Percent\ \ plant\ establishment = \frac{No.of\ establishment\ \ plantlet}{Total\ no.of\ \ plantlets} \times 100$$

3.6 Statistical analysis of data

The data for the parameters under present study were statistically analyzed wherever applicable. The Duncan's Multiple Range Test (DMRT) compared the analysis of variance for different parameters.

CHAPTER IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

The objectives of the present study was to develop an efficient reproducible protocol that can be used for future improvement programs. Thus proliferation of calli from different explants and subsequent regeneration of complete plantlets could play a vital role. The experiment was conducted with three varieties of Lentil (*Lens culinaris* Medik) namely BARI Masur-3, BARI Masur-4, and BARI Masur-5 and two types of explants were used for regeneration purpose. The present study was primarily concerned with the process of callus production and plant regeneration. Remarkable variations were observed in callus induction and plant regeneration in respects of the used explants with different treatments. The results of the present study are presented and discussed under the following sub-headings:

4. INITIATION OF CALLUS:

Effect of different explant on Callus induction

The first step of successful plantlet regeneration through tissue culture in lentil is the induction of calli from explants. To achieve the goal, leaf and nodal segment part of 5-7 days old germinated seedlings of the varieties were cultured on MS medium supplemented with different combination and concentrations of different growth regulators such as 2, 4-D, BAP and NAA. The results of different combinations in callus induction as observed in the present study are described below.

No. of explant showing callus

Analysis of variance for no. of explant showing callus showed significant mean square values for variety and treatment and their interaction showed highly significant (Table 2).

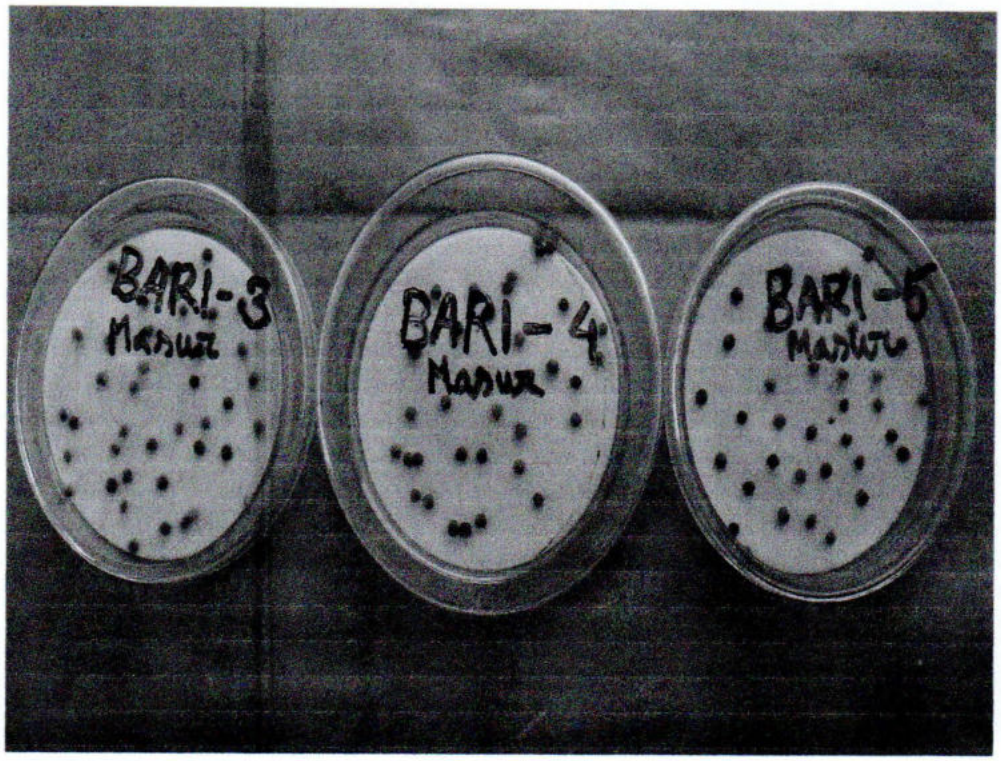

Plate 1: Seeds of three varieties of lentil (BARI Masur-3, BARI Masur-4, BARI Masur-5)

Table 2: Analysis of variance of three characteristics of Lentil (*Lens culinaris* Medik) for callus induction

Sources of variation	Degrees of freedom (df)	Explant	Mean Square (MS)		
			Total no. of explants showing callus	% callus induction	Days required for callus initiation
Variety	2	Leaf	1.000 *	69.472 *	1.593 *
		Nodal segment	0.333 *	23.148 *	0.481 *
Treatment	2	Leaf	4.778**	331.877**	29.481*
		Nodal segment	4.111**	285.691**	26.037*
Variety x treatment	4	Leaf	0.111 **	7.724 **	0.370 *
		Nodal segment	0.444 **	30.877 **	0.593 *
Error	18	Leaf	0.370	25.730	0.778
		Nodal segment	0.222	15.444	0.704
Total	26	–	–	–	–

** Indicates significant at 1% level of probability and * indicates significant at 5% level of probability

Percentage of callus induction

Analysis of variance for percentage of callus induction showed significant difference for variety and treatment and their interaction also showed highly significant.

Days required to callus initiation

Days required to callus initiation showed a significant difference against variety, treatment and their interaction (Table 3).

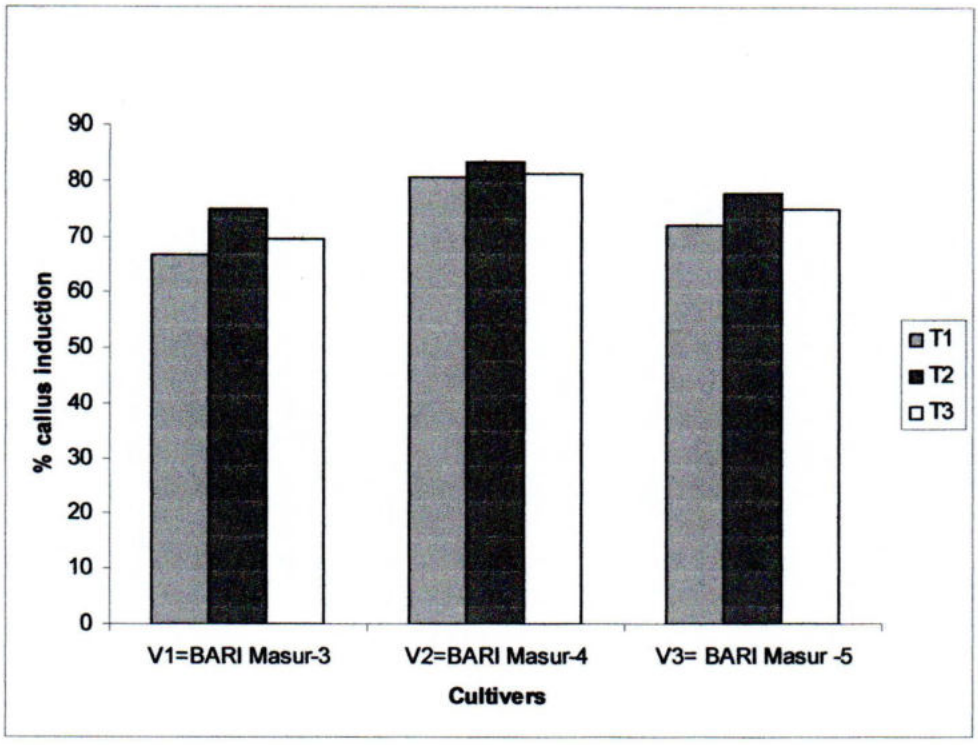

Fig. 1 : Combined effect of different treatments on percent callus induction from leaf explants of three varieties of Lentil (*Lens culinaris* Medik)

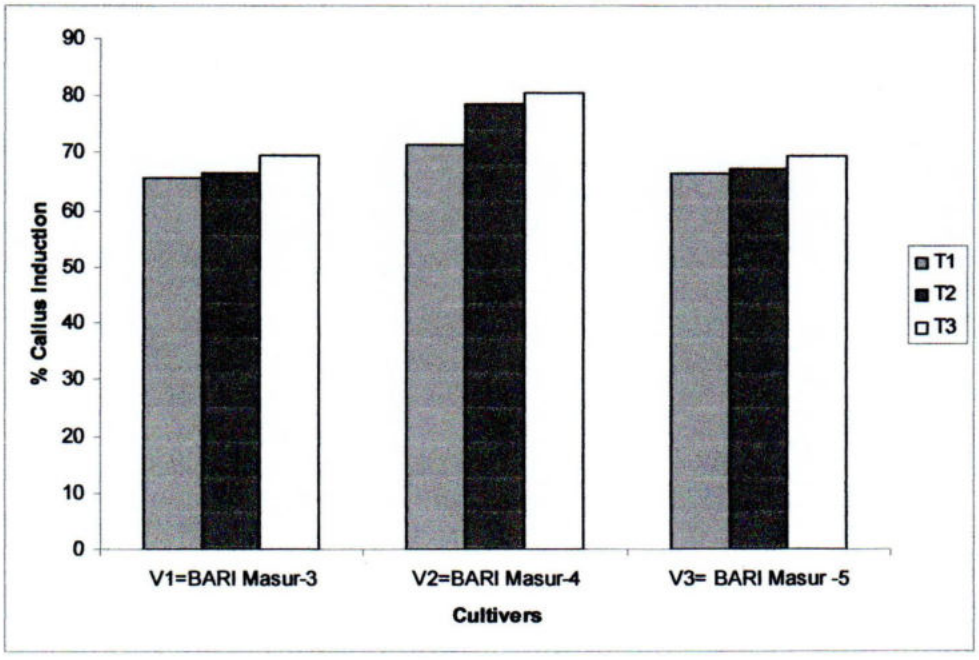

Fig. 2 : Combined effect of different treatments on percent callus induction from nodal segment explants of three varieties of Lentil (*Lens culinaris* Medik)

4.1.1 Effects of varieties

Mean values of varieties on callus inducing characters like total no. of callus induction, % callus induction and days to callus initiation were found statistically significant. The results of the different genotypes observed in the present study are presented in Table 3. Leaf explants started callus initiation by changing their shape after seven days of incubation, and callus formation was completed within 16-21 days of incubation.

Using Leaf as Explant

The highest percentage (82.40%) of callus induction was found in the genotype of BARI Masur -4 followed by BARI Masur -5 (75.00%) and BARI Masur -3 (70.37%). BARI Masur -4 started callus initiation early (17.22 days) in comparison to other genotypes such as BARI Masur -5 (20.11 days) and BARI Masur -3 (20.56 days).

From the above results, it may be concluded that BARI Masur -4 showed the best performance on callus induction by using leaf disc culture.

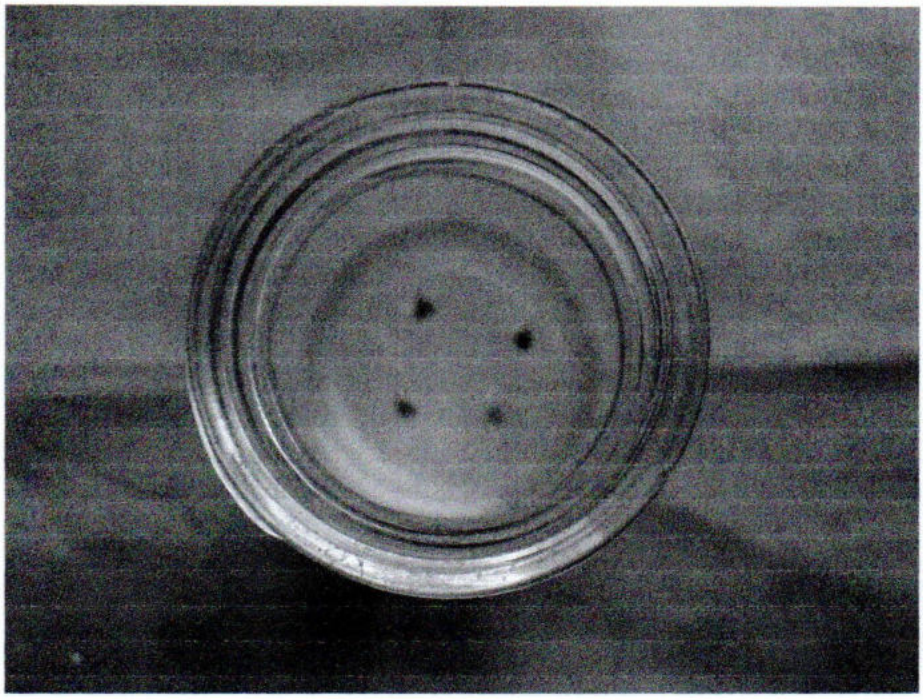

Plate 2 : Leaf disc explant of BARI Masur-4

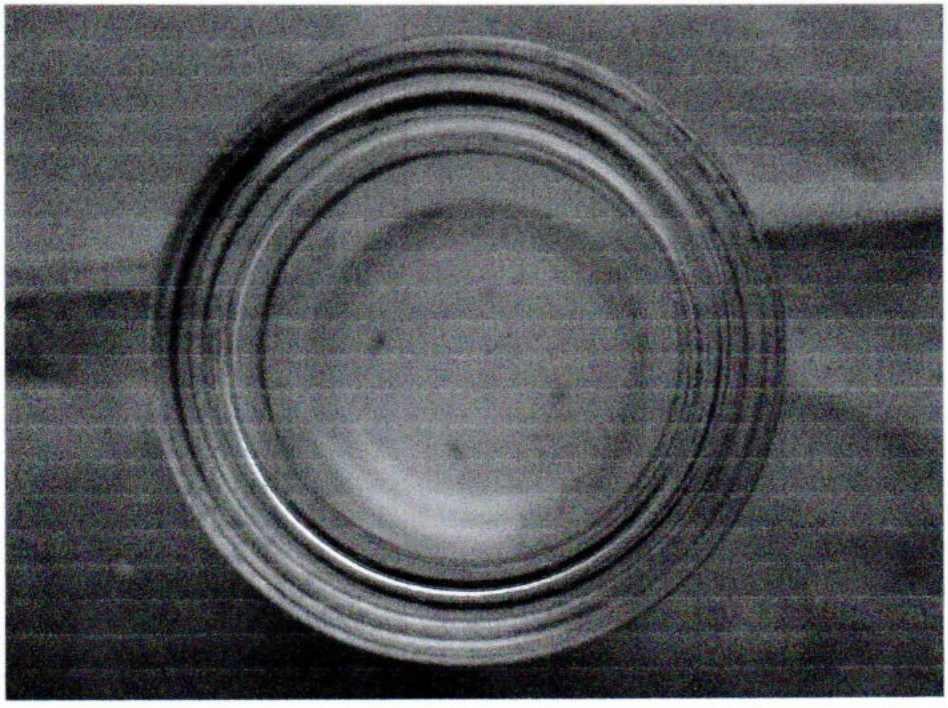

Plate 3 : Nodal segment explant of BARI Masur-4

Table 3: Effects of varieties on callus induction of Lentil (*Lens culinaris* Medik)

Variety	Explants					
	Leaf			Nodal segment		
	Total no. of Callus induction	% callus induction	Days required	Total no. of Callus induction	% callus induction	Days required
BARI Masur-3	8.444 b	70.37 b	20.56 a	8.111 b	67.59 b	20.22 a
BARI Masur-4	9.889 a	82.40 a	17.22 b	9.333 a	77.78 a	17.22 b
BARI Masur -5	9.000 b	75.00 b	20.11 a	8.222 b	68.51 b	20.11 a
LSD at 0.05%	0.6024	5.024	0.8736	0.4666	3.892	0.8310
CV (%)	6.68	6.68	4.57	5.51	5.51	4.37

Using Nodal Segment as Explant

The highest percentage (77.78%) of callus induction was found in the genotype of BARI Masur -4 followed by BARI Masur -5 (68.51%) and BARI Masur -3 (67.59%). BARI Masur -4 started callus initiation early (17.22 days) in comparison to other genotypes such as BARI Masur -5 (20.11 days) and BARI Masur -3 (20.22 days).

From the above results, it may be concluded that BARI Masur -4 showed the best performance on callus induction by using nodal segment culture.

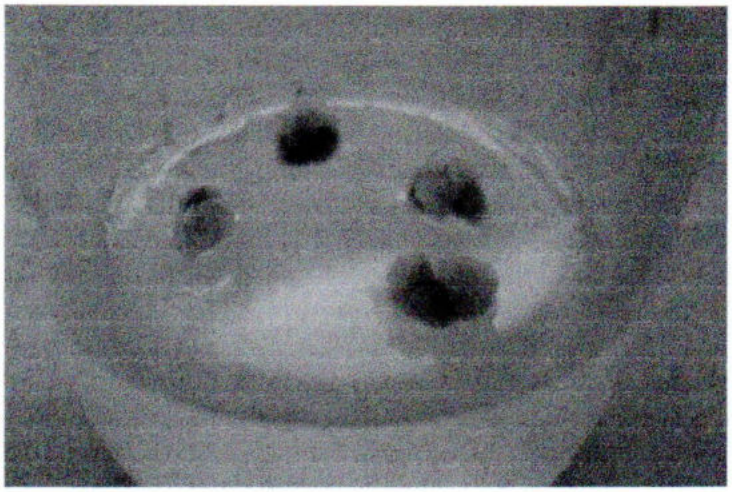

Plate 4 : Development of callus from the variety of BARI Masur-4 with MS +1.5 mg/L 2, 4-D + 0.5 mg/L BAP using leaf as explant

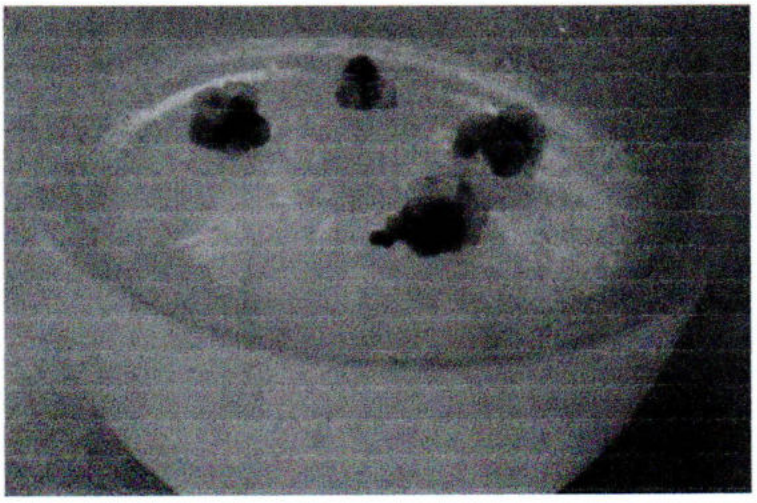

Plate 5 : Development of callus from the variety of BARI Masur-4 with MS +2 mg/L 2, 4-D + 0.5 mg/L NAA using nodal segment as explant

4.1.2 Effects of Treatment:

Total no. of callus induction, percent callus induction and days required for callus initiation showed significant differences among the hormonal (T_1, T_2 and T_3) treatments (Table 4).

Callus induction from leaf discs:

Total number of callus induction was highest (9.444) with high percentage (78.70%) of callus induction in T_2 (MS +1.5 mg/L 2, 4-D + 0.5mg/L BAP) treatment with minimum days (19.78 days) followed by T_3 (MS +2 mg/L 2, 4-D + 0.5 mg/L NAA) (75.92 %) and T_1 (MS +1 mg/L 2, 4-D) (73.14 %) (Table 4).

From the above results, it may be concluded that T_2 (MS +1.5 mg/L 2, 4-D + 0.5mg/L BAP) showed the best performance on callus induction from leaf discs.

Callus induction from nodal segment:

The maximum number of explants showing callus (8.667) was found with highest percentage (72.22%) of callus induction in T_3 (MS +2 mg/L 2, 4-D +0.5 mg/L NAA) treatment followed by in T_2 (MS +1.5 mg/L 2, 4-D + 0.5 mg/L BAP) treatment and T_1 (MS +1 mg/L 2, 4-D) (Table 4).

From the above results, it may be concluded that T_3 (MS +2 mg/L 2, 4-D + 0.5 mg/L NAA) showed the best performance on callus induction from nodal segment as explant.

Table 4: Effects of treatment on different characteristics of callus induction of Lentil (*Lens culinaris* Medik) genotypes

Treatment	Explants					
	Leaf			Nodal segment		
	Total no. of Callus induction	% callus induction	Days required	Total no. of Callus induction	% callus induction	Days required
T_1 (MS+1 mg/L 2, 4-D)	8.778 b	73.14 b	21.00 b	8.333 b	69.44 b	21.00 b
T_2 (MS+1.5 mg/L 2, 4-D + 0.5 mg/L kinetin)	9.444 a	78.70 a	19.78 ab	8.467 ab	70.22 ab	20.11 ab
T_3 (MS+2.0 mg/L 2, 4-D + 0.5 mg/L NAA)	9.111 ab	75.92 ab	20.11 a	8.667 a	72.22 a	19.44 a
LSD at 0.05%	0.6024	5.024	0.8736	0.4666	3.892	0.8310
CV (%)	6.68	6.68	4.57	5.51	5.51	4.37

4.1.3 Treatment × variety interactions on callus induction:

Treatment × variety interactions on different parameter such as, number of explants with callus, percent callus induction and days required for callus initiations are presented in the Table 5. All the parameters were found significant for treatment × variety interactions.

Table 5: Treatment and Variety interaction on callus induction parameters of Lentil (*Lens culinaris* Medik)

Treatment × Variety		Name of explants	Number of explants showing Callus	% callus formation	Days required for callus initiation
T_1 MS+1 mg/L 2, 4-D	BARI Masur-3	Leaf	8.000 d	66.66 d	21.00 a
		Nodal segment	8.000 b	66.66 b	21.33 a
	BARI Masur-4	Leaf	9.667 ab	80.55 ab	17.33 b
		Nodal segment	8.533 b	71.44 b	17.33 b
	BARI Masur -5	Leaf	8.667 b-d	72.22 b-d	19.67 a
		Nodal segment	8.333 b	69.44 b	19.33 a
T_2 MS+1.5 mg/L 2, 4-D +0.5 mg/L BAP	BARI Masur-3	Leaf	9.000 a-d	75.00 a-d	20.00 a
		Nodal segment	8.000 b	66.66 b	20.00 a
	BARI Masur-4	Leaf	10.00 a	83.33 a	17.67 b
		Nodal segment	8.667 a	78.55 ab	17.00 b
	BARI Masur -5	Leaf	9.333 a-c	77.78 a-c	20.67 a
		Nodal segment	8.333 b	69.44 b	20.33 a
T_3 MS+2.0 mg/L 2, 4-D +0.5 mg/L NAA	BARI Masur-3	Leaf	8.333 cd	69.44 c-d	20.67 a
		Nodal segment	8.333 b	69.44 b	20.33 a
	BARI Masur-4	Leaf	9.00 a-d	81.33 ab	16.67 b
		Nodal segment	9.667 a	80.55 a	17.33 b
	BARI Masur -5	Leaf	9.000 a-d	75.00 a-d	20.00 a
		Nodal segment	8.000 b	66.66 b	20.67 a
LSD at 0.05%		Leaf	1.043	8.701	1.513
		Nodal segment	0.8082	6.741	1.439
CV (%)		Leaf	6.68	6.68	4.57
		Nodal segment	5.51	5.51	4.37

Callus induction from leaf discs:

The variety BARI Masur -4 showed best performance with T_2 (MS +1.5 mg/L 2, 4-D + 0.5 mg/L BAP) showing maximum number of explants showing callus (10.00) and percent callus formation (83.33%) followed by T_3 (MS +2 mg/L 2, 4-D + 0.5 mg/L NAA) and T_1 (MS +1 mg/L 2, 4-D).

Days required for callus initiation) (17.67 days) was early in T_2 (MS+1.5 mg/L 2, 4-D + 0.5 mg/L BAP) and callus initiation was late (21.00 days) in T_1 (MS +1 mg/L 2, 4-D).

Callus induction from nodal segment:

The maximum number of explants showing callus (9.667) and percent callus formation (80.55%) were found in BARI Masur -4 genotype with T_3 (MS +2 mg/L 2, 4-D + 0.5 mg/L NAA) followed by T_2 (MS +1.5 mg/L 2, 4-D + 0.5mg/L BAP) and T_1 (MS +1 mg/L 2, 4-D).

The treatment T_3 (MS +2 mg/L2, 4-D + 0.5 mg/L NAA) also took minimum days (17.33 days) and shoot initiation was late in T_1 (MS +2 mg/L 2, 4-D + 0.5 mg/L NAA) (21.00 days).

The present findings showed conformity with Mathur and Prakash (1997).

4.2 Maintenance of callus

One of the most important objectives of the present study was to obtain regenerants from unorganized calli. To achieve this objective, proliferation of callus and regeneration of plantlets are necessary. So, calli raise from all of the genotypes were maintained through subculture at an interval of four weeks. Subculture was done on MS medium supplemented with different combination and concentrations of different growth regulators such as Kn, BAP and NAA.

After one week of subculture, the calli exhibited variation in color, texture and shape i.e., calli become large and greenish in color. The sub-cultured calli then started regeneration by shoot initiation.

4.3 IN VITRO SHOOT REGENERATION

4.3.1 Organogenesis via callus

The ultimate goal of the present investigation was the establishments of free-living plantlets. For this reason, the explants were placed in culture medium, produced calli, shoots and finally the development of roots thereby plantlets. Leaf and nodal segments of three Lentil (*Lens culinaris* Medik) species were cultured on MS medium supplemented with different concentrations as T_1 (MS medium + 1.5 mg/L Kn), T_2 (MS medium + 2.0 mg/L Kn + 0.2 mg/L NAA) and T_3 (MS medium + 0.5 mg/L BAP+ 0.25 mg/L Kn) in order to induce shoot from unorganized calli. The various morphogenic response of calli to different concentration of growth regulators in the medium has been observed and results are presented below:

Different genotypes showed variation in shoot regeneration and days required for shoot initiation (Table 6). Among the three genotypes, BARI Masur -4 showed highest percentage of shoot regeneration.

No. of callus with shoot

Analysis of variance for No. of callus with showing callus showed significant mean sum of square for variety. Treatment and variety interaction also showed significant (Table 6). Treatment showed highly significant.

Percentage of shoot regeneration

Analysis of variance for percentage of shoot regeneration showed significant for variety. Treatment and variety interaction also showed significant. The mean square value showed highly significant for interaction.

Table 6: Analysis of variance of three characteristics of Lentil (*Lens culinaris* Medik) for shoot induction

Sources of variation	Degrees of freedom (df)	Explant	Mean Square (MS)		
			Total number of calli with shoot	% Shoot regeneration	Days required for shoot initiation
Variety	2	Leaf	1.370*	95.122 *	2.259**
		Nodal segment	0.333 *	23.130 *	2.259**
Treatment	2	Leaf	5.593**	388.135**	12.926**
		Nodal segment	4.111**	285.266**	25.037**
Variety x treatment	4	Leaf	0.037 *	2.567 *	0.648 *
		Nodal segment	0.444 *	30.840 *	2.148**
Error	18	Leaf	0.407	28.276	0.333
		Nodal segment	0.222	15.420	0.333
Total	26	–	–	–	–

** Indicates significant at 1% level of probability and * indicates significant at 5% level of probability

Days required to shoot initiation

Days required to shoot formation showed highly significant difference against variety, treatment and their interaction showed both highly significant and significant interaction incase of two explants.

4.3.2 Effects of varieties:

Varieties showed statistically variations for all the characters of shoot regeneration: total no. of shoot, percent shoot regeneration and days required for shoot initiation (Table 7).

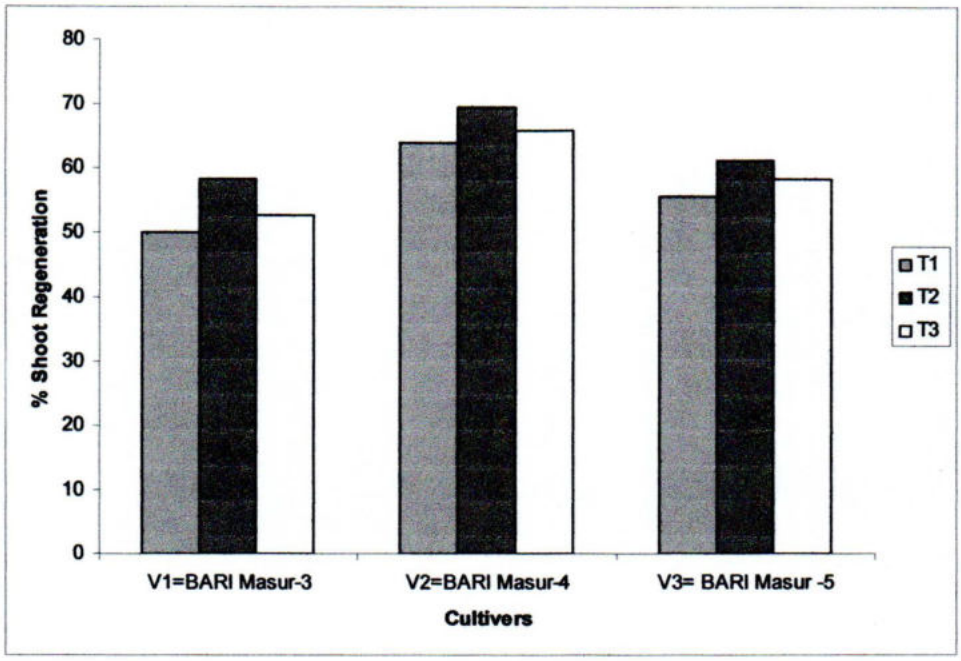

Fig. 3 : Combined effect of different treatments on percent shoot regeneration from leaf explants of three varieties of Lentil (*Lens culinaris* Medik).

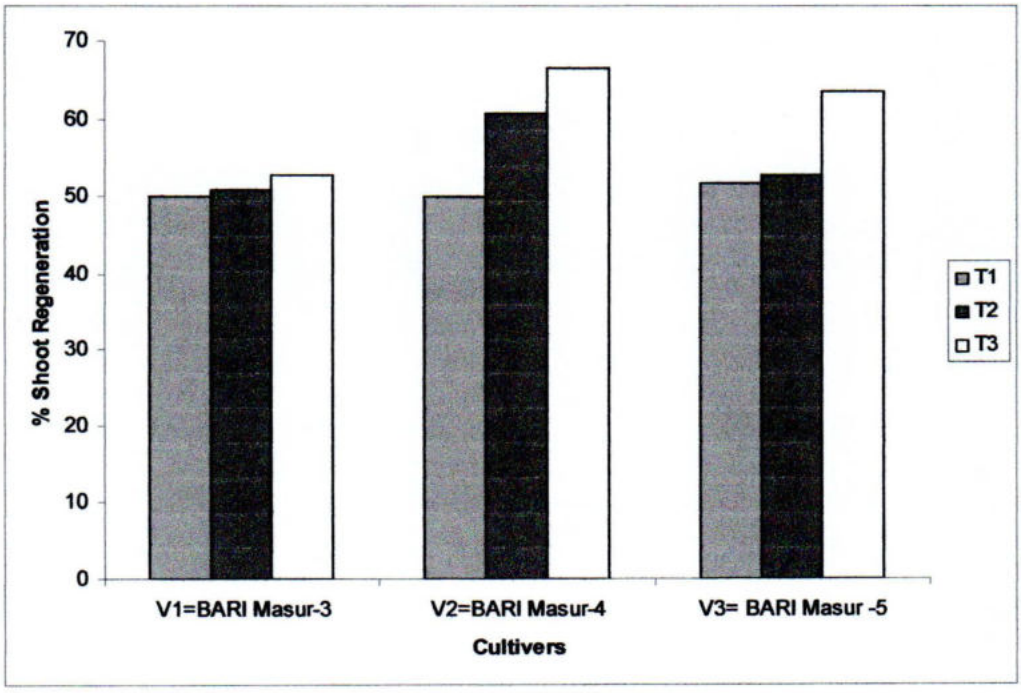

Fig. 4 : Combined effect of different treatments on percent shoot regeneration from nodal segment explants of three varieties of Lentil (*Lens culinaris* Medik).

Using Leaf as Explant

Among the three genotypes, BARI Masur -4 showed best performances (66.66%) on percent shoot regeneration (Table 7). In contrast, BARI Masur -3 showed lowest performance (53.70%) on percent shoots regeneration. Days required for shoot initiation was early in BARI Masur -4 (25.67 days) and shoot initiation was late in case of BARI Masur -3 (27.89 days).

After considering the performance, it may be concluded that BARI Masur - 4 showed the best performance on shoot regeneration.

Table 7: Effect of varieties on shoot induction of Lentil (*Lens culinaris* Medik)

Variety	Explants					
	Leaf			Nodal segment		
	Total number of calli with shoot	% Shoot regeneration	Days required for shoot initiation	Total number of calli with shoot	% Shoot regeneration	Days required for shoot initiation
BARI Masur-3	6.444 b	53.70 b	27.89 a	6.111 b	50.93 b	28.22 a
BARI Masur-4	8.000 a	66.66 a	25.67 b	7.333 a	61.11 a	25.33 b
BARI Masur -5	7.000 b	58.33 b	27.56a	6.222 b	51.85 b	28.20 a
LSD at 0.05%	0.6318	5.266	0.5715	0.4666	3.889	0.5715
CV (%)	8.93	8.93	2.14	7.19	7.19	2.12

Using Nodal Segment as Explant

Incase of nodal segment, BARI Masur -4 showed best performances (61.11%) on percent shoot regeneration (Table 7) followed by BARI Masur -4 (51.85 %) and BARI Masur -3 (50.93%). Days required for shoot initiation was early in BARI Masur -4 (25.33days) and shoot initiation was late in case of BARI Masur -3 (28.22 days).

From the above results, it may be concluded that BARI Masur-4 showed the best performance on shoot regeneration.

4.3.3 Effects of Treatment:

Different treatments showed significant variations for total no. of shootm, percent shoot regeneration and days required for shoot initiation. The results were presented in the Table 8.

Using Leaf as Explant

Among the treatments, T_2 (MS+2.0 mg/L Kn + 0.2 mg/L NAA) showed highest percentage of shoot regeneration (62.96%). Shoot regeneration was lowest (56.48%) in T_1 (MS medium + 1.5 mg/L Kn) (Table 8). Days required for shoot initiation was minimum (26.56 days) in the T_2 (MS+2.0 mg/L Kn + 0.2 mg/L NAA) and maximum (27.58 days) in T_1 (MS medium + 1.5 mg/L Kn).

From the above discussion, it may be concluded that T_2 (MS + 2.0 mg/L Kn + 0.2 mg/L NAA) showed the best performance on shoot regeneration.

Using Nodal Segment as Explant

Out of three treatments, T_3 (MS medium +0.5 mg/L BAP+ 0.25 mg/L Kn) showed highest percentage of shoot regeneration (55.55%) with minimum days (26.78 days) and shoot regeneration was lowest (52.48%) in T_1 (MS medium + 1.5 mg/L Kn) (Table 8) with maximum days (27.78 days).

Table 8: Effects of treatment on different characteristics of shoot induction of Lentil (*Lens culinaris* Medik) genotypes

Treatment	Explants					
	Leaf			Nodal segment		
	Total number of calli with shoot	% Shoot regeneration	Days required for shoot initiation	Total number of calli with shoot	% Shoot regeneration	Days required for shoot initiation
T_1 (MS+1.5 mg/L Kn)	6.778 b	56.48 b	27.58 a	6.333 b	52.78 b	27.78 a
T_2 (MS+2.0 mg/L Kn + 0.2 mg/L NAA)	7.556 a	62.96 a	26.56 b	6.567 ab	55.50 ab	27.22 ab
T_3 (MS+0.5 mg/L BAP+ 0.25 mg/L Kn)	7.111 ab	59.26 ab	27.00 ab	6.967 a	55.55 a	26.78 b
LSD at 0.05%	0.6318	5.266	0.5715	0.4666	3.889	0.5715
CV (%)	8.93	8.93	2.14	7.19	7.19	2.12

From the above results, it may be concluded T_3 (MS medium +0.5 mg/L BAP+ 0.25 mg/L Kn) that showed the best performance on shoot regeneration.

4.3.4 Treatment × variety interaction on shoot regeneration

Results related to treatment × variety interaction for the characters of shoot regeneration such as percent shoot regeneration and days required for shoot initiation in different concentrations of growth regulators showed significant variations. The results are presented in Table 9.

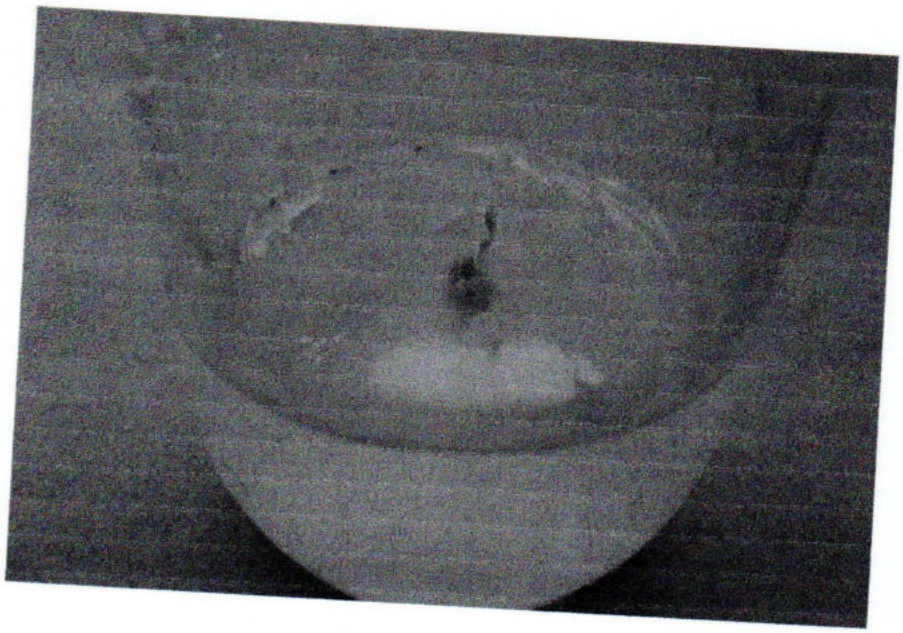

Plate 6: Initiation of shoot from the callus of BARI Masur-4 genotype with MS+2.0 mg/L Kn + 0.2 mg/L NAA using leaf as explant

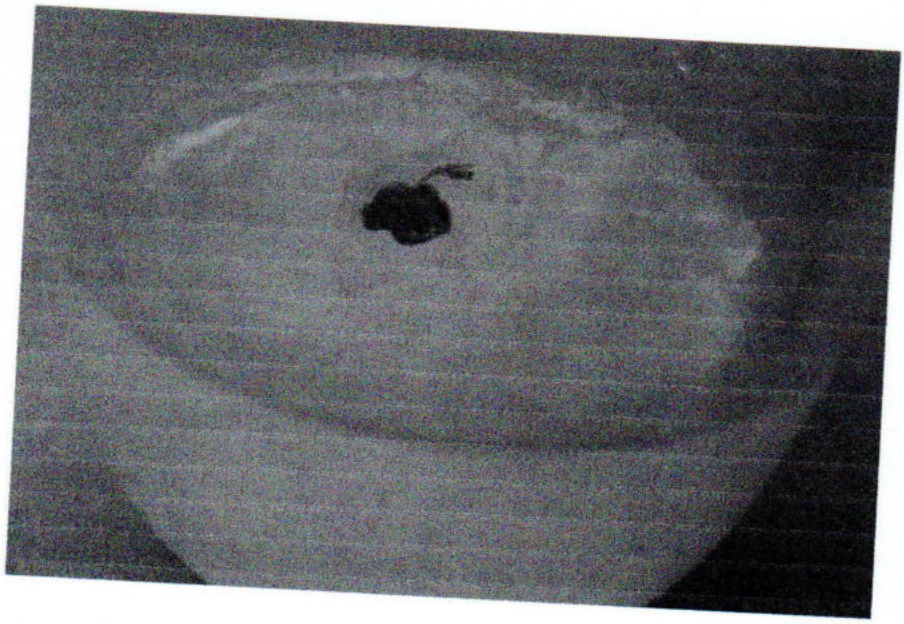

Plate 7: Initiation of shoot from the callus of BARI Masur-4 genotype with MS+0.5 mg/L BAP+ 0.25 mg/L Kn. using nodal segment as explant

Using Leaf as Explant

Best interaction was found in BARI Masur – 4 on percent shoot regeneration in T_2 (MS+2.0 mg/L Kn + 0.2 mg/L NAA) (Table 9) followed by BARI Masur -5 and BARI Masur -3 showed lowest performance (50.00%) on percent shoot regeneration in T_1 (MS medium + 1.5 mg/L Kn).

Time needed for shoot initiation was minimum (25.67 days) on the interactions of T_2 (MS+2.0 mg/L Kn + 0.2 mg/L NAA) with BARI Masur -4 and maximum (28.67days) on the interactions T_1 (MS medium + 1.5 mg/L Kn) with BARI Masur -3 (Table9). All the genotypes showed satisfactory results against T_2 (MS+2.0mg/L Kn + 0.2 mg/L NAA) treatment.

From the above results, it may be concluded that BARI Masur – 4 with T_2 (MS+2.0mg/L Kn + 0.2 mg/L NAA) showed the best performance on shoot regeneration.

Using Nodal Segment as Explant

BARI Masur – 4 also performed superior result on percent shoot regeneration (66.66 %) with T_3 (MS medium +0.5 mg/L BAP+ 0.25 mg/L Kn) (Table 9) followed by BARI Masur -5 and BARI Masur -3 showed lowest performance (50.00%) on percent shoot regeneration in T_1 (MS medium + 1.5 mg/L Kn).

Shoot initiation time was minimum (25.30 days) on the interactions of T_3 (MS medium +0.5 mg/L BAP+ 0.25 mg/L Kn) with BARI Masur -4 and maximum (29.33 days) on the interactions T_1 (MS medium + 1.5 mg/L Kn) with BARI Masur -3 (Table 9). All the genotypes showed satisfactory results against T_3 (MS medium +0.5mg/L BAP+ 0.25 mg/L Kn) treatment.

In the above investigation, it may be concluded that T_3 (MS medium +0.5 mg/L BAP+ 0.25 mg/L Kn) showed the best performance on shoot regeneration.

Similar results also reported by Singh and Raghuvansi (1989).

Table 9: Treatment and Variety interaction on shoot induction parameters of Lentil (*Lens culinaris* Medik)

Treatment × Variety		Name of explants	Total number of calli with shoot	% Shoot regeneration	Days required for shoot initiation
T_1 MS+1.5 mg/L Kn	BARI Masur-3	Leaf	6.000 e	50.00 e	28.67 a
		Nodal segment	6.000 b	50.00 b	29.33 a
	BARI Masur-4	Leaf	7.667 a-c	63.88 a- c	25.67 d
		Nodal segment	6.667 b	50.00 b	25.33 d
	BARI Masur -5	Leaf	6.667 c-e	55.55 c-e	28.67 a
		Nodal segment	6.333 b	52.78 b	28.00 bc
T_2 MS+2.0 mg/L Kn + 0.2 mg/L NAA	BARI Masur-3	Leaf	7.000 b-e	58.33 b-e	27.00 c
		Nodal segment	6.000 b	50.00 b	28.67 ab
	BARI Masur-4	Leaf	8.333 a	69.44 a	25.67 d
		Nodal segment	7.607 a	60.88 a	25.33 d
	BARI Masur -5	Leaf	7.333 a-d	61.11 a-d	27.00 c
		Nodal segment	6.333 b	52.78 b	27.00 c
T_3 MS+0.5 mg/L BAP + 0.25 mg/L Kn	BARI Masur-3	Leaf	6.333 de	52.78 de	27.33 bc
		Nodal segment	6.333 b	52.78 b	29.00 ab
	BARI Masur-4	Leaf	8.000 ab	63.88 a	25.30 d
		Nodal segment	7.667 a	66.66 ab	25.67 d
	BARI Masur -5	Leaf	7.000 b-e	58.33 b-e	28.00 abc
		Nodal segment	6.000 b	63.66 a	28.33 c
LSD at 0.05%		Leaf	1.094	9.122	0.9899
		Nodal segment	0.8082	6.736	0.9899
CV (%)		Leaf	8.93	8.93	2.14
		Nodal segment	7.19	7.19	2.12

4.4 Root regeneration

Regeneration of roots was found in the regeneration medium, which is different in concentrations and combination of growth regulators such as hormone free 0.5 strength MS medium and MS medium supplemented with 15 mg/L IBA and 10 mg/L IAA. From this observation, it was found that induction of roots from regenerated shoots showed wide variations according to varieties and different treatments in the medium. The results are presented below:

No. of shoot with root

Analysis of variance for No. of shoot with root showed a significant mean sum of square for variety and treatment and their interaction also showed a significant difference (Table 10).

Percentage of root regeneration

Analysis of variance for Percentage of root regeneration showed a significant difference in case of treatment, variety and their interaction also showed significant interaction (Table 10).

Table 10: Analysis of variance of Lentil (*Lens culinaris* Medik) for root regeneration

Sources of variation	Degrees of freedom (df)	Explant	Mean Square (MS)		
			No. of shoots with root	% root formation	Days required root formation
Variety	2	Leaf	0.862*	59.33*	1.092*
		Nodal segment	0.763*	52.69*	1.022*
Treatment	2	Leaf	1.000*	69.389*	9.037**
		Nodal segment	1.000*	69.389*	10.815**
Variety x treatment	4	Leaf	0.565*	56.481*	1.059*
		Nodal segment	0.540*	55.382*	1.063*
Error	18	Leaf	0.222	15.420	0.481
		Nodal segment	0.222	15.420	0.407
Total	26	–	–	–	–

** Indicates significant at 1% level of probability and * indicates significant at 5% level of probability

Days required to root initiation

Days required to root initiation showed significant mean square values for variety and variety, treatment interactions on root induction also showed significant mean sum of square. Treatment showed highly significant interaction.

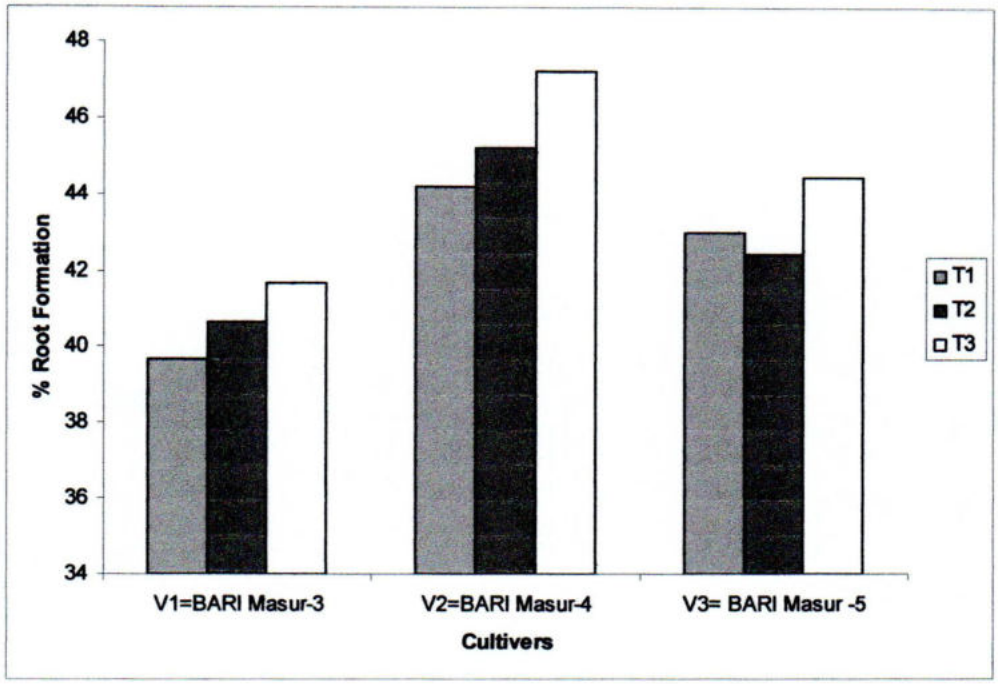

Fig. 5 : Combined effect of different treatments on percent root formation from leaf explants of three varieties of Lentil (*Lens culinaris* Medik)

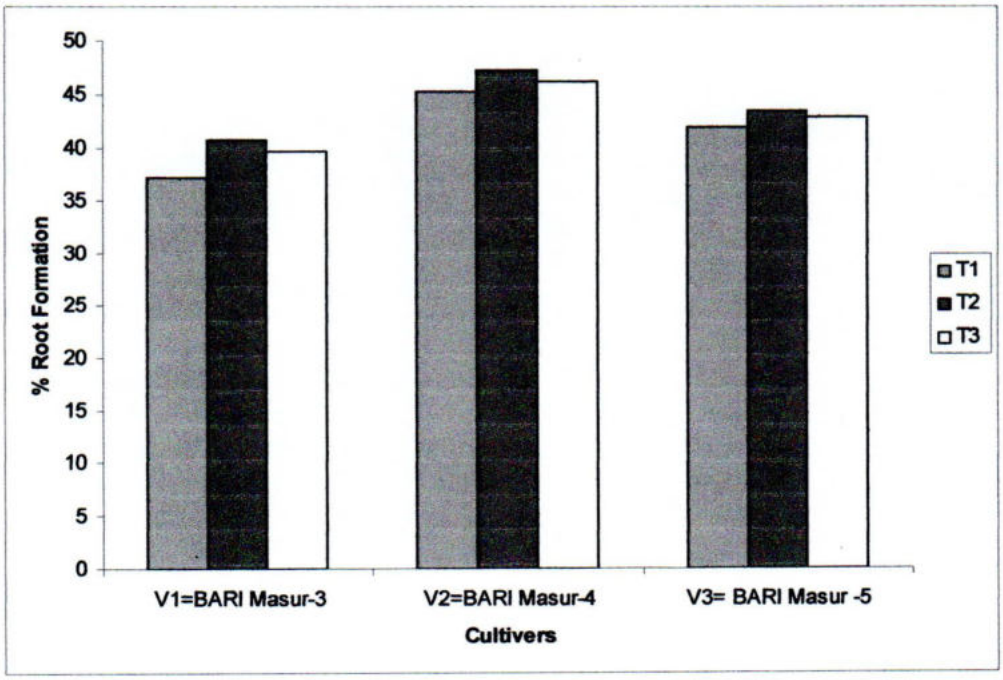

Fig. 6 : Combined effect of different treatments on percent root formation from nodal segment explants of three varieties of Lentil (*Lens culinaris* Medik)

4.4.1 Effcts of varieties:

Using Leaf as Explant

Different varieties showed significant variation in producing root BARI Masur -4 showed the highest percentage (47.25%) of root formation and days required for root initiation was minimum (15.44 days) and BARI Masur -3 showed lowest percentage (40.70%) of root formation and days required for root initiation was maximum (17.44days) (Table 11).

Table 11: Effect of varieties on root formation of Lentil (*Lens culinaris* Medik)

Variety	Explants					
	Leaf			Nodal segment		
	No. of shoots with root	% root formation	Days required root formation	No. of shoots with root	% root formation	Days required root formation
BARI Masur-3	4.300 c	40.70 bc	17.44 a	3.000 c	40.67 c	17.45 a
BARI Masur-4	5.967 a	47.25 a	15.44 c	5.667 a	47.22 a	15.44 b
BARI Masur -5	5.633 ab	44.48 ab	16.33 b	4.333 ab	44.45 ab	15.67 b
LSD at 0.05%	0.4966	3.919	0.6869	0.4666	3.889	0.6318
CV (%)	8.84	8.83	4.23	8.84	8.83	3.94

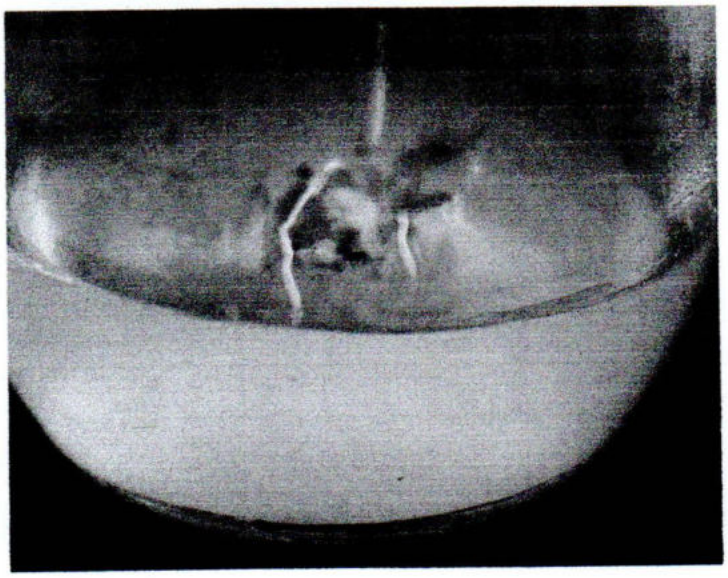

Plate 8 : Root initiation from regenerated shoot of BARI Masur-4 in MS +10 mg/L IAA using leaf as explant

Plate 9 : Root initiation from regenerated shoot of BARI Masur-4 in MS +15 mg/L IBA using nodal segment as explant

Using Nodal Segment as Explant

Among three varieties BARI Masur -4 showed the highest percentage (47.22%) of root formation with earliest days (15.44 days) and BARI Masur -3 showed lowest percentage (40.67%) of root formation with maximum days required for root initiation (17.45 days) (Table 11).

4.4.2 Effects of treatment:

Using Leaf as Explant

Out of three different treatments ; T_3(MS medium +10mg/L IAA) performed best in percentage of root formation (47.45%) with shortest days (16.02 days) followed by T_2 (MS medium +15mg/L IBA) and T_1 (hormone free 0.5 strength MS medium) (Table 12).

Using Nodal Segment as Explant

Root initiation varied in a wide range due to difference in treatments and their concentrations and combinations. The percentage of root initiation (48.45%) was found highest in T_2 (MS medium +15 mg/L IBA) followed by T_3 (MS medium +10mg/L IAA) and T_1 (hormone free 0.5 strength MS medium) with BARI Masur – 4 and also the days required for root initiation was minimum (16.11 days) (Table 12).

Table 12: Effects of treatment on root formation of Lentil (*Lens culinaris* Medik)

Treatment	Explants					
	Leaf			Nodal segment		
	No. of shoots with root	% root formation	Days required root formation	No. of shoots with root	% root formation	Days required root formation
T_1 (Hormone free 0.5 strength MS medium)	4.033 bc	40.45 c	16.56 a	3.433 c	42.45 b	16.44 a
T_2 (MS+15 mg/L IBA)	5.333 ab	44.45 b	16.40 a b	5.833 a	48.45 a	16.11b
T_3 (MS+10 mg/L IAA)	5.733 a	47.45 a	16.02 b	4.633 bc	45.45 ab	16.02 b
LSD at 0.05%	0.4666	3.789	0.6869	0.2666	3.689	0.6318
CV (%)	8.84	8.83	4.23	8.84	8.83	3.94

4.4.3 Treatment × variety interaction on root induction

Results related to treatment × variety interaction for the characters of root regeneration such as percent root formation and days required to root formation in different concentrations treatments showed significant variations. The results are presented in Table 13.

Table 13: Treatment and Variety interction on root formation of Lentil (*Lens culinaris* Medik)

Treatment × Variety		Name of explants	No. of shoots with root	% root formation	Days required root formation
T_1 (Hormone free 0.5 strength MS medium)	BARI Masur-3	Leaf	4.000 c	39.67 c	17.67 a
		Nodal segment	3.000c-d	37.27c-d	17.33 a
	BARI Masur-4	Leaf	5.667 a	44.22 a	15.67 c- d
		Nodal segment	5.637 a	45.22 a	15.67 b
	BARI Masur -5	Leaf	4.990 b	43.01 b	16.67 a-c
		Nodal segment	4.303 ab	42.90 ab	15.67 b
T_2 (MS+15 mg/L IBA)	BARI Masur-3	Leaf	5.000 c	40.67 c	17.60 a
		Nodal segment	3.020 c-b	39.67 c	17.67 a
	BARI Masur-4	Leaf	5.667 a	45.22 a	15.33 d
		Nodal segment	5.687 a	47.20 a	15.33 b
	BARI Masur -5	Leaf	5.323 b	42.45 b	16.33 b-d
		Nodal segment	5.310 ab	43.45 ab	16.00 b
T_3 (MS+10 mg/L IAA)	BARI Masur-3	Leaf	5.030 c	41.67 c	17.00 a-b
		Nodal segment	3.000 c-d	40.67 ac	17.33 a
	BARI Masur-4	Leaf	5.697 a	47.23 a	15.30 d
		Nodal segment	5.667 a	46.22 a	15.34 b
	BARI Masur -5	Leaf	5.363 ab	44.45 b	16.00 b-d
		Nodal segment	4.333 ab	43.45 b	15.36 b
LSD at 0.05%		Leaf	0.2082	6.236	1.190
		Nodal segment	0.5082	6.536	1.094
CV (%)		Leaf	8.84	8.83	4.23
		Nodal segment	8.84	8.83	3.94

Using Leaf as Explant

The interaction of T_3(MS medium containing 10mg/L IAA) with BARI Masur-4 was found more effective one in percent root regeneration (47.23%) followed by T_2 (MS medium containing 15mg/L IBA) and T_1 (hormone free 0.5 strength MS medium) with BARI Masur-3 (39.67%).

The time required for root initiation was minimum (15.30 days) on the interaction of T_3 (MS medium containing 10 mg/L IAA) with BARI Masur-4 and maximum (17.67 days) on the interaction T_1 (Hormone free 0.5 strength MS medium) with BARI Masur-3 (Table 13)

From the above results, it may be concluded that T_3 (MS medium containing 10 mg/L IAA) with BARI Masur-4 showed the best performance on root regeneration.

Similar results also reported by Anju and Pawan (1992).

Using Nodal Segment as Explant

Among the three varieties, BARI Masur-4 showed best performance (47.23%) on percent root regeneration in T_2 (MS medium containing 15 mg/L IBA). But in contrast, BARI Masur-3 showed the lowest performance (37.27%) on percent root regeneration with T_1 (Hormone free 0.5 strength MS medium).

Days required for root initiation was minimum (15.33 days) on the interaction of T_2 (MS medium containing 15 mg/L IBA) with BARI Masur-4 and maximum (17.33 days) on the interaction T_1 (Hormone free 0.5 strength MS medium)with BARI Masur-3 (Table 13).

From the above discussion, it may be concluded that T_2 (MS medium containing 15 mg/L IBA)) with BARI Masur-4 showed the best performance on root regeneration.

The investigation is similar with those of Das *et al.* (2002), Geetha *et al.* (1997).

4.5 Establishment of plantlet:

After proper root development, small plantlets were fit to transfer to ground or pots. All the agar extracts washed off properly. This washing was done with running tap water. Maximum care was taken so that no roots got damaged. The soil, to which the plantlets would be transplanted (soil: sand: cow dung = 1: 2: 1) should be placed in a small pot, plantlets forced on this soil gently, it demanded proper amount of watering by Hogland's solution.

Table 14: Comparative survivability rate of regenerates obtain from leaf discs and nodal segments of the three varieties of Lentil (*Lens culinaris* Medik.)

Planting condition	Name of the genotypes	Number of plants transplanted	Number of plants survived	Survival rate (%)
In pot	BARI Masur-3	10	4	40
	BARI Masur-4	8	5	62.50
	BARI Masur -5	10	6	60

The pots were then covered with clear polyethylene bag to maintain high humidity conditions and kept in the growth chamber for proper hardening. Gradually the plantlets were adapted to soil and established. The survived plants then gave vigorous growth with proper leaf development. The comparative survivability role of regenerates obtained from leaf and nodal segments of three varieties of lentil is given in Table 14. Among the three varieties BARI Masur – 4 showed the highest survival rate for plantlet establishment (62.50%) and it was followed by BARI Masur – 5. The variety BARI Masur – 3 showed the lowest performance (40%) for plant establishment.

Plate 10 : Hardening of regenerated plant of BARI Masur-4 after transplantation into small plastic pot derived from leaf explant

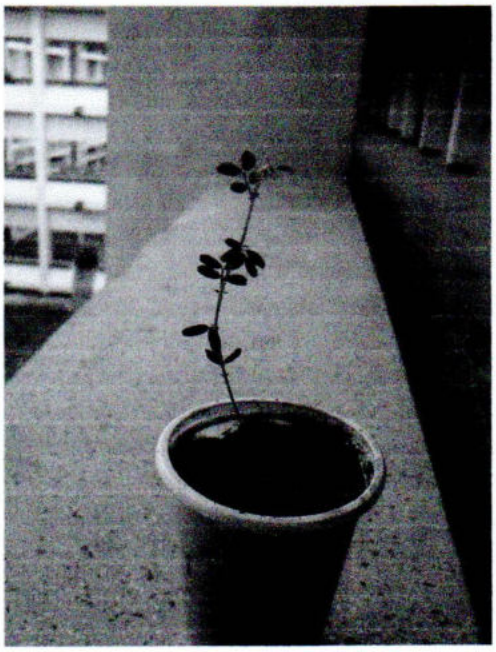

Plate 11 : Survival of plant after hardening of BARI Masur-4 derived from leaf explant

CHAPTER V

SUMMARY AND CONCLUSION

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SUMMARY AND CONCLUSION

An experiment was carried out during the period from November, 2009 to April 2010, in the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science & Technology University, Dinajpur. Three varieties of Lentil (*Lens culinaris* Medik) were used in the present investigation for observing their callus induction and subsequent plantlet regeneration ability by using leaf discs and nodal segments as explant.

Leaf disc and nodal segments of three (*Lens culinaris* Medik) genotypes of Lentil *viz.* BARI Masur -3, BARI Masur -4 and BARI Masur -5 were cultured on MS medium supplemented with different concentrations and combinations of growth regulators for callus induction, shoot regeneration, and root initiation . A wide range of variation was observed by the explants and varieties in different media.

The callus induction was varied with explants in different media. Leaf disc and nodal segment explants were cultured on MS medium supplemented with 1 mg/L 2, 4-D , 1.5 mg/L 2, 4-D + 0.5 mg/L BAP and 2 mg/L 2, 4-D + 0.5 mg/L NAA .

BARI Masur – 4 showed best performance on both leaf segment; where percent callus induction was the highest (83.33%) with minimum days (17.67 days) with treatment T_2 (MS +1.5 mg/L 2, 4-D + 0.5 mg/L BAP) and incase of nodal segment, T_3 (MS +2 mg/L 2, 4-D + 0.5 mg/L NAA) showed best result with highest percent of callus induction (80.55%) taking shortest time (17.33 days). BARI Masur – 3 took maximum time (21.33 days) with lowest percentage of callus induction (65.66%) with T_1 (MS +1 mg/L 2, 4-D) in leaf segment and it also showed poor performance on nodal segment with the same treatment.

While the callus formation was completed, the calli were subcultured on MS medium supplemented with 1.5 mg/L Kn, 2.0 mg/L Kn + 0.2 mg/L NAA and 0.5 mg/L BAP+ 0.25 mg/L Kn to observe the regeneration capability of the calli.

Shoot induction was also highest (69.44%) with BARI Masur–4 in T_2 (MS+2.0 mg/L Kn + 0.2 mg/L NAA) using leaf explant with minimum days (25.67 days) and also with the nodal segment the shoot percentage was maximum (66.66%) with T_3 (MS medium + 0.5 mg/L BAP + 0.25 mg/L Kn) having minimum initiation days (25.30 days).

Hormone free ½ MS medium (T_1), MS medium containing 15 mg/L IBA, 10 mg/L IAA were used to observe the rooting responses of regenerated shoots. The maximum root (47.23%) observed in BARI Masur – 4 with T_3 (MS medium + 10 mg/L IAA) having shortest days (15.30 days) with leaf segment and by using nodal segment the superior performance of root initiation (47.20%) with T_2 (MS medium containing 15 mg/L IBA) with minimum days (15.33 days).

After proper root development, the small plantlets were removed from the culture media and transplanted in small plastic pots in growth chamber for proper hardening of the plantlets. The survival rate of the plantlet in pot was highest in BARI Masur -4 (62.50) followed by BARI Masur -5 (60%) and BARI Masur -3 (40%). The plantlets were successfully survived.

From the above discussion, it can be concluded that among three varieties, BARI Masur -4 showed best performance followed by BARI Masur -5 and BARI Masur -3. When the combined effect of explant were considered, leaf showed better performance than nodal segment. The performance of growth regulators were differ incase of explants. Incase of callus induction T_2 (MS +1.5 mg/L 2, 4-D + 0.5 mg/L BAP) and T_3 (MS +2 mg/L 2, 4-D + 0.5 mg/L NAA), for shoot induction T_2 (MS+2.0 mg/L Kn + 0.2 mg/L NAA) and T_3 (MS medium +0.5 mg/L BAP+ 0.25 mg/L Kn), for root initiation T_3(MS medium +10 mg/L IAA) and T_2 (MS medium containing 15 mg/L IBA) were best with leaf disc and nodal segment explant respectively.

In this experiment, *in vitro* regeneration potentiality of three lentil genotypes has been observed by using leaf discs and nodal segment as explant. Since genetic engineering developed the efficient methods for the regeneration of viable shoots from cultured tissues, this protocol can be followed for genetic manipulation for improvement of lentil genotypes. Further study is needed to determine suitable explant and hormonal combination and concentration for other variety of Bangladesh and by changing the hormonal concentrations and composition, type of media and trying by adding additional growth regulators could make a detailed investigation.

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