DEVELOPMENT OF DISEASE RESISTANT LENTIL (*LENS CULINARIS* MEDIK) LINES THROUGH *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION

Ph.D. THESIS

BY

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DEVELOPMENT OF DISEASE RESISTANT LENTIL (*LENS CULINARIS* MEDIK) LINES THROUGH *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION.

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Dedicated
To
My Respected Parents

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– The Author

CERTIFICATE

This is to certify that the thesis entitled "**Development of disease resistant lentil (*Lens culinaris* Medik.) lines through *Agrobacterium*-mediated genetic transformation"** submitted by Subroto Kumar Das has been carried out under our supervision in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. A part of the investigation was also carried out in Plant Transformation Laboratory, ICGEB, New Delhi. This is further to certify that it is an original work and suitable for submission for the award of Ph.D. in Botany.

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ABSTRACT

Agrobacterium-mediated genetic transformation was carried out to integrate fungal diseases resistant gene in microsperma varieties of lentil (*Lens culinaris* Medik.) cultivated in Bangladesh. As an integral part of *Agrobacterium*-mediated genetic transformation *in vitro* regeneration studies were carried out using various explants from Barimasur (BM) varieties, namely, BM - 1, BM - 4, BM - 5 and BM - 6. Among these explants cotyledon attached decapitated embryo (CADE) from BM - 6 appeared to be the best responsive explant towards *in vitro* regeneration compatible to *Agrobacterium*-mediated genetic transformation. Highest percentage (96%) of multiple shoots as well as healthy roots were obtained through direct organogenesis from CADE explant on MSB_5 medium supplemented with 1.0 mg/l zeatin and 0.1 mg/l NAA. More than 90% of such developing explants produced effective, elongated and healthy roots when they were transferred to MS medium without any hormonal supplement. On the other hand, *in vitro* regenerated shoots those failed to develop roots were exploited to induce *in vitro* flower as well as seed to overcome the constraints created in producing *in vitro* roots in lentil to obtain complete plantlets. MS and half-strength of MS medium supplemented with various concentrations and combinations of IAA, IBA and NAA were employed for this purpose. The best responses regarding the development of *in vitro* flowers and pods were observed on half-strength of MS medium containing 20 mg/l IBA and 0.5 mg/l NAA. Healthy seeds obtained from this pods produced seedlings which were successfully transplanted to soil for further growth and development.

Transformation experiments were performed using three strains of *Agrobacterium tumefaciens*, namely, LBA4404 harboring binary plasmid pBI121 containing GUS and *npt*II gene (Strain I), EHA105 harboring plasmid pGIIMH (strain II) containing *bar* gene resistant to phosphinothricin and also *chitinase* gene (as an antifungal gene) and the third one was LBA4404 containing binary plasmid pCAMBIA2300 (strain III) conferring *npt*II gene resistant to kanamycin and AFP gene as an antifungal protein gene. Strain I was mainly used for the optimization of transformation protocol and to select the suitable lentil variety as well as explant for transformation. Transformed shoots were selected using 2.0 mg/l phosphinothricin (Strain II) whereas 200 mg/l kanamycin was used to select transformed shoots in case of strain I and III. Transformation efficiencies for strain I, strain II and strain III was 2.15, 0.36 and 0.47% respectively. Stable integration of desired gene within the lentil genome was confirmed through PCR, RT-PCR, Southern and Northern hybridization techniques. Seedlings developed through transformation were successfully transferred to soil for the development of transformed progenies in lentil.

1. INTRODUCTION

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1.1 General information about legumes and pulses

During the early days of agriculture, legumes became major food crop and also a source of feed for domestic animals. Today's agriculture also continues to depend on legume crops because they all have high energy and high protein content for human and animal nutrition as well as amino acid profiles complementary to those of other crops mainly cereals. The unique symbiotic ability of legumes is to use atmospheric nitrogen for plant growth makes them preferable crops for sustainable agriculture. In addition, legumes are also diverse in both their adaptations to most of the world's agricultural and natural habitats (Oram and Agocaoili 1994, ICARDA 1998, 2000, Wheeler 2000).

Grain legumes (also called pulses or food legumes) are plants belonging to the family Leguminasae (alternatively Fabaceae). The leguminase is a diverse and important family of angiosperms (Young et al. 2003). This family is the third largest in higher plants and comprises almost 700 genera and 1800 species (Polhill and Raven 1981). Pulses are cultivated in tropics, sub-tropics and temperate regions of the world where they are cultivated in 61.3 million hectares of land (production of 78.91 million metric tons) in 2002 (source-FAOSTAT 2002). Among the major food crops in the Asia-Pacific regions, particularly South, East and Southeast Asia, pulses as nutritionally rich food, play an important role in improving the diet of the people. The countries of this region grow a dozen of summer and winter pulses to meet the dietary requirements, particularly for the poorer section of the society, to whom animal protein is less accessible (Fig. 1). Pulses provide a large variety of food alternative and are a source of income and livestock feed, matching perfectly the requirements of small-scale, low-

income farmers in developing countries. Economically legumes represent the second most important crop plants after Poaceae (grass family), accounting for approximately 27% of the world's crop production (Graham and Vance 2003). In many developing countries of the world, 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 (Bressani 1973). This makes the grain legumes to be considered as the "Meat of the poor".

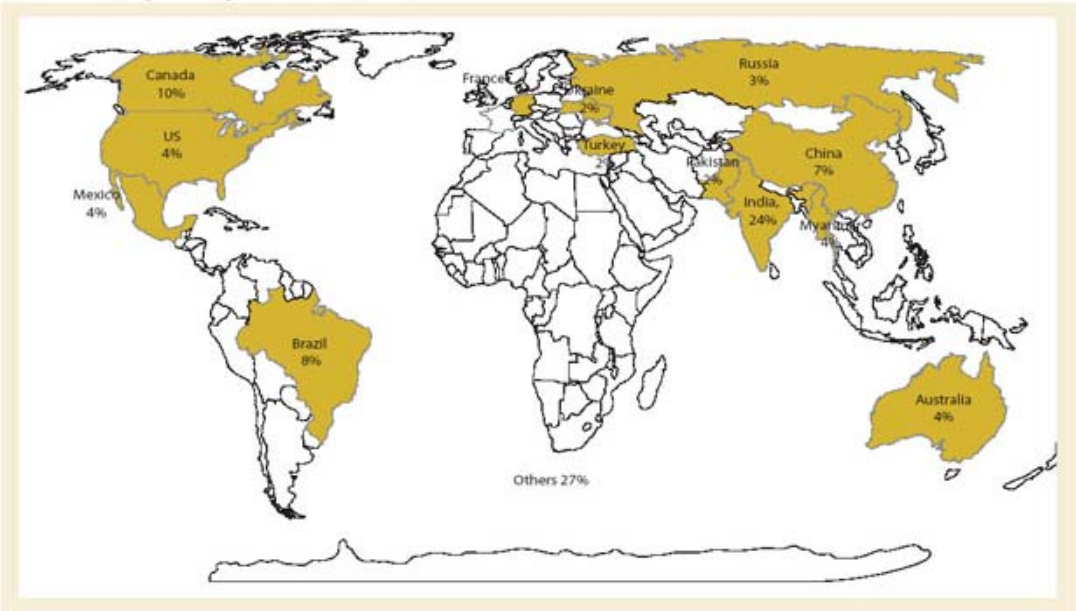


Fig. 1. Production of the pulses in the different countries of the world (Source- Indian agriculture review_Ghana_2009_10_outlook_20091127_16.JPG).

In Bangladesh, a number of pulses are cultivated. Pulses are vital components in diversification predominantly rice-based cropping system in Bangladesh. The main pulses in terms of production in Bangladesh are grass pea, lentil, chickpea, black gram and mungbean. It is worth to mention here that pulses occupy less than 5% of the total cultivated land and have been reported to contribute about 2% of the total food grain

production in Bangladesh (Gowda and Kaul 1982). However, lentil (locally known as "Masur") is one of the major crops of Bangladesh (major crops are those that are grown one per cent or more of the gross-cropped area, GCA) and it occupies about 1.54% of the total crop acreage (14.61 million hectare). Lentil is one of the most important pulse crops in Bangladesh in terms of area and production (Table 1) in the year 2013 (FAO STAT 2014).

1.2 Lentil - an important pulse crop

Lentils have been the part of the human diet since the aceramic (before pottery) Neolithic times, being one of the first crops domesticated in the Near East and they are still one of the most important cool season annual grain legumes or pulse crops throughout the world. Lentil is the only one legume where most of the species convert nitrogen from the atmosphere into nitrogen in the nodules on the plant roots. It is preferred over the other pulses by consumer's preference all over the world. This important grain legume gained worldwide economic importance as a source of protein for human and animal nutrition. The importance of lentil lies in the fact that it is a major source of good quality protein in the common diet as the protein content can reach up to 24-30%. In fact 100 grams of lentil has as much protein as 130 grams of meat in addition to beneficial dietary fibers. Most recently, lentil has assumed the role as a valuable food for health and improvements in athletic performance. Areas with limited rainfall and dry growing season prove to be the most suitable for lentil production. These characteristics make lentil an important crop and ensured its survival to the present day.

Despite its importance as a source of protein through ages, this crop has received little attention to improve its quality. Organized collection of germplasm (Table 2) and crop improvement programs have only started at early 1980's with the establishment of international agriculture centre's including ICARDA (International Centre for Agricultural Research in Dry Areas).

Table 1. Pulses production in Bangladesh in the year 2013.

Name of pulse	Production in ton	Area harvested in Ha
Lentils	89840	93000
Beans-dry	60000	56000
Pea-dry	13269	12622
Chickpea	8000	7500
Pigon pea	500	430

Source- http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor

Table 2. Lentil germplasm collection centre for breeding and research.

Gene bank	Accessions	website
ICARDA	10800	http://www.icarda.org/GeneBank.htm
ATFCC	5250	http://biofire34.pbcbasc.latrobe.edu.au:8080/atfcc_qm
USDA ARS	2798	http://www.ars-grin.gov/npgs/

1.2.1 Nomenclature

Lentil is known by various names in different parts of the world. The most common names are Masur (Bengali), Lentil (English), Adas (Arabic), Mercimek (Turkey), Messer (Ethiopia), Masser/Massur (Hindi), Heramame (Japanese). Other names mentioned in literature are Mangu/Margu (Persian), Masura/Renuka/Mangalaya (Sanskrit).

1.2.2 Historical background

Lentil may have been one of the first agricultural crops grown more than 8,500 years ago. Production of this cool season annual crop spread from the Near East to the Mediterranean area, Asia, Europe and finally the Western Hemisphere (Oplinger et al. 1990). Lentils were probably introduced into the United States in the early 1900's. They have been grown in the Western United States and Western Canada since 1930's mainly in rotation with wheat. It is probably the oldest grain legumes to be domesticated (Bahl et al. 1993), although it is impossible to be tell when domestication of grain legumes exactly began. Small lentil seeds, dating from around 10000 years, have been found in archeological excavations of pre-agricultural sites in Syria, but these may have been wild seeds that were gathered rather than domestication, including a large store of lentils found in Northern Israel that dates to around 8800 years. In some cases it is said that the oldest times of domesticated lentil varieties in the Near East dates to 6000 BC. Cultivation had already spread to the Mediterranean regions and Central Europe by the Neolithic age about 4000 BC (Fig. 2).

1.2.3 Botanical description

Lentil is shaped like a lens. In fact, *Lens* is the latin word for lentil. All *Lens* species are diploid herbaceous annuals with 2n = 14 chromosomes, plant height ranges from 25 - 75 cm with soft hairy branches with pinnately compound leaves and numerous oval leaflets.

Lentil is under the order Rosales, suborder Rosineae, family Leguminosae, subfamily Papilionaceae and tribe Vicieae. After a complex taxonomic history, lentil eventually placed in the genus *Lens* Miller. Analyzing previous findings based on origin and distribution, morphological, cytological, cytogenetical observations and more recently on the basis of isozyme and molecular studies (Ferguson and Robertson 1996), *Lens* was re-classified by Ferguson et al. (2000) into seven taxa split into four species:

Lens culinaris Medikus

Subsp. Culinaris

Subsp. Orientalis (Boiss) Ponert

Subsp. Tomentous (Ladiz.) Ferguson et al. (2000)

Subsp. Odemensis (Ladiz.) Ferguson et al. (2000)

Lens ervoides (Brign.) Grande

Lens nigricans (M. bieb.) Godr.

Lens lamottei Czefr.

This latest classification is accepted by most of the lentil researchers (Sarker and Erskine 2006). *Lens orientalis* is the presumed progenitor of cultivated *Lens culinaris* Medik. The last name is for Medikus, a German botanist-physician who has given the name of the plant in 1787.

The detailed characteristic features of *Lens* species are as follows:

The wild species *L. nigricanes* is a sylender, densly pilose having semi-hastate stipulates, conspicuously aristate peduncles and mauve flowers. The pods are glabrous and small usually with two tiny lenticular seeds. It is morphologically very closed to cultivated species *L. culinaris*. The wild species *L. ervoides* is very sylender with semi-hestate stipulates and long piliform peduncles. It has very small puberulent pods with lenticular seeds. Like *L. nigricans* it is morphologicaly related to cultivated lentil. However, it can be separated from cultivated lentils by traits like structure of stipulates and peduncles, size on pod and seed and flower shape. The wild species *L. orientalis* is slender, pilose and has a very strong resemblance to *L. culinaris* with respect to vegetative growth and structure of the flower and pod. The stipulate are entire, obliquely lanceolate and unappendged. The pods are glabrous with small lenticular seeds. Overall, *L. orientalis* looks like a miniature version of *L. culinaris.*

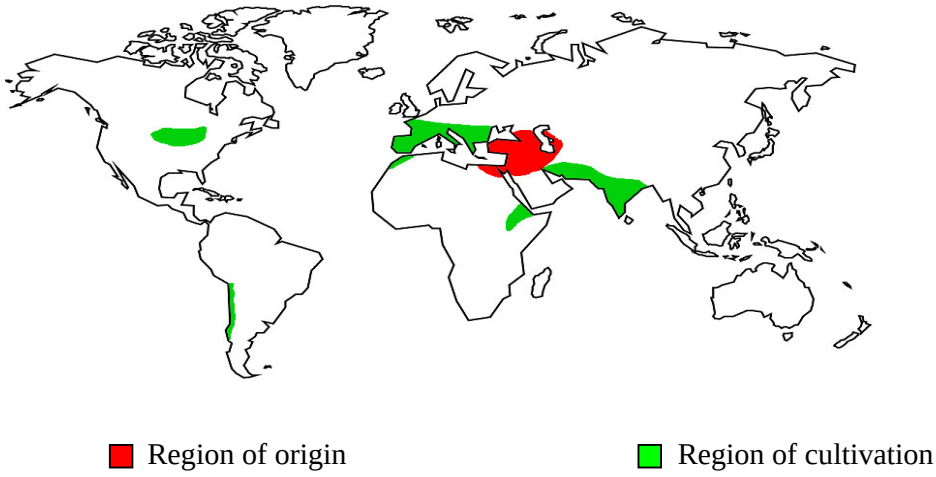


Fig. 2. World map showing centre of origin for lentils and its spread. An ancestral form is unknown. (http://en.wikipedia.org/wiki/Lentil)

Source: Bock D., MPI Köln. (http://www.cilr.uq.edu.au/UserImages/File/Lentil%20S14.pdf

1.2.4 Growth habits

Lentil plants are typically short, slender; semi erect, annuals with compounds leaves 4 to 7 pairs of leaflets. Plants normally range from 30 to 50 cm long depending on the genotype and environmental conditions. Individual plants may bear single stems or may be multi-branched depending upon the population in the field (Opliger et al. 1990). Branches may directly rise from the main stem or from the cotyledonary node below ground or they may rise from other branches depending on the available space in the field and environmental condition (Saxena and Hawtin 1981).

Roots are generally taproots and there is a mass of fibrous lateral roots. Depending on the texture and type of soil, various types of root system can be formed ranging from shallow branched roots to deep taproots (Nezamuddin 1970). The tap roots and the lateral roots in the upper layers of the soil carries numerous small round or elongated nodules which start to decline before onset of flowering (Saxena and Hawtin 1981).

The lentil has thin, ribbed, herbaceous, and weak stems. The base turns into a woody structure as the plant grows old. The stem has a varying pubescence from quite hairy to glabrous. Also pigmentation can vary, anthocyanin can be present only in the basal part or present on the whole. Generally leaves are alternate, compound and pinnate. They are small compared to other legumes. The leaves can be ovate or elliptic each about 1 to 4.5 cm long; some genotypes can form tendrils in early stages of growth. The first two leaves are simple, scale-like and largely fused with two lateral scale-like stipules. The following leaves are bifoliate and subsequent ones are multifoliate.

Generally a single, sometimes two or more flowers originate from short peduncles from upper nodes. Flowers are papilionaceous and 4 to 8 mm long. Color of flower may be white, lilac or purple depending on genotype. Flowering in lentils is acropetal, from the

bottom of the plant to the top. Flowers are predominantly self-pollinated or may be cross pollinated by small insects. In general, cold temperature and long day promote early flowering and good pod set. The corolla wilt within 3 days after opening and pods are visible 3 to 4 days later. Pods contain 1 or 2 seed, they are flattened and 1 to 2 cm long (Fig. 3).

Lentil seed is classified in two categories described by Barulina (1930), as macrosperma and microsperma. Macrosperma have larger pods (15 - 20 × 7.5 - 10.5 mm), generally flat, with large flat seeds (6 - 8 mm dia). Cotyledones are yellow or orange. Flowers are large, white with veins occasionally light blue. Peduncles have 2 - 3 flowers. Calyx teeth are long, leaflets are large (15 - 27 × 4 - 10 mm) and oval (length: width = 3: 3.5). Plant height ranges from 25 - 75 cm, commonly grows in the Mediterranean, Africa and Asia Minor. On the other hand microsprma have small to medium pods (6 - 15 × 3.5 - 7 mm) which are convex. Seeds are flatterened subglobose; small to medium (3 - 6 mm dia). Flowers are small and violet in colour with variable patterns. Leaflets are small (8 - 15 × 2 - 5 mm), elongated, linear or lanceolate. Height of plant varies from 15 - 35 cm. Lentils imbibe more than 100% of the initial air-dry weight. The germination is hypogeal. In the field conditions emergence occurs in 25 to 30 days with winter sowing and 7 to 10 days with spring sowing.

1.2.5 Agronomic information

Agronomical requirements of lentil depend on the agro-ecological conditions throughout the world. In Bangladesh lentil is grown during the winter on the soil moisture conserved during the proceeding monsoon season. Several countries such as

Turkey, Syria, Iraq, Cyprus and Chile experiencing Mediterrian climate, crop is raised during in the wet winters. In the high elevation areas such as USA and Canada this crop is grown during the spring season on the conserved soil moisture.

Under optimum environmental conditions, lentil completes their life cycle in three to four months. In most of spring-sown lentil these conditions are available but in winter-sown lentil growth delays up to 30 to 60 days, because of the suboptimal temperatures. Seeds can be germinated at temperature above 0°C but best temperature for lentil seed germination is in the range between 15 - 25°C. Temperature above 27°C is harmful for its growth. Optimum temperature for growth and yields are around 24°C.

Fig 3. Lentil plants showing different parts (e - flower, g,h,I - petal, l - sepal, k - anther, f - calyx, m.o - pod, n,p - seed, b,c - leaf, d - stipule, a - stem)

Source- http://commons.wikimedia.org/wiki/File:Lens_culinaris_Sturm8.jpg

Hypogeal germination makes lentil resistant to freezing, wind grazing and insect damage since cotyledonary nodes remain below the ground. In any case of damage in young shoot new buds can be initiated easily from the nodes below the ground. Also the crop is said to be drought tolerant, throughout the world, most of the lentil growing areas, are semiarid that depends on water conserved in the soli after fall and winter rains.

Lentils show adaptability to a wide range of soil types. It can be grown in sandy loam soils, alluvial soils, and black cotton soils or in heavier clay soils (Nezamuddin 1970). On soils with very high natural fertility and excessive soil moisture, the crop might take excessive vegetative growth but reduce seed yield (Saxena and Wassimi 1980).

Lentil grows well on slightly acidic soil (pH 5.5 to 6.5) and moderately alkaline soils (pH 7.5 to 9.0) (Bharadawaj 1975). Delayed nodulation and decreased yields have been obtained when the pH of the soil increased beyond 9.0. Most genotypes of lentil are very sensitive to soil salinity especially in the irrigated lentil growing areas, this become a major constraint in obtaining proper yields.

1.2.6 Chemistry of lentil

Lentil has been regarded as a clean crop, relatively free from anti-nutritional factors and low flatulence. Protein concentration in lentil ranged from 22 - 35% and provides energy of about 353 Kcal whereas eggs, fish and chicken provide an amount of 181, 97 and 109 Kcal of energy respectively. Among the dried vegetables lentil is second only to the soybean in protein content. It has been investigated that about 90% of the proteins in lentil is present in the cotyledons, 4% in the seed coat and 3% in the embryo (Singh et al. 1968). Among the cool season legume crops, lentil is the richest in the important amino acids (lysine, arginine, leucine and sulphur containing amino acids)

(Williams et al. 1994). Lentil is deficient in two essential amino acids, methionine and cysteine (http://www.ag.ndsu.edu/pubs/alt-ag/lentil.htm). However, sprouted lentils contain sufficient levels of all essential amino acids, including methionine and cysteine(http://www.bitterpoison.com/protein/11248). In addition, it is high in carbohydrate, calories, fibre, vitamin A, calcium, starch, iron, phosphorous, copper and manganese (Table 3). Lentil is a very good source of cholesterol- lowering fibre and is rich in dietary fibre, both the soluble and insoluble type. Lentils also have "anti-nutritional factors" such as trypsin inhibitors and relatively high phytate content. Trypsin is an enzyme involved in digestion and phytates reduce the bioavailability of dietary minerals. The phytates can be reduced by soaking the lentils in warm water overnight.

1.2.7 Human consumption

People like eating lentil as evidenced by production increases from about 1 million tons in 1960 to over 4 million tons to date. Lentil is often eaten as a product "Dhal", which is a split and de-hulled seed used as a main dish, side dish or salads. Only red cotyledon type is used as food in Bangladesh, where it is boiled into soup-like "Dhal" and eaten with flat bread (Roti) or rice. Khichuri is another popular dish, which is made from a mixture of split lentil seeds and rice. Together rice and lentils make a quickly prepared meal that is well balanced nutritionally. It may also be used as side dish or salads. Lentil seeds can also be fried or seasoned. As a good source of cholesterol-lowering fiber, not only do lentils help lower cholesterol, they are of special benefit in managing blood-sugar disorders since their high fiber content prevents blood sugar levels from rising rapidly after a meal. Lentil's nutrition is a contribution to heart health that lies not just in their fiber, but in the significant amounts of foliate and magnesium.

Table 3. Lentils, raw (dry weight) nutritional value per 100 g (3.5 oz); percentages are relative to US recommendations for adults.

Nutrient	Amount	Daily value (%)
Energy	353 calories	
Total Fat	1.1 g	1
Saturated fat	0.2 g	1
Polyunsaturated fat	0.5g	
Monounsaturated fat	0.2g	
Cholesterol	0	0
Sodium	6 mg	0
Potassium	955 mg	27
Total Carbohydrate	60 g	20
Dietary fiber	30 g	120
Sugar	2 g	
Protein	26 g	52
Vitamin C		7
Vitamin B_6		25
Calcium		5
Iron		41
Magnesium		30

Source:

https://www.google.co.in/?gws_rd=cr&ei=kFJvUrGuOseVrgfz7YE4#psj=1&q=lentil+nutrition

Lentil is a good vegetable source of iron. Iron is particularly important for adolescents and pregnant women, whose requirements for it are increased. It is also important for those who have Celiac disease, in whom iron is a common deficiency.

There are some traditional medicinal uses of lentil too. Lentil is supposed to remedy constipation and other intestinal afflictions. In India, Lentil is poultice onto the ulcers that follow smallpox and other slow-healing sores (Duke 1981).

An interesting recent discovery is that lentils may also contain nutritionally significant level of selenium. Plants do not need it, but humans do. Adequate dietary selenium is important for enzyme activity, antioxidants, and protective physiological pathways that are associated with cancer suppression, HIV treatment, and suppression of free radical induced diseases, and protection from toxic heavy metal toxicity. Besides, Lentil is also considered as the main source of protein for livestock feed and inland fish production. Moreover, like other pulses it has the unique ability to fix nitrogen symbiotically in association with *Rhizobium leguminosarum* group of bacteria and thus improve soil fertility.

From the above discussion, it is evident that lentil is highly nutritious. Because of its nutritional value, cooking quality and easy digestibility, the demand for this crop has been steadily increasing in the Indian subcontinent. On a global scale, lentil consumption is rising at a rate more than twice that of the human population growth. Among the cool season pulses, it is by far the fastest growing crop. Many of the others are actually declining. We expect that by 2030, world lentil consumption will be double.

1.2.8 Lentil production in the world and in Bangladesh

In world the major lentil producing regions are Asia and the west Asia-North Africa region. Lentil is the most important pulse crop in Bangladesh and Nepal, where it significantly contributes to diet. In 2012 the world production of lentils was 4.55 million metric tons. World lentil production has been relatively stable over the last 13 years. Global lentil production recently peaked at 4.76 million metric tons in 2010 whereas in Bangladesh, lentil production peaked at 164372 tonnes in the year 2001 (Table 4).

Table 4. World lentil production in the year 2001-2013.

Year	Production (tonnes)		Year	Production (tonnes)	
	World	Bangladesh		World	Bangladesh
2013	4951720	93000	2006	3338487	115370
2012	4706351	80125	2005	4035716	121065
2011	4423901	80442	2004	3588791	122200
2010	4722774	71100	2003	2980187	116000
2009	3931824	60537	2002	2884279	115000
2008	2824688	71535	2001	3252102	126000
2007	3297791	116810			

Source- http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor

Lentil is produced in over 50 different countries. About 97% of total production primarily coming from top 10 lentil producing countries in the world (Fig. 4). Among them Canada, India, Turkey and Australia typically combine to produce about 75 percent of total world lentil production.

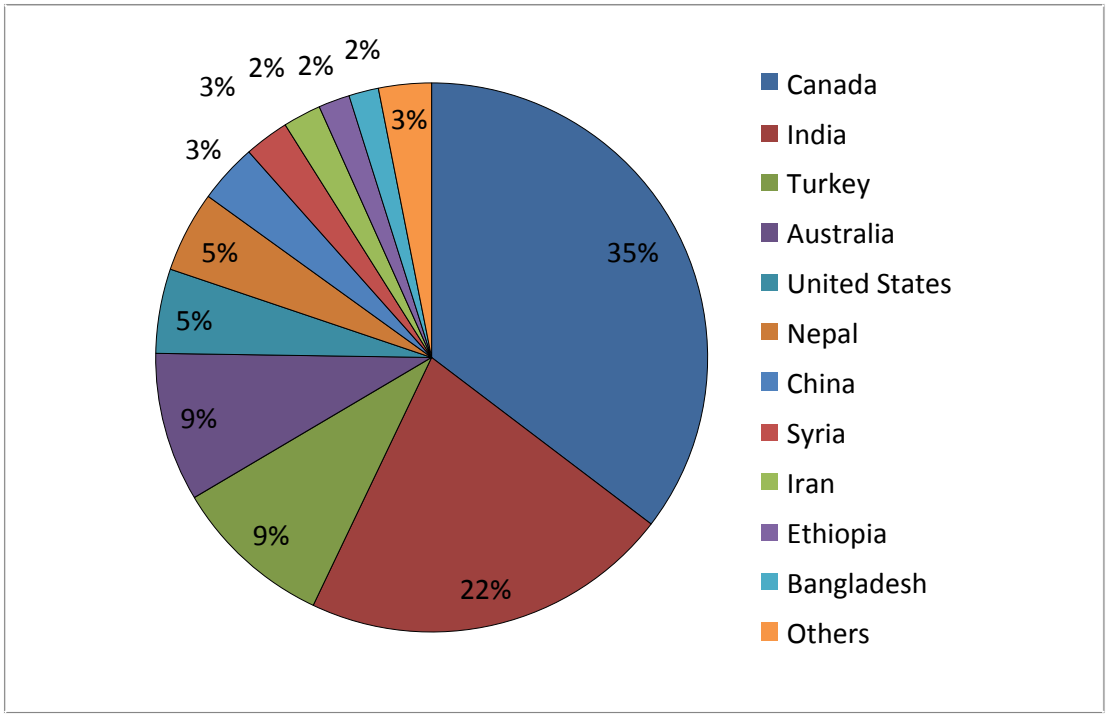


Fig. 4. World lentil production in per cent by country

Source http://www.factfish.com/statisticcountry/bangladesh/lentils,%20production%20quantity

Among the lentil producing countries in the year of 2013, Bangladesh is in tenth position in the world (Table 5). In that year total production of lentil was 93,000 tonnes in an area of 89,840 hectares.

In Bangladesh lentil is largely grown in the northern and northern-southern regions. Beginning in the late 1971's there is a steady increase in the sown area and production of lentil, with the implementation of a utilization of fallow areas. But with the increase

of industry from 1990's the cultivated area and total production begins to decrease (Figs 5 & 6). But with the increase of population and as the main source of dietary protein, the demand of lentil is increasing day by day. Annual demand of lentil in Bangladesh is 1.8 to 2.0 million tonnes whereas average production in last five years (2007 to 2011) was 0.082250 million tonnes per year. For this Bangladesh mostly depends on imported lentil and the trends of lentil import increasing day by day because the local production can meet the country's requirement of two to three months, only 10 - 15 per cent demand is met by local production. Imports increased from 122785 MT in 2003 to 350000 MT in 2011. As a result in last few years Bangladesh stands first as the largest importing country in the world. In 2011 two largest importing countries was Bangladesh (350000MT) and Egypt (300000 MT). According to FAO (2008) Bangladesh ranked first in terms of average import of lentil from the year 2001 to 2005 (Table 6).

1.2.9 Constraints of lentil production - biotic and abiotic stress

Although lentil is considered as an important pulse crop for many parts of the world but its production in most countries is usually characterized by low yield potential. Several factors are supposed to be responsible for the lower production of this important crop which includes susceptibility to disease, pests, fungi, massive flower drop, post harvest loss, and management problem (Erskine 1984). Diseases of lentil at various stages of growth are caused by fungi, bacteria, viruses and nematodes. Among these constraints fungal diseases cause the maximum damage. A total of 17 diseases of lentil (Table 7) have been identified in Bangladesh (Bakr 1994) of which fungal diseases are most devastating and may occur at various stages of development.

Table 5. Top lentil producing country in the world in the year 2013.

Rank	Country	Production (tonnes)
1.	Canada	1880500
2.	India	1134000
3.	Turkey	417000
4.	Australia	324100
5.	United States	227658
6.	Nepal	226931
7.	China	150000
8.	Ethiopia	129833
9.	Syria	125000
10.	Bangladesh	93000

http://www.factfish.com/statistic/lentils%2C%20production%20quantity

Table 6. Top 10 lentil importer in the world in the year 2001-2005 (FAO 2008)

Rank	country	Average import (ton)
1.	Bangladesh	117306
2.	Egypt	97574
3.	Srilanka	93172
4.	Algeria	57288
5.	Pakistan	55078
6.	Colombia	53956
7.	Spain	49990
8.	India	49638
9.	Turkey	31929
10.	Italy	30397

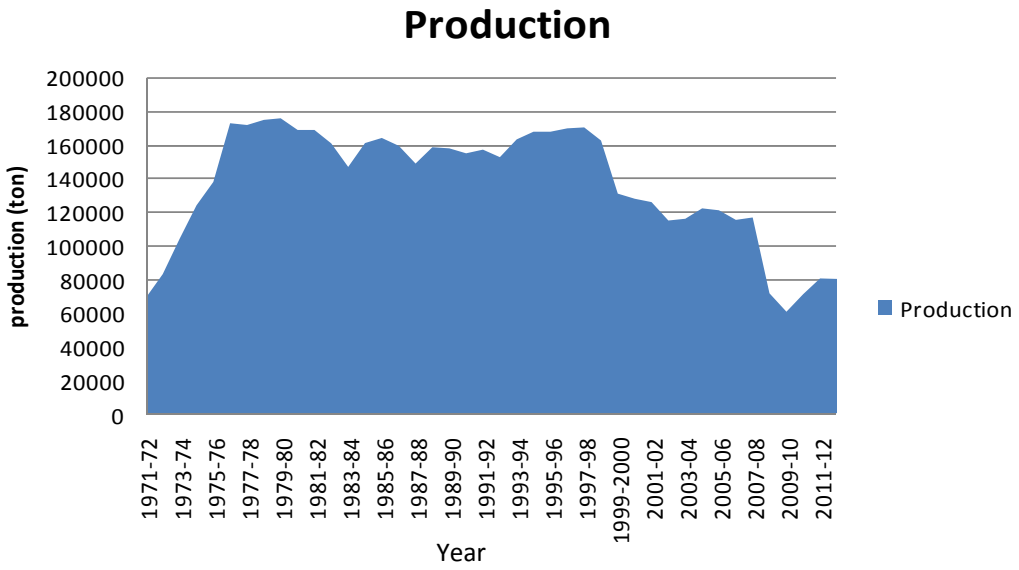


Fig. 5. Lentil production in Bangladesh from 1971 - 2012.

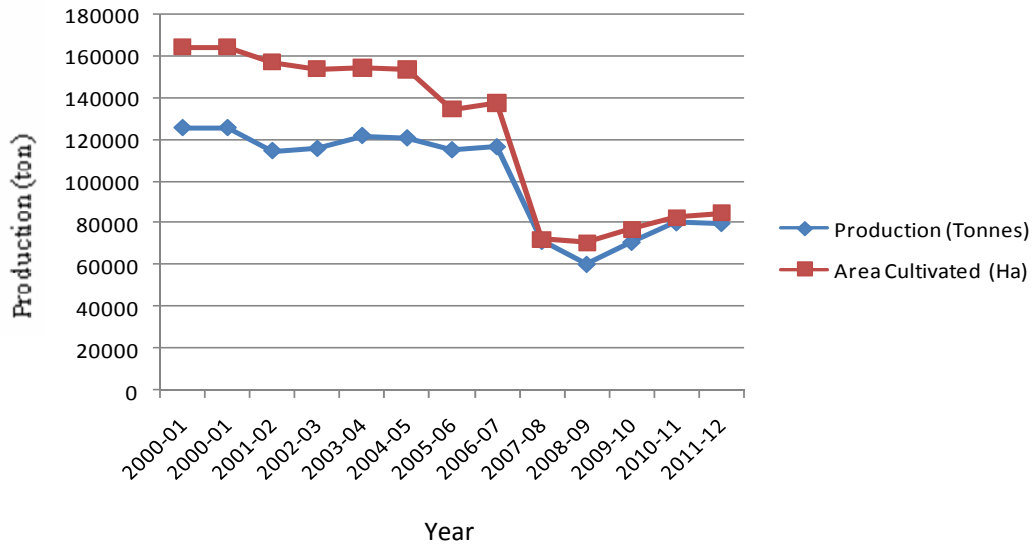


Fig. 6. Lentil production and area cultivated in Bangladesh from 2001 - 2012.

However, among all the diseases rust and stemphylium blight are considered as most frequent. Both diseases can spread rapidly in local cultivars causing in excess of 60% yield reduction. Stemphylium blight is a major threat to lentil in South Asia and North America reported with up to 80% production loss (ICARDA 2004). This pathogen causes a leaf blight, plant defoliation and death.

Rust caused by *Uromyces fabae* is the most important foliar disease of lentil specially in Asia, causes up to 80% to complete yield loss (Beniwal et al. 1993). It is characterized by lesions on the stems and leaves, leaf drop and premature plant death (Ahmad and Moral 1996, Ahmad et al. 1996). Losses from the disease, estimated up to 70% to complete yield loss have been reported. (Erskine and Sarker 1997, Negussie et al. 1998). Collar rot is becoming a serious threat at seedling stage, especially in saturated soil.

Ascocyta blight is also prevailing in the major lentil producing countries, including Argentina, Australia, Canada, Ethiopia, India, Pakistan etc. and this cost about 40% of yield loss (Gossen et al. 1986, Ye et al. 2002, Regan et al. 2006). Ascocyta blight starts with light gray to tan spots occurring on the leaflets, stems and pods, but will have a dark margin around the spot. The centers of the spots turn light-colored and develop small black spots in them. The crops look lighted in appearance.

Fusarium which is responsible for wilt disease produces major economic losses in parts of South America, the Mediterranean basin and South Asia (Erskine et al. 1994, Bayaa et al. 1995). Seedling disease, namely root rot in lentil occurs due to the invasion of sclerotium (Pavgi and Upadhyay 1967) as well as collar rot. While mold of lentil occurs from early flowering to pod setting, usually in highly productive fields with tall, dense stands of lentils. This disease is favored by wet and cool conditions specially on

lower ground where dense canopies usually develop (http.//www.whitemoldresearch. com/HTML/lentil.cfm).

Besides this, several abiotic stresses such as cold, drought, heat, salinity, nutrient deficiency and nutrient toxicity adversely affect lentil yields worldwide (Monti et al. 1994, Saxena 1993, Slindard et al. 1994). Among them temperature and water logging are considered most serious factors (Turner et al. 2001). Low temperature is a factor that limiting production, but is less important than low moisture availability. High temperature is encountered by lentil in major production regions mainly during the reproductive stage of growth. The early stage of vegetative growth is restricted by low radiation and temperature.

Heat stress often accompanies drought causing and their effects on lentil growth and yield. There is general agreement that heat effects the distribution of dry matter to reproductive growth and that high temperatures have been adverse effect on lentil yields.

Salinity problem with lentil is not wide spread but can be acute in certain regions of Indian sub-continent, the Nile delta of Egypt and in some areas in Turkey. Canada also has some difficulty in high salinity areas of Saskatchewan. Of the legumes, lentil is more salt sensitive compared to faba bean and soybean (Katerji et al. 2001). Salt stress can adversely affect nodhulization and N_2 fixation (Rai and Singh 1999, Rai et al. 1985) presumably by restricting growth of the root hairs and the potential sites of infection by *Rhizobium.*

Nutrient deficiencies and toxicities are of lesser importance worldwide but important in localized regions. The acute yellowing symptomatic of iron deficiency is introduced in some areas of Indian sub continent and Ethiopia (Erskine 1997).

Table 7. Major biotic constraints of lentil production in Bangladesh (Bakr 1994).

Serial no	Biotic constrain	Causal agent
Fungi		
1	Ascochyta blight	*Ascochyta lentis*
2	Fusarium wilt	*Fusarium oxysporum* f. sp. *lentis*
3	Collar rot	*Sclerotium rolfsii*
4	Root rot	*Rhizactonia solani*
5	Stem rot or blight	*Stemphylium sarciniformae*
6	Rust	*Uromyces fabae*
7	Powdery mildew	*Erisiphe polygoni*
8	Downy mildew	*Pernospora lentis*
9	Anthracnose	*Colletotrichum truncatum*
Insects		
10	Leaf weevil	*Episomus lacerta*
11	Cutworms	*Psodoptera axigua*
12	Pod borers	*Heliothis armigera*
13	Aphids	*Aphis craccivora*
14	Bugs	*Nezara viridula*
15	Grain beetle	*Callosobruchus anails*
16	Thrips	*Megaluro-thrips distalis*
17	Bruchids	*Callosobruchus chinensis, C. maculates*

Boron toxicity has also been problematic and therefore appears to be some tolerant germplasm (Yau and Erskine 2000). Moreover, pH of soil also plays an important role in lentil growth and nutrient availability.

Weeds are also a playing factor to reduce lentil production. Weed control in lentil is important because lentil is relatively non-competitive crop and is characterized by having short shoot and does not form a dense canopy until flowering. Most annual grass and broadleaf wood species can compete effectively with lentil throughout the growing season. Reduction in yield due to weed competition was estimated to be 20 - 30% and the critical period lies between and 30 - 60 days after sowing (Khatib et al. 2007). Herbicides for the control of certain broadleaf weeds (Canada thistle, perennial sow thistle and dandelion) either are not available or provide less than acceptable control. Lentils must be sown to fields free of difficult to control perennial weeds such as Canada thistle, perennial sow thistle.

1.2.10 Problem associated with the improvement of lentil through conventional breeding

From the above discussion it is clear that the yielding ability of existing lentil varieties is not adequate. Thus to obtain desired performance, improvement of this crop is essential. There is a need to increase productivity, enhance the nutritional value and other desired agronomic quality of this crop. Cultivars resistant to biotic and abiotic stresses and better protein quality and quantity are required. Improvement of this cannot be achieved without the incorporation resistant genes against various biotic and abiotic stresses (Agrios 1997).

Conventional breeding methods including hybridization techniques and selection are being carried out to develop improved varieties of many crops with high stable yield, better quality, wide adaptability and disease resistance. In the past, several attempts have been undertaken to develop disease resistant as well as high yielding varieties of lentil through distant hybridization and mutation breeding. But in lentil, the progress of improvement through conventional breeding is hampered due to lack of genetic variability which caused by a predominantly high degree of self pollination and absence of resistance gene/s of interest in the existing germplasm. Moreover, crossing in lentil is tedious because of small size of flowers. Moreover, hybridization between *microsperma* and *macrosperma* is hampered due to asynchronous flowering. Genetic variation towards the improvement of any crop can also be obtained through introduction and gene mutation. But all attempts in making genetic variability in lentil through introduction, selection, hybridization and mutation did not yield the desired results. Several other important traits, such as biomass yield, pod shedding, nitrogen fixation, resistance to pea leafweevil (*Sitona* sp.), aphids, and the parasitic weed broomrape (*Orobanche* sp.), cannot currently be addressed by breeding because of insufficient genetic variation.

1.2.11 Application of biotechnology for improvement of lentil

Since conventional breeding has several limitations it is imperative that we have to look for some other methods to induce genetic variability in lentil. Beside the conventional breeding techniques, there are many ways of creating variability including induction of somaclonal variation through tissue culture, somatic hybridization and genetic engineering. According to Scowcroft et al. (1987) tissue

culture technique can play a significant role for enrichment of genetic variability by creating variation (somaclonal variation) or mutation (by applying radiation or chemical mutagens to *in vitro* cultured plant materials) at an unexpectedly high rate and may be novel sources of genetic variability in many plant species. But these were found to have a limited application in many crop species including lentil. It is therefore; genetic engineering technique can be a method of choice and be utilized effectively in developing desirable breeding lines of lentil.

With the recent advances in genetic engineering of plants, it is now feasible to introduce genes into crop plants. Genetic engineering thus has broadened the genetic variability in certain cases where the natural variability within a species is not sufficient. Under these circumstances modern techniques of biotechnology, commonly known as “genetic transformation”, can be applied for the improvement of the crop.

Genetic transformation, a comparatively new and exciting tissue culture base technology, has opened new ways for the use of recombinant DNA technology and useful in completing the conventional breeding programs. Moreover, this approach can accelerate the development of new plant varieties which is not possible through breeding and tissue culture alone (Gardner 1993). Genetic transformation is a tissue culture based technology that offers the potential for the introduction of defined genetic information from any organism (related or unrelated plant or animal species) into existing elite plant lines. Such crops evolved by this transgenic technique are called genetically modified or GM crops. This technology is regarded as a pre-breeding method that can provide a solution to certain constraints that limit crop production and quality. Since 1987, numerous potentially useful transgenic plants of cotton, maize, potato, tobacco, rapeseed, raspberry, soyabean, pea, tomato, rice, Hawaiian papaya,

squash (*Zucchini*), sugar cane, sugar beet etc. have been generated (Fisk and Dandekar 1993). Notably, Bangladesh approved Bt brinjal/eggplant for the first time on 30 October 2013, and in record time - less than 100 days after approval - commercialization was initiated on 22 January 2014 when 20 very small farmers planted their first crop of Bt brinjal; a total of 120 farmers planted 12 hectares of Bt brinjal in 2014. Innate™ potato, another food crop, was approved in the US in November 2014. Also in November 2014, a new biotech alfalfa (event KK179) with up to 22% less lignin, which leads to higher digestibility and productivity, was approved for planting in the US. The global hectarage of biotech crops has increased more than 100-fold from 1.7 million hectares in 1996 to 181.5 million hectares in 2014 (ISAAA Brief 49-2014: Executive Summary). In 2014, countries that grew 95% of the global transgenic crops were the United States (40%), Brazil (23%), Argentina (13%), India (6%), Canada (6%), China (2%), Paraguay (2%), Pakistan (1.5%) and South Africa (1.5%).

Since genetic transformation is a tissue culture based technology thus a reproducible, reliable transformation system could enable us to insert genes of interests in lentil lines, which are unavailable in present lentil genotypes. Thus, it might be possible that, genetic transformation method combined with traditional breeding techniques, could aid in improving both the quality and yield of lentil.

Transformation of higher plants has been accomplished by different methods (Grasser and Fraley 1989). For example, direct methods, such as particle bombardment, microinjection, electroporation, chemical method and indirect method such as, *Agrobacterium*-mediated genetic transformation method have been used for transformation. In dicotyledonous species the most efficient method used for the

transfer of desired foreign genes is through the soil bacterium *Agrobacterium tumefaciens* (Grant et al. 1998, Zambyski 1992).

The key transformation events in grain legumes can mainly be focused on *Agrobacterium*-mediated transformation, biolistics for gene delivery, electroporation and /or polyethylene glycol (PEG) treatment. Stable transformation has been reported for a number of legumes using common indirect transformation method with *Agrobacterium tumefaciens* or *A. rhizogenes*, otherwise direct gene transfer method like particle bombardment (Gulati et al. 2002, Masood et al. 1996, Öktem et al. 1999) and electroporation of protoplasts (Christou 1997, Atkins and Smith 1997), PEG-mediated gene transfer (Bohmer et al. 1995, Maccarrone et al.1995).

1.2.12 Worldwide plausible transformation studies on lentil

It is believed that biotechnology will lead the next revolution in agriculture and sustainable economic development. Biotechnology industry has used the issue of world hunger as a cornerstone (White et al. 2004) and legumes are playing one of the main roles to minimize world hunger. It may be mentioned here that though lentil is a source of dietary protein and is an important pulse crop still there are limited literature on lentil genetic engineering. Among the pioneers of *Agrobacterium*-mediated transformation of lentil was Warkentin and McHughen (1991). In this report of lentil transformation they showed that four diverse strains of *Agrobacterium tumefaciens* i.e. C58, Achh5, GV3111 and A281 were capable of inducing tumors at a high frequency on inoculated stems of lentil *in vivo*, and on excised shoot apices *in vitro*. Tumor formation and opine production are indicative of plant cell transformation.

Virtually in all of the *Agrobacterium*- mediated transformation studies (Warkentin and McHughen 1992, 1993, Lurquin et al. 1998, Oktem et al. 1999, Mahmoudian et al. 2002) p35GUSINT vector containing kanamycin resistance gene (NOS-NPTII-NOS) and intron containing GUS gene was used. The vector was regenerated by cloning GUS gene cassette with cauliflower mosaic virus 35S promoter and terminator from pGUSINT plasmid into Stratagene pBS vector (Vancanneyt et al. 1990).

As an initial step for developing of transgenic plants, it is useful to demonstrate that tissues of those species are which capable of expressing a transferred reporter gene. Lentil shoot apex, epicotyle, and root explants were capable of expressing an intron containing GUS gene after inoculation with the disarmed *Agrobacteriun* strain. Expression occurred at all wound sites on these explants except at the end of root explants proximal to the cotyledonary node (Warkentin and McHughen 1992).

Lentils seedling root protoplasts were tested for transient expression system through electoporation and PEG treatment (Maccarrone et al. 1995). Transient GUS activity has been detected in lentil protoplasts and cotyledonary nodes following the delivery of the gene *via* liposomes (Maccarrone et al. 1992) or particle bombardment (Oktem et al. 1999). Chowrira et al. (1995, 1996) in their studies used pea, soyabean, and cowpea beside lentil. The technique applied was electoporation-mediated transformation and they have used intact nodal meristems as explants and GUS as a reporter gene. The shoot developed from the nodes was chimeric but they formed successfully transgenic seeds. GUS expression has also been observed after inoculation of longitudinary sliced embryogenic axix of lentil with different *Agrobacteriun* strains (Lurquin et al. 1998).

Lentil cotyledonary nodes are some of the most regenerative tissues in legumes. Potential of lentil cotyledonary node explants for transformation by *Agrobacterium* was

investigated by Warkentin and McHughen (1993). In this study octopine-type strain GV2260: 35SGUSINT was used and effect of wounding is also examined. In another study of Oktem et al. (1999) cotyledonary node explants were subjected to particle bombardment. Circular pBSGUSINT was used for transformation. Eighty per cent of the bombarded tissues expressed GUS gene and 2% of the shoots emerged from cotyledonary nodes were found to express patches of GUS staining.

As vacuum infiltration has been reported to enhance transformation frequency in *Agrobacterium*-mediated gene transfer in some legumes (Trieu et al. 2000), attempts were made to transformation them by vacuum infiltration was also used. The first report of a vacuum infiltration *Agrobagterium*- mediated transient expression system on lentil cotyledonary nodes was by Mahmoudian et al. 2002. In this study transformation of cotyledonary node explants was infected by *Agrobacterium* strain GV2260::pGUSINT. Results showed that infiltration increases the efficiency of transformation but low number of regenerated shoots exhibit GUS expression. There was no reported transgenic lentil plant in any of these studies.

The only plausible report about genetically engineered lentil was published by Gulati et al. in 2002 with viable F_1 inheriting the transgene with Mendalian ratio. Lentil cotyledonary nodal segments were transformed by bombardment of pBUC19 plasmid with chimeric SuRA/SuRB hra actolactate gene (ALS) from tobacco, conferring resistance to sulfonylurea herbicides.

Bayrac (2004) investigated the regeneration of several tissues on various media *via* indirect organogenesis. He then carried out *Agrobagterium*-mediated transformation using peeled cotyledonary nodes, cotyledonary petiols, shoot tips and roots as expalnt.

Shoot tips showed the highest percentage of GUS expression. Root formation was only achieved in media with NAA/IAA.

Dogan et al. (2005) compared tumor and root formation ability of several tissues from different lentil cultivars after inoculation with *Agrobagterium rhizogens* and *Agrobacterium tumefaciens* strains. The frequency of tumor formation from cotyledonary node explants was higher compared to shoot meristems. Rooting was only observed in cultivar Erzurum 89 while *Agrobagterium rhizogens* mediated hairy roots were induced only in dark.

Production of disease resistant lentil would help to increase its production as it is susceptible to many biotic stresses. Fungus resistant lentil was developed by Hashem (2007) by transforming decapitated embryos with one cotyledon with Ri-pgip gene coding polygalacturonase inhibitory protein, conferring resistance against fungal pathogens followed by an optimized regeneration system, with a very high transformation efficiency of about 35%. Micrografting was used for rooting transformants.

For optimization of lentil transformation, Akcay et al. (2009) used a combination of several treatments with three *Agrobacteriun tumefaciens* strains i.e. EHA 105, C58C1 and KYRT1 to deliver T-DNA into cotyledonary node tissues. As compared to EHA105 and C58C1, KYRT1 was found about three folds more efficient for producing GUS expression on cotyledonary petiols. Fertile transgenic plants were obtained through grafting transgenic shoots on rootstocks. The transgenic insertion and expression were confirmed through PCR, RT-PCR and Southern hybridization, and the transgene were segregated in Mendelian fashion.

Recently, in order to enhance drought and salinity tolerance, Khatib et al. (2011) introduced DREB1A gene driven by the rd29A promoter into lentil decapitated embryo explants followed by shoot regeneration from the apical meristems of cotyledonary buds *via* direct organogenesis. Subsquently basta-resistant putative transgenic explants were micro-grafted onto non-transformed rootstocks to establish transgenic plants. Transgenic insertion and inheritance to the progeny were evaluated through PCR and Southern blot analysis. Expression of DREB1A gene in transgenic plants was introduced by salt stress was confirmed through RT-PCR.

During production of transgenic lentil, micro-grafting has been extensively used to recover transformed plants and there is a need to develop an efficient and reproducible regeneration system. Without passing through the laborious system of micro-grafting. In 2011 Chopra et al. developed a simple and genotype independent *in vitro* regeneration system of lentil capable of root induction. They then used it for transformation using sonication-assisted *Agrobagterium tumefaciens* (SAAT) transformation. A supervurulent *Agrobagterium tumefaciens* strain EHA 105 was employed for transferring the T-DNA containing *npt*II and *uidA* genes into whole seeds using sonication and vaccum infiltration, and 40% of the kanamycin resistant shoots produced through direct shoot organogenesis were able to produce root on a medium with IBA and kanamycin. Transgenic insertion and activity in leaves and roots were detected by PCR and GUS histochemical assay. Examples of some recent reports on gene transfer in lentil *via Agrobacterium*-mediated transformation are listed below (Table 8).

Table 8. Recent studies on *Agrobacterium*-mediated genetic transformation in lentil (Atif et al. 2013)

Agrobacterium strain	Explant	Gene used	Transformation efficiency (%)	Reference
C58, EHA 105	Half embryo	GUS	16.3 - 41.2 based on transient expression	Lurquin et al. (1998)
GV2260	Cotyledonary node	*npt*II, GUS	95 based on transient expression	Mahmoudian et al. (2002)
LBA4404	Cotyledonary node (CN), decapitated embryo (DE),	*npt*II, GUS	0.2 (DE)	Sarker et al. (2003)
A281, A15834	Cotyledonary node, epicotyle, shoot meristem	GUS	0 - 90 based on tumour production	Dogan et al. (2005)
EHA101	Half embryo attached cotyledon(HE), decapitated embryo (DE)	GUS	25-60 (HE), 80-100 (DE), based on transient expression	Hassan et al. (2007)
AgL0	2 days old seedlings	GUS, bar	0.11 - 0.43	Khatib et al. (2007)
EHA105, C58C1, KYRT1	Cotyledonary node	*npt*II, GUS	2.3	Akcay et al. (2009)
EHA105	Whole seed	*npt*II, GUS	0.9	Chopra et al. (2011)

(http://www.springer.com/cda/content/document/cda_downloaddocument/9783642309663c1.pdf?SGWID=0-0-45-1350313-p174514894.)

1.2.13 Strategies for the development of fungal disease resistance transgenic plants

Fungal diseases are rated as one of the most important factors contributing to yield loss in lentil. It can partly be controlled by wide spread application of fungicide. The economic cost and negative environment impact associated with this application have led to recent search for alternative strategies. As conventional breeding does not offer satisfactory results quickly, the heterologous expression of broad spectrum antifungal hydrolytic enzyme that enhances defense system might offer a solution. According to Punja (2001) and Grover and Gowthaman (2003) overall antifungal transgenic approaches can be grouped into seven categories (Islam 2006) such as follows:

1. Expression of genes, e.g. chitinase and glucanases in the plant cells that cause hyphal lysis thereby inhibiting fungal growth.
2. Over expression of genes that produce compounds e.g. pathogenesis related proteins (PR) and phytoalexins which are directly toxic to pathogens or reduce their growth.
3. Expression of gene products e.g. peroxidase and lignins, that can potentially enhance structural defense in the plants.
4. Expression of resistant gene (R) products involved in hypersensitive response (HR), for their interaction with avirulence (Avr) gene.
5. Expression of genes e.g. elicitor, hydrogen peroxidase (H_2O_2), salicylic acid (SA), and ethylene (C_2H_4), that regulate signals to control plant defense.
6. Binding of inactivation of fungal toxins thus stopping invasion of fungus by expression of R gene.
7. Other strategies are production of RNAi, RNase and Isozymes. In such cases genes isolated from sources apart from plants are exploited.

1.2.13.1 Application of chitinase gene in developing fungal disease resistance

Among various fungal resistant genes, chitinase genes are potentially most promises as the enzymes degrade the substance chitin found in fungal cell wall and they showed activity against a wide range of fungus (Lorito 1998). Hence, genetic engineering of plants with chitinase gene is attractive for fungal disease control mechanism. Chitinase also known as poly [1, 4-N- acetyl-D-glucosamine] glycan hydolase is a low molecular weight pathogenesis related (PR) protein that is often extracellular, acid soluble and protease resistant. It catalyzes the hydrolysis of chitin (β-1, 4 linked polymer of N-acyl -D- glucosamine), which is a major component of the cell wall of most fungi. Chitin [$(C_8H_{10}NO_5)_n$] is one of the most abundant natural polymers next to cellulose [$(C_6H_{10}NO_5)_n$], consists of linear chains of β-1,4 linked sugar residues (Fig. 7).

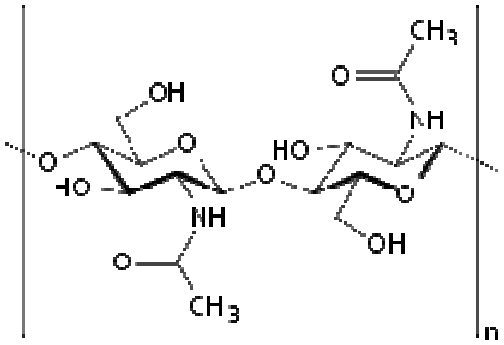


Fig. 7. Chemical structure of chitin. (http://en.wikipedia.org/wiki/Chitin)

Chitin is found in the cell walls of fungi (20%), exoskeleton of arthropods (30%), the shells of crustaceans such as crabs, lobsters, and shrimp (70%), nematodes and insects (37%). It has different developmental, morphological and physiological roles like degrading the old cuticle of insects and crustaceans, pathogen related defense in higher plants, nutritional and parasitism roles in bacteria and fungi, pathogen defense in human and daughter cell separation in yeast (Carstens et al. 2003, Renkema 1995).

Chitinase is classified accoding to its mode of action into two classes i.e.

- Endochitinase (EC 3.2.1.14) which cleave and hydrolyse chitin randomly at internal sites of β -1,4-glucoside bonds producing chitotetrose, chitotriose and diactylechitobiose.
- Exochitinase which has activity on the non-reducing end of the chitin chain and has two subclasses of chitobiosidases (EC 3.2.1.29) and β-1,4-N-acyle glucosainidases (EC 3.2.1.30, which now includes EC 3.2.1.52 as b-L-N-acetylhexosaminidases) cleaving the oligomeric products of endochitinases and chitobiosidases and thus generating monomers of GlcNAc. There is another pathway where chitin deacetylase converts chitin to chitosan, which is degraded by chitonase (EC 3.2.1.132) to glucosamine residues as demonstrated in Fig. 8 (Dahiya 2005, Li 2000).

Chitinase also classified according to amino acid sequences and similarity of catalytic domains into three families 18, 19 and 20. These families are different in the 3D structure. Family 18 contains endochitinase (classes III and V). Family 19 contains plant chitinases (classes I, II and IV) and *Streptomyces* chitinase. Familly 20 contains chitinase from human, *Dictostelium discoideum,*and *Vibrio harveyi*. Most chitinases consist of the following main domains: signal peptides, catalytic domain, chitin-binding domain and fibronectin type III domain. Class I is plant chitinase with a cystein rich domain (Chitin binding domain) at N- terminus and found in rice, tobacco and potato. Class II is mainly found in plants (*Arabidiopsis*, barly and tobacco), fungi, and bacteria and has a similar structure to class I but missing the cystein rich domain and N-terminus. Class III has similar regions to prokaryotic chitinases and found in cucumber.

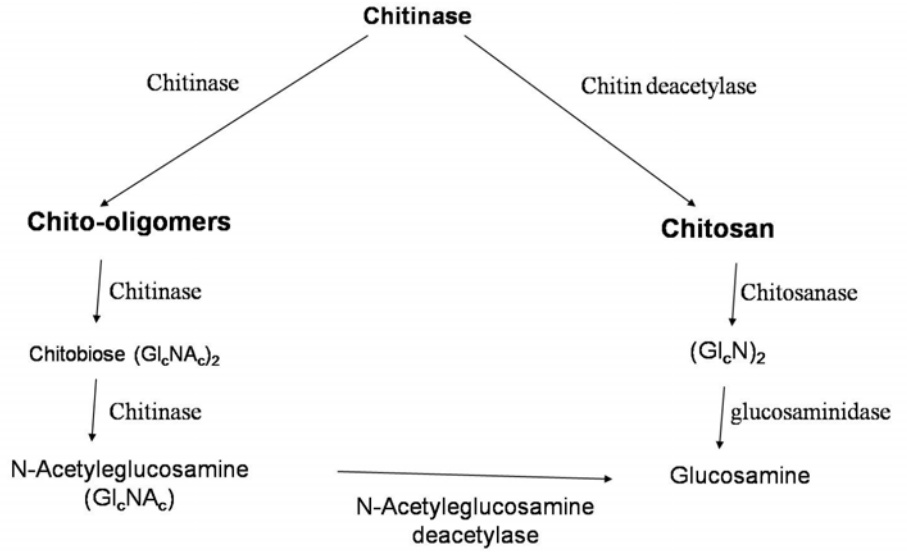


Fig. 8. Chitin degredation pathways in nature (Dahiya 2005, Li 2000).

Arabidiopsis, tobacco and chickpea. Class IV has same structure as class I but it is smaller due to deletions in cystein-rich domain and catalytic domain and found in bean, sugar beet and rape. Class V is similar to bacterial chitinase and found mainly in tobacco (Fukamizo 2001, Neuhaus 1991, Nagasaki 1997, Patil 2000). Some chitinases have two chitin-binding domain like *Brassica juncea* chitinase (Chye 2005, Van den Bergh et al. 2004) and *Aeromonas* sp. No. 10S-24 familly 19 chitinase (Kojima et al. 2005).

As chitinase are one of the most important protein families showing antifungal activity *in vitro*, several reviews and research article have stressed the advantages of using chitinase gene into plants to enhance resistance to fungal pathogens which are summarized in Table 9.

Table 9. Reports on different crops transformed with chitinase gene

Name of the gene	Source	Vector	Host plant	Reference
chi 1	*Rhizopus oligosporous*	pBI121CH	Tobacco	Terakawa et al. (1997)
Chi	Tobacco	pBI121-pBtex	Peanut	Rohini and Rao (2001)
Chi	Bean	pBRC	soyabean	Li et al. (2004)
Chi	Bean	pBI121-BCH	Cotton	Tohidfar et al. (2005)
Chi11	Rice	pNSP3	Rice	Sridevi et al. (2008)
Chit30	*Streptomyces olivaceoviridis*	pGII 0229	Pea	Hassan et al. (2009)
Chit383	Wheat	pCAMBIA1300	Carrot	Wally et al. (2009)
Chit42	*Trichoderma atroviride*	pBIKE3	Potato	Esfahani et al. (2010)
Chi 11	Rice	pHyg-Chi.1	Finger millet	Ignacimuthu and Ceasar (2012)
Chi	-	pBI121	Sugarcane	Khamrit et al. (2013)

1.2.13.2 Application of Antifungal Protein (AFP) gene in developing fungal disease resistance

Antifungal proteins from different sources showed *in vitro* activity by inhibiting hyphal and fungal growth as reported by many scientists (Asao et al. 1997, Bolar et al. 2000, Rajasekaran et al. 2000). Antifungal proteins consist of pathogenesis related (PR) proteins, ribosome inactivate proteins (RIPs) where plant RIPs inactive foreign ribosomes by removing an ademic residue from 28S rRNA. So the 60S ribosomal subunit cannot bind to elongation factor 2 resulting in protein elongation inhibition, small cystein rich proteins such as chitin-binding proteins, plant defensins, heveins and theonins, lipid transfer proteins (LIPs). Storage albumins have a dual role in storage

and defense, polygalactouronase inhibitor proteins (PGIPs), antiviral proteins and non plant antifungal proteins (Faize et al. 2003, Gao et al. 2000). The introduction of AFP gene into plants to enhance resistance to fungal pathogens is shown in Table 10.

Table 10. Reports on different crops transformed with AFP gene

Name of the gene	Source	Vector	Host plant	Reference
AFP	*Aspergillus giganteus*	pCAMBIA1300	Rice	Coca et al. (2004)
AFP-PIN	Prawn	pPin 35S	Finger millet	Latha et al. (2005)
alfAFP	Alfalfa	pEAFP	Tomato	Chen *et al* (2006)
ap24	Tobacco	Phap24	Strawberry	Vellicce et al. (2006)
AFP	*Aspergillus giganteus*	p35SAcS	Pearl milllet	Girgi et al. (2006)
AFP-PIN	Prawn	pPin 35S	Pearl milllet	Latha et al. (2006)
alfAFP	Alfalfa	pEAFP-Chi	Tomato	Chen et al. (2006)
Ca-AFP	Chickpea	pCAMBIA1301	Tobacco	Islam et al. (2007)

1.2.14 An overview of lentil transformation and regeneration problem in lentil

In vitro regeneration and genetic transformation procedure for lentil are not well established compared to the success achieved in other grain legumes from Europe and North America. *Agrobacterium*-mediated genetic transformation in lentil is hampered due to lack of proper regeneration system. In the last 20 years, techniques were progressively improved, the first partial success being reported by Bajaj and Dhanju (1979). They obtained *in vitro* lentil regeneration from meristem tips. Later Williams and Mchughen (1986) described a protocol for regeneration of lentils from the hypocotyle and epicotyle derived callus cells. Upon exploration of high regeneration

capacity of lentil explants, high concentration of cytokinin is used in tissue culture media. However, during the rooting procedure it was understood that the impact of cytokinin usage was depressive to root regeneration. This issue was adequately covered in the study by Polanco and Ruiz (1997) that showed the effect of BAP on root formation from lentil shoots regenerated on BAP containing media. Although Sarker et al. (2003) got a small number of roots from the regenerated shoots produced from BAP containing media. In this case root developed when high concentration of auxin is used. But these induced roots did not come from the base of the cut end and after some days it behaved abnormally, callus deposited at the tip of the roots and afterwards the shoots failed to survive.

This dilemma of rooting problem versus the demand for higher number of putative transgenic shoots from transformation studies of lentil were clearly resolved by Gulati et al. (2001). In their study, lentil shoots regenerated on BAP containing media were micro-grafted onto 5 - 6 days old rootstocks with 96% success efficiency. The success of micro-grafting was noted to be indepented of the nature and concentration of growth regulator used in shoot initiation medium and the time period for induction of shoots. In a similar study by Khawar and Ozcan (2002) cotyledonary node explants from 21 different lentil genotypes were cultured on MS medium containing 0.225 µM TDZ. The regenerated shoots were reported to be micro-grafted with 100% efficiency.

Although the above reports give micro-grafting success as high as 100% efficient but in regeneration procedure micro-grafting is not that much easy. To maintain success a rate above 90% there should be a perfect match between the stock and scion. Still there are other factors associated with micro-grafting can crackdown such high rates of regeneration success. Therefore, success of 100% through micro-grafting is possible as

long as one have either very low number of samples or you have achieved the perfect match between the rootstocks and the scion. Also another important step that is strongly tied to micro-grafting is acclimatization, which is usually introduced drastically decline the success rates of micro-grafted based regeneration. Regarding the factors involved in regeneration through micro-grafting, type of grafting, grafting stem height and the scion health were analyzed in a study of Kamci (2004).

Another main problem of lentil transformation is related with the choice of suitable expalnts. It was shown that the explants which is very good response in regeneration system, showed low response in transformation studies. Sarker et al. 2003 observed in a study that cotyledonary node explants showed above 90% of shoot regeneration but no recovery of transformed shoots in transformation studies. Whereas decapitated embryo showed comparatively less response in regeneration system but showed very high response in transformation studies.

Besides these, another most important difficulty in legumes is integrated of foreign gene. Various methodologies that are utilized for the introduction of foreign DNA into leguminous crops also illustrate the fact that no single technique is optimal for the transformation of all legumes because of the species and frequently cultivar specify of the methods (Atkins and Smith 1997). The transformation efficiency is also low and most of this plant transformation rate may not exceed 1% of transformation efficiency.

Recently limited progress has been made both in regeneration and transformation of microsperma lentil varieties of BM-1, BM-2, BM-3 and BM-4 cultivated in Bangladesh (Sarker et al. 2003a, b, Hassan et al. 2007). Reporter gene like GUS and neomycin phosphotransferase (*npt*II) has been reported in local lentil varieties but recovery of the transgenic plants is still very limited due to lack of genotype independent *in vitro* regeneration system.

Objectives of the present study

Based on this study, the objectives of the present investigation were

1. Development of genotype independent *Agrobacterium*-mediated genetic transformation system for local varieties of lentil using selectable and screenable marker genes.
2. Introduction of desired fungal disease resistance gene constructs into microsperma varieties of lentil.
3. Generation of pre-breeding lines for lentil with resistance to fungal diseases prevalent in Bangladesh using genetic transformation techniques developed during the project.

2. MATERIALS

2. MATERIALS

2.1 Plant materials

Four varieties of lentil (*Lens culinaris* Medik) were used in the present study. These lentil varieties were: i. Barimasur -1 (BM - 1), ii. Barimasur - 4 (BM - 4), iii. Barimasur -5 (BM - 5) and iv. Barimasur - 6 (BM - 6). The seeds of these varieties are presented in Fig. 9. These varieties are belongs to microsperma sub species with chromosome number, 2n=14.

2.1.1 Source

Seeds of these four varieties of lentil (*Lens culinaris* Medik) were collected from Pulse and Oilseed Research Division of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. The materials were stored and maintained in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka.

2.1.2 Descriptions

The important characteristics of these four varieties of lentil are described below:

i. Barimasur - 1 (BM - 1)

This variety was released by the National Seed Board of Bangladesh in 1991 for commercial cultivation. The plant is medium statured (45 - 50 cm), semi erect cultivar with basal primary branch. Leaves are dark green in color with slightly pubescence. Flowers are white; the pods and leaves turn to straw after maturation. Its seed coat is ash in color and testa pattern is dotted with smooth seed surface and cotyledon is red in

color. Duration of life cycle is 105 - 110 days and average production is almost 1700 kilogram/hectare.

ii. Barimasur - 4 (BM - 4)

This variety was released by the National Seed Board of Bangladesh in 1996 for commercial cultivation. This is semi erect and medium statured cultivar with plant height of 42 - 44 cm. The plant is dark green in color; flowers are white; and the pods and leaves turn to straw color after maturation. Size of the seed is larger than that of local varieties, slightly flattened and reddish brown in color. Seed coat color is dark grey and cotyledons are bright orange. Duration of life cycle is 105 - 115 days and average production is almost 2000 kilogram/hectare. Amount of protein and carbohydrates are 25.8% and 59.8% respectively.

iii. Barimasur - 5 (BM - 5)

This variety was released by the National Seed Board of Bangladesh in 2006 for commercial cultivation. It is a semi erect and medium statured cultivar with plant height of 38 - 40 cm. The plant is dark green in color, bushy and small tendrils are present at the tip of leaves. Flowers are blue in color; seeds are larger than local varieties in size, slightly flattened and reddish brown in color. Maturation period is 110 - 115 days and average production is almost 2200 kilogram/hectare. Amount of protein and carbohydrates are 26% and 59.8% respectively.

iv. Barimasur - 6 (BM - 6)

This variety was released by the National Seed Board of Bangladesh in 2006 for commercial cultivation. It is a semi erect, medium saturated and bushy cultivar with

plant height of 35 - 40 cm. The plant is dark green in color; flowers are light blue. Seeds are larger in size compared to the local varieties, deep brown in color and cotyledons are bright orange in color. Maturation period is 110 - 115 days and average production is almost 2500 kilogram/hectare. Amount of protein and carbohydrates are 26% and 59.8% respectively (Alam et al. 2010).

2.1.3 Explants

Seven different types of explants, namely cotyledonary node (CN), decapitated embryo (DE) and cotyledon attached decapitated embryo (CADE)**,** cotyledon (C)**,** hypocotyl (Hypo)**,** nodal segment (NS) and shoot tip (ST) were used in the present investigation. These explants are shown in Fig. 10.

2.2 *Agrobacterium* strain and vector plasmids

Three different genetically engineered *Agrobacterium tumefaciens* strains were used in transformation experiments. The properties of these strains and plasmid vector carried by these strains are mentioned below.

2.2.1 Strain I (LBA4404)

Agrobacterium tumefaciens strain LBA4404 contains plasmid pBI121 (binary vector). This binary vector have two genes within the right border (RB) and left border (LB) region of the gene construct (Fig. 11a):

(i) The *udi*A gene (Jefferson et al. 1987) encoding GUS (β-glucuronidase), driven by CaMV 35S promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation.

(ii) The *nptII* gene (Herrea-Estrella et al. 1983) encoding neomycin phosphotransferase II conferring kanamycin resistance, driven by NOS promoter and NOS terminator.

(iii) The bacterium also contains plasmid pAL4404 which is a disarmed Ti plasmid containing the virulence genes.

The reporter gene GUS can be used to assess the efficiency of transformation. The GUS gene has an intron in the coding sequence which can express GUS activity in the plant cell but not in the cells of *Agrobacterium.* In this strain, the transformed tissue as well as transgenic plant can be selected by using kanamycin.

2.2.2 Strain II (EHA105)

The hypervirulent *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) harboring the pGreenII 0229 derivative binary plasmid containing a bar gene and the pSoup helper plasmid,(pGreen website: HYPERLIK "http://www.pgreen.ac.uk/" http://www.pgreen.ac.uk), (Hellens et al. 2000). This binary vector contains following genes within the right border (RB) and left border (LB) region of the construct (Fig. 11b):

(i) The *bar* gene encoding phosphinothricin-acetyl transferase driven by NOS promoter and NOS terminator which conferring phosphinothricin resistance is present as selectable marker.

(ii) The *chitinase* gene had been isolated from *Streptomyces olivaceoviridis* (ATCC 11238). Chimeric constructs were developed by fusing the *Arabidopsis thaliana* leader peptide (NCBI, AY081519) to the chitinase gene behind the stilbene synthase promoter (pGIIvstN-Chitin) from grape (Wiese et al. 1994).

2.2.3 Strain III (LBA4404)

Agrobacterium tumefaciens strain LBA4404 contains plasmid pCAMBIA2300 enh35S AFP. This binary vector contains *npt*II and AFP genes within the right border (RB) and left border (LB) region of the construct (Fig. 11c). This strain is constructed by V. K. Nguyen, V. S. Reddy on 14/12/2006. For the preparation of this plasmid 1298 bp fragment of cassette "35S enhancer + 35S promoter + AFPF gene + poly A" was digested from pFF19AFPF plasmid with EcoRI + Hind III enzymes. This fragment was cloned into pCAMBIA2300 at same sites, giving pCAMBIA2300enh35SAFPF. The insertion was confirmed by PCR using AFPcaBamintron and AFPC2RS specific primers, and EcoRI + Hind III digestion of pCAMBIA2300enh35SAFPF plasmid.

2.3 Chemicals

2.3.1 Growth media

Substance	Molecular weight	Company
MS Basal Salt mixture	476	DUCHEFA, Netherlands
B5 vitamin	484.47	DUCHEFA, Netherlands
Plant Agar		DUCHEFA, Netherlands
Sucrose	180	SIGMA, USA
Gelrite		SIGMA, USA

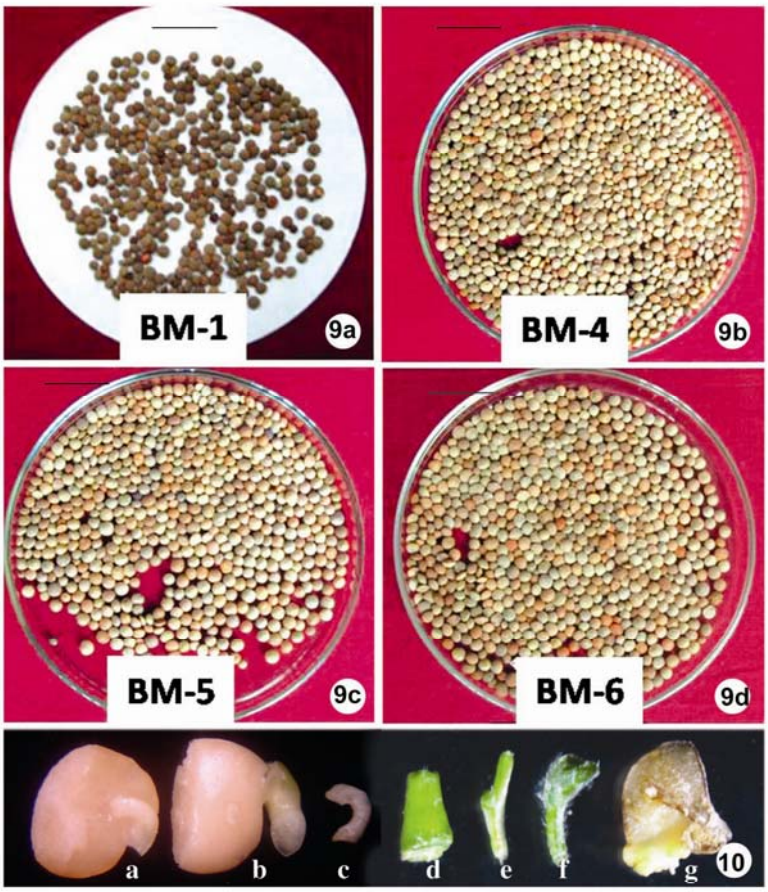


Fig. 9. Seeds of four different varieties of lentil (*Lens culinaris* Medik.) used in the present investigation. (a) BM-1 (b) BM-4 (c) BM-5 and (d) BM-6. Bar = 2 cm.

Fig. 10. Stereomicroscopic view of different explants from BM-4 variety of lentil used in *in vitro* regeneration. ×9. (a) cotyledon attached decapitated embryo (b) cotyledonary node (c) decapitated embryo (d) hypocotyle (e) nodal segment (f) shoot tip (g) cotyledon.

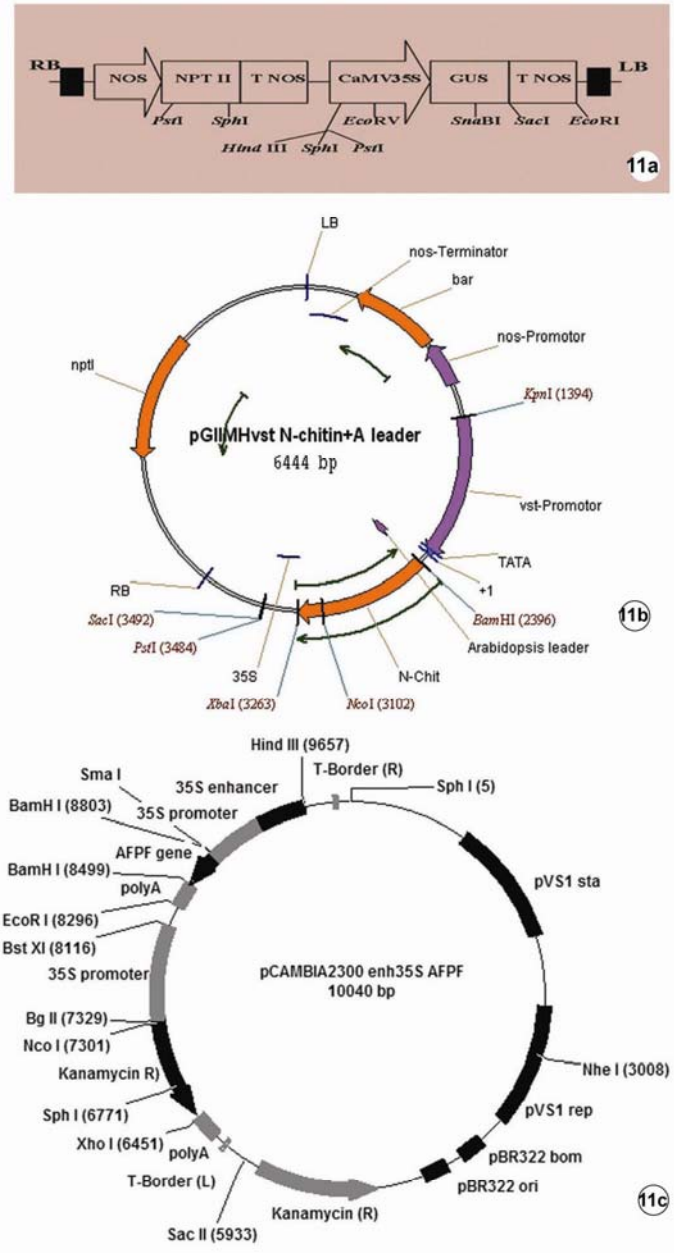


Fig. 11. Diagrammatic representation of *Agrobacterium tumefaciens* strains used in the present investigation. (a) *Agrobacterium tumefaciens* strain LBA4404 containing plasmid pBI121 (Strain I) showing a part of T-DNA between left and right border. (b) *Agrobacterium tumefaciens* strain EHA105 containing plasmid pSOUP-pGII-VST-N-CHIT (Strain II) (c) *Agrobacterium tumefaciens* strain LBA4404 containing plasmid pCAMBIA2300 enh35S AFP (Strain III).

2.3.2 Antibiotics

Substance	Molecular weight	Company	Solvent
Combactam	582.6	Pfitzer, USA	ddH_2O
Ticarcillin	428.4	DUCHEFA, Netherlands	ddH_2O
Cefatoxim	455.47	ALKEM, India	ddH_2O
Kanamycin	484.49	DUCHEFA, Netherlands	ddH_2O
Streptomycin	1457.4	DUCHEFA, Netherlands	ddH_2O
Rifamcin	822.94	DUCHEFA, Netherlands	ddH_2O

2.3.3 Plant hormones and additives

Substance	Molecular weight (MW)	Company	Solvent
BAP	225.3	DUCHEFA, Netherlands	1[N] NaOH
Kinetin	215.2	DUCHEFA, Netherlands	1[N] NaOH
GA_3	346.4	DUCHEFA, Netherlands	1[N] NaOH
Tyrosin	181.2	DUCHEFA, Netherlands	1[N] NaOH
Zeatin	219.25	SIGMA, USA	1[N] NaOH
IAA	175.18	DUCHEFA, Netherlands	1[N] NaOH
IBA	203.2	DUCHEFA, Netherlands	1[N] NaOH
NAA	186.2	DUCHEFA, Netherlands	1[N] NaOH
2,4-D	221.6	DUCHEFA, Netherlands	1[N] NaOH
Acetosyringone	196.2	ROTH, Germany	DMSO
PPT	198.16	Riedel de Haen, USA	ddH_2O

2.3.4 GUS-assay buffer

(i) 100 mM sodium phosphate buffer (pH 7.0)

(ii) 0.5 mM potassium ferrocyanide

(iii) 10 mM EDTA

(iv) 1 mM (0.5 mg/ml) X-*GLUC* (dissolved in DMSO before adding it to Gus buffer)

2.3.5 DNA markers

DNA marker	Concentration	Company
Gene Ruler^{TM} 100 bp DNA ladder	0.5 mg/ml	MBI Fermentas
Gene Ruler^{TM} 1 kbp DNA ladder	0.5 mg/ml	MBI Fermentas

2.3.6 Solvent, sterilizers and others

Compound	Company
Dimethyle sulfoxide (DMSO)	SERVA, Germany
KOH	Carl Roth, Germany
NaOH	Carl Roth, Germany
NaOCl	Riedel de Haen,USA
EtOH	Roth, Germany

2.3.7 Primers

Primer	Sequence (5'-3')	Product	Company
GUS	CCT GTA GAA ACC CCA ACC CG TGG CTG TGA CGC ACA GTT CA	500bp	MWG Biotech
nptII-F nptII-R	GAAAAACTCATCGAGCATCA TTGTCCTTTTAACAGCGATC	700bp	MWG Biotech
Bar 447-F Bar 447-R	GATTTCGGTGACGGGCAGGA TGCGGCTCGGTACGGAAGTT	447bp	MWG Biotech
Chit 555-F Chit 555-R	GGTGACATCGTCCGCTACAC GGTGTTCCAGTACCACAGCG	555bp	MWG Biotech
AFP-F AFP-R	CGCGGATCCATGGCGAGGTGTGAGAATTTGGCT GAGTCCTCGAGACAATTTTTGGTGCACCAACAAC	190bp	Invitrogen
Kan-F Kan-R	TCGACCATGGGGATTGAACAAGATGG ATTCGAGCTCTCAGAAGAACTCGTCAAGAAGGC	700bp	Invitrogen

3. METHODS

3. METHODS

3.1 Preparation of stock solutions for different culture media

Different stock solutions were prepared for the preparation of medium used in different steps of plant regeneration and selection of putatively transformants. The stock solutions of various constituents of the medium were prepared for ready use during the preparation of various medium.

3.1.1 Stock solution A (Macro nutrients) for MS medium

This stock solution was made in such a way that its strength was 40 times more than the final strength of the medium in 1000 ml distilled water. For this purpose, 40 times the weight of different salts required for 1 litre of medium were weighed accurately and were sequentially dissolved one after another in a 1000 ml volumetric flask with 650 ml of distilled water. The final volume of the solution was made up to 1000 ml by further addition of distilled water. The solution was filtered through Whattman No.1 filter paper to remove all the solid contaminants like dust, cotton etc. and was poured into a clean plastic container. After labeling, the solution was stored in a refrigerator at 4˚C for several weeks.

3.1.2 Stock solution B (Micro nutrients) for MS medium:

Two different stock solutions were prepared for this constituent of the medium:

(i) Stock solution B_1 (all micro-nutrients except iron source)

This stock solution was made with all the micro-nutrients except $FeSO_4.7H_20$ and Na_2-EDTA. This was made 100 times the final strength of necessary components in

500 ml of distilled water as described for the preparation of stock solution A. The prepared solution was filtered and was stored at 4˚C.

(ii) Stock solution B_2 (Iron chelate solution)

This stock solution was made 100 times the final strength of $FeSO_4.7H_20$ and Na_2-EDTA in 500 ml distilled water in a conical flask and heated slowly at a low temperature until the salts were dissolved completely. Finally the solution was filtered and stored in a refrigerator at 4˚C for several weeks.

3.1.3 Stock solution C (Organic constituents) for MS medium

It was also made 100 times of the final strength of the medium in 500 ml of distilled water. This solution was also filtered and stored at 4˚C for future use.

3.1.4 Stock solution D (B_5 vitamin) for MSB_5 medium

For the preparation of B_5 vitamin following components were added in distilled water.

Components	Amount needed for 1000 ml medium
Myo inositol	10.0 gm
Thiamin	1.0 gm
Nicotinic Acid	0.1 gm
Pyridoxin HCl	0.1 gm

This was made 100 times the final strength of necessary components in 500 ml of distilled water as described in the section 3.1.1. The solution was filtered and stored at 4˚C.

3.1.5 Stock solutions for growth regulators

The following growth regulators and supplements were used in the present study:

(i) Auxins:

Indole -3 - acetic acid (IAA), Indole -3 - butyric acid (IBA) and α - naphthalene acetic acid (NAA).

(ii) Cytokinins:

6 - benzylaminopurine (BAP), 6 - furfurylaminopurine (Kinetin/Kn), Zeatin and 2, 4-D.

(iii) Gibberellins:

Gibberellic acid (GA_3).

To prepare any of the above mentioned hormonal stock solution, 50 mg of the hormone was weighted and dissolved in required amount of appropriate solvent (mentioned in section 2.3.2). The mixture was then diluted with distilled water and collected in a 50 ml sterilized falcon tube. It was then made up to 50 ml with the addition of distilled water. Therefore the final concentration was 1.0 mg/ml. The solution was then filtered and poured into clean plastic container/ another 50 ml in sterilized falcon tube and stored in a refrigerator at 4ºC for several weeks for future use.

3.1.6 Stock solution for tyrosine

To prepare this stock, 110 mg of tyrosine were carefully weighed and dissolved in 1[N] NaOH. The mixture was then diluted with distilled water and collected in a 50 ml measuring cylinder. It was then made up to 40 ml with the addition of distilled water. Therefore the final concentration was 2.75 mg/ml. The solution was then filtered and poured into a 50 ml sterilized falcon tube and stored in a refrigerator at 4˚C for future use.

3.1.7 Preparation of MS/ MSB_5 medium

To prepare one litre medium following steps were carried out successively:

(i) For the preparation of MS/ MSB_5 medium, 30 g of sucrose was dissolved in 500 ml of distilled water in a litre of volumetric flask.

(ii) 25 ml of stock solution A, 10 ml of stock solution B and 10 ml of stock solution C were added to this 500 ml distilled water and mixed well. Stock solution D was added instead of stock solution C for the preparation of MSB_5 medium.

(iii) When increased amount of macro-salts were used as a constituent of the medium, appropriate amount of solutions from micro-salt stock were added in to the medium. When additive tyrosine was required, from the tyrosine stock appropriate amount of solutions were added in to the medium.

(iv) Required amount of hormonal supplements were taken from the stock solution of hormone and added to the medium either individually or in combinations and were mixed thoroughly. Since each of the hormonal stock solution contained 20 mg of the chemical in 20 ml of solution, the addition of 10 ml of any hormonal stock solution will make 1 litre of medium resulted in 1mg/l concentrations of that hormonal supplement.

(v) The whole mixture was then volume up to 1litre with distilled water.

(vi) pH of the medium was adjusted to 5.8 with a digital pH meter (TOA, Japan) with the help of 1[N] NaOH and 1[N] HCL. Before that, the pH meter was calibrated with two buffer solution having pH 4.01 and 6.86 respectively.

(vii) To solidify either 8.0 g (at 0.8%) of phytoagar (Duchefa, Netherlands) or 2.0 g (at o.2%) of phytagel (Sigma, USA) was added to the medium. To dissolve solidifying agent, the whole mixture was heated in a microwave oven (National, Japan).

(viii) To make liquid medium the last step (vii) of media preparation was omitted.

3.1.8 Preparation of MS medium for plants using MS powder

Occasionally commercially available dry powdered MS medium (Duchefa Biocheme, Netharlands) containing all the constituents of MS medium (inorganic salts, vitamins and amino acids) except sucrose and agar were used to prepare medium for *in vitro* regeneration of plants. Appropriate amount of media powder is dissolved in distilled water (10% less than the final volume of the medium), and after adding sucrose (3%), the pH was adjusted to 5.8. To prepare agar solidified media, 0.8% agar was added.

3.1.9 Culture media for *in vitro* shoot regeneration and elongation

For callus/shoot initiation and development, MS medium (Murashige and Skoog 1962) or MSB_5 medium supplemented with/without various combinations and concentrations of hormones - BAP (6- benzyl aminopurine), Kn (Kinetin), GA_3 (Giberellic acid), 2, 4-D, Zeatin, NAA and additive - tyrosine were used.

3.1.10 Culture media for *in vitro* root induction

For the initiation of roots from *in vitro* grown shoots, full and half strength of MS medium supplemented with different concentrations and combinations of IBA, IAA and NAA were used.

3.1.11 Culture media for *in vitro* flowering and seed formation

For the induction of *in vitro* flowering and seed formations following media were tried.

(i) MS (Murashige and Skoog, 1962)

(ii) ½ MS (half strength of macronutrients, micronutrients, and vitamins of MS)

In both cases, the hormonal supplements were IBA (10 - 25 mg/l), IAA (10 - 25 mg/l) and NAA (0.5 - 1.0 mg/l) were used with their various concentrations and combinations.

3.1.12 Preparation of *Agrobacterium* culture medium

Liquid YEP, YEM and LB were used to grow *Agrobacterium tumefaciens* which were used as bacterial suspension for genetic transformation. Solid YEP, YEM and LB were used as maintenance media for different strains of *Agrobacterium*.

3.1.12.1 Preparation of YEP (Yeast Extract Peptone) medium

For the growth of *Agrobacterium tumefaciens*, YEP medium was prepared in the following manner:

Components	Amount needed for 500 ml medium
Bactopeptone	5.0 g
Bacto Yeast extract	5.0 g
NaCl	2.5 g

All of these ingredients of the medium were added in 250 ml distilled water and mixed properly. Then the final volume was made up to 500 ml by adding distilled water. The pH was adjusted at 7.0 - 7.2.

For the preparation of working culture medium (to make bacterial suspension), liquid medium was used. For preparing maintenance medium 7.5 g agar was added to the medium before autoclaving. Filter sterilized antibiotic kanamycin (100 mg/l), streptomycin (50 mg/l) and rifampicin (10 mg/l) was added to the autoclaved liquid medium (working) prior to bacterial inoculation and to the autoclaved maintenance medium when the medium was cooled down enough. The medium was then poured into petri plates. After solidification, the media were ready for bacterial culture. When required these culture plates were stored at 4˚C for further use.

3.1.12.2 Preparation of YEM (Yeast Extract Mannitol Broth) medium

For the preparation of YEM medium all of these ingredients were used by following the methods described in the section 3.1.12.1

Components	Amount needed for 1000 ml medium
Yeast Extract	0.4 gm
Manitol	10.0 gm
NaCl	0.1 gm
$MgSO_4.7H_2O$	0.2 gm
K_2HPO_4	0.5 gm

3.1.12.3 Preparation of LB (Liquid Broth) medium

For the preparation of YEM medium all of these ingredients were used by following the methods described in the section 3.1.12.1

Components	Amount needed for 500 ml medium
Bactotryptone	5.0 g
Yeast extract	2.5 g
NaCl	5.0 g

3.1.13 Preparation of Co- culture media

For the purpose of co-cultivation, shoot regeneration medium as well as MS media without hormonal supplement were used.

3.1.14 Preparation of medium for selection of putatively transformed plantlets

For the selection of transformed tissues/plantlets two antibiotics namely kanamycin (Duchefa, Netherlandss) and phosphinothricin (Duchefa, Netherlands) were used, as the *Agrobacterium* strains used in the present investigation contained *nptII* (kanamycin resistance) and *bar* (phosphinothricin resistance) genes respectively. After preparation the regeneration medium was autoclaved. The medium was cooled down to 50˚C and appropriate antibiotics were added at a desired rate to a particular regeneration medium inside laminar flow cabinet. Medium was then poured into suitable culture vial and allowed to solidify. Apart from these antibiotics as and when required other antibiotics such as ticarcillin, combactam and cefatoxime were also used to control the overgrowth of bacteria.

3.2 Seed germination and seedling development

Three types of germination medium were used for the germination of lentil seeds. The seeds were first washed with a detergent and few drops of tween-20 under running tap water until the detergent washed out completely. The floating seeds were discarded. The seeds were then treated with 70% alcohol for 1 minute followed by washing with distilled water for three to four times. Seeds were then treated with 0.1% $HgCl_2$ solution for ten to fifteen minutes inside the laminar flow cabinet with constant agitation. Then the seeds were washed five times with sterilized distilled water. After that the surface sterilized seeds were inoculated on the following media:

(i) MS (Murashige and Skoog 1962) medium with 2% sucrose and 0.8% agar.

(ii) Water agar medium with 2% sucrose.

(ii) Autoclaved cotton soaked with sterile distilled water.

3.2.1 Explants preparation

Cotyledonary node (CN), decapitated embryo (DE), cotyledon attached decapitated embryo (CADE), shoot tip (ST), hypocotyl (hypo), nodal segment (NS) and cotyledon (C) explants were used in this investigation. Cotyledonary nodes, shoot tip, hypocotyls and nodal segment were excised from aseptically grown three days old seedlings. For the culture of decapitated embryo and cotyledon attached decapitated embryo surface sterilized seeds were grown on distilled water-agar medium overnight and explants were excised on the following day. The explants were then cultured on MS/MSB_5 media supplemented with different concentrations and combinations of BAP, Kn, GA_3, zeatin, 2,4-D and additive amino acid tyrosin for *in vitro* regeneration of shoots.

3.2.2 Subculture of regenerated explants

Two weeks old regenerated explants were transferred into flasks or small bottles containing fresh media. Explants were sub cultured regularly with an interval of 15- 21 days for maintenance and were routinely examined.

3.2.3 Root induction of regenerated shoots

For root induction, 2 to 4 cm long regenerated shoots were excised and transferred to test tubes (25× 150 mm) containing 25 ml of freshly prepared MS medium as well as half strength of MS medium with different combinations and concentrations of IBA (10 - 25 mg/l) and NAA (0.5 - 1.0 mg/l). The test tubes were then incubated in the growth room under 16/8 h light/dark cycle at 25 ± 2°C.

3.2.4 *In vitro* flowering and pod formation

To obtain *in vitro* flower and pods, 2 - 3 cm long regenerated shoots were separated individually and cultured in test tubes containing 25 ml of MS medium as well as half strength of MS medium with different hormonal supplements i.e. IBA (10 - 25 mg/l), IAA (10 - 25 mg/l) and NAA (0.5 - 1.0 mg/l) in their various concentrations and combinations. Cultures were maintained in the growth room until flower and pod formation.

3.2.5 Pollen viability and observation of pollen tube growth

To examine pollen grain viability, *in vitro* flowers were excised. Sepals and petals were removed. After that anthers were transferred to a slide and squashed in a solution containing 0.01% (w/v) fluorescin diacetate (FDA) and 20% (w/v) sucrose followed by

incubation at room temperature for 30 min (Heslop-Harrison and Heslop-Harrison 1970). Following this incubation period, debris was removed and pollen grains were observed under a fluorescence microscope.

To study the growth of the pollen tubes, the self- pollinated pistils from *in vitro* grown flowers were collected, fixed and softened in a mixture of acetic acid : alcohol (1 : 3, v/v) at 60º C for 10 minutes and thereafter stained with 0.1% (w/v) decolourized aniline blue solution. After staining, pistils were mounted in a drop of 50% (v/v) aqueous solution of glycerol prior to microscopic examination. Stained pistils and pollen grains thus observed under a fluorescence microscope fitted with an incident UV illumination system (Nikon, Microphot; excitation 450 - 490 nm) according to Kho and Baer (1968).

3.2.6 Pollen Germination

In vitro germination of pollen grains was observed in the media containing 10% sucrose and 0.01% boric acid in double distilled water, pH 5.9. For this purpose pollens were dusted in a drop of this media on a clean slide. Pollen germination was observed under fluorescent microscope (Nikon, H600L) every 45 minutes interval.

3.2.7 Germination of *in vitro* grown seeds

Mature seeds developed from *in vitro* flowers were harvested and dried under sunlight for 2 - 3 days. Air dried seeds were surface-disinfected and placed on water agar medium or directly to the pot containing equal ratio of soil, sand and vermiculite for germination.

3.2.8 Transplantation and acclimatization of plantlets

Two to three weeks old seedlings were transplanted into 10 × 6 cm plastic pots containing sterilized peat moss. The roots were gently washed to remove agar. Pots were then covered with transparent perforated polythene bags. Inner side of these bags was moistened with water to prevent desiccation. The plants were acclimatized in the culture room for 1to 2 weeks and then further maintained in net house for flower and pod development.

3.3 Preparation of *Agrobacterium* culture for transformation

For *Agrobacterium* culture, two different culture media were used for each bacterial strain: one for maintaining *Agrobacterium* stock and the other for infection of explants. For maintenance, one single colony from previously maintained stocks was streaked into freshly prepared YEP/LB/YEM medium having appropriate antibiotics. The Petri dish was sealed with parafilm and kept in 37˚C temperatures in the incubator for at least 48 hours. This was then kept at 4˚C to check overgrowth. The culture was sub-cultured regularly each week in freshly prepared media to maintain the stock.

For infection of explants, bacterial suspension was prepared. For this, a single colony from the bacterial stock was taken with the help of inoculation loop and was inoculated into a conical flask containing liquid medium with required antibiotic. This culture was allowed to grow overnight at 28˚C on a rotary shaker to get optimum population for infection and co-cultivation of explants.

3.3.1 Infection and incubation of explants

The overnight grown *Agrobacterium* culture was centrifuged for 10 minutes at 5000 rpm and the pellet was re-suspended in liquid MS medium (pH 5.8) to make the

Agrobacterium suspension. This *Agrobacterium* suspension was used for infection and incubation. Prior to this, "Optical Density" (O.D.) of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). After that the explants were dipped in bacterial suspension for different incubation periods before transferring them to co-cultivation medium.

3.3.2 Co-cultivation of infected explants

All the infected explants were soaked in filter papers for few minutes to remove excess bacterial suspension and co-cultured in regeneration medium for 2 - 4 days at 25±2˚C.

3.3.3 Sub-culture of *Agrobacterium* infected explants and selection of putative transformed shoots

Following co-culture, explants were washed with distilled water three to four times until no opaque suspension was seen, then washed for 10 minutes with distilled water containing 300 mg/l ticarcillin/cefatoxim/combactam and then finally washed with distilled water once again. The explants were then dried on a sterile Whatman filter paper and transferred to regeneration medium with 100 mg/l ticarcillin/cefatoxim/combactam. After 7 - 10 days, the regenerated shoots were then sub-cultured in selection medium containing 50 mg/l kanamycin and 100 mg/l ticarcillin. Regenerated shoots were sub-cultured regularly with an interval of 15 - 21 days and the concentration of selection agents was gradually increased up to 200 mg/l in case kanamycin on selection medium. In case of phosphinothricin, 0.5 mg/l phosphinothricin was used as preliminary selection pressure and the concentration of selection agents was gradually increased up to 2.0 mg/l. Shoots survived on selection

medium were sub-cultured on flowering medium for *in vitro* flowering and seed formation. As control, non-infected explants were cultured in normal regeneration medium. 7 - 10 days old shoots were sub-cultured in selection medium to detect the effect of selection agents on this control shoots. These controls were maintained with each set of transformation experiments to perform various comparative studies.

3.4 Collection of plant sample for molecular analysis

For the isolation of genomic DNA, *in vitro* regenerated putative transgenic plantlets and control leaves were collected. The materials were washed in distilled water and dried on tissue paper to remove any components of medium nutrients.

3.4.1 Preparation of Stock Solutions and Working Solutions used for DNA Isolation

The following chemicals were used for plant genomic DNA isolation.

3.4.1.1 1 M Stock Solution of Tris HCl pH 8.0 (100 ml)

12.14 gm of Tris base was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4º C.

3.4.1.2 0.5 M Stock Solution of EDTA pH 8.0 (100 ml)

18.61 gm of EDTA was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 gm of NaOH pellets was added to adjust the final pH to 8.0. Final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.4.1.3 5 M Stock Solution of NaCl (100 ml)

29.22 gm of sodium chloride (NaCl) was dissolved in 75 ml of distilled water. Then it was volume up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.4.1.4 β-Mercaptoethanol

β - Mercaptoethanol was available at strength of 14.4 M solution and was stored in a dark bottle at room temperature.

3.4.1.5 Ribonuclease A stock solution

10 mg RNAase A was dissolved in 1 ml of deionized distilled water and was store in -20^0 C.

3.4.1.6 Tris-HCl Saturated Phenol

It was prepared by the following procedure

(i) The crystal phenol was melted in a water bath at 65º C for 30 minutes.

(ii) Then 100 ml of melted phenol was taken and same volume of Tris-HCl (pH 8.0) was added and kept on a magnetic stirrer for 10 minutes.

(iii) After proper mixing, the solution was kept for 5 minutes. At this stage, two distinct phases were visible, colorless upper phase and colored lower phase.

(iv) The upper phase was removed with the help of a dropper.

This step was performed for six times to obtain pH 7.75. After saturation, the phenol became the half of the initial volume. As phenol is very much corrosive and highly toxic, protective measures (Apron, Gloves, and Musk) were adopted.

3.4.1.7 Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) (100 ml)

50 ml of Phenol, 48 ml of Chloroform and 2 ml of Isoamyl alcohol were mixed properly using vortex mixture. The solution was then stored at 4º C. The Phenol: Chloroform: Isoamyl alcohol mixture is caustic and produces fumes. So, was used only in a fume hood wearing gloves and eye protection.

3.4.1.8 70% Ethanol (100 ml)

30 ml double distilled water was added in 70 ml absolute ethanol.

3.4.1.9 Stock Solution of TE (Tris-HCl EDTA) Buffer pH 8.0 (100 ml)

1 ml of 1 M Tris-HCl was added with 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.4.1.10 3 M Sodium acetate pH 5.2 (100 ml)

40.824 g of sodium acetate was dissolved in 70 ml of ddH_2O and was adjusted to 100 ml in volume adding ddH_2O and was sterilized by autoclaving.

3.4.2 Extraction Buffer (Homogenization buffer)

Following components were used for preparing DNA extraction buffer.

Name of the chemical	Molecular Weight	Stock con.	Reference con./working con.	Working Volume	
				100 ml	1000 ml
CTAB			2%	2 g	20 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (p^H 8)	372.24	0.5 M	20 mM	4 ml	40 ml
Trisbase (p^H 8)	121.1	01 M	100 mM	10 ml	100 ml
β-Mercaptoethanol		14.4 M	100 mM	700 µl	7 ml

Extraction Buffer was Prepared as the following steps (100 ml):

(i) 10 ml of 1 M Tris-HCl (pH 8.0) was taken in a 250 ml conical flask.

(ii) 28 ml of 5 M NaCl was added to it.

(iii) 4 ml of 0.5 M EDTA (pH 8.0) was added next.

(iv) The solution was then autoclaved.

(v) After autoclaving 1 gm PVP and 2 gm CTAB was added and was stirred in the magnetic stirrer.

(vi) 800 µl β-mercaptoethanol was added prior to use and was mixed by glass rod.

(vii) The pH of the solution was adjusted at pH 5 with HCl and was made up to 100 ml by adding sterile de-ionized distilled water

3.4.3 CTAB method for genomic DNA isolation

Plant genomic DNA was isolated according to Doyle and Doyle (1990) with slight modifications. The steps of DNA isolation are as follows:

(i) Single plantlet of required source was taken and grind in liquid nitrogen using pestle and mortar.

(ii) 800µl of extraction buffer was added and grinded the leaf until it became homogenous paste.

(iii) The paste was transferred to an eppendorf and incubated at 60ºC water bath for 30 min.

(iv) 700µl of chloroform: Isoamyl alcohol (24:1) was added to the extract and centrifuged at room temperature for 5 min at 13000 rpm. This process was repeated twice.

(v) The supernatant was collected and DNA was precipitated with 2/3 vol. chilled Isopropanol.

(vi) Then the suspension was centrifuged for 10 min, 13000 rpm at room temperature.

(vii) Supernatant discarded and the pellet was washed with 70% ethanol for 3 times.

(viii) The DNA was then resuspended in 40 - 50µl of TE buffer.

3.4.4 Qualification and Quantification of Isolated DNA

Measurement of isolated DNA concentration can be done by comparing DNA with the standard DNA on agarose gel electrophoresis or by estimating the absorbance of DNA

by spectrophotometer at 260 nm. Both the methods were carried out during this investigation.

3.4.5 Measurement of DNA Concentration and Quality by Agarose Gel Electrophoresis

3.4.5.1 Preparation of Stock Solutions Used for Gel Electrophoresis

3.4.5.1.1 50 × TAE Buffer (p^H 8.3) (1 litre)

242 g Tris base was dissolved into 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (pH 8.0) was added in it. The p^H of the solution was adjusted by mixing concentrated HCl at p^H 8.3. The final volume of the solution was adjusted to 1000 ml.

3.4.5.1.2 10 × Loading Dye

For 10 ml of 10x loading dye, 40 mg of bromophenol blue (i.e., the final concentration was 0.4%), 40 mg of xylene cyanol FF (i.e., the final concentration was 0.4%) and 5 ml of 98% glycerol (i.e., the final concentration was 50%) were added to 4 ml of sterile de-ionized distilled water. The final volume was adjusted to 10 ml with sterile de-ionized distilled water and was stored at 4º C.

3.4.5.1.3 Ethidium Bromide Solution

For 1ml solution, 10 mg of Ethidium Bromide was dissolved in 1 ml of sterile de-ionized distilled water. It was mixed by hand shaking. The solution was transferred to a dark bottle and was stored at room temperature.

3.4.5.2 Agarose Gel Electrophoresis

The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described by Sharp et al. 1973.

(i) 1.0 gm of agarose was melted into 100 ml of TAE buffer, ethidium bromide was added (10 µg/ml) and poured into gel-tray fixed with appropriate combs.

(ii) After the gel was solidified it was placed into gel-running kit containing 1 xTAE buffer.

(iii) Digested plant DNA solutions were loaded with 6x gel loading dye and elecetrophoresis was continued until DNA fragments were separated well.

3.4.6 Polymerase Chain Reaction

3.4.6.1 Preparation of purified taq DNA polymerase

(i) 2µl of purified Taq DNA Polymerase was transferred to an eppendorf (fresh and autoclaved).

(ii) 4µl of storage buffer was added.

(iii) Dilution of the enzyme was 50 fold.

3.4.6.2 Preparation of storage Buffer

For the preparation of storage buffer 0.0174g of PMSF was first dissolved in minimal volume of isopropanol (~lml). Then 1.21 g of Tris, 0.745g of KCL, 0.0074g of EDTA and 0.03084 g of DTT were added and mixed thoroughly with deionized water after adjusting the pH to 7.9, the final volume was made 75ml with ddH_2O and filter sterilized through 0.2/µm Millipore. Meanwhile, 80% glycerol was prepared from

commercially available glycerol (98%) and autoclaved. 125ml of the 80% glycerol was added to 75ml of the mixture of storage buffer and stored at 4°C.

Components	Molecular Weight	Concentration
Tris	121.14	50.0m M
KCL	74.50	50.0mM
EDTA	372.20	0.1 mM
DTT	154.20	1.0mM
PMSF	174.20	0.5m M
Glycerol	---------	50.0%

3.4.6.3 Preparation of the Master Mixture

Master mixture was prepared by mixing all of the PCR component e.g. buffer, dNTPs, Mg^{2f}, Primer- F & R, etc. except the component against which the optimization strategy was intended. In each reaction, the volume of PCR buffer was used $\frac{1}{10}$ th of the total reaction volume which was 25µl. After through mixing and momentary spin of the master mixture, it was transferred to different eppendorf tubes. The PCR component in question was then added. The final volume was made 25µl by adding varying amounts of sterilized ultra-pure water. Taq DNA Polymerase was added just before the start of the reaction. Finally, the tubes were subjected to momentary spin and transferred to thermo cycler for the amplification reaction (eppendorf Mastercycler gradient).

3.4.6.4 Thermal Cycling Profile used in PCR

The working concentration of the template DNA was 100 ng for each sample. The PCR condition was used for gene amplification are described below:

The PCR condition for GUS and *npt*II gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	5 min	1 (first)
Denaturation	94°C	1 min	35
Annealing	54°C	1 min	
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1 (last)

The PCR condition for Kanamycin and *AFP* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	4 min	1 (first)
Denaturation	94°C	30 sec	35
Annealing	50°C	30 sec	
Elongation	72°C	30 sec	
Final elongation	72°C	5 min	1 (last)

The PCR condition for bar and *chitinase* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	5 min	1 (first)
Denaturation	94°C	1 min	35
Annealing	59°C	1 min	
Elongation	72ºC	1 min	
Final elongation	72°C	5 min	1 (last)

3.4.7 Analysis of PCR product

3.4.7.1 Sample Preparation from PCR Product

To increase the amount of all particular kind of PCR product 4 batches of the same reactions were carried out. After completion of the reaction same variety of DNAs were mixed and then volume was reduced by ethanol precipitation.

*The dried DNA samples were dissolved in minimum amount of TE (~0-20µl).

*Note that the PCR products used for optimization of reaction condition were not precipitated.

3.4.7.2 Visualizing the PCR Product

3 µl of DNA dye was added to the PCR amplified DNA. After a momentary spin the PCR products were loaded in wells of 0.80% agarose gel containing ethidium bromide (0.05µl/ml). Electrophoresis was accomplished at 40 volts and the PCR products were visualized under UV transilluminator and photographs were taken for documentation.

3.4.8 Southern blot analysis

Plant genomic DNA (30 µg) was digested with appropriate enzymes for overnight. Digested DNA fragments were separated on the 0.8% agarose gel in TAE buffer, containing ethidium bromide (0.5 µg/ml) at 40 volt for 6 hours. The gel was treated twice with 0.2 N HCl by gentle agitation for 15 minutes each for depurination of the DNA. After which, gel was washed twice by gentle agitation (15 minutes each) with combination of NaOH 0.5 M + NaCl 1.5 M. Following, the gel was neutralized the denaturing solution by washing twice (10 minutes each) with ammonium acetate (0.5M) and transferred onto nylon hybridization membrane. The gel was placed upside down on a glass plate covered by Saran wrap. The nylon membrane was cut to the exact size, placed onto gel. Three sheets of Whatman paper No.1 were soaked in ammonium acetate (0.5 M), was placed onto membrane. The filter paper towels 10 x 15 cm in size were placed onto Whatman papers. Following, glass plate was placed on the top. 400 g weight was placed on glass plate. Transfer of digested DNAs from gel to membrane was left for overnight, fixed on to membrane by UV- cross linker and stored until required (Sambrook et al. 1989)

3.4.9 Total RNA isolation from transgenic plant leaf tissue

Mortars and pestles were kept immersed in DEPC water over night, autoclaved twice and treated with chloroform for 10 minutes. Leaf tissue was ground with liquid nitrogen to fine powder. 750 µl of RNA extraction buffer was added to ground tissue and transferred to an eppendorf, and mixed by vortex for 1 minute. Sodium acetate pH 4.2 (100 µl), phenol (600 µl), and 200 µl of chloroform: Isoamylalchohol (24:1) were added to mixture. Vortex samples for 2 minutes and kept on ice for 20 minutes. The mixture was centrifuged at 4°C for 25 minutes at 10000 rpm.

Composition of the buffers and solutions used for Southern Hybridization

1. 10X TBE		
a. Tris	107.8 g	Total volume was made up to 1000 ml with distilled water and pH adjusted to 8.2.
b. EDTA	8.41 g	
c. Boric Acid	559 g	
d. Distilled water	600 ml	
2. Denaturation Solution		
a. Nacl (1M)	29.22 g	Total volume was made up to 500 ml with distilled water.
b. NaOH (0.5 N)	10 g	
c. Distilled water	400 ml	
3. Neutralisation Solution		
a. Nacl	43.83 g	The final pH was adjusted to 7.5 with concentrated HCl and then final volume was made up to 500 ml.
b. Tris	30.28 g	
c. Distilled water	350 ml	
4. 20X SSC		
a. NaCl	175.3 g	The final pH was adjusted to 7.0 with concentrated HCl and then final volume was made up to 1000 ml.
b. Na Citrate	88.2 g	
c. Distilled water	800 ml	
5. Maleic Acid buffer (10X)		
a. 1 M maleic acid	116 g	Final volume was made up to 1000 ml with NaOH (solid) to pH 7.5.
b. 1.5 M Nacl	87 g	
6. Washing buffer		1X maleic acid buffer pH7.5; 0.3% (v/v) Tween 20.

The supernatant was taken and transferred to a fresh eppendorf. The sodium acetate pH 5.2 (60 µl), and cold isopropanol (700 µl) was added. The mixture was kept at -20°C for overnight. The pellet of total RNAs was collected by centrifugation at 4°C, 10000 rpm, for 25 minutes and washed with ethanol 70% by centrifugation for 10 minutes at 4°C. Total RNA was air-dried and re-dissolved in 100 µl DEPC water, then added 25 µl LiCl2, mixed gently and kept it on ice. The mixture was dipped in liquid nitrogen for 1 minute, kept at room temperature to thaw. Pellet RNA was collected by centrifugation at 4°C for 25 minutes, 10000 rpm, washed once with ethanol 70% by centrifugation 10000 rpm, for 10 minutes at 4°C. Total RNA was air-dried and re-dissolved in 50 µl DEPC water. To determine the yield, total RNA concentration was measured by spectrophotometry at 260 nm.

Total RNA extraction buffer (for 500 ml)

Guanidium isothiocynate (4 M): 236.333 g

Sodium citrate (2.5 mM): 3.666 g

Sarkosyl (0.5 %): 8.333 ml

RNA loading buffer

Formamide: 720 µl

3-[N-Morpholino] propanesulfonic acid (10X): 160 µl

Formaldehyde (37%): 260 µl

Glycerol (80%): 100 µl

Saturated bromophenol blue solution (0.2 M): 40 µl

Ethidium bromide (10 mg/ml): 15 µl

3.4.10 Northern blot analysis

Fifteen μg RNA was used for preparing a northern blot. Loading dye (10 μl) was added to RNA and denatured by placing at 70°C in the multi-block heater for 10 minutes, then immediately placed on ice for 2 minutes. RNA samples were loaded on the 0.9% agarose gel, prepared in 1 X buffer MOPS (3-[N-Morpholino] propanesulfonic acid) and 5% formaldehyde. The gel was run at 40 volts for 2 hours. Before blotting, the gel was washed twice with DEPC treated sterile water to remove formaldehyde. The gel was soaked in 10 X SSC for 10 minutes, and kept upside down on the Saran wrap. Nylon membrane was pre-soaked in 10X SSC solution was placed onto gel followed by 3 Whatman paper sheets, paper towels (10 cm in height), glass plate and finally weight (400 g) were kept and left for overnight to allow transfer RNA onto membrane. The membrane was air-dried, UV cross-linked and stored until required (Sambook et al. 1989).

3.4.11 Pre-hybridization/hybridization

The membrane was incubated in pre-hybridization buffer containing 200 ng/ml heat denatured salmon sperm DNA (Ausubel et al. 1995). Pre-hybridization was carried out at 65°C with shaking for 4 - 6 hours. Probes (kanamycin) were prepared by nick translation method using the Amersham DNA labeling kit, 200 ng of probe gene fragment; dNTPs minus CTP, dCTP32 were used for labeling. The reaction was performed at 15°C for 90 minutes. Probe was denatured by boiling for 10 minutes and then immediately placed on ice for 2 minutes before adding it to the pre-hybridization solution. Hybridization was carried out for overnight with constant shaking at 45 rpm.

After hybridization, buffer was discarded and membrane was washed in 2 X SSC, 0.1% SDS solution twice (10 minutes each) at room temperature. Then membrane was washed in 0.5 X SSC, 0.1% SDS at 65oC twice (15 minutes each). Finally, membrane was washed in 2X SSC for 10 minutes at room temperature (Sambrook et al. 1989), and was covered with Saran wrap. Autoradiography was carried out in a phosphoimager cassette for overnight, and subsequently scanned in Typoon 9210 or Storm 980. Pre-hybridization buffer (per 1 liter) was prepared as follows:

SSC (20 X): 250 ml
Dextran sulphate (50%): 100 ml
Sodium phosphate (1 M, pH 7.2): 50 ml
Denhardts solution (50X): 100 ml
EDTA (0.5 M): 05 ml
SDS (20%): 20 ml
Sterile water: 475 ml
(Divided into 100 ml aliquots and stored at -20^{o}C).

3.4.12 Semi quantitative RT-PCR

Transgenic expression analysis of PCR positive plants was done by two step reverse transcriptase polymerase chain reaction (RT-PCR) method. Total RNA from transgenic and control lentil plants were isolated using TRIZOL RNA Isolation Protocol and two step RT- PCR using the protocol described in Affinity Script QPCR cDNA Synthesis Kit. Total RNA isolation and RT PCR reaction were done as per the manufactures instruction. Total RNA so isolated was quantified using spectrophotometer at 260 nm. Equal amount of total RNA from both transgenic and control lentil plants were used for RT-PCR reaction and reaction mixture were done as per manufactures instruction (AffinityScript QPCR cDNA Synthesis Kit). All synthesized cDNA is utilized as a

template for second step (cDNA-PCR). PCR reaction was set as previously described, using cDNA as the template with variable cycles. PCR products were taken out from the PCR reaction mix after 20 cycle of amplification at regular intervals and were run on the agarose gel.

Amplification conditions for RT PCR as follows

	Temperature	Duration (min)
Initial template denaturation	65°C	5
Prevention of template RNA renaturation	0°C	2
Hexamer primer annealing	25°C	10
First strand DNA synthesis	42°C	60 – 90
Enzyme inactivation	85°C	5
Storage	-20°C	Until PCR

Amplification conditions for cDNA PCR

	Temperature	Duration	No. of cycles
Initial template denaturation	94°C	2 min	1×
Template denaturation	94°C	15 sec	
Primer annealing	65°C	10 sec	35×
Primer elongation	68°C	30 sec - 5 min	

RT-PCR product was fractioned by 1 per cent agarose gel electrophoresis and visualized in gel documentation system.

4. RESULTS

4 RESULTS

The main objective of the present investigation was to transfer fungal disease resistant gene in the genome of microsperma types of lentil (*Lens culinaris* Medik.) varieties growing in Bangladesh through *Agrobacterium*-mediated genetic transformation. The whole research work was carried out mainly in two phases. The first phase of the investigation was confined to establish an efficient transformation compatible *in vitro* regeneration system for lentil. In the second part attempts were made to transfer the antifungal gene successfully into locally grown lentil varieties through *Agrobacterium*-mediated genetic transformation. Apart from these, experiments were also conducted to induce *in vitro* flower and pod formation from the *in vitro* regenerated shoots of lentil. A series of experiments were conducted to fulfill the above objectives. The results of the present investigation obtained from different experiments are presented in the following sections.

4.1 *In vitro* regeneration of shoots

Genotype, explants source and growth conditions are considered to be important factors for *in vitro* regeneration of plantlets. In this study responses of four varieties of lentil (*Lens culinaris* Medik.) namely, Barimasur-1(BM-1), Barimasur-4 (BM-4), Barimasur-5 (BM-5) and Barimasur-6 (BM-6) were used for *in vitro* regeneration of shoots from different explants on MS medium supplemented with different concentrations and combinations of auxins and cytokinins. A series of experiments were carried out to regenerate shoots directly from the explants or *via* callus induction. Experiments were also conducted to induce functional roots. The effects of various concentrations and combinations of growth regulators were also investigated for this purpose.

4.1.1 Direct regeneration via organogenesis

Regeneration studies were carried out without intervention of any callus. Experiments were designed to develop multiple shoot regeneration directly from different types of explants.

4.1.1.1 Determination of suitable explants for shoot induction

A variety of explants, namely, cotyledonary node (CN), shoot tip (ST), hypocotyle (Hypo), nodal segment (NS), decapitated embryo (DE), cotyledon attached decapitated embryo (CADE) and cotyledon (C) were used to examine their respective regeneration potential and to find out suitable explant for *in vitro* regeneration. Sarker et al. (2003) obtained an adequate frequency of *in vitro* shoot regeneration in microsperma types of lentil varieties using MS media supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.1mg/l GA_3 and 5.5 mg/l tyrosine. Following their report in the present investigation this media combination was employed to select the suitable explants towards *in vitro* shoot regeneration. Among the various explants CN, DE and CADE exhibited positive responses towards multiple shoot regeneration through direct organogenesis. Responses of different explants towards regeneration of shoots are shown in Table 11.

Shoot regeneration from cotyledonary nodal explant : For induction of shoots cotyledonary nodes from BM - 1, BM - 4, BM - 5 and BM - 6 were cultured on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosine. In this combination, shoots were found to initiate from each explants after 4 - 6 days of culture (Figs 12 - 15) and the number of regenerated shoots was found to increase following the maintenance of these shoots in the same medium. It was possible to develop 3 - 6 elongated shoots per explant from cotyledonary node. It was also observed that at the base of the regenerated shoots several new shoot buds were developed at later stages of the culture. Such shoot buds were found to develop new

Table 11. Responses of various explants of lentil towards multiple shoot formation

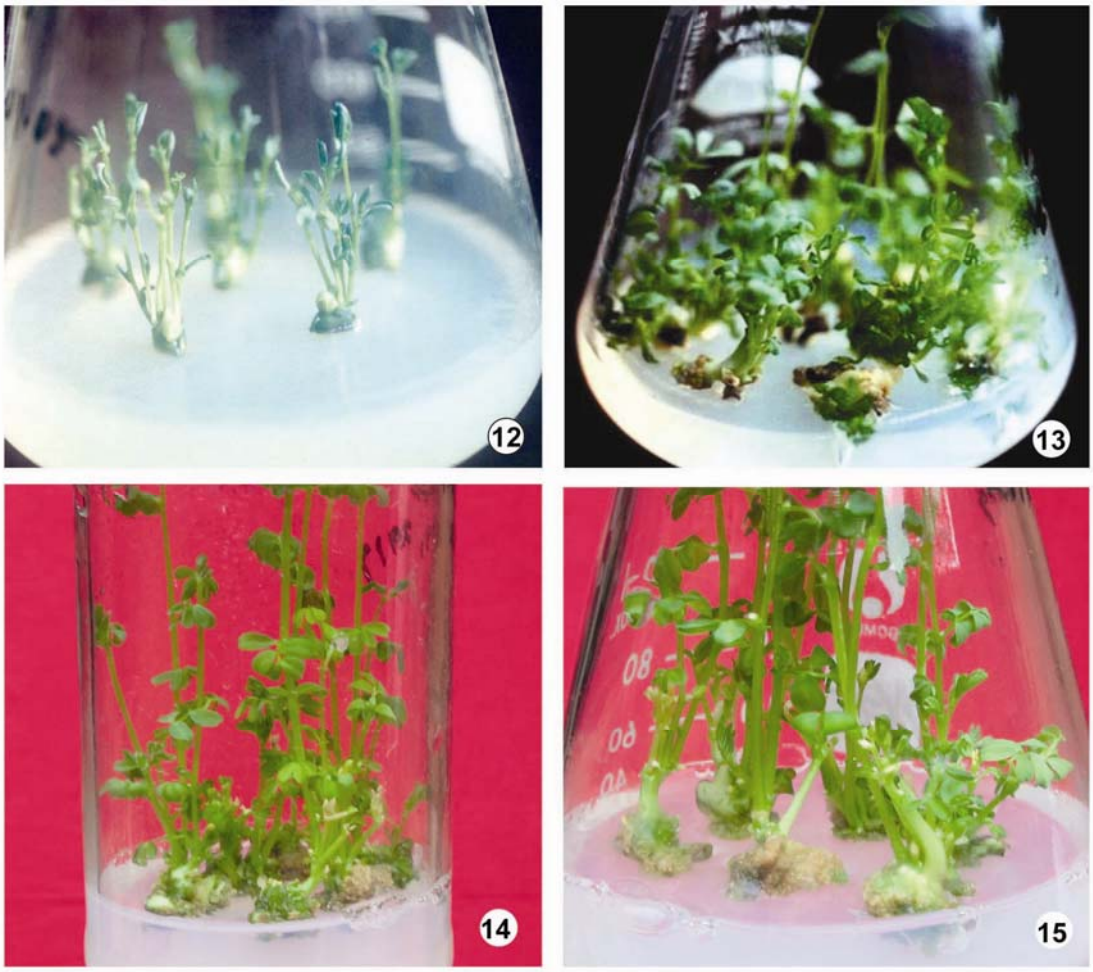
Variety	Source of explant	No. of explant inoculated	No. of responsive explants	% of responsive explants	Time to start regeneration (days)	No. of shoot per explant (mean ± Sd)
BM - 1	CN	150	134	89.33	4-6	5.85 ± 1.25
	DE	113	49	44.59	12-16	1.45 ± 0.20
	CADE	110	80	72.72	5-7	4.25 ± 1.0
BM - 4	CN	142	126	88.73	4-6	6.85 ± 1.75
	DE	124	58	46.0	12-16	3.45 ± 0.60
	CADE	130	97	74.6	5-7	5.75 ± 1.12
BM - 5	CN	130	118	90.7	4-6	7.25 ± 2.27
	DE	160	68	42.5	12-16	2.95 ± 0.83
	CADE	265	197	74.3	5-7	4.95 ± 0.94
BM - 6	CN	130	121	93.0	4-6	8.0 ± 2.05
	DE	105	42	40.0	12-16	3.1 ± 0.85
	CADE	280	216	77.1	5-7	6.15 ± 1.07

CN= cotyledonary node, DE= decapitated embryo, CADE= cotyledon attached decapitated embryo

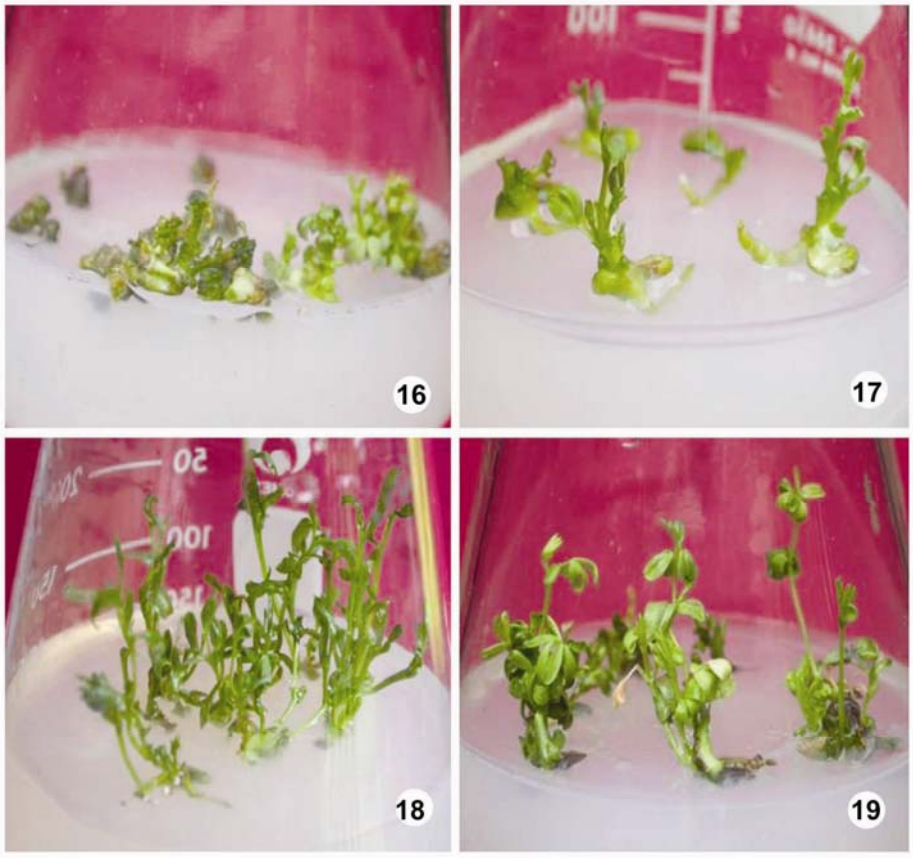
shoots on these media and finally the number of regenerated shoots was found to increase. Regenerated shoots from this explant were healthy, green with numerous leaves. No remarkable variation was obtained among the varieties regarding shoot formation from this explant.

Shoot regeneration from decapitated embryo: MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosine were used for multiple shoot formation from decapitated embryo in four varieties of lentil. In this experiment, the number of initially regenerated shoots varied from 1 - 2 per explants following 12 - 16 days of culture (Figs 16 - 19). The number of shoots did not increase significantly upon their subculture on the same medium. Most of the regenerated shoots were stunted in growth and failed to elongate further and mostly giving a bushy appearance. However, no remarkable variation was obtained among the varieties regarding shoot formation using this explant.

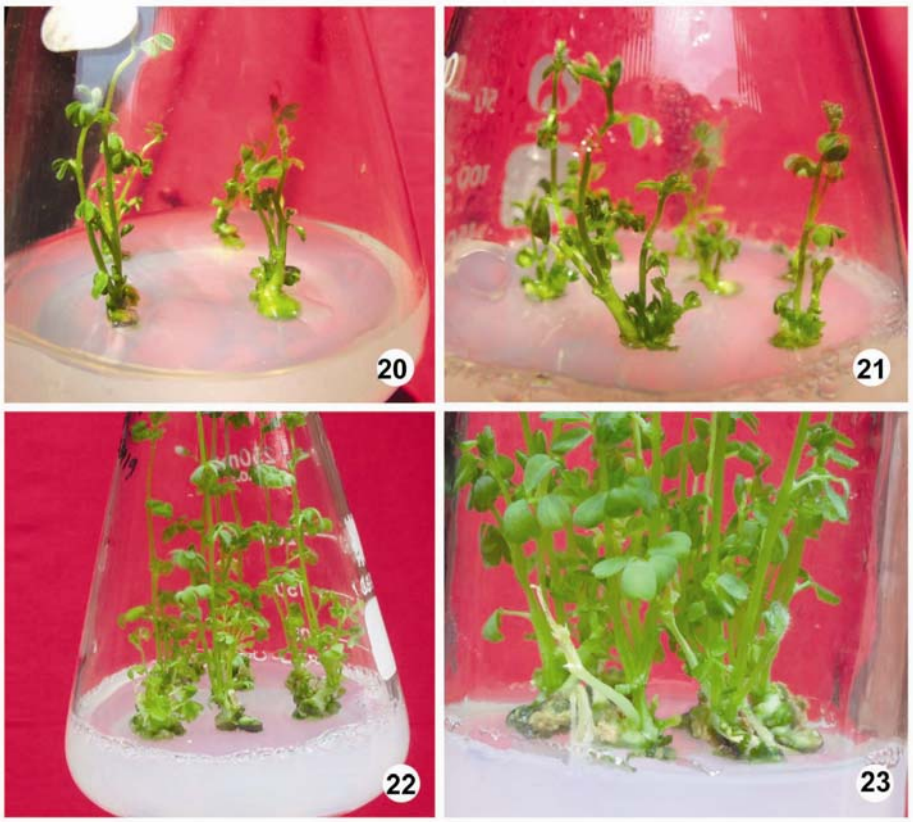
Shoot regeneration from cotyledon attached decapitated embryo: The regeneration ability of cotyledon attached decapitated embryo explants were tested to find out their shoot induction ability on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosine. The shoots were found to initiate within 5 - 7 days of culture (Figs 20 - 23). It was possible to develop 2 - 5 elongated shoots from this type of explants following the maintenance of these cultures in same medium. Like CN explants several new shoot buds were developed at later stages during the culture. Such shoot buds were further developed into new shoots on these media and finally the number of regenerated shoots was found to increase. No considerable variation was also noticed among the varieties regarding shoot forming capability using this explant.



Figs 12 - 15. *In vitro* regeneration of multiple shoots of cotyledonary node (CN) explants from different lentil varieties on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosin. 12. Multiple shoot regeneration in case of variety BM-1 of lentil. 13. Same as Fig. 12 but in case of variety BM-4. 14. Same as Fig. 12 but in case of variety BM-5. 15. Same as Fig. 12 but in case of variety BM-6.



Figs 16 - 19. *In vitro* regeneration of multiple shoots from decapitated embryo (DE) explants of different lentil varities on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosin. 16. Multiple shoot regeneration in case of variety BM-1 of lentil. 17. Same as Fig. 16 but in case of variety BM-4. 18. Same as Fig. 16 but in case of variety BM-5. 19. Same as Fig. 16 but in case of variety BM-6.



Figs 20 - 23. *In vitro* regeneration of multiple shoots of cotyledon attached decapitated embryo (CADE) explants from different lentil varities on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosin. 20. Multiple shoot regeneration in case of variety BM-1 of lentil. 21. Same as Fig. 20 but in case of variety BM-4. 22. Same as Fig. 20 but in case of variety BM-5. 23. Same as Fig. 20 but in case of variety BM-6.

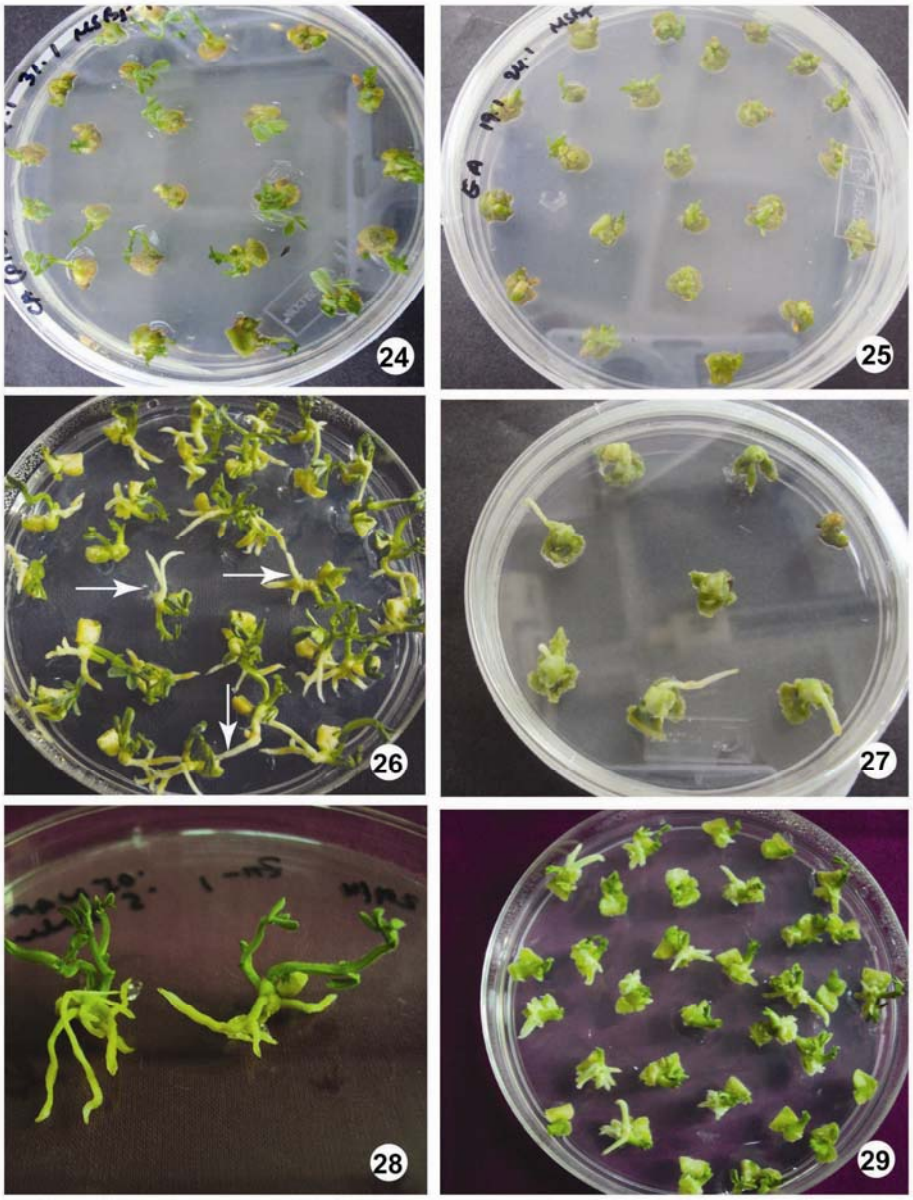
From the above experiments it was observed that cotyledonary node explant showed the best response towards multiple shoot induction. Next to cotyledonary node, cotyledone attached decapitated embryo showed comparatively better response than decapitated embryo explants.

4.1.1.2 Effect of growth regulators on shoot induction

Since CN and CADE were found to be suitable for multiple shoot regeneration therefore, the effect of cytokinins like BAP, Kn and zeatin were investigated using these two types of explants on shoot regeneration from BM-6 variety of lentil. When BAP in combination with NAA was used the explants responded in producing green mass of shoot primodia along with a few thin elongated shoots (Fig. 24). Kinetin (Kn) in combination with NAA also showed more or less identical observation towards shoot regeneration (Fig. 25). But when zeatin in combination with NAA was used multiple shoot induction having profuse rooting at the base of the regenerated shoots was observed (Figs 26 & 27). Zeatin in combination with NAA showed a spectacular result towards shoot induction. For this reason several experiments were carried out using different concentrations of zeatin (0.5 mg/l - 2.0 mg/l) and NAA (0.05 mg/l - 1.0 mg/l) to find out its effect on shoot regeneration in lentil. From this experiment high percentage of multiple shoot induction having profuse rooting at the base of the regenerated shoots was observed in all combinations of zeatin and NAA used in the present investigation for shoot regeneration (Table 12). Highest number of multiple shoots with healthy roots was observed on MSB_5 medium supplemented with 1.0 mg/l zeatin in combination with 0.1 mg/l NAA (Fig. 28). It was also observed that with the increase in concentration of zeatin and NAA decreased the shoot length and resulted in stunted shoots with huge amount of callus mass (Fig. 29). No remarkable variation was obtained between CN and CADE explant towards shoot formation from BM-6 variety of lentil.

Table 12. Effects of different concentration of zeatin and NAA supplemented MSB_5 media on *in vitro* regeneration from CADE explants of BM-6 variety of lentil.

MSB_5 with hormonal supplement (mg/l)		Percentage of responsive explants towards multiple shoot regeneration	No. of shoots/explant (mean ± Sd)	Percentage of responsive explants towards root development
Zeatin	NAA			
0.5	0.05	98	2.96 ± 0.13	70
	0.1	82	2.84 ± 0.13	78
	0.5	92	2.44 ± 0.01	76
	1.0	94	2.38 ± 0.13	78
1.0	0.05	78	2.79 ± 0.21	73
	0.1	98	3.04 ± 0.57	96
	0.5	98	2.45 ± 0.27	86
	1.0	98	2.22 ± 0.21	96
2.0	0.05	82	2.95 ± 0.01	68
	0.1	98	2.54 ± 0.05	94
	0.5	96	2.23 ± 0.11	82
	1.0	96	2.34 ± 0.16	80



Figs 24 -29. Effect of hormonal supplements on *in vitro* morphogenesis. 24. Induction of shoot from CADE explants of BM-6 of lentil on MSB_5 medium supplimented with 1.0 mg/l BAP + 1.0 mg/l NAA. 25. Same as Fig. 24 but MSB_5 medium supplimented with 1.0 mg/l Kn + 1.0 mg/l NAA. 26. Same as Fig. 24 but MSB_5 medium supplemented with 1.0 mg/l zeatin + 1.0 mg/l NAA. Note, the induction of roots (arrows) as well as shoots. 27. Same as Fig. 26 but in case of CN explant. 28. Enlarged view of Fig. 26 showing the development of healthy roots. 29. Same as Fig. 26 where MSB_5 medium supplimented with 2.0 mg/l zeatin + 1.0 mg/l NAA. Note that the higher concentrations of zeatin and NAA inhibited the morphogenesis.

4.1.2 Shoot regeneration via callus development

In this part of investigation attempts were made to regenerate shoots through callus development using the explant which did not respond through direct regeneration. A total of 15 different media composition containing different growth regulators were employed to examine the regeneration ability of four different explants namely, shoot tip (ST), nodal segment (NS), hypocotyle (Hypo) and cotyledon (C) via callus induction. These experiments had been carried out in two phases. In the first phase, callus induction from four different explants of BM-6 variety of lentil was cultured on MSB_5 with and without various concentrations and combinations of BAP, Kn, NAA, zeatin and 2, 4-D. In the second phase, shoot induction and acclimatization of regenerated shoots was tried using various culture media.

4.1.2.1 Effect of hormonal supplements towards callus induction

To find out the effect of hormonal supplements towards callus induction, four different explants were cultured on nine different combinations of hormonal supplements as described in Table 13. After four weeks of culture noticeable difference was observed in case of callus induction among the various combinations of hormonal supplements used in this study. The highest amount of callus was observed on MSB_5 medium containing 1.0 mg/l NAA and 1.0 mg/l zeatin. In this media, explants first developed a large number of callus and then rhizogenesis occured with whitish and thick root from this developed callus. Explants inoculated on the medium containing 1.0 mg/l NAA and 1.0 mg/l BAP produced a small amount of hard compact and smooth callus. Explants cultured on media containing 2, 4-D alone and in combination with other hormonal supplements (Media E, F, G, H and I) produced very soft friable and watery callus. Amount of callus was very little when the explants were cultured on MSB_5 medium supplemented with 1.0 mg/l NAA and 1.0 mg/l Kn. Result obtained from the responsive explants towards callus induction has been presented in Fig. 30.

Table 13. Combinations and concentrations of hormonal supplements used in callus induction and shoot regeneration from different explants of BM - 6 variety of lentil.

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

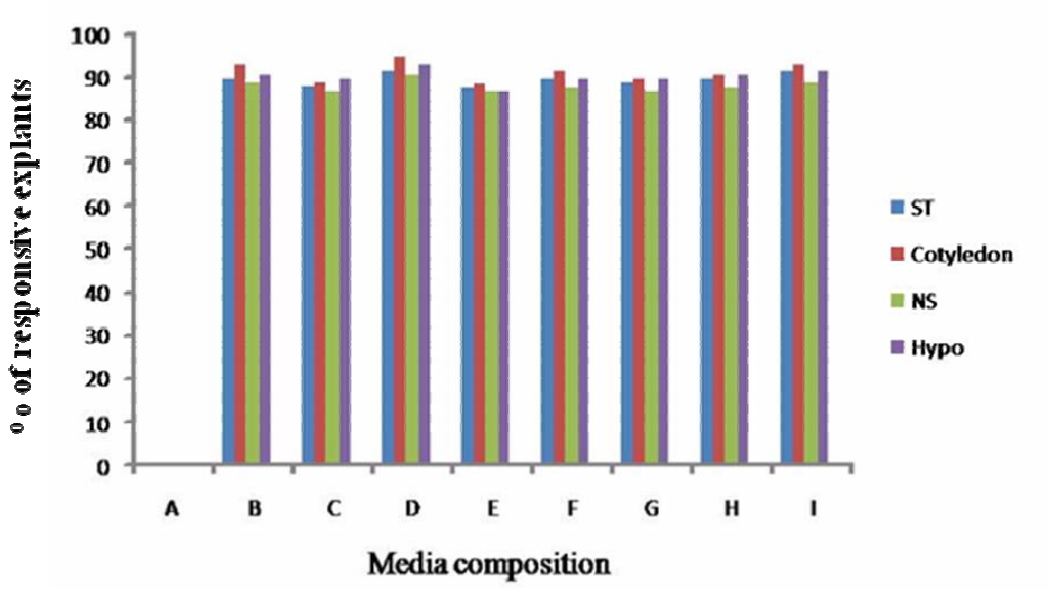


Fig. 30. Responses of different explants towards callus induction on various media combination after 4 weeks of culture.

4.1.2.2 Effect of explants on callus induction

The effect of various explants towards callus induction on MSB_5 medium supplemented with different concentrations and combnations of 2, 4-D, zeatin, BAP, Kn and NAA has been presented in Table 14.

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

Table 14. Effects of various hormonal supplements and explants towards callus induction in BM - 6 variety of lentil.

Media composition	weight of callus in g (mean ± sd)			
	ST	C	Hypo	NS
MSB_5	0.04±.01	0.05±.01	0.04±.01	0.02±.01
MSB_5 + 1.0 mg/l NAA + 1.0 mg/l BAP	0.13±.03	0.16±.02	0.09±.01	0.06±.01
MSB_5 + 1.0 mg/l NAA + 1.0 mg/l Kn	0.06±.01	0.10±.03	0.05±.01	0.02±.01
MSB_5 + 1.0 mg/l NAA + 1.0 mg/l zeatin	0.10±.01	0.17±.02	0.07±.02	0.06±.02
MSB_5 + 0.5 mg/l 2, 4-D	0.07±.01	0.11±.02	0.06±.02	0.04±.02
MSB_5 + 1.0 mg/l 2, 4-D	0.10±.01	0.18±.02	0.07±.01	0.05±.01
MSB_5 + 1.0 mg/l 2, 4-D + 1.0 mg/l BAP	0.11±.02	0.14±.02	0.07±.03	0.05±.02
MSB_5 + 1.0 mg/l 2, 4-D + 1.0 mg/l Kn	0.10±.03	0.15±.01	0.05±.02	0.05±.01
MSB_5 + 1.0 mg/l 2, 4-D + 1.0 mg/l zeatin	0.11±.03	0.15±.02	0.07±.02	0.06±.02

4.1.2.3 Nature of callus

Distinguishable variation was observed in the nature of callus induced from different explants. Callus produced from ST explants was found to be semi hard, friable and pale white on all types of media combination except 2, 4-D containing media after 9 -12 days of culture. Considerable number of shoot primordia was also observed from this explant in all types of media composition used in the present investigation (Fig. 31).

Cotyledon explants responded by hard, compact and green callus with numerous number of roots after 7 -10 days of culture (Fig. 32). Following 12-15 days of culture semi hard and compact callus was observed from NS explant (Fig. 33).

In most of the cases semi hard, friable, whitish and massive embryo like structures were found when hypocotyle explant was used for callus induction (Fig. 34). The nature of developing callus is presented in Table 15.

4.1.2.4 Development of shoot from induced callus

In this part of investigation attempts were made to regenerate shoots from the callus developed from various explants. The induced calli were transferred to MS media supplements with different combinations and concentrations of hormones (as mentioned in Table 13) to observe their effects on shoot regeneration. Among the various explants tested only cotyledon explants responded to shoot regeneration on three media combinations of hormonal supplements, namely, J, K and L (Fig. 35**).** Although ST and Hypo explants formed numerous shoot primordia but these shoot primordia did not show any sign of shoot regeneration and failed to survive.

It was interesting to note that the composition of callus initiation media played important role on the success of shoot induction. Calli produced on the media contain

2, 4-D alone or in combinations with other hormones showed no response on shoot induction. This calli continued to grow on these media without producing any shoot or root (Fig. 36)**.** The overall responses towards shoot regeneration *via* callus derived from different explants are presented in Fig. 37.

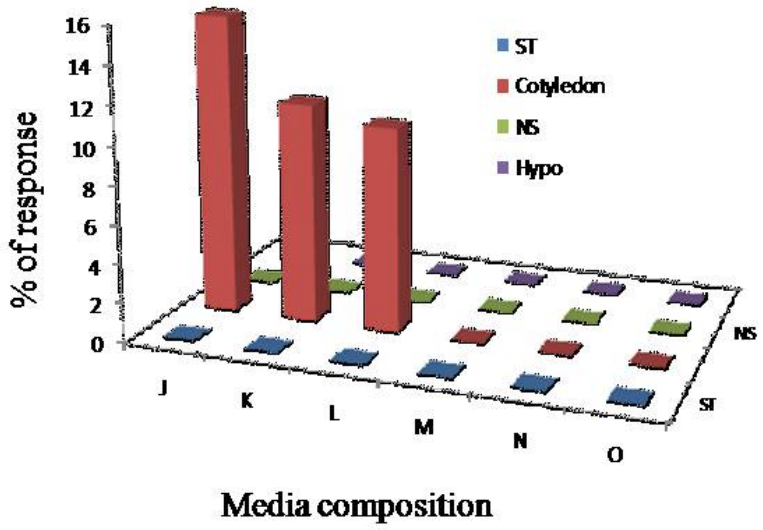
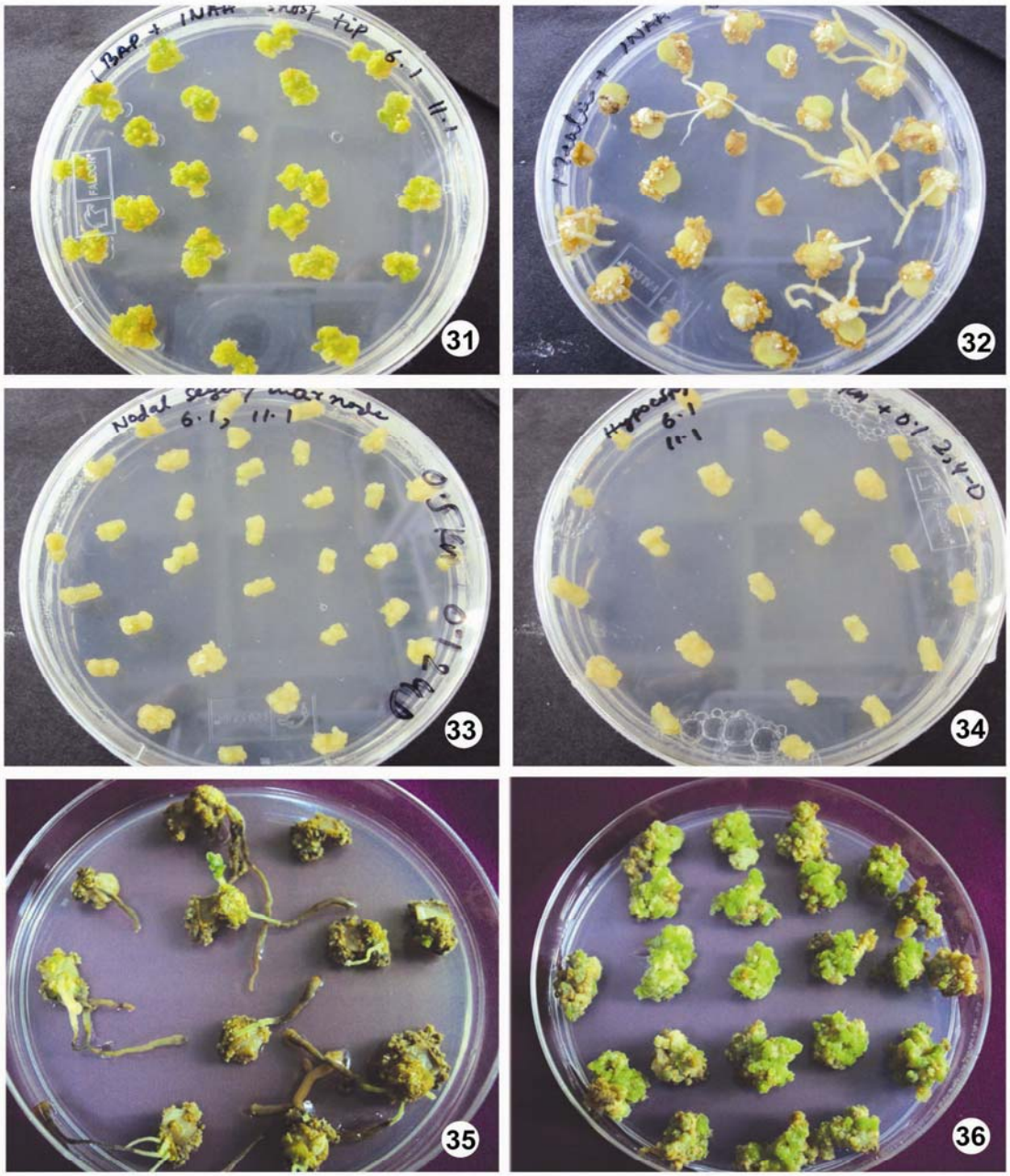


Fig. 37. Responses of induced callus from different explants towards shoot regeneration on MS media without any hormonal supplement.

Table 15. Effect of different media compositions on the nature of induced callus.

Composition of media	Nature of callus			
	ST	Cotyledon	Hypo	NS
MSB_5+1 mg/l NAA + 1 mg/l BAP	Semi hard, friable, green	Hard, compact, green	Semi hard, friable, whitish, embryonic	Hard, compact, brown
MSB_5+1 mg/l NAA + 1 mg/l Kn	Semi hard, friable, green	Hard, compact, green	Semi hard, friable, whitish, embryonic	Hard, compact, brown
MSB_5+1 mg/l NAA +1 mg/l zeatin	Semi hard, friable, green	Hard, compact, green, rooted	Semi hard, friable, whitish, embryonic	Hard, compact, brown, rooted
MSB_5+0.5 mg/l 2, 4-D	Watery, friable, green	Watery, compact, green	Watery, friable, whitish, embryonic	Watery, compact, whitish
MSB_5+1 mg/l 2, 4-D	Watery, friable, green	Watery, compact, green	Watery, friable, whitish, embryonic	Watery, compact, whitish
MSB_5+1 mg/l 2, 4-D + 1 mg/l BAP	Watery, friable, green	Watery, compact, green	Watery, friable, whitish, embryonic	Watery, compact, whitish
MSB_5+1 mg/l 2, 4-D + 1 mg/l Kn	Watery, friable, green	Watery, compact, green	Watery, friable, whitish, embryonic	Watery, compact, whitish
MSB_5+1 mg/l 2, 4-D + 1 mg/l zeatin	Watery, friable, green	Watery, compact, green	Watery, friable, whitish, embryonic	Watery, compact, whitish



Figs 31 - 36. Development of *in vitro* shoots through callus induction. 31. Callus developed from shoot tip (ST) explant on MSB_5 medium supplemented with 1.0 mg/l BAP + 1.0 mg/l NAA. 32. Callus developed from cotyledon (C) explants on MSB_5 medium supplemented with 1.0 mg/l zeatin + 1.0 mg/l NAA. 33. Callus developed from nodal segment (NS) explant on MSB_5 media supplemented with 0.5 mg/l Kn + 0.1 mg/l 2, 4-D. 34. Same as Fig. 33 but in case of hypocotyle explant. 35. Initiation of shoots from the developed callus on zeatin containing media from cotyledon explants after transfer to MS medium without any hormonal supplement. 36. Calli developed on 2, 4-D containing medium continued to grow without the initiation of shoot/root after transfer to MS medium without any hormonal supplement.

Among all the media tested the medium J (MS without any growth regulator) showed best response towards shoot induction. On this media the percentage of shoot regeneration was found to be 15.6 from cotyledon explant. However, in case of media K and L the percentage of response was 11.4 and 10.6 respectively. Although no remarkable variation was observed regarding percentage of shoot regeneration on media L and K, however, the shoot health was better on medium L than K. Explants cultured on media M, N and O did not exibit any shoot regeneration capability. In these media newly transferred callus became dark green, increase in volume but did not show any sign of shoot development.

4.2 Induction of roots from *in vitro* regenerated shoots

Induction of roots from regenerated shoots is considered to be very important to obtain complete plantlet. In the present study most of the explants produced high number of hairy roots in shoot induction medium (MSB_5 media supplemented with zeatin and NAA) and subsequently these hairy roots were found to produce healthy effective root system when they were transferred to MS basal medium (Figs 38 & 39). After 3 - 4 weeks the ideal rooted shoots were carefully taken out from the culture tube and were successfully transplanted to plastic pots containing the equal quantity of soil, sand and vermiculite. About 95% transplanted plantlets survived which bore phenotypically normal flowers and pods (Fig. 40).

Although root formation was observed in zeatin containing shoot induction media but no sign of root formation was observed when the shoots regenerated through the application of other cytokinin (BAP or Kn). To induce roots from the regenerated shoots, 2 - 4 cm long individual shoots were excised and cultured on MS as well as half MS media with different concentrations of IBA and NAA. A negligible rooting from the *in vitro* raised regenerated shoots of lentil was

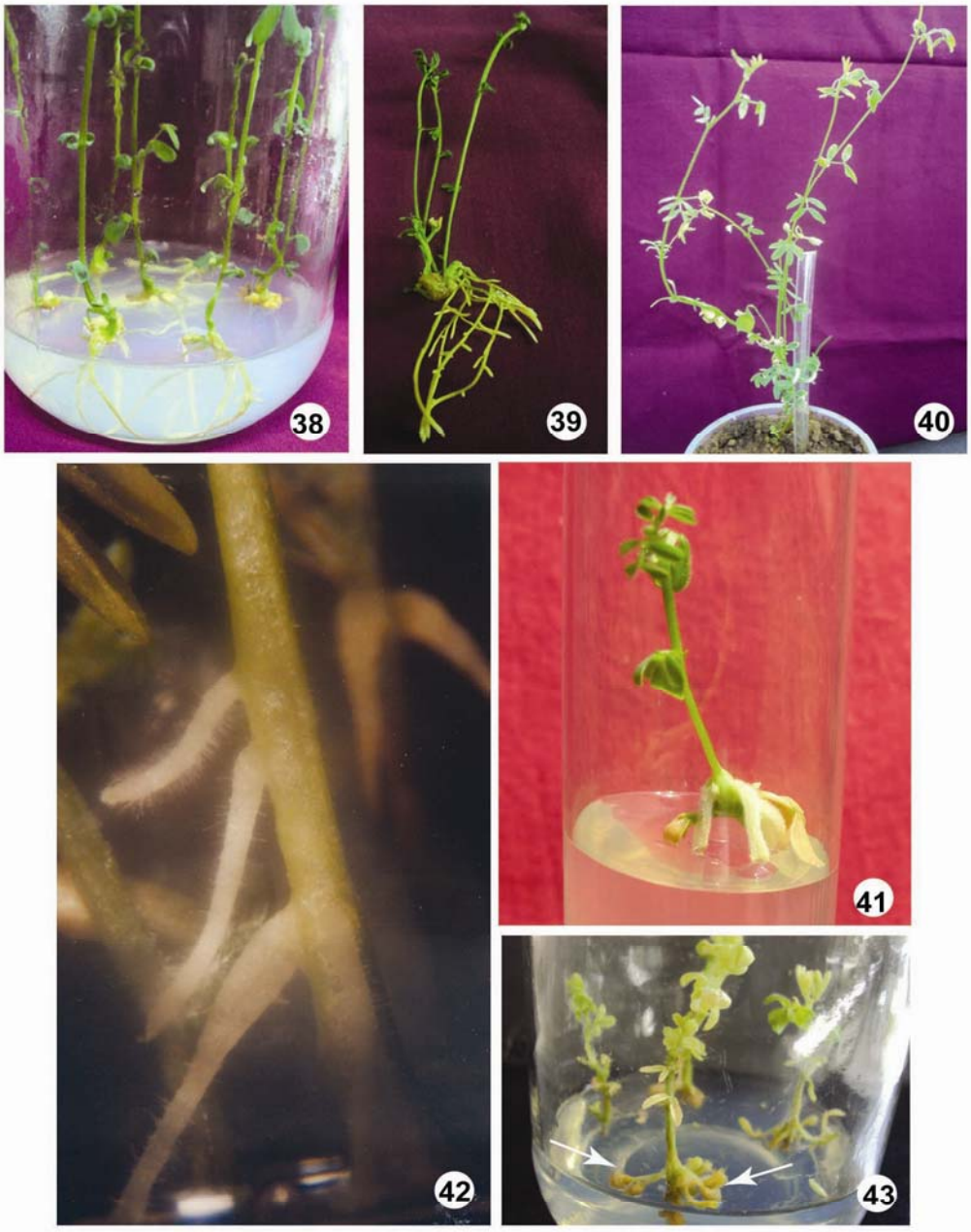
obtained on MS medium supplemented with 20 and 25 mg/l IBA (Table 16). However, these roots did not develop from the base of the shoots; rather they develop slightly above the excision point and failed to integrate effectively with the shoot vascular system. Furthermore, freshly developed roots formed callus when their tips in contact with the medium (Figs 41 - 43). Besides these experiments, a number of media combinations using half and full strength of MS supplemented with IBA and NAA were tried. Moreover, quick deep of excised shoots had also been tried using 1000 mg/l IBA for 1 minute and then transferred this treated shoots to rooting media used in the present study. But no significant results were obtained regarding root initiation. The responses regarding root induction were almost identical for all the four varieties of lentil.

4.3 *In Vitro* flowering and pod formation

In the present study *in vitro* rooting in microsperma type of lentil was also found to be difficult and appeared to be a major constraint in obtaining fully regenerated plantlets. Under these circumstances, attempts were made to initiate *in vitro* flowering and fully developed seed formation from the *in vitro* regenerated shoots to overcome the problems of *in vitro* effective root development in obtaining plantlets through *in vitro* techniques. This technique of *in vitro* flowering was considered as an alternative to regeneration system escaping the methods of root formation from regenerated shoots of lentil. *In vitro* development of pods and seeds following the *in vitro* flower formation was considered as one of the alternatives in obtaining plant progenies particularly in obtaining effective seed formation from the transformed shoots.

Table 16. Effects of auxin on root induction from the regenerated shoots of four varieties (BM-1, BM-4, BM-5 and BM-6) of lentil.

Media composition	Variety	No. of shoot inoculated	No. of shoot responded	Shoots with root (%)	General observation
MS + 10 mg/l IBA	BM-1	22	0	0	No root formation
	BM-4	50	0	0	
	BM-5	50	0	0	
	BM-6	55	0	0	
MS + 15 mg/l IBA	BM-1	20	0	0	
	BM-4	45	0	0	
	BM-5	55	0	0	
	BM-6	55	0	0	
MS + 20 mg/l IBA	BM-1	30	8	26.6	Root initiated from slightly higher than the cut end and callus formed when reached to the medium
	BM-4	35	11	31.4	
	BM-5	65	19	29.2	
	BM-6	60	16	26.6	
MS + 25 mg/l IBA	BM-1	25	9	36.0	
	BM-4	40	15	37.5	
	BM-5	75	24	32.0	
	BM-6	80	24	30.0	
MS + 25 mg/l IBA + 0.5 mg/l NAA	BM-5	70	24	34.2	*In vitro* floweing ovserved
	BM-6	70	22	31.4	
½ MS + 25 mg/l IBA + 0.5 mg/l NAA	BM-5	65	20	30.7	
	BM-6	60	20	33.3	



Figs 38 - 43. Development of *in vitro* roots. 38. Adventitious roots developed from the base of the elongated shoots (developed on MSB_5 medium supplemented with zeatin and NAA on MS media without any hormonal supplement. 39. Enlarged view of Fig. 38 showing healthy roots developed from the base of regenerated shoot. 40. Fully developed plantlets growing in pot containing soil, sand and vermiculite (1:1:1). 41. Induction of roots (slightly above from the cut end) from the regenerated shoots (developed on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosin) of BM-4 following the treatment of high concentration of IBA (25 mg/l). 42. Stereomicroscopic view of adventious roots developed from the body of the regenerated shoots. 43. Same as Fig. 41 showing the formation of callus (arrows) from the tip of the developing roots.

For *in vitro* flowering and pod formation, *in vitro* derived lentil shoots was transferred to MS medium supplemented with several concentrations and combinations of IAA or IBA plus NAA to induce flowering and seed setting.

Experiments were conducted to select suitable media composition and hormonal supplements for *in vitro* flowering using cotyledonary nodal explants derived shoots of lentil as this explants showed best response towards multiple shoot regeneration (section 4.1.1.1). Two different types of media combinations such as MS and half strength of MS with different concentrations and combinations of auxins (IBA, IAA and NAA) as well as MS without any hormonal supplements were employed for this purpose.

4.3.1 Effect of different concentrations of IBA to initiate flower *in vitro*

In these experiments MS medium supplemented with different concentrations of IBA (10 - 25 mg/l) was used to observe the effect of IBA on flower initiation from *in vitro* regenerated shoots of BM-4 varieties of lentil. Results of these experiments are shown in Table 17.

Flower initiation was found to increase with the increase of IBA concentrations but the developing flowers were dried out and failed to develop seed. Shoots that cultured on MS without any hormonal supplements were also monitored to observe the initiation of flowering. Shoots regenerated through this experiment were healthy in appearance and flower bud formation was also observed but these induced flower buds failed to open and remain unopened for several weeks (Fig. 44). Percentage of flower initiation was highest (14.81) on MS media supplemented with 20 mg/l IBA and maximum number of flower (1 - 4) was observed on MS media supplemented with 25 mg/l IBA. Results of these experiments are shown in Table 17.

Table 17. Effects of IBA on *in vitro* flower initiation from the regenerated shoots of BM-4 variety of lentil.

Media used	No. of shoot inoculated	No. of shoot with flowers	% of shoot with flowers	Range of flowers per shoot
MS	570	60	10.52	-
MS + 10 mg/l IBA	580	40	6.89	1
MS + 15 mg/l IBA	310	30	9.67	1
MS + 20 mg/l IBA	405	60	14.81	1 - 3
MS + 25 mg/l IBA	364	39	10.71	1 - 4

4.3.2 Effect of different concentrations of IBA with NAA in initiating *in vitro* flower

Several experiments were also conducted to induce *in vitro* flowers from the *in vitro* regenerated shoots through the application of various concentrations of IBA and NAA in full and half strength of MS medium. For this purpose, three different concentrations of IBA (10, 20 and 25 mg/l) and three different concentrations of NAA (0.2, 0.5 and 1.0 mg/l) in 12 different combinations were used for *in vitro* flowering. The overall responses due to the effect of different combinations IBA and NAA are shown in Table 18.

Among the hormonal combinations tried, the best response regarding flower formation was obtained on half strength of MS medium supplemented with 20 mg/l IBA and 0.5 mg/l NAA while the maximum number of flower was obtained on a medium comprising MS with 20 mg/l IBA and 0.5mg/l NAA. During this study *in vitro* flower buds were initiated within 7 to 10 days of culture of the shoots. Number of flower was found to be increased following regular sub

culturing of these *in vitro* flower bearing shoots on the media having the similar hormonal supplements. Using the above hormonal composition 2 - 10 flowers per shoot was observed. Results of this experiment have been shown in Table 18. It was observed from Table 18 that increasing amount of NAA in the medium increased the percentage and number of flower bud formation per shoot. The increased flower bud formation was recorded with the increase of NAA concentration up to 0.5 mg/l. But the NAA concentrations beyond 0.5 mg/l NAA found to decrease the number of flower buds per shoot.

Table 18. Effects of different combinations and concentrations of IBA and NAA in MS medium on *in vitro* flowering from cotyledonary node derived shoots of lentil.

Media used	Hormonal supplement		No. of shoot inoculated	No. of shoot with flowers	% of shoot with flowers	Range of flower per shoot
	IBA (mg/l)	NAA (mg/l)				
MS	10	1.0	500	300	60.0	1 - 5
	10	0.5	640	440	68.75	1 - 8
	10	0.2	400	130	32.5	1 - 3
	20	1.0	510	210	41.17	1 - 3
	20	0.5	540	345	63.38	1 - 10
	20	0.2	380	142	37.36	1 - 4
	25	1.0	480	160	33.33	1 - 3
	25	0.5	500	184	36.8	1 - 4
	25	0.2	320	102	31.87	1 - 3
½ MS	20	1.0	560	274	48.9	1 - 6
	20	0.5	585	462	73.35	1 - 6
	20	0.2	340	152	44.7	1 - 5

4.3.3 Effect of different concentrations of IAA and NAA to initiate *in vitro* flower

Two different concentrations of IAA (10 and 20 mg/l) and three different concentrations of NAA (0.2, 0.5 and 1.0 mg/l) in nine different combinations were used to obtain *in vitro* flower on half and full strength of MS. The overall responses of the shoots towards flowering due to the effect of these two hormonal supplements are presented in Table 19.

Table 19. Effects of different combinations and concentrations of IAA and NAA in MS medium on *in vitro* flowering from cotyledonary node derived shoots of lentil.

Media used	Hormonal supplement		No. of shoot inoculated	No. of shoot with flowers	% of shoot with flowers	Range of flower per shoot
	IAA (mg/l)	NAA (mg/l)				
MS	10	1.0	510	90	17.64	1 - 2
	10	0.5	450	150	33.33	1 - 2
	10	0.2	400	42	10.5	1
	20	1.0	312	30	9.62	1 - 2
	20	0.5	525	225	42.85	1 - 4
	20	0.2	380	72	18.94	1 - 2
½ MS	20	1.0	500	180	36.0	1 - 3
	20	0.5	525	255	48.57	1 - 4
	20	0.2	310	89	28.7	1 - 3

Among the hormonal combinations tried, half strength of MS medium supplemented with 20 mg/l IAA and 0.5 mg/l NAA showed the best response in flowering. Maintaining such culture condition 1 - 4 flowers were initiated within 10 - 20 days. Frequencies of *in vitro* flowers were increased when the flowering shoots were sub cultured on the same medium.

4.3.4 Responses of various explant derived shoots towards *in vitro* flowering

Among all the media and hormonal supplements tested half strength of MS medium with 20 mg/l IBA + 0.5 mg/l NAA showed the best response towards *in vitro* flowering. Using this media and hormonal supplements responses of various explants were tested for *in vitro* flowering.

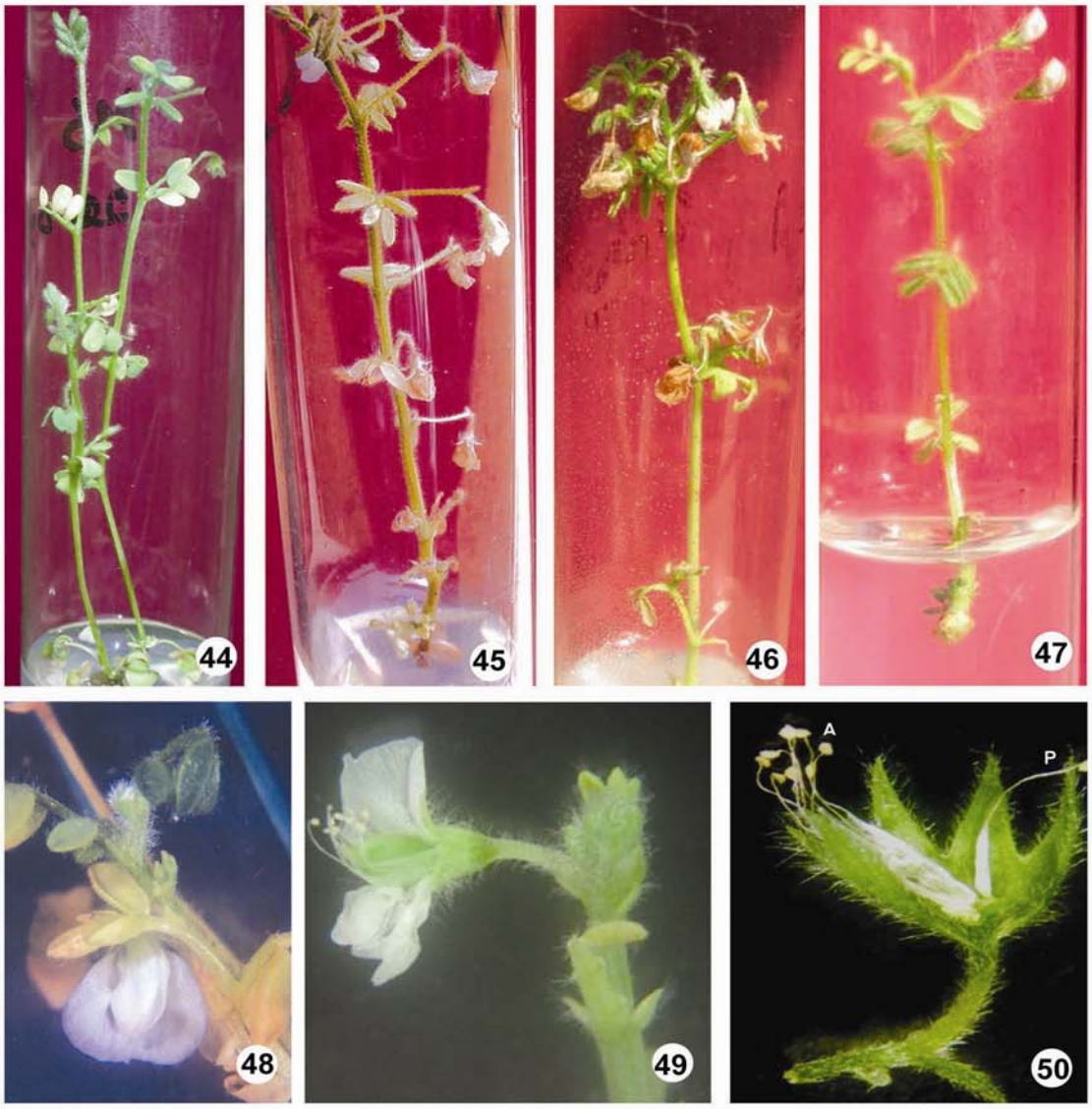
In this case, single shoots that derived from cotyledonary nodes (CN), cotyledon attached decapitated embryo (CADE) and decapitated embryo (DE) explants were inoculated on half strength of MS medium supplemented with 20 mg/l IBA and 0.5 mg/l NAA. These shoots were found to initiate 1 - 6 flowers within 8 - 10 days after inoculation in case CADE explants (Fig. 45), 1 - 8 flowers within 10 - 15 days in case on CN explants (Fig. 46) and 1 - 3 flowers within the same days in case of DE explants (Fig. 47), respectively. These *in vitro* flowers were very much identical to the flowers developed under the field condition (Figs 48 & 49). Stereomicroscopic view of this *in vitro* flower is shown in Fig. 50. Maintaining such culture condition numbers of flowers were increased but at least 50% of flower eventually died after some days in case of the shoots derived from CN and DE explants.

Among the three explants studied cotyledonary node derived shoots showed better response towards *in vitro* flowering. The overall responses of these explants are presented in Table 20.

Table 20. Comparison of the responses of *in vitro* shoots derived from different explants towards *in vitro* flowering cultured on half strength of MS medium supplemented with 20 mg/l IBA and 0.5 mg/l NAA.

Variety	Source of shoots	No. of shoots inoculated	No. of shoots with flower	% of shoots with flower	Days to initiate flowers	No. of flowers per shoot (mean ± Sd)
BM-1	CN	400	280	70.0	12-15	4.95 ± 1.63
	CADE	390	231	59.2	8-10	3.05 ± 1.03
	DE	125	48	38.4	8-10	1.8 ± 0.79
BM-4	CN	420	340	77.0	12-15	5.8 ± 2.14
	CADE	330	210	64.7	8-10	3.65 ± 1.93
	DE	125	50	40.0	8-10	2.1 ± 0.79
BM-5	CN	440	300	68.0	10-12	5.3 ± 1.74
	CADE	400	220	55.0	8-10	4.85 ± 1.53
	DE	150	51	34.0	8-10	2.55 ± 1.16
BM-6	CN	360	305	80.3	10-12	6.0 ± 2.24
	CADE	400	250	62.5	8-10	4.4 ± 1.78
	DE	160	75	46.8	8-10	2.75 ± 1.16

CN= Cotyledonary node CADE= Cotyledone attached decapitated embryo DE= decapitated embryo



Figs 44 - 50. Development of *in vitro* flowers from the regenerated shoots. 44. Induction of *in vitro* flower buds (arrows) from cotyledon attached decapitated embryo derived regenerated shoots of BM-5 on MS medium without any hormonal supplement. 45. ***In vitro*** flowers developed from cotyledon attached decapitated embryo derived regenerated shoots of BM-5 on half strength of MS medium supplemented with 20 mg/l IBA + 0.5 mg/l NAA. 46. Same as Fig. 45 but in case of cotyledonary node derived shoots of BM-5. 47. Same as Fig. 45 but in case of decapitated embryo derived regenerated shoots of BM-5. 48. Enlarged view of developing *in vitro* flower in case of BM - 4 variety of lentil. 49. Same as Fig. 48 but in case of BM - 5 variety of lentil. 50. Enlarged view of fully developed *in vitro* flower of BM - 6 showing different floral parts including pistils (P) and anthers (A). ×16.

4.3.5 Test of pollen viability

Anthers from *in vitro* grown flowers contained a large number of pollen grains (Figs 51 & 52). Viability of pollen grains from these *in vitro* grown flowers were determined on the basis of the degree of staining achieved after soaking the pollen grains in a solution containing 0.01% FDA and 20% sucrose for 30 min. Stained pollen grains were observed using a fluorescent microscope (Nikon, Japan). Fluorescent microscopic observation indicated that about 80% of the pollen grains were viable (Fig. 53).

4.3.6 *In vitro* germination of pollen grains

For the assessment of *in vitro* pollen germination ability, pollen grains from fully developed *in vitro* flowers were placed on MS medium (a liquid culture medium without agar) supplemented with 0.01% boric acid and 10% sucrose. From this experiment it was observed that some of the pollen grains germinated and produced pollen tubes within approximately 90 min after being placed on germination medium but the maximum germination was recorded after 135 min (Fig. 54). It was also observed that not all the viable pollen grains produced pollen tube (Table 21). The pollen grains viability was found to be 80%, whereas only 20% pollen grains showed pollen tube development. No considerable variation was observed in pollen viability and germination ability among the lentil varieties used in this experiment.

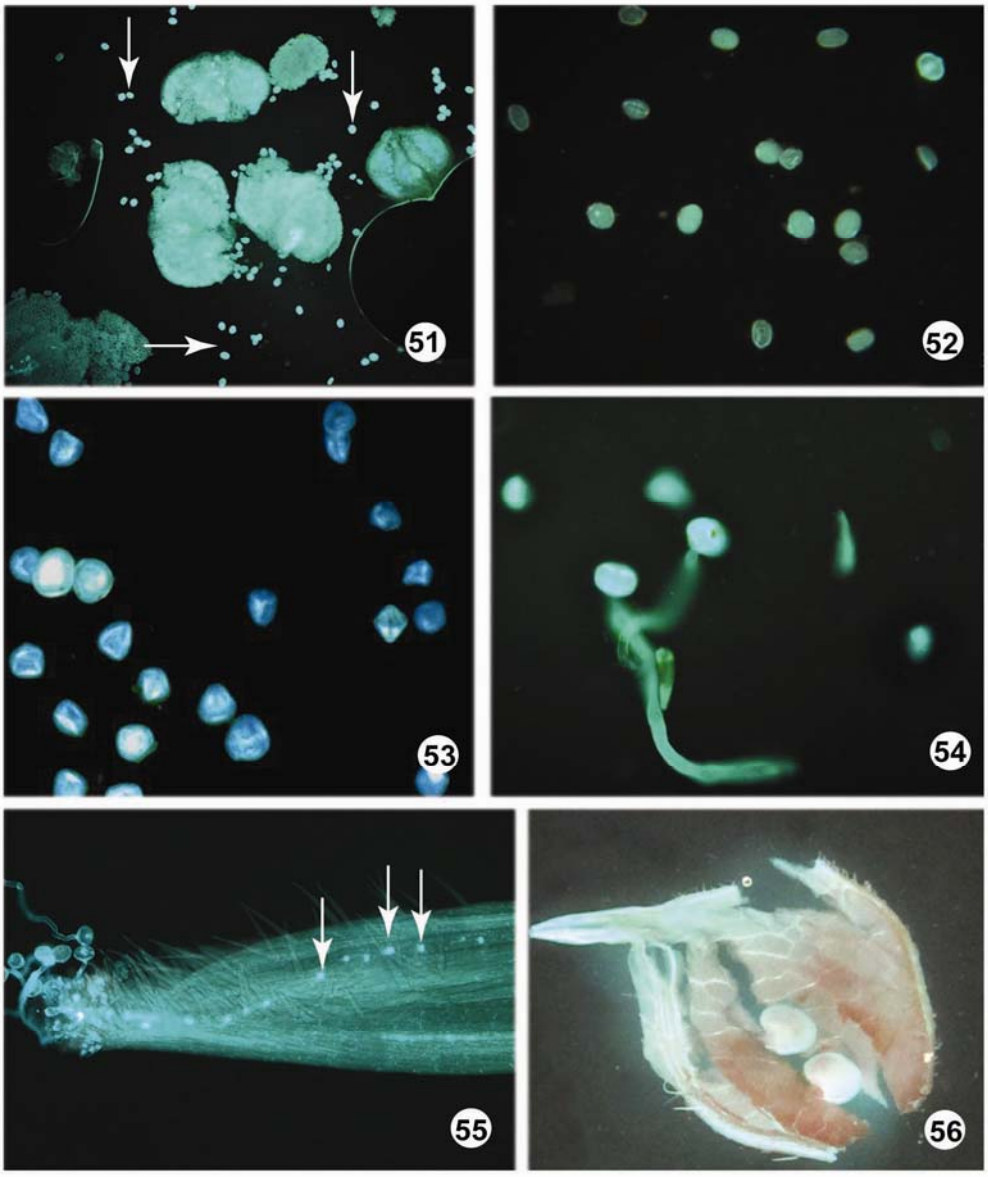
Self pollinated pistils were also examined to monitor *in situ* growth of the pollen tubes within the stylar tissue. Callose invested healthy pollen tubes were observed under epifluorescence within pistilar tissue of the *in vitro* flowers (Fig. 55). After such self-pollination the ovules were found to develop into seeds (Fig. 56).

Table 21. Viability and *in vitro* germination ability of pollen grain.

Variety	No. of observations	No. of pollen grains	No. of stained pollen	% of pollen viability	No. of pollen grain germinated (time interval in min)				Germination (%)
					45	90	135	180	
BM-5	1	36	30	83.33	-	4	8	9	20.22
	2	44	32	72.72	-	4	9	9	
	3	37	28	75.67	-	3	6	6	
BM-6	1	42	32	76.19	-	5	9	9	19.17
	2	36	29	80.55	-	3	6	6	
	3	36	30	83.33	-	3	7	7	

4.3.7 *In vitro* seed formation

For *in vitro* seed formation flower bearing shoots derived from cotyledonary nodes, cotyledon attached decapitated embryo and decapitated embryo explants were maintained in the medium containing same hormonal supplements used for *in vitro* flower formation. During this period after 15-20 days of flowering, seeds were found to develop from healthy flowers. Effects of different concentrations of auxins such as IAA (10 - 20 mg/l), IBA (10 - 25 mg/l) and NAA (0.2 - 1.0 mg/l) were tested. From these experiments it was observed that about 12 to 20 days after flowering mature pods were found to develop from healthy flowers. Best response regarding the number of pods per shoot was obtained on half-strength of MS medium supplemented with 20 mg/l IBA and 0.5 mg/l NAA. About 58 % flowers produced pods and the range of pods per shoot was 1 - 4 (Table 22). Number of seed increased following further subculture in same medium. Media containing IAA and NAA also showed positive responses regarding *in vitro* pod



Figs 51 - 56. Fluorescent micrographs of pollen grains and pollinated pistils. 51. Anthers from *in vitro* derived flowers of BM - 4 showing a large number of pollen grains (arrows). ×14. 52. Same as Fig. 51 but showing an enlarged view of pollen grain. ×10. 53. Fluorescent microscopic views of pollen grains following FDA staining showing viable pollen grains. ×10. 54. *In vitro* germination of pollen grains and pollen tube formation following 180 minute of incubation in 10% sucrose and 0.01% boric acid. 55. Callose invested pollen tube (arrows) growing within the stylar tissue following self-pollination in BM - 6 variety of lentil. ×10. 56. Stereomicrospic view of the developing seeds within the ovary after *in vitro* self-pollination. ×6.

formation (Table 23). Maintaining of such culture condition 1 - 3 pods were initiated within 10 - 20 days of inoculation. From these experiments it was also observed that 25% of flowering shoots responded to pod formation when the shoots maintained in only IBA containing media. Using this hormonal composition only one pod per shoot was observed (Table 24). The seeds obtained from these shoots were germinated for further development of plantlets. Experiments were also conducted to monitor the event of *in vitro* pod formation using hormone free MS medium as control.

Table 22. Effects of different combinations and concentrations of IBA and NAA in MS medium on *in vitro* pod formation from cotyledonary node derived flowering shoots of lentil.

Media used	Hormonal supplements (mg/l)		No. of shoots with flower	No. of shoots with pod	% of shoots with pod	Range of pods per shoot
	IBA	NAA				
MS	10	1.0	300	75	25.0	1
	10	0.5	440	100	22.7	1
	10	0.2	130	17	13.07	1
	20	1.0	210	60	28.5	1
	20	0.5	345	45	13.04	1- 2
	20	0.2	142	27	19.01	1
	25	1.0	160	7	4.37	1
	25	0.5	184	14	7.6	1
	25	0.2	102	7	6.86	1
½ MS	20	1.0	274	117	42.7	1 - 3
	20	0.5	462	266	57.57	1 - 4
	20	0.2	152	41	26.9	1 - 2

Table 23. Effects of different combinations and concentrations of IAA and NAA in MS medium on *in vitro* seed formation from cotyledonary node derived flowering shoots of lentil.

Media used	Hormonal supplement (mg/l)		No. of shoots with flower	No. of shoots with pod	% of shoots with pod	Range of pods per shoot
	IAA	NAA				
MS	10	1.0	90	0	0	0
	10	0.5	150	30	20.0	1
	10	0.2	42	3	7.14	1
	20	1.0	30	0	0	0
	20	0.5	225	60	6.66	1
	20	0.2	72	5	6.94	1
½ MS	20	1.0	180	31	17.22	1 - 3
	20	0.5	255	105	41.17	1 - 3
	20	0.2	89	9	10.11	1 - 2

Table 24. Effects of IBA on *in vitro* pod formation from the cotyledonary node derived regenerated shoots of BM-4 variety of lentil.

Media used	No. of shoots with flower	No. of shoots with pod	% of shoots with pod	No. of pod per shoot
MS	60	0	0	0
MS + 10 mg/l IBA	40	0	0	0
MS + 15 mg/l IBA	30	0	0	0
MS + 20 mg/l IBA	60	15	25	1
MS + 25 mg/l IBA	39	0	0	0

Among all the media and hormonal combinations tested half strength of MS medium with 20 mg/l IBA + 0.5 mg/l NAA showed best response towards *in vitro* flowering and seed formation in all four varieties of lentil. A comparative observation due to effect of various culture media containing different concentrations and combinations of auxins towards *in vitro* flowering and seed formation is presented in Fig. 57.

4.3.8 Determination of suitable explant derived shoots towards *in vitro* seed formation

Experiments were carried out to examine the ability of pod setting of various *in vitro* grown flowering shoots obtained from BM-1, BM-4, BM-5 and BM-6 variety of lentil. Cotyledonary node (CN), cotyledon attached decapitated embryo (CADE) and decapitated embryo (DE) derived shoots were used for this purpose. Shoots developed from different explants were maintained in the best responsive medium (half strength of MS medium with 20 mg/l IBA + 0.5 mg/l NAA) where *in vitro* seed set were obtained. Effect of different explants derived shoots towards *in vitro* pod formation has been shown in Table 25.

In this case, single excised shoots that derived from cotyledon attached decapitated embryo were inoculated on half strength of MS medium supplemented with 20 mg/l IBA and 0.5 mg/l NAA. These shoots were found to initiate 1-5 seeds within 10-15 days after inoculation (Figs 58 & 59).

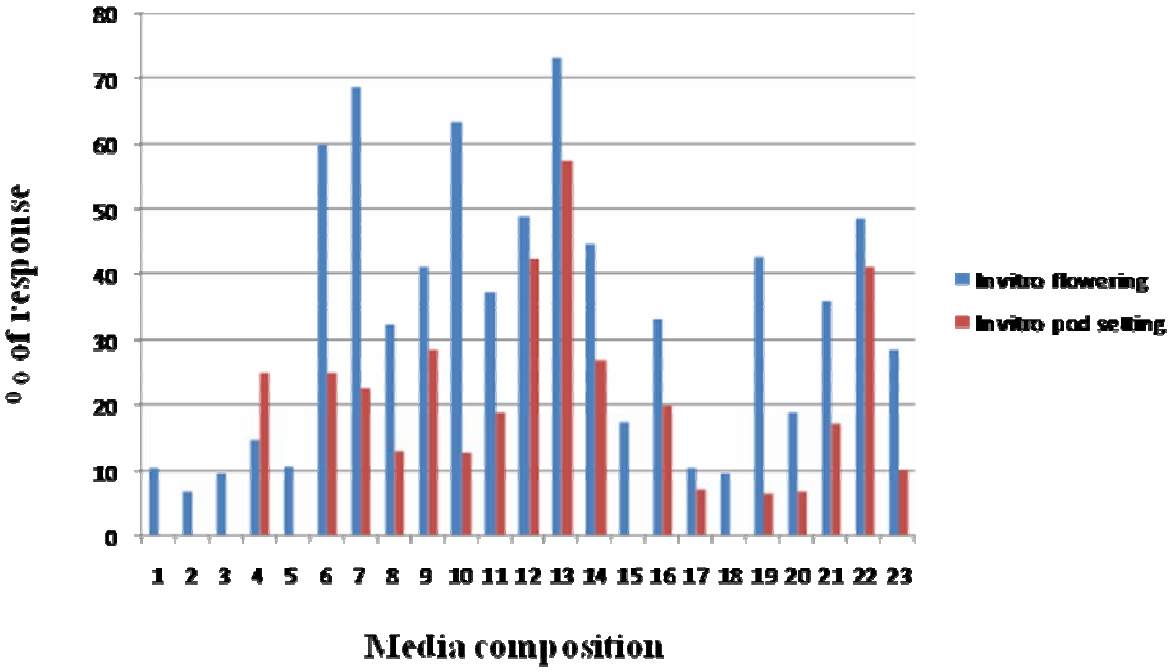


Fig. 57. Effects of various auxin supplements on *in vitro* flowering and pod formation in BM-4 variety of lentil.

1= MS
2= MS+10 mg/l IBA
3= MS+15 mg/l IBA
4= MS+20 mg/l IBA
5= MS+25 mg/l IBA
6= MS+10 mg/l IBA+1.0 mg/l NAA
7= MS+10 mg/l IBA+0.5 mg/l NAA
8= MS+10 mg/l IBA+0.2 mg/l NAA
9= MS+20 mg/l IBA+1.0 mg/l NAA
10= MS+20 mg/l IBA+0.5 mg/l NAA
11= MS+20 mg/l IBA+0.2 mg/l NAA
12= ½ MS+20 mg/l IBA+1.0 mg/l NAA
13= ½ MS+20 mg/l IBA+0.5 mg/l NAA
14= MS+25 mg/l IBA+0.2 mg/l NAA
15= MS+10 mg/l IAA+1.0 mg/l NAA
16= MS+10 mg/l IAA+0.5 mg/l NAA
17= MS+10 mg/l IBA+0.2 mg/l NAA
18= MS+20 mg/l IAA+1.0 mg/l NAA
19= MS+20 mg/l IAA+0.5 mg/l NAA
20= MS+20 mg/l IAA+0.2 mg/l NAA
21= ½ MS+20 mg/l IAA+1.0 mg/l NAA
22= ½ MS+20 mg/l IAA+0.5 mg/l NAA
23= ½ MS+20 mg/l IAA+0.2 mg/l NAA

Table 25. Effects of different explants derived flowering shoots on *in vitro* seed formation in half strength of MS medium supplemented with 20 mg/l IBA + 0.5 mg/l NAA.

Variety	Source of shoots	No. of shoots with flower	No. of shoots with pod	% of shoots with pod	Days required to initiate pod	No. of pods per shoot (mean ± Sd)
BM-1	CN	280	120	42.85	15 - 20	2.45 ± 0.63
	CADE	231	88	38.09	12 - 15	2.65 ± 0.93
	DE	48	9	18.75	8 - 10	1.1 ± 0.33
BM-4	CN	340	170	50.0	15 - 20	2.85 ± 1.09
	CADE	210	100	47.62	12 - 15	3.1 ± 1.38
	DE	50	11	22.0	8 - 10	1.18 ± 0.41
BM-5	CN	300	120	40.0	12 - 16	3.05 ± 1.51
	CADE	220	80	36.36	10 - 12	3.65 ± 1.93
	DE	51	13	25.5	8 - 10	1.23 ± 0.44
BM-6	CN	305	170	55.7	15 - 20	3.55 ± 1.91
	CADE	250	140	56.0	10 - 12	3.75 ± 1.51
	DE	75	19	25.3	8 - 10	1.42 ± 0.51

CN= Cotyledonary node CADE= Cotyledone attached decapitated embryo DE= decapitated embryo

Such cotyledonary nodes derived shoots found to initiate 1 - 4 seed within 10 - 20 days when inoculated in the above mention medium (Fig. 60). In this case pods were developed only on the upper part of the shoot and in most of the cases about 50% of developing flowers failed to produce pods (Fig. 61).

A few numbers of pods was found to develop from the flowering shoots derived from decapitated embryo explants (Fig. 62). Among the three explants studied shoots that derived from cotyledon attached decapitated embryo showed best response towards *in vitro* seed formation. Stereomicroscopic view of these *in vitro* seed is shown in Fig. 63.

4.3.9 *In vitro* germination of seeds and establishment of plantlets into soil

The *in vitro* grown pods were matured fully within 20 to 30 days under *in vitro* condition. Pods were then harvested and dried under sunlight (Fig. 64). Seeds were then surface sterilized and were germinated on water agar medium (Fig. 65). The percentage of germination of the *in vitro* raised seeds was found to be 60, 68, 53 and 57 in case of BM - 1, BM - 4, BM - 5 and BM - 6 respectively.

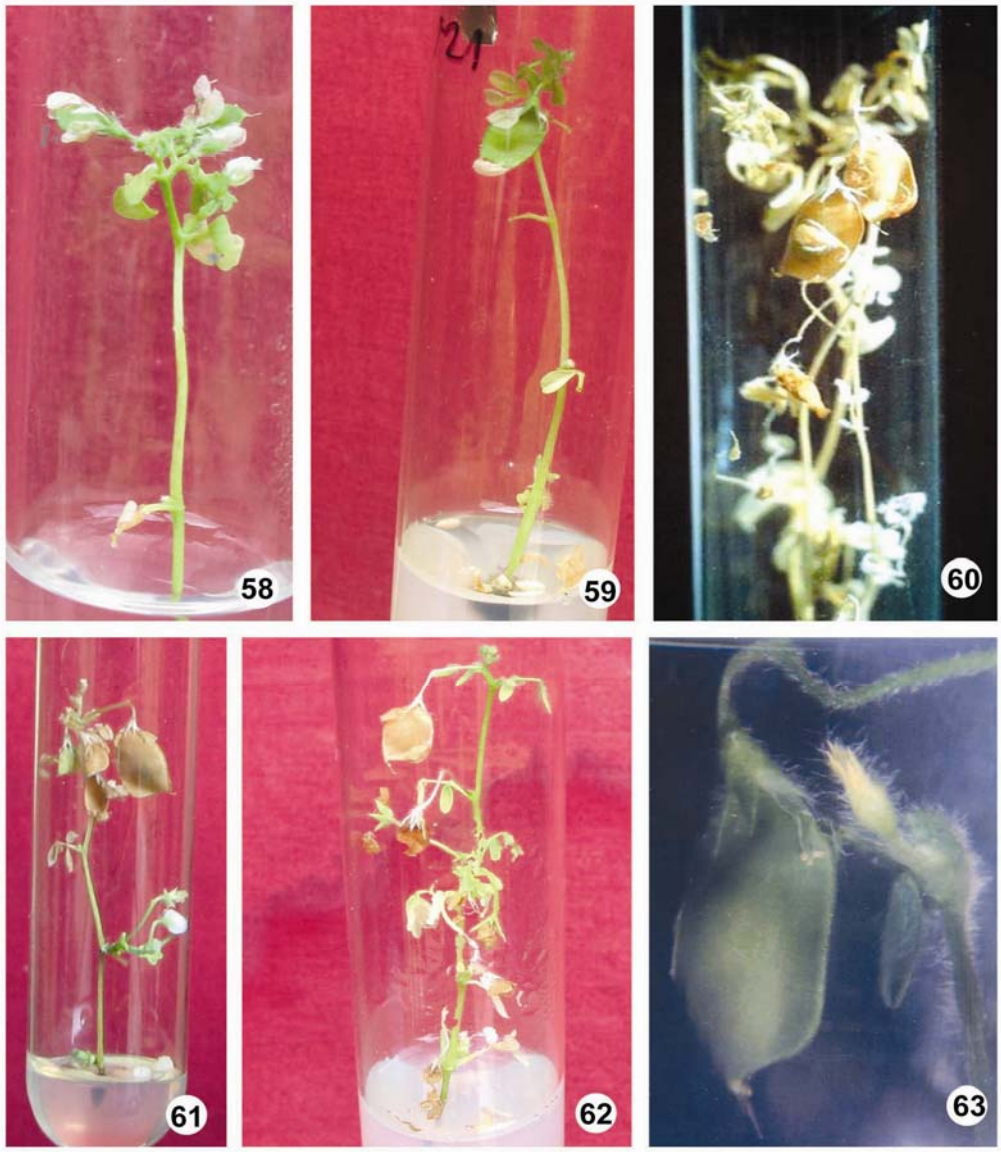
These germinated seedlings were then transplanted into soil for further development and it was observed that most of these transplanted plants eventually developed flowers and seeds (Figs 66 & 67).

At the same time seeds harvested from *in vitro* raised pods were also directly sown in plastic pots containing sterilized peat moss. Pots were then kept in culture room for two weeks and then further maintained in a net house for flower and pod development. In this experiment it was observed that seeds that directly sown in plastic pots containing sterilized peat moss showed better response towards germination compared to the seeds that placed on water agar medium. The overall rate of germination and seedlings survivability is showed in Table 26.

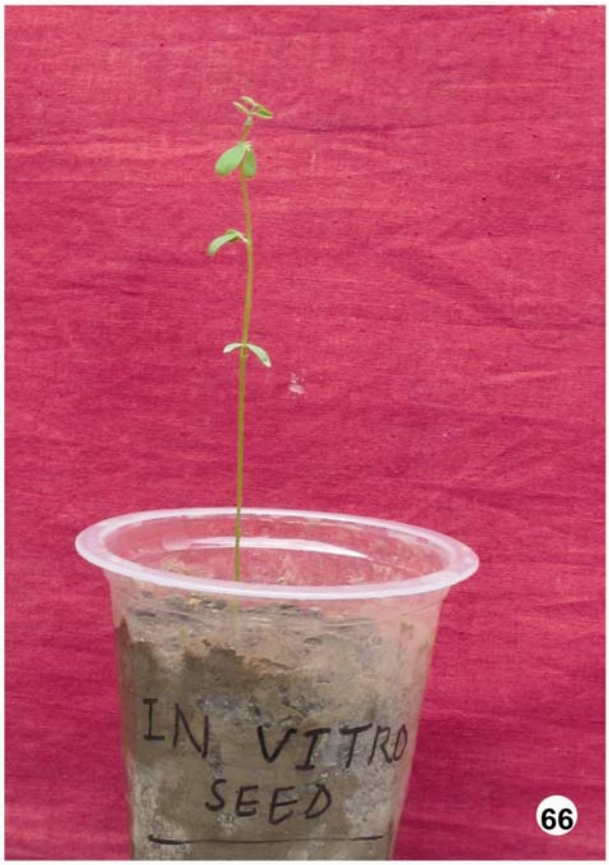
Table 26. Germination and transplantation of seedlings.

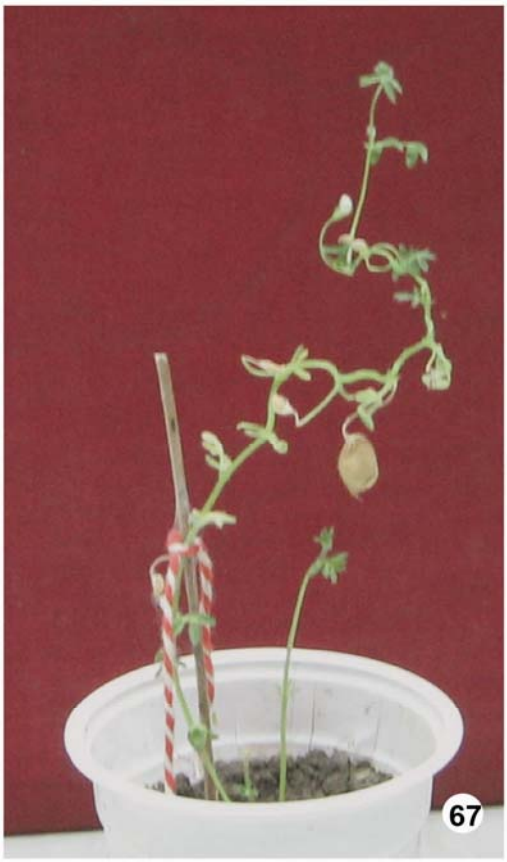
Variety	No. of seed inoculate for germination		No. of seed germinated		% of seed germinated		No. of seedlings transferred to soil		No. of survived seedlings		% of survived seedlings	
	WA	PS	WA	PS	WA	PS	WA	PS	WA	PS	WA	PS
BM-1	50	-	30	-	60.0	-	30	-	11	-	36.7	-
BM-4	50	-	34	-	68.0	-	32	-	14	-	43.8	-
BM-5	30	20	16	13	53.33	65	15	13	7	7	46.7	53.9
BM-6	30	20	17	16	56.66	80	17	16	7	11	41.2	68.8

WA= Water agar medium PS= plastic pots containing sterilized peat moss



Figs 58 - 63. Development of *in vitro* pod from the regenerated shoots. 58. Initiation of *in vitro* pods from CADE derived regenerated shoots cultured on half strength of MS medium supplemented with 20 mg/l IBA + 0.5 mg/l NAA. 59. Development of *in vitro* pods from CADE derived flowering shoots of BM-5 on MS medium supplemented with 20 mg/l IBA. 60. Same as Fig. 59 in case of CN derived shoots of BM-6 variety of lentil. 61. Same as Fig. 60 but in case of BM-5 variety of lentil. 62. Same as Fig. 61 but in case of DE derived regenerated shoots of BM-5. 63. Same as Fig. 58 but showing a magnified view of developing pods. ×7.





Figs 64 - 67. Germination of seeds and transplantation of *in vitro* developed seedlings. 64. Mature harvested *in vitro* developed pods. 65. Germination of *in vitro* developed seed in water agar medium. 66. Plantlet developed from *in vitro* raised seeds transferred to plastic pot containing sterilized peat-moss. 67. Same as Fig. 66 but showing the formation of flowers as well as pods.

4.4 *Agrobacterium*-mediated genetic transformation

For a successful genetic transformation a genotype independent and effective plant regeneration protocol is a prerequisite. During the present study plant regeneration was achieved through organogenesis either directly from the explant or through intervention of callus. However, direct organogenesis from various explants was found to be more effective. Therefore, all the transformation experiments were carried out following the method of direct organogenesis (section 4.1.1). Transformation experiments were undertaken using three different explants of three lentil varieties, namely, Barimasur-4 (BM - 4), Barimasur-5 (BM - 5) and Barimasur-6 (BM - 6). In the present study three different genetically engineered *Agrobacterium* strains with their respective gene constructs, namely, LBA4404/pBI121 (strain I), EHA105/pGII-pSOUPvstN-chitin (strain II) and LBA4404/pCAMBIA2300 enh35S AFP (strain III) were used to transform the Barimasur varieties of lentil. Results of these experiments are presented below in different headings.

4.4.1 Genetic Transformation using marker strain (strain I)

In the present study genetically engineered *Agrobacterium* strain LBA4404/pBI121 (strain I) was used as marker strain which contains GUS gene as screenable marker and *npt*II gene as selectable marker conferring kanamycin resistance. Marker gene was used to investigate the transformation ability of various explants of different varieties of lentil. Generally transient assay for such marker genes were routinely performed as a preliminary step to identify the conditions required for successful transfer of desired gene.

4.4.1.1 Optimization of different parameters influencing transformation efficiency of explants

Available reports on *Agrobacterium*-mediated genetic transformation indicate that, transformation efficiency is influenced by several factors such as optical density (O.D.) of *Agrobacterium* suspensions, incubation and co-cultivation period for the explants. Optimizations of these conditions were done by monitoring transient expression of the GUS reporter gene after co-cultivation of explants in *Agrobacterium* strains LBA4404 containing binary plasmid pBI121 (strain I).

4.4.1.1.1 Influence of optical density (O.D.) and incubation period of *Agrobacterium* suspension on transformation

Agrobacterium suspension prepared from the overnight grown *Agrobacterium* culture (section 3.3) was used to infect the explants. In this experiment the relationship between optical density and incubation period of *Agrobacterium* suspension as well as transformation efficiency of explants were studied. For this purpose optical density (O.D.) was measured at 600 nm and bacterial suspension with optical density of 0.5, 0.8, 1.0 and 1.2 having incubation period of 30, 45 and 60 and 75 minutes were used in these experiments.

Transformation efficiency was found to increase with the increased optical density. Maximum percentage of transformation was observed at O.D of 1.0 and 1.2. At O.D of 1.2 the percentage of GUS positive explants was 80, whereas minimum transient GUS expression (15 %) was recorded at O.D. of 0.5 (Table 27).

These experiments also exhibited that the percentage of GUS positive explants was increased with the increase of incubation period but increase of the percentage of GUS positive explants was not remarkable beyond 45 minutes of incubation.

The maximum percentage of GUS positive explants was observed at 45 and 60 minutes of incubation at O.D of 1.2 (Table 27). It was also observed that when the duration of incubation and optical density of the bacterial suspension was more than 60 minutes and 1.0 respectively then overgrowth of bacteria in the co-cultured plates was observed which hampered desired regeneration capability of the explants. In some cases overgrowth of bacteria was noticed following one or two subcultures when the explants were treated with the above mentioned conditions. This undesired overgrowth of bacteria can be checked when bacterial suspension had an O.D. of 1.0 with incubation period of 45 min. Therefore, the optimum incubation period for the explants was found to be 45 minutes with optical density of 1.0.

4.4.1.1.2 Influence of co-cultivation period on transformation

Four different co-cultivation periods ranging from 2 - 5 days were tried to find out the optimum co-culture period. For strain I it was found that, percentage of transformation could be increased with the increase of co-cultivation period at a constant optical density (1.0) of bacterial suspension and a constant incubation period of 45 minutes. Although percentage of GUS positive explants increased with the increase of co-cultivation period, it was also observed that a co-cultivation for more than 3 days sometimes promoted overgrowth of bacteria (Fig. 68). As a result good number of explants in co-culture media was found to suffer from poor health and became brown thus failed to regenerate (Fig. 69). Therefore, 3 days of co-cultivation period was found to be optimum for transformation with strain I.

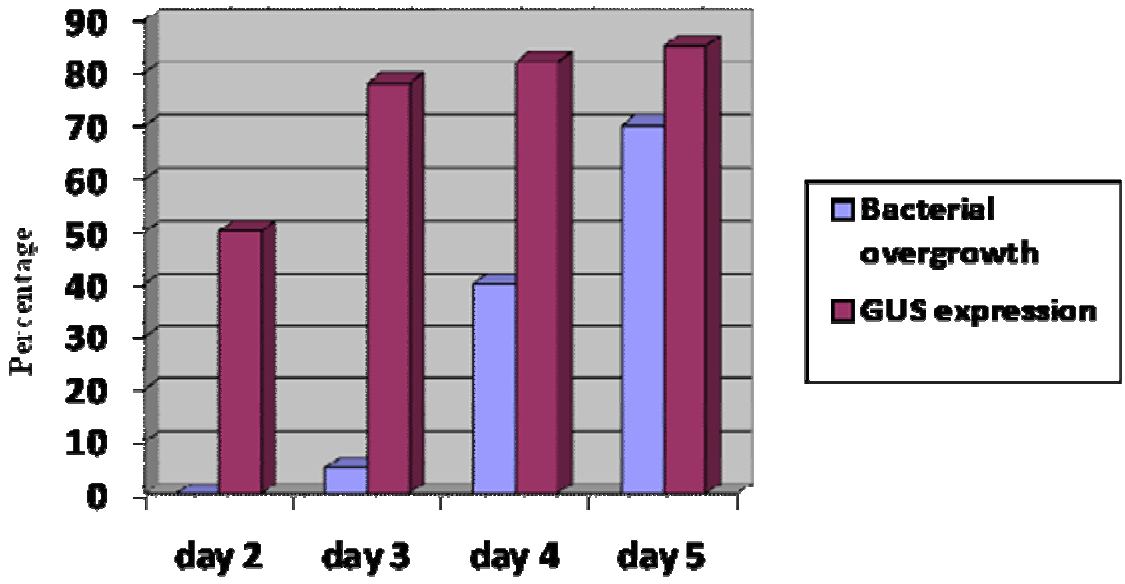


Fig. 68. Interaction between bacterial overgrowth and GUS expression in context with co-culture period of explants in bacterial suspension.

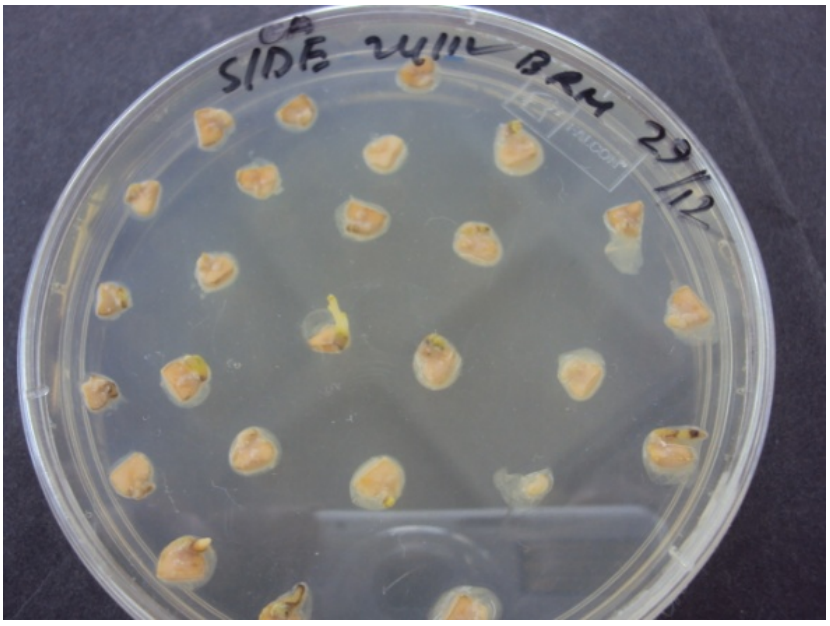


Fig. 69. Overgrowth of bacteria within the explant for a period of 5 days of co-cultivation.

Table 27. Influence of optical density (measured at 600 nm) of *Agrobacterium* suspension and effect of different incubation periods on *Agrobacterium*-mediated transformation of various explants of BM - 5 variety of lentil analyzed through transient GUS histochemical assay.

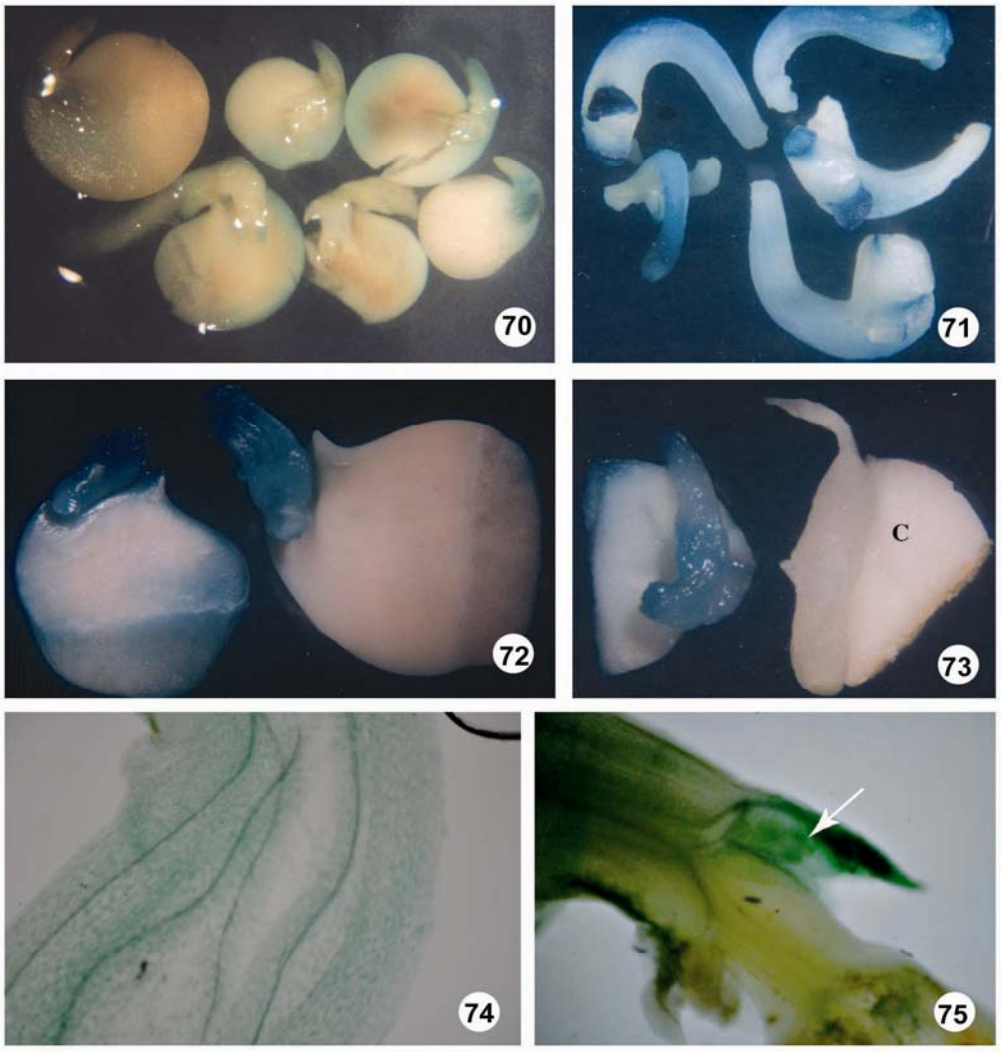
$O.D_{600}$	Incubation period (min)	No. of explants infected	No. of explants assayed for GUS	No. of GUS+ve explants	% of GUS+ve explants
0.5	15	50	20	3	15
	30	50	20	4	20
	45	50	20	5	25
	60	50	20	7	35
0.8	15	50	20	6	30
	30	50	20	6	30
	45	50	20	8	40
	60	50	20	9	45
1.0	15	50	20	6	30
	30	50	20	9	45
	45	50	20	14	70
	60	50	20	14	70
1.2	15	50	20	10	50
	30	50	20	13	65
	45	50	20	16	80
	60	50	20	16	80

4.4.1.2 Responses of various explants of lentil towards *Agrobacterium*-mediated genetic transformation

Transformation ability of various explants, namely, DE, CN and CADE of BM-4, BM-5 and BM-6 variety of lentil was monitored through the expression of the GUS gene following incubation for a period of 45 minutes with *Agrobacterium* strain LBA4404 having an O.D. of 1.0 and co-cultivation for 72 hrs. GUS expression was detected by histochemical assay and such expression was characterized by the formation of indigo blue colour within the transformed cells of the infected explants. Control explants were always maintained in each set of experiment and were subjected to GUS histochemical assay in order to understand the difference between transformed and non transformed tissue. Results of these experiments are presented in Table 28.

A good number of co-cultured explants showed positive to GUS staining. GUS positive regions were visualized mostly at the peripheral area of the cut surfaces as well as within the internal tissues of various explants (Figs 70 - 73).

Prominent blue coloured (GUS+ve) zones within co-cultured explants were visualized under stereomicroscope. In some cases the whole explant appeared to be blue in colour (Fig. 74) but in other cases this blue colour was localized in some specific areas of the explant (Fig. 75). It was evident from Table 28 that, DE explants showed the best response towards transformation with LBA4404 strain and the percentage of GUS positive DE explants was 86.9 in case of BM-6 variety of lentil. Next to DE explants CADE explants showed better responses towards transformation and in this case the percentage of GUS positive explants was found to be 78.18. In case of CN explants highest percentage of GUS positive explant were 58 in case of BM-6 which appeared to be the lowest among the three explants studied.



Figs 70 - 75. Histochemical GUS expression of infected explants with *Agrobacterium* strain LBA4404. 70. Histochemical localization of GUS activity in explants of CN of BM - 4. × 20. 71. Same as Fig. 70 but in case of DE explant × 20. 72. Same as Fig. 70 but in case of CADE explant. × 20. 73. Same as Fig. 72 but in case of BM - 5 variety of lentil. Note that the absence of GUS activity in the control (C) explant. 74. Magnified view of internal tissue showing positive GUS expression conspicuous blue color in whole explant. 75. Same as Fig. 72 showing localized blue color within the internal tissue (arrows) of CADE explant.

Although transformation efficiency was comparatively low in CN explants but its regeneration capacity was found to be better among the three different types of explants studied. Therefore, this explant was included for further transformation experiments. On the other hand, in spite of better transformation efficiency exhibited by DE further transformation experiments were not carried out using this type of explant since the regeneration capability of this explant was found to be very low. Thus, further transformation experiments were carried out only with CN and CADE explants.

Table 28. Responses of various explants of BM - 4 variety of lentil towards GUS histochemical assay following co-cultivation.

Variety	Explants	No. of explants assayed for GUS	No. of GUS+ve explants	% of GUS+ve explants
BM-4	CN	95	53	55.79
	CADE	85	72	77.70
	DE	90	70	84.70
BM-5	CN	146	47	32.19
	CADE	126	105	75.38
	DE	120	91	83.33
BM-6	CN	150	58	38.60
	CADE	138	120	78.18
	DE	110	86	86.90

CN= Cotyledonary node CADE= Cotyledone attached decapitated embryo DE= decapitated embryo

4.4.1.3 Shoot regeneration from transformed explants

To obtain regeneration of shoots co-cultivated explants were washed with 300 mg/l ticarcillin for 10 minutes and then transferred to suitable regeneration medium (MS medium with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosine) with 100 mg/l ticarcillin. Shoot regeneration media supplemented with 100 mg/l ticarcillin were used for the elimination of all unwanted bacteria but no selectable agents were applied immediately after co-cultivation. The regenerating shoots with 2 - 3 leaf stage was sub-cultured on the same media containing selectable agents. For strain I kanamycin was used as selectable agent. In each set of experiment, regenerated control explants were also maintained to perform various comparative studies between transformed and non transformed tissues as well as plantlets.

4.4.1.4 Determination of optimum kanamycin concentration for selection

The shoots developed from both infected and non infected (control) CN and CADE explants were transferred to selection medium. To determine the optimum selection level non infected explants (those served as negative control) were subjected to selection pressure. For this experiment after the initiation of regeneration the explants were transferred to suitable regeneration medium containing 50 mg/l kanamycin. Then the concentration of kanamycin was increased gradually in the following manner: 50,100,150 and 200 mg/l.

From this experiment it was revealed that with the increase of kanamycin concentration the percentage of survived shoots were found to decrease. Due to the effect of kanamycin the shoots first became albino and finally died. The effects of different concentrations of kanamycin on regenerated shoots from control explants have been shown in Fig. 76. In this study it was observed that all the non infected explants (negative control) died in presence of 200 mg/l kanamycin within 15 days of inoculation. Therefore, shoots developed from infected explants

surviving in regeneration medium with 200 mg/l kanamycin for more than 15 days were considered to be transformed.

4.4.1.5 Selection of transformed shoots

For successful transformation a proper and efficient selection procedure is required in obtaining desired transformed shoots/plantlets. To select the transformed shoots initially 50 mg/l kanamycin was used as the selection pressure. After 14 days, only green and healthy shoots were subcultured on fresh regeneration medium with 100 mg/l kanamycin.

Then the kanamycin concentration was raised up to 150 mg/l in the third subculture and then 200 mg/l in the fourth subculture. During each subculture, the albino and deep brown dead shoots were discarded and only green shoots were sub-cultured on fresh medium containing the next higher concentration of kanamycin. From this experiment it was observed that with the increase of kanamycin concentration the percentage of survived shoots were found to decrease. The results of this experiment have been shown in Fig. 77. Finally those shoots that survived on this selection medium (regeneration medium with 200 mg/l kanamycin) for more than 15 days and remained green and healthy were selected as possible transformed shoots (Figs 78 - 82). It was observed that, none of the CN explants derived shoots were able to continue their growth in presence of higher concentration (200 mg/l) of kanamycin (Fig. 83), whereas a few CADE explants derived shoots were recovered after such selection. A total of 31 kanamycin resistant shoots out of approximately 1896 infected explants in case of BM-4, 12 out of 570 in case of BM-5 and 22 out of 1020 in case of BM-6 were recovered in final selection medium with LBA4404 strain of *Agrobacterium* containing binary plasmid pBI121. Therefore, the frequency of recovery of putative transformed shoots was about 1.63 in case of BM-4, 2.10 in case of BM-5 and 2.15 in case of BM-6 (Table 29).

4.4.1.6 *In vitro* flower and pod formation from the transformed shoots of lentil

Shoots that survived in the kanamycin selection medium were separated and transferred to half strength of MS medium containing 20 mg/l IBA, 0.5 mg/l NAA and 50 mg/l kanamycin. It was observed that in higher concentration of kanamycin (100 mg/l) about 50% of flower bud failed to open and in 200 mg/l of kanamycin no flower bud formation was observed. After 2 - 3 weeks *in vitro* flower formation was observed in the healthy shoots that transferred to half strength of MS medium containing 20 mg/l IBA, 0.5 mg/l NAA and 50 mg/l kanamycin (Fig. 84). It was found that out of 65 survived shoots 20 shoots responded to flowering and the maximum number of flower per shoot was 3. It was also found that after 12 - 15 days of flowering, 7 out of these 20 flowering shoots produced viable and healthy pods (Fig. 85) under *in vitro* condition and the range of seed per shoot was only 1 - 2. Results of these experiments are presented in Table 30.

Table 29. Effect of kanamycin on shoots.

Variety	Explant	No. of explants infected	No. of shoots inoculated	No. of survived shoots in regeneration media with kanamycin (mg/l)				% of survive shoots
				50	100	150	200	
BM-4	CN	480	1066	1010	323	53	0	0
	CADE	1896	3125	3072	1011	307	31	1.63
BM-5	CN	410	1020	940	234	13	0	0
	CADE	570	1162	1125	460	256	12	2.10
BM-6	CN	400	1009	920	201	22	0	0
	CADE	1020	2982	2062	490	56	22	2.15

CN= Cotyledonary node CADE= Cotyledone attached decapitated embryo

Table 30. Response of CADE derived survived shoots towards *in vitro* flowering and pod formation and their transformation efficiency.

Variety	No. of shoots subjected for selection	No. of shoots survived in 200 mg/l kanamycin	No. of shoots with flower	No. of flower/ shoot (mean ± Sd)	No. of shoots with pod	No. of pods/ shoot (mean ± Sd)	No. of PCR + ve plants	Transforma-tion efficiency (%)
BM-4	3072	31	6	2.0 ± 1.06	2	1.0 ±0	0	0
BM-5	1125	12	4	1.93 ±1.65	2	1.33 ±0.58	2	0.35%
BM-6	2062	22	10	1.6 ± 1.07	3	1.66 ±0.58	4	0.39%

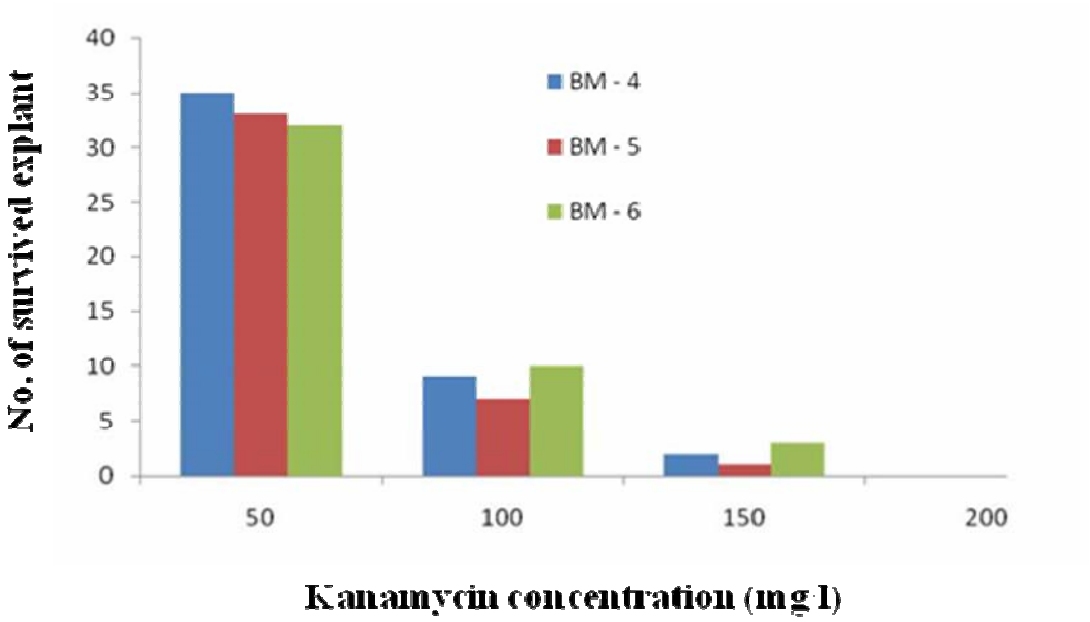


Fig. 76. Effect of kanamycin on shoots regenerated from non infected CADE explant.

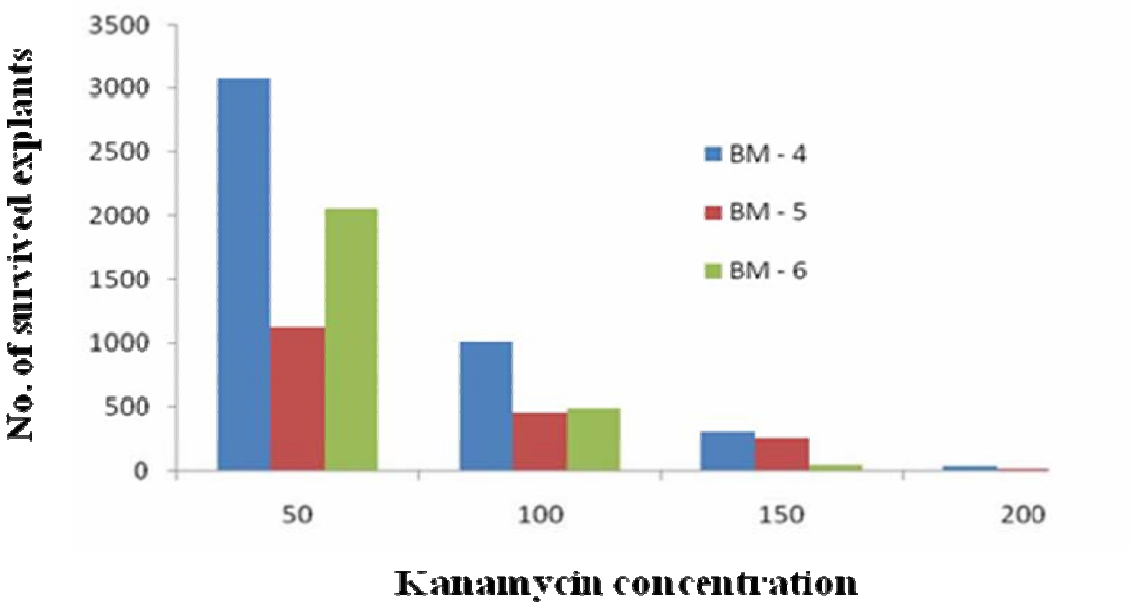
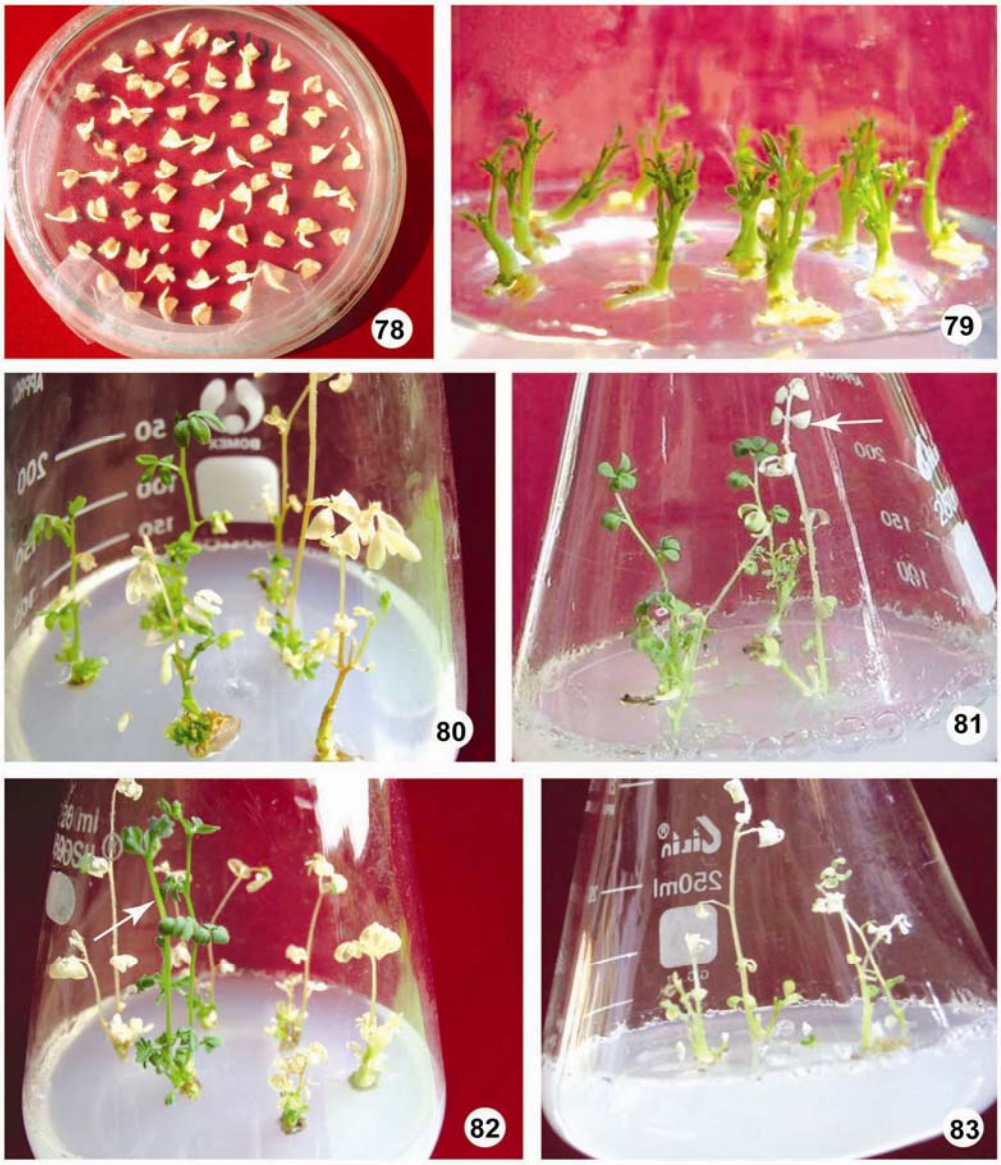


Fig. 77. Effect of kanamycin on shoots regenerated from infected CADE explants.



Figs 78 - 83. Selection of putative transformants. 78. Explants of CADE co-cultivated in regeneration medium following infection with Agrobacterial suspension. 79. Shoots regenerates from infected CADE explants cultured on regeneration medium without any selection pressure following co-cultivation. 80. Same as Fig. 79 but under the selection pressure of 100 mg/l kanamycin. 81. Same as Fig. 80 but under the selection pressure of 150 mg/l kanamycin. Note, the non transformed shoots (arrow) failed to survive. 82. Putative transformed shoots of BM-4 (arrow) survived on the selection medium containing 200 mg/l kanamycin, while non transformed shoots failed to survive after 14 days of culture. 83. Shoots derived from CN explants of BM-4 variety showing albinism (leading to death) on selection medium containing 200 mg/l kanamycin.

4.4.1.7 Establishments of transformed plantlets to soil

Healthy *in vitro* seeds developed from the selected shoots were harvested and dried under sunlight (Fig. 86). Seedling developed from these *in vitro* raised seeds was successfully transplanted into soil for development of transformed progenies (Figs 87 & 88).

4.4.1.8 GUS assay of the fully developed transformed plantlets

Histochemical GUS assay (Jefferson 1987) was performed to detect the expression of GUS gene in developing shoots and leaves at the time of each subculture during selection of transformants. Leaves and small branches of the transformed plantlets were subjected to GUS histochemical assay. Such assay demonstrated the presence of blue coloured zones on a few selected shoots and leaves (Figs 89 - 92). It was also found that, although a number of randomly selected shoots from initial selection medium showed positive response towards GUS expression, but very few of them could survive on the final selection pressure. Chimeric expression of GUS gene was also observed in a number of transformed shoots during the present study. It may be pointed here that in a number of events all shoots that survived in the final selection pressure (200 mg/l kanamycin) did not show positive to GUS expression.

4.4.1.9 Analysis of transgene integration

The transgenic nature of the putative transformed shoots was confirmed by amplification of GUS and *npt*II genes present within the genomic DNA of the transformed shoots. For this purpose total genomic DNA was isolated from the leaf of putative transformants as well as control lentil shoots. This isolated DNA was subjected to PCR for the amplification of *gus* and *npt*II genes.

At the same time plasmid DNA of a maintained genetically engineered *Agrobacterium* (strain I) was isolated to use as positive control during PCR analysis. Specific primers were used for this purpose as mentioned in the materials (Section 2.3.7).

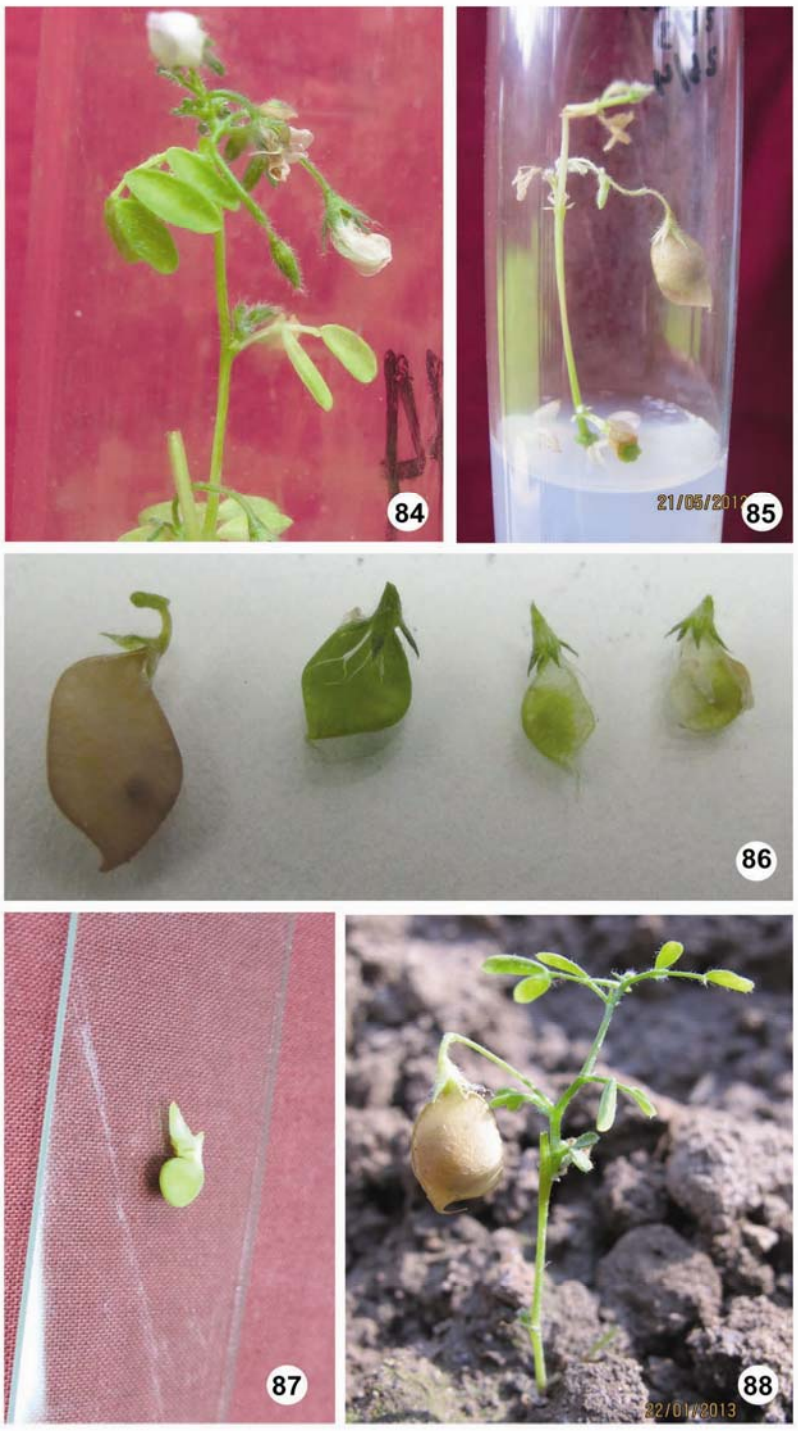
The amplified DNA was analyzed through agarose gel electrophoresis. The results obtained following PCR analysis have been shown in Figs 93 & 94. This observation indicated that presence of transgene within the DNA of plant shoots.

4.4.2 Genetic transformation using antifungal gene construct

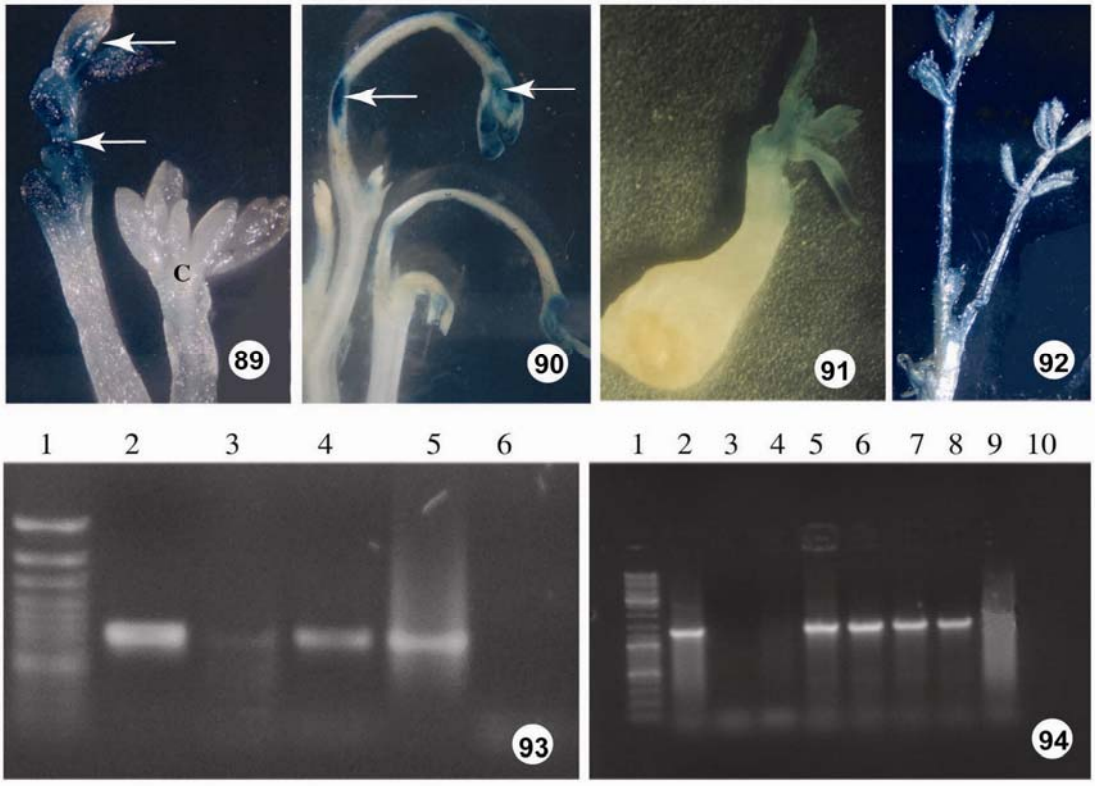
Agrobacterium-mediated genetic transformation was carried out using two other genetically engineered *Agrobacterium* strain, namely, EHA105/pGII-pSOUPvstN-chitin (strain II) and LBA4404/pCAMBIA2300 enh35SAFP (strain III) to integrate fungal disease resistant gene in lentil varieties. Strain II containing bar gene as selectable marker that conferring phosphinothricin (PPT) resistance and chitinase as antifungal gene (the gene of interest) whereas strain III containing *npt*II gene as selectable marker that conferring kanamycin resistance and Anti Fungal Protein gene (AFP) as the gene of interest.

4.4.2.1 Transformation of lentil with EHA105/pGII-pSOUPvstN-chitin strain (strain II)

Transformation experiments were carried out following the results of transformation experiments with marker gene described in the section 4.4.1 only with CADE explants from BM-4, BM-5, and BM-6 varieties of lentil.



Figs 84 - 88. *In vitro* flower and pod formation from the putative transformants of lentil. 84. *In vitro* flower developed from the transformed shoots of BM - 4 on half strength of MS medium supplemented with 20 mg/l IBA and 0.5 mg/l NAA with 50 mg/l kanamycin. 85. Same as Fig. 84 but showing fully developed healthy pod. 86. Mature harvested pod obtained from putatively transformed shoots. 87. Germination of *in vitro* raised seed. 88. Plantlets developed in soil producing normal pod identical to that of control plants.



Figs 89 - 94. Histochemical localization of GUS expression in different parts and molecular confirmation of the transformants. 89. Expression of GUS gene in developing shoots and leaves (arrows) following selection pressure of 200 mg/l kanamycin. Note, the control shoot without the expression of blue color (C). 90. Same as Fig. 89 showing the differential expression of GUS gene in various parts of shoots (arrows). 91. Same as Fig. 89 but in case of young regenerating shoots. 92. Same as Fig. 89 but showing conspicuous expression of indigo blue colour all over the shoot. 93. PCR amplification of GUS gene from transformed shoots of lentil (lane 1- 1kb ladder; lane 2- plasmid DNA of pBI121 as positive control; lane 3- negative control; lanes 4 and 5- genomic DNA of transformed shoots; lane 6- water control). 94. Same as Fig. 93 but for *npt*II gene (lane 1- 1kb ladder; lane 2- plasmid DNA of pBI121 as positive control; lanes 3 - 8 genomic DNA of putative transformed shoots; lane 9- negative control; lane 10- water control).

4.4.2.1.1 Optimization of different parameters influencing transformation efficiency using *Agrobacterium* strain EHA105

In this experiment the effect of optical density (O.D.) of *Agrobacterium* suspension, incubation and co-cultivation period of CADE explants with *Agrobacterium* suspension towards transformation were studied. For this purpose optical density (O.D.) was measured at 600 nm and bacterial suspension with optical density of 0.5, 0.8, 1.0 and 1.2 having incubation period of 10, 20 and 30 minutes were used.

It was observed that more than 20 minutes of incubation with optical density of 1.0 or above of bacterial suspension resulting overgrowth of bacteria in the co-cultured plates and this kind of overgrowth hampered proper growth of the explants. Negligible overgrowth was observed when the explants where infected with the *Agrobacterium* suspension having an O.D of 1.0 or less with incubation period of 20 minutes or less than this (Fig. 95). Therefore, the optimum incubation period was found to be 20 minutes with optical density of 0.8 - 1.0.

Duration of co-cultivation was also played an important role to recover explants after transformation. Four different co-cultivation periods ranging from 2 - 5 days with O.D of 0.8 and 1.0 were tried to find out the optimum co-culture period. It was observed that a co-cultivation for more than 3 days occasionally promoted overgrowth of bacteria and 5 days of co-cultivation period a remarkable overgrowth of bacteria was observed which covered the whole culture plate (Fig. 96). As a result most of the explants in co-culture media suffered from poor health, became brown and failed to regenerate. Based on the above results it was demonstrated that 3 days of co-cultivation period with optical density of 1.0 is optimum for transformation with strain II.

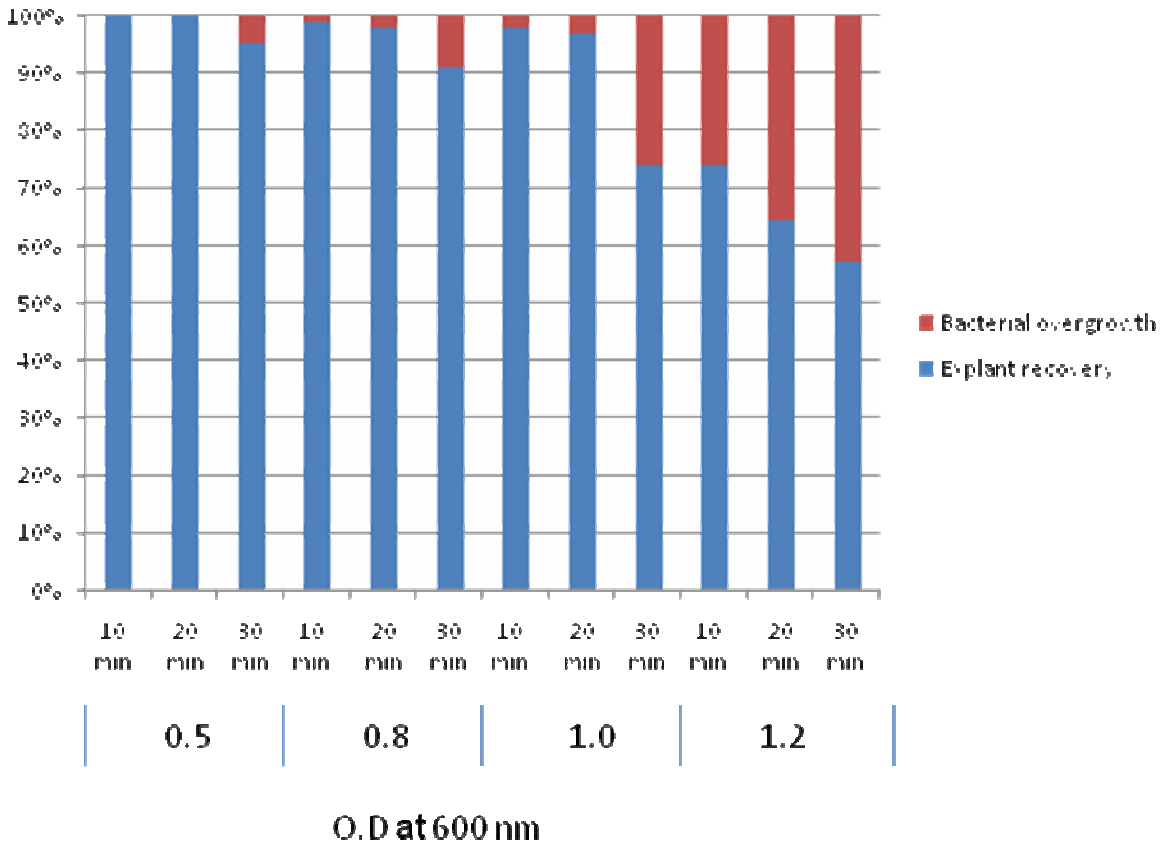


Fig. 95. Effect of O.D. and incubation period of explants with *Agrobacteriun* strain EHA105 towards explant recovery.

Fig. 96. Interaction between bacterial overgrowth and explants recovery in context with co-culture period of explants in bacterial suspension EHA105.

4.4.2.1.2 Determination of optimum phosphinotrycin (PPT) concentration for selection of transformed shoots

For strain II phosphinotrycin (PPT) was used as selective agent as the strain contained bar gene which is resistant to PPT. To find out the appropriate concentration of selection agent different concentration (0.5 - 2.0 mg/l) of PPT were used.

From this experiment it was revealed that with the increase of PPT concentration the percentage of survived shoots were found to decrease. The effects of different concentrations of PPT on regenerated shoots of control plants have been shown in (Fig. 97). At 1.0 mg/l PPT 80 per cent of the explants showed necrosis. Further increase in the level of PPT led to a corresponding decrease in the shoot proliferation and in 2.0 mg/l PPT caused almost inhibition of regeneration and the explant failed to survive within 10 days of inoculation (Fig. 98). Therefore, this concentration (2.0 mg/l PPT) was used for selection of shoots.

4.4.2.1.3 Selection of transformed shoots

For the selection of transformed shoots, following co-cultivation for 3 days explants were washed with 300 mg/l ticarcillin for 10 minutes and then transferred to MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosine and with 100 mg/l ticarcillin for the elimination of all unwanted bacteria (Fig. 99). At this stage no selectable agents were used in the medium.

The regenerating shoots with young leaves were sub cultured on the same media with 1.0 mg/l PPT for selection. After 14 days, only green and healthy shoots were sub cultured on fresh medium with 1.5 mg/l PPT (Fig. 100). To select transformed shoots, PPT concentration was raised up to 2.0 mg/l in the third subculture and then again in 2.0 mg/l in the fourth sub-cultures

(Figs 101 - 103). During each sub-culture, deep brown dead shoots were discarded and only green shoots were sub-cultured on fresh medium containing the next higher concentration of PPT. From this experiment it was noticed that with the increase of PPT concentration the percentage of survived shoots were found to decrease (Fig. 104).

Finally shoots that survived on regeneration medium containing 2.0 mg/l PPT for two rounds (each for 15 days) and remained green and healthy were selected as possible transformed shoots. A total of 5 shoots survived on 2.0 mg/l PPT out of approximately 1390 infected explants in case of BM-4, 7 out of 2040 in case of BM-5 and 17 out of 4680 in case of BM-6 were recovered through selection. Therefore, the frequency of recovery of putative transformed shoots was about 0.36% in case of BM-4, 0.34% in case of BM-5 and 0.36% in case of BM-6 (Table 31).

In each set of experiments, regenerated control explants were also maintained to perform various comparative studies between transformed and non transformed shoots. It was observed regularly that when the growth of the developing shoots under PPT selection was very poor compared to that of the shoots grown without PPT selection pressure.

Under this background several transformation experiments were carried out without using selection pressure (PPT) in the media. In this phase following co-cultivation, explants were washed with 300 mg/l ticarcillin for 10 minutes and then transferred to suitable regeneration medium (MS medium with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosine) supplemented with 100 mg/l ticarcillin. Green and healthy shoots were transferred to same media after every 21 days interval. Since there was no PPT in the regeneration media, the growth of the developing shoots were better compared to the one grown on selection media. After 2/3 subcultures plantlets were transferred to flowering media. Results of these experiments are presented in Table 31

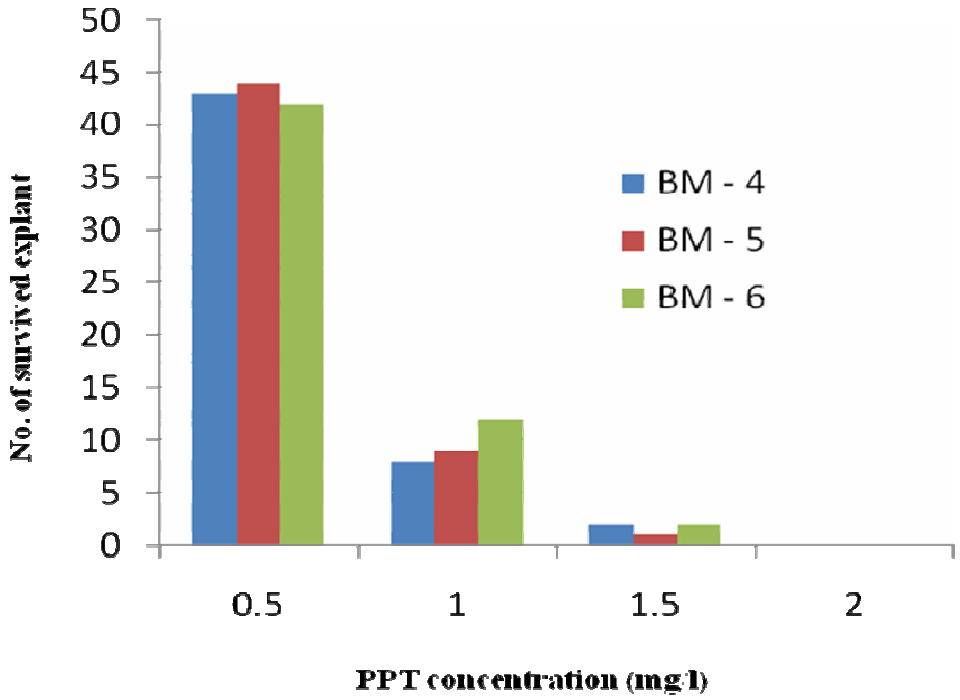


Fig. 97. Effect of PPT on shoots regenerated from non infected CADE explant of BM - 4, BM - 5 and BM - 6.

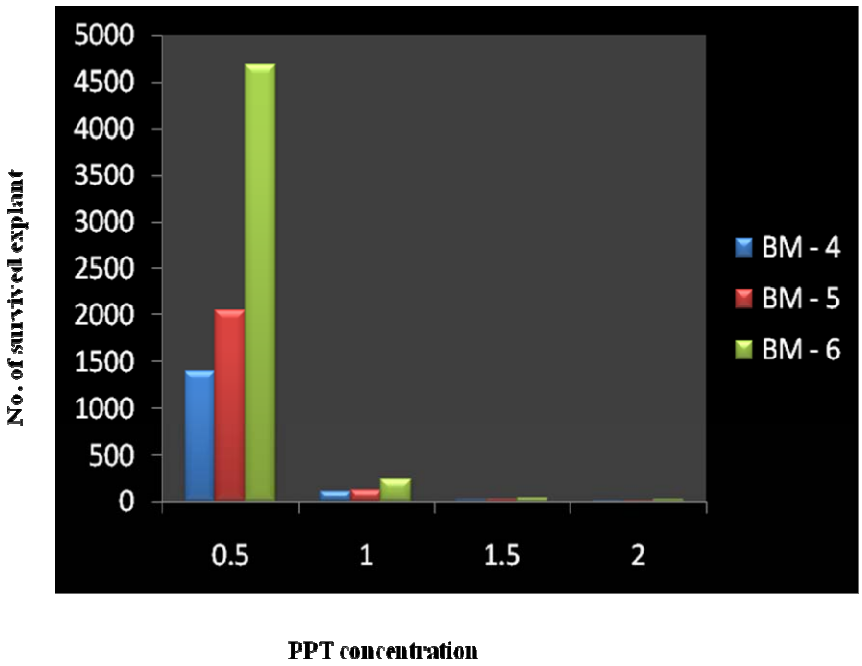


Fig. 104. Effect of PPT on shoots regenerated from infected CADE explant of three lentil varieties of BM - 4, BM - 5 and BM - 6.

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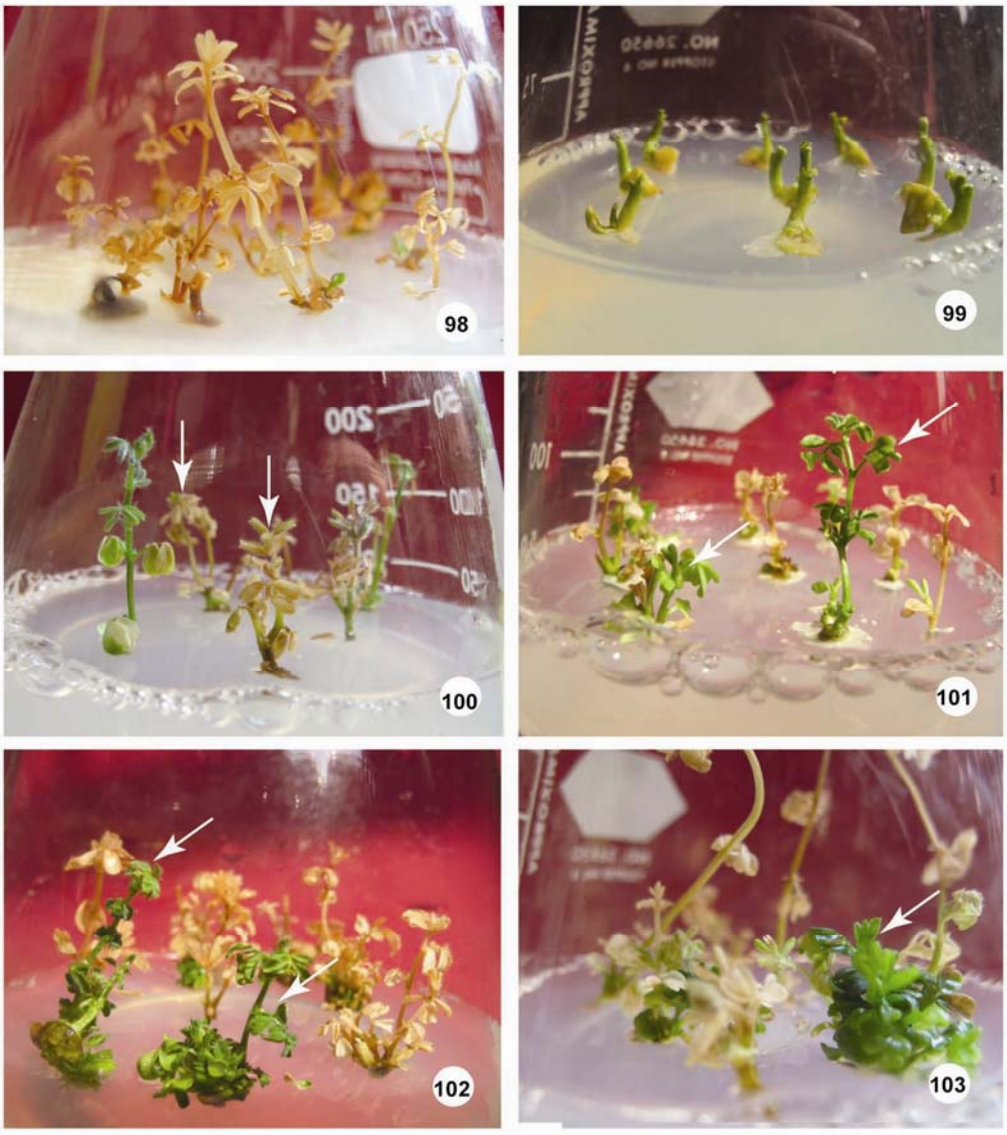


Figs 98 - 103. Selection of putative transformants. 98. Control shoots of BM - 5 regenerated from CADE explants showing necrosis (leading to death) following selection pressure of 2.0 mg/l phosphinothricin (PPT). 99. Shoots regenerates from infected CADE explants cultured on regeneration medium without any selection pressure following co-cultivation. 100. Same as Fig. 99 but with selection medium containing 1.5 mg/l PPT. Note, the non-transformed shoots (arrow) failed to survive in presence of 1.5 mg/l PPT after 15 days of culture on selection medium. 101. Putatively transformed shoots of BM - 6 (arrow) survived on the selection medium containing 2.0 mg/l PPT. 102. Same as Fig. 101 in case BM - 5 variety of lentil. 103. Same as Fig. 101 in case BM - 4 variety of lentil.

Table 31. Effect of PPT on developing shoots.

Variety	Total experi-ment done	Total no. of Explant infected	No of explant transferred to regenera-tion media	Use of selection pressure	No of shoot survived (mg/l PPT)			No of shoots transferred to flowering media	% of survive shoots
					1.0	1.5	2.0		
BM-4	6	1390	1303	Yes	107	15	5	5	0.36
	2	300	260	No	-	-	-	170	67.50
BM-5	9	2040	1980	Yes	123	21	7	7	0.34
	4	780	630	No	-	-	-	522	66.92
BM-6	20	4680	4540	Yes	243	33	17	17	0.36
	8	1670	1390	No	-	-	-	1127	67.49

4.4.2.1.4 *In vitro* flower and pod formation from the transformed shoots of lentil

Shoots that survived in the PPT selection medium as well as media without any selection pressure were separated and transferred to half strength of MS medium supplemented with 20 mg/l IBA and 0.5 mg/l NAA. After 2 - 3 weeks *in vitro* flower formation was observed on the healthy shoots and eventually pod formation was occurred from the shoots that were growing on regeneration media without any selection pressure. Bud formation and flowering were also observed in some of the plants that were recovered through selection pressure; however, these flower failed to produce pods (Fig. 105).

From this experiment it was also found that 690 out of 1848 survived shoots (without selection pressure) responded to flowering and the maximum number of flower per shoot was 3 (Fig. 106). It was also found that after 15 - 20 days 134 out of these 690 flowering shoots produced viable and healthy pods (Fig. 107) under *in vitro* condition and the range of pods per shoot was only 1 - 2. No pod formation was observed from the shoots that recovered through selection pressure. Results of these experiments are presented in Table 32.

4.4.2.1.5 Molecular analysis for the integration of chitinase gene

Molecular analysis through PCR amplification confirmed the presence of bar and chitinase gene in putative transformants. The DNA was isolated from putative transformed shoots as well as control plants for PCR amplification. Plasmid of pGII was used as template DNA. 10 (9 from BM-6 and 1 from BM-5) out of 29 surviving shoots recovered through selection pressure was subjected for PCR analysis. Among them 6 survived shoots showed positive response towards the integration of bar and chitinase genes. Rest of the recovered shoots (19) cannot be characterized as the leafy material was not sufficient to collect DNA due to very poor health condition of these plantlets.

Besides this, a total of 134 pod bearing shoots from 14 transformation experiments (without applying any selection pressure) were also analyzed at the molecular level. 5 of these T_0 shoots in case of BM-6 and 1 in case of BM-5 were PCR positive for the gene of interest. Therefore, the percentage of transformation was about 0.13 in case of BM-5 and 0.30 in case of BM-6. None of the pod bearing shoots of BM-4 showed positive response towards PCR amplification.

Table 32. Response of CADE derived selected shoots following *Agrobacterium* infection towards *in vitro* flowering and pod formation.

Variety	No. of shoots transferred to flowering media	Use of selection	No. of shoots respond to flowering	% of shoots respond to flowering	No. of flowers/ shoot (Mean ±Sd)	No. of shoots responded to pod formation	Days required to develop pod	No. of pods/ shoot (mean ± Sd)
BM-4	5	Yes	0	-	-	0	0	0
	170	No	58	34.12	2.5 ± 0.71	9	15.52	1.0 ± 0
BM-5	7	Yes	1	14.29	1.0± 0	0	0	0
	522	No	185	35.44	2.5 ± 0.58	34	18.38	1.0 ± 0
BM-6	17	Yes	3	17.65	1.3 ± 0.57	0	0	0
	1127	No	443	39.31	2.8 ± 0.46	91	20.54	1.01 ± 0.1

The suitable primer for bar and chitinase gene were used for PCR amplification (section 2.3.7). The presence of amplified band at 0.46 kb for bar gene and 0.55 kb for chitinase gene in transformed shoots confirmed the presence of bar and chitinase gene (Figs 108 & 109).

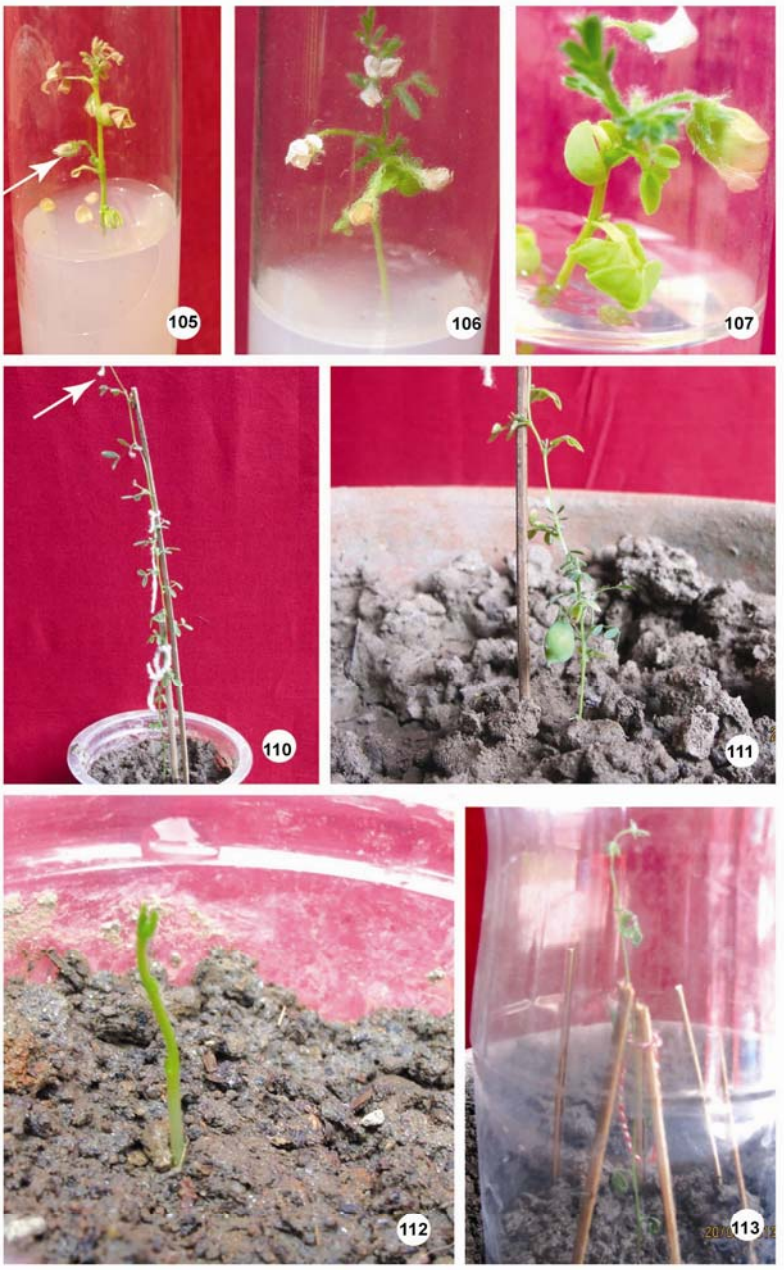
4.4.2.1.6 Progeny analysis

Pods that developed on PCR positive T_0 shoots were harvested dried under sunlight and were subjected for germination in plastic pot containing sterile peat moss in net house along with controls (non-transformed) following the method described in section 3.2.8. The T_0 seeds germinated and gave rise to T_1 plants (Figs 110 & 111) as well as the T_1 seeds germinated and gave rise to T_2 plants (Figs 112 & 113). Results of these experiments are presented in Table 33.

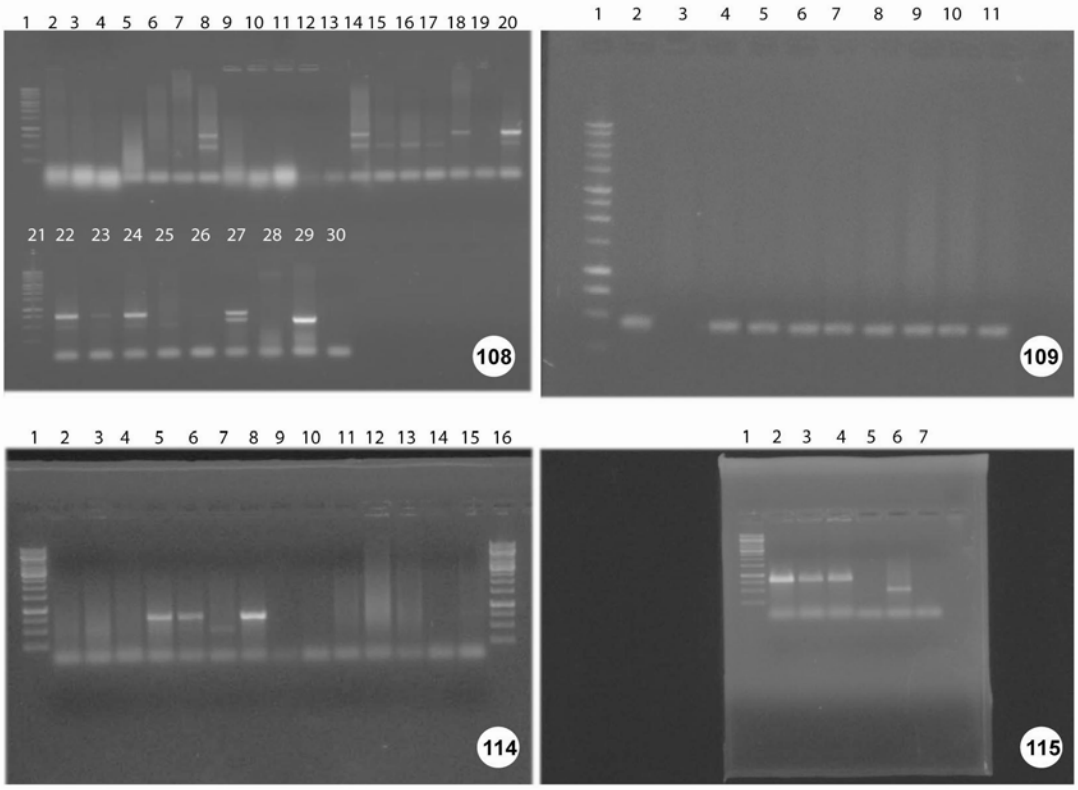
The non-transformed controls were used to compare the growth and were the source for negative control. It was found that branching in T_n (n = number of denoting progeny) was comparatively low, resulting in less leaf material. Otherwise no significant differences were noticed. DNA isolation and PCR amplification for chitinase gene was also done from the T_1 and T_2 plants. The results of PCR amplification of T_1 and T_2 plants are shown in Figs 114 & 115.

Table 33. Assessment of transformed Progenies obtained from BM - 6 variety of lentil.

Variety	No. of explant infected	Selection	T_0 shoot				T_1 Plant			T_2 plant	
			Survived	Flowering	Pod set	PCR +ve	Survived	Pod set	PCR +ve	Survived	PCR +ve
BM-4	1390	Yes	5	0	-	-	-	-	-	-	-
	300	No	170	58	9	-	-	-	-	-	-
BM-5	2040	Yes	7	1	-	0 (1)	-	-	-	-	-
	780	No	522	185	34	1	1	-	-	2	-
BM-6	4680	Yes	17	3	-	6 (9)	-	-	-	-	-
	1670	No	1127	443	91	5	4	1	2	1	1



Figs 105 - 107, 110 - 113. Development of transformed lentil progenies. 105. *In vitro* flower bud (arrow) formation from the regenerated shoots survived on selection medium containing 2.0 mg/l PPT. 106. *In vitro* flower developed from shoots derived from infected CADE explants of BM - 6 without application of any selection pressure. 107. Same as Fig. 106 showing fully developed pod. 110. T_1 Plant growing in pot showing the development of flower (arrow). 111. Same as Fig. 110 but showing the formation of pod. 112. Germination T_1 seed. 113. T_2 plant growing in soil.



Figs 108 - 109, 114 - 115. PCR amplification of chitinase and bar gene of putative transformants. 108. PCR amplification of chitinase gene from T0 plant of lentil. Note that lanes 8, 14, 18, 20, 22, 23, 24 and 27 produced corresponding band, identical to the band obtained from positive control (lane 29); no amplification from negative control (lane 28) water control (lane 30). Lanes 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 15, 16, 17, 19, 25 and 26 are non transformed plantlets. Lane 1 refers for 1kb ladder. 109. Same as Fig. 108 in case of bar gene (lane 1- 1kb ladder; lane 2- plasmid DNA as positive control; lane 3- negative control; lanes 4 - 11 genomic DNA of putative transformed shoots; lane 12- water control). 114. PCR amplification of chitinase gene from T_1 plants, note that lanes 5 and 6 comes from lane 24 of T_0 plant produced corresponding bands, identical to the band obtained from positive control (lane 8); no signal from negative control (lane 11) water control (lane 10) and non transformed plants (lanes 2, 3, 4,7 and 9). 115. Same as Fig. 114 in case of T_2 plants, note that lanes 3 and 4 comes from lane 5 of T_1 plant produced corresponding bands, identical to the band obtained from positive control (lane 2); no signal from negative control (lane 6) and water control (lane 5).

4.4.2.2. Transformation of lentil with LBA4404/pCAMBIA2300 enh35S AFP (strain III)

This part of the transformation experiment was conducted to integrate AFP gene in the genome of lentil varieties. For this purpose transformation experiments were carried out following the protocol developed in transformation experiments with marker gene described in the section 4.4.1. In this case CADE explants from BM-6 variety of lentil and the *Agrobacterium tumifaciens* strain LBA4404 containing the plasmid vector pCAMBIA2300 were used.

Apart from the other techniques following four different methods of explant wounding were followed to achieve the high frequency of transformation in lentil:

i) In first method (method "A") the explants (CADE) were incubated in bacteria suspension for 45 minutes without doing any extra injury.
ii) In second method (method "B") the CADE explants were sliced longitudinally with the help of surgical blade and incubate in bacterial suspension for 45 min.
iii) In third method (method "C") explants were subjected to vacuum infiltration for 1 to 3 minutes along with bacterial suspension using the vacuum pump and
iv) In fourth method (method "D") explants were injured with small glass bids for 20 minutes in 200 rpm and then followed the same procedure described in method A.

Following different wounding techniques the explants were maintained in coculture medium (MSB_5 media supplemented with 1.0 mg/l zeatin, 0.1 mg/l NAA) for 2 - 4 days in dark condition at 25 ± 2° C. Among the different method tested, wounding of explants by mild injury (method "B") followed by 3 days of cocultivation showed highest percentage of regeneration and survibility of explants, while massive injury (methods "C and D") and longer period of cocultivation reduced regeneration and survibility of explants (Fig. 116). It was also observed that none of the explants able to survive when injured with vacuum infiltration pump (Method "C").

Following three days of cocultivation the survived explants were transferred to MSB_5 media supplemented with 1.0 mg/l zeatin, 0.1 mg/l NAA, 100 mg/l kanamycin and 500 mg/l cefataxime for the selection of putative transformants. In this case selection pressure (100 mg/l kanamycin) was applied following cocultivation and this selection method was repeated twice with four weeks interval to decrease the number of the false transformants. Shoots those remained green and healthy under this selection pressure were considered as putative transformants. From this experiment it was observed that 24 out of 610 in case of method "A", 62 out of 1490 in case of method "B", 16 out of 565 in case of method "D" explants survived through second round of selection (Table 34). It was observed that a small number of survived shoots recovered through kanamycin selection responded towards root induction (Fig. 117). Most of the survived explants failed to initiate roots in kanamycin containing medium (Fig. 118).

The putative transformed shoots were then transferred to MS media for the development of induced roots (Fig. 119) and after four weeks the rooted plantlets were successfully transferred to small pots containing equal ratio (1:1:1) of soil, sand and vermiculite. From this experiment it was observed that 2 out of 5 transferred shoots developed flower and pod (Fig. 120). The number of flower varied from 4 to 5 in each of the transferred shoots, whereas only 1 pod/shoot was formed (Table 35). Rest of the flower failed to develop pod and dried eventually.

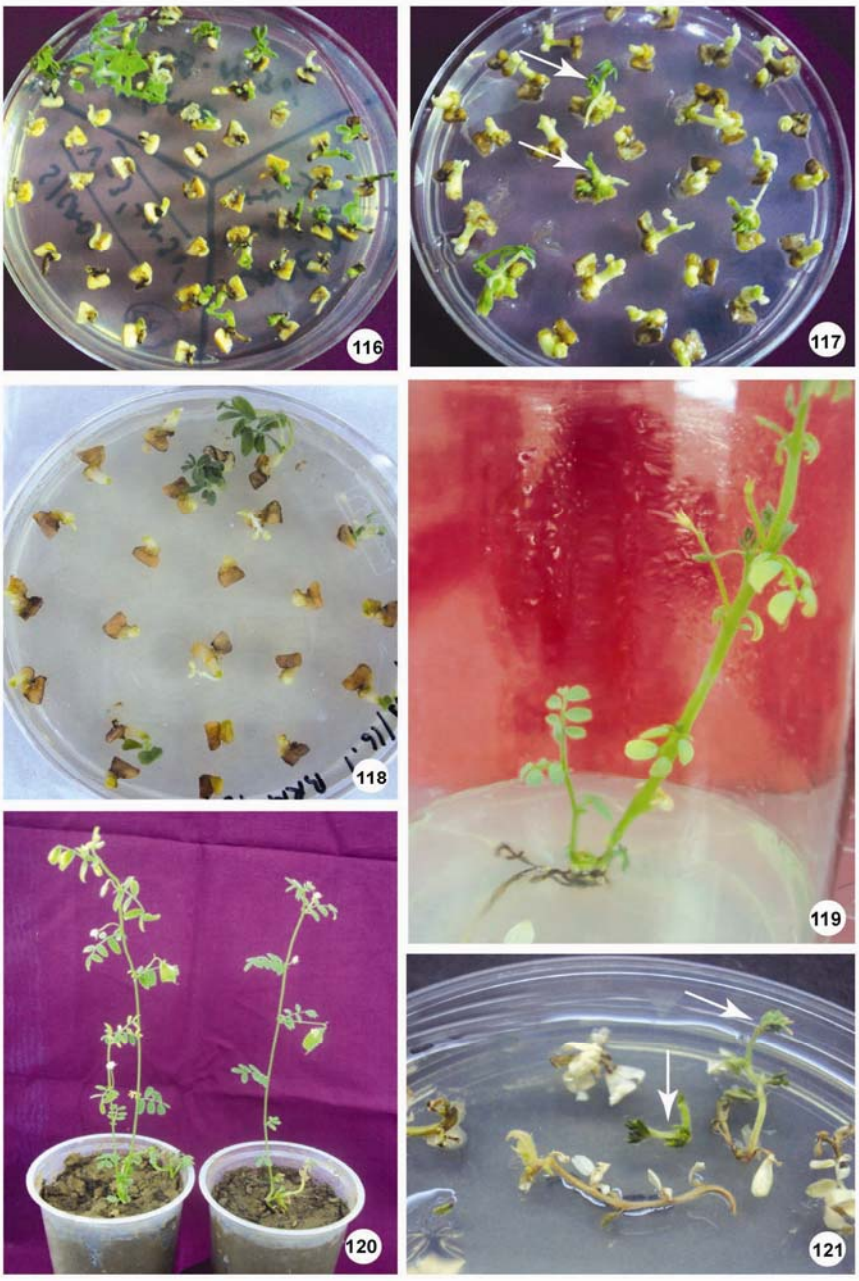
To select the true transformants shoot tips isolated from the well developed putative transformants and placed vertically in 200 mg/l kanamycin containing regeneration medium. From this experiment it was observed that the transformed shoots remain healthy and green in presence of kanamycin whereas the false transformants became albino within 10 days of culture (Fig. 121)

Table 34. Effect of four different explant wounding methods on transformation efficiency.

Method of wounding of explant	No. of explants infected	No. of explants recovered in the selection media after 2 round of selection	No. of shoots rooted in the presence of selective agents	No. of plantlets positive to desired gene follwing PCR analysis		transformation efficiency (%)	No. of transgenic plants transferred to soil
				With root	Without root		
"A"	610	24	1	1	0	0.16	1
"B"	1490	62	12	3	4	0.47	3
"C"	600	0	0	0	0	0	0
"D"	695	30	3	1	1	0.28	1

Table 35. Analysis of reproductive growth of the transgenics developed from different transformation method.

Method	No. of transgenic plants		No of plants responded to flowering		No. of plants responded to pod formation		No. of flowers per plant	No. of pods per plant
	With root	Without root	With root	Without root	With root	Without root		
"A"	1	1	1	0	1	-	1	1
"B"	3	4	1	1	1	-	2 - 5	1
"D"	1	-	0	-	-	-	-	-



Figs 116 - 121. Development of transformants using *Agrobacteium* strain LBA4404 harboring binary plasmid pCAMBIA2300 35Senh AFP. 116. Initiation of shoot morphogenesis from the CADE explants following different types of wounding by culturing the explants on MSB_5 media supplemented with 1.0 mg/l zeatin and 0.1 mg/l NAA following infection with strain III. 117. Selection and development of putatively transformed shoots (arrows) followed by method "B" of wounding on MSB_5 media supplemented with 1.0 mg/l zeatin, 0.1 mg/l NAA, 100 mg/l kanamycin and 500 mg/l cefatoxime. 118. Same as Fig. 117 but the regenerating shoots failed to develop roots under selection pressure. 119. Development of adventious root from the putatively transformed shoot on MSB_5 medium without any hormonal supplement. 120. T_0 plant in a small pot containing soil showing the formation of healthy flowers and pods. 121. Screening of putatively transgenic plants on regeneration medium supplemented with 200 mg/l kanamycin. Note, the true transformed shoots remain green (arrows) whereas the non transformed shoots became albino (leading to death).

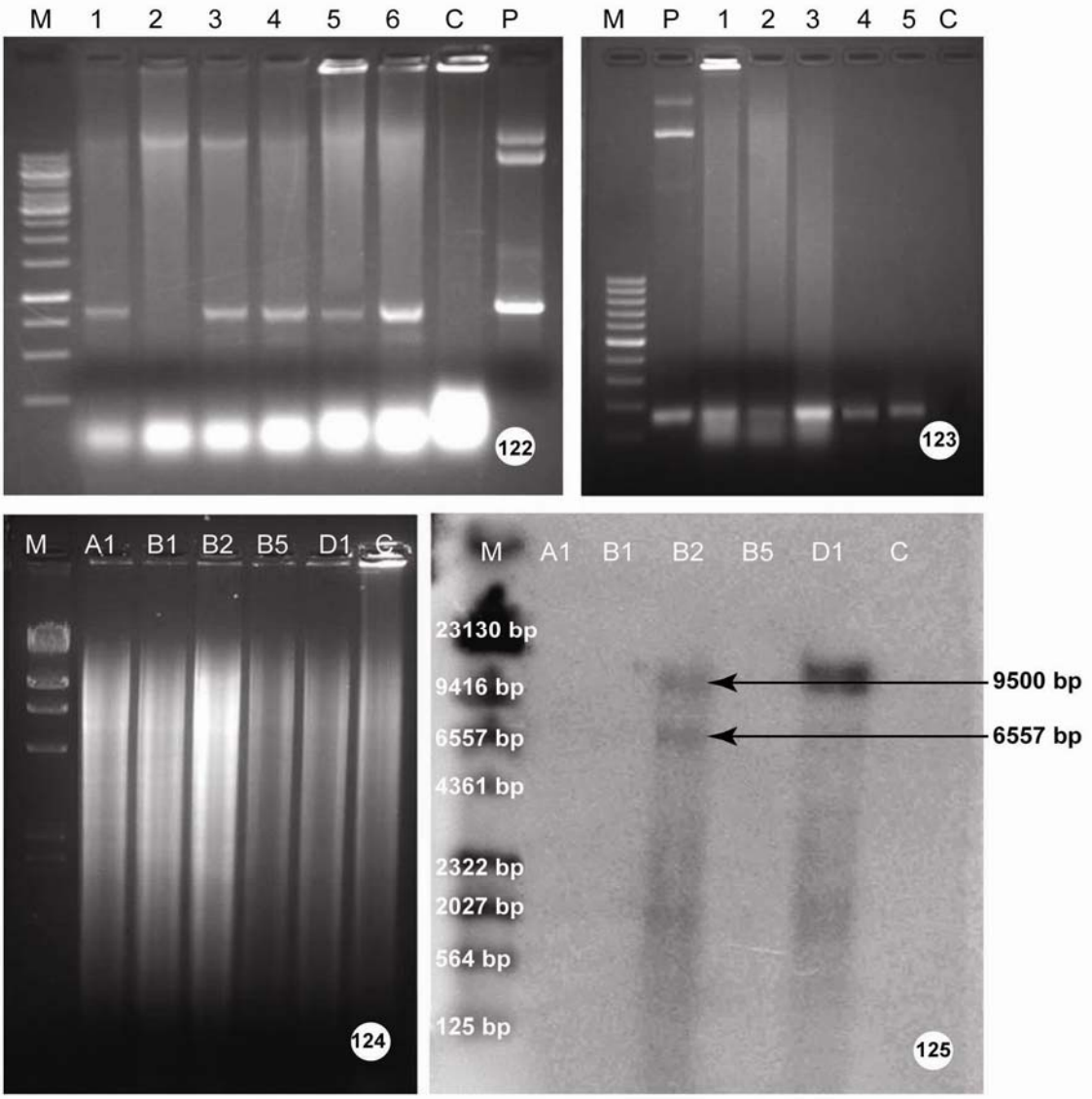
4.4.2.2.1 Molecular analysis of the putative transformants

All the survived plants were subjected to PCR analysis with the specific primers to detect the presence of *npt*II and *AFP* genes. Among the survived plantlets one (A1) out of 24 in case of method "A", 7 (B1 to B7) out of 62 in case of method "B" and 2 (D1, D2) out of 30 in method "D" showed positive response in PCR analysis (Figs 122 & 123). Therefore, the overall transformation efficiency was 0.16 in method "A", 0.47 in method "B" and 0.28 in method "D". If we consider the individual event from where the transgenic developed then the efficiency varied from 0.65 to 1.14 (average 0.87) in method "B", 0.43 to 0.48 (average 0.46) in method "D" and 0.34 in method "A".

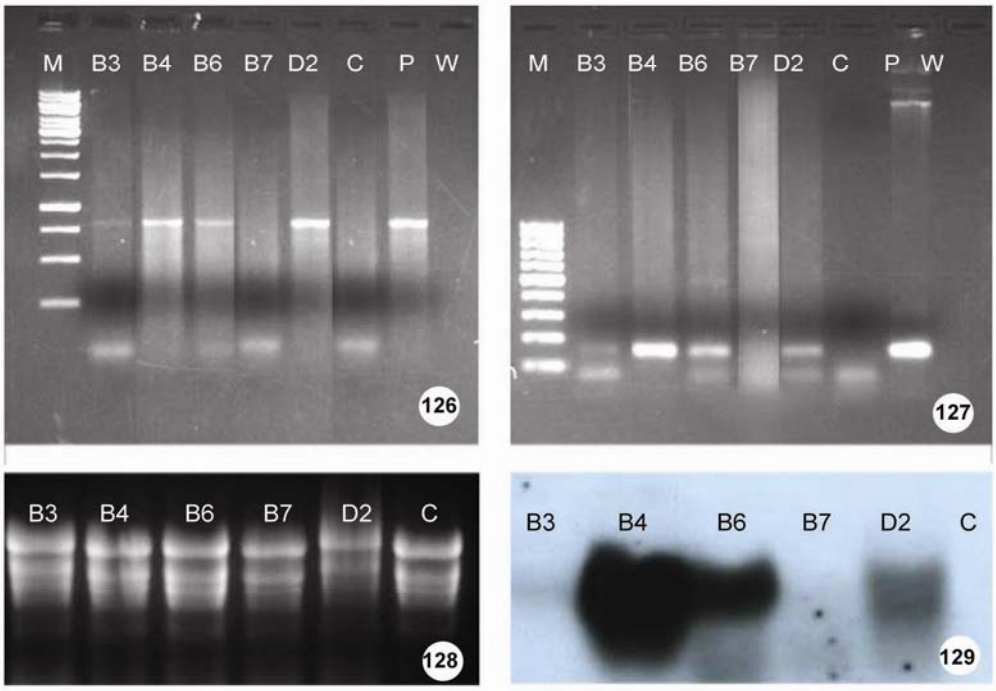
Southern blot analysis was carried out with five rooted transgenic (A1, B1, B2, B5 and D1) and a control plant to determine the stable integration and copy number of transgene integrated into genome of lentil lines (Fig. 124). Genomic DNA digested with EcoRI enzyme that cut only once within the T-DNA was used for hybridization with a 32P-labeled DNA *npt*II gene as probe. Southern hybridization results confirmed the integration of the transgenes into genome of transgenic lentil lines. These results showed that a single hybridization signal of 9.5 kb molecular size was obtained in line D1 and two insert (9.5 and 6.5 kb) was obtained from line B2 (Fig. 125).

Reverse Transcription PCR (RT-PCR) was performed on cDNAs synthesized from total RNAs of this five (B3, B4, B6, B7, D2) non-rooted PCR positive plants. The same base sequences are used as primers that used in regular PCR reactions. In the present investigation *npt*II primers amplified expected 750 bp product and AFP primers amplified 190 bp products from the total cDNAs of four (B3, B6, B7 and D2) PCR positive plants (Figs 126 &127).

Northern hybridization was performed using 15 µg of total RNA (Fig. 128) from five PCR positive non rooted transformed lines (B3, B4, B6, B7, D2). From this experiment, three (B4, B6 and D2) out of this five PCR positive lines showed the expression of *npt*II gene (Fig. 129). Two PCR positive lines (B3 and B7) failed to show the expression of insertion of gene in RNA level.



Figs 122 - 125. PCR amplification and Southern hybridization analysis from the transformed shoots of BM - 6 variety of lentil. 122. PCR amplification of *npt*II gene in T_0 Plants (lane M- 1 kb ladder, 1,3,4,5 and 6 - T_0 lentil samples, 2- non transformed shoots, C- negative control and P- positive control). 123. PCR amplication of AFP gene in T_0 Plants where lane M refers to 100 bp ladder, lane 1 to 5 putatively transformed shoots, lane P and C refers to positive and negative control respectively. 124. Agarose gel electrophoresis of digested genomic DNA. DNA was digested with *Eco*RI. 125. Southern blot hybridization of PCR positive T_0 lines for *npt*II gene. Lanes correspond to λ-*Hind*III marker (M), genomic DNA from untransformed control plant (C) and DNA from five PCR positive plants (A1, B1, B2, B5 and D1).



Figs 126 -129. Reversse transcription PCR (RT-PCR) and northern hybridization analysis from the transformed shoots of BM - 6 variety of lentil. 126. Amplification of *npt*II gene determined by RT-PCR. Note the *npt*II gene specific band (750 bp) was observed in four lines (lane B3, B4, B6 and D2) whereas there was no amplification in negative control (C). Here, lane P shows amplification of plasmid vector and lane W refers to water control. 127. Same as Fig. 126 but in case of AFP gene. 128. Agarose gel electrophoresis of total RNA of five PCR positive T_0 lines and a non transformed control plant (C). Each lane contains 15µg of total RNA. 129. Northern blot analysis for *npt*II gene of different PCR positive T_0 lines (lane B3, B4, B6, B7 and D2) and non transformed control plant (lane C).

5. DISCUSSION

5. DISCUSSION

The present investigation was undertaken to establish an efficient protocol for developing fungal disease resistant lentil (*Lens culinaris* Medik.) lines through *Agrobacterium*-mediated genetic transformation. Four microsperma types of lentil varieties cultivated in Bangladesh, namely, Barimasur - 1 (BM - 1), Barimasur - 4 (BM - 4), Barimasur - 5 (BM - 5) and Barimasur - 6 (BM - 6) were used in the present investigation. In the first phase of the experiments, *in vitro* regeneration of plantlets had been tried with/without the intervention of callus. In the second phase, a series of experiments were conducted to establish a suitable protocol for *in vitro* flowering and pod formation to overcome the difficulties of *in vitro* rooting in lentil to obtaining complete plantlets. In the third phase of the experiments three strains of *Agrobacterium tumefaciens*, namely, LBA4404 harboring the binary plasmid pBI121 conferring β-glucoronidase (*GUS*) and *nptII* gene (strain I), *Agrobacterium* strain EHA105 contained plasmid pGIIvstN-chitin conferring *bar* and *chitinase* gene (strain II) and LBA4404 harboring the binary plasmid pCAMBIA2300 enh35SAFP conferring AFP and *npt*II gene (strain III) were used to integrate the desired gene in lentil genome. Among these strain I was used for the optimization of suitable condition of transformation in lentil whereas strain II and III was used for the integration of antifungal gene into lentil genome.

The transformants developed during this investigation were characterized through polymerase chain reaction (PCR), reverse transcription PCR (RT PCR), Southern and Northern hybridization techniques to study the integration of desired genes in lentil

plantlets. The results of different experiments conducted during this study are discussed below.

In vitro regeneration of plantlets

An efficient and reproducible *in vitro* regeneration system is a prerequisite for the development of transgenic plants through genetic transformation (Gardner 1993). Therefore, several attempts were made in establishing an *in vitro* regeneration protocol for microsperma types of lentil varieties before conducting genetic transformation experiments. Among the various important crop plants, grain legumes have been considered as recalcitrant due to their passiveness to *in vitro* techniques (Bajaj and Ghosal 1981, Mroginski and Karth 1984). Moreover, routine transformation protocols are not available in most of the grain legumes due to their poor *in vitro* regenerating ability especially *via* callus culture. Apart from this lack of a compatible and efficient gene delivery system also hampering the development of desired transformed plants in grain legumes. As a result, success of transformation in leguminous crops is limited (Nisbet and Webb 1990). In spite of this fact, several reports have been available on *in vitro* regeneration of plantlets for different grain legumes including pea (Schroeder et al. 1993), chickpea (Jayanand et al. 2003, Aasim et al. 2011), common bean (Aragão et al. 2002), cowpea (Ikea et al. 2003), soybean (Hinchee et al. 1988), mungbean (Jaiwal et al. 2001) and peanut (Kartha et al. 1981). Although many legumes have been regenerated using tissue culture techniques, very few efficient regeneration protocols are presently available to exploit them in transformation experiments. Moreover, only a few reports are available on lentil *in vitro* regeneration especially for microsperma types compared to other grain legume crops (Williams and McHughen 1986, Khanam et al. 1995, Sarker et al. 2003, Saxena and King 1987, Singh and Raghuvanshi 1989,

Malik and Saxena 1992, Polanco and Ruiz 1997, Gulati et al. 2001, Sevimoy et al. 2005) etc.

Nevertheless, during the present study direct regeneration through organogenesis was carried out using three different types of explants, namely, cotyledonary node (CN), decapitated embryo (DE) and cotyledon attached decapitated embryo (CADE) from microsperma types of lentil varieties grown in Bangladesh. Highest number of healthy multiple shoots were obtained from CN explants 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. In the past Sarker et al. (2003) also used same explants for *in vitro* regeneration in microsperma lentil cultivating in Bangladesh and obtained similar results. A number of authors also previously reported that cotyledonary nodes were the most responsive explant for the induction of multiple shoots through organogenesis in soyabean (Kaneda et al. 1997), pea (Jackson and Hobbs 1990), chickpea (Subhadra et al. 1998), lentil (Warkentin and McHughen 1993, Oktem et al. 1999, Mahmoudian et al. 2002) and *Vigna mungo* (Saini et al. 2003).

Next to cotyledonary node (CN), cotyledon attached decapitated embryo explants (CADE) showed better responses on *in vitro* shoot regeneration. Decapitated embryo (DE) exhibited the lowest responses towards multiple shoot regeneration. Use of embryo or modified embryo has been reported for *in vitro* regeneration in pea (Schroeder et al. 1993) and chickpea (Tewari-Singh et al. 2004, Jayanand et al. 2003) in achieving optimum *in vitro* regeneration. Halbach et al. (1998) used half embryos with or without cotyledon and found that embryo attached with cotyledon responded better for regeneration. Findings of the present investigation were more or less identical to the findings of the above workers.

No remarkable variation was observed among the four different varieties of lentil in case of multiple shoot regeneration used in the present investigation. Using lentil varieties growing in Bangladesh, Khanam (1994) also found the similar response in multiple shoot regeneration while Sarker et al. (2003) found better response for shoot regeneration in the varieties of BM - 2 and BM - 4 of lentil.

Among the three different explants used in the present investigation CN and CADE explants showed better response towards multiple shoot regeneration through organogenesis on MS medium supplemented with BAP and Kn. A series of experiments were also conducted to investigate the effect of different hormonal supplements such as BAP, Kn, zeatin and NAA on *in vitro* regeneration of shoots. Various concentrations of these hormones were used separately alone or in combinations with other hormones for regeneration of shoots. From these experiments higher percentage of multiple shoot induction having profuse rooting at the base of the regenerated shoots was observed on the media containing zeatin in combination with NAA. Zeatin has cytokinin activity and it has been used in many plant species to enhance the regeneration efficiency. Yadav and Sticklen (1995) reported that the culture of leaf explants in medium containing zeatin or zeatin riboside for six days and then sub-cultured to a medium containing zeatin riboside (1.0 mg/l) only caused shoot regeneration in high number in chickpea. Perl et al. (1992) and Fahmy et al. (2003) reported that zeatin riboside in combination with NAA promoted the shoot initials to plantlets in wheat. In the present investigation comparatively higher responses towards multiple shoot regeneration were obtained using MSB_5 media supplemented with 1.0 mg/l zeatin in combination with 0.1 mg/l NAA.

It was also noticed that with the increase in concentration of NAA in the medium inhibited the shoot elongation. Similarly in Chickpea, Aasim et al. (2011) reported that presence of NAA in culture medium showed negative effect on shoot elongation and resulted stunted growth of the regenerating shoots.

In the present study regeneration of shoots via callus formation was also tried using different explants as well as a range of hormonal supplements. It is generally believed that in several instances plant regeneration from different explants such as cotyledon, hypocotyle segments, mature embryo axes, etc. *via* callus is more difficult, time consuming, cost and labour intensive (Wang and Holl 1998).

However, in this study efforts were made to regenerate shoots *via* callus using cotyledon, hypocotyle, nodal segments and shoot tip explants. Among these explants cotyledon explants exhibited positive responses towards callus induction as well as shoot regeneration. Other explants showed positive responses towards callus induction but failed to induce shoots from the callus derived from these explants. Earlier Williams and McHughen (1986) described a protocol for regeneration of lentil shoots from hypocotyl and epicotyl derived callus. Altaf et al. (1999) also reported regeneration of shoots from shoot apices derived callus.

Callus of variable nature such as friable, compact and hard was found to obtain from different explants. The colour of developing callus was also found to be different. In most of the cases green, brown and whitish callus was found to develop. From the present investigation it was observed that compact and green callus showed positive responses towards shoot regeneration. It may be mentioned here that Vasil and Vasil (1992) reported about the variable nature of callus development in wheat. In this case the colour of the callus was reported to be white, off-white or pale yellow while the

nature of the callus was either compact and soft or granular and translucent. Among these calli white as well as compact and soft were found to be embryogenic in nature.

The results of the present study also indicated that hormonal supplements of culture medium played an important role on shoot regeneration through callus culture. It was observed that cotyledon explants derived callus responded to shoot regeneration when they were cultured on MSB_5 media containing zeatin and NAA. Bagheri et al. (2012) also obtained similar results in lentil when they used cotyledonary petiole as explant for indirect shoot regeneration. It may be mentioned here that MSB_5 medium supplemented with 2, 4-D alone or in combination with other hormonal supplements produced profuse callus from all types of explants investigated in the present study. However, the callus that produced from 2, 4-D containing medium failed to initiate regeneration of shoots.

To obtain complete plantlets induction of roots from the base of the regenerated shoot is an integral part for *in vitro* regeneration studies. In the present study, development of roots has been tried from the *in vitro* regenerated shoots that failed to develop roots spontaneously in shoot induction medium using a number of hormonal supplements. Development of effective *in vitro* root has been reported to be difficult in lentil and considered as a major limiting factor in obtaining complete plantlet in this plant (Sarker et al. 2003). However, there are a few reports of rooting from regenerated shoots in lentil. Williams and McHughen (1986) reported 11% of rooting efficiency of the lentil shoots on sand bed in a mist chamber. Singh and Raghuvanshi (1989) reported the formation of roots on medium without hormonal supplements from *in vitro* regenerated shoots. Polanco and Ruiz (1997) stated that rooting of *in vitro* regenerated lentil shoots was dependent on nature and concentration of cytokinins. Fratini and Ruiz (2003)

reported a higher percentage of rooting (95.35) using nodal segments of lentil with an axillary bud cultured in an inverted orientation.

Previous reports further indicate that root induction in lentil can be achieved on a medium containing IAA and NAA (Polanco and Ruiz 2001, Polowick et al. 2000). On the other hand Warkentin and McHughen (1993) reported the formation of roots on medium containing IBA and NAA as well as on hormone free medium. Ye et al. (2002) found that NAA is more efficient than IBA for rooting in lentil. Khawar et al. (2003) reported rooting in lentil (25%) using 1.1 µM of IBA. On the contrary, Tzitzikas et al. (2004) used IAA, IBA and NAA for rooting in pea, Amutha et al. (2003) used IBA for rooting in *Vigna radiata.* Following the above mentioned reports, several hormonal combinations containing IBA and NAA were tried in order to induce roots at the base of the regenerated shoots but the hormonal combinations used for root induction did not show positive response. No effective root induction from the base of the regenerated shoot was observed although occasional induction of non-effective roots was noticed from the stem region slightly higher than the cut end of the shoots when cultured on MS medium supplemented with higher concentrations of IBA (20 - 25 mg/l).

To overcome these problems of rooting the techniques of micrografting can be applied (Gulati et al. 2001). However, micrografting of lentil was found to be rather difficult, time consuming, complicated and also rate of success varies. Thus there was a need to develop other efficient system, so that the recovery of regenerated plantlets may be possible.

During the present study most interesting observation was the formation of *in vitro* flower and normal pod set on the media containing IBA and NAA. Gulati and Jaiwal (1990) had also observed more or less similar responses in mungbean. Under these

circumstances *in vitro* flowering and fertile seed formation was considered as an alternative in obtaining plant progenies where *in vitro* root formation was difficult.

***In vitro* flowering and pod formation**

To overcome the limitations caused by poor root development from *in vitro* shoots experiments were conducted to initiate *in vitro* flowering and pod formation directly from *in vitro* regenerated shoots. The present investigation deals with the development of an alternative regeneration system escaping the methods of *in vitro* root formation from regenerated shoots of lentil. Although there is no report on *in vitro* flowering and seed formation in lentil as well as in other grain legumes but a limited number of studies have been conducted in ornamental plants with regard to the relationship of *in vitro* flowering as in the case of bitter melon (Wang et al. 2001), gentian (Zhang et al. 2000), *Panax ginseng* (Tang 2000), *Cichrium intybus* (Demeulemeester and De proft 1999), *Murraya paniculata* (Jumin and Ahmad 1999).

Flowering is considered to be a complex process regulated by a combination of environmental and genetic factors (Bernier 1988, Weigel 1995, Weller et al. 1997). Phytohormone can also influence many diverse developmental processes on *in vitro* flower formation (McCourt 1999). Previous reports indicate that *in vitro* flowering in ornamental plants was achieved on a medium containing BA and Fe^{+2} (Wang et al. 2001, Jumin and Ahmad 1999), GA_3 and NAA (Tang 2000).

Following the above mentioned reports, several hormonal combinations containing IAA, IBA and NAA were tried in order to induce *in vitro* flowering and seed formation in lentil. For this study, at least 500 - 600 shoots were inoculated for each combination of hormonal supplements and *in vitro* flower buds were found to initiate within 7 - 10 days of culture. The highest number of flowering shoots occurred on half strength of

MS medium containing 20 mg/l IBA and 0.5 mg/l NAA. This combination of hormone eventually produced maximum number of flower per shoot. On the other hand the best response towards percentage of *in vitro* flowering was achieved on half strength of MS medium containing 20 mg/l IBA and 0.5 mg/l NAA. MS medium without hormonal supplements has also been found to be effective for flower bud formation but these flower buds failed to develop fully developed flowers in this medium.

Experiments were also conducted to examine the effect of NAA on *in vitro* flowering and seed formation. The number of flower and seed formation per shoots increased significantly with the application of NAA in combination with IBA compared to that of MS along with IBA.

Although number of flower per shoot was higher on MS medium containing 20 mg/l IBA and 0.5 mg/l NAA than half strength of MS containing 20 mg/l IBA and 0.5 mg/l NAA. However, about 50% of total flower per shoot grown on MS medium containing 20 mg/l IBA and 0.5 mg/l NAA failed to develop seed formation. Thus from the present study, it may be concluded that half strength of MS containing 20 mg/l IBA and 0.5 mg/l NAA is the most effective medium for *in vitro* flowering and seed formation. The number of flowers formed could be increased with two weeks of regular sub-culturing of the *in vitro* flower bearing shoots to fresh media having the same hormonal supplements.

Results presented here clearly demonstrated that the shoots derived from cotyledonary nodes (CN) explants showed a better response towards *in vitro* flower formation. It was also noted that there was no significant differences on number of flower and pod formation per shoot among the four varieties of lentil.

In the present study *in vitro* pollen viability and germination ability was tested using the media containing 10% sucrose and 0.01% boric acid and it was observed that pollen viability was always higher than germination ability. The ratio of the pollen viability and germination ability was about 80 and 19% respectively. This finding was more or less identical to that with tobacco. In this plant species Reale et al. (2006), reported the percentage of viability and germination ability as 80 and 55% respectively, whereas, Al-Dehadhem et al. (2004) reported 47 and 25% respectively in the same plant.

The ultimate success of organogenesis depends on the production of complete plantlets with high survival rates. In spite of considerable progress in developing *in vitro* protocol for successful regeneration of complete plantlets, the establishments of plants in soil is considered to be difficult and a few reports are available discussing the problems involved in lentil plant establishment. But in the present investigation establishment of plantlets was achieved by manipulating of potting mixture. The rooted plantlets were successfully transplanted to plastic pots containing the equal quantity of soil, sand and vermiculite with 95% survival rates. To achieve this high survival rates primarily high humid condition were maintained around the plants but after 2 - 4 weeks this conditions were gradually reduced to ambient level. This probably re-commissioned the photosynthetic machinery of the plants, enabling them to withstand the subsequent reduction in the ambient relative humidity and help to survive the plantlets under natural environmental condition. Similar hardening procedure has been adopted in chickpea by Chaturvedi and Chand (2001).

Genetic transformation

The last part of the present investigation dealt with the integration of desired gene through genetic transformation for microsperma types of lentil varieties grown in

Bangladesh. Among the different approaches, *Agrobacterium*-mediated genetic transformation has been considered as the most common and successful method used in various leguminous crop plants such as soyabean (Hinchee et al. 1988, Meurer et al. 1998), chickpea (Fontana et al. 1993, Kar et al. 1996), peanut (McKently et al.1995). Several other methods have also been used for genetic transformation of plants including microprojectile bombardment, electroporation, sonication, chemical method of transformation, etc. All these methods are plant specific and cost intensive. Generally *Agrobacterium*-mediated genetic transformation has been considered as the most convenient and cost effective than other techniques. It is the most popular method for genetic transformation because of high co-expression of introduced genes, potentially low copy number and preferential integration into active transcribable regions (Birch 1997, Gheysen et al. 1998). Therefore, the *Agrobacterium*-mediated genetic transformation technique was followed to insert desired gene in microsperma varieties of lentil.

Lentil is considered to be an underexploited crop since limited research has been done so far to improve it in comparison to other pulses crops. A few reports are available on the production of transgenic lentil plants using *Agrobacterium*-mediated genetic transformation (Warkentin and McHughen 1991, 1992, 1993, Barton et al. 1997, Halbach et al. 1998, Gulati and McHughen 2003, Celikkol et al. 2009). Most of these studies were carried out mainly for the development of a protocol for *Agrobacterium*-mediated genetic transformation of different varieties of lentil. Studies on the *Agrobacterium*-mediated genetic transformation of lentil using the varieties cultivated in Bangladesh have been initiated earlier by Sarker et al. (2003). The transformation

experiments of the present study have been designed following the method reported by Sarker et al. (2003).

For successful transformation, choice of *in vitro* regeneration system is of prime importance. Single cell involved plant regeneration has been considered as a successful event in achieving transformation and through these system of regeneration there are least chances of chimera formation in developing transgenic plants. But regeneration *via* callus development is still an unsolved problem as there is no effective, reproducible protocol for regeneration *via* callus in most of the crops including lentil. In chickpea, Huda et al. (2000) also demonstrated that transformation using callus tissue failed due to limited shoot regeneration capability and thus failed to develop complete plantlets.

On the contrary, in case of direct regeneration large number of multiple shoots proliferated from different types of explants ensuring the maximum genetic uniformity of the resulting plantlets. Thus direct regeneration exhibits advantages over regeneration via callus development in terms of regeneration capability and uniformity of regenerated progenies. Therefore, in the present investigation direct regeneration method was followed for genetic transformations studies. Integration of gene through direct regeneration protocol has also been reported in some other crops like soyabean (Donaldsons and Simmonds 2000), pigeon pea (Lawrence and Koundal 2001), *Vigna* species (Jaiwal et al. 2001).

In this part of investigation transformation experiments were performed using three different explants, namely cotyledonary node (CN), decapitated embryo (DE), and cotyledon attached decapitated embryo (CADE) from Barimasur - 4 (BM - 4), Barimasur - 5 (BM - 5), and Barimasur - 6 (BM - 6) varieties of lentil. These explants

showed greater potential in regenerating sufficient number of healthy shoots. Sarker et al. (2003) also demonstrated efficient regeneration in lentil using decapitated embryo explant. Hassan et al. (2007) and Chopra et al. (2011) also noticed that this type of explants were efficient for transformation in lentil.

Among all the explants studied, decapitated embryo explants showed the best response towards transformation with bacterial strain LBA4404. This result is similar to the earlier results reported by Sarker et al. (2003) and Hassan et al. (2007). Next to decapitated embryo (DE) explants, cotyledon attached decapitated embryo (CADE) explants showed good response towards transformation. This explant showed variable nature of GUS expression. In some cases, much greater areas of explant exhibited GUS expression while in other cases only a small portion of the wounded tissue were competent for transformation. Similar results regarding the expression of the GUS gene in lentil tissue have been reported by Warkentin and McHughen (1992).

Agrobacterium-mediated genetic transformation procedure is believed to be influenced by several factors (Mansur et al. 1993). Factors that influence successful transformation, such as type of *Agrobacterium* strain, genotype (host) compatibility and responsiveness of explants toward *Agrobacterium* infection, optical density (O.D.) of *Agrobacterium* suspensions, incubation and co-cultivation period were optimized in conducting transformation experiments. Moreover, the efficiency of transformation and transgenic plant production depends on the establishment of suitable protocols including the co-cultivation of host cell/tissue, regeneration and selection of transgenic plantlets.

Transformation frequency and duration of co-cultivation have been reported to be directly correlated as has been described in peas (Kathen and Jacobson 1990). In most

of the transformation experiments 2 to 3 days of co-cultivation period was used as has been reported in tobacco (Horsch et al. 1985). However, Arundhati (1999) reported increased frequency (47.8%) of transformation when leaf discs explant of pigeon pea were co-cultured for a period of 4 days. These results revealed that duration of infection and co-cultivation are genotype specific and has great influence on transformation frequency.

In the present studies, during optimization of regulatory factors maximum number of explants found to be transformed with bacterial suspension having an optical density (O.D.) of 1.0 with 45 minutes of incubation and 3 days of co-cultivation in case of *Agrobacterium* strain LBA4404. In *Agrobacterium* strain EHA105 bacterial suspension having an (O.D.) of 1.0 with 10 minutes of incubation and 3 days of co-cultivation was found to be optimum for transformation of cotyledon attached decapitated embryo (CADE) explants for all three varieties of lentil.

In chickpea Krishnamurthy et al. (2000) incubated mature embryo explants for 20 minutes and then co-cultivated the explants for 3 days and were able to obtain transgenic plants. Tewari-Singh et al. (2004) employed the same co-cultivation period of 3 days but incubated explants in bacterial suspension for 1- 2 hours and got transgenic plant. In the present study it was found that, such longer period of incubation (more than 45 minutes in case of strain I & III and more than 10 minutes for strain II) and co-cultivation (more than 3 days) reduced survivability of explants and lead bacterial overgrowth in culture medium thus hampering the proper growth of infected explants. Warkentin and McHugen (1992) reported that, they were able to observe transient GUS expression from inoculating lentil epicotyl explants incubated only for 10 - 15 minutes, but they did not mention the information on transformation frequency.

In the same report they also mentioned that longer co-culture period was capable of enhancing the explant area for GUS expression. Although the inoculation period differs but their observation supports present results regarding the expression of GUS gene.

The composition of co-culture media particularly the hormonal supplements is considered as an important factor in obtaining regeneration from infected explants. Schroeder et al. (1993) reported that in case of *Pisum sativa* presence of growth regulators in the co-cultivation media enhanced recovery of putative transgenic plants. In chickpea, Kar et al. (1996) reported that absence of growth regulators in co-cultivation media greatly reduced transformation efficiency and recovery of transgenic plants. Similarly in the present study presence of growth regulator in co-culture media also found to enhance the induction of adventitious shoots and found to improve the health of regenerated shoots.

Virulence of the bacterial strain also reported to influence the transformation efficiency in lentil and it was confirmed with four different *Agrobacterium* strains such as C58, Ach5, GV3111 and A281 using shoot apices explants by Warkentin and McHugen (1991). Celikkol et al. (2007) found *Agrobacterium* strain KYRT1 was more efficient (to be on average 2.8 fold) than both EHA105 and C58C1 for producing transient GUS gene expression on cotyledonary petioles. Halbach et al. (1998) and Hassan et al. (2007) found *Agrobacterium* strain EHA101 was more efficient than LBA4404 while using decapitated embryo (DE) explants. Working with different strains of *Agrobacterium* in case of pea transformation, Nadolska-Orczyk (2000) also found higher transformation efficiency with EHA105 than that of $C_{58}C_1$ and LBA4404. Two different *Agrobacterium* strains were also used in the present investigation for the integration of desired gene in lentil genome. Among them the capability of GUS

expression was investigated only with the strain of LBA4404 while that of EHA105 was not conducted since this strain did not possessed screenable marker gene in its T-DNA region. Therefore, comparative efficiency of these two strains was not analyzed.

Selection of transformed cells is an important component of any plant transformation system. In the absence of proper selection system one would face with the option of screening every shoot regenerated from the infected explants. In the transformation experiment, a selectable marker gene was introduced into the plant transformation vectors and a suitable selecting agent was added to the culture medium which favors the growth of only transformed cells.

The *Agrobacterium* strains I and III used in these experiments (LBA4404) contains *npt*II gene within its left and right border of T-DNA. This gene confers kanamycin resistant during the selection of transformed cells. Therefore, selections of the transformants were carried out using various concentrations of kanamycin. In most of the reports kanamycin was applied after co-cultivation for the selection of transgenic shoots/cells (Srinivasan et al. 1991, Chopra et al. 2011). However, in the present study it was found that presence of kanamycin greatly hampered the growth of the explants. This observation was identical to the results obtained in other plants such as flax, alfalfa, potato and chickpea (Pezzoti et al. 1991, McHughen et al. 1989, Cardi et al. 1992, Tewari-Singh et al. 2004). Senthil et al. (2004) opined that explants must be allowed to proliferate multiple shoots and then selection pressure should be applied.

In the present study, co-cultivated explants were cultured initially in a medium without selective agents. After 10 - 12 days the regenerated shoots were sub-cultured in the same media but with selectable agents. Initiation of multiple shoots was found to be retarded in presence of kanamycin. Therefore, a pre-culture period and a delayed

selection with kanamycin were followed in obtaining regeneration from explants with high rate of transformation efficiency. Similar observation during selection of transformed shoots was also reported in other plant species such as *Medicago* (Pezzoti et al. 1991), alfalfa (McHughen et al. 1989), chick pea (Kar et al. 1996) and pea (Polowick et al. 2000) etc.

It was observed that, all control shoots died in the selection medium in presence of 200 mg/l kanamycin. For this purpose kanamycin concentration was gradually increased from 50 to 200 mg/l during the selection of transformed lentil shoots. Sarker et al. (2003) also applied kanamycin concentrations from 50 to 200 mg/l gradually for BM - 2 and BM - 4 varieties of lentil. Celikkol et al. (2009) also increased kanamycin concentrations gradually from 100 to 300 mg/l to select transformed shoots of lentil. In chickpea Kar et al. (1996) applied two concentrations of kanamycin for the selection of transformed shoots (25 and 50 mg/l), Eapen and George (1994) increased kanamycin concentrations gradually from 50 to 100 mg/l in peanut. Therefore, in the present study the shoots that survived in the medium containing 200 mg/l kanamycin were considered as transformed. In this concentration the non transformed shoots first became albino and finally died whereas the putatively transformed shoots remain green and healthy. The number of survived shoots regenerated from cotyledon attached decapitated embryo explants in the final selection pressure was extremely low (1.63 - 2.15%). It may be mentioned here that till now the transformation efficiency in legumes is very low 0.03 - 5.1% (Yan et al. 2000, Senthil et al. 2004). Using kanamycin as a selectable marker Chopra et al. (2011) got the transformation efficiency of 0.9%, Celikkol et al. (2009) got the transformation efficiency of 2.3% in case of lentil, Pniewski and

Kapusta (2005) got the transformation efficiency of 4.1%, Nadolska-Orczyk and Orczyk (2000) got the transformation efficiency of 3.6% in case of pea.

The integration of antifungal gene via genetic engineering techniques to combat fungal diseases has been shown to be an effective strategy for rapid development of resistant to pathogens. Recently, several antifungal genes have been identified which are involved in plant defense against fungal infection. The sensitivity of fungal cell wall to lytic enzymes has been exploited in the present study to over express of chitinase, thereby providing enhanced resistant to fungal infections. For this purpose, in the present study *Agrobacterium* strain EHA105 /pGIIvst-N-chitin (Strain II) and LBA4404 pCAMBIA2300 enh35SAFP (strain III) was used as antifungal strains. Strain II contains *bar* gene within its T-DNA and as it confers phoshinothricin (PPT) resistantance and strain III contain *npt*II gene which is resistant to kanamycin. Thus PPT and kanamycin was used as selectable agent in case of strain II and III respectively. Like kanamycin it was found that the presence of PPT greatly hampered the growth and regeneration of the explants.

From this experiment, it was observed that all control shoots died in the selection medium in presence of 2.0 mg/l PPT. Therefore, the shoots that survived in the medium containing 2.0 mg/l PPT were considered as transformed. For this purpose PPT concentration was gradually increased from 0.5 to 2.0 mg/l during the selection of transformed lentil shoots. The number of survived shoots regenerated from cotyledon attached decapitated embryo explants in the final selection pressure was extremely low (0.36%). This result is very much identical to Khatib et al. (2007) where the transformation efficiency was 0.01 to 0.43%.

Comparing the results of these two different selective agents, it was observed that the transformation efficiency was higher for kanamycin selection (0.89%) than that of PPT (0.36%). In pea transformation Nadolska-Orczyk and Orczyk (2000) got different transformation efficiency like 0.8 to 3.4% for kanamycin vs 1.47% for PPT, Grant et al. (1998) got different transformation efficiency like 8.2% for kanamycin vs 3.6% for PPT.

One of the essential requirements in *Agrobacterium*-mediated gene transfer system for the production of transgenic plants is the application of an efficient wounding treatment. Wounding the plant material before co-cultivation allows better bacterial penetration into the tissue, facilitating the accessibility of plant cells for *Agrobacterium* and possibly stimulates the production of potent *vir* gene inducers like phenolic substances such as acetosyringone and hydroxyacetosyringone (Stachel et al. 1985).

In the present investigation transformation experiments were also carried out to enhance the transformation efficiency in lentil using different wounding techniques. From this experiment it was observed that wounding of explants by mild injury followed by 3 days of co-cultivation showed higher percentage of regeneration and transformation efficiency. Using this technique the final transformation efficiency was found to increase from 0.3 to 0.8% which is three fold better than the techniques without doing any extra injury.

Shoots that survived in higher concentration of selection medium but failed to initiate root were allowed to develop *in vitro* flowers in the flowering medium (half strength of MS + 20 mg/l IBA + 0.5 mg/l NAA). It was found that only two out of 10 kanamycin survived shoots were responded to flowering. On the other hand none of the shoots that survived in the phosphinothricin selection medium were responded to flowering,

whereas, 25% of the survived shoots were responded to flowering and pod formation without use of any selection pressure. This result is comparatively very low compared to shoots without selection pressure. It may be due to the effect of antibiotic in flowering media. Puonti-Kaerlas et al. (1992) also reported that infertility may occur in the transformants as side effect due to the application of antibiotics.

One of the common ways to detect the integration of transgene is through polymerase chain reaction (PCR) analysis. In the present investigation the transgenic nature of the transformed plantlets of T_0, T_1 and T_2 were confirmed through the application of specific molecular techniques like polymerase chain reaction (PCR) analysis. The DNA isolated from both of transformed and non-transformed shoots was subjected to PCR for the amplification of *npt*II gene present in case of *Agrobacterium* strain I, *bar* and *chitinase* gene in case of *Agrobacterium* strain II, *AFP* and *npt*II gene in case of *Agrobacterium* strain III. PCR amplified DNA was analyzed through agarose gel electrophoresis. From the gel it was observed that the single band formed (with product size 750 bp for *npt*II, 500 bp for GUS, 447 bp for bar, 555 bp for chitinase and 190 bp for AFP) in each of the transformed plantlets were identical to the respective amplified DNA of bacterial strain (positive control). This result indicated that the respective target gene was inserted in the genomic DNA of transformed plantlets. In some cases it has been found that although the T_0 clone were positive with bar gene but some of them turned out negative in the PCR of *chitinase* gene. This could be the result of incomplete T-DNA transfer as the transfer initiated from right border got aborted before reaching the left border. It is well known that T-DNA transfer to plant cells occurs in a defined direction, starting from the right border to the left border (Becker et al. 1992, Zambyski 1992). Hassan (2006) has reported similar phenomenon in the study of pea

transformation. In some cases it was also observed that the T_0 clone were positive both with bar and *chitinase* gene but it turned occasionally negative in T_1 progeny.

Southern hybridization was necessary for better confirmation of transgene integration and to monitor the number of introduced genes in transgenic plant (Dai et al. 2001, Kumar et al. 2005 Rai et al. 2007, Maruthasalam et al. 2007). Transgenes expression in transgenic plants could depend much on number of introduced genes. Generally, a number of insertion sites of an introduced gene could lead to silencing of transgenes in plants. Therefore, it is required to know copy number of transgenes that are introduced into genome of plants. Considering its importance of the copy number of transgene, Southern hybridization was used all through in our study. In the present study, 32P-labeled DNA probe was used, corresponding to intact full length genes of kanamycin for hybridization. Number of transgene copies identified in lentil transgenic plants. In lentil a total of five transgenic lines were tested using southern hybridization. Out of them only one transgenic line (D1) contained one copy of introduced gene; while another one transgenic line (B1) contained two copies of introduced gene.

At the transcription level, expression was confirmed by RT-PCR of the selected PCR positive plants in T_0 generation. Stable expression of transgene is of important concern in crop improvement through gene manipulations. From the present study it was observed that 4 out of 5 selected PCR positive T_0 plants showed different expression of *npt*II gene.

Northern blotting analysis was performed to investigate transcript levels of transgenes in non rooted lentil plants transformed with plasmids carrying *npt*II and AFP gene. Total RNA was isolated from leaf tissue of both untransformed plant and transformed lines and hybridized with a 32P-labeled DNA probe, corresponding to intact full length

genes of kanamycin. The results showed that transgenic lines exhibited varying degrees of mRNA transcripts. Similar results were reported with transgenic plants transformed with transgenes under 35S promoter (Selvapandiyan et al. 1998, Nguyen et al. 2004).

From the foregoing discussion, it may be concluded that during the present investigation it has been possible to optimize the *in vitro* regeneration system for four varieties of lentil growing in Bangladesh. One notable findings of the present investigation was to develop *in vitro* flowering and subsequent *in vitro* seed formation from the *in vitro* regenerated shoots of lentil. Through this investigation it has been possible to bypass the *in vitro* rooting system in lentil which was very difficult to induce. Moreover, this technique of *in vitro* flowering and pod formation help in achieving plantlets comparatively in shorter period of time. Finally it has been possible to develop transgenic lentil plantlets using both marker and antifungal genes which were confirmed through molecular characterization based on PCR and RT- PCR analysis as well as Southern and Northern blotting techniques. Available literature indicated that this may be the pioneering report on the successful development of transgenic microsperma group of lentil plants. However, the frequency of transformation using both marker and antifungal genes was rather low which needs to be addressed in the future work of lentil genetic transformation.

6. REFERENCES

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7. APPENDIX

7. APPENDIX – A

ABBREVIATIONS

The following abbreviations have been used throughout the text:

A.	:	*Agrobacterium*
AFP	:	Anti fungal protein gene
BAP	:	6-benzylaminopurine
B_5	:	B_5 basal medium
bar	:	Phosphinothricin acetyltransferase
BARI	:	Bangladesh Agriculture Research Institute
BBS	:	Bangladesh Bureau of Statistics
BM	:	Barimasur
bp	:	base pair
C	:	Centigrade / Celsius
$CaCl_2$	:	Calcium chloride
CaMV	:	Cauliflower Mosaic Virus
cDNA	:	Complementary DNA
CH	:	Casein hydrolysate
cm	:	Centimeter (s)
CTAB	:	Cyle tetramethyl ammonium bromide
dNTP	:	Deoxy nucleoside tri-phosphate
e. g.	:	Example gratia, for example
et. al.	:	et alil and others
etc	:	et cetra, and the rest
FAO	:	Food and Agriculture Organization
Fig/s	:	Figure / Figures
FW	:	Fresh weight
GCA	:	Gross-cropped area
GM	:	Genetically modified
g	:	gram (s)
GUS	:	ß-glucoronidase
Ha (s)	:	Hectare
Hr (s)	:	Hour (s)

HCL	:	Hydrochloric acid
$HCCL_3$	:	Chloroform
$HgCl_2$	:	Mercuric chloride
IAA	:	Indole- 3 – acetic acid
IBA	:	Indole- 3 – butyric acid
ICARDA	:	International Center for Agriculture Research in Dry Areas
i. e.	:	id est = which to say in other words
Kan	:	Kanamycin
Kb	:	Kilo base pair
Kcal	:	Kilocalorie
Kn	:	Kinetin (6- furfurylaminopurine)
KNO_3	:	Potassium nitrate
l	:	Litre
LB	:	Liquid Broth
lb / sq. inch	:	Pound per square inch
m	:	Meter (s)
M	:	Molar
mM	:	Millimolar
mm	:	Milimeter
mg	:	Milligram
mg / l	:	Milligram per liter
min (s)	:	Minute (s)
ml (s)	:	Milliliter (s)
MS	:	Murashige and Skoog Medium 1962
MT	:	Murashige and Tucker Medium
MT	:	Metric tonne
MW	:	Molecular weight
NAA	:	α- naphthalene acetic acid
NaOH	:	Sodium hydroxide
Na_2 – EDTA	:	Sodium salt or ferric ethylene diamine tetra acetate
NH_4NO_3	:	Ammonium nitrate
No.	:	Number
NOS	:	Nopaline synthase
nm	:	Nanometer
*npt*II	:	Neomycine phosphotransferasae II

OD	:	Optical density
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of Hydrogen
PPT	:	Phosphinothricin
rpm	:	Rotation per minute.
RT-PCR	:	Reverse transcription polymerase chain reaction
sec.	:	Second
Sp. / Spp.	:	Species
t	:	Ton
T_0, T_1, T_2	:	Transgenic lines (First and second generation inbreed progeny)
T- DNA	:	Transfer DNA
TDZ	:	Thiadiazuron
US	:	United States
USDA	:	United States Department of Agriculture.
US$	:	United States dollar
UV	:	Ultraviolet Wavelength
Var. (s)	:	Variety (s)
Vir	:	Virulence region
Viz	:	Namely
v / v	:	Volume by volume
WHO	:	World Health Organization
Wt.	:	Weight
w / v	:	Weight by volume
X – gluc	:	5-bromo-4-chloro-3-indolyl glucoronide
YEP	:	Yeast Extract Peptone
YMB	:	yeast extract Mannitol Broth
µ	:	Micron
µM	:	Micromole
µl	:	Micro liter
µg	:	Microgram
1 N	:	1 Normal
2, 4-D	:	2, 4-dichlorophenoxy acetic acid
%	:	Percentage
+ve	:	Positive

APPENDIX - B

Murashige and Skoog (MS) Medium 1962

Components	Concentration **(mg/l)**
Macronutrients	
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
Micronutrients	
$FeSO_4.7H_2O$	27.80
Na_2-FeEDTA	37.30
$MgSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30,000.00

pH adjusted to 5.8 before autoclaving.

APPENDIX- C

MSB_5 medium

Macro and Micronutrients of MS medium and B_5 Vitamins

(Gamborg *et al*.1968)

Components	Concentration **(mg/l)**
Macronutrients	
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
Micronutrients	
$FeSO_4.7H_2O$	27.80
Na_2-FeEDTA	37.30
$MgSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.\ 2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Nicotinic acid	1.00
Pyrodoxine-HCl	1.00
Thiamine-HCl	10.00
Inositol	100.00
Sucrose	30,000.00

PH adjusted to 5.8 before autoclave