OPTIMIZATION OF A REGENERATION AND TRANSFORMATION SYSTEM FOR LENTIL (*Lens culinaris* M., cv. Sultan-I) COTYLEDONARY PETIOLES AND EPICOTYLS

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ABSTRACT

OPTIMIZATION OF REGENERATION AND TRANSFORMATION SYSTEM FOR LENTIL COTYLEDONARY PETIOLES

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In this study, optimization of a transformation and regeneration system via indirect organogenesis in cotyledonary petiole tissue of lentil (*Lens culinaris* Medik.) was investigated. Eight different medium types differing in their plant growth regulator compositions were employed to examine the callus induction potency of cotyledonary petiole. Except two, all other tested medium yielded more than 80% callus induction. Nine different medium types were studied to test the potencies of callus structures for shoot induction. Only the callus induced in medium H (1 mg/L Zeatin riboside + 1 mg/L Naphthalane acetic acid) yielded shoots at 8 to 40 %

frequency. The most responsive medium was MS basal medium with no growth regulators. Also five and three different medium types were employed to examine callus induction potency of epicotyl tissues respectively. Each medium type yielded 90% callus induction. Only the callus induced in medium H yielded shoots At 6 to 26% frequency.

Preliminary studies were carried out for somatic embryogenesis in cotyledonary petiole. Effects of salicylic acid on somatic embryogenesis were also investigated. Salicylic acid at 200µM was found to enhance the percentage of somatic embryos by 25 % and reduce the necrosis 24 %. However none of the globular and heart shape embryos were able to regenerate.

Transient GUS expression efficiencies of roots, shoot tips, and cotyledonary petioles were tested after *Agrobacterium*-mediated transformation. Transformation frequencies were 26, 74, and 38 % for cotyledonary petiole, shoot tips, and roots respectively.

Keywords: Lentil, indirect organogenesis, cotyledonary petiole, epicotyl, *Agrobacterium tumefaciens*

ÖZ

MERCİMEK KOTİLEDON PETİOLLERİNDE REJENERASYON VE TRANSFORMASYON SİSTEMLERİNİN OPTİMİZASYONU

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Bu çalışmada mercimek (*Lens culinaris* Medik.) kotiledon petiolü ve epikotil dokularının transformasyonu ve indirekt organogenesis yolu ile rejenerasyonu incelenmiştir. Farklı bitki büyüme düzenleyicileri kompozisyonuna sahip sekiz farklı besiyerinin kallus oluşturma kapasiteleri arştırılmıştır. Test edilen besiyerlerinden iki tanesi dışında hepsi % 80'den fazla kallus oluşturmuştur. Oluşan kalluslar dokuz

farklı besiyerine aktarılarak sürgün verme kapasiteleri test edilmiştir. Sadece besi yeri H'de (1 mg/L Zeatin ribosid + 1 mg/L Naftala asetik asid) oluşan kalluslar %5 ile 40 arasında değişen oranlarda sürgün vermiştir. Bitki büyüme düzenleyicisi içermeyen MS bazlı ortam % 40 ile en çok sürgün veren besiyeri olarak bulunmuştur. Bunun yanında epikotil dokusunun kallus oluşturma kapasitesi beş farklı besiyerinde araştırılmış ve oluşan kalluslar üç farklı besiyerinde sürgün verme kapasitesi için test edilmiştir. Bütün besiyerleri % 90 kallus oluşturmuş. Sadece besiyeri H'de oluşan kalluslar % 6 ile 26 arasında değişen oranlarda sürgün vermiştir.

Kotiledon petiollerinin somatik embriyogenezi için öncül çalışmalar yapılmıştır. Salisilik asidin somatik embriyogenez üstündeki etkileri ayrıca araştırılmıştır. Salisilik asidin 200µM derişimde embriyo oranını % 25 arttırdığı ve nekrozu %24 düşürdüğü görülmüştür. Buna karşın hiçbir globüler ve kalp-şekilli embriyo rejenere olmamıştır.

Kök, sürgün ucu ve kotiledon petiollerinin geçici GUS ifadeleri verimi *Agrobacerium*-yollu transformasyon sonrası test edilmiştir. Transfromasyon oranları kotiledon petiolünde %26; sürgün ucunda %74 ve köklerde %38 olarak bulunmuştur.

Anahtar Kelimeler: Mercimek, indirekt organogenesis, kotiledon petiolü, epikotil, *Agrobacterium tumefaciens*

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LIST OF ABREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	Analysis of variance
BA	Benzylaminopurine
CaMV35S	Cauliflower Mosaic Virus 35S Promoter
GA	Gibberellic Acid
GUS	β-glucuronidase
IAA	Indole-3-acetic acid
KIN	Kinetin
MES	2-[N-Morpholino] ethanesulfonic acid
MS	Murashige and Skoog
NAA	α-Napthaleneacetic acid
NAA	Naptalen acetic acid
SA	Salicylic acid
T-DNA	Transferred DNA
TDZ	Thidiazurone
Ti	Tumor inducing
YEB	Yeast Extract Broth
ZEA	Zeatin riboside

CHAPTER I

INTRODUCTION

1.1. Lentil as an Important Pulse Crop

Lentils, one of the oldest food crops of mankind, originated in the Near East 8500 years ago and they are still one of the most important cool season annual grain legume or pulse crop through out the world. Lentils are legumes that convert nitrogen from the atmosphere into nitrogen in the nodules on the plant roots. As food they provide a valuable protein source. Areas with limited rainfall and drier growing season prove to be most suitable for lentil production. These characteristics make lentil an important crop and ensured its survival to the present day.

1.1.1. Historical and Taxonomic Perspectives

Lentils are shaped like a lens. In fact, lens is the Latin word for lentil (*Lens culinaris* Medik.). The size and appearance of lentils varies depending on the variety. Lentils were first grown more than 8500 years ago in the Near East, and production later spread to Mediterranean basin, Asia, Europe, and finally the Western Hemisphere. Lentils were probably introduced into the United States in the early 1900s. They have been grown in the western United States and western Canada since the 1930s, mainly in rotation with wheat.

Lentil is called as *Lens culinaris* Medik. Medik is for Medikus; a German botanist-physician who has given name to the plant in 1787.

Lens culinaris Medik. belongs to the division *Anthophyta*, sub-division *Dicotyledonea*, order *Rosales*, suborder *Rosineae*, family *Leguminosae*, subfamily *Papilionaceae*, and tribe *Vicieae*. There are four wild species of lentil: L. *orientalis*, *L. nicricans*, *L. ervoides*, and *L. odemensis.* Morphological similarities in pollen grain morphology, similarities in plant type show that that *L. orientalis* is the wild progenitor of cultivated lentil *L. culinaris* (Williams *et al.*, 1974; Zohary, 1972). In Turkey there are five lens species; *L. montbretii*, *L. nigricans*, *L. ervoides*, *L. orientalis*, *and L. culinaris.* (Davis, 1985)

1.1.2. Description and Growth Habits

Lentil is typically much-branched, short, and light green annual herbaceous plant. It is generally 15 to 75 cm tall depending on the genotype. The plant generally has slender stems that can be single or multi-branched (Figure 1.1.). Depending on the available space in the field of growth branches can directly arise from the main stem, from cotyledonary node below ground or from other branches (Saxena and Hawtin, 1981).

Roots of lentil are generally taproots and there is a mass of fibrous lateral roots. Depending on the texture and type of soil, various types of root systems can be formed ranging from shallow branched roots to deep taproots (Nezamuddin, 1970). The tap root and the lateral roots in the upper layers of the soil, carries numerous small round or elongated nodules which start to decline before the 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 grow 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 plant height is 15 to 75 cm but it is highly influenced by environment. Not only the height but also the whole course of growth in plant height is affected by genotype as well as environment.

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 three and rarely four 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 temperatures at planting, warm growing temperatures and long days promote early flowering and good seed 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. Lentil seed is classified in to two categories described by Barulina (1930), as macrosperma and microsperma. Macrosperma is also called as “Chilean” and are found in the New World and Mediterranean. They are large seeds ranging from 6 to 9 mm in diameter. Microsperma is also called as “Persian” and found mainly throughout the India and Near East. They are small seeds ranging from 2 to 6 mm in diameter (Duke, 1981).

Figure 1.1. Organs of lentil.

Lentils imbibe more than 100% of the initial air-dry weight. The germination is hypogeal. In the field conditions emergence occur in 25 to 30 days with winter sowing and 7 to 10 days with spring sowing.

1.1.3. Agronomic Information

Agronomical requirements of lentil changes depending on the agro-ecological conditions throughout the world. In India crop is grown during the winter on the soil moisture conserved during the proceeding monsoon season. In countries such as Turkey, Syria, Jordan, Iraq, Lebanon, Cyprus, and Chile

experiencing Mediterranean climate, crop is raised during the wet winters. In the high elevation areas of Turkey and Iran, and also in USA and Canada the crop is growing during the spring season on the conserved soil moisture.

Under optimum environmental conditions lentils complete their lifecycle 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. 15 to 25°C is optimum for germination of lentil but at any temperature above freezing seeds can germinate. Optimum temperature for growth is approximately 24°C (Ibrahim *et al.*, 1979; Salih, 1979)

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 shoots new buds can be initiated easily from the nodes below 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 soil after fall and winter rains.

Lentils can easily grow on slightly acidic soils (pH 5.5 to 6.5) and moderately alkaline soils (pH 7.5 to 9.0) (Bharadawaj, 1975).

1.1.4. Nutritional Value of Lentil

Lentils contain 25 percent protein second only to soybeans as a source of usable protein. An excellent source of vitamin A, lentils also provide fiber, potassium, B vitamins, and iron. Unlike meat, poultry, fish and eggs, this protein source contains no cholesterol and virtually no fat. However, lentils must be teamed with a grain, such as rice, pasta, or barley, to complete and enhance their protein availability to the body.

In 100 grams of lentil there exist 340 calories, 24,7 g protein, 1,1 g fat, 79 mg calcium, 6,8 mg iron, 0,37 mg vitamin B1, 0,22 mg vitamin B2, 2 mg niacin, 60 I.U. vitamin A. Also in 100 g of lentil aminoacid ingredient is; 0,216 g tryptophan, 0,896 g threonin, 1,104 g phenylalanine, 1,316 g isoleucine, 1,760 g leucine, 1,364 g valine, 1,528 g lysine, and 0,180 g methionine (Orr and Watt, 1972).

Husks of lentil contain 13% protein so that it is a valuable animal food. Also stems of lentil contains 1,8 % protein, 4,4% carbohydrate, and 50% fiber and it can be used both as dry and wet feed for animals.

1.1.5. Diseases and Pests of Lentil and Their Control

Main diseases in lentils are ascochyta blight, anthracnose, fusarium root rot, rhizoctonia root rot and sclerotinia. Anthracnose and ascochyta blight can cause severe yield loss in lentils.

Anthracnose can be first noticed as white to gray or cream-colored spots develop on the leaflets and stem. They usually appear on the base of the stem and move up the plant canopy. Leaf drop occurs as the disease progresses.

Ascochyta blight also 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 crop will look blighted in appearance. Both diseases can be managed by using a foliar fungicide program.

Crop rotation (growing lentils only once in four years), and the use of certified, disease free seed will help to minimize the disease.

Root rots become evident at any stage from emergence to maturity. Individual plants become stunted, turn yellow, and die. Essentially the root system

has been destroyed. Crop rotation may help. However, the wide host range of these diseases makes this a less than effective option.

Lentils are attacked by insects wherever they are cultivated. Most important insect pests of lentil worldwide are given in Table 1.1.

Table 1.1. Important insect pests of lentil worldwide.

Seedcorn maggots	*Delia platura*
Wireworms	*Limonius* spp. *and Ctenicera* spp.
Cutworms	*Arotis* spp.
Larvae of weevils	*Sitona* spp.
Thrips	*Frankliniella* spp.
Aphids	*Aphis craccivora and Acyrthosiphon pisum*
Leaf weevils	*Sitona lineatus*
Lepidopterous larvae	*Helicoverpa and Spodoptera* spp.
Lygus bugs	*Lygus* spp.
Bruchid beetles	*Bruchus* spp. *and Callosobruchus* spp.
Lepidopteran pod borers	*Helicoverpa armigera and Cydia nigricana*

When heavy infestation develops, chemical control with insecticides containing *Bacillus thrungiensis*, a naturally occurring bacterium that infects lepidopterous larvae and other pest insects, can be used. Also some insect pests such as aphids have many natural enemies, including ladybird beetles, parasitic wasps and lacewings, but chemical control may be necessary if these insects do not keep aphids at low numbers (Homan *et al*., 1991)

1.1.6. Weeds of Lentil and Their Control

Weed control in lentils is important because lentils are a relatively non-competitive crop. 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 and perennial sow thistle.

1.1.7. Lentil Production in the World and in Turkey

In 2002, world lentil production was nearly three million metric tons. Lentils are produced in over 48 different countries. India and Turkey typically combine to produce nearly one half of total world lentil output (Figure 1.2). Canada is also a major producer of lentils with 12% share of world output. World lentil production has been relatively stable over the last ten years (Table 1.2). Global lentil production peaked in 2000 at about 3.4 million metric tons but in 2002 had declined by 13% to about 2.9 metric million tons.

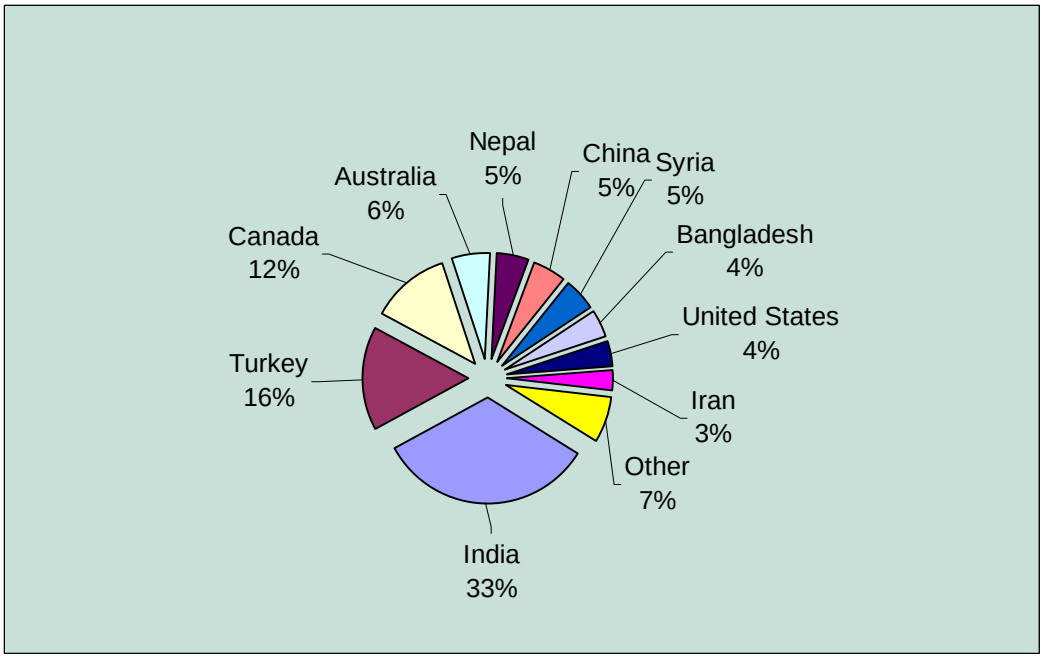

Figure 1.2. Percentage of world lentil production by country (2002)

Table 1.2. Global lentil production from 1993 to 2002.

Year	Production in Metric Tons
1993	2,755,580
1994	2,784,295
1995	2,842,859
1996	2,761,474
1997	2,745,092
1998	2,783,101
1999	2,885,897
2000	3,366,439
2001	3,161,593
2002	2,938,037

In recent years, just over one third of the world's production of lentils has been traded internationally. Approximately one million metric tons of the world's production was exported in 2002. In 2001, the three largest importing countries were Egypt, Turkey, and Sri Lanka. Collectively, these three countries account for around 28 percent of world lentil imports. Imports of lentils are spread among many different countries.

In Turkey there is 78 million hectares land area and 25 million hectares (32%) of this land area is cultivated. 19 million hectares (79%) of this cultivated area is sown and remaining 6 million hectares (21%) is left fallow. Lentil utilizes approximately 5 % of the total area and is the most important food legume. Average lentil consumption in Turkey is 2-3 kg yr^{-1} per person and total domestic demand is 250,000 tones yr^{-1} (Bayaner *et al*., 1997).

Beginning in the late 1970's there is a steady increase in the sown area and production of lentil. In 1982 with the implementation of a utilization of fallow areas project there is a significant increase in the lentil production. But with the problems in the national price policy in 1989 the production begins to decrease. Also the Turkish Grain Board (TMO), which is the principle buyer of the exported lentil, stop purchasing and exporting lentil (Bayaner *et al*., 1997).

Countries like Canada and the Australia are now leaders of lentil trading in the world market although they are at the third and fourth place in production. Turkey therefore needs to take remedial measures to hold its current position in the international market and also to supply national needs. To be able to win this competition Turkey has to promote and support high quality and standardized production, post harvest losses need to be prevented and better disease, pest and weed control mechanisms has to be developed (Bayaner *et al*., 1997).

1.2. Plant Tissue Culture Techniques

Plant tissue culture is the production of whole plant, an organ or a metabolite from a whole plant, cell (meristematic cells, suspension or callus cells), tissue (explant), or an organ (apical meristem, root *etc*.) in an artificial medium, aseptically. Tissue culture techniques are applied to improve existing species. Practically any plant transformation experiment relies at some point on tissue culture. There are some exceptions to this generalization, but the ability to regenerate plants from isolated cells or tissues *in vitro* underpins most plant transformation systems.

Two concepts, plasticity and totipotency, are central to understanding plant cell culture and regeneration. Plants have greater ability to endure extreme conditions and predation than animals. Most of the processes involved in plant growth and development adapt to environmental conditions. This plasticity allows plants to alter their metabolism, growth and development to best suit their environment. Particularly important aspects of this adaptation, as far as plant tissue culture and regeneration are concerned, are the abilities to initiate cell division from almost any tissue of the plant and to regenerate lost organs or undergo different developmental pathways in response to particular stimuli. When plant cells and tissues are cultured *in vitro* they generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type. In this way, whole plants can be subsequently regenerated.

This regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant. This maintenance is called as totipotency and identifying the culture conditions and stimuli required to reveal this totipotency can be extremely difficult.

Culture media used for the *in vitro* cultivation of plant cells are composed of three basic components:

(1) Essential elements, or mineral ions, supplied as a complex mixture of salts;

(2) An organic supplement supplying vitamins and/or amino acids; and

(3) A source of fixed carbon; usually supplied as the sugar sucrose.

For practical purposes, the essential elements are further divided into the following categories:

(1) Macroelements (or macronutrients);

(2) Microelements (or micronutrients);

A culture media generally consist of macroelements, microelements, organic supplements, carbon source, gelling agents and growth regulators.

Macroelements as name implies are required in large amounts for plant growth and development. Nitrogen, phosphorus, potassium, magnesium, calcium and sulphur are usually regarded as macroelements. These elements usually comprise at least 0.1 % of the dry weight of plants Nitrogen is most commonly supplied as a mixture of nitrate ions (from the KNO_3) and ammonium ions (from the NH_4NO_3) (George, 1993). Phosphorus is usually supplied as the phosphate ion of ammonium, sodium or potassium salts. High concentrations of phosphate can lead to the precipitation of medium elements as insoluble phosphates (Franklin and Dixon, 1994).

Microelements are required in trace amounts for growth and development and have many diverse roles. Manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc usually comprise the microelements, although other elements such as nickel and aluminium are frequently found in some formulations. Iron is usually added as iron sulphate, although iron citrate can also be used (Gamborg and Philips, 1995).

Vitamins have a catalytic activity on enzyme reactions. The most important vitamins for plant tissue culture are thiamin (B_1), nicotinic acid (B_3), pyridoxine (B_6), and myoinositol. Also pantothenic acid (B_5), ascorbic acid (C), folic acid (M), α-tokopherol (E), retinol (A), riboflavin (B_2), and cholecalciferol (D_3) have some specific applications.

Amino acids are also commonly included in the organic supplement. The most frequently used is glycine, but in many cases its inclusion is not essential. Amino acids provide a source of reduced nitrogen and like ammonium ions; uptake causes acidification of the medium. Casein hydrolysate can be used as a relatively cheap source of a mix of amino acids.

As a carbon source sucrose is cheap, easily available, readily assimilated and relatively stable and is therefore the most commonly used carbon source. Other carbohydrates (such as glucose, maltose, galactose and sorbitol) can also be used, and in specialized circumstances may prove superior to sucrose.

Media for plant cell culture *in vitro* can be used in either liquid or 'solid' forms, depending on the type of culture being grown. For any culture types that require the plant cells or tissues to be grown on the surface of the medium, it must be solidified. Agar, produced from seaweed, is the most common type of gelling agent, and is ideal for routine applications. Also other gelling agents such as agarose, alginate, phytagel (Gelrite), slicagel, gelatin, and starch are being used in specific applications.

These components are the basic chemical necessities for plant cell culture media. However, other additions are made in order to manipulate the pattern of growth and development of the plant cell culture.

1.2.1. Plant Growth Regulators

There are 5 main classes of plant growth regulators used in plant cell and tissue culture;

(1) Auxins

(2) Cytokinins

(3) Gibberellins

(4) Abscisic acid

(5) Ethylene

Auxins

The term auxin is derived from the Greek word auxein, which means to grow. Compounds are generally considered auxins if they can be characterized by their ability to induce cell elongation in stems and otherwise resemble indoleacetic acid (the first auxin isolated) in physiological activity (Arteca and Wickremesinhe 1996; Mauseth, 1991). Auxins promote both cell division and cell growth. The most important naturally occurring auxin is IAA (indole-3-acetic acid), but its use in plant cell culture media is limited because it is unstable to both heat and light. It is more common to use stable chemical analogues of IAA as a source of auxin in plant cell culture media. 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used auxin and is extremely effective in most circumstances. Auxins are listed in Table 1.3.

Table 1.3. Most commonly used auxins (abbreviations and their chemical name) in tissue culture.

Abbreviation/name	Chemical name
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
Dicamba	2-methoxy-3,6-dichlorobenzoic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MCPA	2-methyl-4-chlorophenoxyacetic acid
NAA	1-naphthylacetic acid
NOA	2-naphthyloxyacetic acid
Picloram	4-amino-2,5,6-trichloropicolinic acid

Cytokinins

Cytokinins are compounds with a structure resembling adenine, which promote cell division and have other similar functions to kinetin. Kinetin was the first cytokinin discovered and so named because of the compounds ability to promote cytokinesis. The most common form of naturally occurring cytokinin in plants today is called zeatin, which was isolated from corn (*Zea mays*). Cytokinins are listed in Table 1.4.

Table 1.4. Most commonly used cytokinins (abbreviations and their chemical name).

Abbreviation/name	Chemical name
BAP	6-benzylaminopurine
2iP (IPA)	[N6-(2-isopentyl)adenine]
Kinetin	6-furfurylaminopurine
Thidiazuron	1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea
Zeatin	4-hydroxy-3-methyl-trans-2-butenylaminopurine

Giberellins

There are numerous, naturally occurring, structurally related compounds termed gibberellic acids. They are involved in regulating cell elongation, and are agronomically important in determining plant height and fruit-set. Only a few of the gibberellins are used in plant tissue culture media, GA_3 being the most common.

Abscisic Acid

Abscisic acid (ABA) inhibits cell division. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis.

Ethylene

Ethylene is a naturally occurring, gaseous plant growth regulator most commonly associated with controlling fruit ripening. Ethylene is a particular problem in tissue culture, because if it builds up sufficiently by explants can easily inhibit the growth and development of culture.

1.2.2. Somatic Embryogenesis

Somatic embryogenesis is a developmental pathway in which embryos have been induced to form from a somatic cell or group of somatic cells. Somatic embryogenesis can occur from cells of the explant tissue without an intervening callus phase (direct somatic embryogenesis). However generally somatic embryos are developed from a proliferated callus (indirect somatic embryogenesis) (Ritchie and Hodges, 1993).

Since the first observation of somatic embryo formation in carrot (*Daucus carota*) cell suspension (Steward *et al.,* 1958) the potential of plants for somatic embryogenesis is showed in wide range of plant species. New species and modified methods are continuously reported so somatic embryogenesis can probably achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed.

Somatic embryos are used for studying regulation of embryo development, but also as a powerful tool for large-scale vegetative propagation. Somatic embryogenesis is a multi-step regeneration process starting with formation of proembryogenic masses, followed by somatic embryo formation, maturation, desiccation, and plant regeneration.

Plant regeneration via somatic embryogenesis includes five steps;

1. Initiation of embryogenic cultures by culturing the primary explant on the medium supplemented with plant growth regulators, mainly auxins (usually 2,4-D) but often also with cytokinins.
2. Proliferation of embryogenic cultures on solidified medium or in liquid medium supplemented with plant growth regulators.
3. Prematuration of somatic embryos in medium lacking plant growth regulators; this inhibits proliferation and stimulates somatic embryo formation and early development.
4. Maturation of somatic embryos by culturing on medium supplemented with ABA and/or reduced osmotic potential.
5. Development of plants on medium lacking plant growth regulators.

Somatic embryogenesis potentially offers a promising system for plant regeneration because of the high proliferation capacity and the probable single cell origin, which may avoid the risk of chimeric plants.

1.2.3. Organogenesis

Organogenesis is a developmental pathway in which shoots or roots have been induced to differentiate from a cell or group of cells. *In vitro* plant regeneration by organogenesis usually involves induction and development of a shoot from the explant tissue followed by transfer to a different medium to induce root formation. If shoots and roots are directly induced and developed from an explant without undergoing a callus this is termed as *direct organogenesis*. If organ development is occurring after an initial phase of callus development this is called as *indirect organogenesis*. Indirect organogenesis is more advantegeous system than direct organogenesis for transformation studies because selection of transformed cells can easily be achieved by indirect organogenesis. Since the plantlet will be formed by transformed cells, chimerism will not be a problem.

With a good selection procedure and indirect organogenesis a one can obtain non-chimeric plants even if transformation efficiency is low.

Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium. In particular, it is the auxin to cytokinin ratio of the medium that determines which developmental pathway the regenerating tissue will take. It is usual to induce shoot formation by increasing the cytokinin to auxin ratio of the culture medium. These shoots can then be rooted.

1.3. Gene Transfer Techniques for Plants

The refinement in plant regeneration from cultured cells, efficient vector constructs and availability of defined selectable marker genes and various methods of transformation have resulted in the production of transgenic plants in more than 100 species (Wimmer, 2003).From the large number of strategies that have been developed, only a few have been used successfully with many plant species (Lindsey, 1992).

1.3.1. Agrobacterium Mediated Gene Transfer

Members of the genus *Agrobacterium* are ubiquitous components of the soil microflora, the vast majority of which are saprophytic, surviving primarily on decaying organic matter. However, several species of *Agrobacteria* cause neoplastic diseases in plants, including *Agrobacterium rhizogenes* (hairy root disease), *Agrobacterium rubi* (cane gall disease), *Agrobacterium tumefaciens* (crown gall disease) and *Agrobacterium vitis* (crown gall of grape). Crown gall and hairy root have been described as a form of ‘genetic colonization’ in which the transfer and expression of a suite of Agrobacterium genes in a plant cell causes uncontrolled cell proliferation and the synthesis of nutritive compounds that can be metabolized specifically by the infecting bacteria. Thus, infection effectively creates a new niche specifically suited to Agrobacterium survival.

As part of this sophisticated parasitism, *Agrobacterium* transfers a discrete portion of its DNA (T-DNA) into the nuclear genome of the host plant. Most of the machinery necessary for this T-DNA transfer resides on a tumor-inducing (Ti) plasmid. This Ti plasmid includes the T-DNA itself, delimited by 25 bp imperfect repeats [known as the right and left borders (RB and LB, respectively)] that define the boundaries of the T-DNA and ~35 virulence (*vir*) genes, clustered together into a *vir* region. The combined action of the *vir* genes achieves the delivery of the T-DNA to the nucleus of the host plant cell (Zupan *et al.*, 2000; Sheng and Citovsky, 1996). The T-DNA contains the genes for inducing tumor formation and opine biosynthesis, and these genes, even though they are bacterial in origin, have evolved to function only in plant cells.

Agrobacterium-mediated transformation systems take advantage of natural plant transformation mechanism. Removal of all the genes within the T-DNA does not impede the ability of *Agrobacterium* to transfer this DNA but does prevent the formation of tumors. T_i plasmids and their host *Agrobacterium* strains that are no longer oncogenic are termed 'disarmed'. There are two key advances that have made *Agrobacterium* transformation the method of choice. These are the development of binary T_i vectors and of a range of disarmed *Agrobacterium* strains (Hellens and Mullineaux, 2000).

It is accepted that *Agrobacterium* mediated transfer requires the activation of two *Agrobacterium* gene families: *chv* and *vir* genes. The *chv* (chromosomal virulence) genes are involved in the recognition and immobilization of the bacteria on the epidermal plant cell surface (Douglas *et al.,* 1982). The *vir* genes are located on an extra-chromosomal DNA replicon, the Tumor-inducing plasmid (T_i). The induction of *vir* genes leads to the transfer of a part of the T_i plasmid: the transferred-DNA (T-DNA). Comprehensive reviews on the DNA transfer process from the bacteria to the plant cells are available (Zupan *et al.*, 2000; Weising and Kahl, 1996).

Phenolic compounds specifically induce the vir genes. First, intermediates of lignin synthesis or phenolic precursors, such as acetosyringone (AS) and hydroxy-AS, are chemo-attractants at very low concentrations, but become *vir* inducers at high concentrations. A variety of other phenols have been described and their *vir* gene induction abilities investigated. Furthermore, other families of phenolic compound such as; hydroxycinnamides are known to act as *vir* gene inducers. (Sangwan *et al.*, 2002) Furthermore, opines and flavonoid compounds may be involved in *vir* gene induction (Zerback *et al.,* 1989)

The method of *Agrobacterium* mediated transformation of intact cells or tissues is developed by using excised tissue of *Nicotinia* and *Petunia* species (Horsch *et al.*, 1985). Studies with these species established rapid and reproducible procedures, which is further extended on to other species. *Agrobacterium* mediated transformation is found to be not suitable for transformation of graminaceous monocots. This is apparently due to lack of evocation of a wound response in these species (Anderson *et al.,* 2002).

1.3.2. Microprojectile Bombardment Technique

Microprojectile particle bombardment into plant cells was first described by Sanford *et al.* in 1986. Tungsten microprojectiles of approximately 4 μm in size were accelerated into onion epidermal cells and directly visualized using an inverted microscope. Transient gene expression in onion and later in maize (Klein *et al.*, 1988) proved in concept that tungsten microparticles could carry functional DNA into intact plant cells. The β-glucuronidase gene (GUS, Jefferson *et al.*, 1987) or an anthocyanin gene from maize (Ludwig *et al.*, 1990) has furthered the development of improved procedures with biolistic technology because transformed cells can be directly visualized.

Microprojectile bombardment mediated transformation is one of the most promising gene transfer techniques even for those plants, which have proved recalcitrant to genetic transformation by any other procedure. This technique has resulted in the production of transgenic plants of several species, particularly monocotyledons, including orchids (Yang *et al*., 1999; Knapp *et al*., 2000), banana (Becker *et al*., 2000) and cereals such as barley (Weir *et al*., 1998), maize (Rudraswamy and Reichert, 1998) and wheat (Rasco-Gaunt *et al.,* 1999), with the introduction of herbicide tolerance into orchids (Knapp *et al*., 2000), wheat (Zhang *et al*., 2000) and sugar cane (Falco *et al*., 2000).

In this technique, DNA coated microcarriers (usually gold or tungsten particles) are accelerated to a high velocity by a various designs of a particle gun apparatus. Due to acceleration, the microcarriers cross the cell wall and plasma membrane barrier, deliver the foreign DNA inside the cell, and transformants are then regenerated under selection. The transgenic nature of the plants is confirmed by assays of transgenic expression, molecular analysis, and inheritance of the introduced gene in subsequent generations.

1.3.3. Other Techniques

There are also other methods to transform plants such as; microinjection, macroinjection, pollen tube pathway, sonication, electroporation, and dry embryo incubation in DNA solution but they are currently not used frequently.

Microinjection

Isolated zygotic proembryos of soybean (*Glycine soya* L.), cotton (Gossypium hirsudum), sunflower (*Helianthus annus* L.), and *Arabidopsis thaliana* were assumed as competent and multiple microinjections are carried out with marker genes then plants are analyzed for putative primary transgenic

chimeras and sexual offspring (Potrykus, 1990). This method is more suitable for stable transformants than transient expression experiments.

Pollen Tube Pathway

In this method foreign DNA transferred into recently pollinated florets. Pollen tube pathway transformation is first reported by Duan and Chen (1985) in rice. They transferred total DNA from a rice variety with purple coloration to florets of a common variety, by cutting the stigma off the recipient floret and applying a drop of DNA solution to the cut end of the style. Method is repeated by Luo and Wu (1998) using neomycin-phosphotransferase and proved by molecular data.

Electroporation

Electroporation is one of the direct gene transfer methods, which is used extensively for transferring cells of various organisms including bacteria and mammalian cells. Electric impulses cause to open transient pores in the plasma membrane of organisms. DNA moves into target cells through these transient pores. Field strength (voltage) and pulse duration are two main variables affecting the permeabilization of the plasma membrane.

Mainly cells are chosen as a target for introducing foreign genes rather than organized tissues for electroporation mediated gene transfer. A procedure was reported by Dekeyser *et al.* (1990) to electroporate DNA into intact leaf tissue of rice.

1.4. Integration of Plant Tissue Culture Protocols to Transformation Protocols

Various methods of plant regeneration are available to the plant biotechnologist. Some plant species may be amenable to regeneration by a variety of methods, but some may only be regenerated by one method. Also various methods can be used to transform plants. Not all plant tissue is suited to every plant transformation method, and not all plant species can be regenerated by every method. There is therefore a need to find both a suitable plant tissue culture/regeneration regime and a compatible plant transformation methodology.

1.5. Tissue Culture Studies in Lentil

Tissue culture and regeneration studies on lentil are very restricted and there is a limited report on lentil regeneration when compared to other species. First report about lentil tissue culture is regeneration from cultured shoot tips (Bajaj, 1979). This study is followed by culturing portions of shoot meristems and epicotyls on a medium containing kinetin and giberellic acid to induce the formation of callus tissue which is then regenerated shoots and rooted in a mist chamber to yield whole, fertile plants (Williams and McHughen, 1986). In this study calli from cotyledons was also tested, but it was reported that cotyledon calli did not regenerate under the conditions tested.

Although somatic embryogenesis has been demonstrated in numerous species (Williams and McHughen, 1986), plant regeneration of lentil through somatic embryogenesis has been only reported by Saxena, *et al.*, 1987 and no progress has been made to date towards developing an *in vitro* regeneration system based on somatic embryogenesis for lentil.

Polanco *et al.* (1988) reported the influence of some growth regulators and explant type on callus and shoot formation. In this study three different cultivars were used and shoot-tip, first node and first pair of leaves were utilized as explant source. It was reported that, 2,4-D induced callus formation in all explants, but no organ regeneration obtained from this calli. Multiple shoot formation was obtained from explants supplemented with BA and NAA. Root formation was achieved only in media with NAA or IAA.

Singh and Raghuvanshi in 1989 reported a method for fertile plant regeneration from callus obtained from nodal segment and shoot tip explants. Callus, obtained on MS basal medium containing kinetin and 2,4-D was induced to regenerate shoot buds on media containing kinetin. Developed shoots were transferred to MS basal media for plantlet formation and then transferring them to soil produced normal fertile plants.

First protoplast study on lentil has carried out by Rozwadowsky *et al*. in 1990. They have isolated the protoplasts from epicotyl tissue but could not success to obtain calli.

Malik and Saxena (1992) investigated the effects of thidiazuron (TDZ), kinetin, and zeatin riboside for rooting of lentil. They have found that TDZ is the best shooting plant growth regulator in lentil. In this study they have problems in rooting if the shooting duration is long. They were successful in regeneration 40-50% of plantlets into mature plants.

Warkentin and McHugen (1993) used cotyledonary node as an explant and showed that shoots readily regenerate from lentil cotyledonary node explants *in vitro* on a medium containing BA. Also they have 50 % success in rooting of these shoots in hormone free medium.

Ahmad *et al*. (1997) investigated an *in vitro* clonal propagation protocol for lentil nodal segments. The aim was to regenerate shoots *in vitro* from nodal segments without a callogenic phase to minimize somaclonal variation via callogenesis. They succeeded in the clonal propagation by including giberellic acid in combination with BA in MS medium lacking sucrose and obtained rooted plants on NAA containing media.

Polanco and Ruiz (1997) reported the inhibitory effect of BA on rooting. They have used different concentrations and durations of BA in shooting medium and then transplanted plantlets to rooting medium to investigate the inhibitory effect of BA on rooting.

Also in a study, Polanco and Ruiz (2001) described an efficient and simple method for plant regeneration from immature lentil seeds. In this study, culture media included different concentrations of BA, alone or in combination with other phytohormones. After 4 weeks in culture, multiple shoot regeneration was observed using media with BA. Regenerated shoots formed advantegeous roots 30 days after transferring them to a medium containing IAA and NAA.

Khawar and Özcan (2002) tested 21 different genotypes on MS medium containing 0.225 µM TDZ using cotyledonary petioles. They have achieved a 20.6 shoots per explant in Akm 362 genotype and micrografted the shoots on to a cultivar Kayı 91 successfully. Micrografted plantlets were acclimatized to ambient conditions and later established under greenhouse conditions.

In a recent research Fratini and Ruiz (2003) assessed the rooting response of lentil nodal segments in relation to explant polarity, hormone, salt and carbohydrate concentrations of the medium. They have achieved 95.35 % rooting and 2.4 shoots per explant from explants placed in an inverted orientation on MS medium salts with 3% sucrose supplemented with 5 µM IAA and 1 µM KIN.

1.6. Transformation Studies in Lentil

The most important plants in the world for survival of mankind are cereals. After cereals most commonly cultivated plants are legumes and it makes them important for mankind. Cereals like wheat, rice, barley, and corn are receiving much more attention than any legume if we consider transformation (Christou, 1993). The most important reason for this attention is difficulty in both tissue culture and transformation of legumes. Various methodologies that were 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 specifity of the methods (Atkins and Smith, 1997).

Transformation frequencies of legumes are generally low but in literature we see that most important legumes have a stable transformation system. Unfortunately there are few reports on lentil and present reports are generally using *Agrobacterium*-mediated transformation.

First report on transformation of lentil is tumour-inducing capability of four different strains of *Agrobacterium tumefaciens* (C58, Ach5, GV3111 and A281) (Warkentin and McHughen, 1991). Southern blot analysis of DNA from a tumour line indicated that a T-DNA fragment had been transferred into the lentil genome. Also the same group in their subsequent study showed the expression of GUS gene after inoculation with disarmed *Agrobacterium* strain GV2260::p35SGUSINT but no transgenic lentil plants were reported (Warkentin and McHughen, 1992).

Transient GUS and CAT activity was detected in lentil protoplasts following delivery of the genes via liposomes (Maccarrone *et al*., 1992).

Chowira *et al.* (1995; 1996) in their studies used pea, soybean, and cowpea beside lentil. The technique applied was electroporation-mediated transformation and they have used intact nodal meristems as an explant and GUS as a reporter gene. The shoots grew up from nodes was chimeric but they formed successfully transgenic seeds. This is the only report of transgenic lentil in the literature

Another *Agrobacterium* mediated transformation study is cocultivation of half-embryonic axis with *Agrobacterium* carrying a *gus* reporter gene (Lurquin *et al.,* 1998). The system was fast and easy to determine the responses of cultivars to bacterial strains and conditions of transformation.

Potential of lentil cotyledonary node explants for transformation by *Agrobacterium* was investigated by Warkentin and McHughen (1993). In the study Octopine-type strain GV2260::35SGUSINT was used and effect of wounding is also examined. In the study, no transgenic plants were recovered

In a study of Öktem *et al.* (1999) cotyledonary node explants were subjected to particle delivery via microprojectile bombardment. Circular pBSGUSINT plasmid was used for the transformation. 80% of the bombarded tissues were expressing GUS gene and 2% of the shoots emerged from cotyledonary nodes were found to be expressing patches of GUS staining. The frequency of stable integration was low but reproducible, so the method was appeared to be promising.

Also in literature we can find the effect of vacuum infiltration on transformation of cotyledonary node by *Agrobacterium* GV2260::pGUSINT (Mahmoudian, 2000). Results showed that infiltration increases the efficiency of transformation. But low numbers of regenerated shoots exhibited GUS expression.

Khawar and Özcan (2001) studied the *in vitro* induction of crown galls by an *Agrobacterium tumefaciens* super virulent strain A281 in 21 different genotypes of lentil and tumour induction was confirmed by histochemical GUS assay.

The most recent study on lentil transformation is the vacuum infiltration based *Agrobacterium* mediated gene transfer to cotyledonary nodes (Mahmoudian *et al.,* 2002). In this study cotyledonary node meristems rapidly produce transgenic shoots without an intermediate callus phase.

1.7. Aim of the Study

In this study we aimed:

I) Optimization of a reliable protocol for lentil regeneration via indirect organogenesis and somatic embryogenesis.

II) To analyze efficiency of a previously optimized *Agrobacterium tumefaciens* mediated transformation technique (Çelikkol, 2002) on different explants of lentil.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

A Turkish cultivar of lentil (*Lens culinaris* M. cv. Sultan-I) was used in this study. It is a summer sown, green cotyledon, and big seeded (75 g per 1000 seeds) lentil cultivar. The seeds were obtained from the Exporter Unions Seed and Research Company.

2.1.2. Plant Tissue Culture Media

MS (Murashige and Skoog 1962) basal and Modified B5 (Gamborg *et al.* 1968) medium supplemented with sucrose and agar was used in this study. 2,4-dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (BA), Kinetin, α-Naphthalene acetic acid (NAA), giberellic acid (GA), Thidiazuron (TDZ), and Zeatin Riboside (Zea) were used in different combinations and concentrations as a plant growth regulators. Cefotaxime and kanamycin were included in to the medium for selection of transformants and elimination of *Agrobacterium tumefaciens.* Ammonium nitrate, glutamine, and salicylic acid (SA) are also used in the study at somatic embryogenesis stage. Before usage the media were dissolved in distilled water. The pH is adjusted to 5.8 with NaOH, and HCl prior to autoclaving at 121°C for 20 minutes. Plant growth regulators, antibiotics and other

chemicals were filter-sterilized by using 0.2 μm pore sized filters and added to the cooled medium prior to dispersing. Composition of the MS basal and Modified B5 medium was given in Appendix A.

2.1.3. Bacterial Strains and Plasmids

The *Agrobacterium tumefaciens* strain KYRT1 (Torisky *et al*., 1997) was used throughout this study. As a binary plasmid vector pTJK136 (Kapila *et al*., 1997) was used. Vector pTJK136 is a derivative of vector pTHW136 (Vancanneyt *et al*., 1990). It carries a gene coding for streptomycin/spectinomycin adenyl transferase gene as bacterial selection marker and an intron containing GUS gene and *npt*-II gene as plant selection markers (Appendix D). Plasmid pTJK136 is provided by Prof. Dr. Van Montagu.

2.1.4. Bacterial Culture Media

Yeast extract broth (YEB) was used to grow *Agrobacterium* cultures, which were used in the plant transformation experiments. Depending on the purpose, it was supplemented with necessary antibiotics (according to the bacterial strain and bacterial selection marker on binary vector), MES (2-[N-Morpholino] ethanesulfonic acid) and acetosyringone (3',5'- Dimethoxy-4-Hydroxyacetophenone). Compositions of YEB, MMA and other supplementary media were given in Appendix B, and the antibiotic requirements for strain and binary plasmid were given in Appendix D.

2.1.5. Other Materials

Antibiotics (rifampicin, carbenicilin, gentamycin, ampicilin, streptomycin, cefotaxim, kanamycin), GUS histochemical substrate which is abbreviated as X-Gluc (5-bromo-4-chloro-3-indolyl glucoronide), 2-[N-Morpholino] (MES), acetocyringone and all other chemicals used in solutions were supplied from

Merck, Sigma, Aldrich, Difco and Applichem chemical companies. All chemicals and enzymes used in molecular analysis were from MBI Fermentas and primers were prepared by Iontech Company (İstanbul, Turkey).

2.2 Methods

2.2.1 Tissue Culture Studies

2.2.1.1. Surface sterilization and germination of seeds

The seeds were surface sterilized by immersion in 70% ethanol for 2 seconds instantaneously and then in 20% sodium hypochlorite for 20 minutes. After three rises in sterile distilled water, the seeds that were swollen, decolorized and have abnormal colour were discarded. The seeds remaining were kept in sterile distilled water overnight in dark at 23°C for imbibition. Imbibed seeds were inoculated onto MS basal medium supplemented with sucrose and agar (Appendix A). They were germinated at 23°C in dark for 3 days for cotyledonary petiole isolation and 5 days for hypocotyl isolation.

2.2.1.2. Isolation of cotyledonary petiole and epicotyl

From 3 days old etiolated lentil seedlings, cotyledons were removed at a single cut, than seed coat was removed and roots and shoots excised from a distance 1-2 mm to the node. Remaining intact two cotyledonary petiole is divided and each cotyledonary petiole is obtained. Figure 2.1 shows the preparation of cotyledonary petioles.

For hypocotyl explants from 5 days old etiolated seedlings, hypocotyl part is directly excised and chopped into two parts, and parts containing nodes were removed. Figure 2.2 shows the preparation of hypocotyls.

Figure 2.1. Isolation of cotyledonary petiole

Figure 2.2. Isolation of epicotyl

2.2.1.3. Induction and maintenance of callus cultures

For induction of callus cultures, isolated explants were placed on a basal medium composed of MS salts, 3% sucrose, and 0.8% agar supplemented with growth regulators as described in Table 2.1. The pH was adjusted to 5.6 with NaOH and HCl prior to autoclaving at 121°C for 20 minutes. Plant growth regulators were filter-sterilized by using 0.2 µm pore sized filters and added to the cooled medium prior to dispensing.

For induction of callus from cotyledonary petiole 2 auxin (2,4-D, NAA), 3 cytokinines (BA, Kinetin, Zea), and a GA_3 were used in different combinations

and concentrations, which was given in Table 2.1. For induction of callus from hypocotyl an auxin (NAA), 3 cytokinins (BA, Kinetin, Zeatin), and GA_3 were used in different combinations and concentrations, which was also given in Table 2.1. Explants are kept in these medium at 28°C in the dark for 4 weeks. At the end of fourth week each callus developed was weighted individually under aseptic conditions and recorded. Each growth regulator concentration was tested in 2 sets of 4 plates each containing 12 explants.

Table2.1. Hormone combinations and concentrations used in the induction of callus

Tissue	Cotyledonary Petiole	Epicotyl
Plant Growth Regulator Concentrations Used	1 mg/L 2,4-D	2 mg/L BA
	5 mg/L 2,4-D	2 mg/L NAA + 0.4 mg/L Kinetin
	2 mg/L BA	2 mg/L BA + 0.2 mg/ L NAA
	1 mg/L 2,4-D + 0.1 mg/L kinetin	10 mg/L Kinetin + 1 mg/L GA_3
	1 mg/L Zeatin riboside + 1 mg/L NAA	1 mg/L Zeatin Riboside + 1 mg/L NAA
	2 mg/L NAA + 0.4 mg/L Kinetin	
	10 mg/L Kinetin + 1 mg/L GA_3	
	2 mg/L BA + 0.2 mg/L NAA	
	MS Basal	

Table 2.2. Codes given to the medium types

Plant Growth Regulator Combination Used in the Media	Code
2 mg/L NAA + 0.4 mg/L Kinetin	A
2 mg/L BA + 0.2 mg/L NAA	B
2 mg/L BA	C
10 mg/L Kinetin + 1 mg/L GA_3	D
1 mg/L 2,4-D + 0.1 mg/L kinetin	E
1 mg/L 2,4-D	F
5 mg/L 2,4-D	G
1 mg/L Zeatin riboside + 1 mg/L NAA	H
MS Basal	I
1 mg/L BA + 0.1 mg/L NAA	K
1 mg/L TDZ	L
0.1 mg/L TDZ	M
1 mg/l Zeatin riboside	N
0.25 mg/L Zeatin riboside	O
10 mg/L Kin	P
1 mg/L GA_3 + 0.25 mg/L BA + 0.1 mg/L NAA	R

2.2.1.4. Determination of callus growth curve

The cotyledonary petioles isolated were placed on to the best responding medium. Each plate was consisted of 10 cotyledonary petioles and each explant was labelled. Each explant was weighted aseptically after 4, 8, 12, 16, 20, 24, and 28 days and growth curve obtained from 3 independent sets of experiments.

2.2.1.5. Regeneration of lentil via indirect organogenesis

For regeneration, formed calli via indirect organogenesis from the explants were placed on a basal medium composed of MS salts, 3% sucrose, and 0.8% agar supplemented with growth regulators as described in Table 2.3.

For regeneration via indirect organogenesis an auxin (NAA), 4 cytokinines (BA, Kinetin, Zeatin, TDZ), and a GA_3 were used in different combinations and concentrations, which was given in Table 2.3. Explants are kept at these mediums at 28°C in the 16-hour photo period (approximately 30µmol/m^2/s) for 4 weeks. Each growth regulator concentration is tested in 2 sets of 24 explants. After 4 weeks shoots formed were recorded.

Table2.3. Hormone combinations and concentrations used in shoot regeneration

Hormone Concentrations Used
10 mg/L Kinetin
1 mg/L GA_3 + 0.2 mg/L BA + 0.1 mg/L NAA
1 mg/L BA + 0.1 mg/L NAA
1 mg/L Zeatin riboside + 1 mg/L NAA
1 mg/L Zeatin riboside
0.25 mg/L Zeatin riboside
1 mg/L Thidiazuran
0.1 mg/L Thidiazuran
MS Basal

2.2.1.6. Rooting of lentil plantlets and growth of plantlets to maturity

After formation of lentil plantlets for root initiation they were placed into jars containing MS basal medium with 2 mg/L NAA and 0.4 mg/L Kinetin. After formation of first roots in a week they were taken in to a hormone free basal medium without hormone. In 2 weeks plantlets matured and became ready to be taken in to the soil.

2.2.1.7. Acclimatization of plants

For acclimatization of plants mature plants are taken in to the soil pots, which were placed in to the boxes containing water, and covered with the transparent plastic bags (punctured to enable aeration) to avoid desiccation of the plantlets. Plantlets continued their development by further growth and elongation of the shoots and formation of lateral shoots.

2.2.1.8. Preliminary studies for somatic embryogenesis in lentil

For induction of somatic embryogenesis isolated cotyledonary petioles were placed on to the Modified B5 medium containing 500 mg/L ammonium nitrate. One set is supplied with 1,5 mg/L 2,4-D and other with 2,0 mg/L 2,4-D. Each set is composed of 20 petri plates each containing 10 explants. Explants were kept in these medium at 28°C in the dark for 4 weeks. At the end of fourth week each explant was weighted individually under sterile conditions.

For proliferation of somatic embryos, callus were transferred into the Modified B5 medium containing 70 mg/L glutamine. One set was supplied with 200 μM salicylic acid (SA) and other without salicylic acid. Each set is composed of 10 petri plates each containing 10 explants. Explants were kept at 28°C under 16 hour photoperiod for 2 weeks. At the end of second week proliferated callus were recorded and each explant was weighted individually under sterile conditions.

Table 2.4. Codes given to the medium types in somatic embryogenesis.

Hormone Combination Used in the Media	Code
Modified B5 medium + 500 mg/L ammonium nitrate + 1.5 mg/L 2,4-D	S
Modified B5 medium + 500 mg/L ammonium nitrate + 2.0 mg/L 2,4-D	T
Modified B5 medium + 70 mg/L glutamine + 200 μM SA	U
Modified B5 medium + 70 mg/L glutamine	V

For histodifferentiation; proliferated callus were transferred on to the MS basal medium containing 70 mg/L glutamine and 0,25mg/L BA. Differentiated callus were recorded as heart or torpedo shaped at the end of third week of incubation at 28°C under 16 h photoperiod.

2.2.2. Transformation Studies

In transformation studies *Agrobacterium* mediated transformation was used. Four kinds of tissues (peeled cotyledonary node, cotyledonary petiole, shoot tip, and root) were used in transformation.

2.2.2.1. Isolation, peeling and wounding of cotyledonary nodes

For isolation of cotyledonary nodes from 3 days old etiolated lentil seedlings, roots and shoots were excised at a distance of 3-4 mm to the node. Then the cotyledons were removed at single cut. For peeling, firstly cotyledonary petioles were excised as near as possible to the node then remaining part was peeled (trimmed from its 4 sides by cutting from nearly 1 mm to surface) and lastly remaining parts of shoot and roots was excised. Each node was wounded by glass needles 5 times. Figure 2.3 shows the preparation of the explant. Peeled cotyledonary node explants were used for transformation experiments within 1-3 hours after preparation.

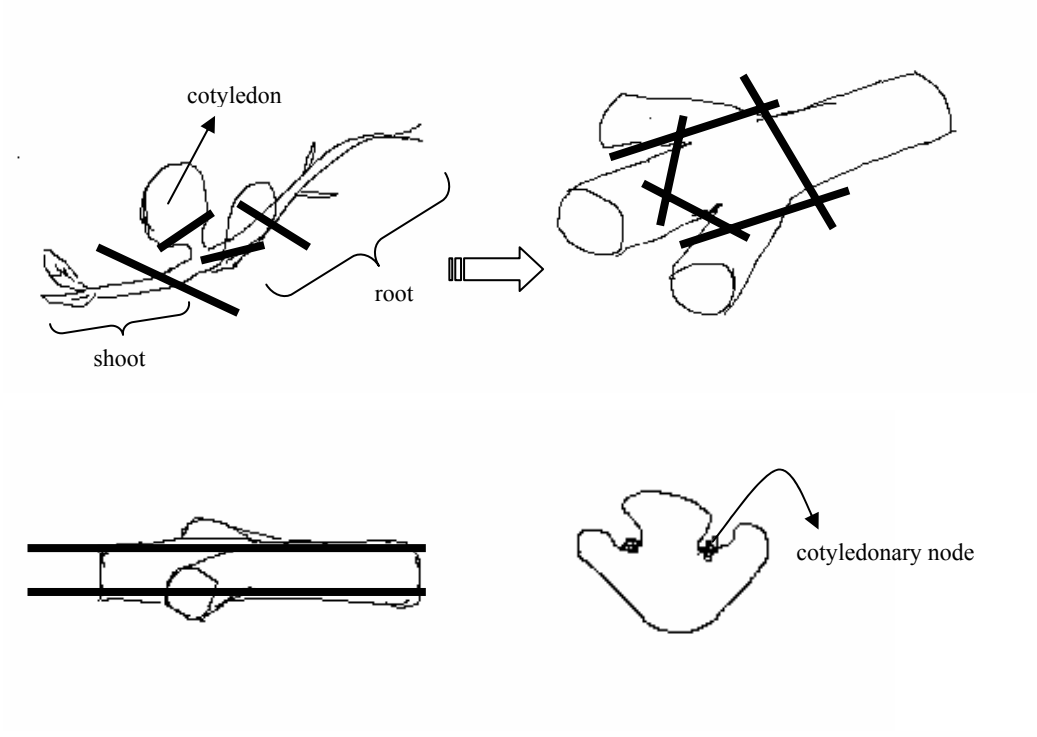

Figure 2.3. Isolation and peeling of cotyledonary nodes. Lines indicate the cuttings.

2.2.2.2. Wounding of cotyledonary petioles

Isolated cotyledonary petioles were wounded 5 times by glass needles. Cotyledonary petioles were used for transformation experiments within 1-3 hours after preparation.

2.2.2.3. Agrobacterium mediated transformation of cotyledonary petioles

To prepare *Agrobacterium* for plant transformation, first a single colony was grown overnight at 28°C in YEB medium supplemented with necessary antibiotics. Then 100 ml YEB medium containing 10 mM MES, 20 μM and necessary antibiotics was inoculated with 100 μl of this overnight grown culture. The culture was grown overnight to OD_{600} of 0.8 at 28°C, 200 rpm. Then the culture was centrifuged for 15 minutes at 3500 rpm, at 4°C. The pellet was resuspended with MMA medium containing 200 μM acetocyringone to a final OD_{600} of 2.4. Finally, the *Agrobacterium* suspension was kept at 22°C for 1 hour at light and then used for transformation of explants (Çelikkol, 2002).

Wounded explants were than taken in to the *Agrobacterium* suspension and cocultivated at 22°C for 20 minute. After this step explants were washed 3 times with sterile distilled water and transferred in to the cocultivation medium, which is composed of MS salts and 200 μM acetocyringone. Explants were hold in cocultivation medium for 3 days; at the end of 3 days they were washed in the liquid MS containing 500 mg/L cefotaxime for elimination of *Agrobacterium*. Each transformation set was consisting of 120 explants. Some of the explants are used for GUS histochemical assay and others are transferred in to the best responding medium (MS Basal consisting 1mg/L Zeatin riboside + 1 mg/L NAA) that was chosen in tissue culture part by addition of 250 mg/L cefotaxime.

2.2.2.4. Analysis of Transformants

GUS histochemical staining was used to analyse transformed bacterial cells and plant tissues.

2.2.2.4.1. GUS Histochemical Assay

GUS histochemical staining was performed according to the procedure of Jefferson (1987). GUS histochemical assay was performed for cotyledonary petiole explants after 3 days of cocultivation, callus formed after 4 week in callus induction medium, and shoots formed after 6 weeks. All explants and tissues were assayed by incubating inside GUS substrate solution for overnight at 37°C. Then the explants were transferred to fixative solution in which they can be preserved for several months. Shoot tissues were transferred to 50 % ethyl alcohol after 4-hour incubation, for decolourization. After 15 minutes in 50 % ethyl alcohol, shoots were transferred to 100 % ethyl alcohol for further decolourization overnight. Finally, GUS expressing regions on explants were examined and counted under microscope, and photographed. Formulation of GUS substrate solution and fixative solution were given in Appendix E.

2.2.3. Statistical Analysis

Least-significant difference test, which is one of the Post Hoc multiple comparisons of one-way ANOVA of Minitab was used to detect variances in means of GUS expression units on explants which were subjected to different experimental treatments. ANOVA tables were given in Appendix C.

CHAPTER III

RESULTS

In production of a transgenic plant, the establishment of a successful regeneration and a good selection system is a prerequisite task. Optimizing a suitable regeneration system that is appropriate for selection is first part of this study. For this purpose indirect organogenesis and somatic embryogenesis of cotyledonary petioles and epicotyls were studied initially in this study. Also a good selection system for the transformants was investigated and a number of parameters effective on increasing efficiency of transformation events were tested.

3.1. Regeneration Studies

Genotype, explant source and growth conditions are important factors in the *in vitro* regeneration of plants. In this study regeneration of a local genotype is studied with two different explants and various growth regulators.

3.1.1. Callus Induction Studies for Indirect Organogenesis

In this part of the study eight different medium differing in their growth regulator compositions, were employed to examine the callus induction potency of cotyledonary petiole. Five different medium were employed to examine callus induction potency of epicotyl.

Callus induction results after 4 weeks of incubation (Figure 3.1) showed that each growth regulator combination except medium I (MS Basal) gave high percentage of callus induction in cotyledonary petiole. The highest response is in the medium H and E with 100 % callus induction. Each combination of plant growth regulators showed different calli formations. Medium A and H first developed a callogenic form and than rhizogenesis begin with whitish and thick roots (Figure 3.15. a, b). Rhizogenesis is probably because of NAA that is present in high amounts in both media. Explants incubated on medium B and C go into a callogenesis and develop a hard, compact and smooth callus, which is probably because of presence of BA. Also other studies carried out showed that BA causes very hard and compact callus in dark incubation. Medium E, F, and G showed a watery and very soft friable calli development that is probably the first stages of embryogenesis. 2,4-D is the responsible hormone for embryogenesis induction in these callus. This response is afterwards used in the somatic embryo studies. Medium D showed the lowest percentage of callus induction. Necrosis percentage was very high in medium D; probably 10 mg/L Kinetin was toxic to cells. In medium I explants did not show any response and necrosis was observed in the second week of incubation.Results of average calli weight in cotyledonary petiole after fourth week are given in Figure 3.2. According to these results the best responding medium was medium H. Medium H showed the highest callus weight because of the rhizogenesis that begin in the second week of incubation. Similarly medium A has the second highest callus weight, again because of the rhizogenesis. Although it seems that medium E, F, and G have the biggest callus mass, their weights was not so high, which means that they have low cell density. Medium B is greater than C in callus weight, they have only difference of 0,2 mg/L NAA in medium B, so NAA even if in low amounts gives results to increase in callus weight. Also differences between each medium were analyzed statistically by One-way ANOVA test as a stack (Appendix A) and box plots (Figure 3.3; 3.4) were drawn accordingly. As seen from the graph and the ANOVA test, there is a significant difference between each medium considering both callus initiation percent and callus weight

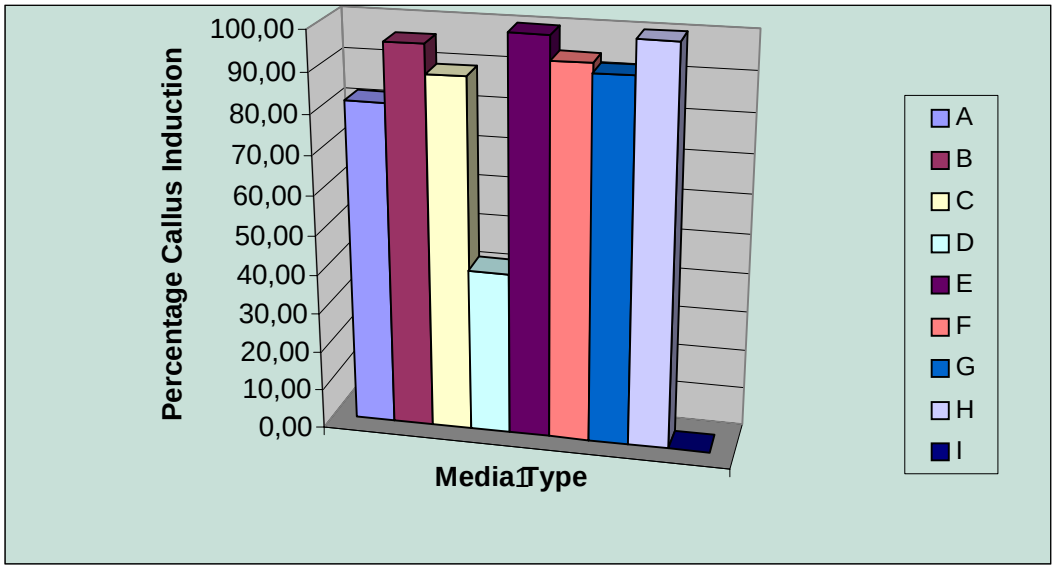

Figure 3.1. Percentage callus induction of cotyledonary petiole in different medium after 4 weeks of incubation.

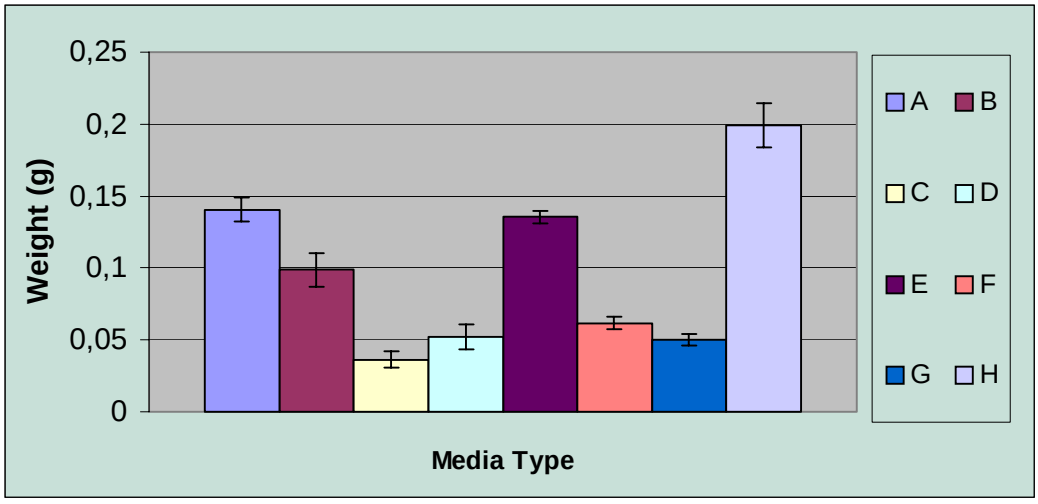

Figure 3.2. Weights of callus (in grams) of cotyledonary petiole in different media after 4 weeks of incubation. Bars indicate the mean weights ± S.E.M.

Table 3.1. One-way ANOVA test of callus weights with each other for cotyledonary petiole (Confidence intervals, 95%). In the table, P-values were given for each medium with different growth regulators combinations. Cells with star (*) indicates the P-values<0.05 meaning the significant difference and cells with two stars (**) where P<0.01, indicates the highly significant difference.

	A	B	C	D	E	F	G	H
A		0**	0**	0**	0,682	0**	0**	0**
B	0**		0**	0**	0**	0,003**	0**	0**
C	0**	0**		0,123	0**	0**	0,056	0**
D	0**	0**	0,123		0**	0,09	0,814	0**
E	0,682	0**	0**	0		0**	0**	0**
F	0**	0,003**	0**	0,09	0**		0,005**	0**
G	0**	0**	0,056*	0,814	0**	0,005**		0**
H	0**	0**	0**	0**	0**	0**	0**	

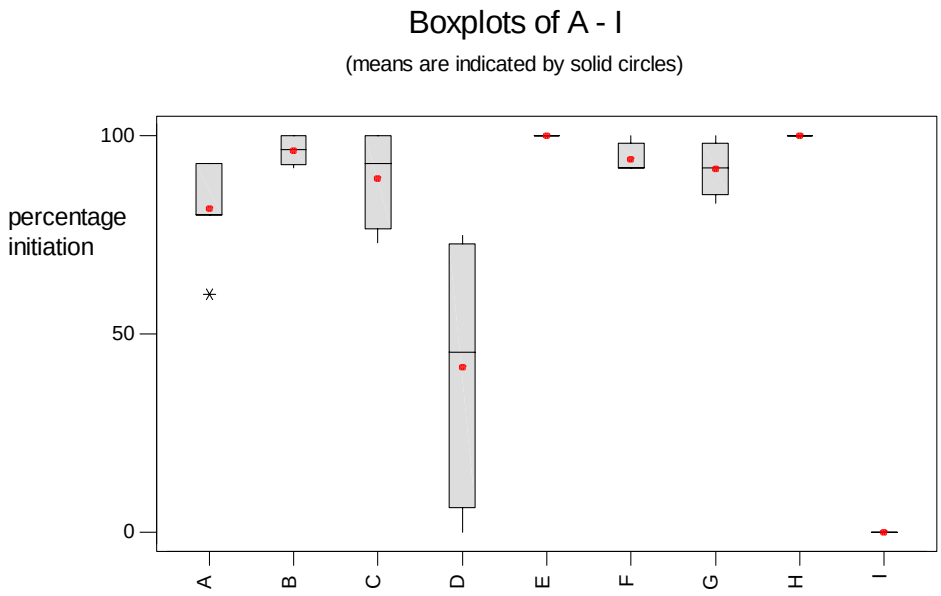

Figure 3.3. Boxplot representation of callus initiation percentages of cotyledonary petiole in different medium.

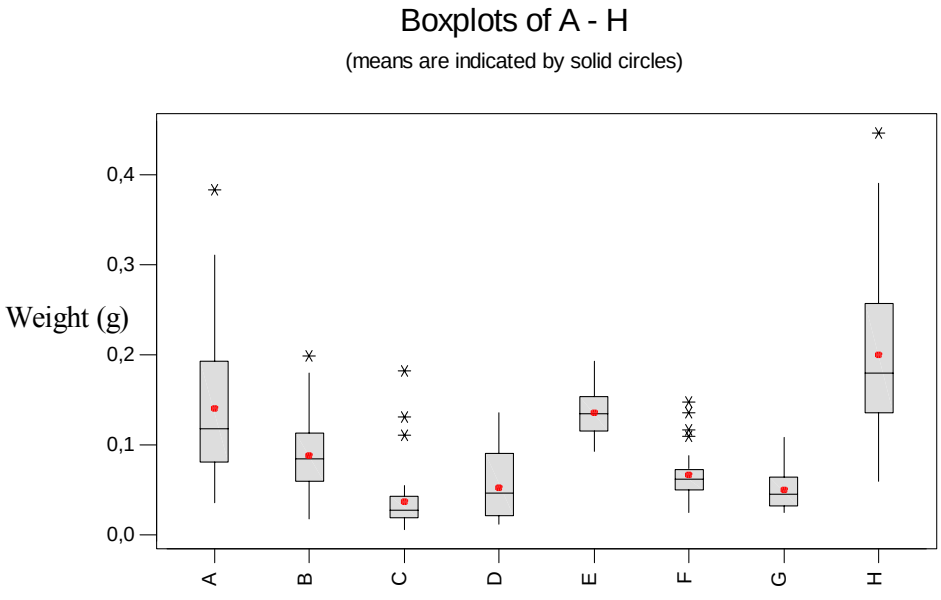

Figure 3.4. Boxplot representation of callus weights of cotyledonary petiole in different medium types.

In tissue culture studies of epicotyl results driven from cotyledonary petiole was used and some nonresponsive media is eliminated and only 3 media A, B, and H is used in callus formation. Each media gave 90% of callus initiation but weights of callus results were different from cotyledonary petiole results (Figure 3.5). The most responsive medium was A, than B, and lastly H. Differences between each medium were analyzed statistically by One-way ANOVA test as a stack (Appendix A) (Table 3.2). As seen from the graph and the ANOVA test, considering callus weight there is a significant difference between each medium except H and B.

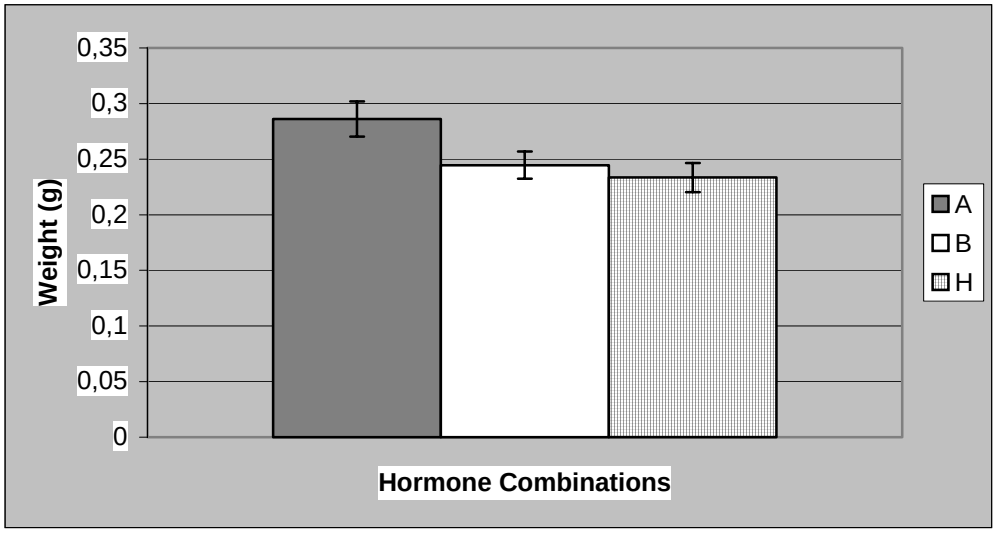

Figure 3.5. Weights of callus (in grams) of epicotyls in different media types. Bars indicate the mean weights ± S.E.M

Table 3.2. One-way ANOVA test of callus weights with each other for epicotyl (Confidence intervals, 95%). In the table, P-values were given for each medium with different growth regulators combinations. Cells with star indicates the P-values<0.05 meaning the significant difference.

	A	B	H
A		0,041*	0,012*
B	0,041*		0,537
H	0,012*	0,537	

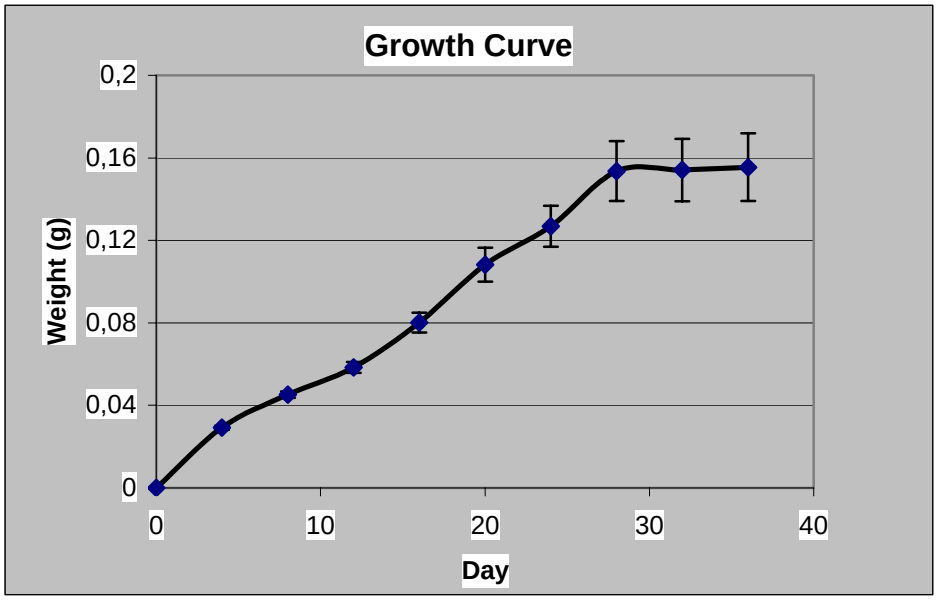

Figure 3.6. Weight of cotyledonary petiole explants in medium H.

3.1.2. Callus Growth Curve

Medium H was the most responsive medium in callus formation in cotyledonary petioles. Thus it was also the only shoot-giving medium afterwards. The callus growth curve of this medium was examined to find the optimum time to stay in callus initiation medium (Figure 3.6). It was seen that after 28 days callus growth reached to equilibrium. Accordingly it was decided that 28 days is an optimum incubation time to reach the maximum callus weight, for cotyledonary petiole explants.

3.1.3. Shooting Success

3.1.3.1. Cotyledonary Petiole

Nine media were evaluated in the part of callus initiation, and eight of these media gave positive response. In shooting part of the study formed callus were transferred into nine different media (namely; H, I, K, L, M, N, O, P, and R) to test their potencies on shoot induction. Results were given in Figure 3.7. It was interesting that callus initiation medium was more important than the shooting medium in shooting response. Only the explants that were in medium H (1mg/L ZEA + 1mg/L NAA) throughout callogenesis gave response in shooting medium (Figure 3.8). Callus induced on the other seven callus initiation media did not give any response when transferred to the shooting medium. Also results showed that most responsive shooting medium was medium I, which means that excluding plant growth regulators resulted in enhanced shooting.

Callus transferred from medium H to medium K, N, O, P, and R did not result in shooting response. Explants in medium K became dark green and after three weeks of incubation necrosis began and in medium N, O, P, and R necrosis directly began without greening of tissue. Any intervention (refreshing medium or chopping of necrotic parts) did not stop necrosis.

The most responsive shooting medium was medium I, without any growth regulator. 40.28 ± 2.78 percentages of shooting was seen in medium I. Shoots formed in the first week of incubation and developed healthy shoots. Also many roots initiated in the callogenesis begin to develop in to more natural form of root. After second week explants were taken from petri plates to jars. In fourth week most of the plantlets were ready to transfer to soil for acclimatization without any rooting procedure *in vitro*. The plantlets that did not develop root transferred into the rooting medium.

Also medium H gave shoots when used as a shooting medium (22.2 ± 1.39) but the percentage was lower than the medium I also some abnormalities was observed during shooting. Firstly roots formed became reddish in color and after second week of transfer the roots became fleshy callus. Since the callus initiation and shooting medium is same in this part, it can be easily derived that light is very important in shooting response. Explants that are further kept in dark did not develop any shoot at all (even etiolated or abnormal).

Medium L and M also exhibited a shooting response in low percentages (15.28 ± 1.39 and 8.33 ± 2.40 percent respectively). Regenerated shoots were pale green in color and not very healthy. Newly established shoots were multiplied and formed a massy structure but did not grow any futher and after a while they undergo necrosis. This shows that Medium L and M (1 mg/L TDZ and 0.1 mg/L TDZ) can be used for further studies to get multiple shoots but further optimization is needed in growth regulator concentrations and incubation period.

As a result in cotyledonary petiole explants we were able to achieve a healthy callogenesis and shooting (Figure 3.9). Also regenerated shoots were able to grow and give healthy roots (Figure 3.10.f) in shooting medium in glass jars, which than transferred to the soil followed by successful acclimatization (Figure 3.11).

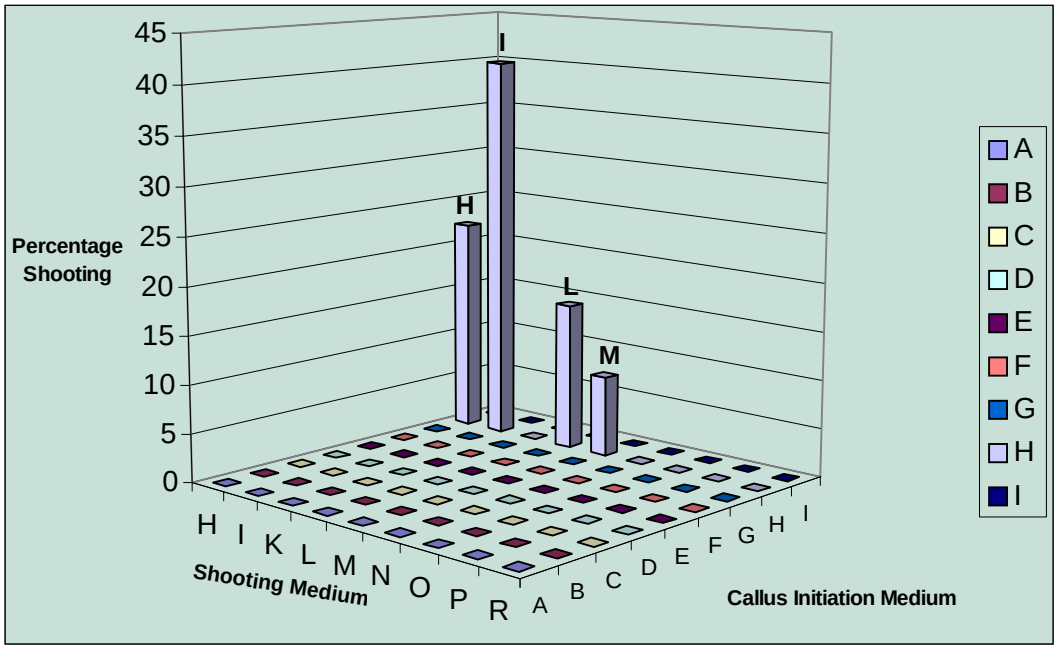

Figure 3.7. Percentage shooting response of cotyledonary petiole in different medium types that are transferred from different callus initiation medium.

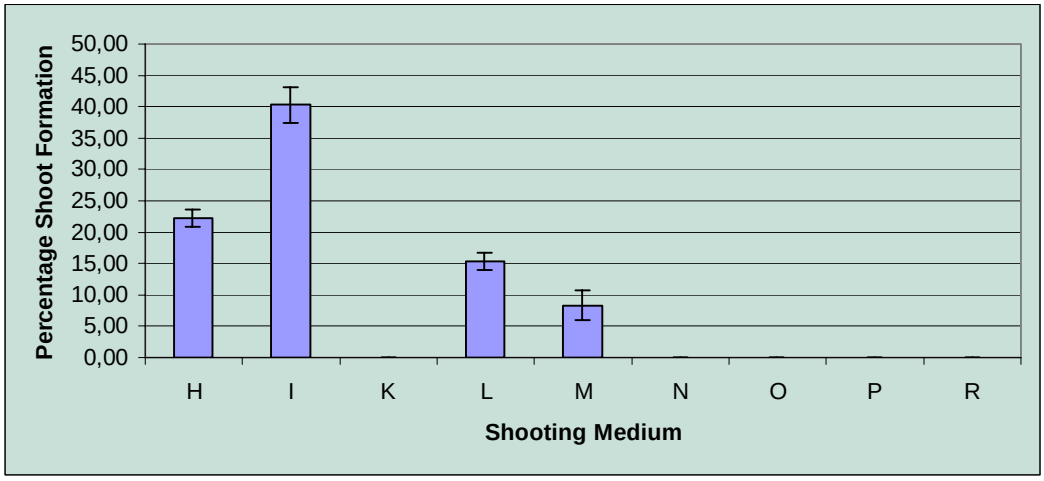

Figure 3.8. Percentages shooting of cotyledonary petiole in different medium types that are transferred from medium H. Bars indicate the mean percentage ± S.E.M.

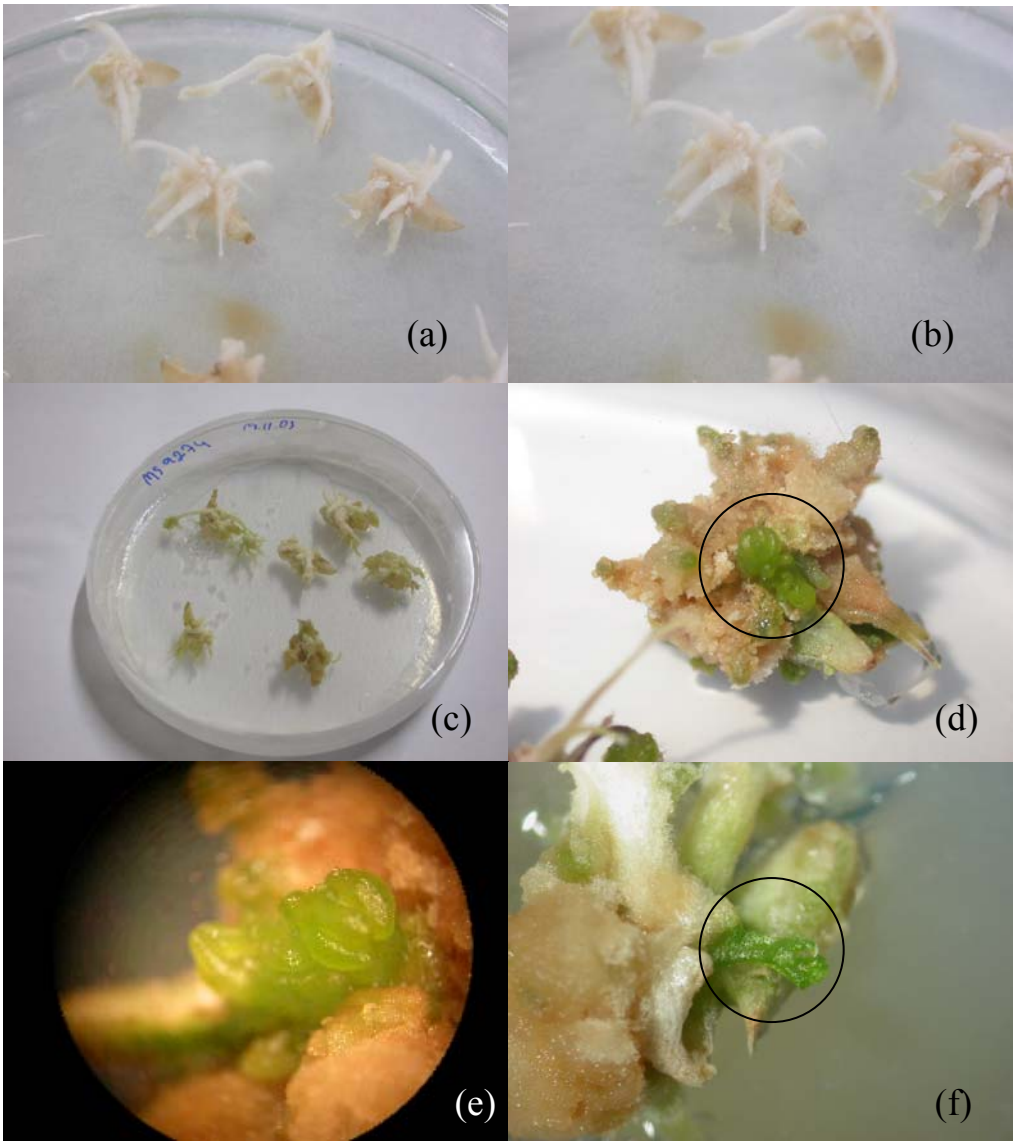

Figure 3.9. Different stages of cotyledonary petiole in medium H and I. Callus formed at the fourth week of incubation at dark (a, b), callus transferred to the medium I for shooting at the end of first week in shooting medium (c), newly emerged shoots in the second week of incubation in shooting medium (d, e, f).

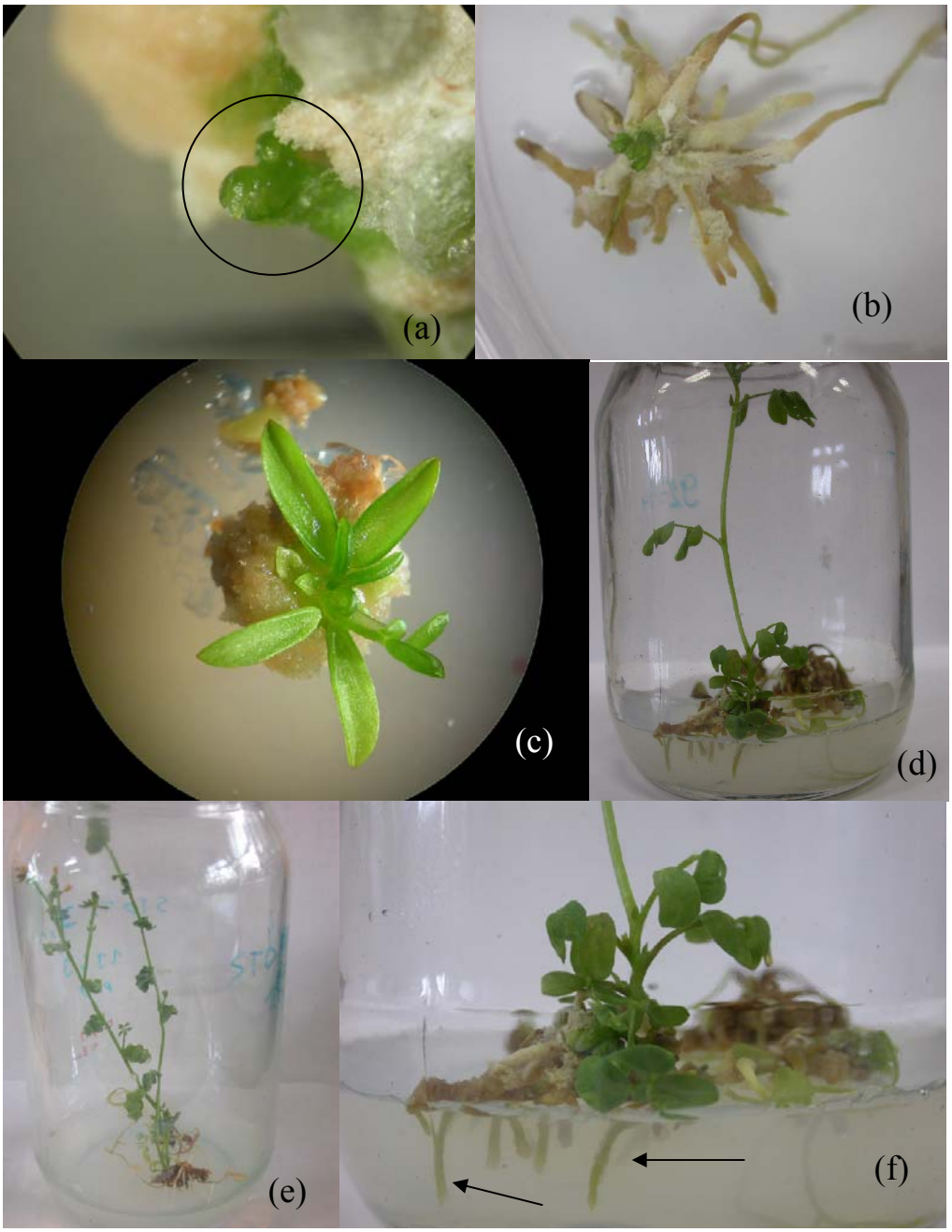

Figure 3.10. Shooting stages of cotyledonary petiole. Newly emerged shoots of cotyledonary petiole (a, b), growing shoot in shooting medium (c), plantlets transferred to the jars (d, e), roots newly formed can be easily seen (f).

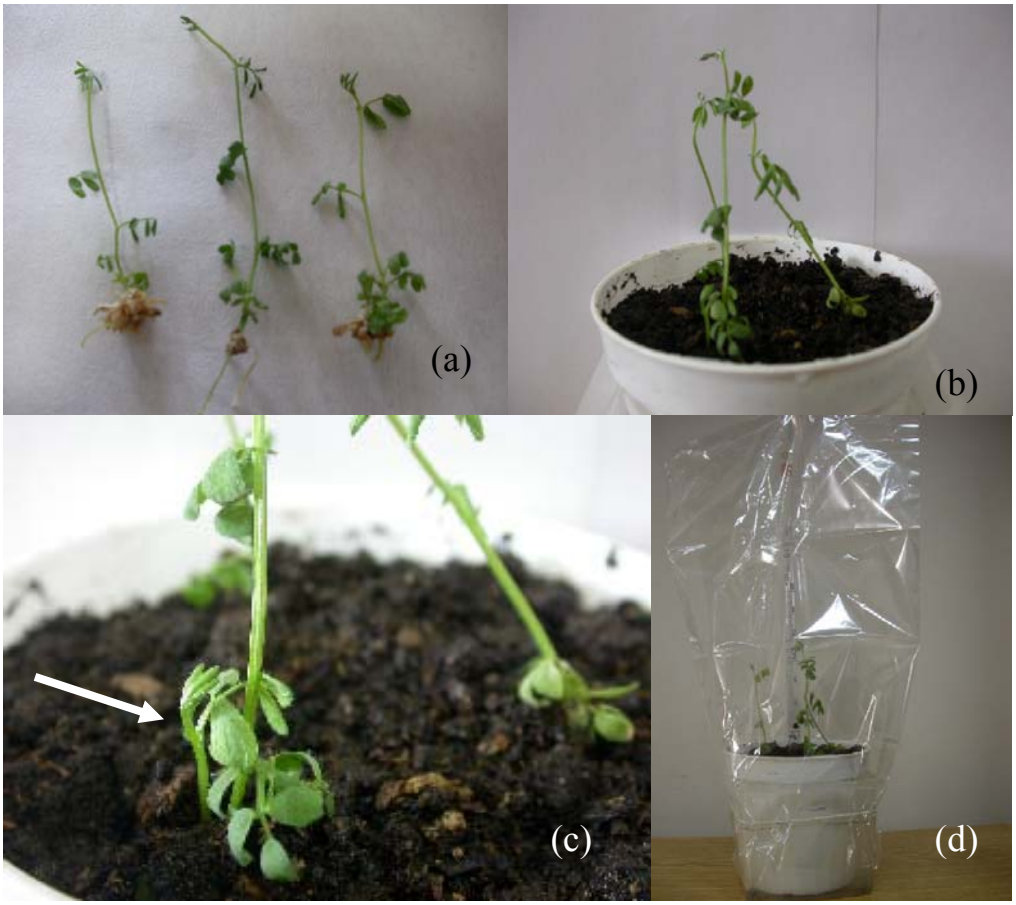

Figure 3.11. Acclimatization of plantlets that are driven from cotyledonary petioles. Plantlets taken from jars (a), plantlets transferred to the soil (b), newly emerging shoots in soil can be seen (c), plantlets in soil at their second weeks (d).

3.1.3.2. Epicotyl

In shooting part of the study, established callus of epicotyls were transferred into the nine different medium types (namely; H, I, K, L, M, N, O, P, and R) for shoot induction. Results are given in Figure 3.12. The results are parallel to the results of cotyledonary petiole. Again only the explants that were in medium H during callogenesis gave response in shooting medium (Figure 3.13). Other 2 medium types (A & B) did not give any response in none of the shooting medium. Medium H derived calli showed similar responses in shooting medium but in this case percentages were lower than cotyledonary petiole. Again the most responsive medium was medium I (26.39 ± 1.39 percentage) with out any plant growth regulator. Also medium H (15.28 ± 1.39 percent), L (9.77 ± 1.39 percent), and M (6.94 ± 1.39 percent) were the responding medium types.

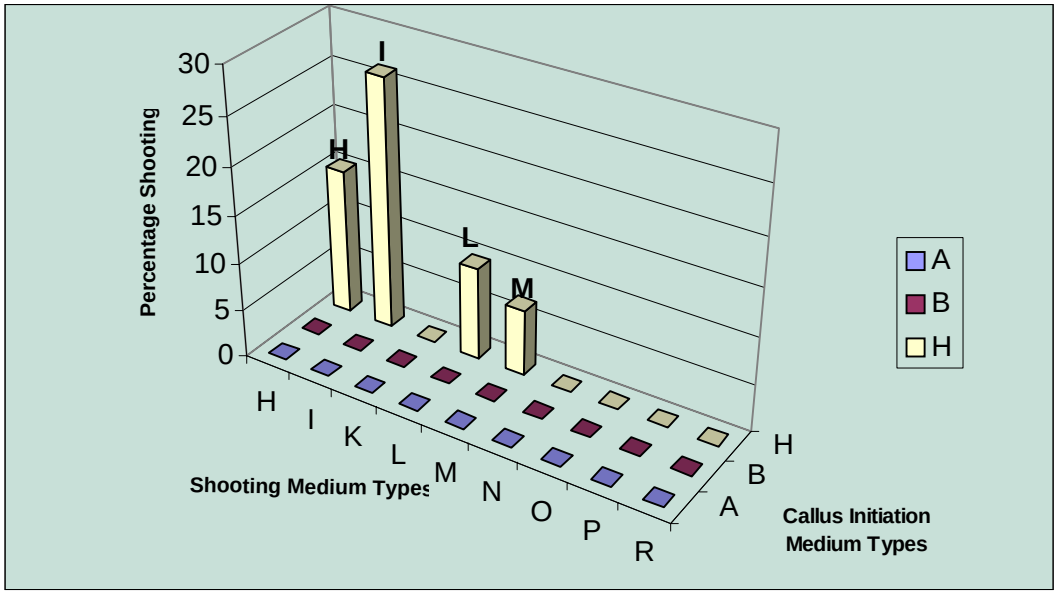

Figure 3.12. Percentage shooting response of epicotyl in different medium types that is transferred from different callus initiation medium.

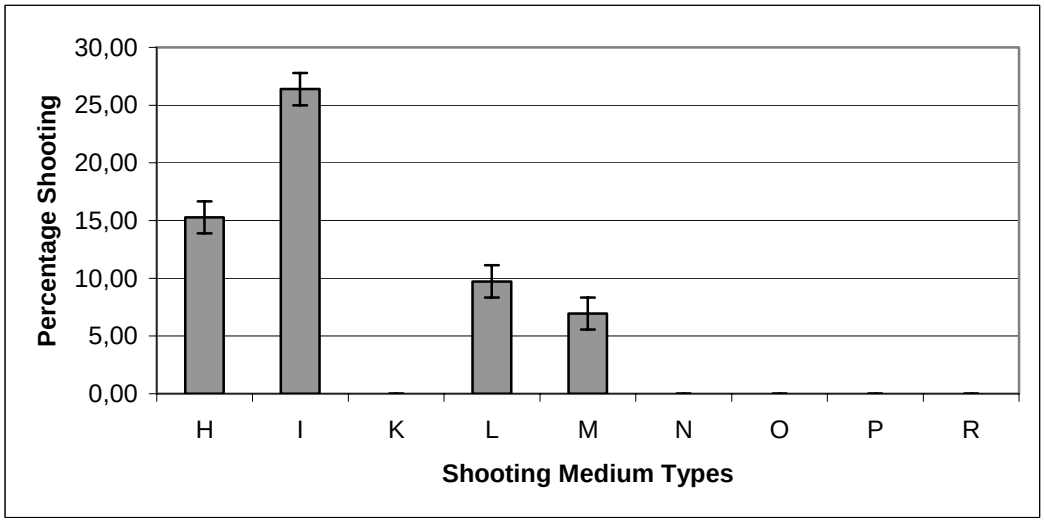

Figure 3.13. Percentages shooting of cotyledonary petiole in different medium types that are transferred from medium H. Bars indicate the mean percentage ± S.E.M.

As a result it is obvious that medium H which is MS basal supplemented with 1 mg/L ZEA and 1 mg/L NAA is the only suitable callus initiation medium type to be able to get shoots in shooting medium for epicotyl explants. Shooting was very successful with 25.27 percent in medium I with no growth regulator. TDZ has the potential of increasing the shoot number that can emerge from callus but needs further optimization. According to the results cotyledonary petiole was found to be more responsive than epicotyl parts.

As a result in epicotyl explants we were able to achieve healthy callogenesis and shooting (Figure 3.14). Also regenerated shoots were able to grow and give healthy roots (Figure 3.14) in shooting medium in glass jars, which were than transferred to the soil followed by successful acclimatization (Figure 3.14).

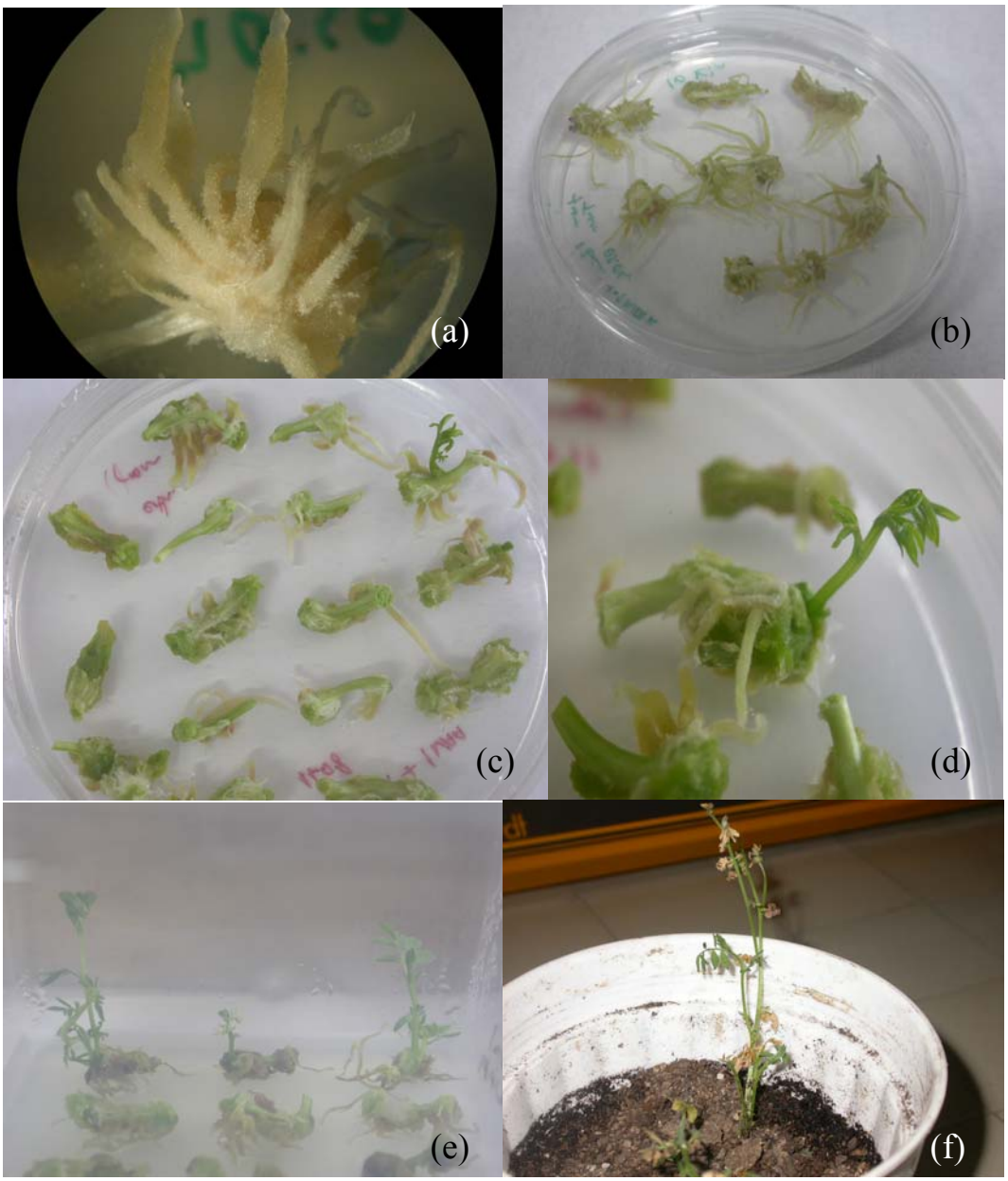

Figure 3.14 Different stages of epicotyl in medium H and I. Callus formed at the fourth week of incubation at dark (a, b), callus transferred to the medium I for shooting at the end of first week in shooting medium (c), newly emerged shoots in the second week of incubation in shooting medium (d), shoots in the third week of incubation in jars (e), shoots transferred to the soil (f).

3.1.4. Rooting of Plantlets

Medium H which is the best responding medium in callus initiation also caused rhizogenesis during callus formation stage (Figure 3.15). These roots were abnormal with high numbers of hairy structures around but after transferred into MS basal medium they formed normal roots (Figure 3.15. c). Most of the regenerated callus had roots when transferred into the shooting medium and these roots developed into healthy roots in shooting medium I. Some plantlets that did not initiate enough root or root with only one terminal were placed in to the medium A for more root initiation. Medium A included 2 mg/L NAA and 0.4 mg/L Kin and this combination quickly resulted in formation of roots in both cotyledonary petiole and epicotyl but the application time of this medium is found to be very important. If callus stays in this medium for more than 5 days newly established roots that are touching the medium goes in to callus formation and these roots stays abnormal afterwards.

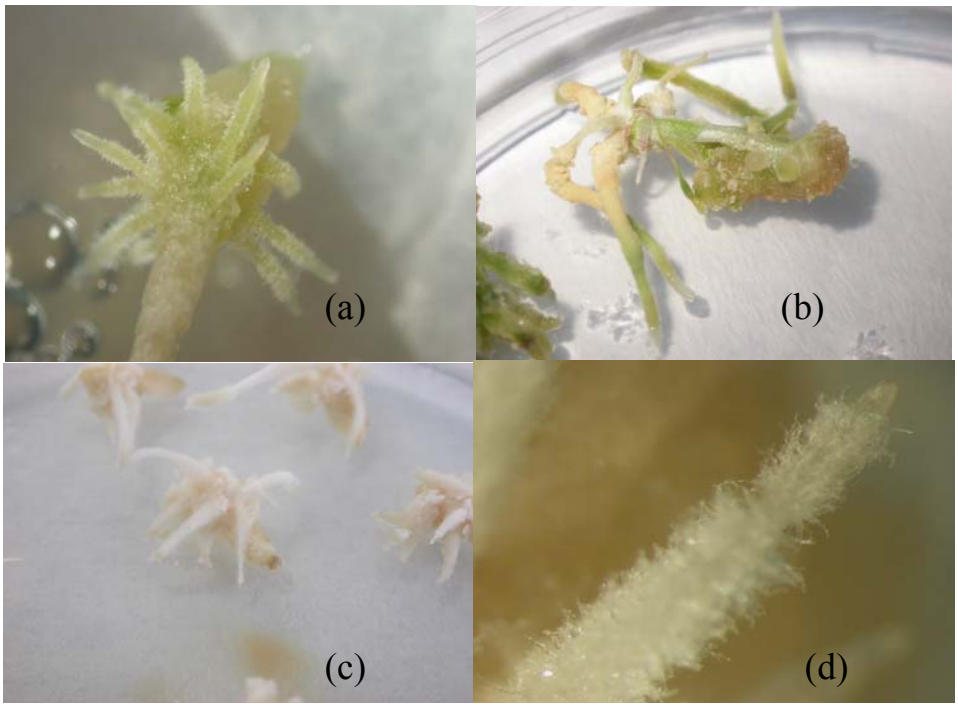

Figure 3.15. Rooting of explants. Formation of secondary rooting (a), multiple rooting of epicotyl (b), multiple rooting of cotyledonary petiole (c), high number of hairy structures around roots (d).

3.1.5. Callus Induction Studies for Somatic Embryogenesis

In this part of the study initiation of somatic embryogenesis was studied. Previous studies that is carried out showed that 2,4-D causes somatic embryogenesis initiation in lentil tissues. Also Saxena and King (1987) showed that medium supplemented with 2,4-D causes somatic embryogenesis in lentil. We have tried of two different concentrations of 2,4-D in mediums S and T with addition of ammonium nitrate as a nitrogen source. Results show that medium T causes more increase in callus weight (Figure 3.16). However, no somatic embryos were developed on the initiated callus.

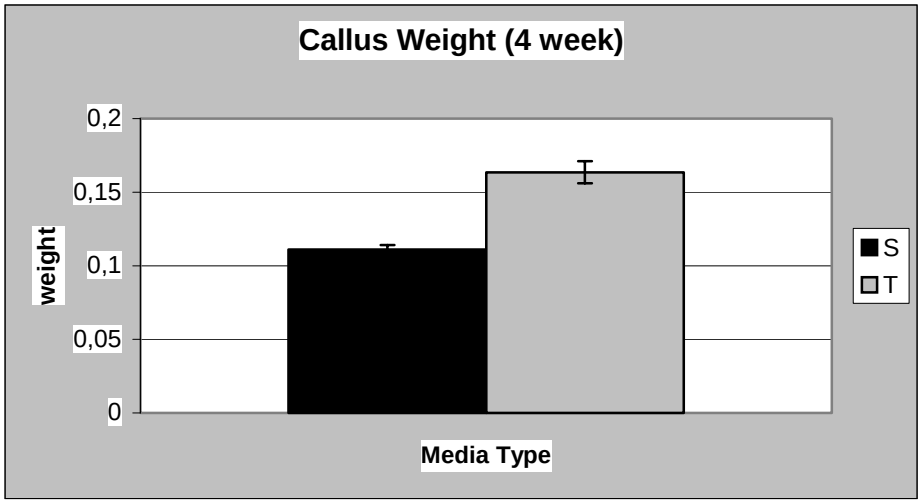

Figure 3.16. Weights of callus (in grams) of cotyledonary petiole in different media for somatic embryogenesis after 4 weeks. Bars indicate the mean weights ± S.E.M.

3.1.6. Effect of Salicylic Acid on Somatic Embryogenesis

Saxena and King (1987) described the somatic embryogenesis of lentil in Laird cultivar and obtained globular and heart stages of somatic embryogenesis. We have carried out the same procedure to get plant regeneration via somatic embryogenesis in Sultan cultivar but our cultivar did not respond like cultivar Laird and no successful regeneration was observed. Therefore in our study we examine the effect of SA on embryogenesis.

In our study the callus that are formed in medium S and T were transferred to medium containing 0 or 200μM SA (medium V and U, respectively). After a month callus were labeled as big (B), normal (R), and necrotic (N) (Figure 3.17). Also stages of embryogenesis were recorded. It was not possible to record the

number of embryos since the embryos were very small and enormous numbers of embryos were established in a calli (Figure 3.18).

Firstly we evaluate the effect of initiation medium whether it is transferred in to medium U or V. Big calli percentage increased from 11.2 % to 28.98 %. But more clear results can be seen in the effect of SA. In callus transferred from medium S, necrosis amount decreased to 14.21 % from 38.48 % also normal callus number increased from 50.20 % to 74.87 %. There was no significant change in the percentage of big callus. In callus transferred from medium T, 34.9 % decrease was observed in necrosis. Also 14.44 % in big callus percentage was observed (Figure 3.17). Most importantly more frequent and organized globular and heart shaped embryos were observed in SA (Figure 3.18).

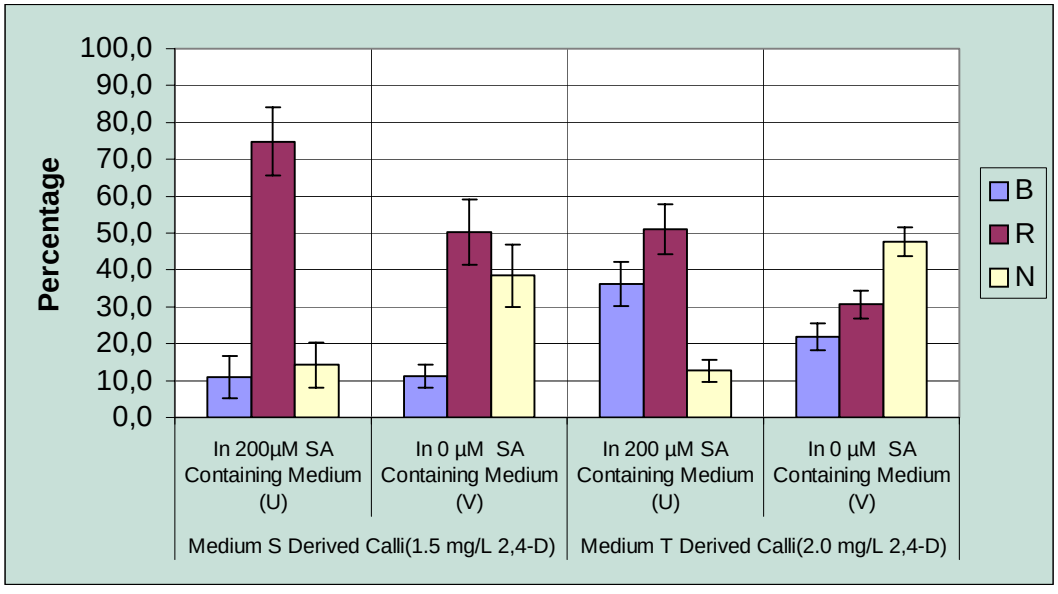

Figure 3.17. Percentage of big (B), normal (R), and necrotic (N) tissues in 0 and 200µM SA containing (V and U) medium that is transferred from medium S and medium T. Bars indicate the mean percentages ± S.E.M

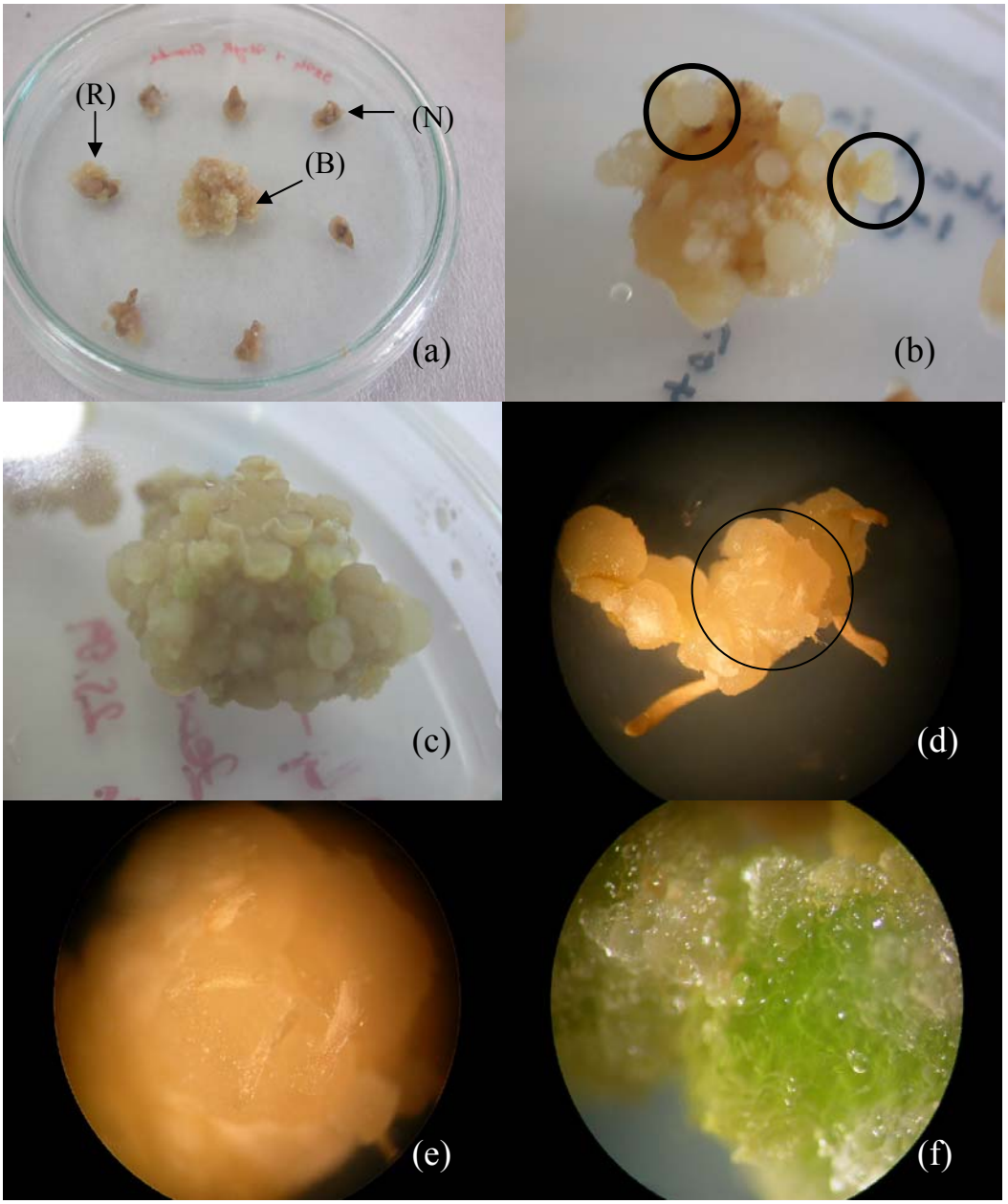

Figure 3.18. Different stages of somatic embryogenesis. Callus types coded as big (B), normal (R), and necrosis (N) (a), globular and heart shaped embryos formed (b), high number of globular embryos formed on an explant (c), first stages of germination of somatic embryos (d, e), cell clusters that are able to form somatic embryos.

3.2. Transformation Studies

In transformation studies *Agrobacterium* mediated transformation was used. Four kinds of tissue (peeled cotyledonary node, cotyledonary petiole, shoot tips, and roots) were used in transformation.

3.2.1. Kanamycin Screening

T-DNA on pTJK136 binary plasmid carries *npt*II gene as a plant selection marker, which detoxifies kanamycin when properly integrated into the plant genome. To be able to find appropriate concentration of kanamycin for selection, different concentrations were included on regeneration medium. Callus weights were recorded at the fourth week of callogenesis. It was seen that even at low concentrations of kanamycin callus weight decreased sharply from 0.20g to 0.07g. However with further increases in concentrations decrease in weight was not significant (Figure 3.19). Also it was seen that even in the lowest concentrations of kanamycin organogenesis is inhibited and no root and shoot formation was observed (Figure 3.20).

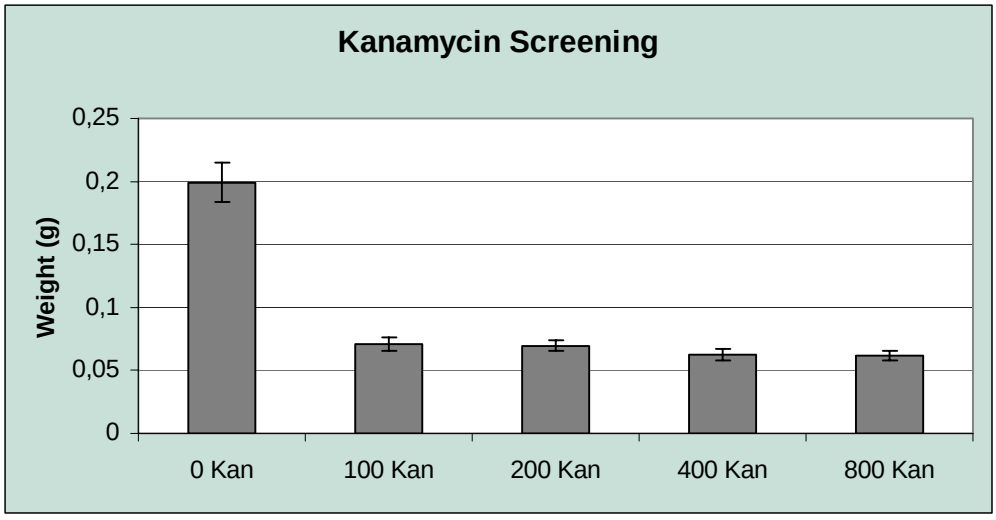

Figure 3.19. Weights of callus in different concentrations of kanamycin containing regeneration medium.

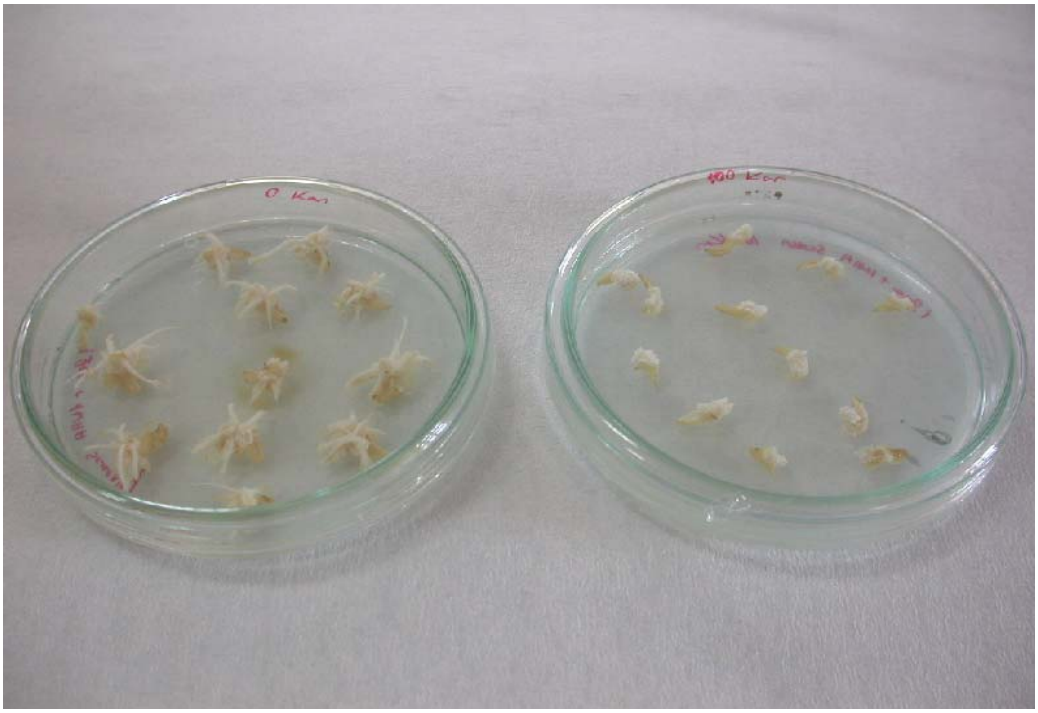

Figure 3.20. Organogenesis of cotyledonary petioles at the third week in 0 and 100 mg/L Kanamycin containing regeneration mediums.

3.2.2. Transformation of Peeled Cotyledonary Nodes

In the transformation studies firstly we tested the transient GUS expression of peeled cotyledonary nodes. Transient GUS expressions were very high in all explants. Since all the surface area of the explant is wounded by peeling action, plant phenolic compound production is very high and this attracts the *Agrobacterium tumefaciens*. We can see easily the entire explant surface has high GUS expression (Figure 3.21). Hundred percent of the explants expressed GUS in peeled cotyledonary nodes. It seems that it is a very promising system for transformation of lentil but this system also have some problems. Peeled cotyledonary nodes have a regeneration system based on direct organogenesis. Shoots emerged from these nodes were not expressing GUS (Figure 3.21), which means that cells forming shoots in organogenesis were not transformed by *Agrobacterium tumefaciens*.

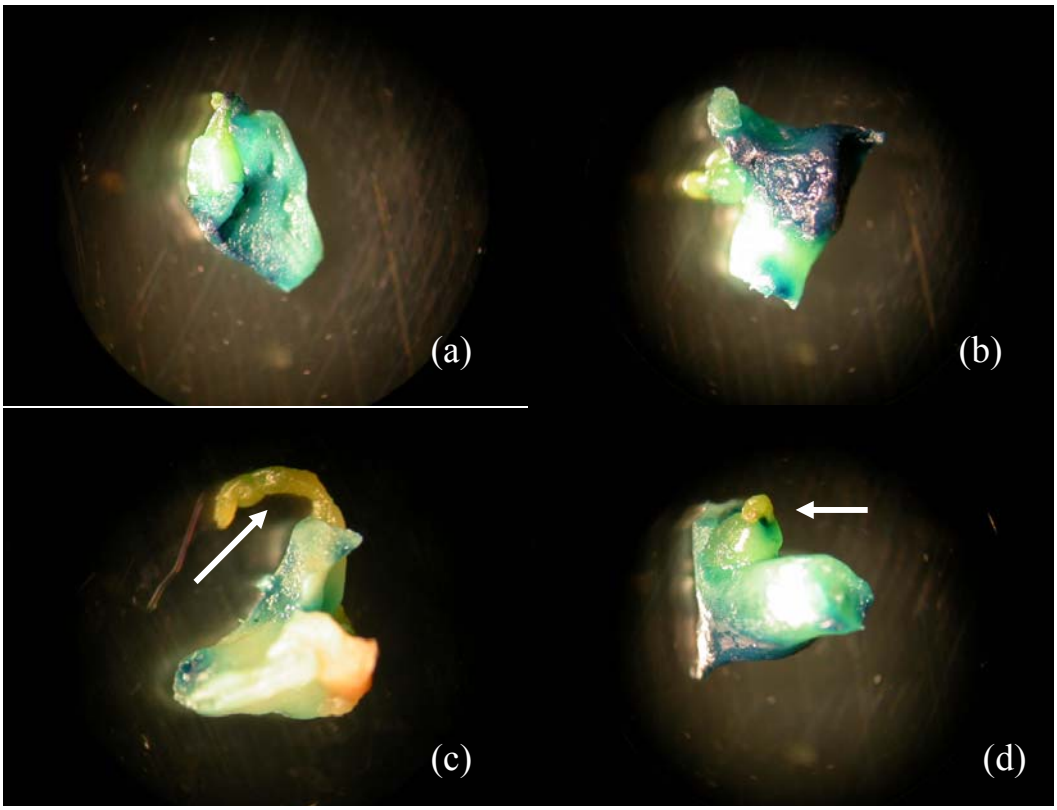

Figure 3.21. Transient GUS expression of the peeled cotyledonary petioles. Transient GUS expression of explants (a, b), GUS expression after 3 day in regeneration medium shoots formed are indicated by arrows (c, d).

3.2.3. Transformation of Different Tissue Types

In this part of the study we tested the transient expression of GUS efficiencies of 3 different tissue types. Roots, shoot tips and cotyledonary petioles were used as an explant in experiments. Tips were most promising explants with 73.7 % GUS expression, also 38 % of the roots and 25.6 % of the cotyledonary petioles expressed GUS (Figure 3.24). In shoot tips and roots percentage of GUS expressing explants are higher than the cotyledonary petioles but we see that cotyledonary petiole express GUS more densely (Figure 3.22, 3.23).

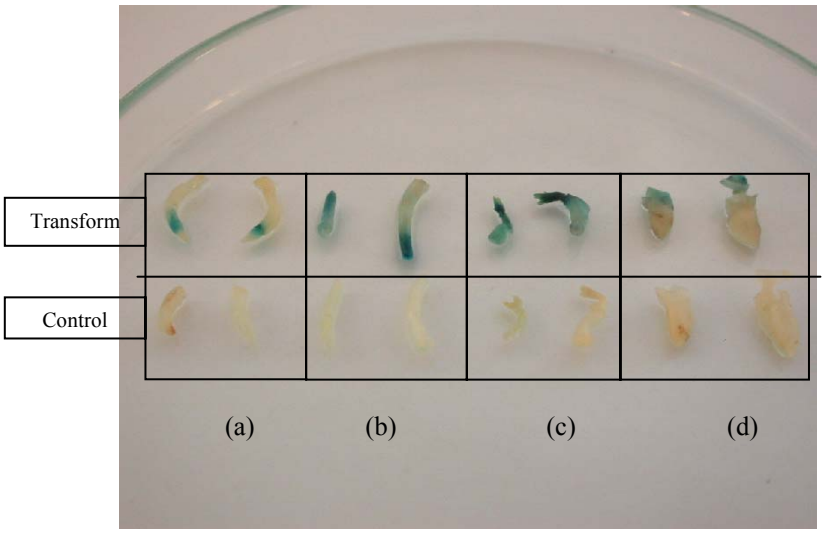

Figure 3.22. GUS analysis of transformed and control explants in different tissue types. (a) root, (b) epicotyl, (c) shoot tip, (d) cotyledonary petiole.

Figure 3.23. GUS analysis of cotyledonary petioles.

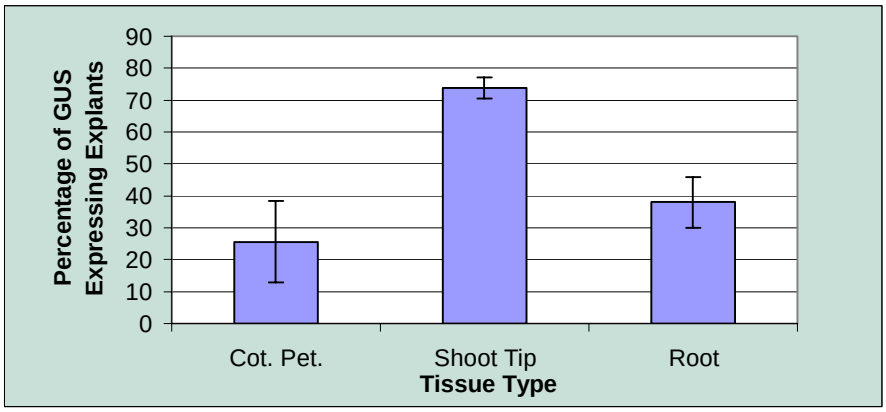

Figure 3.24. Percentage of GUS expressing explants in different tissue types.

CHAPTER IV

DISCUSSION

Cotyledonary nodes of various legumes were used most commonly in the studies of regeneration and transformation. The reason of using cotyledonary node is the potential of producing high numbers of shoots in tissue culture and responsiveness of this tissue to *Agrobacterium tumefaciens.* There are only 3 reports on lentil tissue culture using cotyledonary node. Gulati *et al.* (2001) used cotyledonary nodes and micrografted attained shoots with 96 % efficiency, Khawar and Özcan (2002) used 21 different genotypes and achieved a 20.6 shoots per explant using TDZ, also Fratini and Ruiz (2003) used cotyledonary node in their rooting procedure based on explant polarity. Cotyledonary node is used as explant also in other legumes such as chickpea (Brandt and Hess, 1994; Jayanand *et al.*, 2003), faba bean (Khalfalla and Hattori, 1999), mungbean (Gulati and Jaiwal, 1994), pigeonpea (Sp *et al.*, 1994; George and Eapen, 1994), *Bauhinia vahlii* (Bhat and Dhar, 2000), and acacia (Vengadesan *et al.*, 2002). In most of the studies in which cotyledonary node is used, number of shoots per explant is very high as in the study of Khawar and Özcan (2002) (20.6 shoots per explant) and Vengadesan *et al.* (2002) (30 shoots per explant). It seems that using cotyledonary nodes with *direct organogenesis* is an advantageous way of regeneration of legumes but *direct organogenesis* of cotyledonary node has some problems. The biggest problem of this explant with direct organogenesis is the very low percentage rooting *in vitro*. Shoots formed *in vitro* with high amounts of cytokinin generally have a problem of rooting. BA, KIN and TDZ repress the formation of

roots afterwards if used in shooting medium (Mohamed *et al.*, 1992; Gulati and Jaiwal, 1994; Prakash *et al.*, 1994; Sanago *et al.*, 1996; Polisetty *et al.*, 1997; Polanco and Ruiz, 1997; Fratini and Ruiz, 2003). According to the Polanco and Ruiz (1997) KIN and ZEA in low concentrations regenerate shoots which are more likely to root. In our study using of ZEA and NAA together caused formation of roots even in the late phases of callogenesis. Callus formed roots before regeneration of shots. These roots were abnormal with high numbers of hairy structures around but after transfer they formed normal roots in MS basal medium. Hundred percentage of explants produced roots in our experiments. Since micrografting is very difficult and needs extra hand skills in lentil due to weak and soft stems this procedure makes it easy to regenerate lentil *in vitro*.

Also if we look through the studies of transformation transient expression in this tissue is generally successful. Öktem *et al.* (1999) showed that 50 % of the explant expressed the transferred GUS gene but the regenerated shoots are not so successful in expression of introduced gene, which resulted in chimeric plants, shoots regenerated from cotyledonary nodes exhibits only the patches of GUS staining. Warkentin and McHugen (1993) also were able to produce GUS positive explants transiently but transgenic shoots were not recovered from cotyledonary nodes in selection. This chimerism is the result of direct organogenesis used in the regeneration of this tissue. In our studies we observed that expression of the introduced gene was generally in the wounded parts of explants. But we know that shoots formed from the meristematic tissue in the base of the cotyledonary node. This means that cells that are forming shoots are generally not transformed. To solve this problem instead of increasing transformation efficiency we have tried to solve regeneration problem of lentil. For this reason cotyledonary petioles (which were one of the most responsive explants) were used for optimization of indirect organogenesis based regeneration system. Almost 100 % callogenesis was achieved by this system.

Furthermore we have achieved 40.3 % shooting in MS medium, supplemented with 1 mg/L ZEA and 1 mg/L NAA. Beside cotyledonary petiole epicotyl explants were also studied for regeneration and 26.4 % shooting were achieved. Shooting percentage is lower than the experiments that have carried out direct organogenesis but we shoots are more potent to be a transgenic because of high response of explant to the transformation and good selection through the indirect organogenesis. Integration of plant tissue culture protocols to transformation protocols are very important to be able to produce transgenic plants, so beside finding a good regeneration system researchers have to consider that regeneration system have to be compatible with the selection systems and systems for increasing efficiency of transformation.

Transformation studies showed that the most responsive explant was shoot tips with 73.7 % transient GUS expression. Also roots and cotyledonary petioles showed transient GUS expression 38 % and 25.6 % respectively. Warkentin and McHugen (1992) also studied the different tissue types for their transformation efficiency. Results showed that shoot tips were most responsive explant with 39 to 67 % transient GUS expression. No GUS expression observed in epicotyl while the root wound site had GUS expression. Warkentin and McHugen (1991) studied the transformation efficiency of four different types of *Agrobacterium* in shoot tips and achieved 100 % tumor formation. Khawar and Özcan (2002) also showed that leaves and stem segments are responsive for tumor formation. It is obvious that shoot tips are one of the most responsive tissues in lentil for *Agrobacterium* mediated transformation. In our study we observed that cotyledonary petioles are also transformed by *Agrobacterium* and can be used as an explant to get fertile transgenic plants with suitable regeneration system.

Also preliminary studies were carried out for somatic embryogenesis for further tissue culture studies. Saxena and King (1987) were able to regenerate lentil via somatic embryogenesis but the percentage was very low (3-5% of all the calli showed response). Also we observed in our study that this procedure is

cultivar dependent and it was not suitable for our cultivar. We could not get somatic embryos using this procedure. So we tried to enhance somatic embryogenesis using SA. SA belongs to a group of plant phenolics and considered as a hormone-like substance, which plays an important role in the regulation of plant growth and development (Raskin, 1992). Some studies showed that development of somatic embryos could be promoted by adding exogenous SA in embryogenic cultures (Hutchinson and Saxena, 1996). SA is an inhibitor of ethylene synthesis and promoting effect of SA on somatic embryogenesis could be related to inhibition of ethylene synthesis (Roustan *et al.* 1989; 1990). Inhibitors of ethylene synthesis such as aviglycine and 1-methylcyclopropene have been applied to stimulate somatic embryogenesis in different plants (Kuklin, 1995). In our study we were able to get globular and some heart shape stages but we were not able to generate torpedo shape somatic embryos and plantlets.

CHAPTER V

CONCLUSION

In this study, transformation and regeneration via indirect organogenesis in cotyledonary petiole tissue of lentil was investigated. Eight different medium types differing in their plant growth regulator compositions were employed to examine the callus induction potency of cotyledonary petiole. Only the medium type containing 1 mg/L zeatin riboside and 1 mg/L naphthalene acetic acid resulted in shooting when transferred in to the shooting medium. Other medium types were successful in callus induction but no shooting was observed when they were transferred to the shooting medium. In shooting part of the study, formed callus were transferred into nine different medium types to test their potencies on shoot induction. . The shooting percent was the highest (40.3 %) in MS Basal without any plant growth regulators. Also five different medium types were employed to examine callus induction potency and three different medium types were tested for shooting induction of epicotyl. Again the medium containing 1 mg/L zeatin riboside and 1 mg/L naphthalene acetic acid was the best medium for callus induction and MS Basal medium without any plant growth regulators yielded the best (26.4 %) for shoot induction. Rooting and acclimatization of plantlets was also successful.

Also a preliminary study on somatic embryogenesis was carried out. Globular and heart shaped somatic embryos were observed in the study but further stages can not be achieved. Salicylic acid was tested for its effects on somatic

embryogenesis and it was found that it increases the mass of callus and decreases the necrosis of somatic embryos.

In transformation part of the study, firstly kanamycin screening was tested with optimized regeneration system and it was found that even 100 mg/L kanamycin is enough to prevent organogenesis. Three different tissues (roots, shoot tips and cotyledonary petioles) were investigated for their transient GUS expression efficiencies. Shoot tips showed the highest percentage of GUS expression. For future studies optimized regeneration, transformation and selection systems should be used to obtain fertile transgenic plants.

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APPENDIX A

COMPOSITIONS OF PLANT TISSUE CULTURE MEDIA

Table A.1. Compositions of plant tissue culture media Gamborg B5 and MS

From DUCHEFA Plant Cell Cultures Catalogue	Gamborg B5	Murashige & Skoog
MICRO ELEMENTS	mg/L	mg/L
$CoCl_2.6H_2O$	0,025	0,025
$CuSO_4.5H_2O$	0,025	0,025
FeNaEDTA	36,70	36,70
H_3BO_3	3,00	6,20
KI	0,75	0,83
$MnSO_4.H_2O$	10,00	16,90
$Na_2MoO_4.2H_2O$	0,25	0,25
$ZnSO_4.7H_2O$	2,00	8,60
MACRO ELEMENTS		
$CaCl_2$	113,23	332,02
KH_2PO_4	----	170,00
KNO_3	2500,00	1900,00
$MgSO_4$	121,56	180,54
NaH_2PO_4	130,44	-----
$(NH_4)_2SO_4$	134,00	-----
NH_4NO_3	3051,98	1650,00
VITAMINS		
Glycine	-----	2,00
myo-Inositol	100,00	100,00
Nicotinic acid	1,00	0,50
Pyridoxine HCl	1,00	0,50
Thiamine HCl	10,00	0,10

Table A.2. Compositions of medium types used in the study.

Medium A:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
2 mg/L NAA
0.4 mg/L KIN

Medium B:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
2 mg/L BA
0.2 mg/L NAA

Medium C:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
2 mg/L BA

Medium D:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
10 mg/L KIN
1 mg/L GA

Medium E:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
1 mg/L 2,4-D
0.1 mg/L KIN

Medium F:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
1 mg/L 2,4-D

Medium G:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
5 mg/L 2,4-D

Medium H:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
1 mg/L ZEA
1 mg/L NAA

Medium I:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar

Medium K:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
1 mg/L BA
0.1 mg/L NAA

Table A.2. (continued)

Medium L:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
1 mg/L TDZ

Medium M:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
0.1 mg/L TDZ

Medium N:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
1 mg/L ZEA

Medium O:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
0,25 mg/L ZEA

Medium P:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
10 mg/L KIN

Medium R:
2.2 g MS salts (Sigma 5519)
3% Sucrose
0.8% Agar
1 mg/L GA
0.25 mg/L BA
0.1 mg/L NAA

APPENDIX B

BACTERIAL CULTURE MEDIA

Table B.1. YEB Medium (1 L)

(pH:7.2)

Sucrose	5 g
Nutrient broth	13,5 g
$MgSO_4.7(H_2O)$	2 mM
Yeast extract	1 g
Agar	15 g (if solid medium is required)

Table B.2. MMA Medium (1 L)

(pH:5,6)

Sucrose	20 g
MS salts	4,3 g
MES	1,95 g

APPENDIX C

ANOVA TABLES

Table C.1. One-way ANOVA (stack) test of callus initiation percentages of cotyledonary petiole. P value is 0 indicates the highly significant difference (Confidence intervals, 95%).

Level	N	Mean	StDev	----+---------+---------+---------+--
A	7	81,71	11,21	(-*--)
B	6	96,33	4,03	(--*--)
C	5	89,20	12,19	(--*---)
D	4	41,50	35,20	(---*---)
E	4	100,00	0,00	(---*--)
F	4	94,00	4,00	(---*---)
G	4	91,75	6,95	(--*---)
H	4	100,00	0,00	(---*--)
I	4	0,00	0,00	(---*---)
				----+---------+---------+---------+--
Pooled StDev =		12,72		0 35 70 105

Source	DF	SS	MS	F	P
Factor	8	38038	4755	29,39	**0,000**
Error	33	5339	162		
Total	41	43377			

Table C.2. One-way ANOVA (stack) test of callus weights of cotyledonary petiole. P value is 0 indicates the highly significant difference (Confidence intervals, 95%).

Level	N	Mean	StDev	-------+---------+---------+---------
A	86	0,14072	0,07659	(*-)
B	76	0,08815	0,03774	(-*-)
C	38	0,03623	0,03442	(--*--)
D	19	0,05189	0,03796	(---*---)
E	36	0,13536	0,02440	(--*--)
F	36	0,06681	0,02577	(--*--)
G	33	0,04991	0,02269	(--*--)
H	36	0,19920	0,09303	(--*--)
				-------+---------+---------+---------
Pooled StDev =		0,05432		0,060 0,120 0,180

Source	DF	SS	MS	F	P
Factor	7	0,86908	0,12415	42,07	**0,000**
Error	352	1,03869	0,00295		
Total	359	1,90776			

Table C.3. One-way ANOVA (stack) test of callus weights of cotyledonary petiole. P value is lower than 0.05 indicates the significant difference (Confidence intervals, 95%).

Level	N	Mean	StDev	--+---------+---------+---------+----
A	36	0,28611	0,09465	(--------*---------)
B	36	0,24454	0,07309	(---------*--------)
H	39	0,23339	0,08195	(--------*--------)
				--+---------+---------+---------+----
Pooled StDev =		0,08365		0,210 0,240 0,270 0,300

Source	DF	SS	MS	F	P
Factor	2	0,05689	0,02844	4,07	**0,020**
Error	108	0,75569	0,00700		
Total	110	0,81258			

Table C.4. One-way ANOVA (stack) test of percentage shootings of cotyledonary petiole. P value is lower than 0.05 indicates the significant difference (Confidence intervals, 95%).

Level	N	Mean	StDev	--------+---------+---------+--------
H	3	22,220	2,408	(---*---)
I	3	40,277	4,809	(---*---)
L	3	15,280	2,408	(---*---)
M	3	8,333	4,165	(---*---)
				--------+---------+---------+--------
Pooled StDev =		3,608		12 24 36

Analysis of Variance

Source	DF	SS	MS	F	P
Factor	3	1695,4	565,1	43,41	**0,000**
Error	8	104,1	13,0		
Total	11	1799,5			

Table C.5. One-way ANOVA (stack) test of percentage shooting of epicotyl. P value is lower than 0.05 indicates the significant difference (Confidence intervals, 95%).

Level	N	Mean	StDev	-----+---------+---------+---------+-
H	3	15,280	2,408	(----*---)
I	3	26,390	2,408	(----*---)
L	3	9,720	2,408	(----*---)
M	3	6,943	2,402	(----*---)
				-----+---------+---------+---------+-
Pooled StDev =		2,406		7,0 14,0 21,0 28,0

Analysis of Variance

Source	DF	SS	MS	F	P
Factor	3	665,71	221,90	38,33	**0,000**
Error	8	46,31	5,79		
Total	11	712,03			

Table C.6. One-way ANOVA (stack) test of percentage GUS expressing explants in different tissues. P value is lower than 0.05 indicates the significant difference (Confidence intervals, 95%).

Analysis of Variance

Source	DF	SS	MS	F	P
Factor	2	6624	3312	6,85	**0,009**
Error	13	6287	484		
Total	15	12911			

				Individual 95% CIs For Mean Based on Pooled StDev
Level	N	Mean	StDev	--------+---------+---------+--------
cot pet	6	25,62	31,09	(-------*-------)
tip	5	73,73	7,40	(-------*--------)
root	5	38,00	17,58	(-------*--------)
				--------+---------+---------+--------
Pooled StDev =		21,99		25 50 75

Table C.7. Average and standard error of means (SEM) of callus initiation percentages of cotyledonary petioles in different medium.

	A	B	C	D	E	F	G	H	I
Average	81,71	96,33	89,20	41,50	100,00	94,00	91,75	100,00	0,00
SEM	4,24	1,65	5,45	17,6	0	2	3,47	0	0

Table C.8. Average and standard error of means (SEM) of callus weights of

	A	B	C	D	E	F	G	H
Average (g)	0,1407	0,0988	0,0362	0,0519	0,1354	0,0618	0,0499	0,1992
SEM	0,0082	0,0116	0,0056	0,0087	0,0041	0,0043	0,004	0,0155

cotyledonary petioles in different medium.

Table C.9. Average and standard error of means (SEM) of callus weights of cotyledonary petiole in different media for somatic embryogenesis.

		B	R	N
Medium S Derived Calli (1.5 mg/L 2,4-D)	In 200µM SA Containing Medium (U)	10,92 %	74,87 %	14,21 %
	In 0 µM SA Containing Medium (V)	11,32 %	50,20 %	38,48 %
Medium T Derived Calli (2.0 mg/L 2,4-D)	In 200 µM SA Containing Medium (U)	36,20 %	51,07 %	12,73 %
	In 0 µM SA Containing Medium (V)	21,76 %	30,60 %	47,64 %

Table C.10. Percentage of big (B), normal (R), and necrotic (N) tissues in 200µM and 0µM SA containing (U and V) medium that is transferred from medium S and medium T.

	S	T
Average (g)	0,11098	0,1635
SEM	0,00309	0,0075

Table C.11. Average and standard error of means (SEM) of callus weights of epicotyl in different medium

	A	B	H
Average	0,2861	0,2445	0,2334
SEM	0,0158	0,0122	0,0131

Table C.12. Average and standard error of means (SEM) of percentage of GUS expressing explants in different tissues.

	Cot. Pet.	Tip	Root
Average	25,6	73,7	38
SEM	12,7	3,31	7,9

APPENDIX D

PLASMID MAPS AND PLANT SELECTION MARKERS

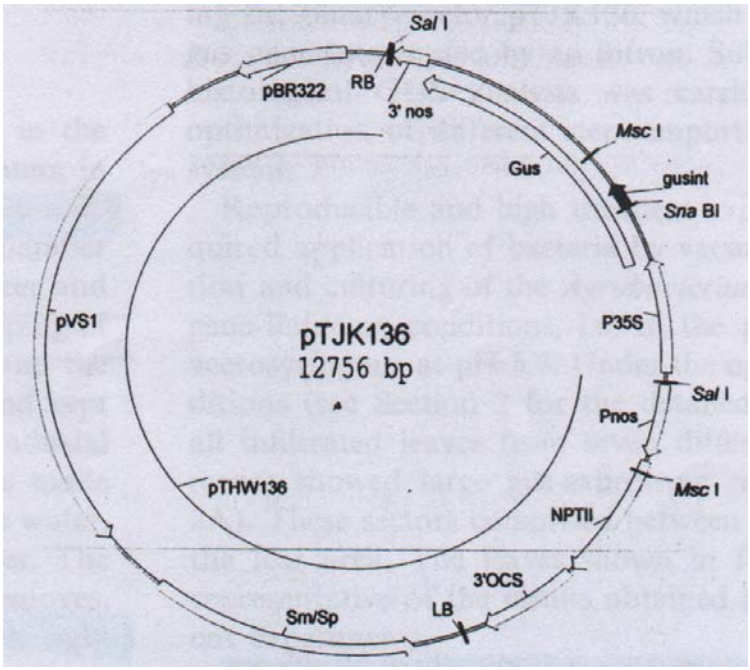

Figure D.1. Map of a pTJK.

Table D.1. Selection Markers Found on Bacterial Strains and Binary Plasmids Used in the Study

Bacterial Strain	Chromosomal/ T_i Plasmid Selection Marker	Plasmid	Bacterial Selection Marker	Plant Selection Marker
KYRT1	Rif^r (100mg/L) Carb^r (100mg/L) Gent^r (40mg/L)	pTJK136	Strep^r (300mg/L) Spect^r (125mg/L)	Kan^r (*npt*II gene) *uid-a* gene

^{r}: resistance character
Rif (Rifampicin), Carb (Carbenicilin), Gent (Gentamycin), Amp (Ampicilin), Kan (Kanamycin), Strep (Streptomycin), Spect (Spectinomycin)

APPENDIX E

GUS HISTOCHEMICAL ASSAY

Table E.1. GUS staining solution.

KPO_4 buffer	0.1 M (pH 7.0)
EDTA	10 mM
K-ferricyanide	0.5 mM
K-ferrocyanide	0.5 mM
5- bromo-(X-Gluc)	1 mM
Triton X-100	10 % (v/v)

Table E.2. GUS fixative solution

Formaldehyde	10 % (v/v)
Ethanol	20 % (v/v)
Acetic acid	5 % (v/v)