REGENERATION OF LENTIL (LENS CULINARIS MEDIK) AND GENETIC TRANSFORMATION BY USING AGROBACTERIUM TUMEFACIENS-MEDIATED GENE TRANSFER

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Ufuk Çelikkol Akçay

ABSTRACT

REGENERATION OF LENTIL (*LENS CULINARIS* MEDIK) AND GENETIC TRANSFORMATION BY USING *AGROBACTERIUM TUMEFACIENS*-MEDIATED GENE TRANSFER

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In this study, the effects of different plant growth regulators on regeneration responses of various lentil explants through direct and indirect organogenesis and through somatic embryogenesis from calli and cell suspension cultures were investigated. Shoot regeneration was obtained in low frequencies from longitudinal embryonic axis explants and nodal buds of epicotyls, however whole plant regeneration was unsuccessful. Conditions provided for indirect organogenesis resulted only in swelling of hypocotyls and root directed ends of internodes and weak callus formation on leaves which were followed by tissue browning and necrosis. In somatic embryogenesis studies, the explants longitudinal embryonic axis and cotyledonary petioles produced soft and friable calli on MS media with Gamborg's vitamins supplemented with 0.75mg/L 2,4-D+0.5mg/L BA. The highest average number of embryos per explant, 12.36 was observed on media containing 0.75mg/L BA +0.5mg/L 2,4-D for cotyledonary petiole explants, whereas 3mg/L BA+1mg/L NAA was the only hormone combination that allowed embryo

development to some extent, in both explants. Somatic callus failed to regenerate despite globular embryo formation and embryo development to some extent.

Combination of sonication treatment with *Agrobacterium* transformation of three lentil explants; cotyledonary nodes, half cotyledons and cotyledonary nodes with intact shoots, had no effect on the improvement of transient *gus* gene expression on explants. Sonication treatment was also unable to form localized wounds on the petiole axils. The best *gus* gene expression on the axil region was obtained when cotyledonary nodes and KYRT1 strain were used in combination with vacuum infiltration and scalpel wounding of the axils. Gradual selection and repeated removal of regenerated shoots between selection cycles increased the number of gus expressing shoots significantly. The regenerated shoots were grafted on root stocks and whole plant regeneration was achieved in greenhouse conditions.

By the use of the optimized *Agrobacterium*-mediated transformation protocol, 4 independent lines were obtained with 2.3% transformation efficiency. Southern blot analysis confirmed the integration of the *gus* gene into the genome of lentil plants. T_0 plants were fertile and all plants showed Mendelian segregation of the *gus* gene in 3:1 ratio to their progenies except one line which carries three copies of the gene. Reverse transcription PCR has confirmed the expression of the genes in T_0 and T_1 generations. T0 plants and the following three generations strongly expressed *gus* gene uniformly in their tissues and the PCR amplifications of both *gus* and *npt-II* genes was successful through generations.

Keywords: *Lens culinaris*, lentil, cotyledonary node, *Agrobacterium tumefaciens*, genetic transformation, regeneration

ÖZ

MERCİMEK (*LENS CULINARIS* MEDİK) BİTKİSİNİN REJENERASYONU VE *AGROBACTERIUM TUMEFACIENS* ARACILIĞI İLE GENETİK TRANSFORMASYONU

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Bu çalışmada farklı bitki büyüme düzenleyicilerinin çeşitli mercimek eskplantlarının doğrudan ve dolaylı organogenez ile, kallus ve hücre sıvı kültürlerinden somatic embriyogenez yoluyla rejenerasyonu üzerindeki etkileri incelenmiştir. Uzunlamasına kesilmiş embriyonik eksen bölgeleri ve epikotil üzerindeki nodal bölgelerden düşük oranda sürgün gelişimi elde edilmiş ancak bütün bitki eldesi sağlanamamıştır. Dolaylı organogenez için sağlanan koşullar hipokotillerde şişme, internodların kök uçlarında ve yapraklarda zayıf kallus oluşumu sağlamıştır fakat bunu dokuların ölümü izlemiştir. Somatik embriyogenez çalışmalarında boylamasına kesilmiş embriyonik eksen ve çenek petiollerinden 0.75mg/L 2,4-D+0.5mg/L BA eklenmiş Gamborg vitaminli MS besi yeri üzerinde yumuşak ve kırılgan kalluslar elde edilmiştir. Eksplant başına en yüksek ortalama embriyo sayısı, 12.36, 0.75mg/L BA +0.5mg/L 2,4-D içeren besi yeri üzerinde kültüre alınan çenek petiollerinde görülürken, 3mg/L BA+1mg/L NAA, iki eksplant için de bir ölçüde embriyo gelişimine izin veren tek hormon kombinasyonu

olmuştur. Somatik kalluslar küresel embriyoların oluşumuna ve gelişimine rağmen rejenere olmayı başaramamıştır.

Sonikasyon uygulamasının çenek boğumları, yarım çenekler ve bütün gövdeli çenek boğumu explantlarının *Agrobacterium* aracılığıyla taransformasyonuyla birleştirilmesi, eksplantlar üzerindeki geçici *gus* geni ifadesinde hiç bir geliştirici etki oluşturmamıştır. Sonikasyon uygulaması petiol eksenlerinde lokal yaralar oluşturulmasında da başarılı olmamıştır. Eksen bölgesinde en iyi *gus* geni ifadesi çenek boğumları ve KYRT1 suşunun vakum infiltrasyonu ve neşterle yaralama ile bir arada kullanılmasıyla elde edilmiştir. Dereceli seçilim ve rejenere olan sürgünlerin her seçilim döngüsünde uzaklaştırılması *gus* ifade eden sürgün sayısını önemli ölçüde arttırmıştır. Elde edilen sürgünler kök stoklarına aşılanarak sera koşullarında bütün bitki eldesi sağlanmıştır.

Yapılan çalışmalarla etkili hale getirilen *Agrobacterium* aracılığıyla transformasyon protokolü kullanılarak % 2.3 transformasyon verimliliğiyle 4 bağımsız hat elde edilmiştir. Southern blot analizi *gus* geninin mercimek genomuyla bütünleştiğini kanıtlamıştır. T_0 bitkileri verimlidir ve geni üç kopya olarak taşıyan bir hat dışında *gus* genini 3:1 Mendel ayrılma oranında sonraki nesillere aktarmışlardır. Ters ifadeli polimeraz zincir reaksiyonu, T_0 ve T_1 nesillerindeki gen ifadesini kanıtlamıştır. T_0 bitkileri ve takip eden üç nesil *gus* genini tüm dokularında aynı biçimde ifade etmiş ve nesiller boyunca *gus* ve *npt-II* genlerinin PZR aracılığıyla çoğaltımı başarılı olmuştur.

Anahtar Kelimeler: *Lens culinaris*, mercimek, kotiledon boğumu, *Agrobacterium tumefaciens*, genetik transformasyon, rejenerasyon

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CHAPTER 1

INTRODUCTION

1.1. Lentil as an Important Legume Crop

Lentils are annual pulse legume plants and their cultivation by humans is as old as the agriculture itself. Although they are very important both economically and as a high protein source for the people of various countries in the world, they received very little research attention for the improvement of nutritional and growth characteristics.

1.1.1. Origin and Distribution

Like wheat and barley, lentil was probably first cultivated in the Near East including Southern Turkey where Neolithic agriculture started to develop about 10 000 years ago. In the next 4000 years the plant had been spread throughout Mediterranean, Asia, Europe and Latin America (Zohary and Hopf 1988).

Lentil is within the order *Rosales*, suborder *Rosineae*, family *Leguminosae*, subfamily *Papilionaceae*, and tribe *Vicieae*. The plant also belongs to the genus *Lens* Miller and the name *Lens* describes the shape of the lentil seed. According to the latest classification by Ferguson (2000), the genous *Lens* has seven taxa divided into four species (Table 1-1).

Archaeological evidence, together with morphological and cytogenetic comparisons, suggests that *L. orientalis* is the wild progenitor of cultivated lentil *L. culinaris*, Medik (Muehlbauer 1995). The name Medik is for Medikus, a German botanist-physician who has named the plant in 1787.

Table1.1. Taxonomical classification of genus *Lens* according to Ferguson (2000).

Lens culinaris **Medikus**	***Lens ervoides*** **(Brign.) Grande**	***Lens nigricans*** **(M. Bieb.) Godr.**	***Lens lamottei*** **Czefr.**
subsp. *culinaris*			
subsp. *orientalis* (Boiss.) Ponert			
subsp. *tomentosus* (Ladiz.)			
subsp. *odemensis* (Ladiz.)			

1.1.2. Botanical Description and Growth Habits

Lentil plants are typically short, slender and semierect annuals varying between 15 to 75 cm tall depending on the genotype and environmental conditions. Branches may arise directly from the main stem or from the cotyledonary node below ground or they may rise from other branches depending on the available space in the field and environmental conditions (Saxena and Hawtin 1981).

Lentil plants have slender taproots and a mass of fibrous lateral roots (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 leaves are alternate, compound and pinnate and are relatively small compared with the trifoliolates of soybean and *Phaseolus* beans. Short peduncles that originate from the upper nodes of the plant generally bear small (4-8mm) self-pollinated flowers that fade within 3 days and the pods are visible 3 to 4 days later (Saxena and Hawtin 1981).

Lens species are diploid plants with $2n = 14$ chromosomes. They all have similar karyotypes consisting of three pairs of metacentric or submetacentric chromosomes, three pairs of acrocentric chromosomes and one satellited pair of chromosomes (Slinkard 1985).

Lentil has a life cycle which lasts 3-4 months. Optimum temperature for the germination of the seeds is in the range of 15-25^{0}C and for the growth and yield, the temperature should be around 24^{0}C.

Lentils show adaptability to a wide range of soil types. They are grown in sandy loam soils, alluvial soils, black cotton soils or in heavier clay soils (Nezamuddin 1970) and they can grow well on slightly acidic soils (pH 5.5 to 6.5) to moderately alkaline soils ranging pH 7.5 to 9.0 (Bharadawaj 1975). Salt tolerance of lentils was reported to be much less than that of most cereals, chickpeas, lupins and faba beans although it is higher than that of *Phaseolus* bean, cowpeas and soybeans (Ivanov 1973). Also the plant is known to be much more tolerant to drought when compared to water logging.

1.1.3. Nutritional Value and Uses

Lentil seeds are very nutritious with protein concentrations averaging 26%. This value is comparable with that of faba bean, higher than that of chickpea and more than double that of wheat (Adsule 1989). However, like other grain legumes, there is a shortage of tryptophan and the sulfur-containing amino acids, methionine and cystine, which are relatively rich in cereals. Also lentils, like chickpeas and faba beans, are good sources of vitamin B, but are poor in carotene and vitamin C. (Adsule 1989).

In addition to its use for human nutrition, residues from threshing of the lentil crop such as dried leaves, stems and fruit walls are also essential for livestock feeding. One cultivar of lentil (Indian head), which has the capability of producing an

abundance of foliar material, is used as a green manure in Canada to improve soil nutritional status. Also to grow lentils in rotation with other crops has the advantage of fixing nitrogen when effectively nodulated, thus reducing the demand for nitrogen fertilizers and depletion of inorganic nitrogen from soil (Muehlbauer 1995).

1.1.4. Factors Limiting Lentil Production

Singh and Saxena (1993) describe the abiotic stresses that affect lentil growth as cold, drought, heat, salinity, nutrient deficiency and nutrient toxicity. Of these stresses, drought and heat are considered the most important world wide (Turner 2001). Muehlbauer (2006) states the importance of cold stress especially in the West Asia-North Africa region whereas salinity is known to be an important stress factor in the Indian sub-continent and to some extent in West Asia-North Africa. Nutrient deficiency and nutrient toxicity is considered as less important world-wide.

Foliar diseases are the most serious biotic stresses affecting lentil crops (Muehlbauer 2006). Ascochyta blight caused by *Ascochyta lentis* is problematic to various degrees in all lentil growing regions of the world, but especially damaging in Canada, Australia and Middle Eastern countries (Ahmed and Morrall 1996, Ahmed 1996). Other major biotic stresses of lentil include Anthracnose caused by Colletotrichum *truncatum*, botrytis grey mold caused by *Botrytis fabae* and *Botrytis cinerea*, stemphylium blight caused by *Stemphylium botryosum*, lentil rust caused by *Uromyces fabae* and sclerotinia white mold caused by *Sclerotinia sclerotiorum* (Muehlbauer 2006).

Weeds are another important limiting factor for the yield of food legumes, which have small initial growth rates and so compete poorly with weeds for light, water and nutrients. If not adequately controlled, weed infestations can reduce yields by

as much as 75 % and in the case of parasitic weed *Orobanche crenata*, complete crop loss can be the result (Knott and Halila 1992).

1.1.5. Lentil Production in Turkey

Lentil is one of the principal food crops cultivated in the semiarid regions of the world, particularly in the Indian subcontinent and in the dry areas of the Middle East (Muehlbauer 1995). India, Turkey, Canada, USA, Australia and Syria can be considered as the major lentil producers. World lentil production has tripled in the last three decades from 1.05 million MT in 1971 to 3.8 million MT in 2004, through a 124% increase in sown area and a 58% increase in average national yield from 611 to 966 kg/ha (FAO 2004).

The total land area of Turkey is 78 million hectares of which about 25 million hectares (32 %) are cultivated, sown area accounts for 19 million hectares (79 %). Of the cultivated land, remaining 5.2 million hectares (21%) is left to fallow. Lentil utilizes approximately 5 % of the total area and is the most important food legume (Bayaner 1997). It is mostly produced for food, as an excellent complement to cereals, and sometimes as a green manure. Average lentil consumption in Turkey is 2-3 kg yr^{-1} per person and total domestic demand is 250,000 t yr^{-1} (Bayaner A. 1997).

Most Turkish lentils are red cotyledon types and are grown as winter crops in the southeastern region where winters are mild. The minority are large, green-seeded lentils which are planted in the spring in Central Anatolia or at higher elevations where winters are severe. Most of the cultivars are, as in chickpea, land-races (Sakar 1992).

In Turkey, a steady increase in the sown area and production of lentil has occurred starting from the late 1970's. However, a major change in Turkey's lentil production took place after 1982 with the implementation of the utilization of

fallow areas project but area sown to lentil and the production has been decreasing since 1989 (Bayaner 1997).

1.2. Tissue Culture and Transformation Studies in Legumes

Leguminous crops are grown throughout the world. With more than 600 genera and 18.000 species, *Leguminosae* is the third largest family of flowering plants, after *Compositae* and *Orchidaceae*. Its species are found in many diverse geographic and climatic areas, including temperate zones, humid tropics, arid zones, highlands, savannas and lowlands (Christou 1993) and it includes important grain, pasture and agroforestry plants.

Among the grain crops, grain legumes (also known as pulses or food legumes) rank third behind cereals and oilseeds in world production and constitute an important dietary constituent for humans and animals. Production of cereals (2029 million metric tones) dominates world food production while pulses (55 million metric tones) play an often-underestimated role as nitrogen fixing crops. Grain legumes are mainly cultivated in developing countries where they accounted for 61.3 million hectares in 2002, compared to 8.5 million hectares in developed countries (Popelka 2004). Legumes associate with nitrogen fixing bacteria and play a central role in low input production systems, particularly on small-scale farms (Graham 2003).

Although very rich in protein content, legumes in general have a shortage of amino acids methionine and cysteine. Also like other crops, legumes suffer from a variety of biotic and abiotic stresses. Although legumes are proven difficult for both genetic modification and regeneration, applications of gene transfer technology started to provide solutions for various constraints that limit production, and also for the improvement of nutritional quality. Various studies on the application of gene transfer techniques for legume plants and development of a model legume plant have also been performed with the aim of understanding the molecular bases and unique metabolic pathways of nitrogen fixation.

1.2.1. Tissue Culture Studies

Plant regeneration from any type of explant involves various phases. De-Klerk (1997) summarizes these stages as dedifferentiation, induction and realization (Figure 1-1). At the first stage, tissues become competent to respond to either organogenic or embryogenic stimuli. During induction stage, cells become determined to form a shoot, root or an embryo. At the final stage, outgrowth of cells to an organ or embryo is completed. First stage in regeneration may involve a period of callus growth in which case the system is called indirect organogenesis. However, usually the cells become competent without a callusing phase and directly turn into an organ or embryo which is called direct regeneration. In recent years, many research groups have been involved in establishing reliable regeneration procedures for agronomically important legumes, since it would be a primary step to facilitate gene transfer and improvement of the crop.

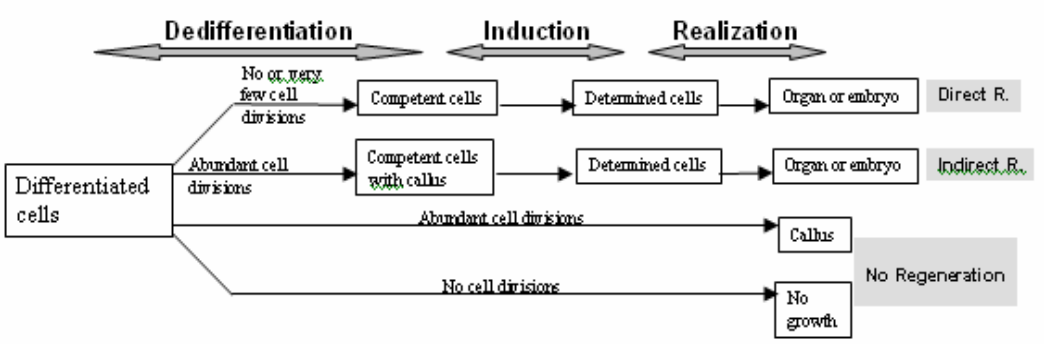


Figure 1.1. Sequence of developmental phases during regeneration (modified from De-Klerk 1997).

1.2.1.1. Direct Organogenesis in Legumes

In direct regeneration systems, the time required for the formation of shoot buds and their complete differentiation into shoots are comparatively short and the recovery of fully differentiated plants is usually higher, making such systems very efficient for genetic transformation work, although several disadvantages also exist.

For example, production of high frequency chimeric plants during genetic transformation followed by a direct regeneration system is mainly the result of targeting mature, differentiated tissue for transformation and easy escape of partially transformed regenerants from selective pressure due to the presence of blocking tissues between media and regenerating tissue. However direct regeneration technique is still very preferable and applied for the regeneration of various legumes.

Literature screening of recent legume tissue culture studies includes efficient direct shoot regeneration from various important legume species including common bean, soybean and chickpea. In most of the studies, cotyledonary nodes, axillary buds on nodal regions, embryo axis and half embryos are the primary explants, although leaves, epicotyls, hypocotyls and immature cotyledons are also used.

A method designed by Cruz de Carvalho (2000) aimed direct shoot regeneration of the commercially important common bean, *Phaseolus* v*ulgaris* L., using the transverse thin cell layers (tTCL) isolated from 2-week-old seedlings which were germinated on 10μM thidiazuron (TDZ). After 2-weeks of culture on 10 μM TDZ, proliferating tTCLs showing shoot organogenesis were excised from the epicotyl. This was followed by a transfer of proliferating tTCLs to a secondary medium of reduced TDZ concentration with 1 μM. On tTCLs excised from the cotyledonary nodes, the frequency was less than 1%. Transverse TCLs from the hypocotyl, cotyledons and roots did not regenerate. An incubation period longer than 2 weeks with 10 μM TDZ concentration resulted in inhibitory effects on the development of shoots and roots. Shoot development was enhanced by 10 μM benzylaminopurine (BA) and 10 μM silver nitrate (AgNO3). Rooting was obtained on MS medium (Murashige and Skoog 1962) containing 1μM a-naphthalene acetic acid (NAA) and 10 μM AgNO3 with 80% of the regenerated plants. The survival rate of rooted plantlets transferred to soil was 95% and the plants were fertile.

A similar protocol was also applied for cowpea (*Vigna unguiculata* L. Walp) cotyledonary nodes by Van Le (2002). The seeds were germinated on media containing MS salts and B5 vitamins (Gamborg 1968) supplemented with TDZ. 10 µM TDZ pre-treatment, shoot tip removal and excision of longitudinal thin cell layers (TCL) at the level of the cotyledonary nodes with subsequent culture on a MSB5 medium supplemented with 1µM indole-3-butyric acid (IBA) and 1µmol/L TDZ were the optimal conditions for maximum bud proliferation. Up to 32.5 regenerated shoot buds were produced per TCL. To promote shoot elongation and rooting, clusters of buds excised from the explants were cultured on a medium containing 1 µM IBA in which the buds developed into shoots then formed roots with a frequency of 80%.

Neves (2001) devised a propagation system for *Medicago truncatula* where the addition of growth regulators was restricted to the induction phase to be able to reduce the risks of epigenetic and somaclonal variation. In the first step, multiple shoots were induced from the cotyledon axillary meristem of pregerminated seeds in MS medium supplemented with 9.3 μM zeatin, 22.2 μM BA or 4.5 μM TDZ. In the second step, the induced shoots were allowed to develop in growth-regulator-free medium. BA at 22.2 μM resulted in the best shoot quality and highest number of shoots. The method provided a continuous supply of embryogenic competent explants that can be readily multiplied before a somatic embryogenesis or transformation experiment.

Tzitzikas (2004) developed a procedure in which plants were regenerated from meristematic tissue on nodal segments of four different pea cultivars. As a first step, stem segments with one node were subcultured on medium containing TDZ with the result of multiple shoot production. When subcultured on the same medium the multiple shoots were able to produce shoots which are fully covered with small buds in a cyclic fashion. When they were cultured on a medium supplemented with a combination of gibberelic acid (GA), BA and NAA, they formed shoots which were rooted on medium supplemented with 0.5 mg/l NAA,

indole-3-acetic acid (IAA) or IBA. Acclimatization of the plants to the greenhouse and development of the plants to maturation were also successful and the system seemed to be genotype independent.

Shan (2005) developed a very similar organogenesis system for soybean in which pretreatment of seeds with TDZ formed multiple bud tissue (MBT) this time at the cotyledonary node explant. Presence of intact axillary buds on the cotyledonary nodes was required for MBT initiation. Pretreatment of the seeds for 1 week on medium supplemented with 0.1 mg/l TDZ which is followed by culture of the cotyledonary nodes on medium supplemented with 0.5 mg/l BA for 4 weeks was the most effective treatment for MBT formation. Culture of the MBT on medium supplemented with 0.1 mg/l TDZ resulted in the proliferation of MBT which was maintained in this way for 12 months. Three hundred thirty six shoots were obtained when 1 g of MBT was subcultured on medium supplemented with 0.5 mg/l BA. Plants were rooted on medium without growth regulators. It was reported that regenerated plants grew normally in the greenhouse but did not set seeds because of the long-day conditions during growth. The regeneration system was recommended for transformation studies since MBT contains much more meristematic cells than a single cotyledonary node.

In another study by Uranbey (2005), an efficient micropropagation system was developed for Persian clover (*Trifolium resupinatum* L.). Three different explants (cotyledonary node, node and hypocotyl) were cultured on MS media containing BA and IBA or BA, Kinetin (KIN) and IBA. The best shooting response in terms of shoot number per explant and percentage of explants producing shoots was observed on media containing 7.1µM BA and 1µM IBA or 14.1µM BA and 1µM IBA with cotyledonary node explants. The highest percentage of rooting (73.33%) and the number of roots per shoot (3.06) were induced on media with 8 µM IBA. Adaptation and survival rate of the plants on greenhouse were also efficient with 77.7 to 100%.

Mundhara (2006) was able to induce shoots from cotyledonary node and epicotyl regions of 3-day-old mung bean (*Vigna radiata*) seedlings germinated on solid minimal media by flooding them in liquid minimal media containing 1μM TDZ. Shooting response of seedlings germinated on solid media with 1μM TDZ followed by flooding with plain liquid mineral media was not as effective as germinating the seedlings on media with 1μM TDZ and flooding with the same media, which was claimed to have a synergistic effect. In a comparative study TDZ (1μM) was found to be more effective than BAP (10μM). The shoots developed on epicotyl had normal morphology and readily rooted on medium supplemented with 1 μM IBA. The plantlets survived in high frequency (70–80%) upon transfer to soil and were able to set seed. It was suggested that in vitro regeneration is a stress-related response and flooding stress is responsible from high shoot induction in this particular study.

In a study by Chakraborti (2005), cotyledons with half embryos were isolated from 2 days-old germinated seedlings of chickpea and used for shoot induction. Among different hormone combinations tested, modified MS medium with 1.5 mg/L BA and 0.04 mg/L NAA induced a maximum number of shoots (26) from a single explant after 20 days of culture. Further culture in modified MS medium containing 0.2 mg/L IAA, resulted in elongation of the shoots (80%) from each regenerating explant in another 20–25 days. After grafting of elongated shoots to rootstocks, 90–95% of the shoots survived in soil and produced seeds.

Another direct shoot organogenesis protocol utilizing the embryo axes explants derived from mature seeds of Bambara groundnut (*Vigna subterranea* L. Verdc.) was developed by Lacroix (2003). MS media with different concentrations of BA or TDZ was tested to initiate multiple shoots. Culture of embryo axes explants on media supplemented with 1 mg/L BA and 1 mg/L NAA increased efficiency of regeneration which was followed by transverse excision of the explants and culturing on a medium containing 1.5 mg/L BA. Shoot regeneration frequency was 100% with up to eight shoots per explant. Histological studies showed the origin of

proliferating buds as the superficial layers of the explants from where proliferation occurred without an intermediate callus phase. Rooting of regenerated shoots was achieved on media containing 1mg/L NAA and plantlets were transferred to greenhouse successfully. Flow cytometric analyses and chloroplast counts of guard cells showed the diploid nature of regenerants which were also morphologically normal and fertile.

A similar explant, shoot apices of 3 day old seedlings was used by Mao (2006) for in vitro regeneration of blackeye cowpea *Vigna unguiculata* L. Walp. The optimum conditions for shoot induction composed of a complex medium including MS salts, B5 vitamins, 8.88 µM BA, 1 g/L casein hydrolysate and 342 µM L-glutamine. The media produced 77% shoot initiation frequency with a maximum of eight shoots per explant. Enrichment of the shoot initiation medium with 14 µM GA was necessary for shoot elongation. Presence or absence of IBA in the rooting medium did not change rooting efficiency. The regenerated plantlets developed normally and produced seeds.

Mohamed (2006) developed a regeneration system for *Phaseolus angularis* non-meristematic epicotyl explants which are not very common components of direct regeneration systems. The explants were isolated from etiolated seedlings and cultured on medium with B5 vitamins containing either 5.0 µM TDZ or BA. Both BA and TDZ at 5.0 µM were equally effective for bud formation with an average of 18 (65%) shoots per explant. Bud formation rate as well as frequency of regenerating explants increased when cultures were pre-cultured in the dark. Medium with 2.5 µM BA was optimal for the conversion of multiple buds into shoots within 3–4 weeks with simultaneous formation of new buds. Media containing 4.0 µM GA in combination with 0.4 µM IBA and 12.5 µM AgNO3 was found to be the most effective for shoot elongation. Pulse treatment of shoots for 10 minutes with liquid medium containing 4.5 µM IBA increased rooting efficiency up to 98%. Pulse treatment was followed by subsequent culture on hormone free MS basal full-strength solidified medium. The rooted plantlets were transferred to the

greenhouse successfully where they grew to maturity with normal flower and pod development.

A more simple procedure for the regeneration of epicotyl explants isolated from 7 days-old axenic seedlings of another grain legume grasspea (*Lathyrus sativus* L.) was described by Barik (2005). Of five different explants (cotyledon, hypocotyl, epicotyl, internode and leaf) tested, epicotyl segments were shown to be the most responsive. MS medium supplemented with 17.76 μM BA and 10.74 μM NAA produced the highest percentage of direct shoot regeneration. Five different cultivars gave different responses among which cultivar IC-120487 showed the highest regeneration frequency with 80 % and a maximum shoot number of 8.2 shoots per explant. Rooting was achieved in half-strength MS medium containing 2.85 μM IAA with 78% efficiency. The plantlets were transferred to soil with a survival rate of 75 %.

Another uncommon explant for direct regeneration, petiolar cut end of primary leaf, was used for the regeneration of pigeonpea (*Cajanus cajan* L. Millsp.) in a very rapid and simple procedure by Dayal (2003). Leaf explants isolated from 5 day old seedlings produced multiple adventitious shoots on MS medium containing 5μM BA and 5μM kinetin with 90% efficiency. Shoot buds, originated from the petiolar cut end of the explants, elongated rapidly on medium containing 0.58 μM GA. Rooting efficiency of elongated shoots on MS medium with 11.42μM IAA was over 80% and the plantlets were transferred to soil with 100% success.

A similar procedure for leaf segments isolated from 10 day old germinated seedlings of pigeonpea was described by Yadav and Padmaja (2003). Unlike the previous procedure the shoot regeneration origin was not petiolar cut ends but the cut sides on the leaf segments. Upon induction with 5mg/L BA 25% of the explants responded with average number of 16.5shoot per explant. MS medium with 1mg/L BA was optimum for shoot elongation and medium with 1mg/L IAA and 0.1mg/L Kinetin was optimum for rooting which produced 84% efficiency and 6.4

roots/explant. Plantlets were transferred to half strength MS medium without growth regulators for one month before transferred into soil/vermiculate mixture (1:1) and finally to the field.

A different type of explant, immature cotyledons which were isolated from mungbean (*Vigna radiata*) embryos were cultured on MS medium with combinations of 1 or 2mg/L BA, 0.1 or 0.5 mg/L TDZ, 0.1 mg/L GA and 0.1 or 0.5 mg/L IAA (Tivarekar and Eapen 2001). Medium supplemented with 2 mg/L BA and 0.5 mg/L IAA produced large number of shoot buds on cotyledons and shoot development continued on the same medium upon subculture. Optimal media for rooting contained 0.1 mg/L IBA and the plantlets were transferred to soil with high efficiency.

Same type of explants, mature and immature cotyledons of soybean (*Glycine max* L.) were used for direct regeneration by Franklin (2004). Green organogenic nodules which were generated at the proximal ends subsequently turned into shoot buds on modified MS medium. The presence of 13.3 μM BA and 4.54 μM TDZ in the medium produced the highest regeneration efficiency which is 84% for mature cotyledons and 55% for immature cotyledons with average shoot number of 19.2 and 7.3 respectively. High regeneration efficiency was attributed to the synergistic effect of two cytokinins since either cytokine was not as efficient alone. The regenerated shoots elongated on 0.29 μM GA and rooted on 2.69 μM NAA. Rooted plants were transferred to soil with 87% efficiency and able to produce viable seeds.

1.2.1.2. Indirect Organogenesis in Legumes

Direct organogenesis usually occurs from the existing meristematic cells which respond with direct adventitious budding upon exposure to the appropriate exogenous cytokinin. Unlike direct regeneration, in indirect organogenesis systems there is the advantage of producing regenerants originated from nonmeristematic

tissues which helps eliminating chimerism problems when subsequently used in transformation experiments. The most common explant types used in indirect regeneration includes hypocotyl, leaf, epicotyl, immature/mature embryo and cotyledons which are usually separated from the proximal ends that contain meristematic tissues.

Sancak (2000) described a shoot regeneration system for immature cotyledons and immature embryo axes of Hungarian vetch (*Vicia pannonica* Crantz). Response of embryo axes was higher than immature cotyledons in terms of regeneration capacity. Highest frequency of shoot regeneration from immature cotyledons was obtained on MS medium containing 20 μM BA and 2.5 μM NAA while immature embryo axes responded on medium containing 5 μM BA and 5 μM NAA. Rooting was achieved on half-strength MS medium containing 5 μM IBA with 50% efficiency and further development of plants on greenhouse was successful with 45% efficiency.

Saafi and Borthaakur (2002) developed two different plant regeneration methods for *Leucaena leucocephala*. First method involved a callus phase in the organogenesis route in which cotyledon, hypocotyl and root segments were cultured on MS medium containing different concentrations of BA, 2,4-dichlorophenoxyacetic acid (2,4-D), and NAA. Callus was obtained only from the cotyledon and hypocotyl explants. The callus generated from the hypocotyl explants did not produce shoots and the root explants died without forming any callus. Only the friable calli generated from the cotyledons formed shoots. In the second method excised cotyledons were cultured on MS medium containing 10–35 mg/L BA and transferred to regeneration medium containing low BA which leaded the explants to direct organogenesis route with 100% efficiency. All of the regenerated shoots formed roots on hormone free basal media.

A regeneration protocol was developed by Sairam (2003) for the cotyledonary nodal callus of three different soybean cultivars. Callus induction and subsequent

shoot formation were initiated from the proximal end of cotyledon explants on modified MS media containing 2.26 μM 2,4-D and 8.8 μM BAP, respectively. Various carbon sources were also tested and sorbitol was found to be the best for callus induction and maltose for plant regeneration. All three cultivars were responsive to the protocol.

Another *in vitro* organogenesis protocol was applied by Amutha (2003) to the cotyledon and hypocotyl explants of *Vigna radiata*. Organogenic calli were obtained on MS medium containing 1.07 μM NAA and 2.22 μM BA for cotyledon explants and 0.90 μM 2, 4-D and 2.22 μM BA combinations for hypocotyl explants. Cotyledon derived calli produced adventitious shoots when cultured on MS medium containing 1.07 μM NAA, 8.88 μM BA and 10% coconut water whereas regeneration of shoots from hypocotyl derived calli was achieved when cultured on MS medium containing 6.66 μM BA, 2.5 μM TDZ and 10% coconut water. GA at a concentration of 1.73 μM helped shoot elongation. Rooting was achieved on half strength MS medium with 4.90 μM IBA followed by successful establishment of the plantlets in field. Throughout the protocol one hypocotyl derived callus produced an average of 7 plants and a cotyledon derived callus produced 15 plants in a period of 3 months.

Among various explants of guar (*Cyamopsis tetragonoloba* L. Taub) tested by Prem (2005), namely the embryo, cotyledons, cotyledonary nodes, shoot tip and hypocotyl, cotyledon and embryo explants were found to be the most amenable to callus induction and regeneration. MS medium supplemented with 10.0 μM 2,4-D and 5.0 μM BA induced green and friable morphogenic callus with 95% of efficiency. Culturing the callus on MS medium containing 13.0 μM NAA and 5.0 μM BA caused 88.4% of the callus clumps produce 20–25 shoots. Half strength MS medium with 5.0 μM IBA was effective for the production of healthy roots from 90% of the shoots.

Different landraces of Bambara groundnut (*Vigna subterranea* L. Verdc) were tested for organogenesis in a study by Kone (2007), in which the cotyledons were again assessed to be one of the most responsive explants. Medium containing 3 mg/L BA and 0.5 mg/L NAA gave the best callusing response for cotyledon explants. Shoots were regenerated from cotyledons with 6% efficiency on media with BA alone (3–5 mg/L) or combined with 0.01– 0.1 mg/L NAA. Roots were produced on 3–5 mg/L BA but at a higher concentration of NAA (0.5 mg/L). The highest callusing with epicotyls was achieved on 3 mg/L BA and 0.5 mg/L NAA and shoots were regenerated with 15–20% efficiency on 3 mg/L BA alone or with NAA at concentrations that depended on the landrace. Rooting was achieved on hormone-free medium and the plants transferred to the greenhouse were morphologically normal and fertile. A flow cytometry data were also supplied which shows the diploid nature of regenerants.

In another study by Khanna (2006), immature embryos of a medicinal legume *Argyrolobium roseum* were used for plant rejeneration. Embryos that were excised from 10 day old pods produced green nodular calli on MS medium supplemented with 0.5 mg/L BA and 0.25 mg/L IAA, with 70 % efficiency. The same medium also supported elongation of shoots initiated from organogenic calli. Rooting was achieved on Gamborg's medium supplemented with 0.5 mg/L IBA with 80 % frequency.

In another study by Rey (2000), regeneration of a legume *Arachis pintoi*, which belongs to the same genius with peanut, was succeeded by using two developmental paths; organogenesis and somatic embryogenesis. Leaf pieces were used as explant source for the induction of organogenic callus cultures on MS medium containing NAA or 2,4-D in combination with BA and kinetin. The best media for callus formation included 10mg/L NAA and 1mg/L BA while MS media with 1mg/L BA was the best for shoot induction. Rooting was obtained on medium supplemented with 0.01 mg/L NAA and the plantlets were transferred to soil with

90% efficiency. Somatic embryogenesis pathway used for this particular study is explained in more detail under the following heading.

1.2.1.3. Regeneration via Somatic Embryogenesis

Somatic embryogenesis is an alternative pathway to organogenesis in which a single somatic cell develops into an embryo that finally forms the whole plant. When compared to organogenesis, it usually requires a much longer culture period. In somatic embryogenesis studies, the explant is taken from a suitable plant part which usually contains tissues rich in parenchyma cells such as pith, cortex, mesophyll or phloem parenchyma from a storage organ, and is placed in a culture medium with appropriate nutrients and usually a synthetic auxin such as 2,4-D (Srivastava 2002). In this medium, the explant produces callus which may have one or more centers of high meristematic activity that called proembryogenic masses (PEMs). PEMs may have hundreds of embryo-like structures and when plated on a medium which lacks exogenous auxins, further development of the embryos continues.

Although somatic embryogenesis is a very important process with many studies conducted for various plant species, its components are still full of unknowns. For example, the role of plant hormones on the conversion of somatic tissues into embryogenic ones and the subsequent maturation of somatic embryos are far from being completely understood. This brings the requirement of trial and error type experimentation to choose the proper conditions, doze and combination of plant hormones that allow efficient induction and development of somatic embryos (Jimenez 2005). Recent articles propose various procedures for somatic embryogenesis in important legume species including soybean, pea and peanut.

Optimization studies for the regeneration of soybean (*Glycine max* L. Merrill) by somatic embryogenesis started at late 80s through the work of Lippman and Lippman (1984), Lazzeri (1987), Finer and Nagasawa (1988) and Parrott (1988). In

the first study, somatic embryos formed when cotyledons from immature soybean seeds were cultured on 0.5 to 1mg/L 2,4-D but subsequent plant regeneration was unsuccessful.

Lazzeri (1987) used 10mg/L NAA for embryo formation/ normality and determined the optimum concentration of sucrose in MS medium as 1.5%. Finer and Nagasawa (1988) cultured the same type of explants on MS media with B5 vitamins containing 6% sucrose and 40mg/L 2,4-D to obtain highly embryogenic callus. Globular embryos formed in suspension culture containing modified MS salts (with 10mM NH_4NO_3 and 30mM KNO_3), 15mM glutamine and 5mg/L 2,4-D which was called FN medium. Globular embryos were regenerated on hormone free MS media with B5 vitamins. Parrott (1988) also used immature cotyledons to induce embryogenesis on MS media, B5 vitamins, 1.5% sucrose and 10mg/L NAA in two weeks. Further culture on hormone free media for six weeks resulted in formation of somatic embryos. All the following studies were done for the perfection of standardized procedures.

Bailey (1993) used the same procedure as Finer and Nagasawa (1988) to choose the best responding cultivar. Genotype effects were different at each stage of the procedure. The most regenerable genotype was chosen as PI417138 for the better embryo yield, germination and conversion capacity.

Samoylov (1998) increased the growth rate of somatic embryos up to 250% over that obtained in FN medium by including lower concentrations of sucrose (87.6 to 29.2 mM) and total nitrogen (from 50 to 35mM). Due to its lowered content, the new medium was designated as FN Lite. The cultures were not sensitive to the changes in ammonium, calcium, potassium and phosphate concentrations. Standard procedure using solid MS media allowed the recovery of an average 3.9 embryos/mg of embryogenic tissue however, when soybean somatic embryos were differentiated in liquid MS medium with 3% maltose, 8.1 embryos/mg of embryogenic tissue were obtained. Also the maturation time of embryos that

developed in liquid medium was only 4 weeks which is 4 weeks less than the standard procedure. Comparison of embryo development in hormone free MS medium with maltose or FN Lite medium containing maltose or an equimolar amount of sucrose showed the superiority of sucrose in promoting embryo differentiation and maturation. Usage of the new protocol allowed the development of transgenic seeds within 9 months following biolistic bombardment, which is 4 months faster than the solid medium based protocol. The same group also tested the effects of polyethylene glycol (PEG) 4000, mannitol and sorbitol as supplements of FN Lite medium (Walker and Parrott, 2001). Addition of 5% PEG or 3% sorbitol improved germination frequency without effecting embryo differentiation efficiency while it reduced the fresh weight of mature embryos by 22%. Addition of 3% mannitol did not reveal any improvement for differentiation or maturation. In another work by Schmidt (2005), it was reported that addition of organic nitrogen supplements, 30mM glutamine and 1mM methionine to FN Lite media helped the accumulation of storage reserves and produced somatic embryos which are more similar to soybean seeds in terms of lipid and protein contents.

Somatic embryogenesis studies in pea (*Pisum sativum* L.) were initiated with the works of Jacobsen and Kysely (1984, 1990). In their first study, leaf-derived calli were cultured in liquid MS medium containing low concentrations of picloram (0.06 mg/L), which resulted in the development of embryoids on the surface of calli. Later on embryoids spontaneously separated from the calli and developed into torpedo-shaped embryos which was followed by the transfer to solid medium although germination of the embryos was not successful on the media tested. In their following study, Jacobsen and Kysely used immature zygotic embryos and shoot apices as explant source and the auxins, picloram and 2,4-D as growth regulators. Again embryogenesis was initiated up to a point and histological examinations confirmed the embryogenic nature of the cultures but the germination efficiency of the embryos was insignificant.

Loiseau (1998) induced somatic embryos from pea shoot apices and cotyledons of immature embryos by culturing them in 4.5μM picloram followed by a culture on 4.5μM zeatin. Immature cotyledons produced more embryos with higher germination (94%) and plant conversion rates (40.7%). Low conversion rates were linked to the lack of protein and starch reserves in the callus which is necessary for the embryo development. Multicellular origin of somatic embryos was also confirmed with histological studies.

Griga (2002) induced embryogenic callus both by the way of indirect and direct somatic embryogenesis. For the first method, immature pea zygotic embryos and shoot apices were cultured on 2.26 μM 2,4-D and 2.5 μM picloram, respectively. Development of embryos took place on the same media or hormone free MS media. For direct somatic embryogenesis, shoot apical meristems were cultured on MS medium with 2.5 μM picloram. Likewise, development of embryos continued on the same medium or hormone free medium. Histological examinations showed the similarity of globular and hearth shaped somatic embryos with their zygotic counterparts whereas torpedo and cotyledonary somatic embryos showed variation mainly on the size, shape and number of the cotyledons.

Somatic embryogenesis optimization studies in peanut (*Arachis hypogaea* L.) started with the use of various explants, immature zygotic embryos (Ozias-Akins 1992, McKently 1995), mature zygotic embryo axes (Baker 1995), immature cotyledons (Ozias-Akins 1989, Durham and Parrott 1992, Ozias-Akins 1992) and leaves from germinating seedlings (Baker and Wetzstein 1992).

Victor (1999) used intact peanut seeds to examine regeneration response under exposure of BA and TDZ. Histological observations showed that structures which formed on cotyledonary node region by 10μM TDZ treatment were somatic embryos while the ones induced by 50μM BA were shoots. The seeds were also exposed to TDZ or BA through vacuum infiltration before culture on MS basal

media. Exposure to TDZ was successful while the explants exposed to BA could not regenerate.

In another study, Little (2000) examined the effects of 11 different auxins (centrophenoxine, chlorogenic acid, *p*-chlorophenoxyacetic acid, *trans*-cinnamic acid, dicamba, NAA, 2,4-D, phenylacetic acid, picloram, 2,4,5-trichlorophenoxy acetic acid, and 2,3,5-triidobenzoic acid) and one cytokinin-like compound *N*-(*c*-chloro-4-pyridyl)-*N*-phenylurea at four concentrations on somatic embryo formation from peanut epicotyls. The best results were obtained with treatment of 83μM picloram and 124.4μM centrophenoxine both in terms of embryo number and percentage of explants responding. Period of embryo conversion into mature plants was 5 months.

Rey (2000) initiated embryogenic calli from leaf pieces of pinto peanut (*Arachis pintoi*) on MS medium containing 20mg/L picloram and 1mg/L BA. Somatic embryo differentiation occurred on 1mg/L BA, while further development occurred on activated charcoal without hormones. The same group also regenerated diploid and triploid pinto peanut plants through somatic embryogenesis from shoot tips and immature leaves of in vitro germinated plants (Rey and Mroginski 2006). Shoot tips and immature leaves from diploid peanut produced the highest number of somatic embryos when cultured on medium containing 10mg/L picloram/0.1mg/L BA, and 10mg/L picloram/0.01mg/L BA, respectively. Triploid peanut responded best on MS medium containing 10mg/L picloram/0.01mg/L BA and 20mg/L picloram/0.01mg/L BA for shoot tips and leaves, respectively. Plant conversion of all somatic embryos took place on MS medium containing 0.01mg/L NAA and 0.01mg/L BA with 25% efficiency.

First of the few studies on cowpea (*Vigna ungniculata* L Walp) somatic embryogenesis reported high frequency regeneration from primary leaves (Anand 2000). Embryogenic calli was formed on MS medium supplemented with 2,4-D and used for the establishment of suspension culture. Development of globular,

heart-shaped and torpedo-shaped embryos was achieved in suspension culture containing 4.52 µM 2,4-D. Media with 0.05 µM 2,4-D, 5 µM abscisic acid and 3% mannitol was used for the maturation of embryos. Embryos were converted into plants with 22% efficiency and the survival rate in the field was 8-10%.

Ramakrishnan (2005) also used primary leaf-derived embryogenic calli of cowpea which was initiated in MS medium with B5 vitamins containing 2,4-D, casein hydrolysate, and L-glutamic acid-5-amide to obtain regeneration through somatic embryogenesis. Liquid MS media with 0.5 mg/L 2,4-D was necessary to obtain embryogenic cell suspensions. Maturation was established in liquid B5 medium containing 0.1 mg/L 2,4-D, 20 mg/L L-proline, 5 µM abscisic acid, and 2% mannitol. The best carbon source for callus induction was sucrose while mannitol and maltose was the best for embryo maturation and embryo germination, respectively. Complete plant development was achieved on half-strength B5 medium containing 3% maltose, 2.5g/L potassium nitrate, and 0.05 mg/L TDZ with 32% efficiency.

The first efficient plant regeneration system for pigeonpea (*Cajanus cajan* L.) somatic embryogenesis has been developed by Sreenivasu (1998). Cotyledon and leaf explants from 10-day-old seedlings were used as explants. Both embryogenic callus induction and somatic embryo development took place on MS medium containing 10µM TDZ. 73.7% of the leaf calli showed embryogenesis while only 45.3% of the cotyledon calli turned into embryos. Maturation and growth of the embryos required the withdrawal of TDZ and occurred with 70% efficiency on MS basal medium. The regenerated plantlets developed normally and produced seeds.

The first liquid suspension protocol for somatic embryo development from pigeonpea was developed by Anbazhagan and Ganapathi (1999). Embryogenic calli was derived from seedling leaf explants on semisolid MS medium containing 6.78 µM 2,4-D, while somatic embryos were developed on MS liquid medium

containing 4.52 μM 2,4-D. The germination of somatic embryos was achieved on hormone-free MS basal medium and the conversion frequency to plants was 5-6%.

In another study, cotyledon segments of mature pigeonpea seeds were used to induce somatic embryos on MS basal medium supplemented with 2.22, 4.44, 13.32 or 22.2 μM BA and 0.45, 1.36, 2.27, 4.54 and 13.62 μM thidiazuron (Mohan and Krishnamurthy 2002). The best response was seen on MS media containing 4.44 μM BA. Development of globular somatic embryos into cotyledonary stage was achieved on medium containing 2.89 μM GA while maturation was achieved on half strength MS medium with 0.38 μM abscisic acid. Germination took place on media containing 0.44 μM BA with 39-54% efficiency.

Singh (2003) observed different effects of various TDZ concentrations on pigeonpea seedlings which were germinated from decoated seeds. Somatic embryos were induced at the cotyledonary node region when the seedlings were germinated on MS media with 10 μM TDZ, whereas, 1 μM and 5 μM TDZ induced multiple shoots and clusters of leafy structures, respectively. Short exposure (16-48 h) of seeds to 10.0 μM TDZ and subsequent transfer to MS medium also caused multiple shoot formation but not somatic embryogenesis. Medium containing 2.5 μM IBA was optimal for root development and rooted plantlets survived with 95% efficiency.

First successful chickpea (*Cicer arietinum* L.) regeneration through somatic embryogenesis was performed by Barna and Wakhlu (1993) from immature leaflets. Globular embryo formation took place on MS medium supplemented with 25 μM 2,4-D and embryos matured on basal MS medium. Subsequent embryo conversion into plantlets was achieved on media with 15μM ABA and 1μM IBA with very low efficiency.

In a following study, again leaf explants of chickpea were used to obtain somatic embryos on MS medium containing 1.25 mg/L 2,4-D and 0.25 mg/L kinetin

(Kumar 1994). MS medium with B5 vitamins which contains 0.125 mg/L IBA and 2 mg/L BA was optimal for embryo maturation. Germination of embryos was obtained on medium supplemented with 0.25 mg/l BA and further development took place on basal medium.

Dineshkumar (1995) also used leaf explants of chickpea for somatic embryo formation on MS medium with B5 vitamins containing 0.25 mg/L 2,4-D, 0.25 mg/L picloram and 0.1 mg/L BA. Maturation of embryos was achieved on modified B5 medium with 2 mg/L BA and 0.1 mg/L IAA. Germination of embryos took place on basal B5 medium containing 0.25 mg/L BA which was followed by development into plantlets on basal B5 medium.

TDZ was used by Murthy (1996) to induce regeneration from chickpea mature seeds by two different routes. Multiple shoots formed when cotyledonary node explants was cultured on medium with TDZ in concentrations ranging from 1 to 100 μM. However when the medium was enriched with L-proline, somatic embryos developed instead of shoots from the same region on the same explant. Germination of somatic embryos was achieved on MS medium and successfully transferred to soil.

Gupta (1997) established direct somatic embryogenesis from seedling leaf segments of winged bean, *Psophocarpus tetragonolobus* (L.). Embryogenic calli was induced on MS medium containing 0.2 mg/L NAA and 2.0 mg/L BA for 28 days as optimum duration through which 43.3% of the explants responded. This treatment was followed by the transfer of embryogenic callus to MS medium containing 0.1 mg/L IAA and 2.0 mg/L BA for embryo development. Culture on MS medium supplemented with 0.1 mg/L ABA for one week and subsequent culture on MS medium containing 0.1 mg/L IBA and 0.2 mg/L BA was necessary for plant conversion with 53.3% efficiency.

Regeneration of a perennial forage legume, *Astragalus melilotoides* through somatic embryogenesis was achieved by Hou and Jia (2004), by the use of protoplasts isolated from hypocotyl-derived embryogenic calli. Hemicellulase, Pectinase and Macerozyme were used in the preparation of protoplasts and the first cell divisions started after 3–7 days from culture initiation. The optimum division frequency were obtained in medium containing 1.0 mg/L 2,4-D, 0.5 mg/L BA, 0.2 mg/L kinetin, 0.2 M glucose, 0.3 M mannitol and 500 mg/l casein hydrolysate. Somatic embryo development from protoplast derived calli occurred on MS medium containing 0.5 mg/L NAA and 1 mg/L BA with 56.3% efficiency. Rooting was achieved on hormone free MS medium and regenerated plants survived in soil with 81% efficiency.

Devi (2004) used mungbean (*Vigna radiata* (L.) Wilczek) explants; mature cotyledons, hypocotyl, nodal segment, and leaf explants to induce somatic embryos. MS medium with 1.809 μM 2,4-D and 3.555 μM BA and incubation under 24/0 hour light/dark cycle was optimal for obtaining embryogenic callus while the highest rate of embryo germination and conversion obtained from leaf explants on media with 3.94 μM IBA. 92% of the plantlets survived in the greenhouse.

Embryos of *Phaseolus angustissimus*, 3 days after pollination, cultured inside pods for 1 week in MS basal media containing 1000mg/L casein hydrolysate and 1000mg/L glutamine, then isolated and cultured further 2 weeks on MS media with 250 mg/L casein hydrolysate, 500 mg/L glutamine and 1.9 μM ABA (Schryer 2005). Embryo germination took place on basal medium containing 0.25 μM BA. 29.3% of the embryos regenerated and fertile plants were recovered.

Embryogenic callus from legume horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.) was obtained from cut slices of the immature cotyledons on MS solid medium including 1.0 mM zeatin and 4.5 mM NAA (Mohamed 2005). During culture for 3 weeks on MS liquid medium containing only 5.6 mM NAA, 26.4 % of the calli were covered with somatic embryoids. Within 5 weeks embryoids

developed into globular, heart and torpedo shaped embryos and further transfer into MS medium without any plant growth regulator achieved maturation. However, only few numbers of somatic embryos (5%) developed into fertile plants.

1.2.2. Transformation Studies

Genetic engineering provides methods to acquire or improve useful and desirable characteristics of cultivars. For this reason, genetic transformation of plants is one of the most developing areas of genetic engineering. The production of transgenic plants through genetic engineering, with the introduction of stress resistance or defense response genes would be very beneficial since it could raise the yield or improve the seed quality of legume plants. Almost all of the legume transformation studies utilize either somatic embryogenesis or direct regeneration systems to produce transgenic plants and the introduced genes range from visual and selectable markers to insect/fungi defense genes or the genes that give the plant edible vaccine properties.

1.2.2.1. *Agrobacterium*-Based Transformation Studies in Legumes

Agrobacterium transformation of legumes is usually described as difficult to perform and this characteristic is mainly attributed to the poor susceptibility of regenerable legume tissues to *Agrobacterium* strains. However, the identification of more virulent strains in recent years and the use of improved transformation protocols made the genetic transformation of many legume species a routine.

Soybean is the most common legume plant which is widely the target in both biolistics and *Agrobacterium*-mediated transformation studies. The early studies on soybean *Agrobacterium*-mediated transformation reported regeneration of fertile transgenic plants by using cotyledonary node (Hinchee 1988, Finer and McMullen 1991, Di 1996) and immature zygotic cotyledon explants (Parrott 1989), but in both systems transmission of the genes to the progeny was unsuccessful. Also the

transformation system was genotype-dependent and applicable to limited number of cultivars. The study performed by Donaldson and Simmonds (2000) aimed to determine the susceptibility of various commercial soybean cultivars to three different wild-type *Agrobacterium* strains (A281, C58 and ACH5) in tumor formation assays. Although the overall susceptibility is very high, the responses were found to be both strain and cultivar dependent. The cultivars were also subjected to transformation with EHA105 carrying pBI121 and only one cultivar responded with stable *gus* gene expression. The overall efficiency of transformation was very low although the explants showed transient expression with a rate of 27-92% depending on the cultivar. The low transgenic recovery rate was attributed to problems in transformation of cells with high regeneration potential and to inefficient selection of transformed cells.

Yan (2000) evaluated three different factors taking part in *Agrobacterium*-mediated transformation of immature zygotic cotyledons of soybean. Cotyledon size was found to be very important since small explants died shortly after co-cultivation. Concentration of *Agrobacterium* cells had no significant effect on transformation efficiency, although concentration of selection antibiotic was also found to be very important. Through somatic embryo development, three transgenic plants were obtained with 0.03% efficiency. The low efficiency was suggested to be the result of inefficient somatic embryo proliferation tissue culture techniques.

Olhoft (2001) investigated the effects of certain thiol compounds, copper chelators and iron chelators on the frequency of *Agrobacterium*-mediated transformation of soybean. Different *Agrobacterium* strains, soybean genotypes and binary vectors produced improved transformation rates in the presence of thiol compounds during co-cultivation period, especially for L-cysteine. The ion chelators and all the other thiol compouns including D-cysteine, dithyotreitol, glutathione and sodium thiosulfate also increased transformation efficiency to some extent. Thiol compounds were suggested to be the inhibitors of plant defense response mechanisms and in that way decrease enzymatic browning and necrosis. Olhoft and

Sommers (2001) also obtained eight independent transgenic soybean lines carrying *gus* and *bar* genes with 2.1% transformation efficiency. Cotyledonary node tissues and AGL1 strain were used during transformation in the presence of L-cysteine in solid co-cultivation medium which was claimed to increase *Agrobacterium* infection in target tissues from 37% to 91%. In 2004, Zeng developed a selection scheme for glufosinate selection of soybean to be able to control the effects of L-cysteine used during cocultivation for which they claimed to reduce the selection pressure throughout regeneration period. Optimal selection conditions of 8mg/L glufosinate applied during first and second shoot initiation stages and 3mg/L glufosinate used during shoot elongation increased the transformation efficiency to 5.9%.

Ko (2003) transformed immature cotyledons by the help of *Agrobacterium* strain KYRT1 and induced embryogenesis under selection. The strain produced transgenic plants for two cultivars among six and was found to be effective with transformation frequencies ranging from 1.1 to 1.7%. Molecular analysis showed stable integration and expression in both T_0 and T_1 plants.

Olhoft (2003), increased transformation efficiency of soybean cotyledonary nodes to 16.4% by combining an efficient hygromycin selection protocol and an optimized co-cultivation medium including mixtures of various thiol compounds, *L*-cysteine, dithiothreitol, and sodium thiosulfate, whereas Liu (2004) used embryogenic tip regeneration system and kanamycin selection with 15.8 % transformation efficiency.

Half cotyledons with dissected embryos were also used as explant source in *Agrobacterium*-mediated transformation of soybean (Paz 2006) and 3.8% transformation efficiency was achieved with the use of EHA105 strain.

The first functional genes transferred to soybean by *Agrobacterium*-mediated transformation were *cryIA* and *pta* which gives the plant insect resistance

characteristics (Dang and Wei 2007). Embryogenic tip explants from seven different cultivars were used in the transformation experiments with KYRT1 strain which is combined with *ppt* selection. Transformation frequency changed from 4.29 to 18% depending on the cultivar and resistance of the transgenic plants to cotton bollworm was significant.

Medicago sativa (alfalfa) and *Medicago truncatula* (barrel medic) are among the most studied legume plants since they were accepted as key model plants in understanding of legume biology and symbiotic plant-bacteria interactions. First transgenic alfalfa plants were expressing *Bacillus licheniformis* alpha-amylase and mangaese-dependent lignin peroxidase (Mn-P) from *Phanerochaete chrysosporium* (Austin 1995). Although expression of alpha-amylase had no effect on phenotype, expression of Mn-P had severe effects on plant development. Any bioassay data for the assessment of resistant characteristics was not provided.

Ziegelhoffer (1999) transformed alfalfa plants with genes encoding thermostable cellulases E2 and E3 of *Thermomonospora fusca* linked to a constitutive promoter, with the aim of producing bioreactor plants. Both E2 and E3 enzymes were expressed stably in alfalfa tissues, but in very low levels (0.01% and 0.001–0.002% of total protein, respectively).

Two strains LBA4404 and C58 were used to transfer a portion of the leukotoxin gene from *Mannheimia haemolytica* to alfalfa, with the aim of developing an edible vaccine against pneumonic pasteurellosis which is widely seen in cattle (Ziauddin 2004). The gene fused with *gfp* as a marker and 30 independent lines expressed the fusion gene in detectable levels. Potential of the produced plants as a vaccine and any assessment of calf-feeding results were not provided.

Montague (2007) used phosphinothricin-based selection for alfalfa transformation with 50% of the explants giving rise to one or more transgenic plants. Leaf painting tests, PCR and ELISA analysis confirmed transgenic nature of plants.

A novel non-antibiotic selection system was applied to *Agrobacterium*-transformed alfalfa plants and the plantlets were regenerated through embryogenesis (Rosellini 2007). The system composed of a bacterial *hemL* gene encoding a mutant form of the enzyme glutamate 1-semialdehyde aminotransferase (GSA-AT) which confers resistance to gabaculine (3-amino-2,3-dihydrobenzoic acid) that is used as selective substance. The enzyme GSA-AT is already expressed in plants in very low levels. Overexpression of the gene produces a more efficient selection system then kanmycin-based selection.

Leaf segments from both *M. sativa* and *M. truncatula* plants were transformed with three different symbiosis-related nodulin promoter-*gus* fusion genes (Trinh 1998). Both plants transformed and regenerated through somatic embryogenesis with high efficiency. Expression patterns of all three fusion genes matched with the expected expression pattern of the wild type nodulin genes which gave an insight for the molecular mechanism of the symbiosis with *Rhizobium*.

High frequency transformation was achieved by the use of floral parts of *M. truncatula* in *Agrobacterium*-mediated transformation with EHA105 strain which is followed by inducing somatic embrogenesis (Kamate 2000).

Efficiencies of different *Agrobacterium* strains were evaluated by Chabaud (2003) in *M. truncatula* transformation. Among AGL1, LBA4404, C58pMP90 and C58pGV2260, AGL1 was found to be the most efficient in terms of both transgenic yield and the time required for plant regeneration.

Araujo (2004) developed a highly embryogenic genotype named as M9-10a from *M. truncatula* cv. Jemalong via somatic embryogenesis and used the line effectively in plant transformation with the strain EHA105 harbouring plasmid constructs containing the oat arginine decarboxylase gene, *Adc* and the GUS reporter gene (p35S*Adc–Gus*) or ELIP-like drought stress protein 22 (DSP22) encoding gene

from *Craterostigma plantagineum* (p35S*Dsp22*). Any bioassay data proving drought resistance characteristics of transgenic plants was not provided.

Zhou (2004) used EHA105 and AGL1 strains with equal efficiencies for genetic transformation of cotyledonary node explants of *M. truncatula* by *gfp* and *gus* genes. Average frequency for the transgenic shoot recovery was 35% and efficiency of rooting was 39%. Stable expression of the transgenes was present also in T_1 generation.

In the most recent study on barrel medic, an effective system was developed for the regeneration of *A. tumefaciens*- transformed root explants and for the *A. rhizogenes*-transformed hairy roots (Crane 2006). T_0 plants obtained from both systems stably expressed the *gus* and bar transgenes.

First report on chickpea (*Cicer arietinum*) transformation documented *gus* expressing transgenic T_0 plants which were obtained by the regenerating adventitious shoots from epicotyl explants (Fontana 1993). Three different groups (Kar 1996, Krishnamurthy 2000 and Tewari-Singh 2004) used embryo axis explants for *Agrobacterium*-mediated transformation of chickpea through a regeneration system depending on multiple shoot formation from embryonic meristem sites. Inheritance of the genes to T_1 progeny was reported in the two of the studies except Kar (1996) which obtained only T_0 plants.

Senthil (2004) reported an efficient transformation method for two chickpea cultivars by using embryo axis slices isolated from germinated mature seeds as explant source and AGL1 as the *Agrobacterium* strain, with 5.1% efficiency. Presence of the transgene in two generations of the progeny was also shown. In a separate study same type of explant was used by (Polowick 2004) with a transformation efficiency of 3.1%. The inheritance of the transgenes in T_0 was proven with southern blot and the inheritance was followed in the next two generations by GUS assay.

Sanyal (2005) transformed chickpea cotyledonary nodes with *Bacillus thuringiensis cry1Ac* gene driven by the CaMV35S promoter with 1.12% frequency by combining preconditioning on medium supplemented with DTT and L-cysteine, sonication, vacuum infiltration and the use of acetocyringone in co-cultivation media. In both T_0 and T_1 generation, significant protection against major pod borer *H. armigera* was reported.

Pea (*Pisum sativum* L.) is one of the first transformed legume plants along with soybean. First genetic transformation of pea was succeeded in 1990 by Kaerlas with the use axenic shoot and seedling epicotyl explants from which hygromycin resistant calli obtained and eventually developed into transgenic plants. The same group also followed the transmission of the transgene successfully in the next two generation (Kaerlas 1992).

In 1993, two groups independently transformed embryogenic axis of immature seeds (Schroeder 1993) and lateral cotyledonary meristems (Davies 1993) in germinating seedlings with *npt-II/gus* and *npt-II/bar* genes, respectively. For both studies, transmission of the integrated gene to the next progeny was confirmed.

Schroeder (1995) transformed pea plants with common bean α-amylase inhibitor linked to another bean seed gene promoter, phytohemagglutinin. The protein stably expressed in next generations including T_5 and was very effective in blocking the development of bruchid larvae including pea, cowpea and Azuki bean weevils.

Gran (1995) used immature cotyledons and Bean (1997) used lateral cotyledonary meristems to transform peas with bar gene. Transmission of the transgene to T_1 generation was proven to be successful in both of the studies.

Orczyk and Orczyk (2000) tested three different *Agrobacterium* strains; LBA4404, C58C1 and EHA105 on the efficiency of pea transformation and selected EHA105 as the most efficient with 8.2% transformation frequency. They also tested four

different selection regimes conferred by the genes; *npt*II, *hpt*, *dhfr* and *bar*. Number of kanamycin resistant plants was significantly higher than phosphinotricin resistant plants and no plant was regenerated on hygromycin or methotrexate containing media.

Grant (2003) tested two strains KYRT1 and AGL1 for the transformation efficiencies on immature pea cotyledons and found KYRT1 as three times more efficient than AGL1.

In the most recent study on pea, two cultivars were transformed with two different antifungal genes coding for polygalacturonase-inhibiting protein (PGIP) from raspberry (*Rubus idaeus* L.) which is controlled by double 35S promoter and stilbene synthase (*Vst*1) from grape (*Vitis vinifera* L.) driven by its own inducible promoter combined with bar gene as selectable marker (Richter 2006). The two transgenes then combined in the same plant by conventional breeding. Developed plants were proven positive for the inhibition assays against fungal polygalacturonases and the stable transmission of the genes into progeny was successful.

First *Agrobacterium*-transformation of pigeon pea (*Cajanus cajan* (L.) Millsp.) was performed by Lawrence and Koundal (2001) by using embryogenic axes explant isolated from germinated seedlings and GV2260 strain containing a cowpea protease inhibitor gene controlled by CaMV35S promoter and *nptII* gene as selection marker. Transformation rate, transmission of the gene to next progeny and insect resistance characteristics of the transformants were not mentioned in the report.

Thu (2003) developed a regeneration system for pigeon pea utilizing cotyledonary node region of germinating seedlings. By using the same regeneration system, transgenic plants were obtained both by biolistics and *Agrobacterium*-mediated

transformation. Although passage of the genes to progeny was shown by PCR and GUS assay, no data was provided on transformation frequency.

Transgenic pigeon pea expressing the 2-kb fragment of hemagglutinin (H) protein of Rinderpest virus was developed by Satyavathi (2003). The study aimed development of stable edible vaccine for wild and domestic bovids against a fatal disease caused by Rinderpest virus. EHA105 strain was used to transform embryonic axis and cotyledonary node explants from germinating seedlings. Southern and western blots confirmed transformation, and a high level of H protein expression (0.49%) was observed in leaves. The transgene was also expressed in the progeny.

Kumar (2004) transformed pigeon pea with rice chitinase (*Rchit*) gene along with *hpt* gene which gives hygromycin resistance. Cotyledonary node explants and C58 strain were used with 2.83% transformation efficiency. Integration of the transgene in T_0 and transmission of the gene to T_1 was confirmed by southern blot and RT-PCR but the effectiveness of the *Rchit* against fungal pathogens was not tested.

Surekha (2005) transformed pigeon pea with a synthetic *cryIE-C* gene, controlled by 35S promoter, which confers resistance against *Spodoptera*. GV2260 strain was used to transform embryonic segments isolated from mature seeds which were later produced shoots under kanamycin selection. Western blot confirmed the presence of cry I E-C protein in T1 and T2 generations and bioassays showed the resistance characteristics of the transgenic plants against *Spodoptera litura* larvae of first and second instar stages.

In another study, *Bacillus thuringiensis cry1Ab* gene was transferred to pigeon pea by using axillary buds of germinating seedlings (Sharma 2006). Cry1Ab protein content changed in different tissues with 0.1% in flowers and 0.025% in leaves, which were calculated by enzyme-linked immunosorbent assay. Potential of the transgenic plants for resistance to insects was not evaluated.

Thu (2007) transformed pigeon pea with a mutant *Nicotiana sylvestris* dihydrodipicolinate synthase gene (*dhdps-r1*), the key enzyme in lysine biosynthesis, to prevent its feedback inhibiton by lysine, using the same protocol described in Thu (2003). The enzyme activity and the free lysine content increased in the seeds as expected, but this did not improve the total seed lysine content. Plants were morphologically normal and passed the gene to next generation as assessed from PCR.

There are only two reports for cowpea transformation in the literature. In both of the studies cotyledonary node explants were used although *Agrobacterium* strain and selection force were different. Popelka (2006) used AGL1 strain carring gus and bar genes with 0.1% efficiency and Chaudhury (2007) used EHA 105 strain with *gus* and *nptII* genes with 0.75% efficiency. Both groups obtained fertile progeny expressing transgenes.

Saini (2003) reported first transformation of blackgram (*Vigna mungo*) from cotyledonary node tissue with EHA105 strain carrying *gus* and *nptII* genes. T_1 progeny inherited the transgenes and the efficiency of transformation was 1%. In 2005, Saini and Jaiwal reported another transformation of *Vigna mungo* with 6.5% efficiency by using the same *Agrobacterium* strain and binary plasmid but changing the explant type from cotyledonary node to embryonic shoot apices isolated from imbibed mature seeds.

First *Agrobacterium*-transformation of Chinese milk vetch (*Astragalus sinicus*) was achieved by the use of *Agrobacterium rhizogenes* DC-AR2 strain carrying *gus* and *nptII* genes by transforming hairy roots (Cho and Widholm 1998). Plant regeneration was achieved through somatic embryogenesis. Molecular tests and *gus* activity confirmed transformation. However, plants exhibited Ri syndrome with thin leaves and short internodes. Later on Cho and Widholm (2002) reported an improved transformation of *Astragalus sinicus* with a more efficient somatic

embryogenesis protocol which resulted in the recovery of the transgenic plants in a higher frequency.

Stiller (1997) developed a transformation and regeneration technique for *Lotus japonicus* by testing various wild type *A. rhizogenes* strains of which 9402 and AR10 were found to be the most effective. Nodulation of transgenic shoots was also achieved to provide a system for the analysis of genes involved in nodulation. Large number of regenerated plants expressed *gus* and luciferase genes in T_0 progeny.

Another transformation method utilizing hypocotyl explants and *A. tumefaciens* carrying bar gene was used to transform *Lotus japonicus* with the production of 5 independent lines stably expressing and transmitting the transgene (Lohar 2001).

Lombari (2003) described a transformation procedure for *Lotus japonicus* using root generated calli for *A. tumefaciens* mediated transformation. With an efficient regeneration protocol, transgenic plants carrying gus and *hpt* genes were obtained in 4 months period.

First *Agrobacterium*-transformation of *Vigna radiata* L. Wilczek utilized cotyledonary node explants from germinated seedlings (Jaiwal 2001). By the use of LBA4404 strain carrying *gus* and *npt-II* genes, transgenic mungbean plants were obtained with 0.9% efficiency. Sonia (2007) used the same transformation procedure with improvements on cocultivation and selection to transform mungbean with *bar* and *α*-amylase inhibitor-1 genes with 1.51% efficiency. However no test was performed on plants to analyze insect resistance characteristics acquired by the transgene.

Cotyledonary meristematic tissue from three different clover species *Trifolium repens*, *T. pratense* and *T. subterraneum* were transformed with selectable marker genes by using AGL1 strain (Ding 2003). Using the same regeneration system, *bar*,

npt-II and *hpt* marker genes produced similar selection efficiencies. Transmission of the genes to the progeny was also successful with an expected segregation ratio of 3:1.

In a single report regarding *Agrobacterium*-transformation of grasspea (*Lathyrus sativus* L.) epicotyl segments were transformed with *gus* and *npt-II* marker genes (Barik 2005). Parameters such as wounding type, *Agrobacterium* concentration and strain type were optimized, reaching 36% transient expression efficiency. Integration and transmission of the transgenes were also successful with expected segregation ratio.

1.2.2.2. Biolistics-Based Transformation Studies in Legumes

Since the first report of microprojectile penetration on plant cells by the utilization of compressed inert gases by Klein in 1987, dozens of plant species were transformed with various genes by the help of particle bombardment method. Although it has several disadvantages such as generation of broken or rearranged transgene copies which cause gene silencing or unstable expression, it has many advantages including the freedom of using cassettes rather than optimized vectors, being devoid of problems associated with cleaning *Agrobacterium* cells off tissues following transformation and ability to transform plastids or mitochondria which are very difficult to achieve with *Agrobacterium*. In spite of its advantages, usage of particle bombardment in legumes is not very common, except for soybean transformation studies which is possibly related with the preference of direct transformation techniques for legume regeneration that are more efficiently applied with macro scale wounding methods.

The first genetic transformation of soybean using immature seed meristems was achieved by the use of particle acceleration with electric discharge (McCabe 1988). Finer and McMullen (1991) and Hadi (1996) used an optimized particle bombardment procedure to transform proliferative embryogenic soybean tissues.

Sato (1993) compared shoot regeneration from shoot tips and somatic embryogenesis from suspension cultures and obtained transgenic plants only when somatic embryogenesis was coupled to particle bombardment. Ponappa (1999) transformed embryogenic soybean suspension cultures for the first time with jellyfish green florescent protein (GFP). Simmonds and Donaldson (2000) screened potential of 18 different short-season soybean cultivars for proliferative embryo formation and potential to be transformed with particle bombardment, of which only five of them responded and only young proliferative cultures regenerated into fertile plants.

First functional gene introduced to soybean via particle bombardment was inverted repeat soybean dwarf virus (SbDV) coat protein (Tougou 2006). Only three T_0 plants carrying SbDV coat protein gene were regenerated through somatic embryogenesis and only one of them set seeds. In T_2 plants presence of SbDV-coat protein-specific siRNA was confirmed by northern blot and the plants did not show any symptoms when inoculated with SbDV by the help of aphids. The same group introduced also the sense SbDV-CP gene to soybean via particle bombardment in 2007. One transgenic line out of six showed viral resistance by RNA silencing through the accidental introduction of the inverted repeat, though other lines had the expression of partial or complete gene which produced resistant plants through vaccination.

Kita (2007) backcrossed Jack cultivar with soybean mutant line QF2, which lacks the major storage proteins glycinin and β-conglycinin and accumulates high levels of free amino acids in seeds. The researchers thought that low amount of endogenous storage proteins would be compensated with the presence of introduced foreign proteins and this would result in equal amounts of total protein in seeds. Since mutant line QF2 was recalcitrant to somatic embryogenesis and transformation, potential of the backcrossed product which also lacks storage proteins was tested for transformation via particle bombardment with the genes *hpt*

and *gfp*. Three transgenic lines were obtained and the potential of the backcrossed breeding lines for genetic modification of soybean was reminded.

Inaba (2007) transformed embryogenic suspension cultures of soybean with *gus* gene coupled to tobacco anthranilate synthase (ASA2) promoter which is expressed as tissue culture dependant manner in tobacco. Similar pattern was observed in soybean with *gus* expression only in tissue cultures, pollen and seeds and with very low levels in vegetative tissues.

Li (2007) applied P1 bacteriophage Cre/*loxP* recombination strategy to develop marker-free soybean plants. Cre recombinase gene controlled by *app1*, embryo specific gene promoter, was placed between two loxP recombination sites along with *hpt* marker gene. *Gus* gene was cloned outside the recombination site as to combine with its constitutive promoter when the excision succeeded. By the use of the system selection was achieved at the early stages of plant development and 30% of the transgenic plants were produced as marker-free.

El Shemy (2007) introduced a modified *Gy1* proglycinin gene with a synthetic DNA encoding four continuous methionines into soybean plants via particle bombardment along with *gfp* and *hpt* genes. Transgenic plants showed higher levels of glycinin, which is an important storage protein of soybean, in their seeds.

In another recent study, Dufourmantel (2007) developed herbicide resistant soybean plants via particle bombardment, expressing a sensitive 4-hydroxyphenylpyruvate dioxygenase (HPPD) gene from *Pseudomonas fluorescens* in their plastids. Transformants showed strong herbicide tolerance by expressing HPPD in a ratio of 5% of total soluble chloroplast proteins.

First stable gene transfer in chickpea (*Cicer arietunum* L.) was achieved by Kar (1997). *cryIA(c)* gene of *Bacillus thuringiensis* was transferred to embryo axis of chickpea plants along with *npt-II* gene. Transformed plants showed resistance

against the chickpea pod-borer, the larvae of *Heliothis armigera* Hubner. Indurker (2007) also transformed chickpea with *cryIA(c)* gene via particle bombardment. The efficiency of transformation was very high with 18% and transgenic plants showed high resistance for *Heliothis armigera* and *Spodoptera litura* larvae.

In another important study concerning chickpea transformation, a non-antibiotic selection marker system utilizing a desensitized aspartate kinase (AK) gene was developed (Tewari-Singh 2004). After bombardment with the desensitized AK gene shoots were regenerated from embryo explants on media containing lysine and threonine. The method offered a safe and practical selection system applicable to other plant species as well.

1.2.2.3. Other Transformation Studies in Legumes

One of the two studies reporting alternative methods for genetic modification of a legume without utilizing either biolistics or *Agrobacterium*-mediated transformation is the transformation of pea axillary meristems via in planta electroporation (Chowrira 1998). The introduced gene which is chimeric pea enation mosaic virus (PEMV) coat protein was expressed and transmitted only by three lines. Gene was tracked until T_4 generation and transgenic plants showed moderate resistance against pea enation mosaic virus.

The second study reported transformation of soybean through the pollen tube pathway (Gao 2007). A simple linear gene cassette with phytase gene, regulated by 35S promoter and terminated by T-nos, was introduced into the pollen tube by dipping the exposed ovaries into 300 µg/ml gene construct solution. 13% of the produced seeds stably expressed the phytase gene and 3-fold expression was obtained in transgenics when compared to control plants. Since the method is tissue culture independent, it can be promising for genetic modification of other legume species which are generally recalcitrant to common tissue culture techniques.

1.3. Tissue Culture and Transformation Studies in Lentil

When compared to other legume crops especially *Medicago* species and soybean, there is much lesser research effort both for the establishment of a regeneration system and for the stable transformation of lentil. The reasons for the little attention received by the plant can be summarized as the recalcitrance to tissue culture and genetic transformation techniques and lack of interest from researchers which are mostly unfamiliar to the crop.

1.3.1. Tissue Culture Studies in Lentil

The first study on lentil culture reports 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 into shoots and rooted in a mist chamber to yield whole, fertile plants (Williams and McHughen 1986). In the study calli obtained from cotyledons was also used in regeneration experiments, but no regeneration was achieved under the conditions tested.

Saxena and King (1987) regenerated lentil plants by the process of somatic embryogenesis from embryo-derived callus cultures. In this study, callus from 2,4-D induced embryonal axes differentiated into embryoid-like structures upon transfer to medium without hormones or with BA and IAA. Further subculture on a glutamine-supplemented medium produced well-organized embryos having cotyledons, shoots and roots which were able to develop into whole plants.

Polanco (1988) investigated the influence of growth regulators and explants on callus and shoot formation from in vitro cultures of lentil. In the study, shoot-tip, first node and first pair of leaves were used from three different lentil cultivars. It was reported that, 2,4-D induced callus formation in all explants, but no organ regeneration obtained from this calli. Also multiple shoot formation was obtained

from explants supplemented with BA and NAA but root formation was achieved only in media with NAA or IAA.

Singh and Raghuvanshi (1989) described a method for fertile plant regeneration through callus obtained from nodal segments and shoot tips. 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 hormone-free MS media for rooting and fertile plants were produced after adaptation to soil.

Rozwadowsky (1990) have isolated viable protoplasts from lentil epicotyl tissue and studied their further development to form calli, but the regeneration of calli was unsuccessful.

Warkentin and McHugen (1993) showed that, shoots readily regenerate from lentil cotyledonary node explants in vitro on a medium containing BA. Approximately 50% of these shoots rooted on hormone free medium. This regeneration protocol was rapid and of relatively high frequency.

Ahmad (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. 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) tested the inhibitory effect of BA on rooting of the shoots regenerated from lentil explants. They changed the BA concentration in the medium inducing shoot regeneration and the time in culture before transplanting the regenerated shoots to rooting medium. The in vivo study of root growth of lentil seedlings demonstrated the strong inhibitory effect of BA on root growth.

Polanco and Ruiz (2001) described an efficient and simple method for plant regeneration from axillary meristems of immature lentil seeds. In the 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 adventitious roots 30 days after transferring them to a medium containing IAA and NAA.

Gulati (2001) used micrografting technique to obtain healthy root development in lentil by grafting newly regenerated lentil shoots on three-days-old germinated lentil seedlings. Shoots were micrografted on rootstocks with 96% efficiency.

A rooting procedure was developed by Fratini and Ruiz (2003) for the nodal segments including the axillary bud and adjacent inter-node segments of lentil and several other hypogeous legumes (pea, chickpea and *Lathyrus*) by using explant polarity. 95% of the explants developed roots and average number of shoots regenerated per explant was 2.4. In the study no data on rooting frequency of nodal segments isolated from seedlings older than several days was presented.

In a recent study, Newell (2006) tested the conclusion of Fratini and Ruiz (2003) which states that shoot orientation has an effect on rooting. In their study, it was observed that when the proximal end of nodal segments was in agar the rooting percentage was low (9-25%) even when the orientation of the micro cutting was altered by inverting the culture tube. However, when the proximal end of the micro cutting was in an aerobic environment (the shoot being placed upside down in agar medium or placed normally or upside down in an aerated medium), rooting percentages were higher (62-100%). The conclusion driven from the study was that, the medium aeration at the proximal end of the micro cutting is more important than shoot orientation for *in vitro* rooting of lentil micro cuttings.

1.3.2. Transformation Studies in Lentil

Transformation frequency of legumes are generally low, much lower than less recalcitrant families, such as *Solanaceae*, and rather similar to the cereals, but for many legume species viable plants and seeds have been recovered and transformations are stable (Atkins and Smith 1997). However, few reports on lentil engineering have been published and the technique used was usually *Agrobacterium*-mediated transformation.

The first study concerning lentil genetic modification reported that four diverse strains of *Agrobacterium tumefaciens* (C58, Ach5, GV3111 and A281) were capable of inducing tumors at a high frequency on inoculated stems of lentil *in vivo* and on excised shoot apices *in vitro* (Warkentin and McHughen 1991). Tumors produced appropriate opines corresponding to the *Agrobacterium* strain. Southern blot analysis of DNA from a tumor line indicated that a T-DNA fragment had been transferred into the lentil genome. In subsequent experiments, lentil shoot apex, epicotyl and root explants were shown to be capable of expressing 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 but no regeneration system originating from protoplasts was developed (Maccarrone 1992).

Another system, electroporation-mediated transformation of intact nodal meristems was described by Chowira (1996). This method was applied to pea, soybean, lentil and cowpea. Transient expression of a chimeric *gus* reporter gene was used to monitor the uptake and expression of the introduced DNA in electroporated nodal axilary buds in vivo. The branches that grew out of the nodal meristems were chimeric, but reported to be successfully formed transgenic seeds. The method

described by the group was not used for transformation of any legume species since the date of the report.

In another study, Lurquin (1998) described the use of half-embryonic axes cocultivated with *Agrobacterium* carrying a *gus* reporter gene. The system was described to be practical and fast, and recommended for determining the transient expression responses of different cultivars, effectiveness of bacterial strains and cocultivation conditions before choosing the most appropriate conditions.

Potential of lentil cotyledonary node explants for transformation by *Agrobacterium* was investigated by Warkentin and McHughen (1993). In the study Octopine-type strain GV2260 carrying 35SGUSINT was used for the inoculation of cotyledonary node explants which were then tested for GUS expression. In explants in which no wounding was made in the axils of the cotyledonary petioles, some small buds or shoots in the axils displayed dark blue sectors. However, GUS expression in the axil region itself was never detected when this region was wounded prior to inoculation. Therefore, it was concluded that this region from which all shoots arose was not readily amenable for transformation and only a small number of cells in the axils are competent for transformation. In the study, no transgenic plants were recovered.

Cotyledonary node explants were also subjected to particle delivery via microprojectile bombardment (Öktem 1999). pBSGUSINT plasmid was used as a vector for transformation. It was observed that almost 80% of the bombarded tissues were expressing the transferred GUS gene as deduced by blue colour formation on the cotyledonary nodes. 2% of the shoots regenerated from bombarded cotyledonary nodes also exhibited patches of GUS staining.

In a study performed by Mahmoudian (2002), *Agrobacterium* GV2260 harbouring pGUSINT was used in assistance with vacuum infiltration to transform cotyledonary node and nodal segment explants of lentil. The efficiency of the

system was assessed by examining GUS expression. Results indicated that cotyledonary node of lentil was amenable to genetic transformation with a high transient gene expression of the foreign gene, and vacuum infiltration improved the efficiency of GV2260/pGUSINT mediated transformation. Unfortunately, low number of regenerated shoots exhibiting GUS expression was obtained from the utilization of this system.

In the most recent study reporting the production of fertile transgenic lentil plants, particle bombardment technique was used to transform cotyledonary node tissues with chimeric SuRA/SuRB Hra acetolactate synthase gene (*ALS*) from tobacco, conferring resistance to sulfonylurea herbicides with 4.5% transformation efficiency (Gulati 2002). Micrografting technique was used to obtain transgenic plantlets. The putative T_0 transformants were tested for herbicide tolerance by using metsulfuron herbicide leaflet painting assay and stable gene transfer was confirmed by PCR and Southern blot analysis.

1.4. Aim of the Study

Although lentil is very important as a highly nutritious food legume plant for Turkey and for the entire Middle Eastern, Latin American and African countries, little research has been done for the improvement of the crop through genetic engineering. Neither of the two studies (Chowira 1996 and Gulati 2002) existed in the literature which reports stable genetic transformation of lentil utilized the most practical and economical gene transfer technique, *Agrobacterium*-mediated transformation. The aim of this study is to develop a reproducible *Agrobacterium*-mediated genetic transformation technique followed by an efficient regeneration system which can together provide stable expression and transmission of the introduced genes to the next progeny by the use of cotyledonary node explants isolated from germinated mature lentil seeds.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

A summer sown, green cotyledon lentil (*Lens culinaris*, Medik) cultivar Sultan-1 was used throughout the study. The seeds were obtained from Agricultural Research Institute, Eskişehir.

2.1.2. Plant Tissue Culture Media

According to the application, MS (Murashige and Skoog 1962) basal medium with MS or B5 vitamins (Gamborg 1968) as 4.4 grams of powder per 1 liter was used in the study. MS medium including either MS or B5 vitamins was supplemented from Duchefa Biochemie B.V., Netherlands. Depending on the usage, the medium was supplemented with 0.4% or 0.8% plant agar, plant growth regulators BA, kinetin, zeatin riboside (ZR), TDZ, 2,4-D, NAA, IBA and IAA, antibiotics cefotaxime and kanamycin, sucrose, mannitol, maltose, trehalose, L-glutamic acid, L-proline, casein hydrolysate or additional ammonium nitrate and potassium nitrate. Before their usage, the media components were dissolved in distilled water and sterilized at 121°C for 20 minutes. Except the carbon sources and agar, all ingredients were prepared as solutions, filter sterilized by passing through 0.2 μm pore-size filters, and added freshly to the sterilized media. Composition of the MS basal medium, MS vitamins and B5 vitamins was given in Appendix A.

2.1.3. Bacterial Strains and Plasmids

The hypervirulent succinamopine type *Agrobacterium tumefaciens* strain KYRT1 (Torisky 1997) harbouring a super-virulent Ti plasmid pTiBo542, a vir helper plasmid pKYRT1 which is derived from pTiChry5 and a binary vector pTJK136 (Kapila 1997) was used for the transformation experiments. Vector pTJK136 is a derivative of vector pTHW136. It carries a gene coding for streptomycin/spectinomycin adenyl transferase as bacterial selection marker, an intron containing *gus* gene and *npt*-II gene as plant selection markers. The map of pTJK136 vector can be found in Appendix B. The plasmid was kindly provided by Prof. Dr. Van Montagu (Appendix C).

2.1.4. Bacterial Culture Media

Yeast extract broth (YEB) was used to grow *Agrobacterium* cultures for plant transformation experiments. The YEB medium with pH 5.6 consists of 13.5 g/L nutrient broth, 1g/L yeast extract, 5g/L sucrose and 2mM $MgSO_4.7H_2O$. Depending on the purpose, it was supplemented with bacterial selection antibiotics, rifampicin (100mg/L), carbenicilin (100mg/L), gentamycin (40mg/L), streptomycin (300mg/L), spectinomycin (125 mg/L), 10mM 2-(N-morpholino) ethanesulfonic acid (MES) and 20 μM 3',5'-dimethoxy-4-hydroxyacetophenone (acetocyringone).

2.1.5. Other Materials

The antibiotics, plant hormones, GUS histochemical substrate X-Gluc (5-bromo-4-chloro-3-indolyl glucoronide), MES, acetocyringone, agar and all other chemicals used in the solutions were supplied from Merck, Sigma, Aldrich, Duchafe and Applichem chemical companies. All chemicals and enzymes used in the molecular analysis were obtained from MBI Fermentas, Favorgen or Roche. The primers for *gus* and npt-II genes were either prepared by Iontech Company, Bursa, Turkey or IDT gene technologies Coralville, Iowa, USA.

2.2. Methods

Experiments were performed throughout the study to be able to assess the responses of lentil explants on different regeneration routes. The most efficient regeneration system was used in *Agrobacterium*-mediated transformation studies with further optimization of the transformation parameters (Figure 2.1).

2.2.1. Tissue Culture Studies

Laminar flow cabinets were used and aseptic techniques were employed throughout the performance of tissue culture studies.

2.2.1.1. Seed Surface Sterilization and Germination

Lentil seeds were rinsed with 70 % alcohol instantaneously for once, alcohol was discarded and the seeds were kept in 20 % Na-hypochloride solution for 8 minutes. The seeds were washed with sterile distilled water for 3 times and the ones with decolorized or swollen seed coats were discarded. The seeds were either imbibed overnight (o/n) in water or placed directly on MS basal medium supplemented with 3% sucrose and 0.4% agar for germination at 23°C in dark for 4 days.

2.2.1.2. Preparation of Explants

Cotyledonary node explants were prepared from 4-days-old etiolated lentil seedlings by excising roots and shoots at a distance of 3-4 mm to the node. Then the cotyledons were removed at a single cut. Cotyledonary node explants were cultured for shoot regeneration in 1mg/L BA containing MS media for 4 days before transformation experiments. For the preparation of half cotyledon explants the cotyledons were kept intact and the explants were cut in two halves from the nodal axis. Longitudinal embryonic axis explants were prepared by dissecting embryonic axis isolated from 1-day-old imbibed seedlings into two halves. Epicotyl

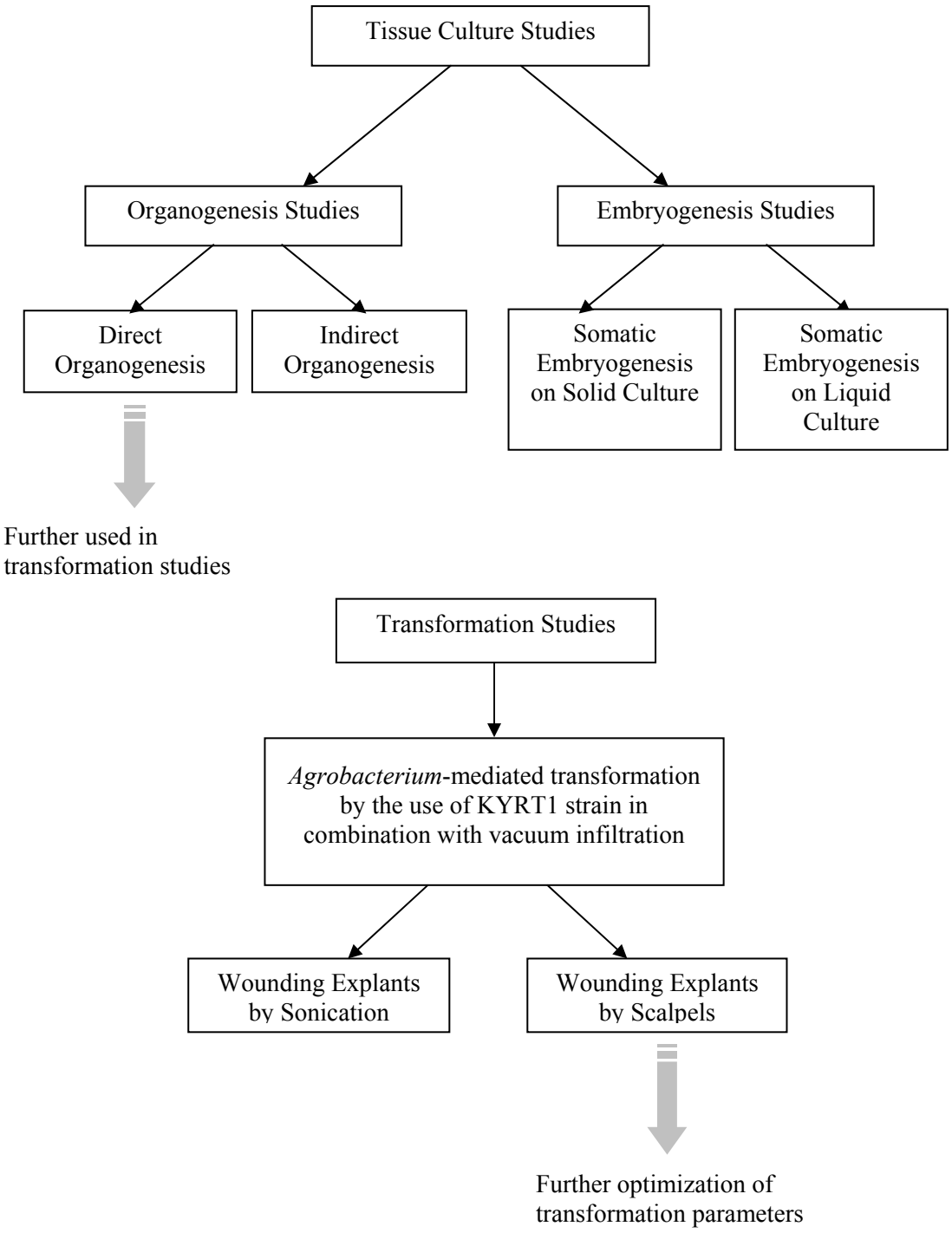


Figure 2.1. The main steps of tissue culture and transformation studies performed.

and hypocotyl explants were prepared by excising the tissues in 0.5-1cm sized segments from 4-days old etiolated lentil seedlings. Internode and leaf explants were prepared from 2-weeks-old lentil seedlings by excising the tissues as 0.5-1cm and 0.5cm^2 sized segments, respectively.

2.2.1.3. Organogenesis Studies

Longitudinal embryonic axis, epicotyl, hypocotyl, internode and leaf explants were cultured on MS medium containing plant hormones; BA, ZR, NAA, IAA and IBA in various combinations. All explants were transferred into regeneration media after 3 weeks in induction media. Responses of explants were recorded after 6 weeks of culture and photographed by using Nikon COOLPIX 4500 digital camera through the Nikon SMZ-800 stereoscopic microscope, when magnification is required.

2.2.1.4. Embryogenesis Studies

For somatic embryo induction trials, different combinations of NAA, BA, 2,4-D, kinetin, ZR and TDZ, presence of 3% mannitol, 3% maltose, 3% trehalose or 3% sucrose, L-proline, L-glutamic acid (LGA) or gluthatione reduced (GSH), casein hydrolysate, ammonium nitrate and potassium nitrate, use of semisolid or solid MS media with MS and B5 vitamins for callus induction and embryo development were assessed mainly on two lentil explants, longitudinal embryonic axes and cotyledonary petiole. Also some preliminary somatic embryogenesis trials were performed by using leaf, root and internode segments. The weights of calli which was formed through different applications were recorded with two week periods.

Suspension culture for lentil was prepared by using 3-weeks-old friable calli from cotyledonary petioles produced on semisolid MS media containing B5 vitamins and supplemented with 0.75mg/L 2,4-D. The cultures were produced by placing nearly 0.5g fresh calli into 20ml liquid MS media with B5 vitamins containing 0.25mg/L 2,4-D. The suspension cultures were established in 3 weeks under 16/8 hours

light/dark cycle with constant agitation at 90 rpm. Liquid culture media was also enriched with 20mg/L proline and 500mg/L ammonium nitrate. The cultures were maintained by transferring 5ml culture into 20ml fresh media every three weeks for a duration of five months.

2.2.2. Transformation Studies

During genetic transformation studies of lentil, cotyledonary node explants were used in *Agrobacterium*-mediated transformation which is combined to vacuum infiltration technique. Sonication was coupled to *Agrobacterium*-mediated transformation studies to test the efficiency of sound waves on wounding lentil explants.

2.2.2.1. Vacuum Infiltration Based *Agrobacterium*-Mediated Transformation

To prepare *A. tumefacies* for plant transformation, 100 ml of YEB medium containing 10 mM MES, 20 μM of acetosyringone and necessary antibiotics were inoculated with the *A. tumefaciens* strain. The culture was grown to OD_{600} of 1 at 28°C, 200 rpm and then centrifuged for 15 minutes at 4000 rpm at 8°C. The pellet was resuspended with MMA medium (4.3g/L MS salts, 10 mM MES, 2% (w/v) sucrose, 200 μM acetosyringone with pH 5.6) to a final OD_{600} of 3 and the suspension was kept at 22°C for 1 hour according to Kapila (1997). Secondary shoots developed from petiole axil region of cotyledonary nodes were removed by the help of sterile scalpel blades and underlining meristematic tissue was wounded by poking 8-10 times just before transformation for 20 minutes under vacuum pressure of 200 mmHg. 30 cotyledonary node explants were used in each independent transformation experiment.

After co-cultivation with *Agrobacterium* suspension on agar (0.8% w/v) solidified MS basal media containing 3% sucrose, at 22°C in the dark for 3 days, the explants were either subjected to histochemical GUS staining or washed one hour in MMA

medium supplemented with 500 mg/l cefotaxim. After washing once with distilled water, the explants were moved to MS basal media containing 1mg/L BA, 200mg/L cefotaxim and 100mg/L kanamycin. The cultures were maintained at 24 °C with 16 hour photoperiod, light provided with florescent lamps at an intensity of 40 μmol m^{-2} s^{-1}. The plants were subcultured on selection media containing 200mg/L kanamycin after a week. After another week on 200mg/L kanamycin, explants were further cultured for four weeks on 300mg/L kanamycin by refreshing the media once in two weeks. During each step of gradual selection and medium refreshment, survived shoots were removed from cotyledonary nodes and small tissue parts were histochemically stained for *gus* expression. GUS positive shoots were immediately micrografted on 5-days-old root stocks prepared from lentil seedlings (Figure 2.1).

2.2.2.2. Wounding Tissues by Sonication

For sonication experiments three different explants; cotyledonary nodes, half cotyledons and cotyledonary nodes with intact shoots were isolated from 5-days-old germinated lentil seedlings and immediately wounded by 55 kHz PC5 model batch sonicator (L&R Company, N.J., USA) for various durations; 0.5 seconds, 1 seconds, 3 seconds, 5 seconds, and 10 seconds inside *Agrobacterium* suspension solution before transformation for 20 minutes under 200 mmHg vacuum pressure.

2.2.2.3. Micrografting and Hardening of Regenerated Shoots

After a series of gradual selection, regenerated shoots originated in any stage of selection were removed from cotyledonary node and checked for *gus* expression. After an overnight incubation on MS basal media, GUS positive shoots were grafted on rootstocks which were prepared by removing the main shoot from 5-days-old seedlings and giving a 2-3 mm perpendicular cut to the tip of 5 mm shoot base (Figure 2.2 a, b) according to Kamçı (2004). After cutting the shoot base with a 45^0 of angle (d), shoots were placed on the rootstocks (e, f). The grafts were located on perlite containing pots wetted with liquid MS media (g). The pots were

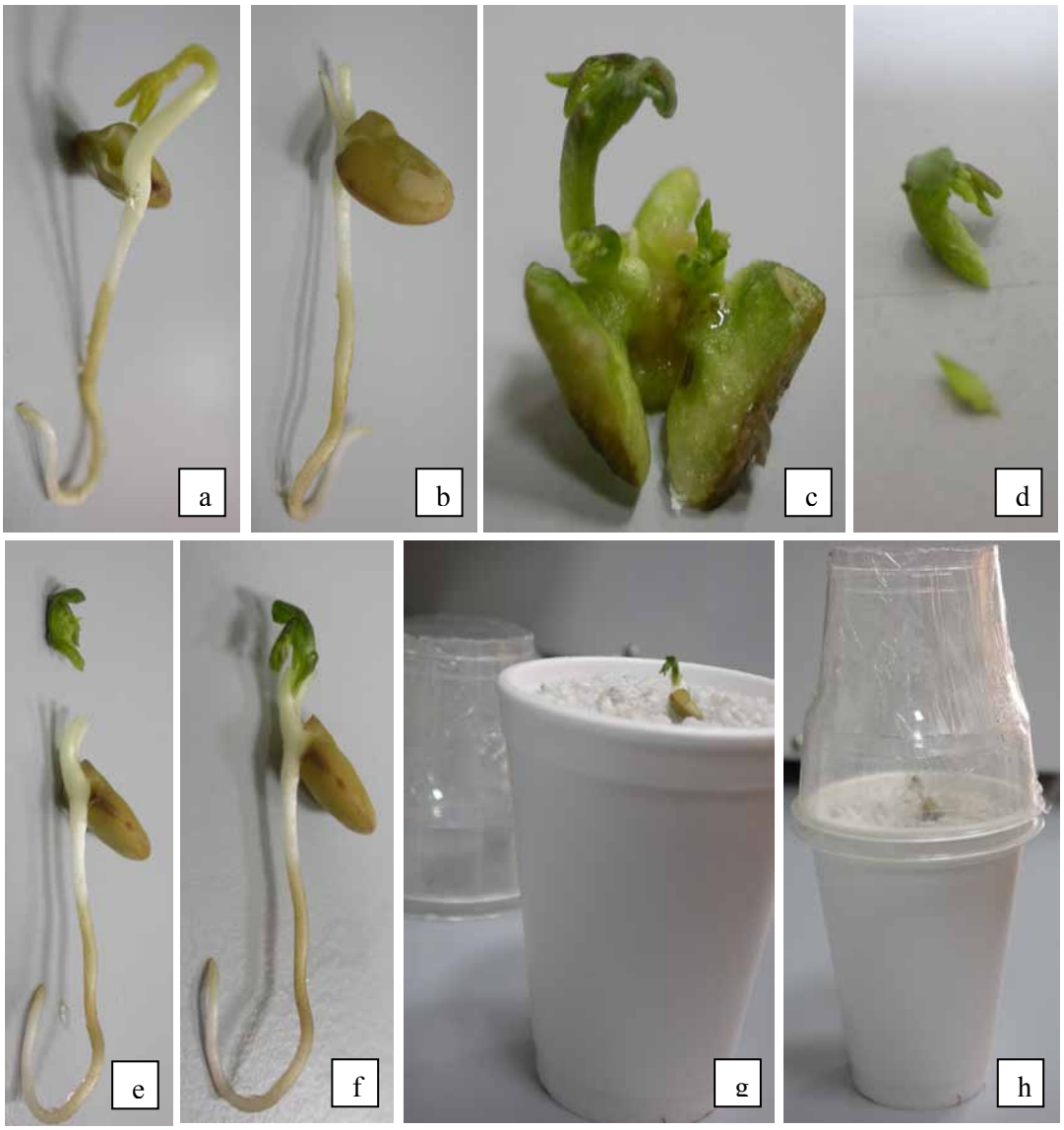


Figure 2.2. Micrografting of lentil shoots into lentil root stocks. 5-days-old ethiolated seedling (a), perpendicular cut on the shoot base of a root stock (b), shoots regenerated from cotyledonary node explants (c), cut with a 45^0 of angle on the base of regenerated shoot (d), bringing vascular tissues in proximity (e, f), a graft placed on a perlite filled pot with a transparent cover (g, h).

immediately covered with transparent plastic cups and placed on tissue culture room with 16 hour photoperiod at 24 °C (h). The cups were punctured continuously during the growth of grafted shoots to acclimatize plants to low moisture conditions. After complete adaptation, plants were hardened in the soil and grown in the greenhouse until maturation.

2.2.3. Analysis of Transformants

Preliminary analysis of transformants and tests for transient expression of transgenes were done by the use of histochemical gus staining throughout the study. Molecular analysis of transgenes was done by using PCR, reverse transcription PCR, real time PCR and Southern Blotting techniques.

2.2.3.1. Histochemical GUS Staining

Histochemical GUS staining was performed according to the procedure of Jefferson (1987) to observe GUS activity on various lentil explants, to evaluate transient *gus* expression efficiency and for the detection of stable *gus* expression on the mature tissues of T_0, T_1, T_2 and T_3 generations. All explants and tissues were assayed by incubation inside GUS substrate solution (0.1 M $NaPO_4$ Buffer, 0.5mM K-ferricyanide and 0.5 mM K-ferrocyanide with pH 7.0, 10 mM EDTA, 1 mM X-Glucoronide and 10% v/v Triton X-100) overnight at 37°C. After incubation, explants were transferred to fixative solution (10% v/v formaldehyde, 20% v/v ethanol and 5% v/v acetic acid) in which they can be preserved for several months. Chlorophyll containing tissues were transferred to 50 % ethyl alcohol for decolorization following 4-hours of incubation on fixative solution. After 15 minutes in 50 % ethyl alcohol, shoots were transferred to 100 % ethyl alcohol for further decolorization overnight.

2.2.3.2. PCR Analysis

For the preliminary analysis of the presence of transgenes in transfomed plant tissues, genomic DNA was isolated both from control plants, which were not inoculated and incubated with *Agrobacterium* suspension and from putative transformants which are already expressing *gus* gene. For genomic DNA isolation, 1g of plant tissue were ground to fine powder inside a pre-cooled mortar by the help of liquid nitrogen. DNA isolations were done by using Favorgen® Plant DNA isolation kit (Favorgen, Taiwan) according to the kit manual. The PCR analysis was performed by using both *npt*-II and *gus* gene primers. The primer set designed for the amplification of *npt-II* gene has the sequences "gaggctattcggctatgactg" for forward and "tacggtatcgccgctcccgat" for reverse primers. The primers amplify the entire *npt-II* gene with 700 base pairs. Sequences for the primers amplifying *gus* gene are "ggtgggaaagcgcgttacaag" and "tggcggaagcaacgcgtaaac" for forward and reverse, respectively. Primers for *gus* gene amplifies 1203 base pair product which is almost equal to the complete *gus* gene sequence including the intron region.

To be able to test the presence of contaminating *Agrobacterium* cells in plant tissues aminoglycoside adenyltransferase gene (*aadA*) specific primers "gctcacgcaactggtc" and "gcactacatttcgctcat" were used for forward and reverse, respectively. The primers were designed to amplify 777 base pair product which is 220bp shorter than the entire gene. The composition and the conditions of the PCR reactions were given in Appendix D.

For visualization of PCR products, 1 % gels were prepared by dissolving 1 gram agarose in 100ml of 0.5X TBE buffer in a microwave oven. 5µl of ethidium bromide solution was added to 50ml of agarose gel solution. The melted gel solution was poured into an electrophoresis gel tray which contains a firmly located comb. After removal of the comb and placing of the gel on 0.5X TBE filled electrophoresis tank, DNA samples and size markers (Fermentas SM331, SM371) were loaded into wells by mixing with 6X loading buffer at a final concentration of

1X. Power supply was adjusted to 75-100 V and the gel was run for 2-4 hours. Finally the bands were visualized under UV light and photographed by using Vilbert-Lismart gel documentation system.

2.2.3.3. Real-Time PCR Analysis

Real-time PCR analysis was performed for T_0 generation by using Roche LightCycler instrument and LightCycler GMO Screening Kit. Total RNA was isolated according to De Graff (1988) by using TRIZOL reagent (a mono-phasic solution of phenol and guanidine isothiocyanate). All the equipment was DEPC treated before the isolation. All the reagents except TRIZOL and chloroform were prepared by using DEPC-treated water.

100mg plant tissue was grinded by using liquid nitrogen, mortar and pestle, mixed with 1ml TRIZOL reagent and vortexed for 15 minutes. After 5 minutes of centrifugation at maximum speed and 4^0C, the supernatant was transferred to another tube and mixed with 180µL chloroform by shaking vigorously for 15 seconds. Following centrifugation under the same conditions, aqueous upper phase was collected and mixed with 200µL chloroform. After 5 minutes of centrifugation aqueous phase is collected and mixed with one volume of isopropanol by gently inverting the tube several times. Following incubation at room temperature for 10 minutes and centrifugation at room temperature for 10 minutes, supernatant was discarded, pellet was washed with 75% ethanol and air-dried for 10 minutes. Finally, the pellet was dissolved in 50µL DEPC-treated water by incubating at 65^0C for 15 minutes and RNA solution was stored at -80^0C until use.

Total cDNA was prepared by using Fermentas First Strand cDNA Synthesis Kit with oligo$(dT)_{18}$ primers according to the instruction manual by using 3µg template RNAs which were extracted according to De Graff (1988).

PCR set-up was prepared according to the Roche suppliers' instructions (Fandke 2002). LightCycler GMO Screening Kit (Roche) which is an optimized kit for the flourometric detection of 35S promoter of cauliflower mosaic virus (CaMV) and the 3' untranslated region (terminator) of the nopaline synthase (NOS) gene of *A. tumefaciens* was used for the real-time detection. Also as a reference and control for DNA extraction efficiency, a primer set for a plant-specific gene is included in the kit. One set of 35S-specific probe is labeled at the 5' end with Light-Cycler–Red 705 and one NOS-specific probe is labeled with LightCycler–Red 640. The other set of 35S- and NOS-specific probes are labeled at the 3' end with fluorescein. After hybridization to the template DNA, two probes for each target come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, fluorescein, the donor fluorophore, is excited by the light source of the LightCycler Instrument and part of the excitation energy is transferred to LightCycler–Red, the acceptor fluorophore. The emitted fluorescence is then measured by the instrument. For the detection of the reference plant gene, a specific pair of probes, one labeled with LightCycler–Red 640 and the other with fluorescein, are utilized (Fandke 2002).

T-DNA of pTJK136 binary vector contains the *gus* gene which is driven by the 35S promoter and ended by nos terminator. Therefore, both of the fluorescent labeled probes of GMO detection kit were suitable for the detection of transgene and used for the analysis of transgenic nature of T_0 generation.

2.2.3.4. Reverse Transcription PCR

Total RNA samples were isolated according to the method of De Graff (1988) and first strand cDNAs were synthesized by using the template RNAs and Fermentas First Strand cDNA Synthesis Kit. Synthesized cDNA strands were directly used in the reverse transcription PCR (RT-PCR) experiments for both T_0 and T_1 generations by the use of same primer sets designed for genomic DNA PCR analysis, both for the amplification of *gus* and *nptII* cDNAs.

2.2.3.5. Southern Blot Analysis

Southern blot analysis was performed by using DIG-High Prime DNA Labeling and Detection Starter Kit II, Roche Applied Sciences, according to the instructions manual. The kit is composed of Klenow enzyme which allows random-primed labeling of DNA templates with DIG-11-dUTP, Anti-digoxigenin-AP conjugate, blocking solution and hybridization granules to be used in the preparation of hybridization buffer. Detection was achieved by the use of CDP-Star reagent from New England Biolabs, MA, USA. Probe was prepared by random primed labeling reaction following the PCR amplification of a partial *gus* gene by the use of primers having the sequences "ctgtcggctttaacctctctttag" and "agtgaagatccctttcttgttacc" for forward and reverse, respectively. Primers for the *gus* gene amplifies 656 base pair product which corresponds to a non-conserved region of the gene which was determined according to "Conserved Domain Search" tool of National Center for Biotechnology Information (NCBI).

To prepare genomic DNA isolated by the use of Favorgen DNA Extraction Kit, two repeats of overnight restriction enzyme digestion were performed by using Hind III enzyme, which has no restriction site inside T-DNA region of the vector construct. To purify and reduce sample volume before loading to agarose gels, sodium acetate precipitation was performed by adding 1/10 volume of 3 M sodium acetate, pH 5.2 and 2.5 volume of ice-cold 100% ethanol to the solution of DNA. After overnight incubation at -20^0C, the solution was centrifuged at a maximum speed for 5 minutes in a bench-top microfuge. DNA pellets were washed with 1ml 70% ethanol once and dried at room temperature until the pellets are no longer visible. Finally the pellets were dissolved in TE buffer (10mM Tris-HCl and 1mM EDTA), pH 8.0 and stored at -20^0C (Moore 2005).

0.8% agarose gel was prepared by dissolving 2.8g agarose inside 350 ml 0.5X TBE buffer. Preparation of TBE buffer and all the solutions used in southern blot analysis were given in Appendix E. The agarose solution was poured into 18x22cm

sized gel tray and solidified for 50 minutes. 30µg of digested genomic DNA fragments were separated by agarose gel electrophoresis overnight, under 25 volts constant voltage. All the steps of gel preparation and southern blotting were performed according to Brown (2005). The gel was washed with depurination solution with constant agitation for 15 minutes and placed immediately in denaturation solution. After 30 minutes incubation inside denaturation solution, the gel was transferred into neutralization solution. After another wash for 30 minutes with constant agitation inside denaturation solution, the gel was washed with sterile distilled water and placed on blot set up which contains 20X SSC wetted Whatman papers located on 20X SSC containing gel tank. The surface of the gel was covered with Hybond-N + nylon membrane (Amersham, Buckinghamshire, England) and the membrane was covered with 3 sheets of dry chromatography papers (Whatman, Maidstone, England) over which tissue papers were lined up at the height of 15cm. A 500g weight was placed at the top of the set up and the capillary transfer took place overnight.

The set up was dissolved and the membrane was washed inside 2X SSC solution for 5 minutes before crosslinking of DNA fragments in automatized crosslinker for 45 seconds. The membrane was dried in an oven at 60^0C for two hours which is followed by hybridization, stringency washes and signal detection that were done according to the kit's instructions manual.

2.2.3.6. Statistical Analysis

SPSS (Statistical Package for Social Sciences, SPSS Inc., Illinois) was used for the determination of mean and standard error of mean for different experimental treatments. The Chi-Square test of SPSS was performed to analyze inheritance patterns of transgenes in three generations.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Tissue Culture Studies for Lentil

Establishment of an efficient regeneration system which can be used in combination with gene transfer studies was one of the primary aims of this study. Different regeneration routes; direct, indirect organogenesis and somatic embryogenesis were tried to be induced by the use of various plant growth regulators, carbon sources and other supplementary compounds.

3.1.1. Studies for Regeneration Through Organogenesis

Throughout the organogenesis trials, different types of tissues which also have the potential to be used in the transformation studies were utilized. The explants included longitudinal embryonic axis, nodal segments, leaves, internodes, hypocotyls and epicotyls.

3.1.1.1. Direct Organogenesis Studies

Direct organogenesis studies were performed to be able to find an effective alternative to the explant cotyledonary node. The cotyledonary node is not only the most common explant used in direct regeneration studies of various legumes, but it was also used for the transformation studies of lentil by Warkentin and McHughen (1993), Öktem (1999) and Mahmoudian (2002). The only stable lentil transformation study through the use of biolistics also reports the use of cotyledonary node explants (Gulati 2002). Throughout this study, longitudinal embryonic axis explants isolated from imbibed lentil seedlings, epicotyl and

hypocotyl explants isolated from 5-days-old germinated seedlings and nodal segments isolated from germinated seedlings of various ages were used for the optimization of direct organogenesis. Shoot formation was observed on longitudinal embryonic axis on all hormone combinations tested except 3 BA+0.5 NAA, with the highest number of shoots (1.72 shoot/explant) on media containing 5mg/L BA (Table 3.1, Figure 3.1, a,b). Elongation of the shoots and rooting was unsuccessful when subcultured both on the same media, hormone-free media, on media containing 1mg/L BA + 2mg/L giberellic acid (GA) or 1mg/L GA alone. Hypocotyl explants were unresponsive for all BA concentrations tested. 9% of the epicotyl explants responded with shoot formation (in average 3.4 shoots/explant) on MS media containing 3, 4 and 5 mg/L BA (Table 3.1, Figure 3.1, c,d), however the origin of shoot formation was the nodal buds which were invisible at the time of explant preparation and also the elongation and rooting on the regenerated shoots was not successful.

For direct shoot regeneration, plant growth regulators, kinetin, zeatin riboside and thidiazuron were also tested on epicotyl, hypocotyl and internode explants. A phenyl-urea derivative TDZ resulted in tissue browning and necrosis in all the explants tested. Khawar (2004) also reported that shoot or callus formation was not achieved from leaf or stem explants of lentil cultivars, Ali Dayı and Kayı-91 in the presence of different concentrations of TDZ (0.25, 0.5, 1.0 and 2.0 mg/L). Epicotyl and internode explants on MS medium with 2 mg/L of kinetin produced roots in the direction of root side with 42 and 23% efficiency, respectively (Figure 3.1, f). Very low amount of root was also formed in the presence of 1mg/L kinetin. Since kinetin, a substituted adenine occurring naturally in plants, is responsible from lateral root initiation in intact plants, the findings is not unexpected. However, rooting was only observed on epicotyl explants and neither of the concentrations induced shoot regeneration. The presence of ZR in three different concentrations (1, 2, 3mg/L) resulted in shoot formation on the nodal buds of epicotyl explants with 2.1, 2.7 and 3.2 shoots/explant, respectively, however the number of responsive explants was very low (1.3%). The regenerated shoots developed and

Table 3.1. Explant types and hormone combinations used for the lentil organogenesis studies. All hormone concentrations are in mg/L level. Superscript numbers in each row represent the prevalent response given by the particular explant.

Explant Type	Hormone Combinations for Induction	Hormone Combinations for Regeneration	Response
epicotyl[1], leaf[2], internode[3]	1 ZR+0.1 NAA	Same media (sm) or (/) 2 ZR	Callus on root side[1], necrosis[2,3]
epicotyl[1], leaf[2], internode[3]	1.75 ZR+0.6 IAA	sm / 2 ZR	Callus on root side[1], necrosis[2,3]
epicotyl[1], leaf[2], internode[3]	1.5 BA+0.5 NAA	sm / 3 BA+0.75 NAA / 3BA	Callus on root side, necrosis[2,3]
epicotyl[1], leaf[2], internode[3]	1.5 BA+0.5 IAA	sm / 3 BA+0.75 NAA / 3BA	Callus on root side[1], necrosis[2,3]
epicotyl[1], leaf[2], internode[3]	1.5 BA+0.5 IBA	sm / 3 BA+0.75 NAA / 3BA	Callus on root side[1], necrosis[2,3]
epicotyl[1], leaf[2], internode[3], hypocotyl[4]	2 BA+1 NAA	sm / 3 BA+0.75 NAA / 3BA	Callus on root side[1,3], swelling and necrosis[2,4]
epicotyl[1], leaf[2], internode[3], hypocotyl[4]	2 BA+1 IAA	sm / 3 BA+0.75 NAA / 3BA	Callus on root side[1,3], swelling and necrosis[2,4]
epicotyl[1], leaf[2], internode[3], hypocotyl[4]	2 BA+1 IBA	sm / 3 BA+0.75 NAA / 3BA	Callus on root side[1,3], swelling and necrosis[2,4]
longitudinal embryonic axis	3 BA+0.5 NAA	1 BA+2 GA / hormone free media (hfm) / 1GA / sm	Tissue swelling, callus formation
longitudinal embryonic axis	3 BA+0.5 IAA	1 BA+2 GA / hfm / 1GA / sm	0.4 shoot/explant, no elongation
longitudinal embryonic axis	3 BA+0.5 IBA	1 BA+2 GA / hfm / 1GA / sm	1.2 shoot/explant, no elongation

Table 3.1 (continued)

epicotyl[1], hypocotyl[2], longitudinal embryonic axis[3]	3 BA	1 BA+2 GA / hfm / 1GA / sm	3.4 shoot/explant[1], 1.1 shoot/explant[3], no elongation[1,3]
epicotyl[1], hypocotyl[2], longitudinal embryonic axis[3]	4 BA	1 BA+2 GA / hfm / 1GA / sm	3.5 shoot/explant[1], 1.2 shoot/explant[3], no elongation[1,3]
epicotyl[1], hypocotyl[2], longitudinal embryonic axis[3]	5 BA	1 BA+2 GA / hfm / 1GA / sm	3.3 shoot/explant[1], 1.7 shoot/explant[3], no elongation[1,3]
epicotyl[1], hypocotyl[2], internode[3]	1 kinetin	sm / hfm	root formation[1,3], necrosis[2]
epicotyl[1], hypocotyl[2], internode[3]	2 kinetin	sm / hfm	root formation[1,3], necrosis[2]
epicotyl, hypocotyl, internode	4 kinetin	sm / hfm	necrosis
epicotyl[1], hypocotyl[2], internode[3]	1 ZR	sm / hfm	2.1 shoot/explant[1], necrosis[2,3]
epicotyl[1], hypocotyl[2], internode[3]	2 ZR	sm / hfm	2.7 shoot/explant[1], necrosis[2,3]
epicotyl[1], hypocotyl[2], internode[3]	4 ZR	sm / hfm	3.2 shoot/explant[1], necrosis[2,3]
epicotyl[1], hypocotyl[2], internode[3]	1 TDZ	sm / hfm	Tissue swelling[1,2,3], necrosis[1,2,3]
epicotyl[1], hypocotyl[2], internode[3]	2 TDZ	sm / hfm	Tissue swelling[1,2,3], necrosis[1,2,3]
epicotyl[1], hypocotyl[2], internode[3]	4 TDZ	sm / hfm	Tissue swelling[1,2,3], necrosis[1,2,3]

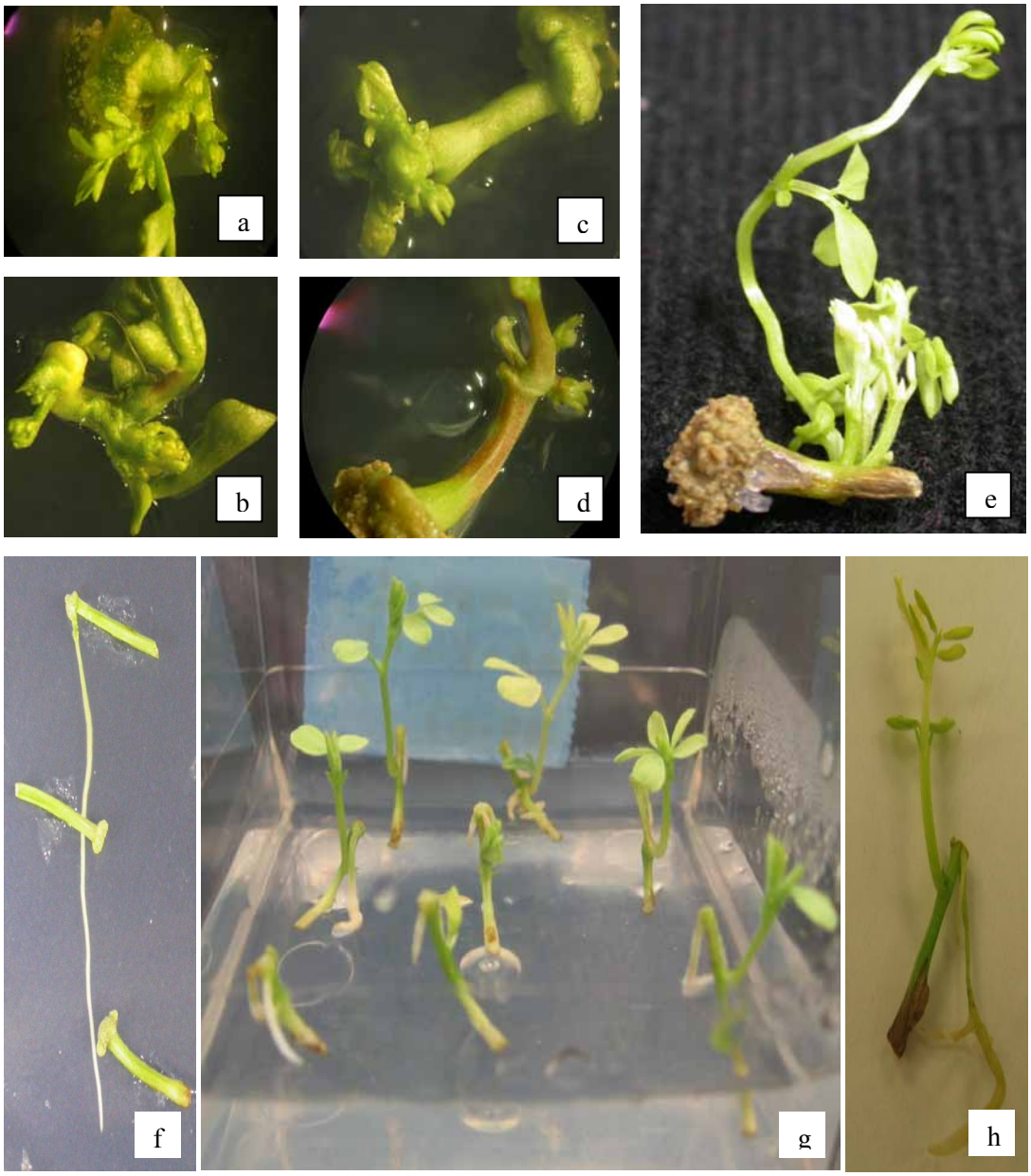


Figure 3.1. Direct plant regeneration studies for lentil. Shoot regeneratin from longitudinal embryonic axis explants in the presence of 5mg/L BA (a,b) and shoot formation on the nodal buds of epicotyls on the same media (c,d). Shoot formation from the nodal bud on an epicotyl explant in the presence of 4mg/L ZR (e). Root formation on an epicotyl explant on MS media with 1mg/L kinetin. Regeneration of nodal segments isolated from 1-week-old seedlings on 1mg/L IAA and 0.2mg/L kinetin (g,h).

elongated on the same media successfully (Figure 3.1, e), however no root formation was observed on either same medium or hormone free medium and the regeneration of the whole plants was not achieved.

Regeneration from nodal segments was achieved according to the method of Fratini and Ruiz (2003) which used Spanish lentil cultivar Verdina. In their study, nodal segments were isolated from 1-week-old in vitro germinated seedlings which were grown under standard light/dark cycle and placed in inverted position on MS medium supplemented with 3% sucrose, 1mg/L IAA and 0.2mg/L kinetin. Rooting percentage obtained in those conditions was 95.35% and the average number of shoots regenerated per explant was 2.4. Under the same conditions, 93% of the explants produced roots and average number of shoot per explant was 1.7 with cultivar Sultan-1 under our laboratory conditions (Figure 3.1, g,h). However, when two-weeks-old seedlings were used as the source of nodal segments, rooting percentage decreased to 24% and the average shoot number to 0.8. After three weeks of seedling growth, none of the isolated explants produced roots and the shoots regenerated from nodal buds were weak and unable to complete their developments without the presence of root tissues. The rooting procedure was also applied to the nodal segments isolated from shoots which were regenerated from cotyledonary node explants. The application was thought to have the potential for propagating transgenic shoots developing from cotyledonary nodal meristems, however it was not possible to obtain any roots from these explants using the method described. Therefore, the application of the method was limited to nodal segments isolated from very young lentil seedlings, which restrict its usage in combination with genetic transformation studies.

3.1.1.2. Indirect Organogenesis Studies

Epicotyl and hypocotyl segments of six days old etiolated seedlings and, internode and leaf segments of 15 days old lentil plants which were grown under standard dark/light cycle were used in indirect regeneration studies. For the various growth

regulators applied, including combinations of cytokinins ZR, BA and auxins IAA, IBA and NAA (Table 3.1), all the explants were unresponsive except swelling of hypocotyls and root directed ends of internodes and weak callus formation in leaves which were always followed by tissue browning and necrosis (Figure 3.2). Utilization of MS medium with MS and Gamborg's vitamins, presence of 3% sucrose, maltose or mannitol as the carbon source and presence of 500mg/L ammonium nitrate as an additional nitrogen source did not result in any improvements.

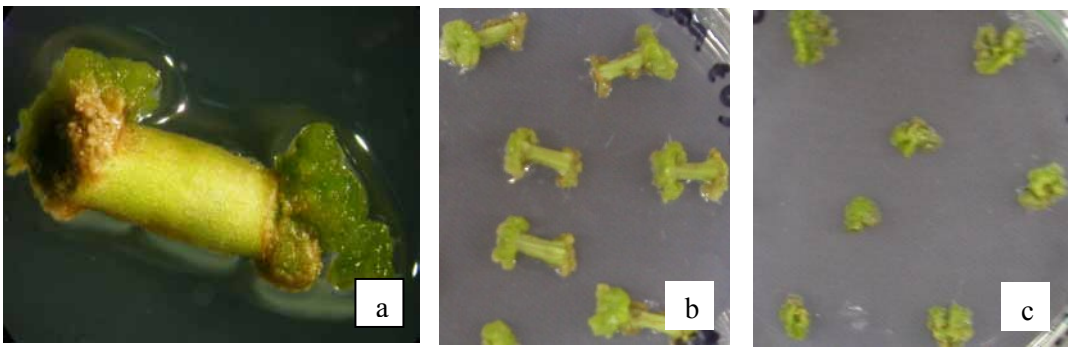


Figure 3.2. Indirect organogenesis studies on lentil. Hypocotyl explant developed on 2 mg/L BA+1mg/L IBA (a), internode and leaf explants on 1 mg/L ZR+0.1 mg/L NAA (b and c, respectively).

3.1.2. Studies for Regeneration Through Embryogenesis

Regeneration of plants via somatic embryogenesis is considered to be one of the most efficient approaches for genetic manipulation applications since regenerants originate from single cell and produced in large quantities. Therefore, optimization studies for lentil regeneration through somatic embryogenesis pathway were also performed. In this context, mainly the cotyledonary petioles isolated from 3-days-old seedlings and embryonic axis explants isolated from imbibed seedlings were used. In addition to these explants, leaf, root and internode segments were also tested in the preliminary embryogenesis studies.

3.1.2.1. Solid Culture

Throughout the study, mainly the effects of three auxins, NAA and 2,4-D in combination with cytokinins, BA, ZR, TDZ and kinetin were tested on cotyledonary petioles and longitudinal embryonic axis explants (Table 3.2). In the preliminary assays, effects of five different hormone combinations; 3mg/L TDZ+0.1mg/L kinetin, 2mg/L NAA+1mg/L kinetin, 2mg/L 2,4-D+0.5mg/L BA, 3mg/L NAA+1mg/L BA and 2mg/L 2,4-D were also tested on leaf, root and internode explants. Weak callus formation was observed only on media containing 3mg/L NAA+1mg/L BA and 2mg/L NAA+1mg/L kinetin. No embryo formation was followed in any of the leaf, root and internode calli throughout the proceeding steps of the culture.

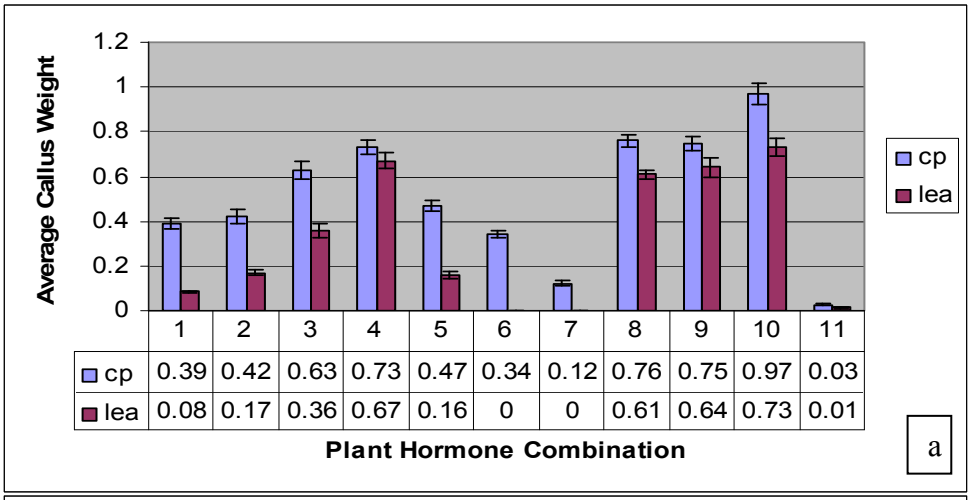
Following the preliminary trials, various hormone combinations were tested by using two explants, longitudinal embryonic axis and cotyledonary petioles which were very responsive for callus formation in many hormone combinations tested especially for 3mg/L NAA+1mg/L BA and 0.75mg/L 2,4-D+0.5mg/L BA. However, on 3mg/L NAA+1mg/L ZR containing media, both explants developed callus which later on turned into root structures followed by darkening and death of tissues. Longitudinal embryonic axis explants also died after 1 week of culture on 1.5mg/L TDZ+0.1mg/L kinetin, whereas cotyledonary petiole explants which carry half cotyledon and half of the embryo, responded with shoot formation and swelling instead of callus formation. 2,4-D+BA combinations usually resulted in callus formation in both explants, except 2 mg/L 2,4-D+0.5 mg/L BA which resulted in very small callus formation on both tissues followed by browning and the death of the explants in two weeks. MS with 0.75mg/L 2,4-D+0.5mg/L BA was the most promising media for both explant types with soft and friable callus development (Figure 3.3, Figure 3.4, d). Cotyledonary petiole explants were also responsive to 0.5mg/L 2,4-D containing media with healthy callus formation, however the same hormone concentration resulted in very weak callus formation on longitudinal embryonic axis explants.

Table 3.2. Different explant types and hormone combinations used in somatic embryogenesis studies. cp is for cotyledonary petiole and lea for longitudinal embryonic axis.

Explant type	Hormone Combinations for Callus Formation	Hormone Combinations for Embryo Induction	Response
1 cp[1], lea[2]	1.5mg/L NAA+ 0.5mg/L BA	1.5mg/L BA+0.5mg/L NAA	callus and embryo formation[1,2]
2 cp[1], lea[2]	2mg/L NAA+ 0.5mg/L BA	2mg/L BA+0.5mg/L NAA	callus and embryo formation[1,2]
3 cp[1], lea[2]	2mg/L NAA+ 1mg/L BA	2mg/L BA+1mg/L NAA	callus and embryo formation[1,2]
4 leaf[1], root[2], internode[3], cp[4], lea[5]	3mg/L NAA+ 1mg/L BA	3mg/L BA+1mg/L NAA	weak callus, no embryo formation[1,2,3] callus and embryo formation[4,5]
cp[1], lea[2]	3 mg/L NAA+ 1 mg/L ZR	3mg/L ZR+1mg/L NAA	callus and root formation followed by necrosis[1,2]
cp[1], lea[2]	1,5 mg/L NAA+ 0,5 mg/L kinetin	1.5mg/Lkinetin+ 0.5mg/L NAA	swelling and death of explants[1,2]
cp[1], lea[2]	1 mg/L NAA+ 0,5 mg/L kinetin	1mg/L kinetin+ 0.5mg/L NAA	swelling and death of explants[1,2]
leaf[1], root[2], internode[3], cp[4], lea[5]	2 mg/L NAA+ 1 mg/L kinetin	2 mg/L kinetin+ 1mg/L NAA	weak callus and no embryo formation[1,2,3,4,5]
cp[1], lea[2]	3 mg/L NAA+ 1 mg/L kinetin	3 mg/L kinetin+ 1mg/L NAA	swelling and death of explants[1,2]
5 cp[1], lea[2]	0.5mg/L 2,4-D	0.1mg/L 2,4-D	callus and embryo formation[1,2]
6 cp[1], lea[2]	1mg/L 2,4-D	0.2mg/L 2,4-D	weak callus formation[1] tissue death[2]
7 leaf[1], root[2], internode[3], cp[4], lea[5]	2mg/L 2,4-D	0.4mg/L 2,4-D	swelling and death of explants[1,2,3,5] weak callus formation[4]
8 cp[1], lea[2]	0.5 mg/L 2,4-D+ 0.75 mg/L BA	0.1 mg/L 2,4-D+ 0.15 mg/L BA	callus and embryo formation[1,2]
9 cp[1], lea[2]	0.5 mg/L 2,4-D+ 0.5 mg/L BA	0.1 mg/L 2,4-D+ 0.1 mg/L BA	callus and embryo formation[1,2]
10 cp[1], lea[2]	0.75 mg/L 2,4-D+ 0.5 mg/L BA	0.5 mg/L 2,4-D+ 0.75 mg/L BA	callus and embryo formation[1,2]

Table 3.2 (continued)

11 leaf[1], root[2], internode[3], cp[4], lea[5]	2 mg/L 2,4-D+ 0.5 mg/L BA	0.5 mg/L 2,4-D+ 2 mg/L BA	weak callus formation followed by necrosis[1,2,3,4,5]
leaf[1], root[2], internode[3], cp[4], lea[5]	3 mg/L TDZ+ 0.1 mg/L kinetin	3 mg/L kinetin+ 0.1mg/L TDZ	swelling and death of explants[1,2,3,4,5]
cp[1], lea[2]	1.5 mg/L TDZ+ 0.1 mg/L kinetin	1.5 mg/L kinetin+ 0.1mg/L TDZ	swelling and shoot formation[1], necrosis[2]



	1	2	3	4	5	6	7	8	9	10	11
cp	0.39	0.42	0.63	0.73	0.47	0.34	0.12	0.76	0.75	0.97	0.03
lea	0.08	0.17	0.36	0.67	0.16	0	0	0.61	0.64	0.73	0.01

	1	2	3	4	5	6	7	8	9	10	11
cp	3.12	4.62	5.82	5.73	3.67	3.36	2.62	7.61	11.7	12.4	0
lea	0	2.14	3.22	4.21	1.63	0	0	3.43	4.13	5.73	0

Figure 3.3. Average weights of calli (a) and number of embryoids produced per cotyledonary petiole (cp) and longitidunal embryonic axis (lea) explant in different hormone combinations tested. Hormone combinations represented by numbers 1 to 11 are defined in the first column of Table 3.2.

Higher concentrations of 2,4-D resulted in complete tissue death for longitudinal embryonic axis explants and reduced the size of calli in cotyledonary petioles. All other hormone combinations resulted in callus formation ranging from very little to moderate in both of the explants. The difference in the responses of cotyledonary petioles and longitidunal embryonic axes might be explained by the presence of intact cotyledons on cotyledonary petiole explants which is an important source of internal growth regulators especially IAA (Srivastava 2002). When combined with the presence of external regulators, the signal could be perceived different by the two explants. Calli weights were recorded only for the different concentrations of the two most promising hormone combinations (3mg/L NAA+1mg/L BA and 0.75mg/L 2,4-D+0.5mg/LBA) for both explants (Figure 3.3).

Although low in number, particular hormone combinations resulted in embryo formation in both longitudinal embryonic axis and cotyledonary petiole explants (Figure 3.3). Although insufficient embryo development was achieved, combinations of 2,4-D and BA were very effective in forming globular embryos on the surface of calli, with the highest average number of embryos/explant 12.36, observed on MS media with Gamborg's vitamins containing 0.75mg/L BA +0.5mg/L 2,4-D (Figure 3.4, a,b,c). 3mg/L BA+1mg/L NAA was the only hormone combination that allowed embryo development to some extent, in both longitudinal embryonic axis and cotyledonary petioles (Figure 3.4, d,e,f,g). However, complete plant regeneration was not observed in either explants.

The only study in the literature that reports somatic embryogenesis from lentil (cultivar Laird) used embryonal axes cultures derived from immature embryos (Saxena and King 1987). The presence of 1 to 5mg/L 2,4-D in B5 medium with 500mg/L ammonium nitrate reported to be resulted in callus growth, organization and maintenance without a significant difference. 2,4-D concentrations above 5mg/L reduced the callus growth and 10mg/L completely inhibited. Transfer of the calli on B5 medium without hormones or 1mg/L BA and 0.25mg/L IAA resulted in 10-20 embryo like structures in one responding callus. Only 3-5% of all calli

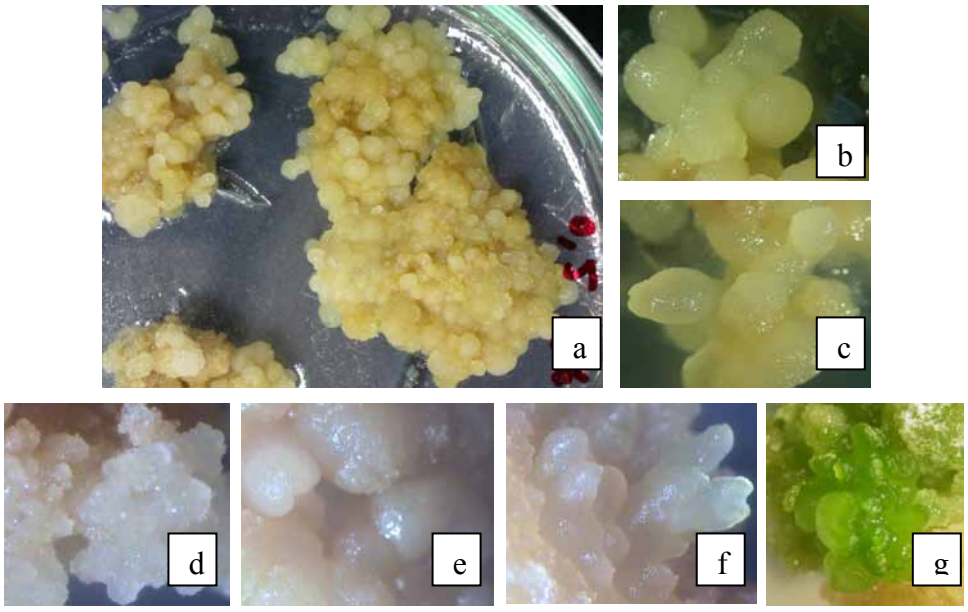


Figure 3.4. Somatic embryogenesis on lentil solid culture. Globular stage embryos (a and b) and development of oblong shaped embryos with possible shoot poles (c) from cotyledonary petioles in the presence of 0.75mg/L BA +0.5mg/L 2,4-D. Formation of friable calli (d), heart shaped (e), and torpedo shaped embryos (f,g) in the presence of 3mg/L BA+1mg/L NAA in cotyledonary petiole explants.

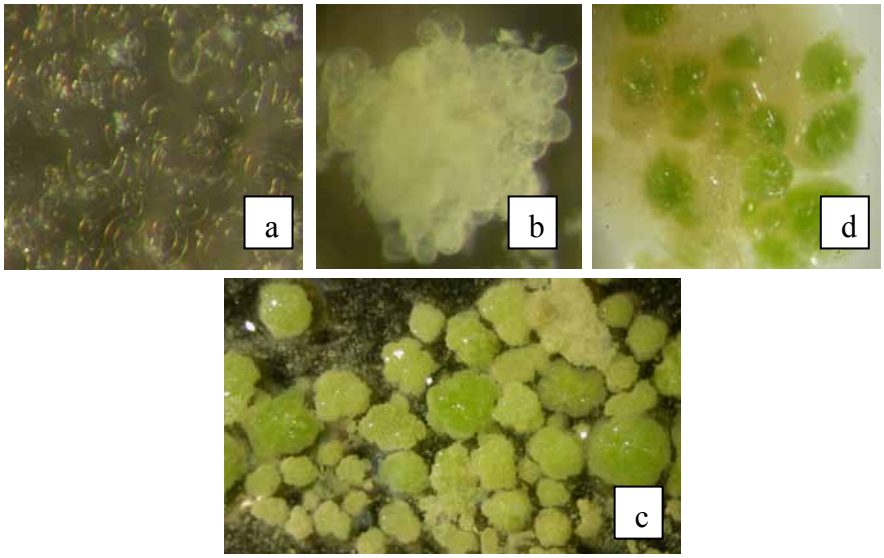


Figure 3.5. Suspension culture trials for lentil somatic embryogenesis. Elongated cells produced on early culture (a), multicellular cell aggregate (b), cell clumps (c) and localized green areas formed on cell clumps (d).

showed any response. Further subculture of the structures on 70mg/L glutamine supplemented B5 medium produced embryos with cotyledons, shoots and roots. In our study, a similar explant, longitudinal embryonic axis isolated from mature embryo of Sultan-1 cultivar exhibited a quite different response when treated with 0.5, 1 and 2 mg/L 2,4-D. Very small calli were formed when the explants were cultured on B5 medium with 0.5mg/L 2,4-D, while 1 and 2mg/L 2,4-D completely inhibited callus formation. On average 1.63 embryo like structures were obtained from longitudinal embryonic axis explants when cultured on B5 medium with 0.1mg/L 2,4-D and none of the embryoids developed into mature embryos. The system described by Saxena and King (1987) had a low efficiency with only 3-5% of calli responded with embryo formation. However, a similar system resulted in the lack of response in our study, which might be due to the cultivar difference.

Hormone combinations 0.75mg/L BA +0.5mg/L 2,4-D and 3mg/L BA+1mg/L NAA were further tested on cotyledonary petioles in combination with MS media containing MS or Gamborg's vitamins, both for callus formation and for further embryo formation and development. The choice of vitamin mixture did not have any significant effect on either the size of calli or the number of globular embryos.

To be able to increase embryogenesis response, effects of 150mg/L casein hydrolysate (CH), 500mg/L ammonium nitrate (AN) and 500mg/L potassium nitrate (KN) as nitrogen sources were assessed on cotyledonary petiole explants. The amounts were determined by preliminary trials to estimate toxic concentration of the particular compound on the explants. The determined concentrations were applied on 30 explants for each different trial. Since CH is a rich source of nitrogen in both free and amino form and for the various free amino acids, a significant effect was predicted, however neither explants nor calli showed any improved response when compared to control in the presence of CH. AN significantly increased calli size formed from cotyledonary petiole explants, however did not have any effect in embryo formation and development. To be able to enhance embryogenesis response, also 100mg/L L-glutamic acid (LGA), 20mg/L L-proline,

50mg/L α-tocopherol and 100mg/L gluthatione reduced (GSH), which were proven to have antioxidant properties in various studies, were included in the embryo induction media. Although proline had a positive effect on the durability of the calli on tissue culture without media refreshment, which increased almost two weeks, neither of the constituents resulted in significant improvement on embryo formation.

Instead of 3% sucrose, different carbon sources, 3% mannitol, 3% maltose or 3% trehalose were also included in MS media both for callus and embryo formation. Presence of mannitol had a negative effect both on the size of calli and the number of embryos appeared. Presence of either maltose or trehalose in the medium did not cause any significant effect on both callus and embryo formation properties.

3.1.2.2. Liquid Culture

Suspension culture for lentil was prepared by using 3-weeks-old friable calli from cotyledonary petioles produced on semisolid MS media containing B5 vitamins and supplemented with 0.75mg/L 2,4-D. The cultures were established on MS media containing Gamborg's vitamins supplemented with 0.25mg/L 2,4-D. The liquid media also contained 20mg/L proline and 500mg/L ammonium nitrate. Elongated cells with a dense cytoplasm were the only cell type observed in the suspension cultures in two weeks (Figure 3.5, a), unlike Ramakrishnan (2005) which observed the presence of two morphologically distinct cells, spherical and elongated both with visible cytoplasm in the suspension cultures of cowpea. They reported that only spherical cells were embryogenic and further developed into heart-stage and torpedo-stage somatic embryos. In our study, some of the cells organized into multicellular aggregates (Figure 3.5, b) and were visible in three weeks of culture. When transferred into media containing 0.1mg/L 2,4-D, cell aggregates formed relatively hard irregular clumps (Figure 3.5, c) and turned light green in color. However, the clumps did not progressed into globular stage embryos or oblong stage embryos which contain root and shoot poles, in both liquid MS media with

0.1mg/L 2,4-D and also in hormone free liquid MS media. Localized green areas were formed on the clumps when they were further cultured on solid MS media without hormones or containing 0.1mg/L 2,4-D (Figure 3.5, d), however, neither organogenesis nor embryogenesis response were obtained and cell proliferation continued without a clear establishment of polarity.

3.2. *Agrobacterium*-Mediated Transformation of Lentil

Literature data shows that like other legume species, transformation studies in lentil were associated mostly with direct regeneration techniques and the most commonly used explant is cotyledonary node. The potential of lentil cotyledonary nodes for transformation by *Agrobacterium* was investigated first by Warkentin and McHughen (1993). In their study GV2260 strain harbouring 35SGUSINT plasmid was used to transform lentil cotyledonary nodes, however only *gus* expression was observed on cotyledonary petioles instead of meristematic axil region. In the study, no transgenic plants were recovered and it was concluded that axil region is not readily amenable for transformation and only a small number of cells in that region are competent for genetic transformation. In a following study, Mahmoudian (2002) used GV2260 strain carrying pGUSINT plasmid in assistance with vacuum infiltration to transform cotyledonary node explants. Usage of vacuum infiltration significantly increased transformation efficiency on petiole region, however low number of regenerated shoots exhibiting GUS expression was obtained and no transgenic plant were regenerated from the utilization of this system.

Various studies concerning transformation of legumes reported the superiority of using succinanamopine *Agrobacterium* strains such as KYRT1, EHA105, and AGL1 when compared to nopaline (C58C1) or octopine (LBA4404 and GV2260) strains. Mahmoudian (2002) which used GV2260 strain in their study also predicted the usage of succinanamopine *Agrobacterium* strains to increase transformation efficiency in lentil. In a comparative study, to evaluate virulence of different strains of *A. tumefaciens* on soybean explants, KYRT1 stain with partially disarmed

pKYRT1 was shown to be more virulent than other commonly used strains, including EHA105, GV3850 and LBA4404 (Torisky 1997, Meurer 1998). Also in a comparison of the efficiency of two *Agrobacterium* strains, AGL 1 and KYRT1, for producing transgenic pea plants, KYRT1 was found to be on average threefold more efficient than AGL 1 (Grant 2003). Recently Dang and Wei (2007) used KYRT1 for the transformation of soybean embryonic tips and reported it to be more effective than EHA105 and LBA4404 in producing shoots.

After determination of the *A. tumefaciens* strain Chry5 is hypervirulent on soybeans by Kovacs and Pueppke (1993), Torisky (1997) constructed a disarmed derivative of pTi-Chry5 by deleting a segment of the Ti plasmid which is designated as pKYRT1. However, a few small gall-like growths were observed by the group on infected plants and this was interpreted as the disarmed derivative still contains one or more oncogenes. Consistent with this observation, Palanichelvam (2000) have reported that pKYRT1 is not truly disarmed and contains a second T-region, TR-DNA of pTiChry5, in addition to about 9 kb of TL-DNA. They have also shown that the TR-DNA of pKYRT1 co-transfers with the binary vector T-DNA at a high frequency during transformation of *Arabidopsis thaliana*. Since such a co-transfer of Ti plasmid T-DNA genes together with T-border genes is undesirable, Palanichelvam et al. (2000) have constructed a new helper plasmid, pKPSF2, lacking both known T-regions and their borders. Ko (2004) compared the efficiencies of *A. tumefaciens* strains harboring pKPSF2 and pKYRT1 on the transformation of soybean somatic embryos and made an interesting observation that KYRT1 with partially disarmed pKYRT1 was much superior in embryo induction.

In the previous studies performed in our laboratory concerning *Agrobacterium* mediated transformation of lentil cotyledonary nodes (Çelikkol 2002), efficiencies of different *Agrobacterium* strains (LBA4404, EHA105, C58C1, KYRT1) and two binary plasmids (pGUSINT and pTJK136), both carrying *gus* and *npt-II* genes, were compared in terms of transformation efficiency. The most efficient strain was

chosen to be KYRT1 harboring partially disarmed Ti plasmid pKYRT1 and binary vector pTJK136, as deduced from the number of *gus* expressing loci in cotyledonary petioles which are in close proximity to petiole axil region that give rise to secondary shoots in the presence of 1mg/L BA. Unlike the study of Warkentin and McHughen (1993) which used GV2260 strain, extensive blue coloring was observed on cotyledonary petioles spontaneously and on the axil region only when scalpel wounding was applied. The explants were very responsive for *Agrobacterium* transformation as observed by Mahmoudian (2002), in which, wounding of axil region was not analyzed. Throughout this study, remarkable *gus* expression was observed on regenerating shoots only when KYRT1 strain was used in combination with vacuum infiltration and scalpel wounding of meristematic axil region which has the potential to allow transformation of meristem tissues from which shoots originate.

3.2.1. Optimization of Transformation Efficiency

In this study, *Agrobacterium* cells were prepared for vacuum infiltration according to Kapila (1997) with some modifications. In the method, yeast extract broth which was used for the growth of bacterial inoculation was supplemented with 20μM acetocyringone and 10mM MES which has the buffering capacity for the acidic medium with pH of 5.6. After sufficient growth of bacterial cells which was determined to be 1-1.2 at OD_{600}, the cells were concentrated by collecting with centrifugation and resuspending in MS based medium MMA supplemented with 200 μM acetocyringone, having pH of 5.6, to be able to obtain a very concentrated *Agrobacterium* suspension solution. It was also important to get rid of five different bacterial selection antibiotics in the explant inoculation media. The bacterial cells were both grown and incubated for 1 hour in low pH media containing acetocyringone as phenolic compound, to induce *vir* gene complex and this treatment had a high potential in improving transformation capacity of *Agrobacterium* cells.

Throuhout the study, all *Agrobacterium* transformations were also performed in the presence of vacuum infiltration. The technique was first used with *Agrobacterium* suspensions by Kapila (1997) for the intact leaves of *Phaseolus vulgaris*. Under *vir* gene inducing conditions all infiltrated leaves from seven different experiments showed large GUS expressing sectors comprising 20-90% of the leaf area. Leaves, which were not infiltrated, but submerged in the bacterial suspension, showed GUS expression only at the wounded sites on the leaves, which were generated during handling. Three different plant species, *P. acutifolius*, poplar and tobacco also presented a similar ability to express GUS activity, thus illustrating a genotype independence of the method. The technique was used in almost all *Agrobacterium* transformation studies recently performed on legumes. By the utilization of the technique, bacterial penetration to deeper tissue layers and greater transformation efficiencies were possible which was also one of the parameters tested in the previous studies performed in our laboratory (Mahmoudian 2002).

After *Agrobacterium* transformation in the presence of vacuum infiltration, a washing step was also included to remove excess *Agrobacterium* cells from the explants, before co-cultivation for three days. In the same way, co-culture period was performed in the dark to prevent bacterial overgrowth over tissues and on the surface of nutrient medium, which can prevent the availability of moisture and nutrient uptake by the explants.

The minimum concentration of selection antibiotic kanamycin in culture media was chosen to be 150 mg/L to completely block shoot regeneration in cotyledonary nodes isolated from non-transformed lentil cultivar Sultan-1 (Çelikkol 2002). Concentrations less than but close to 150mg/L allowed regeneration of one or more shoots from minority of the explants and killed the others. Although very high, this concentration was necessary to affect shoot origins which are seperated from the selective media by the blocking nodal tissue. Therefore, kanamycin was used for the long term selection of transformed tissues at a minimum concentration of 150mg/L throughout this study.

3.2.1.1. Sonication for the Improvement of Transformation Efficiency

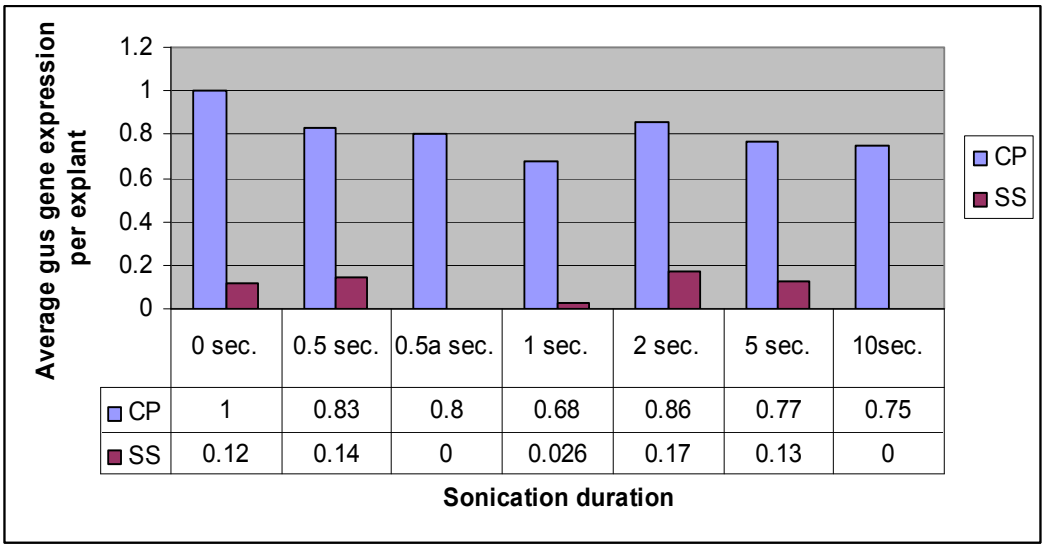
The use of cotyledonary node explants in *Agrobacterium*-mediated transformation to achieve regeneration through direct organogenesis route requires very efficient wounding procedures to be performed. Ordinary methods of wounding such as poking or scratching with a needle or blade are not efficient for many types of tissues, since they usually stimulate wound response of the plants. Defence responses to wounding include deposition of tannins resulting in the browning of wounded tissues and hypersensitive response, which generate a barrier of dead cells on the wound site (Olhoft 2001). This barrier of dead cells prevents efficient transformation of the tissue especially if the *Agrobacterium*-mediated transformation method is used. For this reason, development of new and efficient wounding methods has been required. One of them is bombarding the tissue with uncoated microprojectiles, prior to *Agrobacterium* treatment. Bidney (1992) showed that wounding of tobacco leaves and sunflower meristems by bombardment prior to *Agrobacterium* treatment increased the transformation frequencies. After this study, *Agrobacterium*-mediated transformation of various tissues including banana meristems (May 1995), common and tepary bean meristems (Brasileiro 1996), carnation stem explants (Zuker 1999) and soybean embryogenic clumps (Droste 2000), has been enhanced by the use of this method. In these studies microwounds, resulting from their small size, were reported to prevent intense wounding response and this was enhanced *Agrobacterium* attachment to cells. Although differ upon inoculation of different strain/binary plasmid couples, previous studies performed in our laboratory showed that overall transient expression appeared to be reduced when cotyledonary node tissues of lentil were microwounded by particle bombardment.

Another common wounding method is the sonication assisted *Agrobacterium*-mediated transformation (SAAT). This procedure mechanically disrupts and wound cells via sonic wave effects and is thought to permit more thorough penetration of *Agrobacterium* into the plant tissues. The method was first used by Trick and Finer

(1997) and shown promise for increasing the transformation efficiency of many crops recalcitrant to *Agrobacterium*-mediated transformation including soybean, Ohio buckeye, cowpea, white spruce, wheat and maize. Studies of other groups showed a wide range of plants responsive to SAAT including black locust (Zaragoza 2004), *Pinus pinea* L. (Humara 1999), *Eucalyptus grandis* (Gonzales 2002) and *Chenopodium rubrum* L. (Flores Solis 2003). SAAT allows deep micro-wounds without damaging the neighboring cells. The deeper wounds are advantageous when subsurface tissues such as cotyledonary nodes and shoot meristems are targets for transformation. The studies reported that the size of SAAT-induced microwounds ranged from less than l mm to well over 1 mm, which is large enough to permit *Agrobacterium* to infect the tissues. As the duration of sonication was increased, microwounds became larger and more numerous. This wounding method seemed to hold much promise for increasing transformation rates especially in recalcitrant legumes for *Agrobacterium*-mediated transformation.

In this study, wounding by the help of sonication was integrated into *Agrobacterium* mediated transformation of various lentil explants to asses the method's effects on the transformation efficiency. Sonication duration, explant type and bacterial concentration were among the evaluated parameters. The technique was primarily used on 223 cotyledonary node explants and the *gus* expression on cotyledonary petioles and secondary shoots was quantified (Figure 3.6).

Micro-scale wounds were not observed in the sonicated explants except 10 seconds treatment. The type of wound formed on the explants in this treatment was not microscopic and almost all the explants subjected to 10 seconds treatment died after cocultivation period. Figure 3.7 shows two explants that survived with wound after 10 seconds sonication treatment. Also, the shoots regenerated from the explants showed *gus* expression in highly chimeric manner and there was no significant difference in the expression level and pattern between shoots regenerating from control and sonicated explants. Effect of sonication treatment also did not change when explants were inoculated with half diluted *Agrobacterium* suspension.



	0 sec.	0.5 sec.	0.5a sec.	1 sec.	2 sec.	5 sec.	10sec.
CP	1	0.83	0.8	0.68	0.86	0.77	0.75
SS	0.12	0.14	0	0.026	0.17	0.13	0

Figure 3.6. The change in the average *gus* gene expression per explant on two different parts of cotyledonary node explant according to different sonication durations. Cotyledonary petioles (CP) were scored either as 1 or 0 according to the presence or absence of *gus* expression. Gene expression on secondary shoots (SS) developing from petiole axis was determined by calculating the average number of *gus* expressing shoots (in chimeric manner) per explant.
a: Sonicated explants were inoculated with 1:2 times diluted *Agrobacterium* suspension (OD_{600}=1.2).

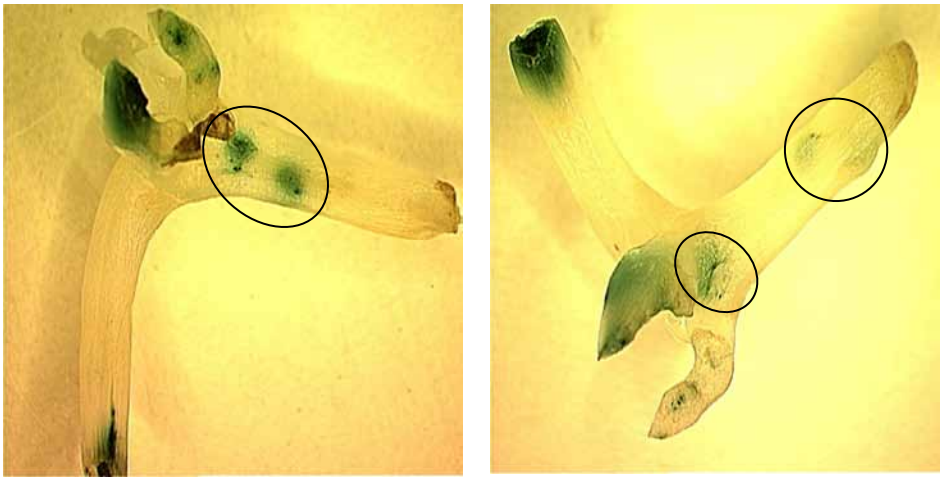


Figure 3.7. Cotyledonary node explants sonicated for 10 seconds. Circles show the wounds formed after the treatment.

By considering the literature data that summarized different responses on different plant species and even the different tissues of the same plant upon sonication treatment, response of another explant, cotyledonary nodes with intact shoots was also examined. 312 explants were subjected to 7 different sonication parameters. After 3 days of cocultivation, shoot apex and nodal regions showed more intense gene expression when compared to non-sonicated, *Agrobacterium* infected control explants, although control explants also showed a week gene expression on the same regions (Figure 3.8). Few shoots that developed on selective media had considerable gene expression (2/67) and one of them was obtained from a non-sonicated, *Agrobacterium*-treated control explant.

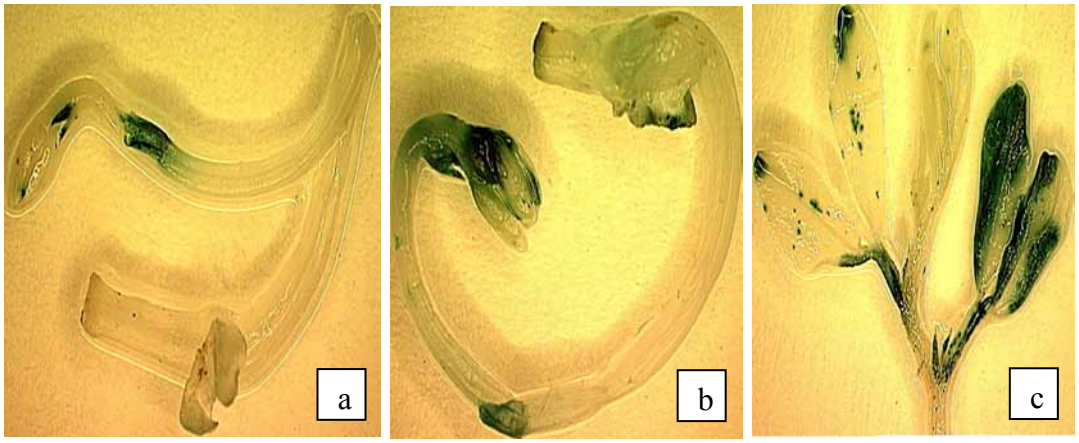
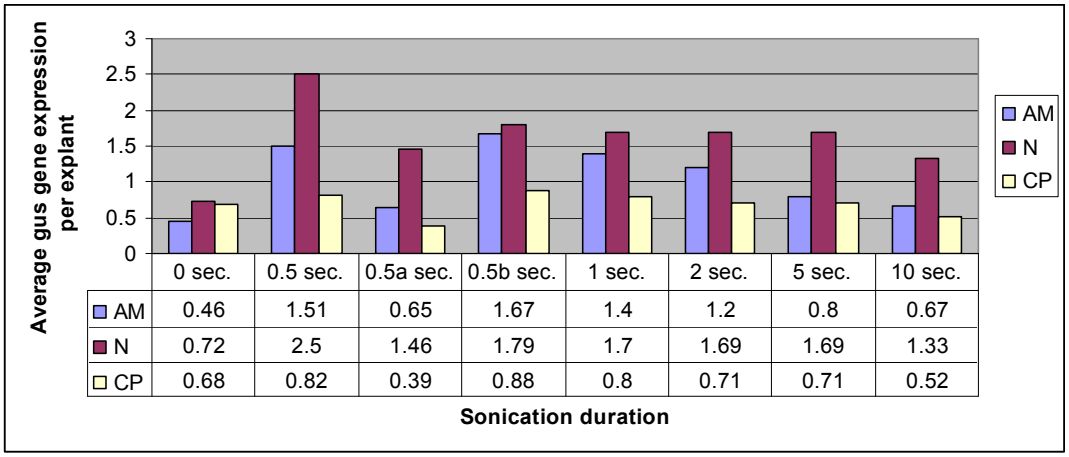


Figure 3.8. Not-sonicated, *Agrobacterium* infected control explant (a), cotyledonary node with primary shoot explant sonicated for 0.5seconds (b) and a chimeric shoot developed from a control explant cocultivated on selective media (c).

Figure 3.9 shows quantified *gus* gene expression on different regions of the explant. All considered regions on the explant contained meristematic tissue and had a potential for regeneration. The most intense gene expression was seen on explants subjected to 0.5 seconds sonication. Neither dehydration of the explants for 10 minutes before SAAT nor dilution of the *Agrobacterium* suspension had any significant effect on the explants' response.



	0 sec.	0.5 sec.	0.5a sec.	0.5b sec.	1 sec.	2 sec.	5 sec.	10 sec.
AM	0.46	1.51	0.65	1.67	1.4	1.2	0.8	0.67
N	0.72	2.5	1.46	1.79	1.7	1.69	1.69	1.33
CP	0.68	0.82	0.39	0.88	0.8	0.71	0.71	0.52

Figure 3.9. Change in the average *gus* expression per explant on different tissues of the cotyledonary node with intact shoot explants subjected to different sonication parameters. Apical meristem (AM) and nodes (N) were scored with 0-3 rating system. Cotyledonary petioles (CP) were scored as either 1 or 0 according to the presence or absence of *gus* expression.

a: Explants were dehydrated for 10 minutes before sonication, by means of removal from MS medium plates.
b: Sonicated explants were inoculated with 1:2 times diluted *Agrobacterium* suspension (OD_{600}: 1.2).

Nodal parts on the developing shoots were used for further shoot development according to the method of Fratini and Ruiz (2003). Nodal segments were isolated and cultured on 5µM IAA, 1µM kinetin and 75mg/L kanamycin containing media. Although weak and short, shoots developed from the nodes of each segment after three weeks on selection media. GUS expression on these shoots was examined and it was seen that the expression was only on the leaf covering nodal region but none of the developing shoots was transformed (Figure 3.10).

Another explant used in sonication experiments was half cotyledons which have the ability to readily regenerate shoots. GUS expression on petiole region was determined by scoring the expression level from 0 to 3 (Figure 3.11). The same rating system was also used for scoring cotyledonary nodes and cotyledonary nodes with intact shoots. Different sonication parameters were applied to 490 explants in addition to scalpel blade wounding of the axil region. On average 6 shoots were

Figure 3.10. Cotyledonary node with primary shoot explant used for the isolation of nodal segments, arrows point excised regions (a), shoot and root development on a nodal segment under selection (b), *gus* staining of a shoot developed from a nodal segment under selection (c).

regenerated from a single half cotyledon explant, independent from the application of sonication treatment. In spite of the strong transient *gus* expression on the petiole region of the explants resulting from different wounding treatments, none of the shoots developed from the axil showed expression (Figure 3.12). On average the most intense expression was on control plants transformed without sonication treatment and the lowest transient expression was observed when the explants were wounded by scalpel blades (Figure 3.13).

Some studies in the literature also reports ineffectiveness of sonication in improving *Agrobacterium* transformation efficiencies. For example, unlike the study of Trick and Finer (1997) which reports effectiveness of the system on soybean immature cotyledons and Santarem (1998) on embryogenic suspention cultures, Meurer (1998) reports the ineffectiveness of the system in stable genetic transformation of 28 soybean cultivars by three different *Agrobacterium* strains. The same group also reports the significant reduction in regenerating shoot number following sonication treatment. The method could not improve transgene expression in *Agrobacterium* mediated transformation of *Trifolium repens* (Christiansen 2000) cotyledons and whole seedling of *Sclerocarya birea* (Mollel

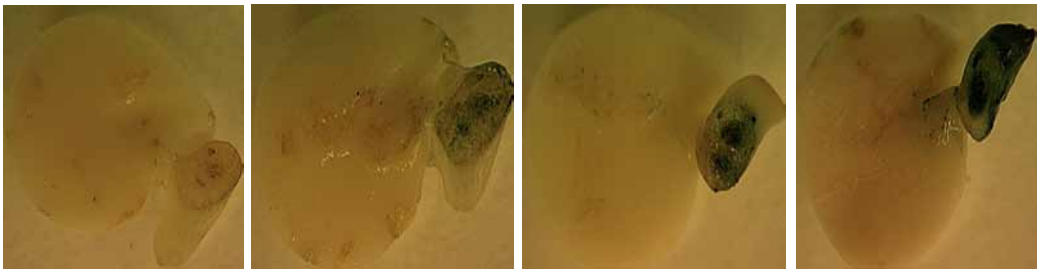


Figure 3.11. Half cotyledon explants with GUS expressing petioles that are scored as 0, 1, 2 and 3 respectively.

Figure 3.12. GUS expression on cotyledonary petiole after 3 days of cocultivation (a). Expression on half cotyledon explants after two weeks on selection media with 150mg/L kanamycin (b).

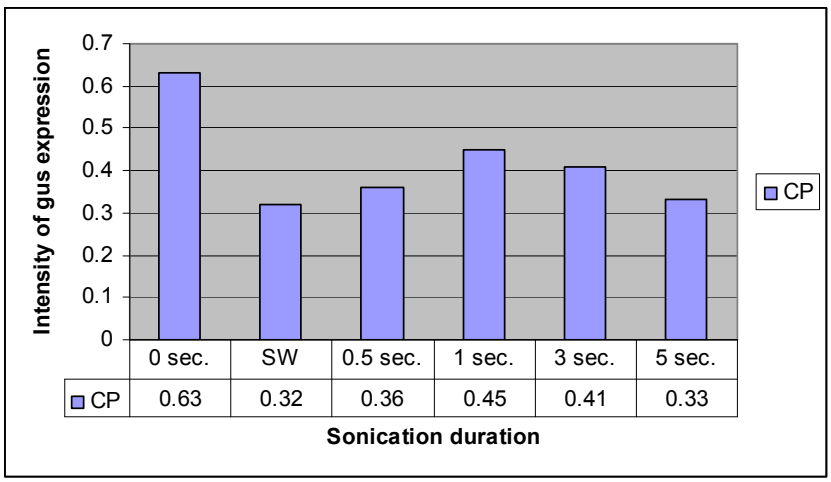


Figure 3.13. GUS expression determined by 0-3 rating system on petiole region (CP) of half cotyledon explants.
SW: Response in scalpel wounded explants.

and Govyaerts 2004). Amoah (2001) investigated the role of SAAT on transformation efficiency of immature wheat inflorescences and observed an increase in the number of transgene expressing explants but decrease on the area of the expression units on the explants which negatively affected overall transformation efficiency. Also Weber (2003) observed the positive effect of sonicaton on transient GUS and GFP expression of sunflower tissues, however the system had no effect on stable genetic transformation of the plants.

Most of the lentil explants used in this study, similarly did not reveal any improvement in *gus* gene expression following sonication treatment. The two explants; cotyledonary node and half cotyledons which have the greatest potential in regenerating high number of shoots responded with decrease in transient gus expression in their petiole tissues which alredy have no potential of regeneration. Localized wounds on the target tissue, meristem axis was never obtained by the use of sonication. Since non of the explants produced any stable gus expressing shoots, to asses the effectiveness of sonication in stable genetic transformation of lentil was not possible.

3.2.1.2. Improving Selection Efficiency

Observation of the ineffectiveness of sonication-mediated wounding system resulted in the use of two explants, cotyledonary nodes and half cotyledons, carrying same region of meristematic tissues, in *Agrobacterium* transformation experiments in combination with wounding by the help of scalpel blades. Although exhibited less transient expression efficiency when compared to control and sonicated explants, the use of scalpels helped the formation of localized wounds on the target meristematic tissues. Both explants were capable of regenerating shoots after wounded by 8 to 10 times by scalpels, the strike powers of which was optimized to form wounds neither too deep nor to shallow which can affect only the tissues on the surface. Explants did not exhibit a severe wounding response after

scalpel wounding. Only minor amount of explants, which were usually over wounded, were unable to survive through selection cycles.

Preparation of the cotyledonary node explants and wounding of the tissues immediately before transformation in combination with vacuum infiltration resulted in the production of large number of shoots which do not express the *gus* gene in any of their tissues. In total, only 7 highly chimeric shoots were regenerated from 1500 cotyledonary node and half cotyledon explants. All of these shoots appeared at the late phases of culture period on the media containing 200mg/L kanamycin and regenerated slowly. Their anatomy was also different, with shorter and/or thinner stems and tiny leaves when compared to the escape shoots which developed faster and grew taller. This observation can be related with the intact axil meristems giving rise to escape shoots and scalpel wounded meristems which has damaged and needed time to form their integrity, giving rise to partially transformed shoots (Figure 3.14).

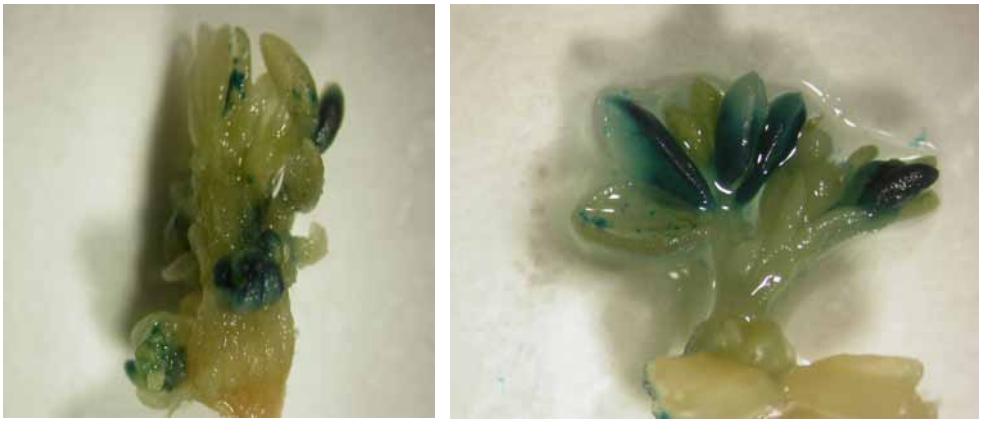


Figure 3.14. Two chimeric shoots regenerated from cotyledonary node and half cotyledon explants.

The results obtained from transformation experiments were not feasible in this form and the possibility of obtaining transgenic plants was extremely low. To be able to increase the efficiency of GUS expressing shoot production, explants were

subjected to gradual selection by refreshing selection media once a week with increasing kanamycin concentrations (starting with 100mg/L, continuing with 200 and 300mg/L). The exactly opposite of this selection regime was used by Gulati (2002) in the transformation of lentil by particle bombardment. In the study, selection herbicide chlorsulfuron was used in 5 nM concentration for the first 4 weeks followed by 2.5 nM, for the remainder of the culture period. The application relieved selection pressure on the explants and provided better regeneration and selection of regenerating shoots. On the other hand, it is known that legumes have a natural resistance to aminoglycoside antibiotics like kanamycin (Christou 1994) and this property makes *npt-II* marker gene an inefficient selection marker when used for legume transformation. Therefore, in our case, a gradually intensifying selection regime was observed to be much more efficient for the selection of transgenic lentil tissues when compared to a regime exerting a gradually decreasing or constant selection pressure intensity.

Culturing of the explants on 1mg/L BA for four days prior to transformation experiment, removing regenerated shoots immediately before wounding of the axil region and application of gradual selection increased GUS expressing sectors significantly. Also removing regenerated shoots after cocultivation, and after each round of selection resulted in the formation of shoots expressing *gus* gene with less chimerism levels or on their entire tissues (Figure 3.15). Improvement of transgene expression upon gradual selection and repeated removal of shoots was observed only in cotyledonary node explants but not in half cotyledon explants. This result was attributed to the presence of the whole cotyledon tissue attached to the target meristematic regions of half cotyledon explants which possibly blocked the access and also decreased the dependence of the regenerating tissues to selective media.

Tissue browning and cell death was not visible in majority of the cotyledonary node explants until the third cycle of the selection. Explants were able to regenerate, in average, 9.2 shoots/explant, during the period of six selection cycles. Although anatomically normal, shorter and thinner stems associated with the transgene

expressing regenerants on the late culture period, in the same way with the weak chimeric shoots obtained from cotyledonary nodes without the application of gradual selection and repeated removal of regenerated shoots in the early experiments. This result may be attributed to the loss of vigor in the meristem tissues exposed to long in vitro culture conditions in the presence of high concentrations of toxic selection antibiotic. However, loss in vigor alone can not explain the increase in the number of strongly GUS expressing shoots in the later phases of culture. One possible explanation can be the presence of meristem tissues that were younger or non-existent at the time of wounding which had a chance to regenerate later in the culture, when the present shoots were removed. Possibly those stably transformed tissues had a capacity to produce shoots with little or no chimerism when compared to the early shoots regenerated from tissues either showed transient expression or which were already mature and organized when the time of wounding.

Figure 3.15. Shoots which were regenerated from cotyledonary nodes expressing *gus* gene in different intensities.

Transformations with the new experimental set up were systematically performed on 6 independent experiments by using 30 explants in each. After 6 weeks of culture on selection media, all regenerated shoots were analyzed for GUS expression without damaging apical dome and nodal buds. 174 explants produced 19 shoots which expressed *gus* gene partially or completely in their tissues. All GUS positive shoots were grafted on root stocks for whole plant regeneration.

3.2.1.3. Grafting and Hardening of Regenerated Shoots

Although cotyledonary nodes have the capability to regenerate roots when cultured on MS basal media, after long term culture on selective media which also contain 1mg/L BA, they completely lose their rooting capacity. Similarly the shoots regenerated from cotyledonary nodes did not show any rooting capacity in rooting trials on hormone free media and media supplemented with various auxins which took place throughout the study. Polanco and Ruiz (1997) also reported significant inhibition of root formation from the shoots cultured on BA containing media more than 4 weeks. This inability becomes a major constraint in the establishment of a successful plant regeneration system both from regenerated shoots and cotyledonary node explants. Therefore, grafting was used to overcome rooting related regeneration problems and also to start hardening of the tissues to reduce the time necessary for regeneration process. Root stocks which were isolated from 3-days-old lentil seedlings, were used directly for the grafting of transgene expressing shoots. Regenerated shoots grafted on the root stocks started to grow within 1 week on perlit containing pots covered with transparent plastic cups. Perlit containing cups were regularly wetted with liquid MS media containing MS vitamins. Adaptation of the plantlets to tissue culture room conditions was achieved by puncturing the transparent cup regularly and finally exposing the tissues completely to the environment (Kamçı 2004).

Unless the grafted shoots were very thin or very short, 100% grafting efficiency were obtained. However, even when the shoots with poor vascular tissue develop-

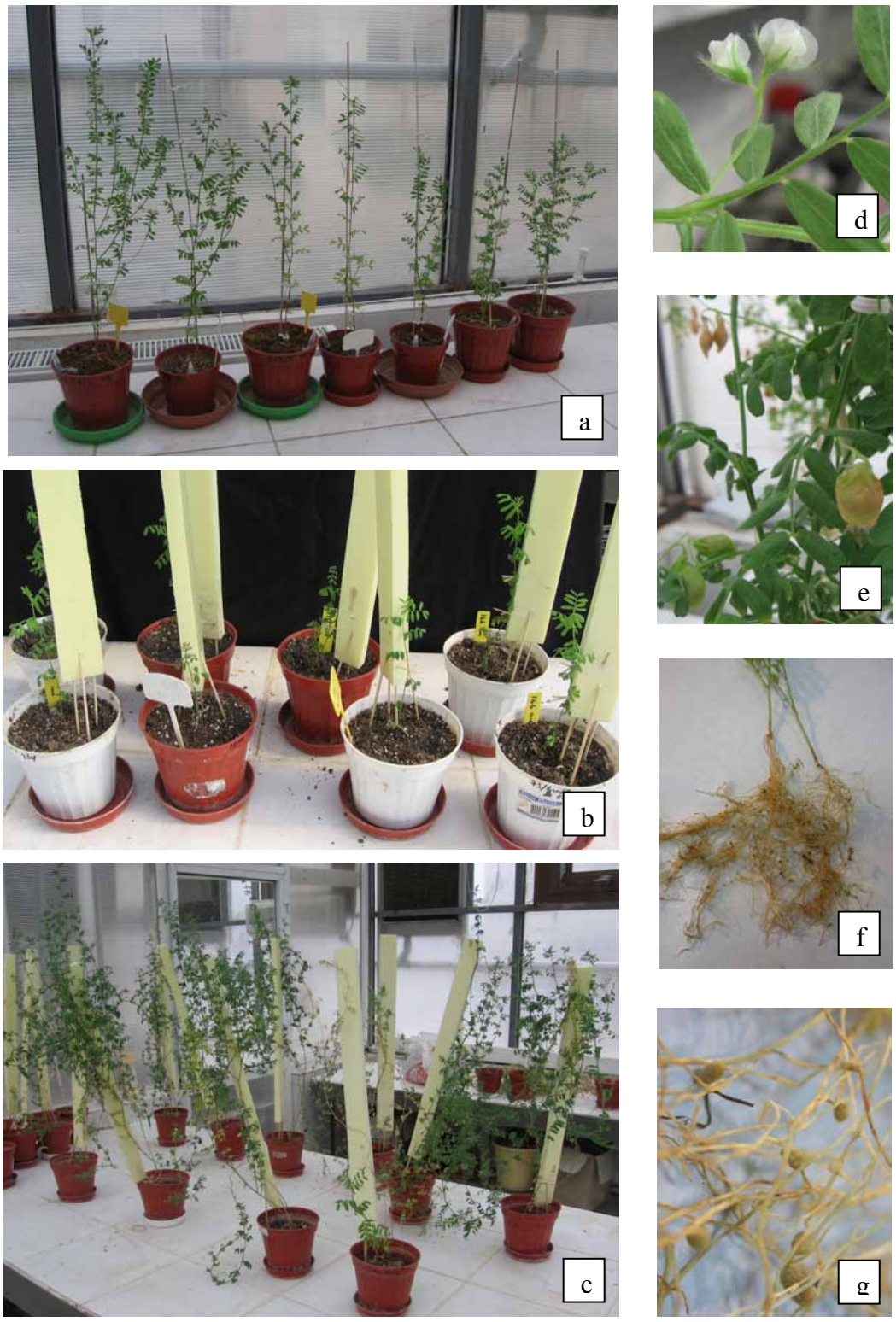


Figure 3.16. T_0 (a), T_1 (b) and T_3 (c) plants developing in greenhouse conditions. Flowering (d), seed set (e) and healthy root (f) and nodule formation (g) in transgenic plants.

ment were successfully grafted and growth was observed to some extent, their adaptation to greenhouse conditions was unsuccessful. Of the 19 shoots grafted on rootstocks, although fused well with mother tissues and elongated to some extent, very weak 5 shoots died after transfer to the greenhouse. Therefore, on average, the growth success of the grafted shoots expressing *gus* gene was 74%. These shoots were grown to maturity in the greenhouse conditions with natural light/dark cycle and temperatures ranging from 10-35 ^{0}C. Plants were watered regularly with tap water containing NPC fertilizer, in the concentration recommended by the producer company. Water was never directly poured on the surface of the soil and only placed on the pot underlay to prevent water-logging stress which is known to be more damaging for lentil when compared to drought stress (Hawtin 1980). Healthy nodulation on roots was obtained in the greenhouse conditions independent of the season. However the seasons affected seed set durations which changed from 2 to 4 months, relatively through summer to winter.

3.2.2. Analysis of Transformation in T_0, T_1, T_2 and T_3 progeny

Transgenic nature of the plants was primarily tested by histochemical GUS staining followed by PCR analysis for each generation. Reverse transcription PCR was performed on both T_0 and T_1 generation. Real time PCR and Southern blot were performed on only T_0 generation. Different numbers of seeds were obtained from T_0, T_1 and T_2 plants which expressed transgenes in different ratios. Chi-Square analysis helped evaluation of observed deviation from expected mohybrid ratio of 3:1 for the inheritance of a single gene.

3.2.2.1. GUS Expression of Transformed Tissues

Histochemical GUS assay was used on every stage throughout the transformation studies. Efficiency of the sonication treatment and scalpel wounding was assessed by examining *gus* gene expression in various tissues. Regenerated shoots, which were produced after optimized transformation experiments, used for micrografting

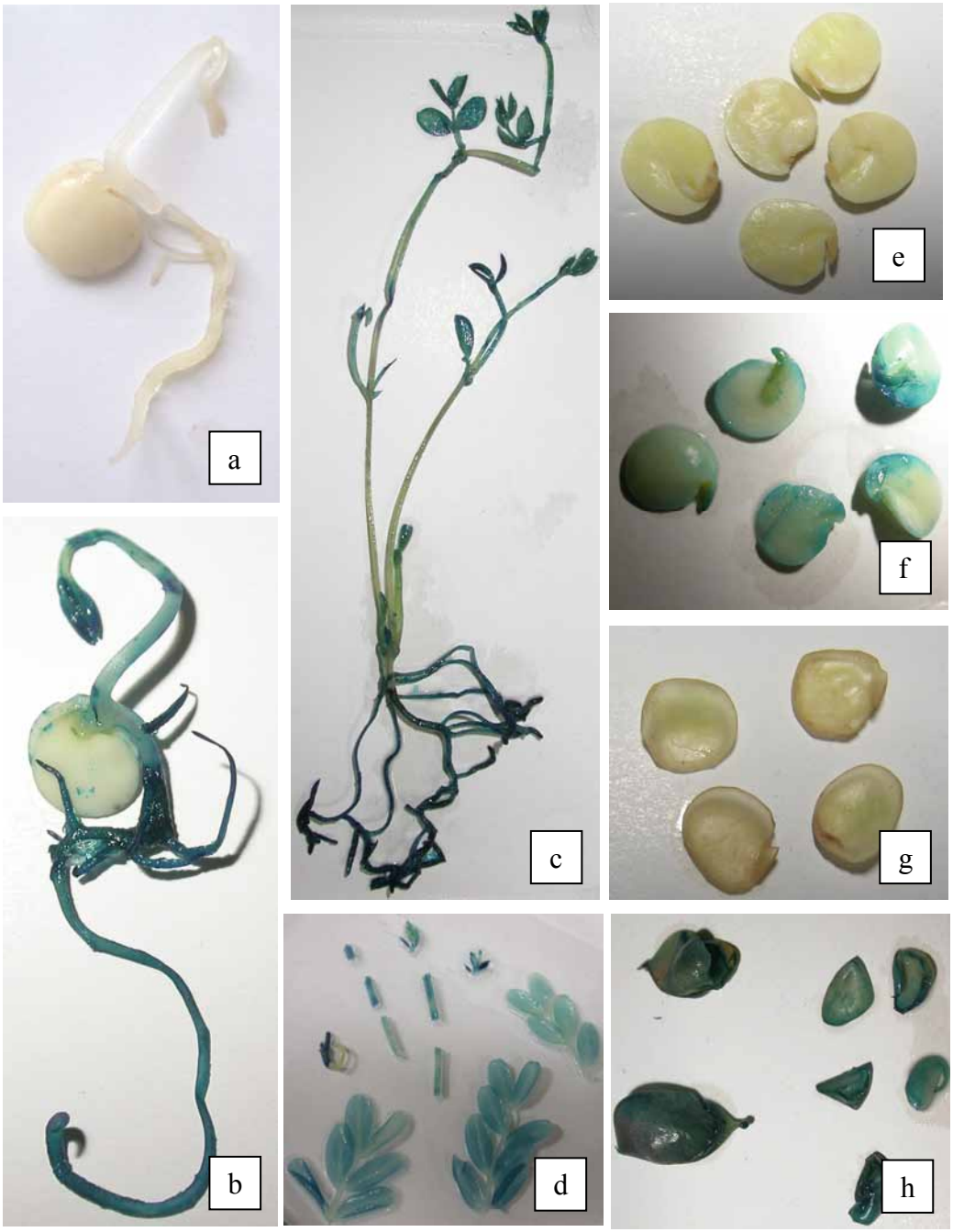


Figure 3.17. *Gus* gene expression on various lentil tissues, control seedling (a), germinating seedling of a T_1 plant (b), 10-days-old T_3 seedling (c), leaf and stem parts from a T_0 plant, imbibed control (e) and T_3 generation transgenic seeds (f), seed coats and pots of control (g) and transgenic T_3 seeds (h).

according to the GUS expression level on their tissues. Stability of transformation in different tissues of the regenerants through generations was tracked also by GUS expression (Figure 3.17). Deduced from the histochemical staining results, of the 14 shoots grafted on root stocks, only 7 stably expressed the *gus* gene uniformly in all sections of their tissues tested, until the end of their life cycles. Independent from the transgene copy numbers which were revealed by southern blot analysis, all 7 plants expressed the *gus* gene in high levels. 2 of the plants that homogenously expressed the gene in all tissues could not inherit the both genes into T_1 generation as revealed by both GUS staining and PCR results. 7 shoots which had shown a chimeric nature prior to grafting could not express the *gus* gene in their actively growing tissues and GUS expression in changing intensities was only observed in 3 of them as limited to the basal leaf tissues. None of these plants were able to inherit the gene to T_1 generation which was also shown by both GUS staining and PCR analysis. Segregation of the *gus* gene in T_2 and T_3 generations was also determined by applying GUS staining on randomly selected tissues from different branches after germination of the seeds on wet perlit. PCR analysis results performed throughout the study on genomic DNAs which were extracted from randomly selected GUS positive and GUS negative individuals were always in consistency with GUS histochemical staining results.

3.2.2.2. PCR Analysis

PCR analysis was performed to detect *gus* and *npt-II* sequences integrated into the plant genome in each generation, when the tissues reached to an appropriate size. Primers were designed to amplify whole gene sequences except the promoter and terminator regions. Amplification of the *gus* gene with an intron region and *npt-II* gene resulted in 1200bp and 700bp products, respectively (Figure 3.18). PCR analysis showed that 2 strongly GUS expressing original lines could not transfer the both transgenes into T1 generation as interpreted both from GUS histochemical staining and PCR amplification of both *gus* and *npt-II* genes (Figure 3.18, c, d, lanes 5 and 7).

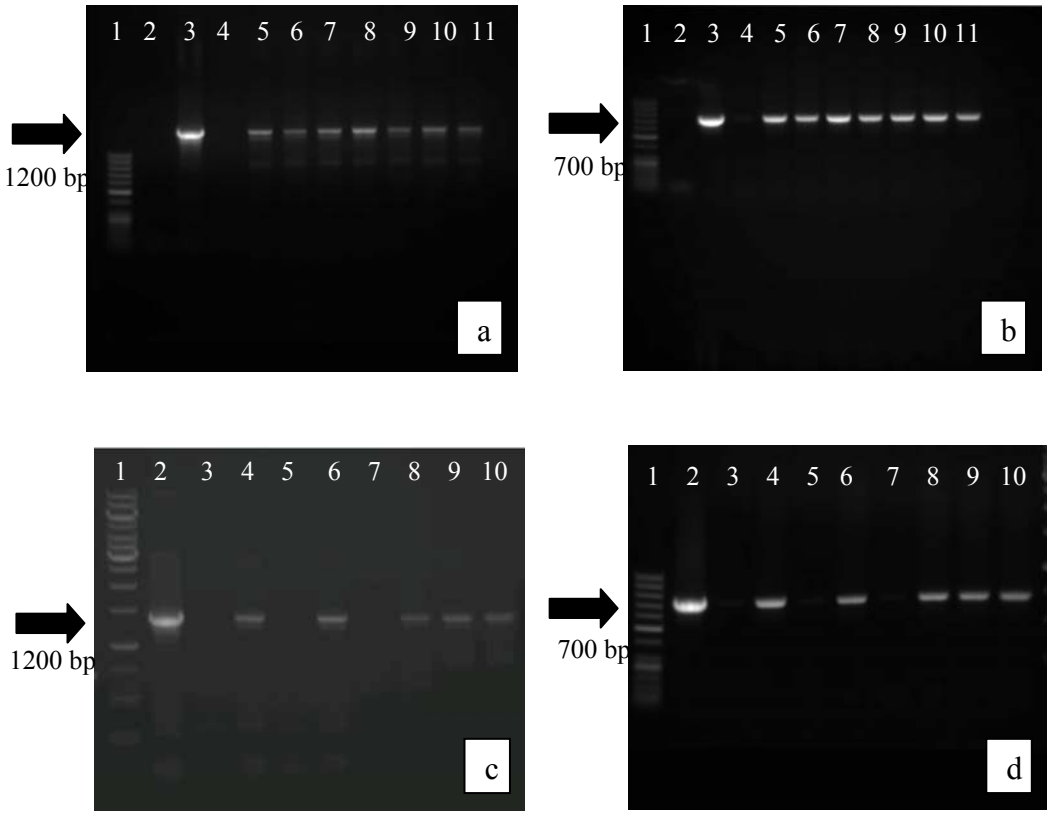


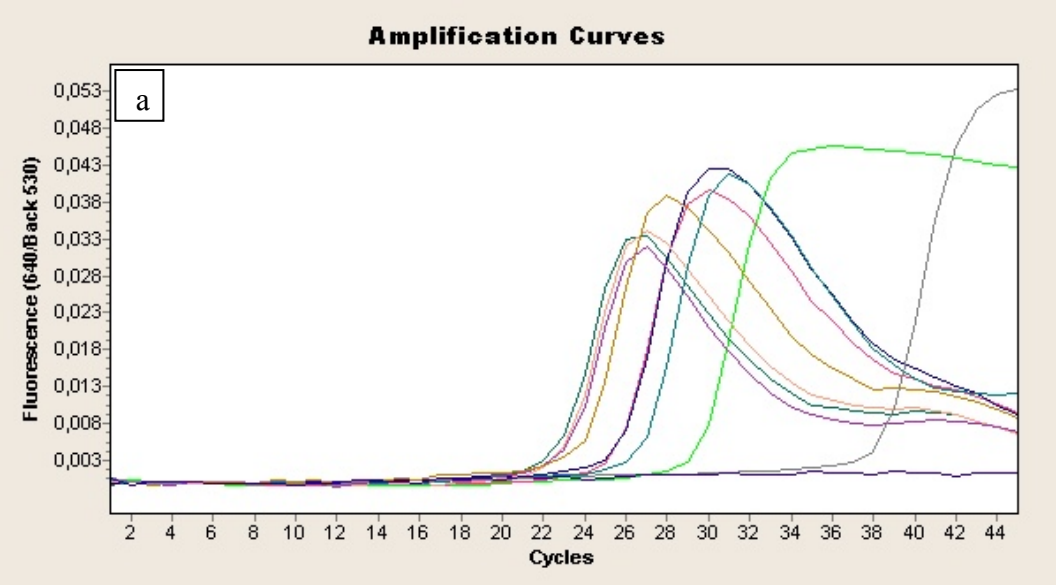
Figure 3.18. PCR amplification of integrated genes in two generations. Amplification of gus gene in 7 independent T_0 plants (a) and amplification of *npt-II* gene in the same T_0 plants (b), lane 2 in a and b does not contain any PCR product, lane four contains PCR product of control plant, lane 3 shows amplification of pTJK136 plasmid. Amplification of *gus* gene in 5 T_1 plants (c) and amplification of *npt-II* gene in 5 T_1 plants (d), lane 3 in c and d contain PCR product of control plant, lane 2 shows amplification of pTJK136 plasmid, lanes 5 and 7 contain PCR products of GUS negative T1 individuals.

Negative results were obtained in PCR assay performed on DNA samples of lentil plants selected randomly from different generations using primers specific for aminoglycoside adenyltransferase (*aadA*) gene which is found outside T-DNA region and gives bacterial streptomycin/spectiomycin resistance. The results avoided suspicion of false positives due to contaminating *Agrobacterium* cells in plant tissues (data not shown).

3.2.2.3. Real Time PCR Analysis

LightCycler GMO Screening Kit (Roche), which is optimized for the flourometric detection of 35S promoter of cauliflower mosaic virus and the 3' untranslated region of the nopaline synthase (NOS) gene of *A. tumefaciens*, was used for the real time PCR analysis. The kit contains one set of 35S-specific probe which is labeled at the 5' end with Light-Cycler–Red 705 and one NOS-specific probe labeled with LightCycler–Red 640. The other set of 35S- and NOS-specific probes are labeled at the 3' end with fluorescein. The emited fluorescence during energy transfer between the two fluorophores, which comes in close proximity after hybridization to template DNA, is measured by the instrument.

To be able to measure the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection which can only measure the amount of product at the end (plateau) phase. The analysis was performed on cDNAs of 7 putative independant transformants which belong to T_0 progeny and a non-transformed control lentil plant. The *gus* gene in T-DNA region of pTJK136 vector construct is driven by 35S promoter and terminated by nos. Therefore, both of the fluorescent labeled probes of Roche LightCycler GMO detection kit were suitable for the detection of the cDNA of *gus* gene prepared from putative transformants. Figure 3.19 (a) shows the NOS terminator amplification signals of 7 GUS positive plants which are collected before cycle 26. Non-transformed control lentil cDNA responded on the 36^{th} cycle which is 8 cycles behind the reference positive control cDNA and 10 cycles later than the signal of putative transformants. Similar amplification curves were obtained from the real time PCR for 35S promoter (3.19, b). cDNAs from 7 putative independent transformants gave amplification signals earlier than that of reference positive control cDNA and signal of non-transformed lentil control cDNA appeared only at the 38^{th} cycle. The results were also in consistency with GUS staining and PCR analysis.



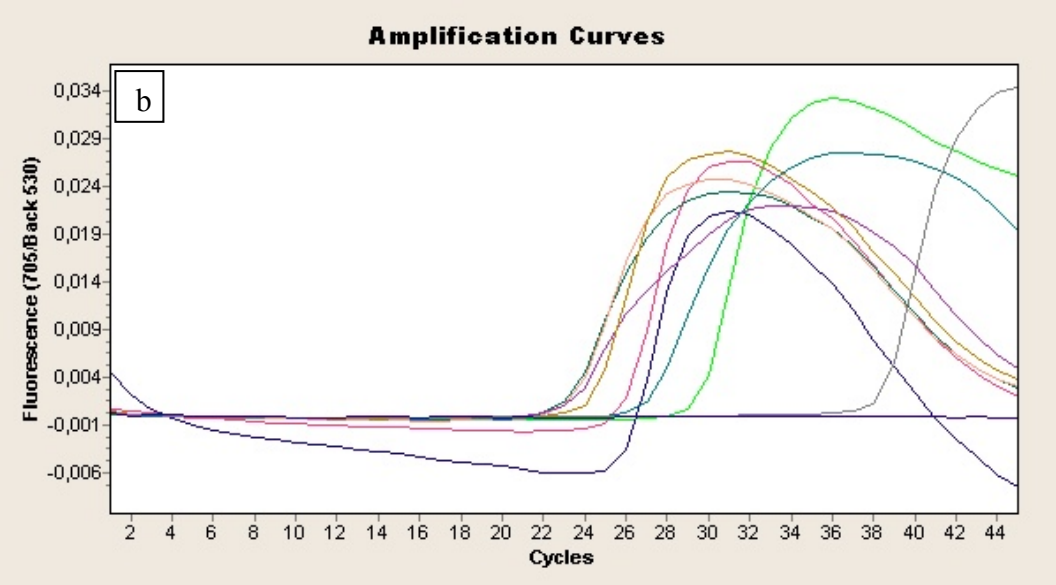


Figure 3.19. Real time PCR analysis for the amplification of nos terminator (a) and 35S promoter controlling *gus* gene (b).

3.2.2.4. Southern Blot Analysis

Southern blot analysis was performed to demonstrate the integration of the *gus* gene in the initial transformants. A non-transformed control plant and five putative transgenic plants which belong to T_0 generation were used for the analysis. Genomic DNA samples were digested with HindIII restriction enzyme which does not recognize any sequence inside T-DNA region of the binary vector. The digested DNA samples were fractionated on a 0.8% agarose gel (Figure 3.20, a) and capillary blotted on a positively charged nylon membrane. After fixation of DNA on membrane by baking at 60 ^{0}C for two hours, the blot was hybridized with a PCR-amplified *gus* fragment labelled with Digoxigenin by the use of random primed labelling of DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche). Probe was designed to have a capacity to bind 656 bp region of the *gus* gene, which corresponds to a non-conserved region of the gene.

While non-transformed control plant did not exhibit any signal, a single hybridization signal of different molecular sizes was obtained in lines B3 (9.5 kb), C4 (18.5 kb) and D5 (6.6 kb). Two lines, A1 and A2 exhibited the same banding pattern shown in Figure 3.20, b. The analysis confirmed that this two sister lines were derived from a single transformation event and regenerated from the same explant. Genomic DNA of both lines carried three inserts of 16.2 kb, 8 kb and 5.9 kb. As the size 5.9 kb is quite close to the T-DNA length of the pTJK136 construct which is 5.4 kb, this transformed line might be carrying tandem repeats of the T-DNA in its genome.

Detection of transgene signals in T_0 lines were also in consistency with the results of GUS histochemical staining, PCR, RT-PCR, real time PCR and chi-square analysis.

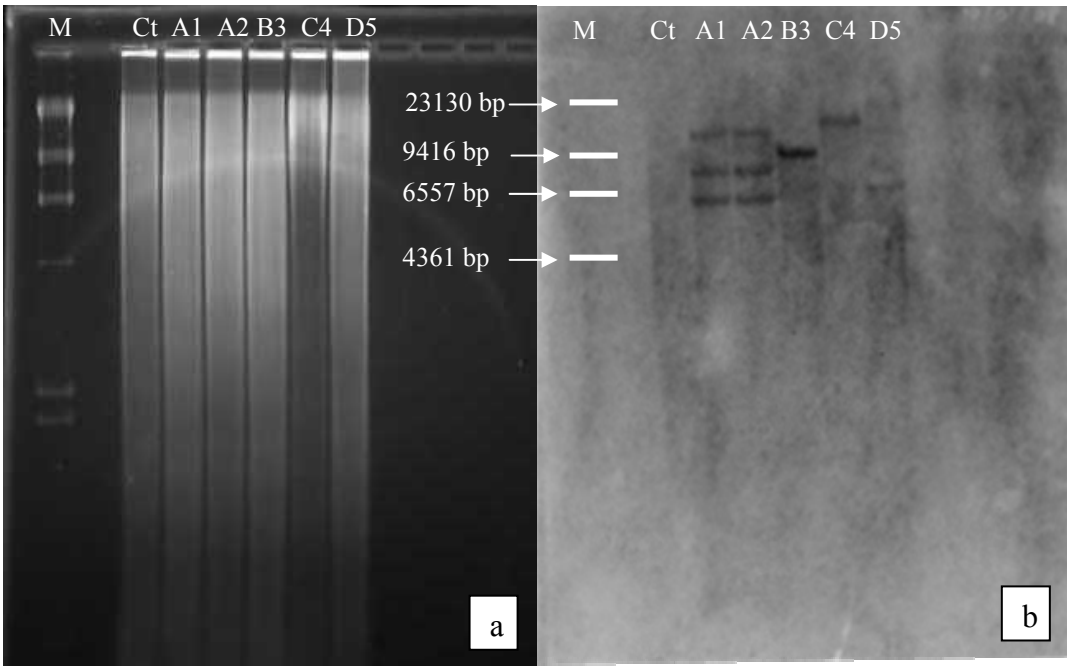


Figure 3.20. Agarose gel electrophoresis of digested genomic DNA (a) and probe signals on nylon membrane blot (b). Lanes on figure a corresponds to λ-HindII marker, control DNA, and DNA from 5 different transgenic lines, respectively. Signals on figure b have the same order as figure a.

3.2.2.5. Reverse Transcription PCR

RT-PCR was performed on cDNAs synthesized from total RNAs of 7 *gus* gene expressing lines from T_0 generation and 5 GUS positive and 2 GUS negative plants from T_1 generation. While *npt-II* primers amplified expected 700 bp product from total cDNAs of GUS positive T_0 and T_1 plants, *gus* primers amplified 1100 bp products which correspond to intronless *gus* gene sequence of complementary DNA. In consistency with the PCR analysis, RT-PCR showed that individuals of T_1 generation that were unable to express *gus* gene also exhibit negative results for the RT-PCR amplification of both c-DNAs (Figure 3.21, c, d, lanes 5 and 7).

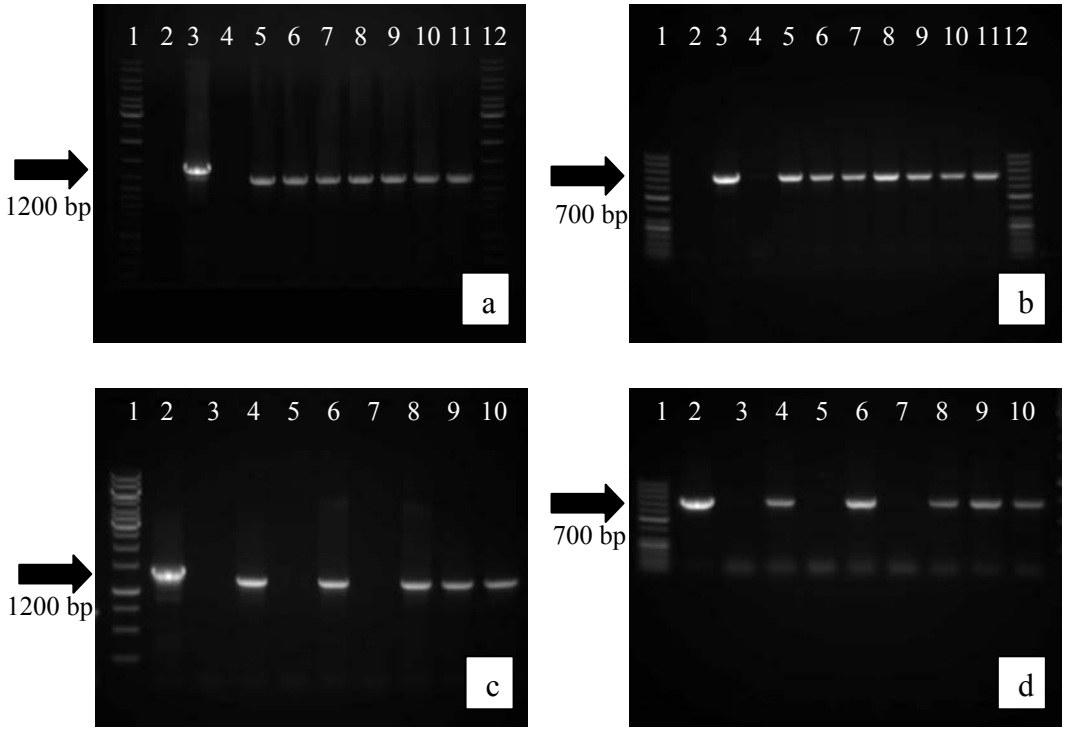


Figure 3.21. Reverse transcription PCR amplification of *gus* and *npt-II* cDNA products. Amplification of *gus* cDNA in 7 independant T_0 plants (a) and amplification of *npt-II* cDNA in the same plants (b), lane 2 in a and b does not contain any RT-PCR product, lane four contains PCR product of control plants, lane 3 shows amplification of pTJK136 plasmid. Amplification of *gus* cDNA in 5 T_1 plants (c) and amplification of *npt-II* cDNA in 5 T_1 plants (d), lane 3 in c and d contain RT-PCR product of control plant, lane 2 shows amplification of pTJK136 plasmid, lane 5 and 7 contains RT-PCR products of *gus* negative T1 plants.

3.2.2.6. Progeny Analysis

After self-pollination of the transgenic lentil T_0 lines, the stable inheritance of the transgenes was tested in four independent T_1 lines and one line with the same origin with A1 by the use of histochemical GUS staining. PCR analysis for *gus* and *npt-II* genes from randomly selected GUS positive T_1 plants always resulted in amplification of the transgene.

Table 3.3 shows the segregation of the *gus* gene in T_1 progeny deduced by the GUS histochemical staining**.** χ^2 values were calculated for the Mendelian ratio of 3:1 for

the segregation of a single dominant gene. Only line A1 had a value larger than 3.84 for 5% probability level. Although within limits A2 revealed a very high χ^2 value "3" in spite of very low sample size. Southern blot analysis explained the deviation from the expected ratio 3:1 by revealing three copies of the transgene in the genomic DNA of two T_0 lines which were also proven to be produced from the same transformation event by exhibiting the same banding pattern on the blot.

Table 3.3. Segregation of *gus* gene in T1 progeny of transgenic lentil plants.

To Plants	Number of Seeds Tested for GUS Expression			Expected Ratio	χ^2 value
	TOTAL	GUS+	GUS-		
A1	17	17	0	3:1	5.669
A2	9	9	0	3:1	3.0
B3	23	15	8	3:1	1.173
C4	36	24	12	3:1	1.33
D5	14	11	3	3:1	0.095

χ^2 (df=1) for p = 0.05 is 3.84

CHAPTER 4

CONCLUSION

To be able to establish an efficient regeneration system which can be used in combination with gene transfer studies, different explants; longitudinal embryonic axis, nodal segments, leaves, internodes, hypocotyls and epicotyls were cultured on MS basal medium including various plant growth regulators for direct organogenesis. Shoot regeneration was achieved in low frequencies from longitudinal embryonic axis explants and nodal buds of epicotyls, however elongation and rooting of the shoots were unsuccessful. Leaf, internode and hypocotyl tissues were unresponsive for all growth regulator combinations tested. Nodal segments isolated from 1-week-old in vitro germinated seedlings produced shoot and roots when cultured in inverted position on MS medium supplemented with 1mg/L IAA and 0.2mg/L kinetin according to Fratini and Ruiz (2003). However, nodal segments isolated from older seedlings and produced from cotyledonary nodes could not regenerate successfully. This observation impeded the method's usage in combination with genetic transformation experiments in this study.

Epicotyl, hypocotyl, internode and leaf segments were also used for indirect organogenesis studies. All the explants were unresponsive except swelling of hypocotyls and root directed ends of internodes and weak callus formation on leaves which were followed by tissue browning and necrosis.

In somatic embryogenesis studies, the most responsive explants for callus and embryo formation were longitudinal embryonic axis and cotyledonary petioles. MS basal media with B5 vitamins supplemented with 0.75mg/L 2,4-D+0.5mg/L BA was the most promising media for both explant types with soft and friable callus

formation. The highest average number of embryos/explant (12.36) was observed on MS media with Gamborg's vitamins containing 0.75mg/L BA +0.5mg/L 2,4-D for cotyledonary petiole explants, whereas 3mg/L BA+1mg/L NAA was the only hormone combination that allowed embryo development to some extent, in both longitudinal embryonic axis and cotyledonary petioles. Somatic callus failed to regenerate despite the presence of embryo like structures and complete plant regeneration was not observed through somatic embryogenesis in either explants.

Suspension cultures originated from cotyledonary petiole calli were also established on liquid MS media containing B5 vitamins supplemented with 0.25mg/L 2,4-D, 20mg/L proline and 500mg/L ammonium nitrate. Although suspension cultures were maintained stably for months and some multicellular aggregates appeared on media containing 0.1mg/L 2,4-D, the aggregates could not progressed into the stages of embryogenesis.

During the transformation optimization studies, effect of sonication treatment on transformation efficiency of cotyledonary nodes, half cotyledons and cotyledonary nodes with intact shoots was assessed by comparing different sonication durations; 0.5, 1, 2, 5 and 10 seconds. The cotyledonary node and half cotyledon explants which have the greatest potential in regenerating high number of shoots responded with decrease in transient GUS expression in their petiole tissues and localized wounds on the meristematic petiole axis was never obtained by the use of sonication.

Throughout this study, remarkable GUS expression was observed on the petiole axil and regenerating shoots only when cotyledonary nodes and KYRT1 strain were used in combination with vacuum infiltration and scalpel wounding of meristematic axil region which has the potential to allow transformation of meristem tissues from which shoots originate. Culturing of the explants on 1mg/L BA for four days prior to transformation experiment, removing regenerated shoots immediately before wounding of the axil region, application of gradual selection and removing

regenerated shoots after each round of selection increased GUS expressing sectors and reduced chimerism levels on the regenerating shoots, significantly.

Regenerated GUS positive shoots were grafted on lentil root stocks and plants were grown to maturity under greenhouse conditions. Southern blot analysis revealed that over 174 explants used in *Agrobacterium*-mediated transformation experiments, 4 independent lines were obtained with 2.3% transformation efficiency. The analysis also suggested the insertion of *gus* into the lentil genome as a single copy gene in three of the lines and as three copies for one of the transgenic lines. All 4 lines stably expressed *gus* gene and inherited the *gus* and *npt-II* genes into the following three generations. GUS histochemical staining and PCR analysis helped to assess inheritance patterns and reverse transcription PCR has confirmed the expression of the genes in T_0 and T_1 generations. Chi-square analysis indicated that the transgenes were inherited in the progeny of the three independent transgenic plants as a single Mendelian character, however two lines exhibited a different pattern of inheritance. These results were also in consistency with the gene copy numbers determined by southern blot analysis.

To our knowledge this is the first study to date that reports stable genetic transformation of lentil plants by using *Agrobacterium*-mediated transformation and describes the stable integration and the expression of marker genes through three generations of transgenic lentil plants. This method for producing stably transformed plants via *Agrobacterium* will allow new opportunities for lentil genetic improvement studies.

Currently the optimized *Agrobacterium*-mediated transformation technique is being used for the nutritional improvement of lentil which has the shortage of tryptophan and the sulfur-containing amino acids, methionine and cystine in addition to the genetic transformation of the plant with the gene encoding Na^+/K^+ pump controlled by a constitutive promoter to enhance salt/drought resistance characteristics.

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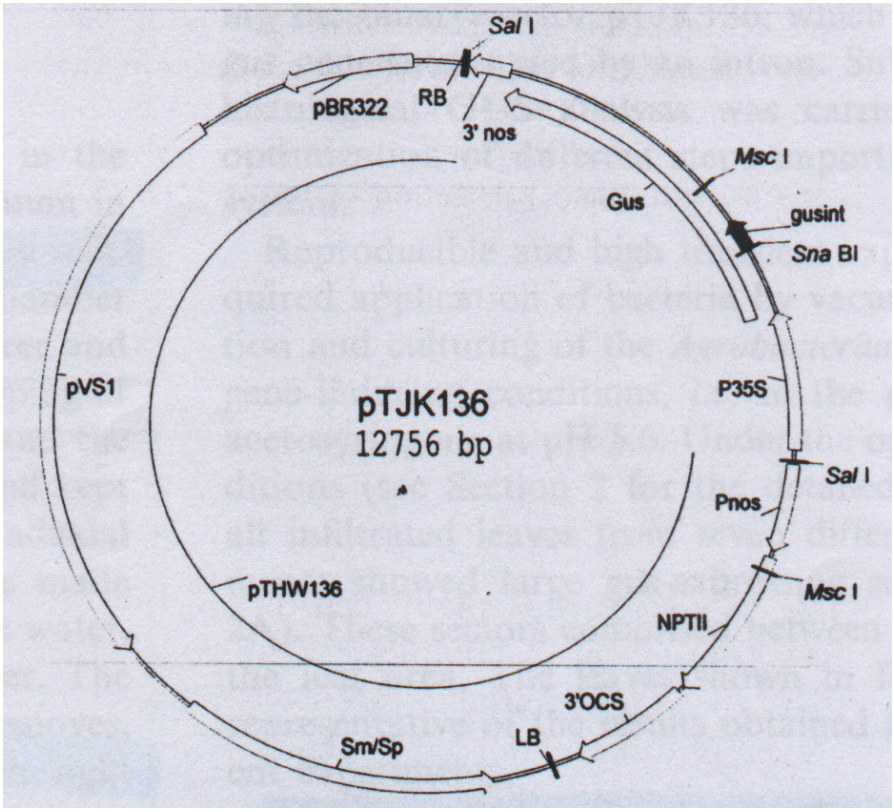
APPENDIX A

COMPOSITION OF MS BASAL MEDIUM WITH MS OR B5 VITAMINS

MACRO ELEMENTS	**mg/L**
$CaCl_2$	332.02
KH_2PO_4	170.0
KNO_3	1900.0
$MgSO_4$	180.54
NH_4NO_3	1650.0
MICRO ELEMENTS	**mg/L**
$CoCl_2.6H_2O$	0.025
$CuSo_4.5H_2O$	0.025
FeNaEDTA	36.7
H_3BO_3	6.2
KI	0.83
$MnSO_4.H_2O$	16.9
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.6
MS VITAMINS	**mg/L**
Glycine	2.0
Myo-Inositol	100.0
Nicotinic acid	0.5
Pyridoxine.HCl	0.5
Thiamine.HCl	0.1
B5 VITAMINS	**mg/L**
Myo-Inositol	100.0
Nicotinic acid	1.0
Pyridoxine.HCl	1.0
Thiamine.HCl	10.0

APPENDIX B

pTJK136 PLASMID MAP



APPENDIX C

PERMISSION LETTER FOR pTJK136 PLASMID

Laboratorium Genetica
Vakgroep Moleculaire Genetica
K.L. Ledeganckstraat 35
B-9000 Gent, BELGIE
Tel. 32(0)9-264 51 70/71
Fax. 32(0)9-264 53 49

Gent, 24.08.00

Dear Prof.Dr.Hüseyin Avni ÖKTEM

Please find enclosed the requested material. I would appreciate if you fill in the declaration and send it by return post to me.

Map included

If you need more information, don't hesitate to contact me.

Yours sincerely,

Christiane Genetello
e-mail: chgen@gengenp.rug.ac.be

APPENDIX D

PCR REACTION COMPOSITION AND CONDITIONS

GUS Gene Amplification Conditions

	Temperature	Time
Denaturation:	94°C	1 min.
Annealing:	60°C	45 sec.
Extension:	72°C	45 sec.

Cycle number: 35

*npt*II and *aadA* Gene Amptification Conditions

	Temperature	Time
Denaturation:	94°C	1 min.
Annealing:	55°C	45 sec.
Extension:	72°C	30 sec.

Cycle number: 35

REACTION COMPONENT	**AMOUNT**
10X PCR Buffer	2.5µl
$MgCl_2$, 25mM	2µl
dNTP, 2.5µM	2µl
Taq DNA Polimerase, 5u/µl	0.2µl
Primer (forward), 10µM	1.5µl
Primer (reverse), 10µM	1.5µl
DNA, 0.5-1 µg/µl	0.5µl
dH_2O	14.8µl
Total Volume	25µl

APPENDIX E

BUFFERS AND SOLUTIONS USED IN SOUTHERN BLOT

TBE Electrophoresis Buffer
For 10X stock solution, 1L

108g Tris Base (0.089M)
55g Boric Acid (0.089M)
4mL 0.5M EDTA, pH 8

SSC Buffer
For 20X stock solution, 1L, pH 7

175g NaCl (3M)
88g Na_3citrate.$2H_2O$ (0.3M)

Depurination Solution
For 1L solution

15.3ml HCl (250mM)

Denaturation Solution
For 1L solution

88g NaCl (1.5M)
29g NaOH (0.5N)

Neutralisation Solution
For 1L solution, pH 7.5

88g NaCl (1.5M)
79g Tris-Cl (0.5M)

CURRICULUM VITAE

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EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biotechnology	2002
BS	METU Department of Biology	1999
High School	50. Yıl High School, Ankara	1994

WORK EXPERIENCE

Year	Place	Enrollment
2002-Present	METU Department of Biology	Research Assistant
1998 June-July	TÜBİTAK Gene Research Laboratory	Intern Biology Student

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

1. Mahmoudian, M., Celikkol, U., Yucel, M. and H.A. Oktem, 2002. "Vacuum infiltration based *Agrobacterium* mediated gene transfer to lentil tissues" Biotechnology and Biotechnology Equipments, Vol. 16, pp. 24-29.

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