OPTIMISATION OF *AGROBACTERIUM* MEDIATED GENE TRANSFER AND MICROGRAFTING SYSTEMS IN LENTIL (LENS CULINARIS MEDIK)

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

OPTIMISATION OF *AGROBACTERIUM* MEDIATED

GENE TRANSFER

AND

MICROGRAFTING SYSTEMS IN LENTIL

(*LENS CULINARIS* MEDIK)

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In this work *Agrobacterium* (KYRT1::pTJK136) mediated gene transfer to lentil (*Lens culinaris* Medik.) embriyo apex and regeneration through micro-grafting in lentil was studied.

In micro-grafting the root stock stem height and two different types of root stock preparations were optimized. According to the results for the root stock stem height half stem length was found to be more successful then the full stem length.

For the root stock type the lentil root stock was found to be more successful then the chickpea root stock. The types of root stock preparations studied were designated as Z and M. The Z type root stock was found to be superior then the M type, when the micro-grafting, hardening and green-house stages of the experiments were concerned.

In study of *Agrobacterium* mediated gene transfer to lentil embryo apex the effect of the following parameters on the transformation efficiency were addressed; the type and intensity of injury, the type of pre-incubation media for injured explants, the effect of evacuation, the effect of L-cysteine during co-cultivation and *Agrobacterium* incubation duration. According to the results crushing type of injury was found to be superior over the poking and sonication type of injuries. Following the injury Hogland`s solution was used as pre-incubation media prior to *Agrobacterium* infection. The effect of evacuation parameter was found not to be significant whereas the effect of L-cysteine during co-cultivation was found to be negative on the transformation efficiency. According to the *Agrobacterium* incubation duration studies, 240 minutes, followed by 120 minutes of *Agrobacterium* incubation were the most efficient in terms of transformation efficiency. However there was no significant difference among the two so 120 minutes was chosen to be the optimum bacterial incubation duration.

Key words: Lentil, regeneration, micro-grafting, gene transfer, *Agrobacterium*

ÖZ

MERCİMEKTE (*LENS CULINARIS* MEDIK.) *AGROBAKTERİ*

YOLUYLA GEN TRANSFERİ

VE

MİKRO AŞILAMAYA DAYALI REJENERASYON

SİSTEMLERİNİN OPTİMİZASYONU

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Bu tezde *Agrobakteri* (KYRT1::pTJK136) yoluyla mercimek (*Lens culinaris* Medik.) embriyo apeks bölgesine gen transferi ve mikro aşılama yoluyla rejenerasyon optimizasyonu calışmaları yapılmıştır.

Mikro aşılama çalışmalarında kök stoğu gövede boyu ve kök stoğu çeşidinin yanısıra iki değişik aşı sitili optimizasyonu yapılmıştır. Sonuçlara göre kök stoğunda yarı gövde boyu tam gövde boyuna göre ve mercimek kök sistemi nohut kök sistemine göre daha başarılı bulunmuştur.

Kök stoğu hazırlanışına göre Z ve M olarak adlandırılan iki değişik aşı sitilinden Z tipi aşı, mikro aşılama, aklimizasyon ve sera koşulları göz önüne alındığında M tipine göre daha başarılı bulunmuştur.

Agrobakteri yoluyla mercimek embriyo apeks bölgesine gen transferi çalışmalarında ise yaralama çeşidi ve yogunluğu, yaralamadan sonra ön inkübasyon ortamı tipi, evakuasyon süresi, *Agrobakteri* ile inkübasyon süresi ve L-sistinin içeren besiyerinde kültivasyon parametrelerinin transformasyon verimine etkileri araştırılmışıtr. Elde edilen sonuçlara göre ezme suretiyle yaralama sistemi iğneleme veya sonikasyona göre daha etkin bulunmuştur. Yaralanan ekspilentlerin *Agrobakteri* ile enfeksiyonu öncesinde ön inkübasyonunda ise Hogland solüsyonu tercih edilmiştir. Evaküasyon faktörünün transformasyon üzerinde pozitif bir etkisi görülmemişken bakteriyle kültivasyon sürecinde L-sistinin kullanılması transformasyon üzerinde negatif bir etki göstermiştir. *Agrobakteri* ile inkübasyon süresi calışmalarında elde edilen sonuçlarda en yüksek geçici gen ifadesi yoğunluğu 240 sonra 129 dakika inkübasyon sürelerinde ölçülmüştür. Fakat bu iki gurup arasında kayda değer bir fark görülmediğinden 120 dakika en entkin süre olarak kabul edilmiştir.

Anahtar Kelimeler: Mercimek, rejenerasyon, mikro aşılama, gen ,transfer.

To my Aunt, Şayeste Altunbaş

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LIST OF ABBREVIATIONS

ANOVA……..	Analysis of variance
BA……...	Benzyladenine
GUS……	β-glucuronidase
MES……	2-[N-Morpholino] ethanesulfonic acid
MS……...	Murashige and Skoog
npt-II ……..	Neomycin phospho transferase type II
OD……...	Optical density
T-DNA……	Transferred DNA
Ti…….	Tumour inducing
vir…….....	Virulence
X-Gluc……	5-bromo-4-chloro-3-indolyl- β-D-glucuronide
YEB……	Yeast extract broth

CHAPTER-I
INTRODUCTION

1.1. GENERAL PERSPECTIVE AND PROPERTIES OF LENTIL

Lentil plant is herbaceous annual pulse (grain legume) crop, which is suggested to be one of the earliest domesticated plants traced back to 8000 BC in Near East including Southern Turkey. *Lens culinaris* is indigenous to the Near East and central Asia. The wild subspecies *Lens culinaris orientalis* is found in Turkey, Syria, Lebanon, Israel, Jordan, Iraq, Iran, Afghanistan and central Asia. Domestication involved selecting for plants that retain their seeds in the pod so that they are not lost before harvest, and selecting for greater seed size. Like many domesticated food plants, *L. culinaris* is mainly self-pollinated which means that it is easy to keep separate breeding lines. (Sauer, J.D. 1993)

The domestication of *Lens culinaris* formed an important part of the agricultural revolution in the Neolithic, along with Wheat and Barley. Although the amount harvested per unit area for Lentil is less than for Wheat and Barley, the high protein content (25%) of Lentil seeds makes them a highly nutritious (and tasty) food source. Small seeds of Lentil have been found in archaeological excavations of pre-farming comunities in Syria dating from 9200 to 7500 BC. It is clear that at this time people were harvesting seeds of wild Lentil, wild Barley and wild Einkorn Wheat. An ancestral form is unknown. The cultivated varieties have been bred by selection and crossing of primitive varieties with only a few firm pods. It is difficult to establish when Lentil started being domesticated because the only way one can determine evidence of domestication from seeds is an increase in seed size which happened only gradually over a long period of time. However, there is clear evidence from a large store of lentils found at an archaeological site in northern

Israel that by 6800 BC Lentil were being cultivated. Lentil seeds are also found abundantly in later Neolithic sites. (Zohary, D. and Hopf, M. 1993)

By the Bronze Age, the plant had been spread throughout the Mediterranean region, Asia and Europe, followed by the New World including Latin America. Today the major lentil producers and exporters are India, Turkey, Canada, USA, Syria, Australia and China. Major lentil importing countries include Spain, Colombia, Egypt, Algeria, Sri Lanka and India. The crop was traditionally important for largely vegetarian populations of South Asia. It is used as human nutrient source or high quality straw as fodder. The seed size varies between 2 to 7 mm (Figure 1.1) and color ranges from light tan to brown, purple and black.

A comparison between the protein content of cereals and lentil reveals that lentil protein content ranges between 20-36 % whereas that of cereals ranges between 8-12 %. However almost all of the species of lentil is noted to be low in methionine content (Christou, 1994/1997)

The crop is also well adapted to all types of soil and has relatively good tolerance to cool weather conditions. Poor yield and susceptibility to insect and fungal diseases (Erskine, 1984) are some crop constrains.

Lentil plants are typically short, slender, semierect annuals varying between 15 to 75 cm tall depending on the genotype and environmental conditions (Figure 1.1). Individual plants may bear single stems or may be multibranched. 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 tap roots and a mass of fibrous lateral roots. The leaves are alternate, compound and pinnate and are relatively small compared with the trifoliolates of soybean and *Phaseolus* beans. They may contain as many as 14 sessile, ovate or ecliptic leaflets (Figure 1.1).

Short peduncles that originate from the upper nodes of the plant generally bear single, sometimes two or three and rarely four flowers. They are self-pollinated and may be white, pale purple or purple blue. The pods are flattened, smooth, 1 to 2 cm long and usually contain 1 or 2, rarely 3, seed (Figure 1.1).

Throughout the world, a large proportion of the lentil crop is grown in semiarid regions without the benefit of irrigation. In most of these regions, agriculture depends on water conserved in the soil after fall and winter rains. Most genotypes of lentil are very sensitive to soil salinity.

Classical breeding techniques helped for isolation of high yielding varieties and greater yields in biomass, tolerance to drought, insects and diseases. Further breeding practices for resistance to lodging, pod shattering and different growth habits increased the yield potential along with facilitation in farming practices (Popelka *et al*., 2004).

After a complex taxonomic history, lentils were eventually placed in the genus *Lens* Miller. The name *Lens* describes the shape of the seed of the cultivated form of lentil, which is nowadays called *Lens culinaris* Medik. The last name is for Medikus, a German botanist-physician who has given the name to the plant in 1787.

Lentil is within the order *Rosales,* suborder *Rosineae,* family *Leguminosae,* subfamily *Papilionaceae,* and tribe *Vicieae.* A new taxonomic perspective about the letils sub species according to Ferguson *et al.,* (2000) is as follows:

L. clunaris Medik.

subsp. culinaris
subsp. orientalis (Boiss) Ponert
subsp. tomentosus (Ladiz) Ferguson *et al*., 2000
subsp.odemensis (Ladiz) Ferguson *et al*., 2000

L. ervoides (Bring.) Grande

L. nicricans (M. Bieb.) Godr.

Figure 1.1: General view of lentil plant (Sultan 1 cv.) **(a)** Stem segment of the plant. **(b,c)** Flowering and pod filling stages of the plant. **(d)** Root system of the plant. **(e)** Seeds of the plant.

1.2. TRANSFORMATION AND TISSUE CULTURE OF GRAIN LEGUMES

1.2.1. Soybean (*Glycine max)*

The first *Agrobacterium* based systems addressed the limitation of severe host range restriction with *Agrobacterium* (Lianzeng *et al*., 1984). The most prominent steps for evaluation of this limitation were development of more virulent *Agrobacterium* strains for improvement of strain-host range and tumorogenesis (Hood et al., 1987) and introduction of phenolic compounds that are known as enhancers of bacterial virulence (Owens of transgenic soybean plants.

Various studies based on protoplast transformation (through *Agrobacterium* co-cultivation or electroporation), resulted with transgenic callus lines, but these soybean callus bodies were failed to regenerate into complete plantlets.

The first successful transformation event of soybean with *Agrobacterium* (Hinchee *et al*., 1988) relies on three critical and basic events of transformation; (1) use of cultivar susceptible to *Agrobacterium* infection ("*cultivar Peking*"), (2) development of regeneration response from explants ("*direct shoot organogenesis from cotyledon region*"),

(3) enrichment of transformed tissues under selectional stress ("*Kan^r and Glyphosate tolerance, also GUS activiy as visual marker*"). Other trials that utilize the same three bases but similar events failed to yield transgenic soybean plants (germinating seed-*Agrobacterium* co-cultivation, ovary microinjection etc...)

Development of new transformation techniques and evaluation of the previous ones moved expectations forward to ideal transformation systems that covers three crucial criteria as follows: First it should be possible to recover large number independently derived transgenic plants for selection of useful levels for gene expression between the extreme expression levels. This was thought to be crucial in order to eliminate unwanted position effects.

Second it should posses a rapid recovery method for transgenic lines to minimize tissue culture based somaclonal variation. Third it should be a genotype independent transformation method (Christou 1994).

A simple genotype independent regeneration protocol based on multiple shoot proliferation from the meristem tissues of soybean embryonic axes was coupled to an efficient foreign DNA delivery method based on acceleration of micro-projectiles with an electric discharge by Christou *et al*., (1990). Optimization of the bombardment parameters for the appropriate meristematic cell layers of the explants that give rise to germline cells yielded transgenic soybean lines with expression pattern of transgene correlated well between the R_0 plants and their progeny. Within the later studies Christou and McCabe (1992) referred this transformation method as an efficient commercial process of genetic improvement for especially species that were recalcitrant to transformation. Following the release of this work the event of "value added crop plants" has been studied by modulating the lysine biosynthesis. Under seed specific promoter and with chloroplast transit peptide signal lysine feedback insensitive bacterial genes dihydrodipicolinic acid synthase (DHDPS) and aspartate kinase (AK) were transformed into soybean demonstrating a 100 fold increase in free lysine content.

The most recent struggles in soybean transformation targets tissues of somatic embryos, embryonic clumps and cotiledonary node segments as explants and transformation on these tissues are being made mainly with *Agrobacterium* strains or bombardment integrated systems. In their work Droste *et al*., (2000) used an integrated bombardment and *Agrobacterium* method for transformation of somatic embryos. Droste et al., (2000) claimed that the success of this method was lower with respect to micro projectile method on the basis of transient expression analysis. Also the study was based on high *Agrobacterium* responsive cultivars which were pointing the strict *Agrobacterium* host restriction during transformation.

Another recent transformation study was reported by Olhoft *et al*., (2003). Their work concentrated on transformation of the high frequency shoot regenerating tissues, the cotiledonary nodes with an effective hygromycin-B selection and addition of a mixture of thiol compounds in order to eliminate host pathogen response during cocultivation. Transgenic lines have been recovered in this work and efficiency was reported well above 15 %.

The most recent two works received on soybean transformation by Ko *et al*., (2003 and 2004) uses cotyledons as explant sources. After transformation with *Agrobacterium* the explants were cultured under hygromycin selection with specific explant orientation. At the end of 10 weeks sub culturing somatic embryos with GUS expression were recovered. Overall transformation efficiency was reported to be 1.1 to 1.7 % and confirmation for the presence of transgene was made with molecular data in T_0 and T_1 plants. In their subsequent work (2004) to increase the efficiency of somatic embryogenesis and transformation Ko *et al*., modulated the helper plasmid, pKYRT1 t-DNA sequences that probably had role in somatic embryogenesis. With this new strain and in conjunction with the synthetic growth regulator 2,4-D viable somatic embryos were handled.

1.2.2. Common Bean (*Phaseleus vulgaris*)

Common bean has been a dominant staple in central and south America for thousands of years and hence shows an extremely high morphological variability and been adopted to wide range of environments plus cultivation methods. Among the domesticated species of the *Phaseleus* genus (*acutifolius, coccineus, lunatus, polyanthus*), *P. vulgaris* occupies approximately 90 % of the total *Phaseleus* farmland. Classical breeding was the faith of the crop improvement programs over the past decade (Popelka *et al*., 2004). Considerable resource investments were served for preserving and expending bean germplasm especially in Central and Latin America (Christou 1994). The crop has several constrains during farming practices, ranging from the major diseases like anthractose rust etc., to insect damage, drought stress, soil toxicities and nutritional deficiencies (Popelka *et al*., 2004).

Development of superior varieties to overcome these problems is far out from the scope of conventional breeding practices within the limitations of required time and investments.

Almost all of the explants from the *Phaseleus* can be cultured *in vitro* for regeneration successfully (Popelka *et al*., 2004). But the transformation events were not that much efficiently coupled with the regeneration. As an example, no successful regeneration and transformation events have been reported on protoplast based direct or indirect DNA transfer methods. The two general approaches namely *Agrobacterium* infection and particle bombardment were marked as viable and productive transformation events.

The initial trials of bean transformation with *Agrobacterium* are tracing back to Lippincott *et al.,* (1968). Lippincott demonstrated crown gall induction on primary leaves of pinto beans (cultivar *pinto*) but no regeneration was conducted from these tumorogenic growths. Later on, Mariotti *et al*., (1989) claimed for the transformation of *P. vulgaris* and *P. coccineus* with *Agrobacterium* on the basis of rifampicine selection and GUS activity evaluation without any molecular genetic interpretation. Many more trials from germinating seed assays to leaf disk methods have been conducted till the demonstration of the strict genotype restriction of bean infection with *Agrobacterium* strains (Lewis and Bliss, 1994/ Aragao *et al*., 1996). Up to date no reports have been confirmed the stable transformation of *P. vulgaris* with *Agrobacterium,* but *P. acutifolius* (which can be hybridized with *P. vulgaris*) can be routinely transformed with *Agrobacterium* (Dillen *et al*., 1997/ De Clercq *et al*., 2002).

Based on the soybean transformation systems through the use of particle bombardment method Aragao *et al*., (1992) transformed bean mature embriyo with brazil-nut methionine rich albumin protein sequence. 24 hours later expression analysis was proven with western blotting. However no stable transformants was recovered. A similar procedure of soybean transformation is adapted to *P. vulgaris* by Russell *et al*., (1993). Meristematic tissues of embryonic axes were targeted during bombardment.

Successful recovery of transgenic plants from the progeny of the chimeric primary transformants was possible. With this method *phaseleus* plants were engineered for β-glucronidase, herbicide and viral resistance. Transgene stability was shown in subsequent generations (Christou 1994)

1.2.3. Pea (*Pisum sativum*)

Pea is an important legume plant and is grown in temperate regions. It is a good protein source and rich in lysine but is a poor source of sulfur containing amino acids; cysteine and methionine (Christou 1997).

Genetic modification approaches of pea focused on *Agrobacterium* based transformation vectors. *A. tumafecience* tumorogenesis and *A. rhizogenes* hairy root formation was demonstrated *in vitro*. Although direct DNA transfer methods like particle bombardment and electroporosis have been studied successful pea transformation was conducted with *Agrobacterium* based transformation systems. The direct DNA transfer into protoplasts through electroporation was carried up to callus phase, from which plant regeneration was not possible (Puanti and Kaerlas, 1992a/b).

Axenic shoot cultures and seedling epicotyls were co cultivated with *Agrobacterium* and cultured on hygromycin or kanamycin containing shoot induction media (Puanti and Kaerlas, 1990). But regeneration was reported to occurre on hygromycin selection (not kanamycin) after several sub culturing steps. Different cultivars showed varied responses of regeneration under different selection pressures. Finally the shoots harvested were rooted and transferred to greenhouse for subsequent analysis. Stable transmission of the foreign gene was demonstrated in the progeny for two subsequent generations (Puanti and Kaerlas, 1992a/b).

One handicap of the methodology is pointed out to be nine months of tissue culture period covering the risk of tissue culture mutagenesis. However following these studies on the same basis Schroeder *et al*., (1993) recovered kan and PPT resistant lines from the embryonic axis of immature seeds.

Also lateral cotiledonary meristems of germinating seeds are transformed with *uidA* gene and GUS expressing lines were recovered (Davies *et al*., 1993; Bean *et al*., 1996). Schroeder et al., (1995) also introduced bean seed α-amylase inhibitor under seed specific promoter into pea with the same *Agrobacterium* incubation method 3 % accumulation of the inhibitor protein in the seed was demonstrated to be sufficient to abolish the pea weevil larvae feeding.

Although *Agrobacterium* based systems were only successful event of transformation in pea its obvious key limitation during transformation event was noted to be strain-genotype (cultivar) specificity (Christou 1997).

1.2.4. Peanut (*Arachis hypogaea*)/ Ground pea

Peanut is grown in all tropical and subtropical regions of the world. Species has two cultivated sub species; *A. hypogaea-hypogea* and *A. hypogaea-fastigiatta.* China accounts for 40 % of the world's production (Popelka *et al.,* 2004). It is a good source of high quality vegetable oil. Peanut also contains arachidic acid and linoleic acids along with glycerides of oleic and linoleic acids (Allen and Allen, 1981). It is processed to wide range of products or roasted and served for direct human consumption. Besides being an excellent live stock with relatively high protein content (up to 32%) it is also valuable cash for small scale farmers and also commercially important for its high oil content-up to 54 % (Popelka *et al*., 2004).

Crop has a narrow germplasm base that strictly restricts conventional practices for development and isolation of superior cultivars at least for resistance to major pathogenic fungi and viruses. Insects as being a central problem have direct impact on yield besides serving as efficient vectors for viruses (Popelka *et al*., 2004).

The early attempts for genetic improvement of the crop are handled by Ozias *et al*., (1993) through bombardment of embriyogenic cultures of peanut and selection on hygromycin. Only PCR screening but no other molecular genetic data were reported for supporting putative transgenics.

Later on Brar *et al,.* (1994) reported successful transformation by bombardment of shoot meristems of mature embryonic axes. From this study GUS positive and BASTA® resistant lines were recovered and transgene expression was confirmed by resistance to herbicide spraying in the progeny.

The early reports for the *Agrobacterium* transformation of peanut targets zygotic embriyo axes of mature seeds (McKently *et al*., 1995). *Agrobacterium* strain *EHA 101* with binary vector engineered for GUS and *npt II* was used. Transformation event for the R_0 plants and the transgene expression for the progeny were confirmed with molecular data (Christou 1997).

For comparison of superior transformation systems optimized, strict host specificity and low levels of transformation frequencies of *Agrobacterium* based systems were put forward as drawbacks against the best suiting bombardment based systems in 1997 reports (Christou 1997). Recent advances in peanut transformation was focusing on bombardment of embriyogenic tissues that regenerate through a callus phase (Livingstone and Birch, 1999) and non tissue culture based integrated bombardment method (Rohni and Rao, 2000).

1.2.5. *Vigna* species; cowpea, blackeyed pea, mungbean, month bean

Various small seeded Asian phaseleus species of the mung group fall into *Vigna* genus (Ohwi, 1969; Verdcourt, 1969). *V. unguiculate* (cowpea/ black-eyed pea), *V. radiate* (mung bean), *V. aconitifolia* (month bean) are represented as the important species of *Vigna* (Christou 1997). The genus is mainly represented by cowpea on global scale in farmlands. It is a widely adopted nutritious grain legume and currently 92 % of the world's production is harvested from Africa, where a huge population consumes cowpea on daily basis. Also in Africa the crop is source of income and livestock feed (Popelka *et al*., 2004).

Crop yield is limited by a range of abiotic and biotic stresses but the major constrain is the insect threat almost at every stage of the development. The natural variation in the germplasm maintains a limited solution against the abiotic and biotic stress factors (Popelka *et al*., 2004).

Early transformation studies utilized both direct and *Agrobacterium* mediated transformation to protoplasts however only callus lines that are expressing various transgenes recovered (Christou 1997). Such transformation results addressed the problems in regeneration from single cell cultures. Regeneration via somatic embryogenesis from the callus cultures of young leaves, mature embryonic axes and immature cotyledons are studied but reported to show low frequency levels of success. So transformation events focused on direct organogenesis cultures developed from hypocotyls, axenic hypocotyls, epicotyls and mature cotyledons (Popelka *et al*., 2004).

Up to date many works have failed transform cowpea; via *Agrobacterium* (Garcia *et al*., 1986; Penza *et al*., 1991; Suzuki et al., 1993) or through direct DNA transfer methods (Kohler *et al*., 1987). Although several *Agrobacterium* strains are shown to infect cowpea the major constrain on the success story become the handicap in regeneration of transformed tissue. One big step towards the transgenic cowpea has been recently registered by Le and Cruz de Carvalho, (2002). They successfully regenerated fertile cowpea plants from cotyledons by 2.4-D application opened the avenue for successful transformation events.

1.2.6. Faba bean (*Vicia faba*)

Vicia genus covers approximately 150 species of the *vetch* family. Many species of the genus is native to Eurasia and USA and distributed to temperate regions of the globe (Allen and Allen, 1981). *Vicia faba* is traditional crop of the Europe. *Minor* and *equine* (for animal feed), *major* (for human feed) are the sub species of the *V. faba* (Austin *et al*., 1990). Faba bean has high protein content and has a potential use as high energy crop and also adopted to give high yield in temperate regions (Selva *et al*., 1989). Because of these properties it is center of concern in crop development programs of Europe. Main problem with the crop is the instable yield linked to the fluctuations in environmental conditions. Belonging to vetch family it also contains amino-glycosidases that are toxic to animals and men (Christou 1994). On the other hand it is rich in L-Dopha which is successful in treatment or Parkinson's disease.

Transformation events of faba bean starts with Ramsay and Kumar, (1990). Cotiledonary tissues and stem segments of eight different cultivars of *V. faba* are infected with *A. rhizogenes* (Ri plasmid was engineered for neomycin phosho transferase). Varied infection response with tissue type and genotype was recorded. Genetic instability of the transformant tissues resulted in polyploidy and plantlet regeneration was not reported.

In the next transformation study (Pickardt et al., 1991) *V. narbonensis*, a close relative of *V. faba* was infected with *Agrobacterium*. The shoot tips and epicotyl segments of the plant were co-cultivated with *A. tumafecience* (containing binary plasmids coding hygromycin phosho transferase). Under hygromycin selection recovery of putative transformants was achieved with an efficiency of 18 %. These callus bodies are driven to regeneration through somatic embryogenesis that resulted in low levels of plant regeneration. Also no transgenic plants were recovered from these studies. In the following years however Pickardt *et al*., (1995) transformed same species with methionine rich 2S albumin from Brazil nut under seed specific promoter. The vector vas *Agrobacterium* EHA101 strain and epicotyl explants were used in transformation. After callus induction under kanamycin selection somatic embryogenesis is performed to yield transgenic plants. Transgene expression in the regenerated plantlets was confirmed by western blotting. 2S albumin compromised 1-4.8 % of the total seed protein. Pickadt *et al*., (1995) concluded this work by showing transgene expression in the progeny with molecular data.

The most recent report available on transformation studies of faba bean focuses on the strain cultivar relationship for an efficient transformation event (Jelenic, 2000). In this study three broad bean cultivars with different morphology and geographic origins were infected with nine different *Agrobacterium* strains of *tumafecience* and *rhizogenes*. Evaluation was made on the basis of relative tumorogenesis of the wild type *Agrobacterium* strains on the stem segments of beans *in vivo*. Also for analysis of increment in peroxidase activity of the transformed tissues, *in vitro* tumor cultures were established.

Different strain-cultivar combinations resulted in different transformation efficiencies and different sizes of tumors also. *A. tumafecience* strains A281, B6S3 and C58C1 (with plasmids pTiBo542, pTiB6S3 and pRiA4b respectively) were found to be most virulent for the cultivars Topolo, Lobab Lippoi and Oslje cultivars respectively. The phenotypic differences between callus cultures of tumors were found to be dependant on the strain used rather then the cultivar. Although the most virulent and susceptible cultivars were coupled *in vivo* the following *in vitro* transformation studies were failed and there still exists *in vitro* regeneration problem.

1.2.7. Chickpea (*Cicer arietinum*)

Chickpea is noted as an ancient crop that probably originated in Anatolia approximately 7000 years ago and spread through Middle East south Asia and North Africa. Two types are being prevalently cultivated through the globe; small seeded *desi* type and less common bigger seeded *kabuli* type. Seed nutrient content is given as approximately 20 % protein, 5 % fat and 55 % carbohydrate. It is a low input crop that ends its life cycle under heat and drought stress. The estimated crop yield is 5 t/Ha however due to drought stress, poor management practices and diseases average crop yield regresses to 0.8 t/Ha. During developmental stage exudation of malic and oxalic acid from the granular hairs covering the plant body distracts aphids and many insects. Major yield threat comes from the pod borer *Helicoverpa armigera* and also during storage chickpea seeds become highly susceptible to burchid beetles (Popelka *et al*., 2004).

Initial regeneration studies was limited to callus (2,4-D induced) driven somatic embryogenesis. Also zeatin and low levels of IAA was shown to promote cotyledon like structures (Shri and Dawis, 1992). Regeneration of multiple shoots from callus phase was marked inefficient due to limited number of shoot production (Huda *et al*., 2000). However callus material was efficiently used to reveal the strain cultivar specificity in *Agrobacterium*-chickpea interaction (Islam *et al*., 1994) and also those later reports confirmed *Agrobacterium* as a potential vector in chickpea transformation

(Altinkut *et al*., 1997). In the transformation events following these findings the method *Agrobacterium* (armed with *gus* and *npt II* genes) co-cultivation of embriyo axes was reported (Fontana *et al*., 1993). The transgenes was successfully traced up to the T_2 generation. Using similar *Agrobacterium* incubation protocols and different genotypes as explant sources shoot induction (on MS and combinations of BAP/ NAA/ Kinetin) from these explants yielded production of primary transgenic plants, however low transformation frequencies and failure in reproduction marked these methods as inefficient transformation systems in chickpea transformation (Popelka *et al*., 2004).

Around these low throughput systems two transformation events that utilized potentially useful transgenes have been reported. One of them that used biolistics as vector, (Kar *et al*., 1996) transformed the explants with *cry IAc* (from *Basilus thrungiensis)-npt II* (as selectional marker) construct. The regenerated plants were traced only up to the T_1 progeny for the transgene and insect feeding trials showed inhibition of larval growth of chickpea pod borer (*H. armigera*). The other method used *Agrobacterium* to transform *desi* type chickpea with seed specific α-amylase inhibitor (αAI1) from P. vulgaris and *npt II* as selectional marker (Sarmah *et al*., 2004). The progeny analysis showed stable transmission of the transgene. High level expression of the transgene was shown to inhibit the growth of cowpea and adzuki bean weevils (*C. maculatus* and *C. chinensis* respectively).

1.3. TISSUE CULTURE STUDIES ON LENTIL

One of the initial studies on tissue culture of lentil was reported by Williams and McHughen, (1986). In this *in vitro* regeneration study 3-4 days old seedlings were used as explant sources for shoot meristems, epicotyls and cotyledons that were cultured in kinetin (KN) and giberellic acid (GA). The yielding callus bodies were further cultured for shoot production and finally from these regenerated shoots root induction was studied. It was revealed that among the explants cultured on MS with KN and GA all gave callus bodies at acceptable frequencies but only epicotyls and shoot meristems were capable of regenerating shoots.

Between the two explants the callus derived from the shoot meristem was reported to produce shoots at higher frequencies with an average rate of 60 %. These regenerated shoots were then subcultured on MS with IAA or NAA but no rooting was observed at all.

Only an 11 % success of rooting was achieved from the shoots regenerated in mist chamber. Against loses during acclimisation and soil transfers it was possible to end the study with viable and flowering plants with very low frequency.

In 1987 Saxena and King studied somatic embryogenesis from the callus cultures of intact lentil embryonic axes (whole lentil embryos) with or without cotyledons. Different macro and micronutrient sources were tested (MS and Gamborg`s B5) for callus induction and somatic embryogenesis. Callus induction with 2,4-D was found to be quicker and more pronounced on whole embryo explants then the cotyledons bound to embryo axis. The callus bodies harvested from the explants were subcultured on B5 medium with different concentrations of auxins, cytokinins or kinetins. Well organized club shaped embriyo like structures raised within two weeks. With the addition of glutamine to the culture media these structures turned into well formed bipolar embriyoids. But somatic embryos derived germinated occasionally.

Factors affecting callus and shoot induction of *in vitro* cultures of lentil is another study that used callus bodies in their regeneration system (Polanco *et al.,* 1988). In this study shoot tip, first node and primary meristematic leaf explants were harvested from the germinated fresh seedlings. With subsequent culturing of the explants in MS salts with Gamborg`s B5 vitamins and 2,4-D or other growth regulators callus induction was maintained. These callus bodies were then subcultured for shoot induction with different growth regulator combinations. In this study no respective effect of the media was shown on callus induction from the explants. NAA and BA combination was found to be superior during high frequency callus induction. 2,4-D induced 100 % callus in all cases with different explants and media used with or without other growth regulators,

but all of the callus bodies were ineffective in induction of morphogenesis and shooting. Variance in callus induction frequency was scored to be less pronounced with different explants. For any concentration of 2,4-D, no morphogenesis obtained. However BA was found to be more effective in shoot induction from the callus bodies or directly from the explants. For the root induction from the regenerated shoots, combinations of BA, IAA and NAA was used. BA alone or in combination with other growth regulators never induced roots. IAA alone induced large but infrequent roots whereas NAA alone induced roots resembled callus and were short. Such inefficient rooting patterns and low rates of success in rooting shielded the underlying progress in whole plant regeneration.

In another tisuue culture study TDZ was reported to be inducing high frequency shoot regeneration from the intact seedling of lentil and two other hypogenous legume species (Malik and Saxena, 1993). Axenic seedling cultures of the species were established on MS with different cytokinins (Zeatin, Kinetin and TDZ). TDZ was reported to be more effective then the other cytokinins for shoot induction at the end of one week in culture. But the emerging shoots were noted to have no apparent vascular connections with the parent tissue. However it was noted that within the next 4-6 weeks of culture *de novo* differentiation of shoots and buds occurred from these explants. Higher concentrations of TDZ (over 50μM) were found to be severely inhibiting germination whereas concentrations between 0.1-30 μM had no significant effect on germination. Along the 3 weeks of culture with TZD an average of 19 shoots per explant was recovered. On continuation of the culture secondary shoots regenerated from the axils and bases of the multiple shoots. On the other hand as expected the rooting was inhibited by the concentration and duration of exposure to TDZ. TDZ concentration higher then 5μM suppressed the primary and secondary root development. Periods of exposure to TDZ longer then 2-3 weeks made rooting difficult on the shoots harvested.

Rooting was carried in MS salts plus B5 vitamins with 2.5 µM NAA. No rooting frequency about the explants was given in the report but 40-60 % efficiency was noted for the maturation of the rooted plantlets.

High frequency-shoot regeneration from the cotiledonary nodal segments of many legume species is a shortcut organogenesis showing less genotype dependence. Regeneration from cotiledonary node explants of lentil with BA and analysis of this explant for transformation by *Agrobacterium* was studied by. Warkentin and McHughen (1993). In this *in vitro* study multiple buds and shoots were reported to be arising from epidermal and sub-epidermal layers of axils of cotiledonary petiole with 100 % response per explant. Rooting of the regenerated shoots were maintained in half strength Gamborg`s B5 medium with or without NAA at a rate of 34-53 % within 1-2 months culture but only a few of them were reported to be brought into fertile whole plants.

Although the regeneration studies of direct organogenesis for lentil and some other hypogeous legumes have been succeeded with an array of growth regulators the inhibitory effects of cytokinin like phytohormones on root development from the shoots regenerated was the limiting factor for the whole plant regeneration in the lentil case also. This handicap was addressed with the study of BAP effect on *in vitro* and *in vivo* rooting of lentil (Polanco and Ruiz, 1997). In this study the freshly germinated seedlings were used to collect explants of shoot tips, first nodes, bractlets, and immature seeds. These explants are cultured on MS with BAP for shoot induction. The effect of BAP concentration and the culture duration within BAP containing media on rooting were tested. Rooting was maintained in IAA containing media. Cause of low frequency rooting response for the regenerated shoots were addressed to the cytokinin concentration used, the nature of the cytokinin used for shoot induction and the time elapsed in cytokinin containing media. Also study of BAP effect on *in vivo* rooting was shown to be drastically reducing in the mitotic index of the root meristems.

Under the light of the initial studies that reveal the rooting handicaps and regenerative potential of the target lentil meristematic tissues, *in vitro* propagation for lentil cultivars was studied by Ahmad *et al.,* 1997. The study was aimed to optimize a procedure of efficient *in vitro* clonal propagation for lentil from nodal segments. Depending on this aim the media components (MS and sucrose concentration) along with the phytormone concentrations were assessed. For efficient shoot regeneration a media composition of full strength MS with no sucrose and 2.89 µM GA_3 plus 1.11 µM BA was determined to be the optimum condition. An average shoot regeneration of approximately 2 shoots per explant was recorded with these optimum conditions.

Root induction from the regenerated shoots on the other hand was optimized in full strength MS with 5.37 µM NAA. Rooting success of the regenerated shoots ranged between 23-47 %. Fallowing the rooting success, during the next step, hardening of the plantlets were carried out in ½ strength MS media but no success rate was stated for this step.

Evaluation of the micrografting technique applied to certain annual legume plants and commitment of the totipotent cell source as cotiledonary nodes, complete plant regeneration at high frequency for lentil become reality with the study of Gulati *et al.,* (2001). In their study multiple shoot regeneration from the cotiledonary nodal segments on MS media with 8.8 µM BA was followed by an efficient micrografting of the shoots to lentil root stocks and successful acclimisation. Compared to the 7-12 days of recovery and elongation of the micro grafts *in vitro* root initiation with plant growth regulators was referred to as least efficient and lengthy process. Both successes in micrografting and soil transfers were given with rates of 90-100 % for the study.

Other than the work of Warkentin and McHughen (1993) an extended study of high frequency-shoot regeneration from the cotiledonary nodal segments of different lentil genotypes that were coupled to regeneration by micrografting was carried out by Khawar and Özcan (2002-b).

Cotiledonary nodal segments from 21 different lentil genotypes were cultured for shoot production with TDZ *in vitro*. Root induction *in vitro* from these shoots was shown to be unsuccessful and micrografting was addressed for efficient plantlet regeneration from the shoots regenerated. The shoot regeneration efficiency for the different genotypes and lines were scored to be in the range 60-100 % with scores over 80% for 14 out of 21 different lentil genotypes. Also mean number of shoots per explant ranged from 3 to 20. The graft settings of shoots derived from 7 different genotypes were demonstrated with varied success rates from 25 to 100 %. And this variance is attributed to the scion and stock incompatibility that can be overcome with selection of better root stock.

Root induction problems of the shoots regenerated from the meristem tissues of lentil and relatively low frequencies of multiple shoot induction from these tissues other then cotiledonary node forced for the expansion of totipotent cell search to intact explants like immature seeds. A study about the case concerns analysis of factors for plant regeneration from *in vitro* cultures of immature lentil seeds (Polanco and Ruiz, 2001). In this study cultivar and immature seed size differences were studied along with the phytohormone concentrations to optimize successful plant regeneration system in an array of lentil cultivars. Buds and shoots were regenerated from the axilary meristems of cotiledonary node region and bigger seed size approximating maturity was found to enhance multiple shoot formation and root initiation. Multiple shoot regeneration was noted to be more pronounced in the media containing BAP then other kinetins but shoots were shorter and required to be treated with auxin for elongation and handling. High percentages of multiple shoot induction was maintained in media containing 1 μM BAP. For the rooting case 10 μM BAP induced shoots were noted to be showing almost no rooting response. Among the shoots recovered a concentration range between 0.1-1 μM or no BAP was noted to have very high frequency of rooting. The cultivar difference in regeneration frequencies was noted not to be dramatic. After 4 weeks of shoot induction in BAP containing culture media shoots harvested were transferred to rooting media with 11.4 μM IAA.

About one months culturing in rooting media root induction was seen at the cut end site of the shoots. Also rooting response was found to be genotype dependent for the optimized rooting media and shoot induction media.

Complete plant regeneration from lentil meristematic tissues were given by the study of Fatini and Ruiz, (2003). A rooting system based on the explant polarity was developed and tested for hypogeous legumes including lentil. The work was submitted as an extensive analysis of the rooting response in lentil nodal segments in relation to explant polarity, nature of hormones, their concentration and different nutrient salts, sucrose concentrations. As an expansion to the previous regeneration studies that were concerning the phytohormones, explant orientation was noted to be the key event of the study.

The nodal segments cultured as their apical ends in the media with 5 μM IAA and 1 μM kinetin resulted in 95 % of root and 2.4 shoot regeneration per explant. Also full strength MS with sucrose (decrease in sucrose amount was strictly correlated to reduction in root formation) shown to have positive effect on root regeneration.

1.4. TRANSFORMATION STUDIES ON LENTIL

1.4.1. Basics of Transformation

A routine transformation system requires some factors that can be summarized as follows; (1) recipients (source of totipotent cells) for DNA delivery, (2) means of DNA delivery to the recipients, (3) a selection and identification system for transformed cells (Somers *et al*., 2003).

Mainly two techniques of gene transfer are currently being reproducibly used; *Agrobacterium* mediated gene transfer and microprojectile bombardment method. Both methods have their own superiorities and handicaps. The transformation ability of *Agrobacterium* is based on its natural ability to infect a range of hosts and induce tumorogenesis. During the infection event a DNA (T-DNA) sequence from the bacterial plasmid called tumor inducing (Ti) plasmid is transferred to the host.

Integration and expression of the T-DNA sequence in plant cells and in their proliferating tumorogenic descendants' results in synthesis of nutrients called opines or octapines for bacteria nutrition. Exploitation of such an ability of this natural engineer released a wide array of transformation systems for a range of hosts. As for the most efficiently used representative of the direct DNA transfer systems microprojectile bombardment method relies on acceleration of DNA coated micro particles (mainly tungsten or gold) by means of gas or electric discharge forces. For a comparison of *Agrobacterium* mediated gene transfer and microprojectile bombardment methods on the basis of direct versus indirect transformation systems we can state that bombardment has a simple methodology since only DNA coated particles are targeted to the respective tissue materials. However the method is costly and requires high price machinery that is not always affordable. On the other hand *Agrobacterium* mediated gene transfer method requires more care during preparation of bacteria and selection of the best plant-cultivar and bacterial-strain couple. For *Agrobacterium* based methods other than enhancement in media pH and acetosyringone addition during bacterial growth it is important to have a specific transformation method for the plant species and even for the explant type that is being used. On the other hand with particle bombardment method the parameters to be optimized are routine and simple; gas pressure optimization, micro projectile particle nature and size optimization, evacuation pressure optimization etc. Although direct DNA transfer method mentioned above is so simple compared to Agrobacterium mediated gene transfer method due to its maintenance and working costs and also due to the scientific curiosity the natural genetic engineer is currently leading the usage in transformation events. *Agrobacterium* mediated transformation methods are developing day by day and new hosts are being explored from animal to plant tissues

1.4.2. Transformation Methods Applied to Lentil

One of the initial transformation studies on lentil that extensively analyzed the transgene expression on an array of lentil tissues is addressed by Warkentin and McHughen, (1992). In their study shoot apex, epicotyl and root explants were shown to be capable of expressing an intron containing GUS gene transformed with the *A. tumafecience* strain GV2260::p35SGUSINT. With both histochemical and fluorimetric GUS assays it was concluded that duration of co-culturing was positively correlated with the percentage of GUS positive explants. Also epicotyl region in the vicinity of the shoot apex was shown to have lower percentage of GUS expression then the shot apex and root meristem whereas other sections of the embryonic axis (excluding the cotiledonary nodal meristem) had no apparent GUS activity at all. Also the tissue zones that are nearer to the active growing centers like shoot, cotiledonary node and root meristems (efficient sink zones for the cotyledon driven nutrients) showed higher GUS activities on the whole. Along with the relative transformation and GUS activities of the various lentil explants also *Agrobacterium* infectivity of the cotiledonary node explant was shown in this study. In the absence of injury low frequency transformed buds and shoots were observed from the meristematic tissues of the explants.

As noted previously regeneration of shoot from cotiledonary node explants of lentil with BA and analysis of this explant for transformation by *Agrobacterium* was studied by Warkentin and McHughen (1993). Transformation experiments were carried out with GV2260::35SGUSINT strain *A. tumafecience*. The transformation study was concluded with the claim that the axils of cotiledonary petiole was not amenable to transformation by *Agrobacterium* due to the observation that the wounding practice to the meristematic zone of the explant did not produce GUS positive shoots whereas in non wounding case shoots regenerated with GUS positive patchy areas at very low frequency.

Other then *Agrobacterium* based transformation systems transformation events with lipofection method was also employed for lentil (Finazzi-Agrò *et al.,* 1992). Multilamelar liposome constructs of dipalmitoyl phosphatidylcholine and stearylamine was used as agents of lipofection after PEG precipitation of GUS and CAT (chloramphenicol acetyl transferase) coding plasmids on to the liposomes. Transient transgene activities were recorded from the 24 hours old lipofected protoplasts.

Another extreme application of transformation to lentil totipotent tissues was referred to as electroporation mediated gene transfer to intact meristems *in planta* (Chowrira *et al.,* 1995). A number of hypogeous legumes including lentil were transformed in this study through electroporation to the intact nodal meristem tissues that give rise to new floral and vegetative parts of the plant. The protocol was launched as an efficient *in vivo* transformation system but suspicious of non reproducibility aroused after the release of the report. GUS reporter along with *bar* gene for Basta selection was used in this study. It was claimed that lipofectin coating of the plasmid DNA for protection increased the transformation rate. The putative transformants and some of their progeny was claimed to be expressing transgene but no representative molecular data given.

Another excavating study from Lurquin *et al.,* (1998) aiming an efficient transformation system for a number of hypogeous legume species used half embriyo cocultivation technique to estimate the susceptibility of explants for *Agrobacterium* transformation. In this study four different lentil cultivars and one pea cultivar was transformed with two *Agrobacterium* strains (C58::pGV2260 and EHA105::pEHA105) with a common binary plasmid p35SGUSINT. Both stems and leaflets were scored to be GUS positive but no staining was recorded for the root sections of the half embryos at all. Also genotypes varied remarkably in their response to *Agrobacterium*. Addition of acetosyringone along with a definite pH for the bacterial growth media was shown to be effective in increasing the transformation activity of the *Agrobacterium.*

One contradiction with the acetosyringone addition to the cocultivation media was noted to be a deteriorus effect on *Agrobacterium* transformation of the explants for the case of lentil cultivar *Eston*. Also remarkable decrease in expression pattern of the reporter gene was shown till 2 weeks of post transformation. Accordingly stable expression analyses were confined to at least 2 weeks post transformation.

Among the studies of transformation by particle bombardment one of the initial studies on lentil cotiledonary nodes was reported by Öktem *et al.,* (1999). pBSGUSINT plant transformation vector on tungsten micro carriers were accelerated towards the cotiledonary node explants that were harvested from two days old lentil seedlings. According to the transient GUS activity scores, after 2 days of transformation 50 % success in number of GUS positive explants were recorded. The stable GUS records showed patchy expression patterns on the regenerated shoots also. Optimization of the bombardment parameters like gas and evacuation pressure was noted to be not critical in enhancing the transient expression rate. Also the regeneration potential of the explant was not effected noteworthy with overall 82 % regeneration potential and 2.2 shoots per explant regeneration capacity.

Although previous studies of transformation and tissue culture noted the cotiledonary node as not amenable to transformation by *Agrobacterium* the potential regenerative capacity of the explant and advances in transformation methods required the re-evaluation of susceptibility of cotiledonary node explants to *Agrobacterium*. Mahmoudian *et al.,* (2002-a) evaluated the transformation of lentil cotiledonary nodes through infiltration of *A. tumafecience* (strain GV2260::pGUSINT) suspension into the explant tissues.

In this study following *Agrobacterium* infiltration, cotiledonary nodes were cultured in BAP containing media for shoot regeneration. Evacuation pressure and time was optimized to be 200 mm Hg and 20 minutes for the study. Dispersed patterns of GUS activity was observed from the axils of cotiledonary node segment with low frequency GUS staining on the regenerated shoots.

An expansion of the preceding transformation work was released at 2002 by Mahmoudian *et al.,* (2002-b). Infiltration of *Agrobacterium* suspension to cotiledonary node and nodal segments were studied. Transformed explants were cultured with their respective phytormones (BA and GA plus Kinetin) in MS media under kanamycin and cefotaxime selection. Transient GUS assays were carried at the end of three days in non selective media whereas stable GUS assays were carried out after 2 weeks of culture in selective media preceded with 1 week of culturing in non selective media. Infiltration done at 650 mm Hg pressure for 20 or 30 minutes resulted in a relative increase of transient GUS expression with time. However 20 minutes infiltration was noted to be the efficient for stable transgene expression. Analysis of GUS expression patterns of the explants revealed that nodal segments were scored to have higher number of GUS positive loci then cotiledonary nodes but GUS staining of the cotiledonary nodes was recorded to be more clumped with mostly at the shoot apices regenerated.

An extensive transformation study on a range of lentil cultivars with *Agrobacterium* strains on the basis of both tumor formation and GUS expression analysis was submitted by Khawar and Özcan, (2002-a). The leaf and stem explants from a variety of 21 different lentil genotypes were inoculated with a super virulent *Agrobacterium* strain (A281). Efficiency in transformation was scored according to percent tumors formation tumor diameter and tumor mass. After 6 weeks of tumorogenesis some genotypes showed no tumor on leaf or stem or both. But on overall scene leaf explant was found to be most susceptible to infection and tumor formation with rates ranging in 78-83 % of explants. The corresponding rates ranged between 0-50 % for the stem explants. Also on the whole tumor dimension and mass variables assigned the leaf portions as the potential explants in transformation.

The most recent transformation study that successfully uses particle bombardment and an efficient selection method combines the high frequency shoot regeneration of cotiledonary nodal segment with micrografting method for recovery of fertile transgenic lentil progeny (Gulati *et al.,* 2002).

As the selective agent for the transformed cells a sulfonylurea urea-derivative herbicide (chlorosulfuron) was used to evaluate the presence of the transgene, (tobacco derived mutant "acetolactate synthase" ALS gene). This selection construct was noted to be efficient over the others since that the herbicide applied was only effective at the active growing centers, in the initial steps of valine and leucine synthesis. After the bombardment of lentil cotiledonary nodes 2 days of culture without selection was followed by 4 weeks of chlorosulfuron selection. During this time the emerging healthy shoots were accepted as escapes and discarded. The subsequent 2 weeks of culture in half concentration of selective agent and phytormones (4.4 µM BAP and 5.2 µM GA) resulted *de novo* regeneration of advantageous shoots that are later micro-grafted. With the optimal conditions of transformation events an average of 6 % transformation efficiency with recovery of fertile transgenic plants reported. The optimal conditions were as follows: 2 days of pre-culturing prior to bombardment; 900 psi rapture pressure for particle acceleration; subsequent decrease in the selective agent from 5 to 2.5 nM chlorosulfuron concentrations during the following 4 and 2 weeks of selection, respectively. The study was supported with positive PCR and southern results for the transgene in both T_0 plants and T_1 progeny.

1.5. AIM OF THE STUDY

The major important drawback in lentil tissue culture is poor rooting response of regenerating shoots. This is also an important reason which limits genetic transformation studies in lentil. For these reasons in this study we aim:

i) To optimize a micrografting based regeneration system for lentil shoots obtained from direct organogenesis studies

ii) To optimize a tissue culture independent transformation system for lentil

CHAPTER-II
MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. MICRO-GRAFTING

2.1.1.1. Plant Materials

Spring sown Turkish lentil cultivar Sultan 1 was used mainly throughout this study as root stock and scion. Some trials have been also carried out with chickpea root stocks of cultivar Gökçe. All other explants were derived from fresh seedlings of above stated cultivars of lentil and chickpea. All seeds were obtained from Exporter Unions Seed and Research Company.

2.1.1.2. Plant Tissue Culture Materials

During micro-grafting experiments for germination and culturing of the sterilized explant sources, MS (Murashige and Skoog, 1962) basal medium supplemented with sucrose and agar was used. The media was sterilized at 121^{o}C and 1.5 atmosphere pressure for 25 minutes.

Seven days old germinates of Sultan 1 cultivar was used as explant source. For regeneration of the shoots that were to be used as scions, the cotiledonary node explants were cultured in MS basal media supplemented with the growth regulator 6-benzylaminopurine (BA) at a concentration of 1mg/lt (Warkentin and McHughen 1993).

During establishment of vascular unity between the root stock and the scion signed by elongation of scion, micro grafts planted into perlit (used as filler) were watered with full strength Hogland's solution. Grafts were maintained in totally closed one liter glass containers to avoid dehydration. Hogland's` solution was sterilized at 121^{o}C and 1.5 atmosphere pressure for 25 minutes.

2.1.2. TRANSFORMATION

2.1.2.1. Bacterial Strain and Plasmid Constructs

Agrobacterium tumafecience strain KYRT 1 with binary vector pTJK136 was used throughout the transformation study. The vector pTJK136 is a derivative of pTHW136 and carries streptomycin and spectinomycin resistance gene encoding for the adenyl-transferase enzyme as bacterial selection marker and carries an intron containing *gus* and *npt-II* genes as plant selection markers (Kapila *et al.,* 1997).

See Appendix A for the plasmid map and selection agents in detail. pTJK136 was provided by Prof. Dr. Van Montagu (see Apppendix B for permission of pTJK136).

2.1.2.2. Plant Materials and Explant Source

Explant source used throughout the transformation studies was seeds of lentil Sultan-1 variety. Explants prepared form overnight imbibed seeds. One of the cotyledons was stripped of leaving the embriyo linked to the other cotyledon through cotiledonary petiole.

2.1.2.3. Growth Media Used

Yeast Extract Broth (YEB) was used as the main nutrient supplement in bacterial culturing. All other culturing media were derived from YEB through addition of necessary components. Streak plating of the strain was made on YEB+MES+Agar media with necessary antibiotics as selection agents. Initialization of liquid culturing from the streak of the strain was carried in liquid YEB medium with antibiotics. Bacterial culturing was made with YEB+MES media with antibiotics plus acetosyringone (as *vir* function activator phenolic compound).

During transformation event for re-suspension of the bacterial cells, media referred to as MMA was used. For transformation, the explants were also inoculated in this suspension.

During co-cultivation and growth Hogland's solution was used as the only plant tissue culture medium.

All chemicals and materials were obtained from Duchefa (Harlem, Netherlands). Bacterial culture and plant growth media compositions were given in detail in Appendix C.

2.2. METHODS

2.2.1. MICRO-GRAFTING STUDIES

2.2.1.1. Preparation for Micro-Grafting

2.2.1.1.1. Surface Sterilization of Explant Sources

For surface sterilization of lentil seeds, 20 % (v/v) sodium hypochlorite (NaOCl) solution was used. After momentarily rinsing in 70% ethanol followed by distilled water, 20 min surface sterilization with NaOCl on orbital shaker at 140 rpm was carried out. Then NaOCl and decolorized seeds were removed through serial washes with sterile distilled water. Lastly on a sterile filter paper seed debris and brown worn out seeds were removed and the healthy seeds were left for overnight imbibition on orbital shaker (140 rpm). Above mentioned sterilization procedure with 5% (w/w) NaOCl for 25 min was carried out for surface sterilization of chickpea seeds.

2.2.1.1.2. Germination, Explant Preparation and Culturing

Surface sterilized overnight imbibed seeds were blotted dry on sterile filter paper. Then healthy seeds were transferred to MS germination media. After seven days of germination in dark at 22^{o} C these etiolated germinates were used either as root stocks or explant sources for cotiledonary nodal segments.

As root stocks, fresh seedlings were cut at the first node above the cotyledons. Shoot was discarded and the rest was used as root stock.

For culturing the cotiledonary node explants to yield shoots that are to be used as scions, the germinated one week old seedlings were cut off from their emerging primary shoot and root at a distance of approximately 5 to 7 mm away from the cotiledonary node region. Also the cotyledons were removed from the rest of the explant leaving the cotiledonary petiole on the explant (Figure 2.1). The cotiledonary nodal segment left in hand was cultured in shooting medium that was oriented according to the gravitational center of explants during culturing period in order to yield linear shoots to be used as scions (Figure 2.2). Culturing was made for one month at 22^{o} C with 16 hr photoperiod. The emerging shoots were harvested and used as scions.

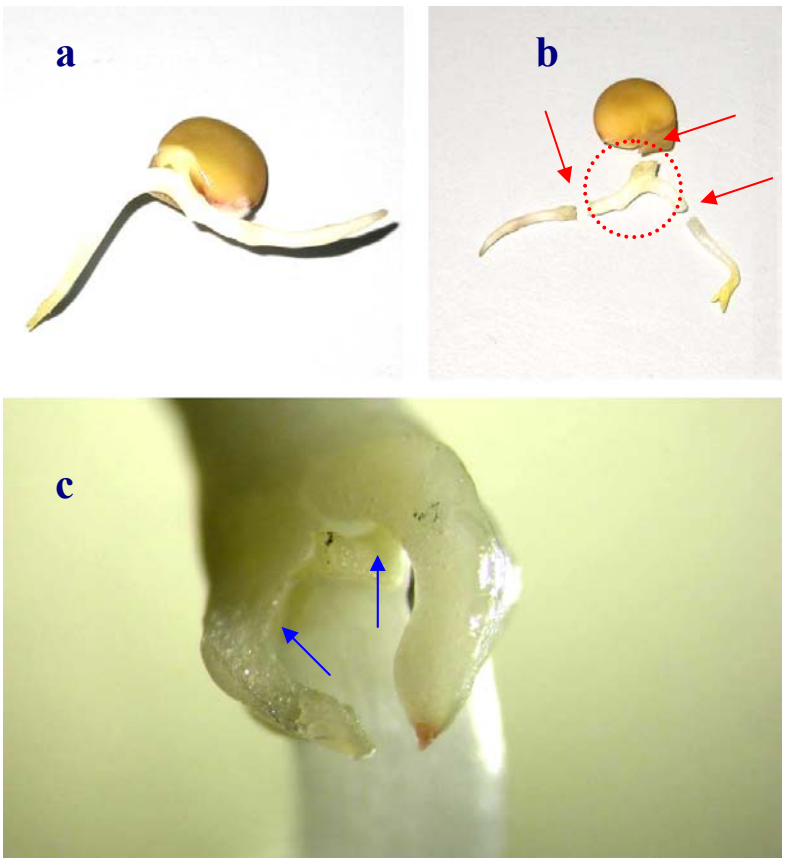

Figure 2.1: Cotiledonary node explant. (**a**) Intact germinated seeds. (**b**) Simple cuts introduced (shown with arrows) yielding the cotiledonary node; enclosed in the circle. (**c**) Isolated cotiledonary node; up side down orientation, arrows showing regions of secondary meristems that rise advantageous shoots.

2.2.1.2. Micro-Grafting

One type of scion preparation versus two types of root stock preparations was tested in the study. Main idea was to introduce a cut that exposes as much as possible vascular bundles on both stem axis of the stock and the scion to be met during uniting. By this way fast recovery and strong branching of the grafts were expected.

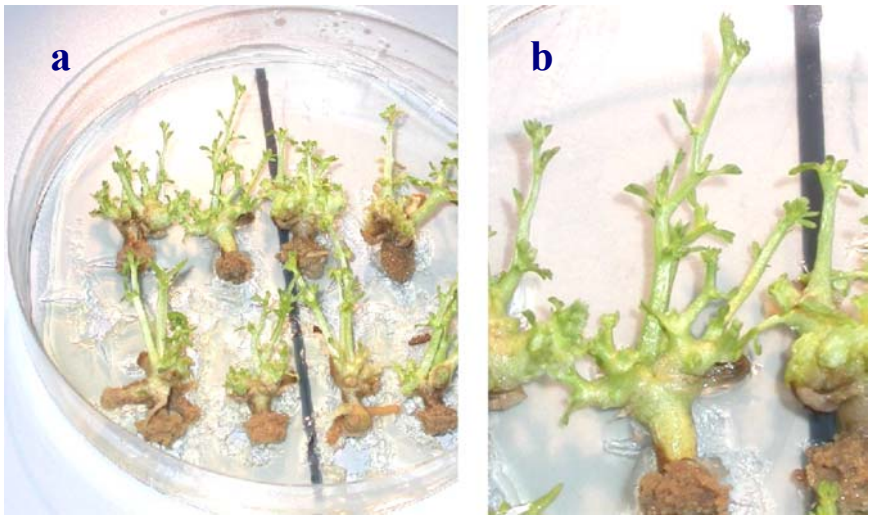

Figure 2.2: Cotiledonary node culture plate. (**a**) Orientation of the cotiledonary node explants on their vertical axis. (**b**) Geotropic elongation and growth of the shoots producing linear scions that is easy to graft.

Two types of root stock preparations were designated with letters M and Z, respectively (see Figure 2.3 and 2.4). During preparation, the shoots of the germinated explants chosen as root stock sources were removed off from the first internodal segment above the cotyledons.

For the M type root stock preparation stem axis of the root stock was split apart into two equal pieces with 3-5 mm deep cut. Then the prepared scion was inserted into this cut exposed stem of the root stock (Figure 2.3).

For Z type of root stock preparation on the other hand, the stem axis was split apart (but not cut off) with a narrow angular degree, that transverse the stem axis with 4-6 mm deep cut. During scion insertion to the Z type of root stocks special care was taken to meet the correct surfaces of vascular bundles of the stock and the scion (Figure 2.4).

For preparation of the scion, the secondary shoots regenerated from the meristems of cotiledonary nodal segment were used. These harvested scions were put into a sealed sterile environment with sterile distilled water to prevent dehydration prior to preparation. For preparation of scion a lateral cut with a steep angle (30-45^{o}) was made along the stem axis on the scions (Figure 2.5).

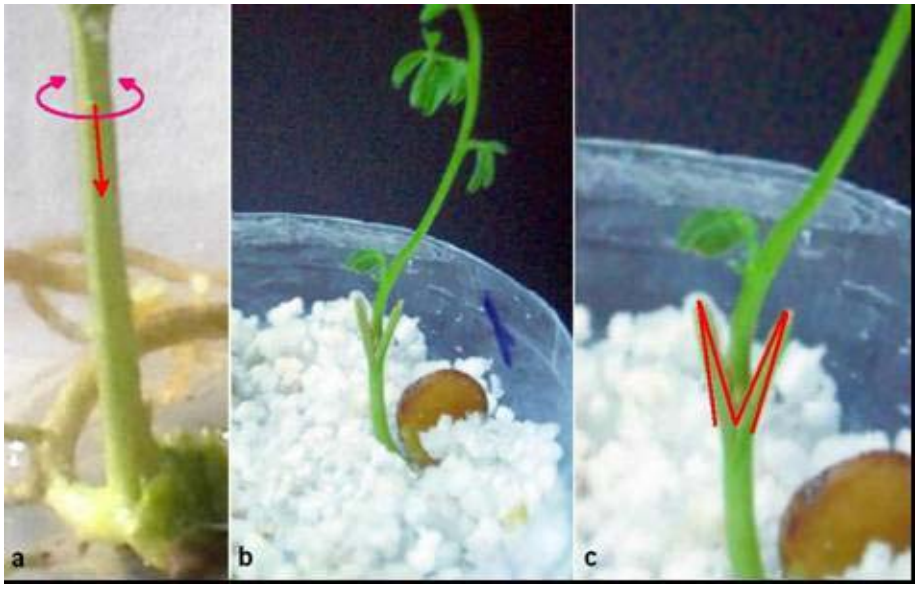

Figure 2.3: MV type Micro Graft. **(a)** Preparation of M type root stock; the red arrows are showing the cut directions to be introduced. **(b)** The union pattern after a successful M/V type graft. **(c)** The uniting scion and stock results in distortion of the stem of root stock that resembles the letter M; shown with red line.

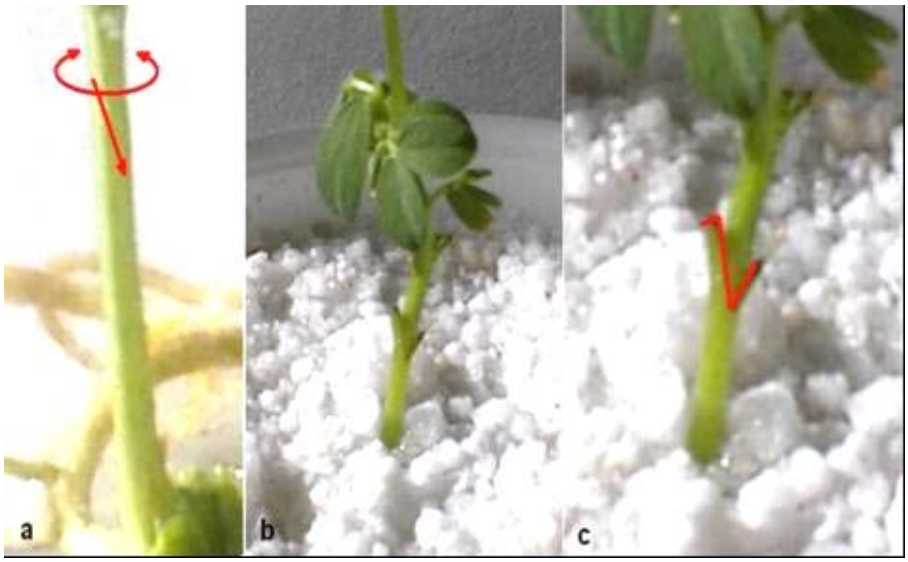

Figure 2.4: ZV type Micro Graft. **(a)** Preparation of Z type rootstock; the red arrows are showing the cut directions to be introduced. **(b)** The union pattern after a successful Z/V type graft. **(c)** The uniting scion and stock results in distortion of the cut exposed sites of the stem of root stock that resembles the letter Z; shown with red line.

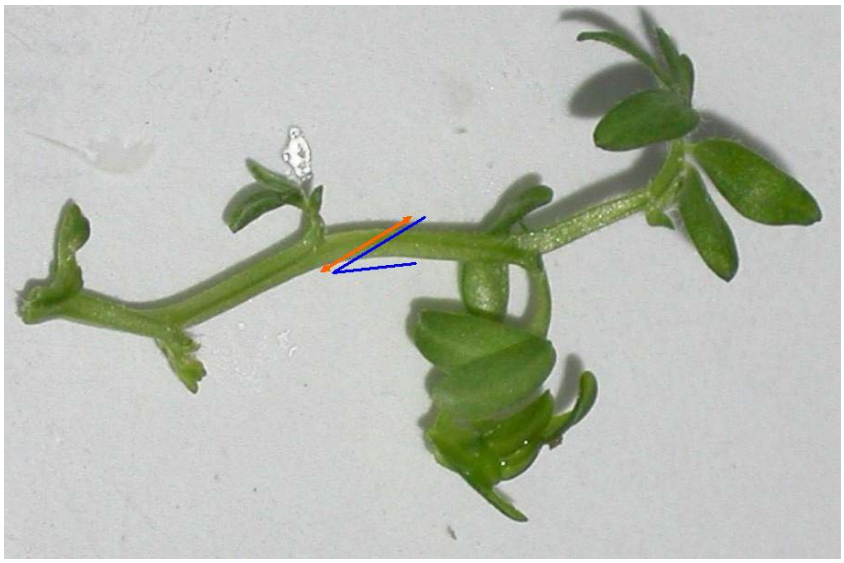

Figure 2.5: Scion preparation. Red arrow shows the cut directions to be introduced; blue lines resembling the letter V. This scion type prepared is used in both of the graft types and named as V type scion.

2.2.1.3. Maintenance and Evaluation

After grafting the micro grafts were planted into perlite with full strength Hogland's solution and maintained in one liter volume jars. These totally sealed jars were incubated at 22^{o}C under 16 hour photoperiod. Micro grafted explants were monitored at 3 day intervals. Advantageous shoots regenerated from the root stocks were removed.

According to the growth behaviors of grafted plantlets, observations and evaluations were carried out at three distinct stages referred to as recovery, acclimisation, hardening and greenhouse growth.

Recovery and acclimisation period extended between two to three weeks starting from the date of micro-grafting. During this period the union region between the root stock and scion responds to recovery with callus formation between the cut exposed surfaces. Following the callus formation and probably union of vascular bundles, growth of the scion takes place. At this phase as long as the apical nodes of the scion gives rise to fresh branches, acclimisation was started through gradually exposing the enclosed micro-grafts to open air.

After full acclimisation of the grafts, hardening was committed in pots with soil at 25^{o} C and 16 hr light photoperiod. Hardening was carried out for seven days.

Following the hardening stage the grafted plantlets were transferred to green-house. Number of flowers and seeds per pot were recorded.

2.2.2. TRANSFORMATION STUDIES

2.2.2.1. Explant Preparation

Explants were surface sterilized with 20 % (v/v) NaOCl for 10 min on orbital shaker then rinsed with sterile distilled water. After that the seeds were blotted dry to remove the brownish unhealthy seeds. Healthy and proper seeds to be used as explant sources were selected and imbibed overnight on orbital shaker.

After overnight imbibition of the seeds, one of the cotyledons was removed and the rest of the explant was used in the experiment (Figure 2.6). The explants were kept wet to prevent dehydration until the injury process.

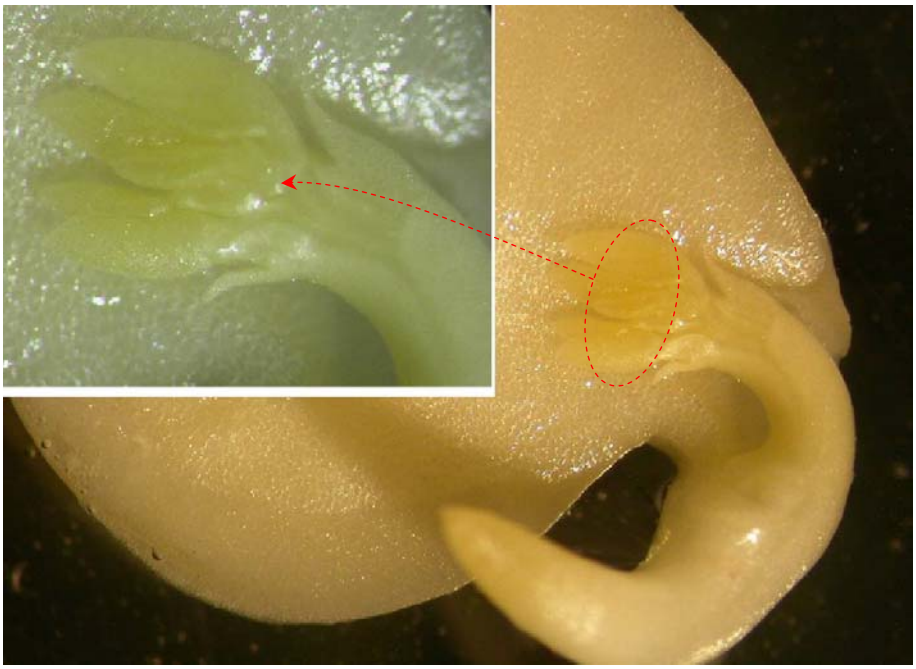

Figure 2.6: Mature lentil Embryo. Circle showing the zone of injury

Prior to transformation the explants were injured under dissecting microscope with the aid of extra fine glassware pins and crushers. The target of injury and hence the transformation was the embryonic apical meristem tissue that gives rise to primary hypogeal growth (Figure 2.6). After the injury the explants were incubated in full strength Hogland's solution for one hour and then used in transformation experiment.

2.2.2.2. Bacterial Culture

For initiation of bacterial culture a single colony of KRYT1::pTJK136 was inoculated into YEB+Antibiotics medium (3 ml) and cultured at 28^{o}C and 180 rpm for two days. After maintenance of growth in starter culture, pilot scale inoculation was made into YEM+MES+Antibiotics+Acetosyringone medium (100 ml). Under the same incubation conditions culture was grown until cell density reaches to OD_{600}=0.8. Then the culture was centrifuged at 4000 rpm for 15 min to recover bacterial cells. The pellet was re-suspended within MMA medium to a cell density of OD_{600}=2.4.

After re-suspension of bacterial cells within MMA medium, in order to activate the virulence machinery, acetosyringone was added and the suspension was kept at 21^{o}C for one hour. Then the *Agrobacterium* suspension was immediately used in transformation experiment.

2.2.2.3. Transformation

Transformation was initiated by transferring the injured explants into the *Agrobacterium* suspension. The incubation procedure was altered according to the designated parameters to be tested. *Agrobacterium* incubation duration was preset as 90 minutes. Infiltration duration of *Agrobacterium* suspension to the injured explants was tested at 200mm Hg (Mahmoudian *et al*., 2002-a) for 0, 20, 40, 60 minutes. Another tested parameter was the type of injury namely crushing versus poking to the apical meristem. The next parameter tested was the *Agrobacterium* incubation time (30, 60, 90, 120, 240 minutes). The effect of the thiol compounds to *Agrobacterium* transformation efficiency was tested with L-cysteine at 0, 400, 800, 1200, 1600 mg/liter concentrations.

2.2.2.3. Transient Gene Expression Analysis

2.2.2.4.1. Histochemical GUS Assay

Histochemical GUS assays were carried out to confirm the success of transformation experiments. For transient gene expression analysis three days old germinates of transformed lentil embryos were used. The emerging primary shoot was excised and used in GUS assays. Faith of *gus* gene expression was also assayed with 2 weeks old regenerated transformants without the root sections or with the leaves harvested from the terminal and the three youngest auxiliary buds of 7 days old and older seedlings. Non-transformed explants, as the control group, were treated in the same manner as transformed ones.

Histochemical GUS assay was first described by Jefferson *et al.* (1987) as a very sensitive assay for visualization of β-glucronidase (GUS) activity, based on the formation of colored precipitates at the sites of GUS activity in plant tissues. Histochemical localization of the GUS expression was performed as described by Jefferson *et al.* (1987).

For preparation of the histochemical GUS assay solution of 20 ml, X-Gluc substrate (5-bromo-4-chloro-3-indoly-β-D-glucuronide) was dissolved in DMSO and then diluted to a final concentration of 1 mM with the addition of the following chemicals. 2 ml 1M NaPO4 buffer (at pH 7), 800µl 0.25 M EDTA (at pH 7), 2 ml 0.005 M Potassium Ferric Cyanide (at pH 7), 2 ml 0.005 M Potassium Ferrous Cyanide (at pH 7), 200µl 10 % Triton X-100 and 12 ml distilled water.

The samples were incubated in the GUS assay solution for 3 days at 37°C. Then tissues were placed in GUS fixative solution which contains 10 ml formaldehyde (10 % v/v), 20 ml Ethanol and 5 ml acetic acid in an aqueous solution of 100 ml. Depending on the maturity and chlorophyll content of the explants fixation was followed by incubation in Ethanol for decolorization (50 % Ethanol for 30 minutes followed by pure Ethanol overnight).

Histochemical GUS assay results were scored under dissection microscope on area base at milimetric scale. See Appendix D for GUS histochemical assay solution and GUS fixative solution.

2.2.3. STATISTICAL ANALYSS

Minitab statistical analysis package program, release 13.1 was used for the analysis of the experimental results.

CHAPTER-III
RESULTS AND DISCUSSION

3.1. MICROGRAFTING

Aim of grafting was to maintain the correct meeting of the vascular bundles and cambium through the stem axis of the uniting scion and root stock. Therefore newly formed vascular connection maintains transportation between the potential root stock and scion which may have favorable superiorities.

3.1.1 *IN VITRO* MICROGRAFTING

The initial studies of the micrografting were carried out under *in vitro* conditions. Both during germination of the root stocks and during micrografting, contamination were arising as a threat for the grafted plantlets. Also one month duration was required for the recovery of the micro-grafts and elongation of the scion. At the end of this period, successful grafts that were transferred to soil for hardening totally wilted and dehydrated (Figure 3.1 a-c). For example in one *in vitro* experiment out of 40 grafts only 8 of them (20 %) were successfully taken out to soil for hardening but none of them survived in soil. An interesting observation among these grafts was that the 2 out of the 8 grafts recovered had higher elongation and higher number of shoots then the rest which had stunned growth. Another difference of this 2 graft was a prominent callus body at the uniting region of the root stock and scion (Figure 3.1-a, b). These callus bodies were also noted in the study by Kahwar and Özcan (2002-b).

The wilting phenotype of the soil transferred grafts for acclimation was correlated with the water stress that arises with the limited vascular connection of the stock and scion after establishment (Wadano *et al.,* 1999). The claim was also supported by detection of increased catalysis of ascorbic acid and glutathione due to the water deficiency and oxidative stress in tomato.

Although in our experiments acclimation is started at the end of one moths period during which a pretty good elongation and development takes place, similar water stress and wilting responses were visually observed on the vegetative parts of the grafts (Figure 3.1-c).

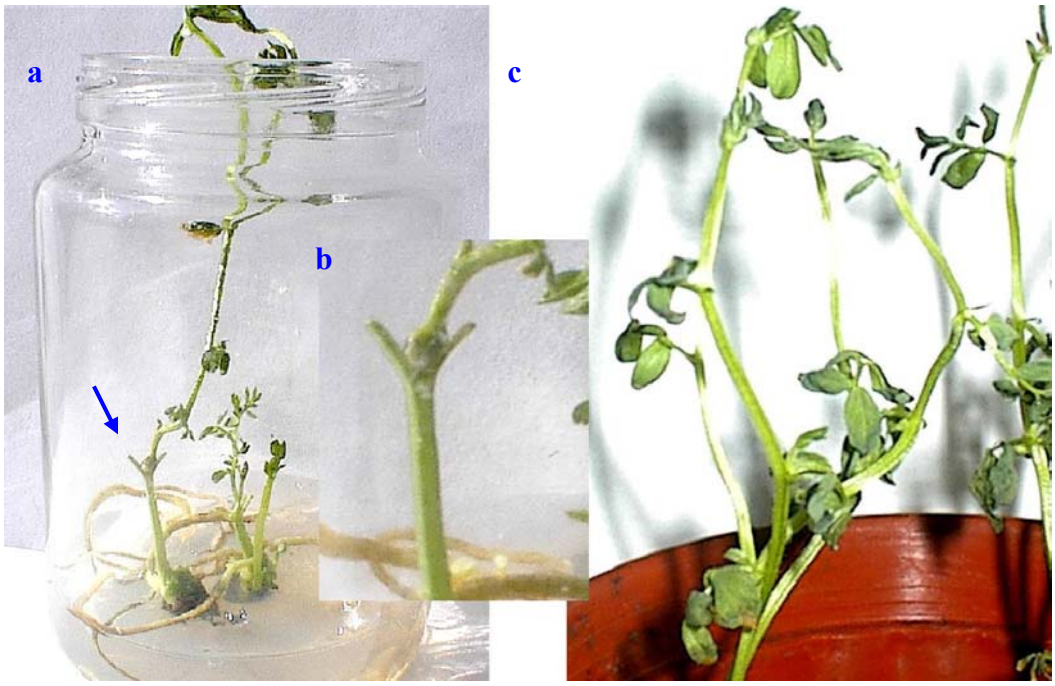

Figure 3.1. *In vitro* grafting. (**a**) Growth after establishment. Arrow showing callus body prominent at the uniting region, signing the establishment. (**b**) Close up of the union. (**c**) Wilting of the soil transferred *in vitro* graft.

As a result of the above observations and difficulties of *in vitro* micrografting along with the extreme care of sterility to avoid contamination in tissue culture we devised an *in vivo* procedure of micrografting. Advantages of this *in vivo* procedure were stated as minimal tissue culture needs and sterility care during germination of stocks and maintenance of grafts made.

3.1.2. IN VIVO MICROGRAFTING

3.1.2.1. Root Stock Stem Height

Stem stock height of the root stock was the first parameter tested in the initial *in vivo* experiments. Above the cotyledons till the first node (excluding node) on the epicotyl the stem length of the root stock was decided to be the full stem length. Grafting is either made to ½ or 1 unit of this length.

Results of two independent sets of experiments demonstrated that the full stem length was found to be superior over the half stem length at the initial step of micrografting, where establishment (evident by elongation) between the stock and scion took place. However, during the following steps of hardening and green-house maintenance, all of the grafts that were made on the full stem length dehydrated and died.On the other hand overall success rate of half stem length grafts was recorded as 15 % (refer to Table 3.1). When the most successful experiments for both full and half stem length was considered, the full stem length had a maximum success of 70 % establishment at the stage of grafting and 0 % success rates in next steps.

Table 3.1. Graft root stock type and stem height

	grafting (1)			hardening (2)				green house (3)			
	# of grafts	# of successful grafts	% success (1)	# of plants	# of successful hardenings	% success	success on 1 & 2	# of plants	# of successful transfers	% success	overall success (1,2 & 3)
stock stem height											
height 1	47	32	68,1	32	0	0	0	0	0	0	0
height ½	13	4	30,8	4	2	50	15,4	2	2	100	15,4
stock type											
lentil	23	21	91,3	21	4	19	17,4	4	0	0	0
chickpea	13	13	100	13	5	38,5	38,5	5	1	20	7,7

On the other hand half stem length had maximum success of 75 % during establishment stage and 50 % success in the next steps of hardening and green-house maintenance.

Depending on these findings concerning the overall success, half stem length was accepted as the optimum root stock stem length. Contrary to our stem length optimization study, Gulati *et al.,* (2001) approached to this parameter in a range of 1 to 1.5 cm stem length over the epicotyl. Also in the study of Kahwar and Özcan (2002-b) grafts were made just above the cotyledons on to a stem length of 2.5 to 5 mm which is closer to the cotyledons then in our study. Probably the closer the graft made to the cotyledons the higher the graft success may take place.

3.1.2.2. Root Stock Type

Unlike the studies of Gulati *et al.,* (2001) and Kahwar and Özcan (2002-b) which demonstrated the effect of cultivar difference for the best efficient root stock selection, in our experiments we have studied the applicability of the grafting between different genera (chickpea root stock and lentil scion). Experiments were evaluated at the initial steps of grafting and establishment. Data and visual observations (Figure 3.2) clearly showed that more successful establishment (91 to 100 % success for lentil and chickpea root stocks, respectively), faster recovery and elevated levels of growth was prominent in the chickpea-lentil compared to the lentil-lentil micro grafts (Table 3.1) Although the establishment between the lentil scion and chickpea stock was prominently more strong and efficient then the lentil-lentil graft these chickpea-lentil grafts were not successfully brought beyond the hardening step up to flowering and seed set in green house. This might be due to incompatibility of the vascular connection between graft counterparts that the scion vegetation increase could not be supported further by the chickpea root stock.

The diagnosis of the deadly behavior of the lentil chickpea grafts was not carried further due to the more vulnerable nature of the chickpea root stock to contamination and water logging compared to lentil root stock.

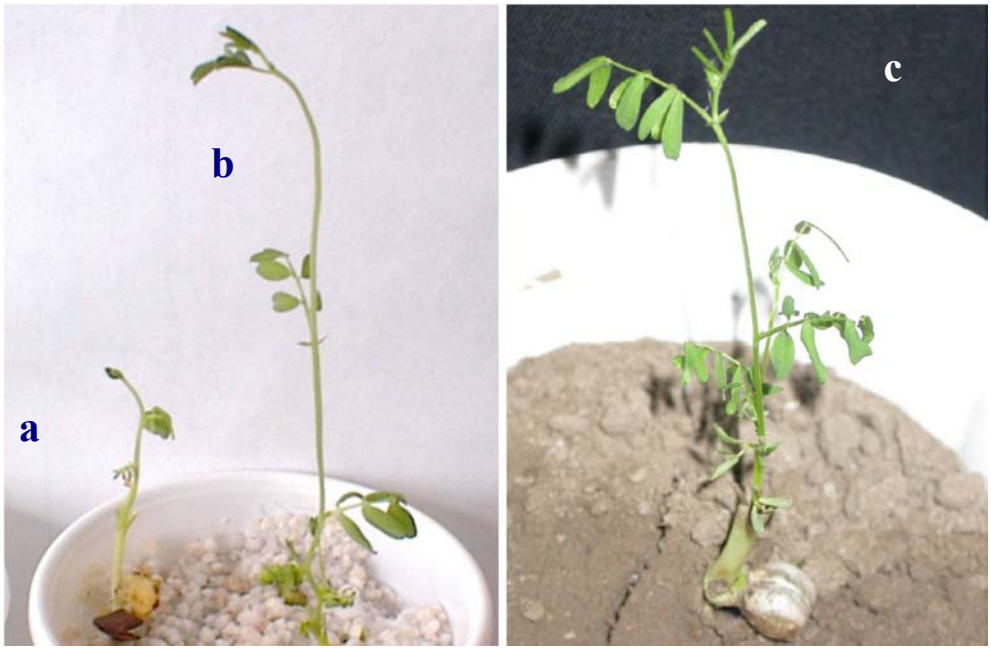

Figure 3.2. Chickpea versus lentil root stock. Two weeks old lentil-lentil **(a)** and chickpea-lentil **(b)** micro graft. **(c)** Successful chickpea lentil graft after hardening stage.

A representative report on micrografting study between different genera was not found on herbaceous annual legumes (possibly due to presumed graft rejection) for comparison of our results, but higher percentage of success of chickpea-lentil grafts might attributed to the bigger stem girt of the chickpea root stock.

3.1.2.3. M-V versus Z-V Type Micro Grafts and Sealing

Another parameter studied was the applicability of different incisions to the stem axis of the root stock. Since the stem girth of the 7 days old lentil root stock was not big enough to compensate any experimental error during microgafting. As a result improper unity between the graft counter parts can hinder graft successes. For evaluation of this handicap and to minimize the difficulty during uniting we aimed to produce different root stock stem cut surfaces for maximum vascular meeting between the stock and scion.

One type of the incision designated with the letter M introduces a vertical cut into the stem axis and splits the stem into two equal pieces while the other designated as Z introduces a diagonal cut with a narrow angle (without chopping off the pieces) resulting in two asymmetric conical portions (refer to Figures 2.3 and 2.4). The Z type stock preparation was presumed to expose much more vascular bundles and be superior over the M type.

Another application tested with the different root stock preparations was sealing the graft at the uniting region of the stock and the scion. Also it was presumed that sealing would enhance the establishment between the graft counterparts (see Figure 3.3).

According to the data collected from three independent experiments for testing the root stock preparation type and sealing parameters it was concluded that with respect to grafting and establishment, hardening, green house stages, the Z-V type of micrografting with sealing is superior over the others with success rates of 73 %, 85 %, 88 % during grafting and establishment, hardening, greenhouse stages, respectively (Figure 3.4).

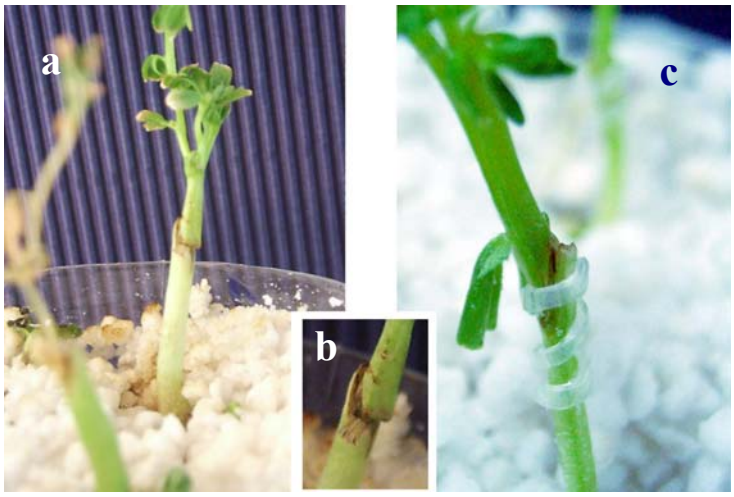

Figure 3.3. Sealing and non sealing of graft uniting region. **(a)** Micro graft without seal. **(b)** Close up of the uniting region. **(c)** Micro graft with seal around the uniting region.

The least successful graft type was recorded as M-V type without seal with rates of 72 %, 75 % and 8.7 % during grafting and establishment, hardening, greenhouse stages, respectively. The superiority of the Z-V type graft with seal was expected to be so throughout the experiment till greenhouse. However, surprisingly at the initial step of the grafting during which the establishment takes place (signed by elongation of the scion), the M-V type graft with seal was recorded to have highest progress (90.3 %) then the rest of the applications.

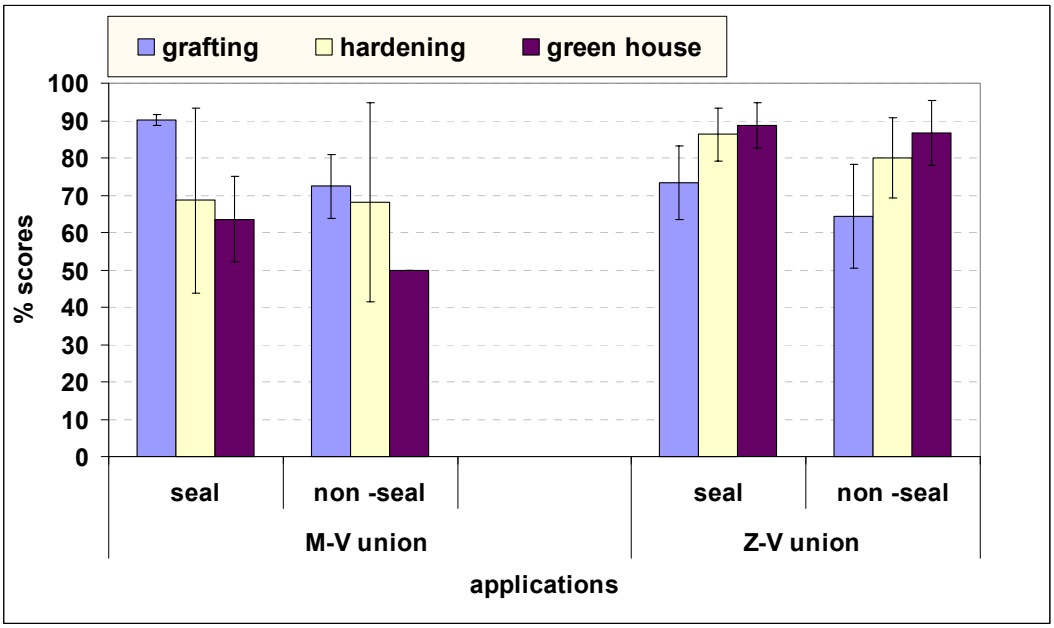

Figure 3.4. Success rates of different grafting applications.

Sealing or non-sealing factor for either of the M-V and Z-V types of micro-grafts did not introduce significant difference statistically. The most significant factor that enhances the success rates of micro-grafts throughout the stages was recorded to be the root stock preparation type

3.1.3. OVERVIEW OF MICROGRAFTING

In literature, up to date there are only two studies that report micro-grafting in lentil. Concerning the age of the root stock used in the previous reports Gulati *et al.,* (2001) stated that grafts were done on to 5-6 days old seedlings whereas in the reports of Kahwar and Özcan (2002-b) the grafting was done onto 3-4 days old seedlings. However, in our study root stock age was ranging between 6-7 days.

Also the graft types in these studies were similar to that of M-V type in our studies. In our experiments the M-V type micro graft with seal had a success rate of 87-92 % at the grafting and establishment stage, which was similar to results presented by Gulati *et al.,* (2001) as 84-96 %.

On the contrary to our study for the effect of the nature of root stock, Gulati *et al.,* Khawar and Özcan studied the effect of nature and genotype of the scions on graft success. In these studies the rates of successful grafts were ranging from 25 to 100 % depending on the nature of the scion. Whereas, in our study the chickpea root stock type was shown to be 100 % successful at the grafting and establishment stage. In our experiments similar phenotypic observations were recorded with that of Gulati *et al.,* (2001) during the recovery and establishment of the grafted plantlets. Elongation and growth of the scion ranged between 10-15 days in our studies whereas it was reported to be 7-12 days by Gulati *et al.,* (2001). Also it was noted in the report that finalization of the graft took 2 weeks whereas in our case it ranged between 2-3 weeks.

For the grafts that were successfully taken to the green house were phenotypically normal, set flowers and pot filling was observed, but the white fly arose as a threat during the season when the M-V type grafts were raised. During the green house conditioning of the M-V type grafts the impact of the white flies resulted in loss of the grafts. Larval and pupa feeding stages of flies was determined to be taking place under the leaves which was fallowed by mold invasion and dead of the plantlets. On the other hand successful flowers and seeds were recovered from the Z-V type grafts as the insecticide application started and depressed the white flies. For the Z-V grafts that are sealed all of the 28 plants transferred to green house set flowers and eventually filled pods.

Also almost all of the Z-V grafts from non-seal group flowered and set seeds (23 out of 24). The acceptable levels of success in lentil plant regeneration through micrografting were presented in our study and in the previous reports. However, a new root regeneration method based on explant polarity from the nodal segments in a number of hypogeous legumes including lentil was reported by Fatini and Ruiz (2003). The rooting success for lentil nodal segments was reported as 95 %. But since the study is confined to *in vitro* regeneration and only uses the intact nodal segments as explants, its use is seemingly limited. Contrary to this stated tissue culture method any shoot regenerated in tissue culture or shoots harvested from *in vivo* nursed seedlings can be grafted to give complete lentil plantlet. Also the micrografting method do not posses the complex tissue culture needs during root initiation along with the possible tissue culture mutagenesis.

3.2. TRANSFORMATION

One of the most important drawbacks in lentil transformation is lentils` poor tissue culture response. The early transformation events that address this difficulty were *in planta* electroporation mediated gene transfer in lentil and in some grain legumes (Chowrira *et al*., 1995/1996) and to some extent; half embryo co-cultivation with *Agrobacterium* in lentil (Lurquin *et al*., 1998). Both of the studies have shown transformation event to some extent in lentil but are far from reproducibility and had low transformation rates.

In our study we devised a different approach for transformation of lentil. Basically in this non tissue culture based system overnight imbibed lentil seeds were injured at the embryo shoot apex region and incubated with *Agrobacterium* suspension. Then the seeds were left for germination and growth. Thus the system does not require any further tissue culture stages. Up to date this system has not been applied to lentil. The only two studies that approach the vision of embryo apex transformation were *Agrobacterium* mediated gene transfer to the shoot apices of a grain legume- (*Lupinus angustifolius*), (Pigeaire *et al*., 1997), and *Agrobacterium* mediated gene transfer to the micro wounded premature embryo derived hypocotyls of sunflower (Lucas *et al*., 2000).

In both of the studies the embryo used as explant was deprived of the cotyledons and for the sunflower study also the root was removed and hypocotyl was dissected in to two equal parts. Although the two systems utilize the regenerative capacity of the embryo shoot apex both require further *in vitro* culture and a subsequent regeneration procedure after handling putative transgenic shoots.

In our study various factors including tissue injury type, infiltration of *Agrobacterium* cells, *Agrobacterium* incubation time and involvement of L-cysteine in co-cultivation media were studied. Successes of experiments were addressed by histochemical GUS analysis.

3.2.1 EFFECT OF EXPLANT INJURY

During injury optimization three different methods of injury were devised. Injuries were made to the lentil embryo apex (Figure 2.6) by means of poking with superfine needles (diameter 1/8 mm), by crushing and by sonication (a single pulse at 2 kHertz). Two different strengths of injuries were applied for each injury applications. These were 10 and 25 times poking, 1 and 2 times crushing and sonication. Impact of the injuries on growth behavior of the embryos was addressed with three distinct observations. These were germination frequencies of 3 days old seedlings, mass length ratio of elongating primary shoot and number of primary leaves at the end of 7 days of germination (Figure 3.5).

Effect of different injury applications on germination was found to be statistically insignificant. In general, for the applications other then sonication-(2) which had 80 % germination, germination rates ranged between 90 to 100 %. As compared to germination rate the mass length index along with the number of primary leaves gave the most representative results about the effect of the injury application. For the mass/length index of the seedlings it was observed that the control group and the poking-(10) group variances were insignificant (Figure 3.5) and statistically, the mass/length index of the two groups were significantly higher than the rest of the injury applications ($p<0.05$). That is, injury with 10 times poking was similar with non injury and was not affecting the growth of embryo at all. The mass/length index of the rest of the group did not exhibit significant difference.

Since our target for injury was meristem tissue at the embryo apex, it was thought that the relative injury intensity to this meristematic region could be demonstrated by the number of embriyogenic leaves emerging after 7 days of germination. According to the results and statistical analysis both control (no injury) and poking-(10) group produced significantly higher number of primary leaves then the rest of the group (also evident from the mass/length index). Sonication-(1) and poking-(25) groups on the other hand produced the second higher number of primary leaves. The injury groups of crushing-(1) and sonication-(2) produced the third higher number of primary leaves. But the most intense injury type and hence, the lowest number of primary leaves were produced with the crushing-(2) type of injury which was also significantly different from the rest of the group.

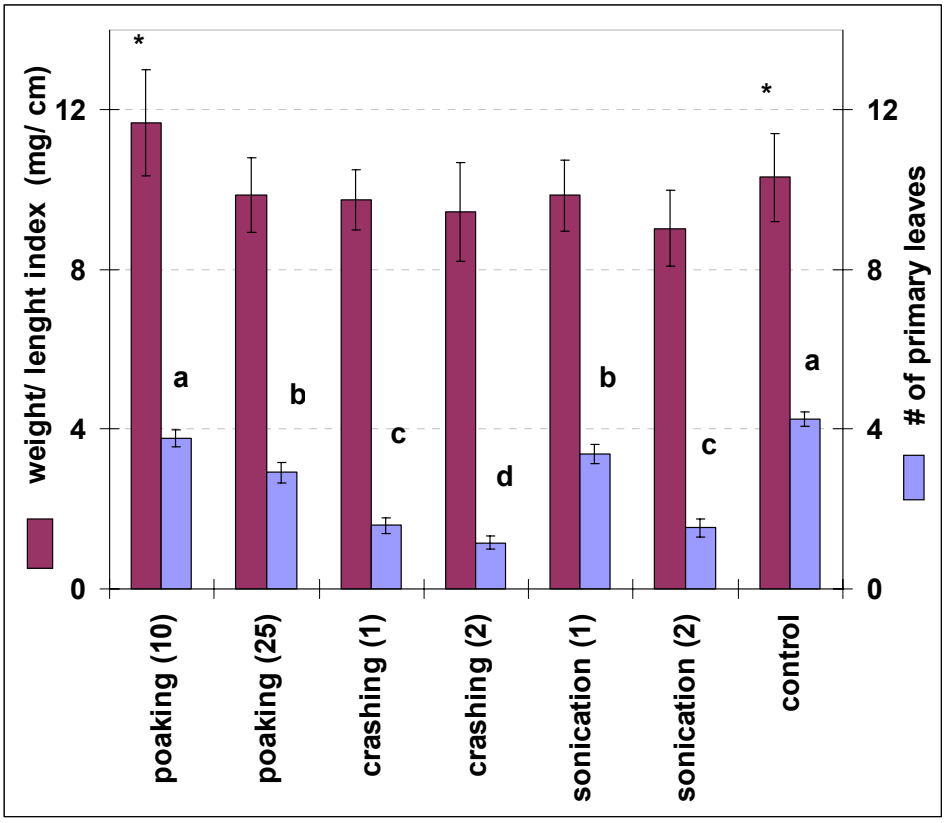

Figure 3.5. Effect of different injuries on growth of lentil embriyos. Different letters a-d and * sing are denoting variances in means. The same letters and sings denoting that the means are in the same 95% CI (one way ANOVA).

As a result two times crushing application (crushing-(2)) to the embriyo apex was found to be significantly injuring this portion of the explant and reducing the number of embryonic leaves whereas the application was not effectively reducing the germination frequency and primary elongation of the shoot (evident by the mass/length). Also this injury application was found to be the least sophisticated and least cumbersome as compared to sonication and poking.

In literature we did not found any report that is dealing with the injury optimization to such extent for lentil. The early transformation events include many injury applications. Some are slashing or abrasion, poking, micro wounding with tungsten or gold particles, infiltration and simple cutting. Only in the report of Gulati *et al*., (2002) which used biolistics for gene delivery, optimization of the rapture pressure for the acceleration of the micro particles was noted to be an important parameter during delivery to the regenerative cell layers in the target tissue. Either means of mechanical injury through micro carriers or with some devised needles, discrete points of injuries are produced and hence scattered GUS spots are observed after transformation. In our study with the optimized crushing type of injury we were aiming to introduce an area based injury to the apex region without depressing the regeneration capacity of the meristem tissue. By this way it would be possible to transform as much as possible meristematic zone on the apex that may rise transformed shoots. Representative data are given in figure 3.6.

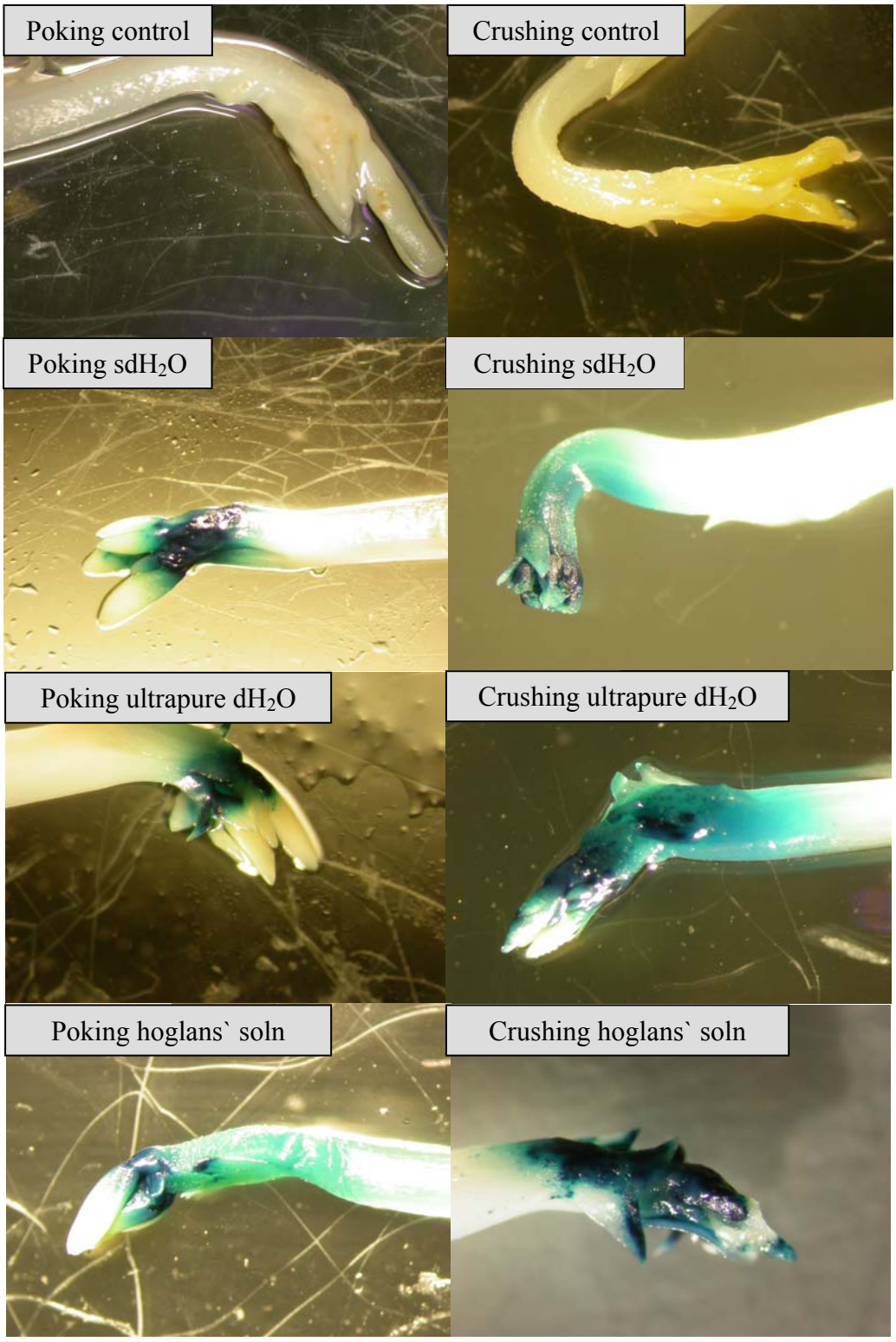

Figure 3.6. Representative data for GUS histochemical stainings of the explants subjected to different injury types

3.2.2 EFFECT OF PREINCUBATION MEDIA

Following the incubation of the injured explants with *Agrobacterium*, germination and cocultivation of the embryos were made with hoglands` solution. In order to simulate the germination media and to prevent dehydration of the injured explants (prior to *Agrobacterium* incubation), embryos were also maintained (pre-incubated) in hoglands` solution. The combined effect of the injury type (crushing and poking) and pre-incubation media on plant wound response and *Agrobacterium* transformation might be an important factor. To test the effect of the pre-incubation on transformation we devised an array of different pre-incubation media which were ultra pure water, sterile distilled water and hoglands` solution.

In tissue culture based transformation systems and especially in transformation events that use particle bombardment, a step of pre-culture optimization was evident. Our attempts on optimization of pre-incubation media might be compared with these studies. In the study of Gulati *et al*., (2002) in lentil an optimum pre-culture duration of two days was noted to be enhancing the survival and shoot regeneration rate of the cotiledonary node explants following bombardment. However, it would be important to state that the studies referred in the above mentioned report use preculturing were also confined to micro projectile bombardment method as means of gene delivery to target tissues. Also in one of the *Agrobacterium* mediated transformation events of lentil (Warkentin and McHughen, 1992) pre-culturing between the injury and bacterial incubation step was noted to be insignificant in enhancing the transient GUS expression. In our study the germination scores of different injury and pre-incubation applications ranged between 92-100 % without any significant difference, implying that none of the applications interfered with the germination (Figure 3.7). Also all of the explants exhibited GUS signals with various degrees (Figure 3.8). As far as injury by crushing was concerned in our experiments no significant difference was observed between the experimental sets. As a result of these findings were injured via crushing and maintained in hogland’s solution.

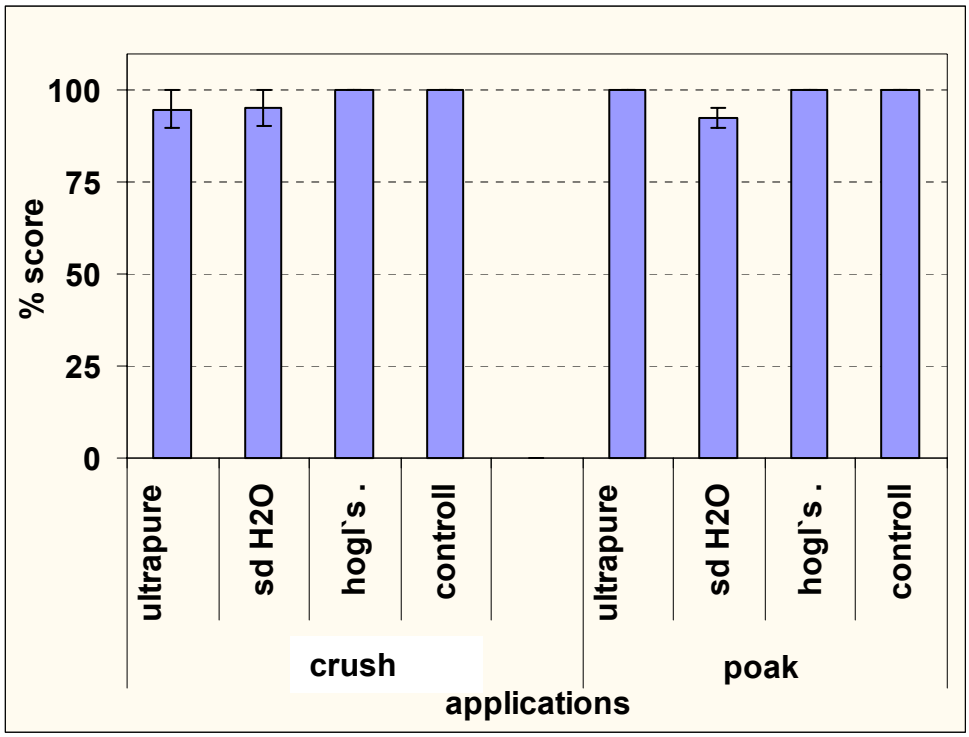

Figure 3.7. Efect of preincubation media and injury on germination

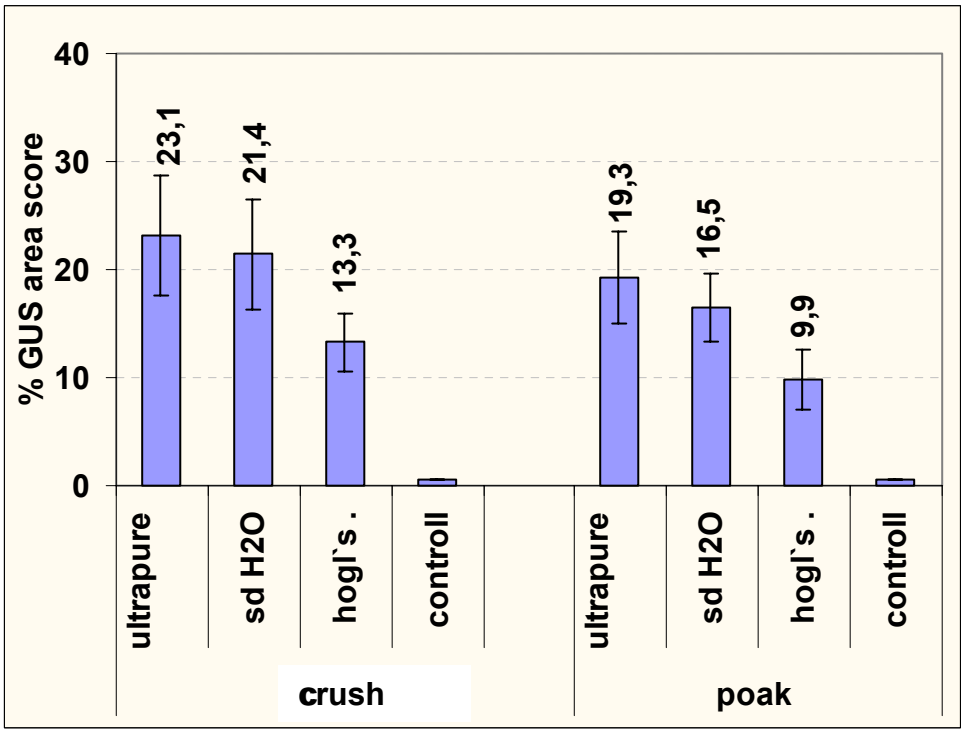

Figure 3.8. Efect of preincubation media and injury on histochemical GUS scores.

3.2.3 EFECT OF INFILTRATION

In the previous studies conducted in our lab Mahmodian, 2000 and Çelikkol, 2002 and reports concerning these studies (Mahmoudian *et al*., 2002-a) evacuation pressure of -200 mmHg was shown to be effective in *Agrobacterium* infiltration to certain explants of lentil evident by enhanced transient GUS activities. Therefore, as another parameter we have tested the effect of infiltration on transient GUS activity and germination of the embryos.

After incubation of injured embryos with *Agrobacterium* suspension the explants were subjected to vacuum at an evacuation pressure of -200mmHg for different durations. Two fold control of this treatment were done to see the combined effect of injury and infiltration. First one was infiltration of *Agrobacterium* suspension to non injured explants. The second one was infiltration of the injured explants in cell suspension media without *Agrobacterium* cells.

For all types of applications germination rates were above 90 % revealing that none of the applications were severely inhibiting the germination (Figure 3.9). Also different durations (0-60 min) of infiltration to the injured explants in *Agrobacterium* suspension did not produce significant difference on percent GUS positive explant scores with rates between 85-90 %. However non injured but infiltrated explants (see Cont-1/ Figure 3.9) had significantly lower GUS score (65 % GUS positive explant score) then the injured and infiltrated (0-60 min) explants.

Among the experimental sets (0-60 min infiltration) other then the control groups no significant difference was observed in percent average GUS area staining scores of shoot apex. Percent average GUS area score of the control-1 group (which was only *Agrobacterium* infiltrated but not injured) scored significantly lower (8.38 %) then the maximum achieving group (40 min) of infiltrated and injured explants (10.81 %) (Figure 3.8).These results imply that infiltration itself could be accepted as an injury for lentil embriyo apex. as a supportive data to our findings in most of the *in planta* transformation studies (cotton pollen, the model legume *Medicago truncatula* and Arabidopsis transformation events) no other means of explant injury were reported to be coupled to infiltration in *Agrobacterium* suspension.

Again according to our experimental results combined effect of mechanical injury and infiltration (at -200mmHg) did not produce significantly higher transient *gus* expression rates compared to only mechanical injuries as evident with the GUS scores of 0, 40 min infiltrations and cont-1 group (Figure 3.9). In the studies of Çelikol (Akçay) (2002), effect of infiltration on transient GUS activities of transformants were stated to be significantly different when different *Agrobacterium* strain plasmid combinations were used. Also in the same study for KYRT1::pTJK136 couple infiltration was noted to be decreasing the transient GUS activity of the transformed cotiledonary nodes. In our experiments with respect to germination rates and transient GUS activities, there was no significant difference between the non-infiltrated and infiltrated tissues. As a result, further transformation studies were carried out without infiltration.

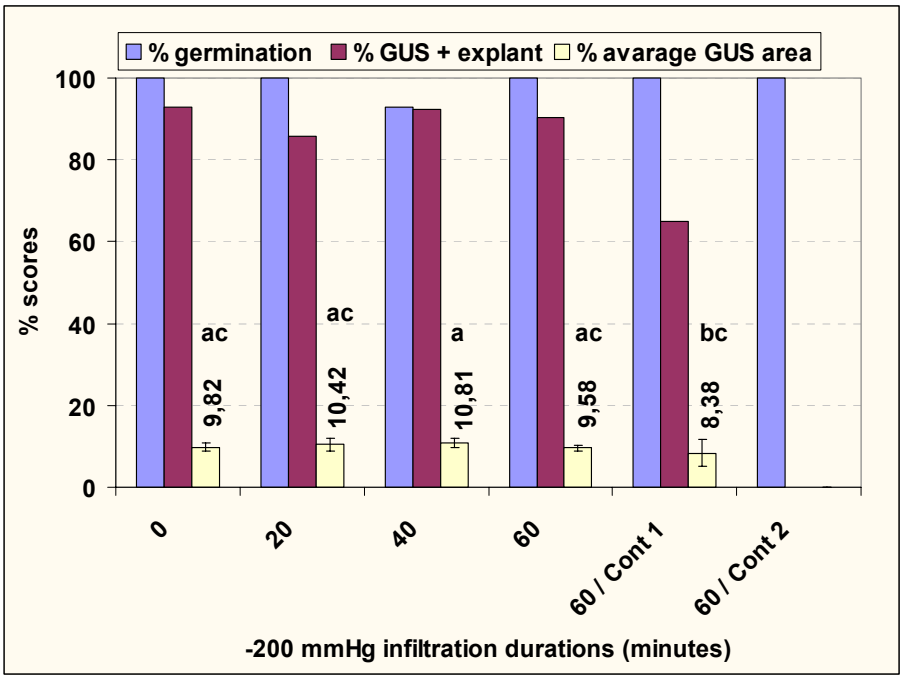

Figure 3.9. Effect of infiltration on the transient GUS scores of embryos. Cont-1 group; *Agrobacterium* infiltrated (60 min) non injured explants. Cont-2; Injured and infiltrated explants without *Agrobacterium.*
Letters a-c are for the variance between the groups, same letters denoting that the scores are not significantly different ($p<0.005$)

3.2.4 EFECT OF L-CYSTEINE

Previous studies demonstrated that, involvement of L-cysteine into the co-cultivation media, significantly increased the transformation frequencies in soybean (Olhoft and Somers, 2001; Olhoft *et al*., 2001). According to these reports the plant pathogen response upon wounding was raised through peroxidases which require metallic co-factors, like iron and cupper. Thiol compounds like L-cysteine were addressed to be ion chelators functioning in elimination of peroxidase-like activities. The effect of L-cysteine has not been tested for lentil transformation. For this reason in our transformation system we have analyzed the effect of different L-cysteine concentrations on germination and transient GUS activities.

Effect of L-cysteine on germination of the transformed embryos were scored not to be significantly different from each other and ranged between 84-95 % (Figure 3.10). The germination rate of the control group (96 %) was not different from the treatment groups. Also percent GUS positive explant number ranged between 90-100 %.

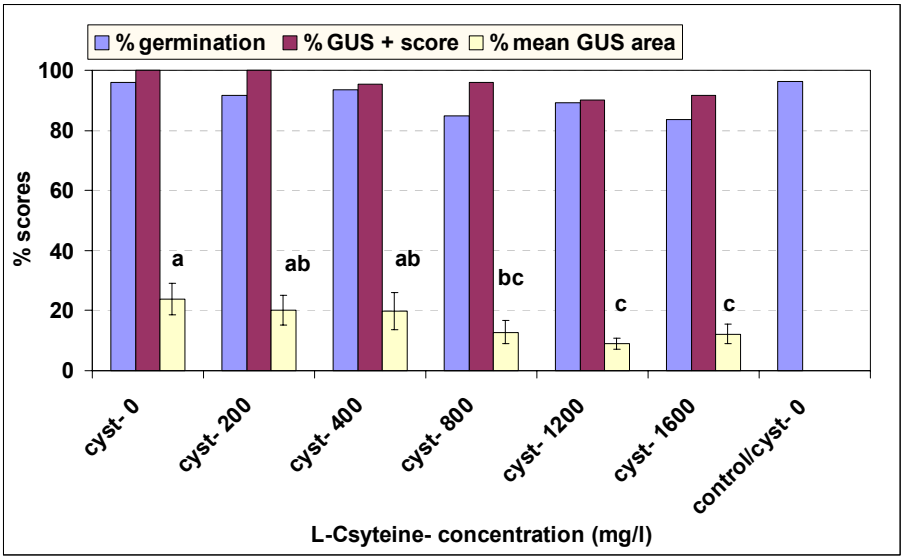

Figure 3.10. Effect of L-cysteine on GUS expression and germination.Letters a-c; for the variance between the groups, same letters denoting means are in the same 95% CI (one way ANOVA)

The highest percent GUS area score was 23.9 % which was observed in the absence of L-cysteine. Percent GUS area score of L-cysteine control was found to be significantly higher then 800, 1200 and 1600 mg L-cysteine treatments, but no significant difference was observed between 0, 200 and 400 mg/l L-cysteine treatment groups.

Although germination rates were not below 80 %, co-cultivation of *Agrobacterium* incubated explants in L-cysteine containing media resulted in severe inhibition of growth (Figure 3.11) evident by the stunned germination of the embryos. So it was presumed that the relative average growth weights of the shoots elongated could reveal such differences. However the growth rates in terms of weight did not perfectly correlate the growth patterns with the L-cysteine content (0-1600mg/lt) in treatment groups. That is, growth rate of the no L-cysteine treatment was not much lower then that of 200/ 400/ 800 mg/l L-cysteine groups (Figure 3.11). The control group on the other hand scored a growth mass of 42 mg/explant which was two times higher then 200 mg/l L-cysteine treatment. The comparison of the treatment and control groups revealed that either L-cysteine or the *Agrobacterium* culture used or the combined effect of both factors depressed the growth rates of the transformed lentil embryos. But, since L-cysteine 0 and control groups which had only *Agrobacterium* as variable factor resulted in highly different growth rates the current growth phenotype should be mainly correlated with the *Agrobacterium* itself. This is also evident by the combined effects of *Agrobacterium* and L-cysteine on growth masses of the treated embryos which were not significantly different from each other but lower then control group.

According to the reports of Olhoft and Somers (2001) combinations of thiol compounds or L-cysteine itself enhanced the rate of transformation in soybean. They were addressing both the increase in percentage of GUS positive explants and number of GUS foci during co-cultivation with thiol compounds.

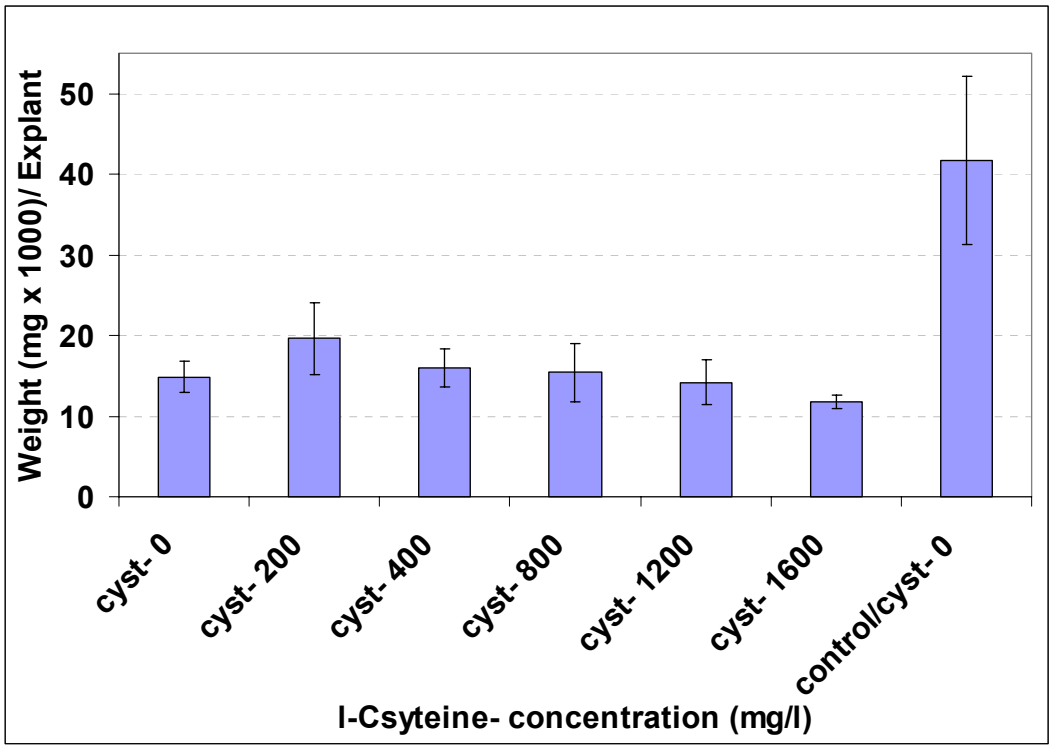

Figure 3.11. Effect of L-Cysteine on the growth masses of transformed explants. Following the *Agrobacterium* incubation of the injured explants three days of co-cultivation media was reproduced with different L-cysteine concentrations. Combined effect of *Agrobacterium* and L-cysteine resulted in growth retardation without affecting the germination rates

But, as it is depicted with the above results it was not the same case with our experimental set up. This could be due to the combined effect of *Agrobacterium* infection and L-cysteine on lentil tissues.

Further experimentation and L-cysteine concentration optimization for lentil is necessary to clarify this observation. With the current experimental setup, according to the results (Figure 3.10) L-cysteine treatment was found to be non-significant in enhancing the GUS expression rates of the transformed lentil embryos in our experimental setup. For this reason L-cysteine was not used in further experiments. Representative data are given in figure 3.12.

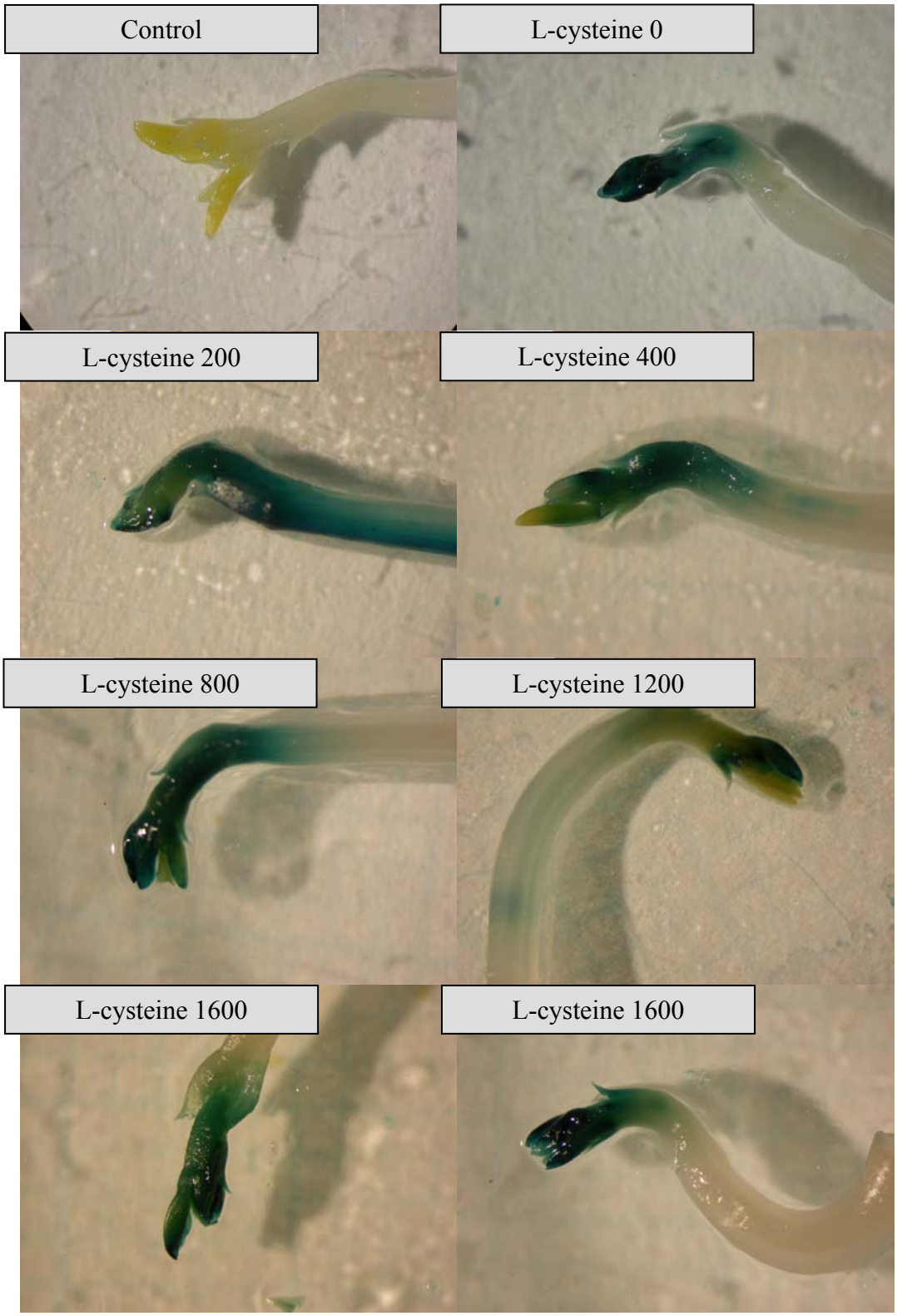

Figure 3.12. Representative data for GUS histochemical stainings of the explants subjected to different L-Cysteine concentrations during co-cultivation

3.2.5 EFFECT OF AGROBACTERIUM INCUBATION TIME

Although the bacterial incubation time was presumed to be 90 minutes depending on the initial trial and error studies, this parameter was also need to be optimized. For this purpose *Agrobacterium* incubation durations of 30, 60, 90, 120, 240 minutes were tested against transient GUS expression levels of transformants. Incubation of the explants with *Agrobacterium* cells for different durations (30-240 min) did not affect the germination of the explants (Figure 3.11). For all of the treatment groups the explants exhibited more then 90 % of germination and all explants were GUS positive (100 %). Up to 90 min incubation time, explants exhibited 8-10 % mean GUS area (Figure 3.12). Longer incubation periods enhanced the transient GUS activity as much as five fold.

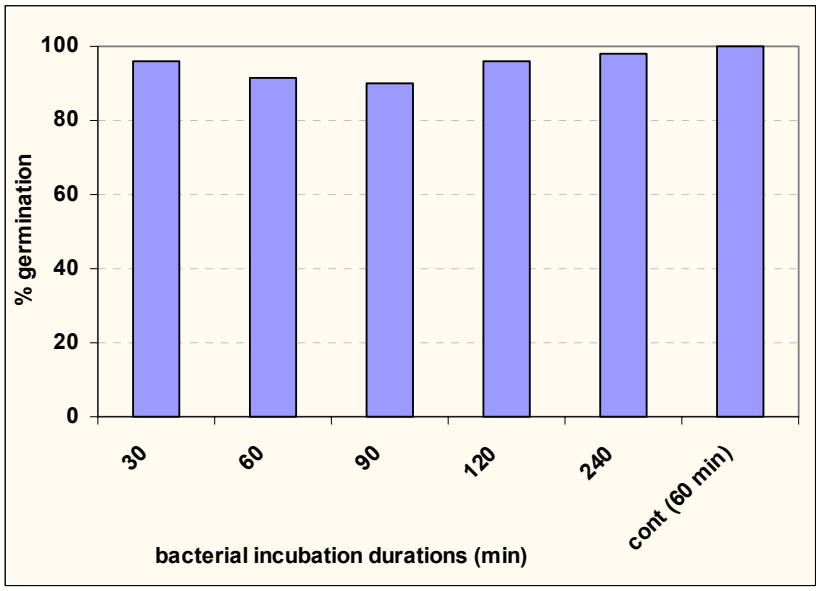

Figure 3.11. Effect of *Agrobacterium* incubation time on germination.

In literature various *Agrobacterium* incubation procedures along with durations are present. For example in sunflower transformation micro wounded immature embryos were inoculated with *Agrobacterium* suspension and directly used in co-cultivation without and intermediary step of incubation time (Lucas *et al*., 2000).

In another study (Pickardt *et al*., 1995) *Vicia narbonensis* explants were incubated in *Agrobacterium* suspension for two days at 120 rpm. Similar procedure was used in transformation study of chickpea by Kar *et al*., (1996). For the *Agrobacterium* incubation time studies in lentil we may refer two representative reports. In the study of Mahmoudian *et al*., (2002-b) cotiledonary nodal segments of lentil seedlings were incubated in *Agrobacterium* suspension for 20 and 30 minutes during evacuation only.

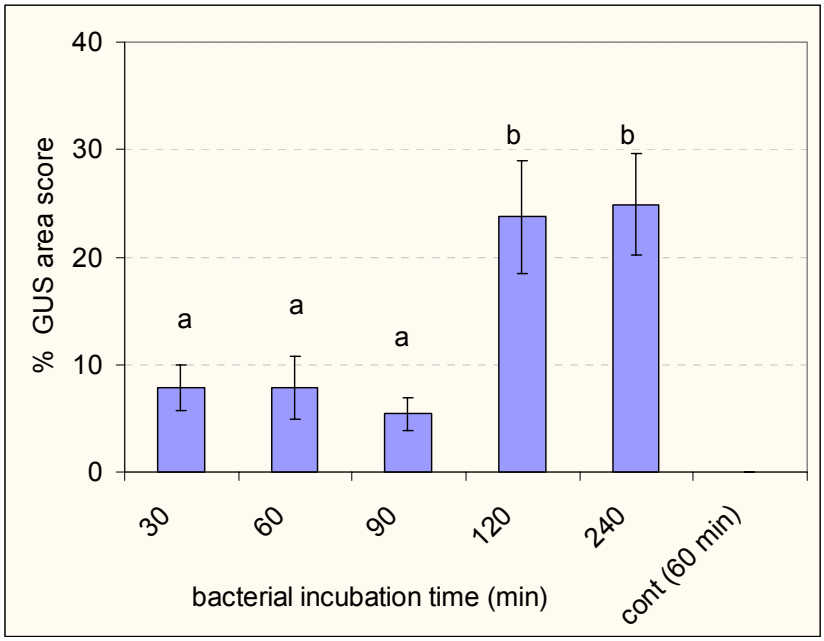

Figure 3.12. Effect of *Agrobacterium* incubation time on transient GUS activity. Different letters are denoting significant difference ($p<0.05$) between the experimental sets.

In another study (Warkentin and McHuhen, 1992) various explants from germinated lentil seedlings were incubated in overnight grown *Agrobacterium* culture for only 10-15 minutes.

In this report co-culture duration was linearly correlated with the percent number of GUS positive explants and also it was stated that coculture duration did not significantly increase the GUS staining area.

When we compare our results with that of Warkentin and McHughens` we can state that our results are not in parallel with their claims. First our incubation duration study exceeded far beyond 15 minutes (240 min) with minimum 30 minutes incubation time. Second the percent GUS positive score for all of our treatment groups were 100 %. Third a non linear increase in GUS area staining was observed with approximately 5 fold increase after 90 minutes of incubation in our experiments.

In all these studies depending on the plant genotype and bacterial strain couple and depending on any tissue culture factor, bacterial incubation time may change considerably. According to our results, since no significant increase in percent GUS area score was present between 120 and 240 minutes *Agrobacterium* incubation time of the injured explants was set as 120 min. Representative is given in figure 3.13.

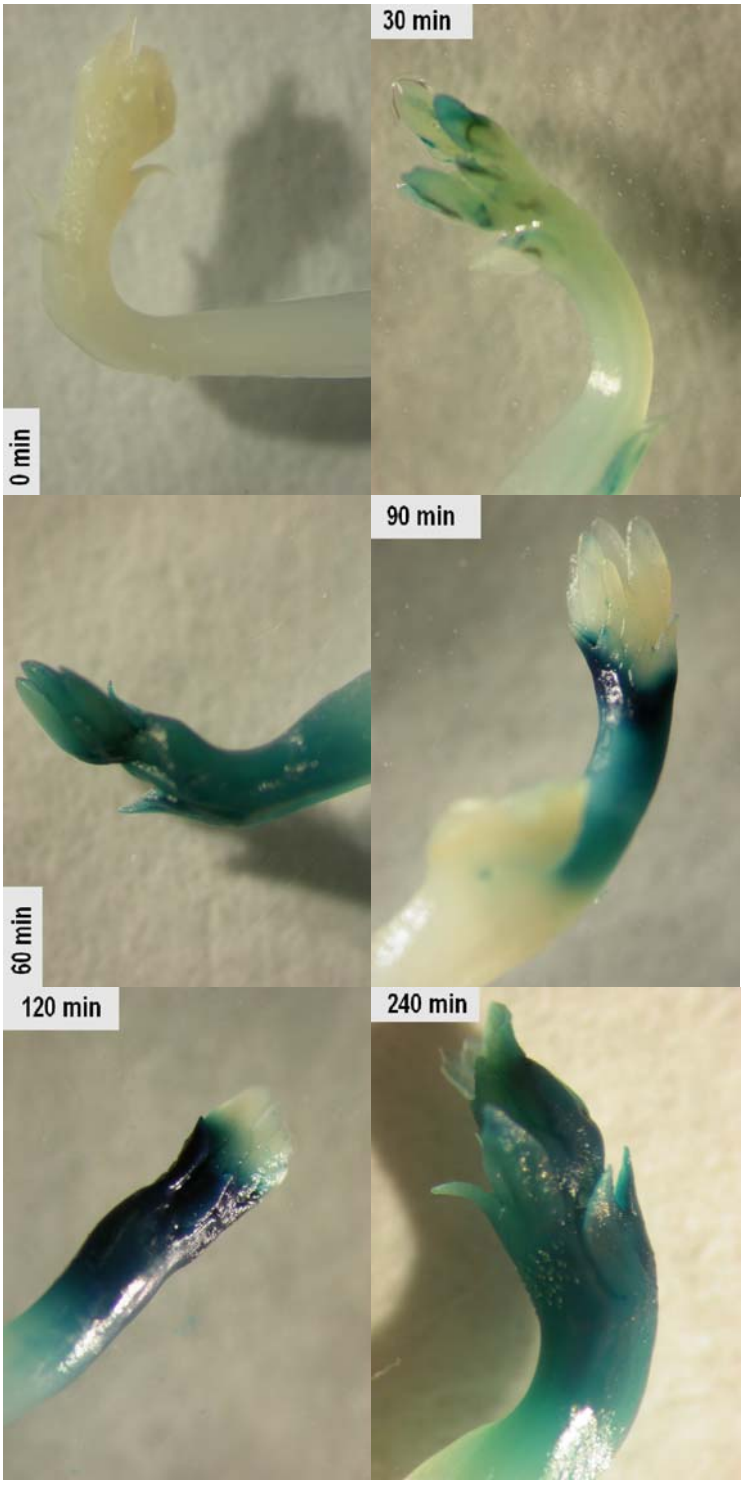

Figure 3.13. Representative data for GUS histochemical stainings of the explants subjected to different *Agrobacaterium* incubation

3.2.6 PROLONGED TRANSIENT GUS EXPRESSSION ANALYSIS

According to the optimization studies given in the preceding sections the following parameters were found to be the optimum: Explants are injured through two times of successive crushing to the embriyo apex. The injured explants are incubated in full strength hogland's solution for one hour. Incubation of the explants with *Agrobacterium* was carried for 120 minutes. No infiltration was carried after the incubation step and during co-cultivation, no L-cysteine was used. Based on these optimized parameters new transformation experiments were carried out to evaluate the stable histochemical GUS stainings beyond 3 days (2 -3 weeks).

In the study of Lurguin et al., (1998) it was stated that GUS expressing sectors were decreasing from 1 week to 2-4 weeks after co-cultivation. That is GUS expressing sectors decrease as plant growth continues. In our study further transient GUS expression analysis were carried out at 14 and 21 days after transformation in order to evaluate the faith of *gus* gene expression histochemically. Since growth patterns of individual plants were different, instead of length, amount of growth was stated in terms of the number of nodes raised. The average growth that was scored as 5 and 7.5 nodes for the 14 and 21 days old transformants, respectively. During presentation of histochemical GUS analysis data on the parts of transformants starting from the cotyledon to the apical meristem nodal segments was numbered as 1-10 (see Figure 3.14). Every GUS spot that occurs in each segment (stem, leaves and buds) was assigned with its node number. GUS scores were given as percentage of GUS positive explants, the percentage of nodes (node number 1, 2, 3 etc.) that expresses GUS and average number of GUS spots per explant. Also GUS scores for leaves stem segments and buds were given.

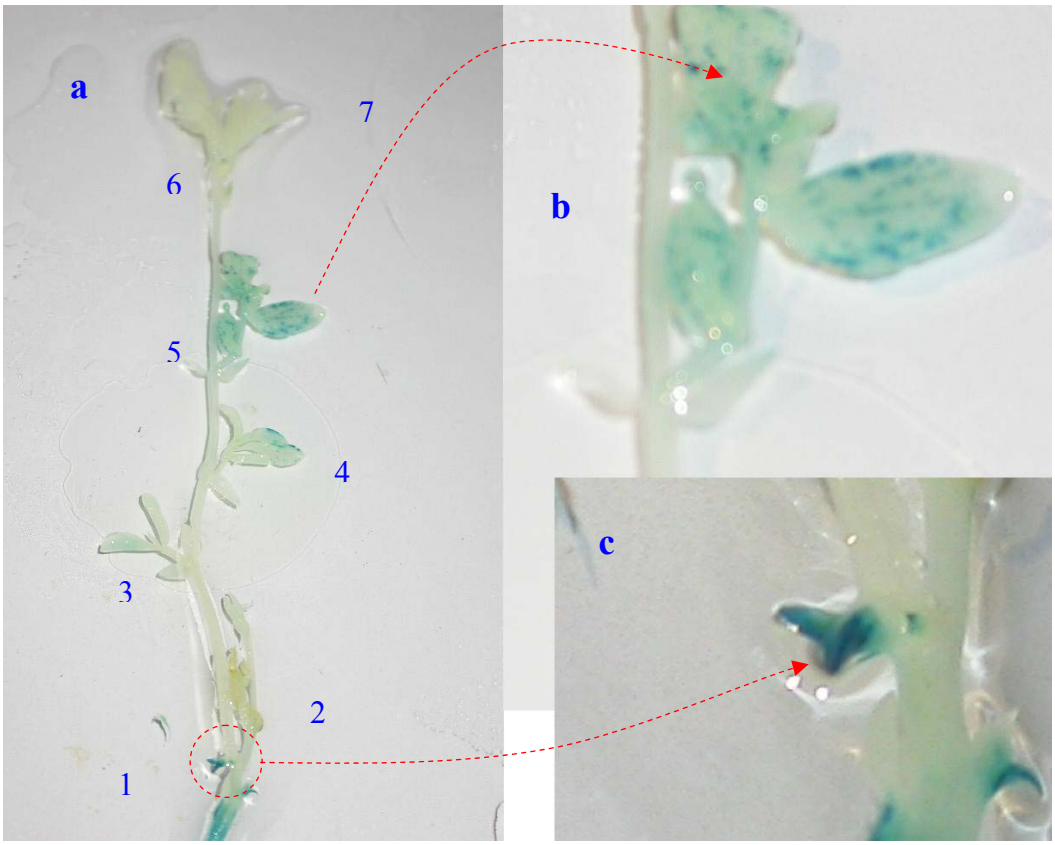

Figure 3.14. GUS histochemical staining of 21 days old transformant. Numbers are indicating the nodes

The highest GUS score (number of GUS spots) was observed on leaves followed by stems and then buds (see Figure 3.15). As expected percent GUS positive scores were declining from 14 to 21 days old stable transformants. Also with elongation and growth (as the number of nodes increases) the GUS expressing nodal segments decreased. Starting from the oldest node (number 1) to the newest (number 6) percent GUS expressing node decreased from 90 % to 8 % (see Figure 3.16).

According to these results the apex tissues of the transformed embryos are hardly transmitting the transgene to the younger segments of the developing transformed plant (see figure 3.15). The possible causes may be as follows.

The crushing type of injury application may not be injuring adequate meristematic layers that give rise to the younger organs, or the crushing type of injury might be so intense that result in dead of the injured regions and growth may be handled by the non-injured and hence, non-transformed meristematic zones.

Also in our study no means of selection was present to depress the growth of non transformed cells at the meristematic regions. In one parallel study, for *Agrobacterium* mediated transformation of the shoot apex of the grain legume *Lupinus angustifolius* (Pigeaire *et al*., 1997) cotyledons along with the primary leaves were stripped of from the embryo during explant preparation.

Following these treatments the injured and transformed apex region was subjected to selection with application of a drop of selection agent. Subsequently culturing of the embryo in a hormone containing media resulted in production of new shoots that were subcultured within the media containing selective agent. It could be possible to use a selection method similar to that of Pigeaire *et al*., in our transformation method.

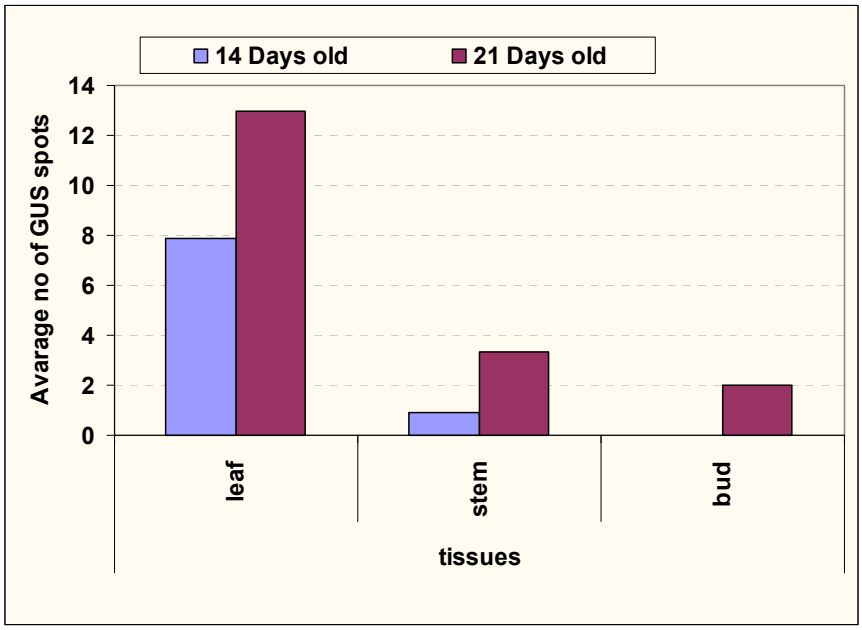

Figure 3.15. GUS histochemical stainings at different tisues of transformed plantlets

Figure 3.15. GUS histochemical staining of 21 days old transformant.

CHAPTER-IV
CONCLUSION

4.1. MICROGRAFTING OPTIMISATION

During micro-grafting optimization study as much as four parameters were tested. These were the root stock stem height, root stock nature, type of root stock preparation and sealing factor for the union region.

After commitment of *in vivo* type of micro-grafting due to the sterility and handling problems of the *in vitro* method the first parameter studied was the root stock stem height. Half or the full stem length effect was not differentiated prominently at the initial stages of micro-grafting but half stem length gave promising results for the next steps of the grafting although this parameter requires re-evaluation.

The root stock nature was only tested with lentil and chickpea. The prominent effectiveness of the chickpea root stock for supporting the growth and development of the lentil scion was at least two fold greater then the lentil root stock. But effectiveness of chickpea in supporting the growth of lentil scion did not continue after the step of hardening. Further diagnosis may be required to exploit the potential of chickpea root stock. However, lentil root stock type was used in the next steps of the study.

The type of root stock preparation and sealing factor was the most effectively studied parameters. In order to maintain a fast recovery and effective connection between the uniting graft counterparts' as much as possible vascular tissue of the scion and stock must be met during grafting. Both of the graft types studied (namely Z-V and M-V) were actually adaptations from the classical grafting methods routinely used in agriculture. Among them M type of the stock preparation was shown to be effective then the Z type at the establishment step. But on the whole Z type stock preparation was found to be more effective then the M type which is routinely observed in the regeneration studies reported.

The sealing factor on the other hand is not frequently used *in vitro* in regeneration with micro-grafting due to its potential of contamination. But it has been successfully used in our experiments and shown to have positive effect on establishment and hardening stages of micro-grafting.

As a result it was concluded that for successful micro-grafting and regeneration of the lentil scions 7 days old lentil seedlings with Z type of stock preparation on to the half stem length of the first internode (above the epicotyl) should be met with V type preparation of the scion under *in vivo* conditions. Sealing is important at the initial 3 days of establishment but can be avoided if grafts are maintained carefully at this stage.

4.2. TRANSFORMATION OPTIMISATION

During transformation optimization study effect of explant injury, pre-incubation media, and effect of infiltration, L-cysteine and *Agrobacterium* incubation time were analyzed.

For optimization of injury the most relevant, easy and least complicated method was evaluated between the poking, crushing and sonication type of injury methods. The crushing type of injury was concluded to be best with respect to effectiveness and ease of application.

The combined effect of injury and pre-incubation media on transient GUS activities was analyzed as the next parameter. Crushing and poking type of injuries were coupled to 3 types of pre-incubation media (ultra pure or Sterile distilled water and hoglands` solution) used for prevention of explants from dehydration. No significant enhancement was observed for the different types of pre-incubation media when crushing type of injury was concerned. Since co-cultivation was carried with hoglands` solution also, the pre-incubation media was selected to be hoglands` solution.

The effect of infiltration tested at -200 mmHg for different duration was found not to be significantly enhancing the transformation rates.

L-cysteine was noted to be enhancing the transformation rate in soybean significantly. This thiol compound did not produce the same effect in our experimental setup. L-cysteine effect on transformation efficiency must be studied further in detail.

The effect of *Agrobacterium* incubation time was tested for the range 0 to 240 minutes. It was observed that longer incubation time (120, 240 min) significantly enhance the transient expression level.

Further experimentation is necessary to demonstrate the applicability of this transformation technique in development of transgenic lentil plants.

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APPENDICES

APPENIDIX A

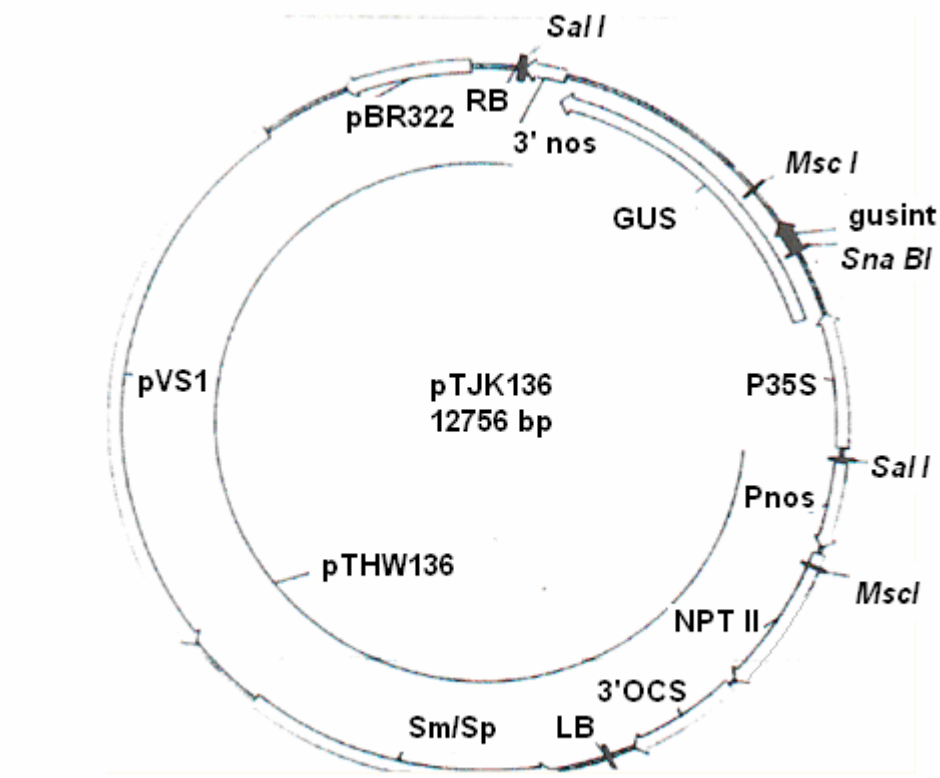
SELECTION MARKERS FOR KYRT1::Ptjk136

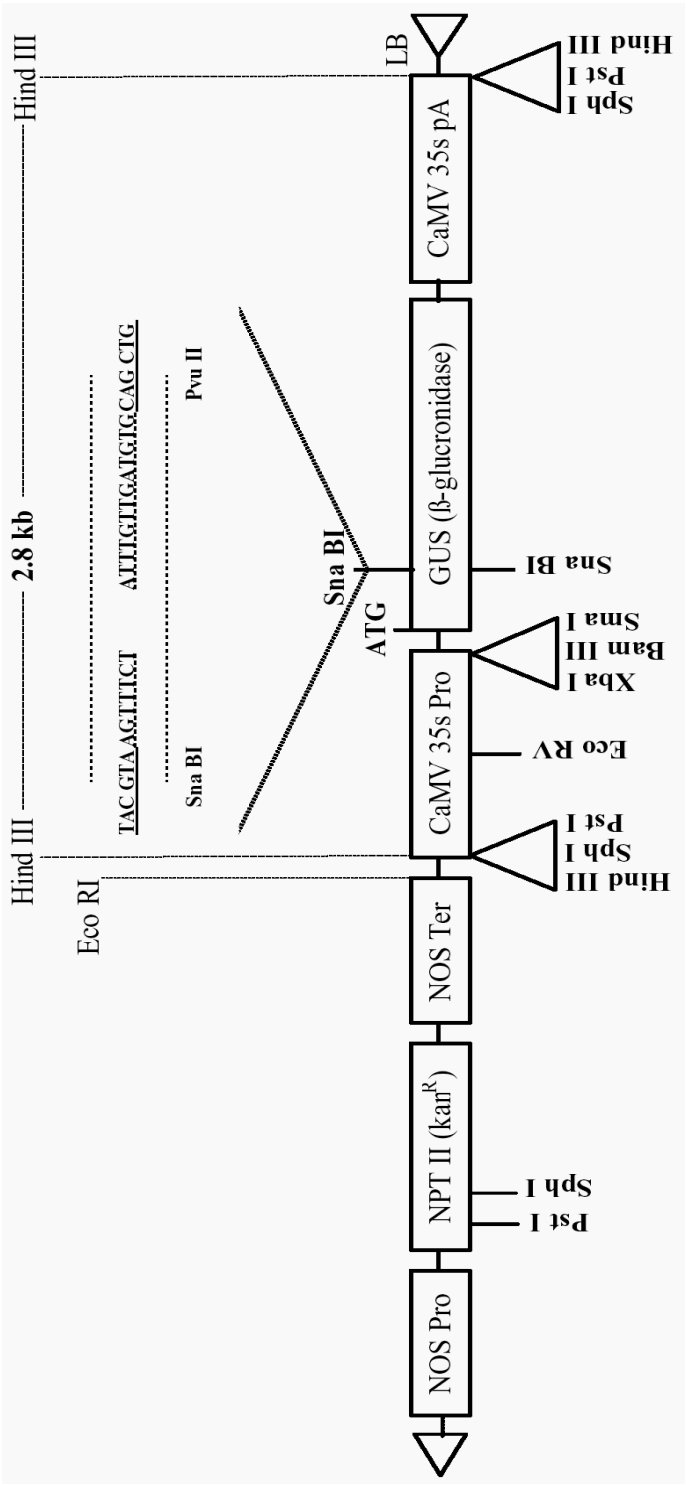
KYRT1: Rif ^{R} (100mg/l), Carb ^{R} (100mg/l), Gent ^{R} (40mg/l)

pTJK136: Bacterial selection markers; Strep ^{R} (300mg/l), Spect ^{R} (125mg/l)

Plant selection markers; Kan ^{R} (*npt II* gene), *uid-A* gene

T-DNA REGION of pGUSINT AND WHOLE pTJK136 PLASMID




Hind III
2.8 kb
Hind III
Eco RI
TAC GTAAGTTTCT
ATTTGTTGATGTGCAG CTG
Sna BI
Pvu II
Sna BI
ATG
LB
NOS Pro
NPT II (kan^R)
NOS Ter
CaMV 35s Pro
GUS (ß-glucronidase)
CaMV 35s pA
Pst I
Sph I
Hind III
Sph I
Pst I
Eco RV
Xba I
Bam III
Sma I
Sna BI
Sph I
Pst I
Hind III

APPENIDIX B

PERMISSION LETTERS FOR pGUSINT AND pTJK136 PLASMIDS

Institut
für Genbiologische
Forschung
Berlin GmbH

Institute für Genbiologische Froschung Berlin GmbH
Ihnestrabe 63 14195 Berlin GmbH

Telefax (030) 83 00 07 – 36
Telefon (030) 83 00 07 – 0
Durchwahi (030) 83 00 07 – 60

Bearbeiler Wi/kr
Berlin. den 24. 08. 1994

Dr. Hüseyin Avni Oktem
Dept. of Biology
Middle East Technical University
061531 Ankara
Turkei

Dear Dr. Öktem,

with respect to your recent letter we send you enclosed the 35S-GUS-INT gene with following restriction sites in the surroundings:

HindIII- sphI - PstI - HincII - 35S promoter - Xbal - BamHI - Smal - GUS- gene -

SstI - KpnI - 35S-3'-end - sphl - PstI - HindIII.

Good luck!

Yours sincerely,

Prof. Dr. L. Willmitzcr

Handetsregister AG Berlin Charlottennburg 96 HRB 21001-USI ID. Nr DE 136629511
Vorsitzender des Kuralenoms Prof. Dr. Gunter Stock Geshaltsfuhrer Prof. Dr. Alex Brennicke and Prof. Dr. Lathor Willrpitzer
Bank verbindong Commerzbank AG Berlin, Konto-Nr 108 703 000 Bankleitzaht 100 400. 00

Laboratorium Genetica
Vakgroep Moleculaire Genetica
K. L. Ledeganckstraat 35
B-9000 Gent, BELGIE 1
Tel. 32(0)9-2645170/71
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

APPENIDIX C

BACTERIAL AND PLANT CULTURE MEDIA

YEB Medium

13.5 g/L Nutrient Broth

1 g/L Yeast Extract

5 g/L Sucrose

2 mM $MgSO_4$. $7H_2O$

10 mMMES

20 mM Acetocyringone

pH 5. 6

MMA Medium

4. 3g/L MS Basal Salts (Sigma M5524)

20g/L Sucrose

l0mMMES

20 uM Acetocyringone

pH5. 6

HOGLANDS SOLUTION

Composition	stock solution	use ml/l
MgSo4.7H2O	24.6 g/100ml	1.0 ml
Ca(NO3)2 4H2O	23.6 g/100ml	2.3 ml
KH2PO4	13.6 g/100ml	0.5 ml
KNO3	10.1 g/100ml	2.5 ml
Micronutrients	see solution below	0.5 ml
Fe EDTA	see solution below (added last)	20 ml

Micro nutrient solution

Addition	Stock
H3BO3	2.86 g/l
MnCl2 4H2O	1.82 g/l
ZnSO4 7H2O	0.22 g/l
Na2MoO4 2H2O	0.09 g/l
CuSO4 5H2O	0.09 g/l

Fe EDTA Solution

Addition	Stock
FeCl3 6H2O	0.121 g/ 250 ml
EDTA	0.375 g/ 250ml

APPENDIX D

GUS HISTOCHEMICAL ASSAY

GUS Substrate Solution

0. 1 M $NaPO_4$ Buffer, pH 7. 0

l0mMEDTA

10. SmM K-ferricyanide, pH 7. 0

0.5 mM K-ferrocyanide, pH 7. 0

1 mM X-Glucoronide (dissolved in dimethylormamide)

10 % v/v Triton X-100

GUS Fixative Solution

10 % v/v Formaldehyde

20 %v/vEthanol

5 % v/v Acetic Acid