OPTIMIZATION OF SELECTION CONDITIONS AND *AGROBACTERIUM* MEDIATED TRANSFORMATION OF CHICKPEA (*Cicer arietinum* L. cv. Gökçe)

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

OPTIMIZATION OF SELECTION CONDITIONS AND *AGROBACTERIUM* MEDIATED TRANSFORMATION OF CHICKPEA (*Cicer arietinum* L. cv. Gökçe)

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The objective of this study was to optimize an efficient selection system and *Agrobacterium* mediated transformation of chickpea (*Cicer arietinum* L.).

Cotyledonary node explants of Turkish chickpea cultivar Gökçe were used to determine the effects of selective agents, two antibiotics (Kanamycin, Hygromycin) and two herbicides (PPT, Glyphosate) as well as four antibiotics (Augmentin, Carbenicillin, Cefotaxime, Timentin) for eliminating *Agrobacterium* on multiple shoot and root induction. Selective agents and antibiotics were applied to explants at different concentrations for one month and numbers of regenerated shoots and roots were recorded. Kanamycin at 100 mg/L, Hygromycin at 20

mg/L, PPT at 3 mg/L and Glyphosate at 5 mg/L were found to be appropriate to select chickpea transformants. Lowest concentrations of all selective agents (50 mg/L Kanamycin, 10 mg/L Hygromycin, 3 mg/L PPT, 1 mg/L Glyphosate) totally inhibited rooting of the regenerated shoots.

Among the *Agrobacterium*-eliminating antibiotics, Cefotaxime and Augmentin each up to 600 mg/L had no adverse effect on shoot induction, whereas Timentin (300 mg/L) significantly increased and Carbenicillin (300 mg/L) significantly decreased shoot induction after four weeks of culture. Augmentin was determined to have no effect on rooting capacities of chickpea shoots. However Cefotaxime at all concentrations significantly decreased root induction. On the other hand only high concentrations of Carbenicillin (300 mg/L) and Timentin (200 mg/L) significantly decreased rooting. Sulbactam in combination with Carbenicillin and Cefotaxime displayed effective inhibition of bacterial growth.

Furthermore, *Agrobacterium* mediated transformation procedure for cotyledonary node explants of Gökçe, was also optimized by monitoring transient *uidA* expression on 4^{th}, 9^{th}, and 16^{th} days after transformation. Transformation procedure was improved via mechanical injury of axillary region of explants and application of vacuum infiltration at 200 mmHg for 40 minutes.

Keywords: Chickpea cotyledonary node; Transgenic selection; *Agrobacterium tumefaciens*; GUS; Transient gene expression.

ÖZ

NOHUTTA (*Cicer arietinum* L. cv. Gökçe) SEÇME KOŞULLARI VE AGROBAKTERİYE DAYALI TRANSFORMASYONUN OPTİMİZASYONU

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Bu çalışmanın amacı, nohutta (*Cicer arietinum* L.) verimli bir seçme sistemi ve Agrobakteriye dayalı transformasyonun optimize edilmesiydi.

Türk nohut çeşidi Gökçe'ye ait kotiledon boğumları, seçici ajanların; iki antibiyotik (Kanamisin, Higromisin) ve iki herbisit (PPT, Glifosat) ile Agrobakteri eliminasyonunda kullanılan dört antibiyotiğin (Augmentin, Karbenisilin, Sefotaksim, Timentin) çoklu sürgün ve kök oluşumu üstüne etkilerinin araştırılmasında kullanılmıştır. Seçici ajanlar ve antibiyotikler eksplantlara farklı konsantrasyonlarda bir ay süre ile uygulanmış ve rejenere edilen sürgün ve kök sayısı kaydedilmiştir. 100 mg/L Kanamisin, 20 mg/L

Higromisin, 3 mg/L PPT ve 5 mg/L Glifosat nohut transformantlarının seçiminde kullanılmaya uygun bulunmuşlardır. Tüm seçici ajanlar kullanılan en düşük konsantrasyonlarında (50 mg/L Kanamisin, 10 mg/L Higromisin, 3 mg/L PPT, 1 mg/L Glifosat) rejenere edilmiş gövdelerin köklenmesini tamamen durdurmuştur.

Agrobakteri-eliminasyon antibiyotikleri arasında Sefotaksim ve Augmentin sürgün oluşumu üstüne herhangi kötü bir etki göstermemiş; bunun yanı sıra dört haftalık kültür sonunda Timentin (300 mg/L) gövde oluşumunu arttırmış, Karbenisillin (300 mg/L) ise düşürmüştür. Augmentin'in nohut sürgünlerinin köklenme kapasitesi üstüne herhangi bir etkisinin olmadığı bulunmuştur. Ancak Sefotaksim kullanılan tüm konsantrasyonlarda kök oluşumunu anlamlı bir şekilde düşürmüştür. Diğer taraftan sadece yüksek konsantrasyonlarında Karbenisilin (300 mg/L) ve Timentin (200 mg/L) köklenmeyi anlamlı bir şekilde düşürmüştür. Sulbaktam'ın Karbenisilin ve Sefotaksim ile kombinasyonları bakteri büyümesi üstünde etkili engellemeler göstermiştir.

Ayrıca Gökçe kotiledon boğumları için Agrobakteriye dayalı transformasyon prosedürü, transformasyonu takip eden 4, 9 ve 16ncı günlerde *uidA* geni geçici ifadesi izlenerek optimize edilmiştir. Transformasyon prosedürü, eksplantların aksiller bölgesinin mekanik olarak yaralanmasıyla ve vakum infiltrasyonun 200 mmHg basınçta 40 dakika uygulanmasıyla geliştirilmiştir.

Anahtar kelimeler: Nohut kotiledon boğumu; Transgenik seçimi; *Agrobacterium tumefaciens*; GUS; Geçici gen ifadesi

To the spirit of my beloved grandmother

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A	Augmentin
Acetosyringone	3',5'-Dimethoxy-4-hydroxyacetophenone
ANOVA	Analysis of Variance
Augmentin	Amoxicillin + Clavulanate
BA	Benzylaminopurine
C	Carbenicillin
Cf	Cefotaxime
CN	Cotyledonary Node
cv	Cultivated Variety
EDTA	Ethylenediamine Tetra Acetic Acid
G	Glyphosate
GUS	β-D-glucuronidase
H	Hygromycin
IBA	Indole Butyric Acid
K	Kanamycin
MES	2-[N-Morpholino] Ethanesulfonic Acid
MS	Murashige and Skoog Basal Medium
nptII	Gene coding for Neomycin Phospho Transferase II
NPT-II	Neomycin Phospho Transferase II
OD	Optical Density
P	PPT (Phosphinotricin)
PCR	Polymerase Chain Reaction
PPT	Phosphinotricin
RAPD	Randomly Amplified Polymorphic DNA

rpm	Revolution per Minute
S	Sulbactam
SEM	Standard Error of Mean
T	Timentin
T-DNA	Transferred DNA
Timentin	Ticarcillin + Clavulanate
uidA (gusA)	Gene coding for β-D-glucuronidase
vir	Virulence genes
X-GlcA	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic Acid
YEB	Yeast Extract Broth

CHAPTER I

INTRODUCTION

1.1. Chickpea

The genus *Cicer*, belonging to the family Leguminosae, comprises 43 species, nine of which are annuals including the cultivated chickpea (*Cicer arietinum* L.), 33 perennials and one unspecified (van der Maesen, 1987; Ahmad, 1999). The cultivated species of chickpea is a cool season annual grain legume believed to have originated and cultivated in south eastern Turkey and adjoining parts of Syria (Singh, 1997). It is grown and distributed from the Mediterranean region to India, Australia, Ethiopia, Mexico, Chile, the cooler parts of the tropics and North America (Robertson *et al.*, 1997).

Chickpea, which is consumed as a dry pulse crop, provides high quality protein. It is also used as feed for livestock and has a significant role in farming as a substitute for fallow in cereal rotations since it can fix atmospheric nitrogen (Singh, 1997). In New Crop Fact Sheet of Purdue University, it is cited that Duke (1981) notes the medical application of acid exudates from leaves of chickpea and also notes that chickpea yields 21 % starch suitable for textile sizing, giving a light finish to silk, wool and cotton cloth (Muehlbauer and Tullu, 1997). All these characteristics make chickpea an important crop both for producers and consumers.

1.1.1. Historical and Taxonomic Perspectives

Chickpea was one of the first grain legumes to be domesticated in the old world. It most probably originated in an area of south eastern Turkey and adjoining parts of Syria (Singh, 1997; Lev-Yadun *et al.*, 2000). Based on seed protein electrophoresis, Ladizinsky and Adler (1976) considered *C. reticulatum* as the wild progenitor of cultivated chickpea, and south eastern Turkey as the center of origin for the crop. Analyses of interspecific hybridization, karyotype, isozyme patterns and randomly amplified polymorphic DNA (RAPD) indicate close relationships between *C. arietinum* and *C. reticulatum* (Ocampo *et al.*, 1992; Singh and Ocampo, 1993; Labdi *et al.*, 1996; Ahmad, 1999; Ahmad, 2000).

C. arietinum L. belongs to the division Anthophyta, subdivision Dicotyledonea, order Rosales, family Leguminosae, subfamily Papilionoideae and monogeneric tribe *Cicereae* Alef. The genus includes 43 species, 9 of which are annuals and chromosome number of annual species is 2n = 16 (van der Maesen, 1987; Ahmad, 1999). Crossability and fertility of hybrids in interspecific crosses have been used as a basis to classify the annuals into 4 crossability groups. Both *C. arietinum* L. and *C. reticulatum* are included in the same crossability group.

Chickpeas are classified as either *desi* or *kabuli* types. These major cultivar types have emerged under domestication. The *desi* types are characterized by smaller, angular, and dark pigmented seeds; whereas the *kabuli* types are characterized by larger seeds that are more rounded and cream or yellow in color. *Kabuli* types are considered relatively more advanced because of their larger seed size and reduced pigmentation achieved through conscious selection.

1.1.2. General Botany and Growth Habits

Chickpea is typically a branched, short, annual herbaceous plant. It has hypogeal seedlings. Plant height ranges between 20 to 100 cm and may exceed 130 cm

under favorable conditions. The foliage is covered with glandular hairs which secrete highly acidic exudates, and is considered important in conferring tolerance to some insect pests. Leaves are unipinnate compound, arranged in an alternate phyllotaxy, and born singly at each node (Singh, 1997). Each leaf consists of 5 to 6 pairs of leaflets and a top leaflet (rachis ending in a leaflet); on a rachis with a small petiole (Figure 1.1).

The chickpea plant has a deep tap root system with a primary long root producing lateral roots. Root system is robust, up to 2 m deep, but major portion up to 60 cm. Association of root hairs of chickpea and a soil bacterium, *Rhizobium*, results in the formation of root nodules which are common to other legumes. With the aid of nodules chickpea is efficient in fixing atmospheric nitrogen in a plant-usable form through biological nitrogen fixation. The crop is highly efficient in uptake of phosphorus from soils containing low amounts of available phosphorus.

Flowers are usually solitary, sometimes 2 per inflorescence, and born in axillary racemes; on a peduncle and pedicel. The corolla may be white, pink, purplish (fading to blue), or blue in color. The ovary is sessile, inflated and pubescent (Cubero, 1987; van der Maesen, 1987). Chickpea is a self-pollinated crop.

Figure 1.1. Chickpea. (A) Organs of chickpea plant. (B) Chickpea seeds.

Seed color can be cream or yellow in *kabuli* type and brown, black or green in *desi* type. Seed shape ranges from rounded to angular. Seed coat is smooth or wrinkled, or tuberculate. Seed is laterally compressed with a median groove around two-thirds of the seed, and anterior is characteristically beaked (Figure 1.1). The weight of 100 seeds varies from less than 8 g to more than 70 g (Singh, 1997).

Chickpea is usually grown as a rain fed, cool weather crop or as a dry climate crop in semi-arid regions. Optimum conditions include 18-26°C day and 21-29°C night temperatures and annual rainfall of 600-1000 mm (Smithson *et al.*, 1985). Chickpea thrives on a sunny site in a cool, dry climate on well-drained soils and grows on a residual moisture in the post-rainy seasons of sub tropical winter or spring of the northern hemisphere (Smithson *et al.*, 1985). Chickpea is generally grown on heavy black or red soils having a pH range between 5.5 and 8.6. Relative humidity of 21-41 % is optimum for seed setting.

Chickpea matures in 3 to 7 months and the leaves turn brown to yellow during maturity. For dry seeds, the plants are harvested at maturity or slightly earlier by cutting them close to the ground or uprooting.

1.1.3. Nutritional Value and Content of Chickpea Seeds

Chickpea is one of the important grain legumes as a source of protein for human as well as animal feeding. Proteins are the major seed component in all grain legumes, and are the reason for their relevant nutritional and socio-economical impact (Duranti and Gius, 1997). Chickpea is valued for its nutritive seeds with high protein content, ranging between 25.3 and 28.9 % (Hulse, 1991). The leaves contain 4-8 % protein (Muehlbauer and Tullu, 1997).

The majority of proteins in legume seeds consist of salt-soluble globulins, or storage proteins, that are synthesized during seed development, stored in protein

bodies, and hydrolyzed during germination to provide nitrogen and carbon for the developing seedling. The remainder is albumins that include many proteins such as lectins, and lipoxygenases. Compared with meat, our main source of protein, legumes in general are deficient in sulfur-containing amino acids (Wang *et al.*, 2003). Wide variation for seed protein content in annual wild *Cicer* species was demonstrated by Ocampo *et al.* (1998). Protein content ranged from 168 g to 268 g per kg and mean content was 207 g per kg (Ocampo *et al.*, 1998).

Chickpea seeds are consumed fresh as green vegetables, fried, roasted, and boiled; as snack food, sweet and condiments; seeds are ground and the flour can be used as soup and to make bread. In many developing countries, animal feed is another use of chickpea. Green or dried stems and leaves are used for stock feed or whole seeds may be milled directly for feed.

Among the food legumes, chickpea is the most hypocholesteremic agent. Germinated chickpea is accounted to be effective in controlling cholesterol level in rats (Geervani, 1991).

Legume seeds in general are also an important source of dietary minerals, with the potential to provide all 15 of the essential minerals required by man (Grusak, 2002). Chickpea seed contains 38-59 % carbohydrate, 4.8-5.5 % oil, 3 % fiber, 3 % ash, 0.2 % calcium, and 0.3 % phosphorus. Digestibility of seed proteins varies from 76-78 % and its carbohydrates from 57-60 % (Huisman and van der Poel, 1994).

Raw whole seeds contain 357 calories, 4.5-15.69 % moisture, 0.8-6.4 % fat, 14.9-24.6 g protein, 140-440 mg calcium (Ca), 190-382 mg phosphorus (P), 5.0-23.9 mg iron (Fe), 0-225 µg β-carotene equivalent, 0.21-1.1 mg thiamin, 0.12-0.33 mg riboflavin, and 1.3-2.9 mg niacin per 100 g (Huisman and van der Poel, 1994).

Calcium (Ca) is an essential nutrient for humans, but is quite limited in diets of low-income sectors and is of particular concern for pre-school children, adolescents, and pregnant and lactating women. Chickpea seeds contain 103 - 259 mg Ca per 0.1 kg dry weight and 70 % of this is in the seed coat. Therefore seeds of chickpea are a potential source of dietary Ca (Williams and Singh 1987; Abbo *et al.*, 2000).

Percent fatty acid compositions for *desi* type is oleic 52.1, linoleic 38.0, myristic 2.74, pactic 5.11, and steatic 2.05; and for *kabuli* type is oleic 50.3, linoleic 40.0, myristic 2.28, palmitic 5.74, stearic 1.61, and arachidic 0.07 % (Muehlbauer and Tullu, 1997).

1.1.4. Chickpea Production in the World and in Turkey

Chickpea is an ancient crop that has been grown in India, the Middle East and parts of Africa for many years. Chickpea is the third most important grain legume after bean and pea in the world and consumption of chickpea is the second only to bean marketed as human food. In 2003 world chickpea production was nearly seven million metric tons. Over the last four years, yield in world chickpea production was around 6,800 to 7,800 hectograms per hectare (Table 1.1). In 2000 to 2003, world chickpea production ranged between 6.9 and 8.3 million metric tons; Asia producing the major part 5.4 to 7.3 million metric tons.

Major chickpea growing countries are India, Turkey and Pakistan in Asia; California and Washington in the U.S.; Ethiopia in Africa; Spain in Europe; Mexico in Latin America; Canada and Australia (Figure 1.2) (See Appendix A for detailed comparison of major chickpea growing countries).

Table 1.1. Chickpea production in the World (Ha: Hectare; Hg/Ha: Hectogram per hectare; Mt: Metric tons) (FAOSTAT, 2004).

Years	Area Harvested (Ha)	Yield (Hg/Ha)	Production (Mt)	Seed (Mt)
1990	9,920,318	6,842	6,787,963	552,319
1995	11,396,760	8,016	9,135,410	611,399
2000	10,100,005	7,860	7,938,601	478,411
2001	9,481,888	7,283	6,905,242	521,477
2002	10,475,071	7,894	8,268,837	517,722
2003	10,374,133	6,866	7,122,650	516,753

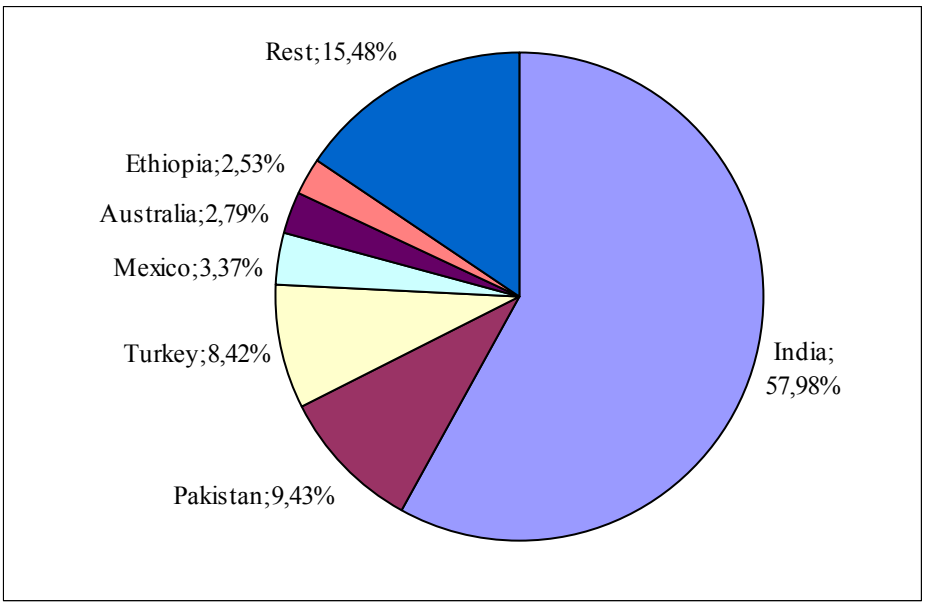


Figure 1.2. Major chickpea producing countries and their percentages of world production in 2003 (FAOSTAT, 2004).

Chickpea may have been grown in Turkey nearly for 7,400 years. Turkey was the second country in 2001 and 2002 and the third in 2003, in chickpea production. In 2003 chickpea production was 600,000 metric tons with a yield of 9,231 hectograms per hectare in Turkey (Table 1.2). According to the chickpea yield as hectogram per hectare, Turkey has always greater yield values than overall world values. Chickpea harvested area in Turkey in 1990 was 877,976 hectares and this value decreased to 636,000 hectares in 2000. Over last four years the cultivated area of chickpea stayed steady. Although annual yield has not changed significantly, total production, which was 860,000 metric tones in 1990, decreased to 548,000 in 2000, probably due to the reduction in cultivated area.

Table 1.2. Chickpea production in Turkey (Ha: Hectare; Hg/Ha: Hectogram per hectare; Mt: Metric tons) (FAOSTAT, 2004).

Years	Area Harvested (Ha)	Yield (Hg/Ha)	Production (Mt)	Seed (Mt)
1990	877,976	9,795	860,000	105,360
1995	745,000	9,799	730,000	93,600
2000	636,000	8,616	548,000	77,400
2001	645,000	8,295	535,000	80,400
2002	670,000	9,701	650,000	78,000
2003	650,000	9,231	600,000	78,000

1.1.5. Factors Affecting The Yield in Chickpea

The main factors affecting chickpea yield are diseases caused by fungi, drought, freezing range (below –1.5°C) temperatures, high temperatures, insects, nematodes, parasites, viruses and salinity. Singh *et al.* (1994) categorized the major abiotic and biotic stresses affecting chickpea production in order of relative

importance (Figure 1.3). Biotic stresses account for nearly 58 % and abiotic stresses account for 42 % impact on crop production.

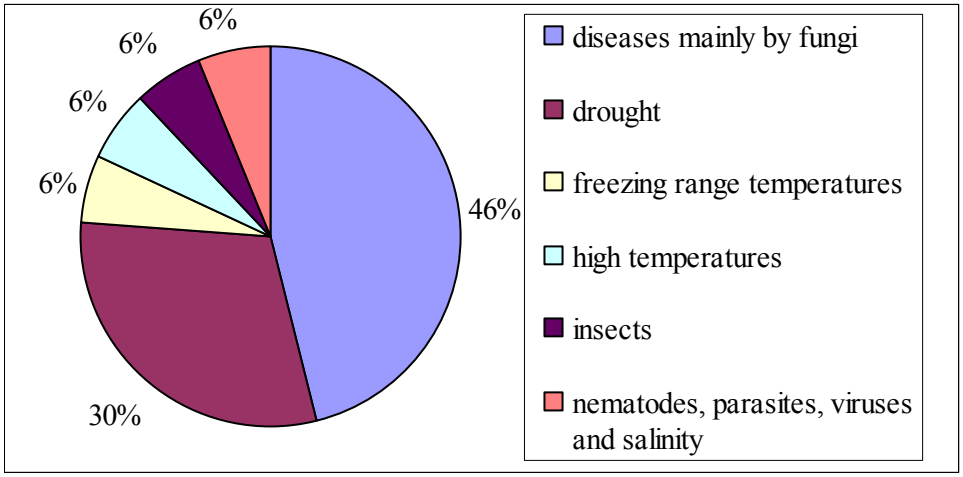


Figure 1.3. Relative importance of biotic and abiotic stresses on chickpea production (Modified from Singh *et al.*, [1994]).

1.1.5.1.Biotic Factors

Several constraints adversely affect the grain productivity of chickpea. Pests and weeds could be considered among the major restrictive factors for chickpea production. Management of pests, weeds and diseases are significant cost components of chickpea production.

The most important fungi that reduce chickpea production are *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. f.sp. *ciceris* [Padwick] Snyd. & Hans. causing the plant to wilt and *Ascochyta rabiei* [Pass.] Lab. causing Ascochyta blight (Smithson *et al.*, 1985). Ascochyta blight is a devastating disease of chickpea, occurring widely in countries, where chickpea is grown, especially in North India, Pakistan, the U.S. and the Middle East. Blight causes brown spots on

leaves, stems, pods and seeds (Kaiser, 1992). Ascochyta blight is also the most serious disease of chickpea in Turkey.

Other fungi known to attack chickpea include leaf spot (*Alternaria* sp.), *Ascochyta pisi*, rust (*Uromyces ciceris-arientini*), Botrytis gray mould (*Botrytis cinera*), powdery mildew (*Leviellula taurica*), *Pythium debar-yanum*, *P. ultimum*, dry root rot (*Rhizoctonia bataticola*), *R. solani*, foot rot (*Sclerotium rolfsii*), *Sclerotinia sclerotiorum*, wilt (*Verticillium albo-atrum*).

Pod borer (*Helicoverpa armigera*), is the most important pest of chickpea, and feeds on leaves and developing seeds (Smithson *et al.*, 1985; Popelka *et al.*, 2004). Cutworms (*Agrotis* sp.), lesser armyworms (*Spodoptera exigua*), leaf miner (*Liriomyza cicerina* Rond.), groundnut aphid (*Aphis craccivora*), pea aphid (*Acyrthsosiphon pisum*), cowpea bean seed beetle (*Callosobruchus maculatus*), and Adzuki bean seed beetle (*C. chinensis*) are also important pests of chickpea. Leaf miner is an important insect pest in Mediterranean region (Singh *et al.*, 1998) causing up to 30 % yield loss (Weigand, 1990).

Another biotic stress factor on chickpea production is cyst nematode caused by *Heterodera ciceri* Vovlas, Greco and Di Vito (Singh, 1997).

In general, estimates of yield losses by individual pests, diseases or weeds range from 5-10 % in temperate regions and 50-100 % in tropical regions (van Emden *et al.*, 1988).

1.1.5.2.Abiotic Factors

Although chickpea is considered to be relatively resistant to drought compared to other crops, drought stands to be the major limiting abiotic factor in major chickpea growing regions; because the crop is grown on residual moisture, and is eventually exposed to drought during pod set and seed filling (terminal drought)

(Johansen *et al.*, 1994). In subtropical areas and Mediterranean-climatic regions chickpea crops are exposed to terminal drought (Leport *et al.*, 1999). Leport *et al.*, (1999) reported that water shortage reduced seed yield by 50 to 80%, due to a reduction in seed number and seed size in field experiments carried out in the Mediterranean-climatic region of Western Australia.

Extreme temperatures can also exert stress on chickpea production. Cold stress is an important stress factor in Mediterranean region (Singh *et al.*, 1998) especially during plant flowering that inhibit pod set (Leport *et al.*, 1999; Srinivasan *et al.*, 1999). Temperatures up to 15°C have been demonstrated to cause flower and pod abortion in parts of the Indian subcontinent and Australia (Srinivasan *et al*., 1998; Clarke, 2001; Croser *et al.*, 2003). Additionally the crops can be exposed to high temperatures during seed filling that limit yields (Buddenhagen and Richards, 1988; Leport *et al.*, 1999).

Salinity is another factor affecting the yield in chickpea production. Katerji *et al.* (2003) grouped some important crops according to their tolerance or sensitivity to salinity using several classification methods. They reported chickpea as sensitive to salt according to the pre-dawn leaf water potential, expressed as water stress day index during the growing period, and according to soil salinity.

1.1.6. Improvement of Chickpea

Among the grain crops, grain legumes rank third behind cereals and oilseeds in world production, but are an important dietary constituent for humans and animals (Popelka *et al.*, 2004). As being an important crop in the world, chickpea production is tried to be improved either by increasing the yield in areas where chickpea has already being produced or by enlarging the chickpea cultivated area via releasing cultivars that can be grown in extreme environments.

Improvement of chickpea is directed to different aspects of seed yield improvement and production stability. Major emphases of improvement studies have been on resistance to diseases and insects; like ascochyta blight in cool and wet climates, fusarium wilt and other root rot diseases in dry climates, leaf miner in Mediterranean climates and pod borers in tropics. Some other goals of improvement are directed to tolerance to environmental stresses like; drought, cold and high temperatures (Singh, 1997; Christou, 1997). Singh *et al.* (1994) indicated that research efforts going toward biotic stress accounts for nearly 80 % and on the other hand abiotic stress receives 20 % of research efforts.

1.1.6.1.Chickpea Breeding

The conventional breeding techniques utilize processes of crossing, back crossing and selection with the requirement of scanning and presence of desired characteristics within the germplasm resources.

For scanning of germplasms, accessions of chickpea available at International Center for Agricultural Research in the Dry Areas (ICARDA) or at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) are evaluated for diversity in response to different stresses.

Singh *et al.* (1998) evaluated 228 accessions of eight annual wild *Cicer* species and 20 cultivated chickpea lines for diversity in response to six of the most serious biotic and abiotic stresses which reduce crop yield and production stability of chickpea, *i.e.*, ascochyta blight, fusarium wilt, leaf miner, bruchid, cyst nematode, and cold. Pair-wise correlations among the six biotic and abiotic stresses showed the possibility of combining these resistances and possibility of usage of wild species as resource. Singh *et al.* (1998) also indicated that multiple stress resistant accessions were predominantly of Turkish origin.

Singh and Ocampo (1997) performed interspecific crosses and their reciprocals between wild and cultivated species of chickpea to increase seed yield. Research lasted for nearly 8 years and 7 generations were grown in field. Nine F_7 lines, which over-yielded the parent cultivated species by up to 39 %, were developed.

Germplasm with some degree of resistance to bruchids has been identified, but it appears to be correlated with undesired physical characteristics of the seed coat. Since dark color, roughness, altered chemical composition and thickness of the seed coat makes bruchid resistant chickpeas less desirable for human consumption, the introduction of unlinked resistance genes via gene transfer technology would be advantageous (Popelka *et al.*, 2004).

It takes five to six generations with a minimum of 7-10 years to transfer a trait into the high yielding locally adapted cultivars through the conventional breeding. And one has to plant and test a large number of progenies to be able to select the plants with appropriate combination of traits before a variety could be identified for cultivation by the farmers (Sharma *et al.*, 2002).

Conventional breeding strategies have shown some success but their effectiveness could be considerably increased with the application of suitable biotechnologies (Ranalli and Cubero, 1997).

1.1.6.2.Chickpea Biotechnology

Biotechnology, which involves the systematic application of biological processes for the beneficial uses, is emerging as one of the latest tools of agricultural research in recent years. It has significantly augmented the conventional crop improvement, and has a great promise to assist plant breeders to meet the increased food demand predicted for the 21st century (Sharma *et al.*, 2002). Unlike conventional breeding which utilizes domestic crop cultivars and related species as a source of genes for improvement of existing cultivars,

biotechnological approaches can transfer defined genes from any organism and in this manner can increase the gene pool available for improvement.

Despite its potential to complement current breeding programs, biotechnology and genetic transformation technology is not yet routinely available for most legumes of importance in developing countries (Popelka *et al.*, 2004). The absence of variety-independent gene transfer methods for major agronomic species has limited the use of biotechnology and recombinant DNA techniques to improve grain legumes (Christou, 1997).

Both for conventional breeding and biotechnological improvements of crops, molecular analysis should be performed. Marker-assisted selection reduces the time required to develop novel improved varieties and it does not require large scale planting of progenies up to crop harvest. Proteins, isozymes, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat length polymorphism (SSR), amplified fragment length polymorphism (AFLP), expressed sequence tag (EST), and single nucleotide polymorphism (SNP) are major classes of biochemical and molecular markers used in improvement programs. Once genomic regions contributing to the trait of interest have been assigned, they can be transferred to high-yielding cultivars by making crosses. The offspring with a desired combination of traits can then be selected using markers (Sharma *et al.*, 2002).

Genetic variations and relationships among perennial and annual *Cicer* species especially grown in Turkey were assessed by using RAPD markers, AFLP fingerprinting, and inter simple sequence repeat (ISSR) variations (Sudupak *et al.*, 2002; Sudupak *et al.*, 2004; Sudupak, 2004).

1.2. Gene Transfer Techniques for Plants

Manipulation of genomes of plants is achieved by the techniques of transgenic plant technology that aims stable expression and transmission of the introduced genes to progeny. These genes can confer resistance to diseases, herbicides and abiotic stresses, improve the yield and quality or make plants produce pharmaceuticals and industrial chemicals.

Efficiency of a transformation protocol depends on several components which are (1) the choice of explant type which can easily regenerate; (2) the presence of a reliable regeneration method for the plant species into which the novel gene is desired to be introduced; (3) the presence of a reproducible and highly efficient gene transfer technique; (4) an effective screening and selection method for the recovery of transformants.

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

The method of DNA delivery into plants can be indirect such as *Agrobacterium* mediated and viral vector mediated delivery or direct such as microprojectile bombardment, electroporation, microinjection, macroinjection, PEG mediated, silicon carbide mediated delivery, and sonication. Methods frequently in use are *Agrobacterium* mediated delivery, microprojectile bombardment, electroporation, microinjection and PEG mediated delivery.

Electroporation is one of the direct gene transfer methods, which is used extensively for transferring cells of various organisms including bacteria and

mammalian cells. Electric pulses cause to open transient pores in the plasma membrane of organisms and DNA moves into target cells through these pores. Field strength and pulse duration are two main variables affecting the permeabilization of the plasma membrane. Mainly cells are chosen as a target for introducing foreign genes rather than organized tissues for electroporation.

Cell wall of plants is an important problem in use of electroporation for transformation of plant cells. Therefore generally employed method is the removal of cell wall and use of protoplasts in electroporation. Ou-Lee *et al.* (1986) reported the expression of bacterial chloramphenicol acetyl transferase gene (*cat*) after electroporation of protoplasts of three important graminaceous plants; rice, sorghum and wheat. Another procedure was reported by Dekeyser *et al.* (1990) to electroporate DNA into intact leaf tissue of rice.

Microinjection employs the use of fine micro-capillaries to transfer a specified number of genes into cytoplasm or directly into nucleus of cells. This method is more suitable for stable transformation rather than transient gene expression but the method is labor intensive, has a high cost and number of transformants produced at the end are relatively low compared to other methods of gene delivery.

Isolated zygotic pre-embryos of soybean, cotton, sunflower and *Arabidopsis thaliana* were assumed to be competent and multiple microinjections with marker genes were carried out. Then the plants were analyzed for putative transgenic chimeras and offspring (Potrykus, 1990). In another study, integration of foreign DNA into genome of tobacco protoplasts following microinjection was reported by Crossway *et al.* (1986).

Agrobacterium mediated transformation and microprojectile bombardment are the methods used frequently for various plant species and explants.

1.2.1. *Agrobacterium* Mediated Gene Transfer

Plant transformation started in the early 1980s with the first conclusive demonstration that the causative agent of crown gall disease, *Agrobacterium tumefaciens*, could be harnessed by researchers to introduce defined fragments of DNA into plant cells (Newell, 2000).

Agrobacterium mediated transformation take the advantage of natural mechanism performed by nature's genetic engineer; *Agrobacterium*. Although the molecular mechanism involved in transfer of DNA to plant and integration of it to genome is not well defined; functions of various genes and proteins are reviewed in detail at a molecular level in reports of Weising and Kahl (1996), Sheng and Citovsky (1996), and Zupan *et al.* (2000).

Removal of all the genes within the transferred DNA (T-DNA) does not impede the ability of bacteria to transfer this DNA but does prevent the formation of tumors (Hellens and Mullineaux, 2000). *Agrobacterium* has become a useful tool in plant transformation when the native genes are removed from T-DNA and foreign genes are inserted instead. These foreign genes include selectable or scorable markers in plants, multiple cloning sites for efficient insertion of various genes, origins of replication and markers that permit efficient plasmid replication and selection in *Agrobacterium* and *Escherichia coli*.

The method of *Agrobacterium* mediated transformation of intact tissues was developed using excised tissues of *Nicotiana* and *Petunia* species (Horsch *et al.*, 1985). Studies with these species established rapid and reproducible procedures, which are further extended to other species.

Research using *Agrobacterium* mediated transfer techniques initially indicated that monocotyledonous species including maize, rice, wheat and barley and some dicotyledonous species including legumes were not amenable to transformation

with *Agrobacterium* due to the natural host range of the bacterium (Newell, 2000) or due to the lack of evocation of wound response in these species (Anderson *et al.*, 2002). A number of economically important cereals such as; rice, maize and wheat have now been transformed by *A. tumefaciens* (Komari *et al.*, 1998).

Many different techniques have been used to improve the efficiency of transformation with *Agrobacterium*, centered on attempts to increase the exposure of bacteria to the plant cells and often involving novel ways of wounding the plant tissue prior to inoculation (Newell, 2000).

Wounded plant cells release low molecular weight phenolic compounds that attract *Agrobacterium* to the wound site and function in the induction of virulence genes (*vir*). Intermediates of lignin synthesis or phenolic compound precursors such as acetosyringone are chemo attractants at very low concentrations but are *vir* gene inducers at high concentrations. Other groups of phenolic compounds, such as; hydroxycinnamides are known to act as *vir* gene inducers (Sangwan *et al.*, 2002). Furthermore opines and flavonoid compounds may be involved in *vir* gene induction (Zerback *et al.*, 1989). Addition of phenolic compounds to the inoculation media, used during transformation procedures, or wounding of explants may increase the *vir* gene induction therefore increasing the transformation efficiency. Warkentin and McHugen (1992) used acetosyringone for induction of *vir* gene expression in lentil (*Lens culinaris* M.) tissue transformations using *A. tumefaciens*. Cheng *et al.* (1997) also pointed out the importance of presence of acetosyringone in inoculation and co-cultivation media in the study reporting the stable transformation of wheat with *Agrobacterium*.

Vernade (1988) demonstrated that low pH conditions increase the background level of *virG* expression, which encodes for a positive transcription factor involved in the activation of transcription of all other *vir* genes, but low pH conditions do not produce any significant *vir* gene induction in the absence of acetosyringone and an osmoprotectant glycine betaine. Decreasing the pH of the

inoculation media may increase the *vir* gene induction and transformation efficiency.

Kapila *et al.* (1997) observed high levels of transient gene expression when *A. tumefaciens*, which was pretreated under *vir* gene inducing conditions, was infiltrated into intact leaf tissues of bean (*Phaseolus vulgaris*).

Mahmoudian *et al.* (2002) demonstrated that vacuum infiltration of *A. tumefaciens* suspension containing the lentil cotyledonary nodes at evacuation pressures of 200 or 400 mm Hg for 20 or 30 minutes yielded in a significantly higher amount of β-D-glucuronidase (GUS) gene expression in infiltrated explants than non-infiltrated ones. Vacuum infiltration is an efficient way of increasing the exposure of bacteria to the plant cells.

During plant-bacteria interaction in *Agrobacterium* mediated transformation, plant cell viability decreases and oxidative bursts increase. In different studies of transformation, use of anti-necrotic compounds resulted in a reduction in browning and necrosis of the plant tissues undergoing co-cultivation with *Agrobacterium*; together with increase in transformation efficiencies. Enriquez-Obregon *et al.* (1999) presented a procedure of Japonica rice transformation which is coupled to treatment with anti-necrotic compounds cysteine and ascorbic acid. Average damage decreased and viability increased in meristematic tissues of rice with the addition of cysteine and ascorbic acid to solid media used for culturing of explants. Olhoft and Somers (2001) observed that addition of L-cysteine to solid co-cultivation medium resulted in decreased necrosis and increased T-DNA transfer in soybean (*Glycine max*) cotyledonary node axillary meristem cells. Olhoft *et al.* (2001) pointed out that by reducing wound and pathogen defense responses in plants, anti-necrotic compounds have the potential to increase the capacity of *Agrobacterium* to infect plant tissues and stably transfer T-DNA and to increase the frequency of infected cells that remain viable and become transformed.

Development of a range of *Agrobacterium* mediated gene delivery techniques and use of various improvements such as wounding, vacuum infiltration, sonication or addition of anti-necrotic compounds to media during procedures, resulted in advances in plant transformation technology and resulted in the use of *Agrobacterium* mediated transformation for a wide range of plant species covering poplar, carrot, potato, monocotyledonous plants such as wheat, rice, and maize; leguminous plants like bean, soybean and pea; and many others.

1.2.2. Microprojectile Bombardment

Microprojectile bombardment involves accelerating metal microcarriers of tungsten or gold, which are coated with DNA, to velocities at which they can penetrate into plant cells. This is achieved usually by utilizing compressed helium or nitrogen. Microprojectile bombardment of plant cells was first described by Sanford *et al.* (1987) using onion, tobacco, corn, and rice. Klein *et al.*, (1987) further proved the use of high velocity particles for nucleic acid delivery into living cells. And transient gene expression in maize proved that tungsten particles can carry functional DNA into intact plant cells (Klein *et al.*, 1988).

The main advantage of microprojectile bombardment is that it is species or cultivar independent, avoids host restrictions of *Agrobacterium*, and avoids regeneration problems encountered in protoplast systems with the result that the DNA to be introduced does not need to contain the sequences necessary for T-DNA replication and transfer (Newell, 2000).

This technique has resulted in the production of transgenic plants of several species, particularly monocotyledonous species including orchids (Yang *et al.*, 1999; Knapp *et al.*, 2000), banana (Becker *et al.*, 2000), sugar cane (Falco *et al.*, 2000) and cereals such as barley (Weir *et al.*, 1998), maize (Rudraswamy and Reichert, 1998) and wheat (Vasil *et al.*, 1992; Altpeter *et al.*, 1996; Rasco-Gaunt *et al.*, 1999) and dicotyledonous species such as lentil (Öktem *et al.*, 1999)

soybean (Christou *et al.*, 1990) bean (Russell *et al.*, 1993) and peanut (Brar *et al.*, 1994).

Various parameters like amount of plasmid DNA, microcarrier concentration, spermidine concentration, acceleration, vacuum pressure, and osmotic pretreatment of target tissues may have significant effects on gene delivery via microprojectile bombardment.

1.3. Tissue Culture Studies in Chickpea

There are several reports of tissue culture and regeneration studies using organogenesis and somatic embryogenesis from chickpea explants; however regeneration efficiency of chickpea is very low. Barna and Wakhlu (1993) reported somatic embryogenesis and plant regeneration from immature leaflet callus cultures of chickpea on MS (Murashige and Skoog, 1962) based media supplemented with 25 μM 2,4-Dichlorophenoxyacetic acid (2,4-D). The frequency of regeneration was quite low and the procedure may not be efficient for all cultivars.

Barna and Wakhlu (1994) developed a protocol indicating whole plant regeneration of chickpea from callus culture via organogenesis. They investigated the effect of 2,4-D, naphthalene acetic acid (NAA), picloram and benzylaminopurine (BA) on callus initiation and growth.

In another study of somatic embryogenesis in chickpea Rao and Chopra (1989) obtained somatic embryos and performed regeneration from leaflet derived chickpea calli but they could not achieve complete plant recovery. MS media containing 0.5 mg/L 2,4-D and 0.5 mg/L BA and dark incubation was found to be best for somatic embryo production. Kumar *et al.* (1994) reported efficient plant regeneration in chickpea via somatic embryogenesis from leaf explants.

Besides somatic embryogenesis; regeneration of chickpea through direct organogenesis without an intermediary callus phase was reported in many research articles. Direct organogenesis is superior to regeneration through a callus phase, because it reduces both the possibility of somaclonal variation, which results in genotype instability and the time required to obtain complete plants *in vitro*.

High frequency shoot regeneration from intact seedlings of pea, chickpea and lentil by culturing mature seeds on MS media supplemented with thidiazuron was reported by Malik and Saxena (1992).

An efficient procedure of somatic embryogenesis induction from mature embryo axes in chickpea bypassing the intermediary callus stage was reported by Suhasini *et al.* (1994). Absence of intermediary callus stage makes the technique superior to other somatic embryogenesis studies reported earlier. Murthy *et al.* (1996) achieved combination of direct organogenesis and somatic embryogenesis in chickpea by culturing mature seeds on MS media containing either thidiazuron or BA. After culturing for 2 to 3 weeks, *de novo* multiple shoot formation at the cotyledonary node regions without an intermediary callus phase was observed.

Polisetty *et al.* (1997) reported multiple shoot induction and complete plant regeneration from seed explants of chickpea with a frequency of 80%. The researchers indicated that shoot differentiation was influenced by type of explant, genotype, concentration of BA and by orientation of explant. Average number of shoots per explant was increased to 20 shoots. The protocol produces sufficient numbers of shoots for transformation events but it is significantly influenced by type and orientation of explant and genotype of cultivar used.

In vitro regeneration of plants from calli derived from internodal explants of chickpea was achieved by Roy *et al.* (2001). Callus induction was performed successfully on both Gamborg's B5 (Gamborg *et al.*, 1968) and MS media

supplemented with different combinations and concentrations of auxins and cytokinins. Complete plantlets were developed through grafting of regenerated shoots onto root stock since rooting could not be achieved *in vitro*. Somaclonal variation, resulting in various seed colors and seed weights, is the obstacle encountered in their study.

Raj *et al.* (2001) reported induction of differentiation in chickpea hypocotyl explants, which were believed to be non-differentiating in *in vitro* culture, by modification of several physiological factors. Orientation of explants also seems to play an important role in inducing shoot bud differentiation from hypocotyl explants.

Direct organogenesis without an intervening callus phase is reported by Shikha *et al.* (2001). Mature embryo axis without shoot apex was used as explant and was cultured on various media for shoot induction. Complete plantlets were developed by root induction from regenerated shoots *in vitro*.

Multiple shoot regeneration from the cut plumular ends of embryo axes of chickpea was evaluated on MS medium having different concentrations of thidiazuron, BA, kinetin or zeatin (Singh *et al.*, 2002). TDZ (0.2 mg/L) was reported to be the most effective cytokinin as it produced multiple shoots in all of the explants from genotypes of various cultivars. 70 % complete plant production was achieved with shoot elongation and rooting on growth regulator-free media.

Jayanand *et al.* (2003) developed an efficient and reproducible protocol for the regeneration of shoots at high frequency by using explants derived from the axillary meristems from the cotyledonary nodes of *in vitro*-germinated seedlings of chickpea. Researchers tried to optimize culture conditions for various stages of adventitious shoot regeneration including the induction, elongation, and rooting of the elongated shoots.

Direct organogenesis of chickpea seems to be more advantageous over other regeneration systems in the aspect of its use after transformation studies. Because direct organogenesis is less labor intensive, less time consuming, and results in less or no somaclonal variation since it eliminates intervening callus phase.

1.4. Genetic Transformation of Chickpea

Grain legumes play a crucial role in food protein supply in developing countries and in the sustainability of agricultural systems. Several constraints that limit crop production or quality have been addressed by conventional breeding, but there are situations where the existing germplasm lacks the required traits. Genetic transformation may provide solutions to certain constraints (Popelka *et al.*, 2004). Despite its potential to complement current breeding programs, genetic transformation technology is not yet routinely available for most legumes of importance. Legumes, with the exclusion of some important species, attract a little research aiming genetic transformation; because of difficulties in both tissue culture and transformation.

To date, only a few reports described genetic transformation of chickpea. Transformation experiments relying on callus (Mohapatra and Sharma, 1991) failed due to limited shoot regeneration (Huda *et al.*, 2000) but demonstrated the potential of *A. tumefaciens* as a transformation vector for chickpea (Islam *et al.*, 1994; Altınkut *et al.*, 1997). Islam *et al.* (1994) evaluated the susceptibility of four genotypes of chickpea to four wild strains of *A. tumefaciens*. Researchers pointed out that chickpea can be infected by *Agrobacterium* and agropine strain A281 was the most effective for tumor induction.

First report of chickpea stable transformation (Fontana *et al.*, 1993) provided molecular evidence for transgene presence in progeny. In this report seed derived embryo axes deprived of the apical meristem were used as explants and adventitious shoot regeneration was performed on MS media supplemented with

1.0 mg/L kinetin. Transformation was carried out by co-cultivation of explant with *A. tumefaciens* strain LBA4404. Whole chickpea plants expressing β-D-glucuronidase (GUS) and neomycin phosphotransferase II (NPTII) were obtained. Presence of transgene in transformants and progeny up to T_2 was demonstrated by southern hybridization. Transformation percentage of the employed procedure is indicated to be 4 %.

Formation of multiple shoots from different genotypes and production of transgenic plants using *Agrobacterium* has been reported employing similar experimental procedures (Kar *et al.*, 1996; Krishnamurthy *et al.*, 2000). Kar *et al.* (1996) reported transgenic plant production using three genotypes of chickpea and a local *desi* variety. Embryo axes devoid of root meristem and shoot apex produced an average of 22.51 shoots per explant with local *desi* variety. Transformation was carried out by co-cultivation of explant with *A. tumefaciens* strain LBA4404. Presence of *nptII* gene in transgenic whole plants was demonstrated by southern hybridization. In the study of Kar *et al.* (1996), transgene presence in progeny was not proven and transformation frequencies were lower, ranging between 1.40 % and 1.96 %, compared to the report of Fontana *et al.* (1993).

Transient expression of marker genes in zygotic embryos of chickpea was demonstrated and conditions for optimum transient expression of *uidA* (*gus*) and *nptII* genes were established by Husnain *et al.* (1997). Using accelerated tungsten particles in transformation procedure; researchers optimized concentration of plasmid DNA, distance of explant, and negative pressure of chamber to obtain optimal transient expression of *uidA (gus)* gene in chickpea embryos. Husnain *et al.* (1997) also performed transformation of zygotic embryos using *A. tumefaciens* and concluded that *A. tumefaciens* strain A281 is more efficient in transformation compared to strain C58 according to the percentage of calluses or tumors expressing GUS activity. The researchers proposed that micro-injury of embryo explants with DNA coated tungsten particles followed by co-cultivation of

explants with *A. tumefaciens* carrying the same DNA portion may increase the efficiency and frequency of transformation.

There are two reports of transgenic plants expressing potentially useful transgenes in chickpea (Kar *et al.*, 1997; Sarmah *et al.*, 2004). Transgenic chickpea plants, produced via biolistic transformation with gold particles, expressed the bacterial *cryIA(c)* gene from *Bacillus thuringiensis* together with *nptII* as the selectable marker (Kar *et al.*, 1997). Explant and regeneration procedure used were the same as the previous *Agrobacterium* mediated transformation method reported by Kar *et al.*, (1996); whereas gene construct and transformation method were different in this report. Microprojectile bombardment technique was used for delivering genes to explants. Molecular analyses including; southern, northern, and western hybridizations, of transformants indicated the presence and expression of transferred functional *cryIA(c)* gene. Insect feeding trials with one primary transgenic plant demonstrated an inhibitory effect on growth of larvae of chickpea pod-borer *Heliothis armigera* Hubner. Transmission of gene to progeny was only demonstrated by polymerase chain reaction (PCR) analysis, although further molecular analyses have not been reported.

Agrobacterium mediated transformation of four accessions of chickpea was carried out using *A. tumefaciens* strains C58C1/GV2260 carrying the plasmid p35SGUSINT and strain EHA101 harboring the plasmid pIBGUS (Krishnamurthy *et al.*, 2000). Researchers performed concentration of bacterial cell suspension through centrifugation and resuspension in MS containing BA to increase the number of bacteria per explant during inoculation. Expression of chimeric *gus* gene was confirmed by histochemical localization of GUS activity in regenerated shoots. T-DNA integration was confirmed with southern blot analysis of putative transgenics. However four individuals of T_1 progeny were only shown to posses the *nptII* gene using PCR analysis. On the other hand these four individuals are found to be GUS-negative according to histochemical staining.

A. tumefaciens strain AGL1 was used to transform *desi* type chickpeas with a seed specific α-amylase inhibitor (*αAI1*) gene from bean (*Phaseolus vulgaris*) and the *nptII* gene as selectable marker (Sarmah *et al.*, 2004). The researchers pointed out that bean *αAI1* was specifically expressed in the seeds of chickpea, accumulated up to 4.2 % of seed protein and was processed to low molecular weight polypeptides as occurs in bean seeds. Stable transmission and expression of the transgenes in subsequent generations was demonstrated using molecular analyses. Transgenic protein was active as an inhibitor of porcine α-amylase *in vitro*. The high level of expression of the *αAI1* gene protected chickpea seeds from insect damage by severely inhibiting the development of cowpea weevils (*Callosobruchus maculatus*) and adzuki bean weevils (*C. chinensis*) in insect bioassays. Researchers concluded that the bruchid resistance afforded by *αAI1* gene will be a useful trait to introduce into chickpeas especially in areas where losses to stored grain pests are high.

In another recent study Senthil *et al.* (2004) reported an *Agrobacterium* mediated transformation method for chickpea using longitudinal slices of embryo axis as explant. According to transient GUS activity, assayed by fluorometric analysis at fourth day of co-cultivation, strain AGL1 was found to be more virulent compared to LBA4404 and C58. A total of 41 confirmed transformed lines were developed, giving an overall transformation frequency of 5.1 % across the three genotypes used. Southern blot analysis, GUS histochemical staining, and leaf paint assay was performed to demonstrate integration and expression of transgenes.

Polowick *et al.* (2004) recently reported another *Agrobacterium* mediated transformation method for chickpea using longitudinal slices from embryonic axes. Researchers recovered transgenic plants with a frequency of 1.3 % and this frequency was improved to 3.1 % with the addition of a shoot elongation medium to the protocol. Gene integration, expression and inheritance were demonstrated by various assays of primary putative transgenics and of their progeny.

Study reported by Tewari-Singh *et al.* (2004) described three different selection systems; namely Kanamycin-based antibiotic selection, PPT-based herbicide selection, and aspartate kinase (AK) gene-based non-antibiotic selection, for use in the production of transgenic chickpea. Transgenic shoots regenerated from embryo explants bombarded with the desensitized *AK* gene were selected on media containing two amino acids, lysine and threonine (LT). Southern hybridization was used to present the gene integration in T_0 plants carrying *AK* gene. Also PPT selection system for *Agrobacterium* mediated chickpea transformation was developed by Tewari-Singh *et al.* (2004). That was the first report of the successful use of AK/LT selection system for the production of transgenic chickpea plants. AK/LT system may be of considerable practical importance in transgenic crop development programmes since it avoids the use of antibiotic selection genes.

1.5. Aim of the Study

Presence of a reliable regeneration method; a reproducible and highly efficient gene transfer technique; and an effective screening and selection procedure for the development and recovery of transformants are three critical prerequisites for transgenic chickpea production.

There are several reports of chickpea regeneration and transformation; however, the frequencies of transgenic plant production are severely low. Therefore, this study concentrated mainly on the development and optimization of efficient transformation, regeneration and selection systems for improvement of locally cultivated and high yielding Turkish chickpea (*Cicer arietinum* L.) cv. Gökçe, using a highly regenerable explant; cotyledonary nodes (CN).

One of the objectives of this study is to optimize an efficient regeneration and selection procedure that can be employed after *Agrobacterium* mediated

transformation using CN of chickpea cultivar Gökçe. Therefore this study is focused on:

I. determination of dose of frequently employed selective agents which are required to inhibit multiple shoot induction and root induction after transformation procedures.
II. investigation of effects of antibiotics, which are commonly used for elimination of bacteria after *Agrobacterium* mediated transformation, on direct organogenesis and rooting.

The second objective of this study is to establish and optimize a reproducible and highly efficient *Agrobacterium* mediated gene transfer technique and to increase the frequency of transgenic plant production by monitoring transient expression of *uid*A gene. Therefore in the context of this study effects of various parameters are inspected. The parameters investigated are:

I. mechanical injury of explants prior to transformation.
II. vacuum infiltration during inoculation of explants with bacteria.
III. addition of L-cysteine to co-cultivation media.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

Throughout the tissue culture and transformation studies of this work, cotyledonary node (CN) explants of chickpea (*Cicer arietinum* L. cv. Gökçe) were used. Cultivar Gökçe is a *kabuli* type with cream or yellow colored large seeds, which is cultivated in Turkey. The seeds were obtained from Exporter Unions Seed and Research Company (İhracatçı Birlikleri Tohumculuk ve Araştırma Sanayi ve Ticaret A.Ş.).

2.1.2. Plant Tissue Culture Media

In this study, MS (Murashige and Skoog, 1962) based media containing MS micro-macro elements and vitamins with additions of sucrose and agar were used for all plant tissue culture media. The composition of MS basal media is given in Appendix B.

For germination of the surface sterilized seeds, half strength MS basal media including only MS micro and macro elements with additions of 1.5 % sucrose and 0.6 % agar at pH 5.6 - 5.8 were used.

In selection system development studies, MS basal media supplemented with 3 % sucrose and 0.8 % agar were utilized. Depending on the experimental purpose, either 1 mg/L of benzylaminopurine (BA) or 0.1 mg/L of indole butyric acid (IBA) and selective agents and/or antibiotics were added to the MS media.

In transformation optimization studies, MMA medium containing MS basal salts without vitamins but with 2 % sucrose, 10 mM MES (2-[N-Morpholino] ethanesulfonic acid) and 200 µM acetosyringone (3',5'-Dimethoxy-4-hydroxyacetophenone) was used for bacterial resuspension after centrifugation and bacterial inoculation of explants. The pH of MMA was adjusted to 5.6. Cocultivation of explants with bacteria was done on solid MS basal medium supplemented with 1 mg/L BA. Kanamycin, Cefotaxime and Carbenicillin were included into MS basal media for selection of putative transgenics and for elimination of bacteria. The compositions and uses of plant tissue culture media are provided in Appendix C.

The media were dissolved in distilled water; pH of the medium was adjusted to 5.6-5.8 and sterilization was done by autoclaving at 121ºC for 20 minutes. Growth regulators, acetosyringone, selective agents and antibiotic solutions were filter sterilized with 0.2 µm pore sized filters and added freshly to the sterilized and cooled media.

2.1.3. Bacterial Strain and Plasmid

During transformation optimization studies, *Agrobacterium tumefaciens* strain KYRT1 (Torisky *et al*., 1997) containing pTJK136 (Kapila *et al.*, 1997) as a binary plasmid vector was used. *A. tumefaciens* KYRT1 carries Rifampin, Carbenicillin and Gentamicin resistance genes on chromosomal genome. Binary vector pTJK136 is a derivative of pTHW136 and it carries a gene coding for streptomycin/spectinomycin adenyl transferase as bacterial selection marker and

also carries an intron containing *uidA* (*gusA*) reporter gene and *nptII* selectable gene as plant selection markers which code for the β-D-glucuronidase (GUS) and neomycin phospho transferase II (NPT-II), respectively (Appendix D). Binary vector pTJK136 was provided by Prof. Dr. Marc van Montagu (Appendix E).

2.1.4. Bacterial Culture Media

Yeast extract broth (YEB) containing nutrient broth, yeast extract, sucrose and magnesium sulphate at pH 7.2 was used to grow *A. tumefaciens* KYRT1. The medium was supplemented with Rifampin (100 mg/L), Carbenicillin (100 mg/L), Gentamicin (40 mg/L), Streptomycin (300 mg/L) and Spectinomycin (125 mg/L) (Appendix D). During the transformation studies, for the induction of *vir* genes of *A. tumefaciens*, YEB was enriched with 20 µM acetosyringone and 10 mM MES and its pH was adjusted to 5.6 and named as YEB+MES. The compositions of bacterial culture media are provided in Appendix F.

2.1.5. Culture Conditions

All plant tissue cultures were incubated at 24±2ºC under fluorescent light at 100 µmol $m^{-2}s^{-1}$ with a 16/8 hour (light/dark) photoperiod. The seeds, on the other hand, were germinated at 24±2ºC under dark conditions. All the bacterial cultures of *A. tumefaciens* were incubated at 28±1ºC with a 180-200 rpm (revolution per minute) in a Sanyo Gallenkamp shaker.

2.1.6. Chemicals

The chemicals used in this study were purchased from Sigma Chemical Company (N.Y., USA), Merck Chemical Company (Deisenhofen, Deutschland), MBI Fermentas (Ontario, Canada), Applichem (Darmstadt, Germany), and Duchefa (Haarlem, The Netherlands). All of the media and solutions were prepared by using distilled water.

2.2. Methods

2.2.1. Tissue Culture Studies

In tissue culture studies of this work; optimization of an efficient and reproducible selection and regeneration system for putative transgenic shoots, emerging from cotyledonary nodes, was the main aim.

2.2.1.1. Seed Surface Sterilization and Germination

The seeds of chickpea were surface sterilized in 3 % sodium hypochloride including 0.1 % Tween-20 for 90 minutes. Then they were rinsed in sterile distilled water four or five times, each lasting for 3 to 5 minutes. They were not remained in water for imbibition. Then the seeds were blotted dry on sterile filter papers immediately after sterilization. Dried seeds were placed onto half strength MS media lacking vitamins and supplemented with sucrose and agar (Appendix C). Germination was carried out in climatized rooms at 24±2ºC in dark for 4 days.

2.2.1.2. Cotyledonary Node Explant Preparation

Cotyledonary nodes were isolated from four days old etiolated chickpea seedlings (Figure 2.1). The radicle and emerging shoot primordium were removed with single cuts leaving 5-6 mm of tissue on both sides of the node (Figure 2.1.B). Then the cotyledons were excised from the cotyledonary node with two cuts, one for each, leaving 2-3 mm of tissue on explant (Figure 2.1.C). Figure 2.1 represents a chickpea seedling and the preparation of cotyledonary node explant. The shoots emerge from the axillary region between cotyledonary petiole and shoot primordium via direct organogenesis.

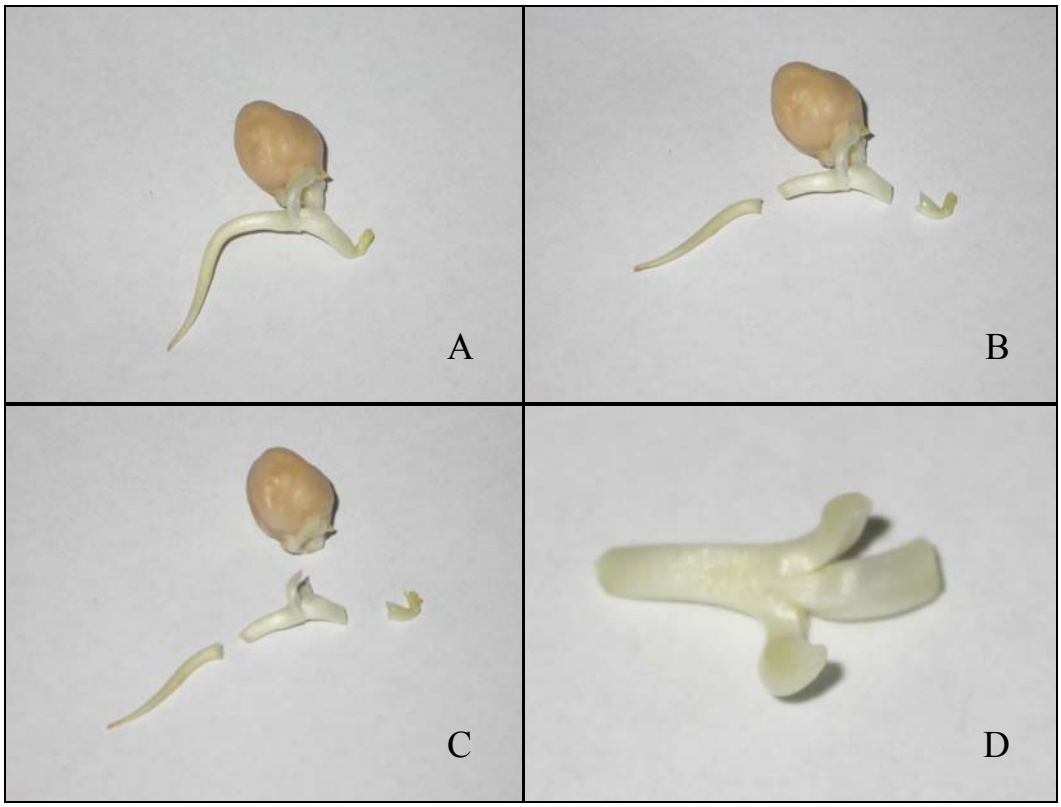


Figure 2.1. Cotyledonary node explant preparation. (A) Four days old etiolated chickpea seedling, (B) removal of radicle and emerging shoot primordium, (C) removal of cotyledons, (D) cotyledonary node explant.

2.2.1.3. Multiple Shoot Induction via Direct Organogenesis

For multiple shoot induction from CNs, the isolated explants were placed onto MS basal media supplemented with sucrose (3 %), agar (0.8 %) and BA. The explants were cultured in normal orientation (basal end in medium). Various concentrations of BA (1 mg/L, 3 mg/L and 10 mg/L) were used to find out the appropriate concentration at which healthy shoots are formed via direct organogenesis. The CNs were cultured for a period of one month at 24±2ºC under fluorescent lights with a 16/8 hour (light/dark) photoperiod. At the end of the month, number of shoots per CN was recorded.

2.2.1.4. Rooting of Regenerated Shoots

CNs on MS basal media enriched with 1 mg/L BA produced shoots. These shoots are removed from the explant and subcultured to MS basal media supplemented with sucrose, agar and IBA. Two different concentrations of IBA (0.1 mg/L and 0.5 mg/L) were applied to find out the rooting response of the shoots. The shoots were cultured for four weeks at 24±2ºC under light with a 16/8 hour (light/dark) photoperiod. At the end of the culture period, the rooting frequency and number of roots per shoot were recorded.

2.2.1.5. Lethal Dose Determination for Selective Agents

Selective agents are used to select the transformants after an event of transformation. Generally employed ones in plant transformations are antibiotics or herbicides. In this study, CNs isolated from 4 day old chickpea seedlings were subjected to selective agents to determine their effect on multiple shoot induction and to find out the lethal dose that can be used during transformation studies of chickpea. Different concentrations of two antibiotics (Kanamycin, Hygromycin) and two herbicides (PPT, Glyphosate) were used for this purpose. The cultivation of CNs with selective agents and 1 mg/L BA was done for 4 weeks and each week number of shoots per CN was recorded.

The shoots emerging from CN were also subjected to selective agents to determine their effect on rooting response of shoots. Cultivation of shoots with agents and 0.1 mg/L IBA was done for 4 weeks and rooting frequency and number of roots per shoot were recorded at the end of 4 weeks. Figure 2.2 summarizes the tissue culture studies done to determine lethal dose for selective agents.

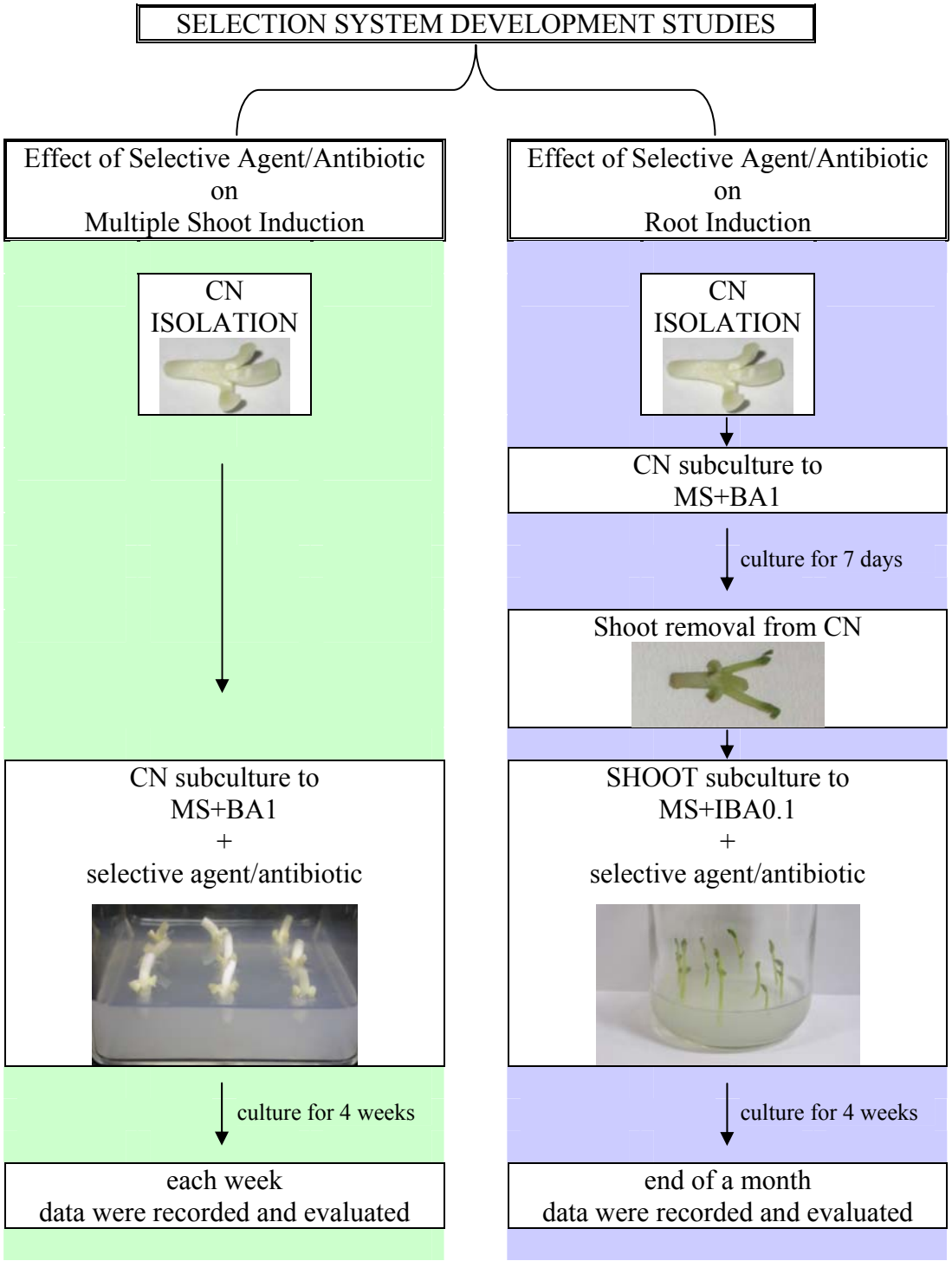


Figure 2.2. Tissue culture studies performed to develop an efficient selection system that can be used after transformation of chickpea. (CN: cotyledonary node; MS: Murashige and Skoog based media; BA1: 1 mg/L benzylaminopurine; IBA0.1: 0.1 mg/L indole butyric acid).

2.2.1.6. Effect of Antibiotics on Direct Organogenesis and Rooting

Different antibiotics are employed to inhibit bacterial growth after *Agrobacterium* mediated transformation of plants. To determine the effect of *Agrobacterium*-eliminating antibiotics on CN regeneration, the isolated explants were cultured with 1 mg/L BA and different concentrations of antibiotics (Carbenicillin, Cefotaxime, Augmentin and Timentin) for 4 weeks and each week number of shoots per CN was recorded.

The shoots emerging from CN were also subjected to 0.1 mg/L IBA and to the antibiotics to determine antibiotic effect on rooting response of shoots. Cultivation was done again for 4 weeks and rooting frequency and number of roots per shoot were recorded. Figure 2.2 summarizes the tissue culture studies done to determine the effect of *Agrobacterium*-elimination antibiotics on multiple shoot induction from CN and root induction from regenerated shoots.

2.2.1.7. Effect of Antibiotics on *Agrobacterium tumefaciens* KYRT1

The antibiotic used during selection of transformants should effectively inhibit bacterial growth. For determination of the effect of *Agrobacterium*-eliminating antibiotics on *Agrobacterium tumefaciens* strain KYRT1; two different methods were used which are antibiotic sensitivity testing and spectrophotometric determination of antibiotic effect on bacterial growth.

2.2.1.7.1. Antibiotic Sensitivity Testing with Agar Diffusion Method

To determine the effect of *Agrobacterium*-elimination antibiotics on bacteria; a modified method of agar diffusion is performed. An overnight grown culture of *A. tumefaciens* strain KYRT1 was first of all diluted to a concentration that is equal to 0.8 at 600 nm. And 100 µL of this bacterial culture was spread on solid YEB media with the aid of a glass spreader. The inoculated agar surface is air dried for

about 5 minutes. Then sterile filter paper discs containing specified concentrations of antibiotics were placed on the agar surface and they were secured by applying a little pressure with the aid of a sterile forceps. The plates were incubated at 28±1ºC for two days in an inverted position. Then diameter of each clear growth inhibition zone around each disc was measured with a millimeter ruler.

2.2.1.7.2. Antibiotic Effect on Bacterial Growth

The CNs isolated from 4 day old etiolated chickpea seedlings were incubated with *A. tumefaciens* KYRT1 for 40 minutes. After incubation, explants were rinsed in sterile distilled water, then they were blotted dry on sterile filter papers and placed onto MS media supplemented with different combinations and concentrations of antibiotics and 1 mg/L BA. The cultivation was carried out for 4 weeks at 24±2ºC under light with a 16/8 hour photoperiod. At the end of the cultivation period, each explant was immersed into liquid YEB in separate tubes and incubated for two days at 28±1ºC with a 180-200 rpm. Then the OD (optical density) of the YEB medium was recorded at 600 nm spectrophotometrically to determine the antibiotic effect on bacterial growth.

2.2.2. Transformation Studies

In transformation studies of this work, optimization of *Agrobacterium* mediated transformation of CN explants of chickpea (*C. arietinum* L.) cv. Gökçe was performed.

2.2.2.1. *Agrobacterium* Mediated Transformation of Cotyledonary Nodes

The utilized transformation procedure is adopted from Mahmoudian *et al.* (2002) and composed of several important stages such as induction of *Agrobacterium vir* genes, concentration of bacterial suspension by centrifugation, inoculation of CN explants with bacteria and co-cultivation of explants with bacteria (Figure 2.3).

A single colony of *A. tumefaciens* strain KYRT1 was grown overnight at 28±1ºC with 180-200 rpm shaking in 3 mL liquid YEB medium (Appendix F) supplemented with appropriate antibiotics. Then 400 mL of liquid YEB+MES medium containing 10 mM MES, 20 µM acetosyringone and necessary antibiotics at pH 5.6 was inoculated with 400-500 µL of this overnight grown initial culture of *A. tumefaciens* KYRT1. The culture was grown overnight at 28±1ºC with 180-200 rpm till OD at 600 nm reaches to 0.8. Then the bacterial culture was centrifuged at 1500 g for 15 minutes at 4 ºC. Then the bacterial cells in pellet were resuspended in MMA medium (Appendix F) which contains 200 µM acetosyringone till OD at 600 nm reaches to 2.4-2.5. Finally the bacterial suspension was incubated at 24±2ºC under fluorescent lights for 1 hour and then used for transformation of CN explants (Figure 2.3).

CN explants isolated from 4 day old etiolated chickpea seedlings were used for *Agrobacterium* mediated transformation. The explants were inoculated with bacteria in MMA suspension for 40 minutes at 24±2ºC under fluorescent lights. Then the explants were blotted dry on sterile filter papers and placed onto MS media containing 1 mg/L BA (Appendix C) for co-cultivation. Co-cultivation of CN explants with bacteria was performed for 4 days at 24±2ºC under light with a 16/8 hour photoperiod (Figure 2.3).

To determine the effect of mechanical injury on transformation efficiency, CN explants were injured with a fine glass needle. Injury was performed by poking 6 to 8 times at the each axillary region of cotyledonary petiole where the secondary shoots develop. Then these injured explants were inoculated with bacteria in MMA suspension for 40 minutes at 24±2ºC under light. Then the explants were blotted dry on sterile filter papers and placed onto MS media containing 1 mg/L BA for co-cultivation for 4 days.

Vacuum infiltration can be applied to bring bacteria and plant tissue in closer contact. For the same purpose vacuum infiltration was coupled to the conventional

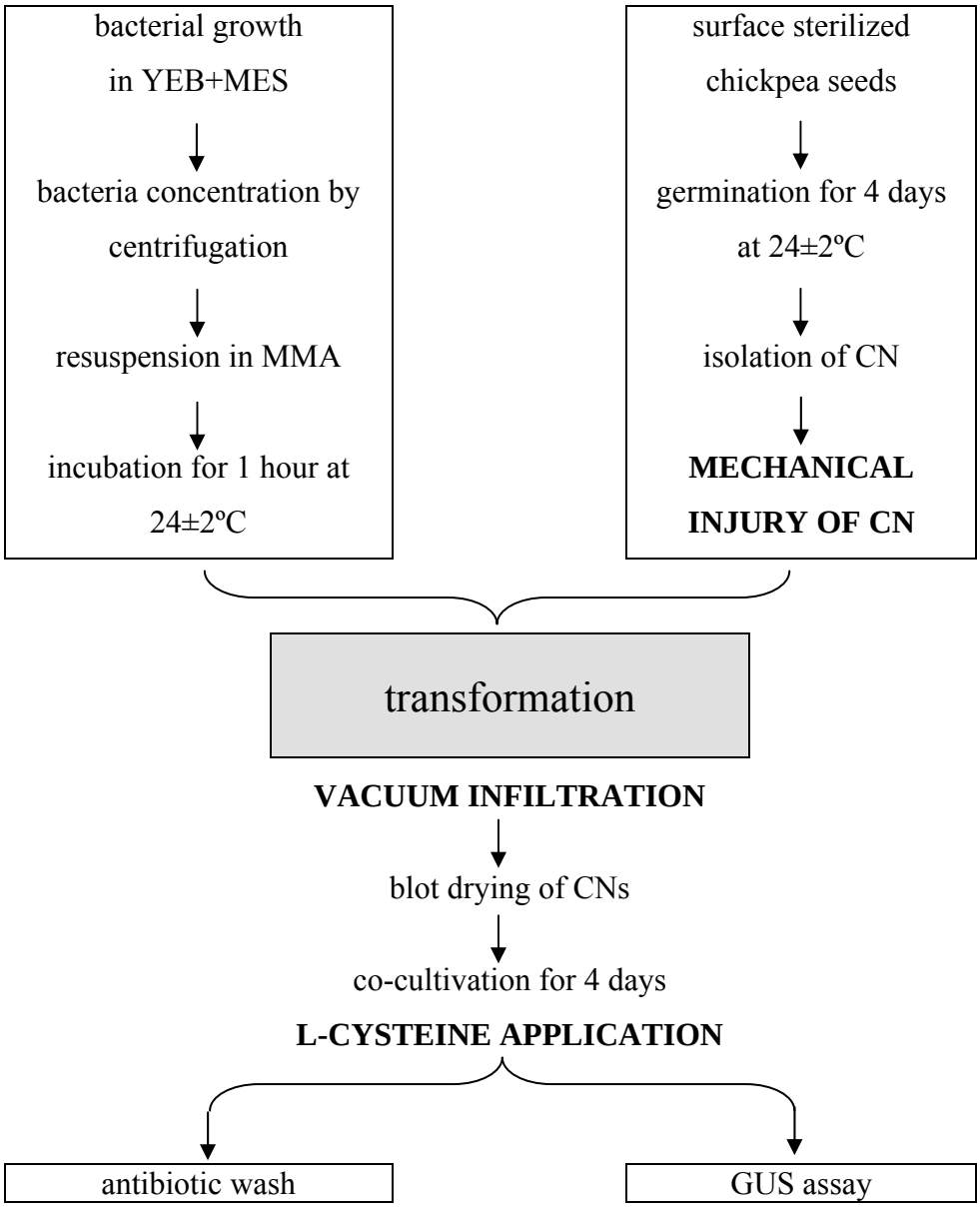


Figure 2.3. Transformation procedure performed during this study. Texts in uppercase and bold indicate the treatments performed to determine their effects on transformation efficiency. See Appendix F for YEB+MES and MMA; and Appendix C for antibiotic wash. (CN: cotyledonary node)

Agrobacterium mediated transformation in this study. For determination of infiltration effect on transformation efficiency, isolated and mechanically injured CN explants were vacuum infiltrated during inoculation of explants with bacteria in MMA suspension. Vacuum infiltration was performed for 40 minutes at evacuation pressures of 200, 400 and 600 mmHg. Then the explants were blotted dry on sterile filter papers and placed onto MS media containing 1 mg/L BA for co-cultivation for 4 days.

For determination of L-cysteine effect on transformation efficiency, various concentrations (100, 200, 400, 800 and 1200 mg/L) of L-cysteine was added to the co-cultivation medium that is used after transformation of chickpea CNs. At the end of co-cultivation period, some of the CNs was used for GUS histochemical staining assay and the others were rinsed in liquid MS medium containing antibiotics.

At the end of the co-cultivation period, for each treatment, some of the CNs, which were randomly selected, was used for GUS histochemical staining assay and the others were rinsed in liquid MS medium containing 400 mg/L Cefotaxime and 200 mg/L Carbenicillin to perform antibiotic wash (Appendix C) and to get rid of bacteria. Transformation procedure performed during this study is summarized in Figure 2.3.

2.2.2.2. Development and Selection of Putative Transgenics

Regeneration and selection are important steps in development of transgenic plants after an event of transformation. During this study, multiple shoot induction from transformed CNs of chickpea was performed on MS media supplemented with 1 mg/L BA and rooting of regenerated shoots was performed on MS media supplemented with 0.1 mg/L IBA. Selection of transformed shoots was performed with the aid of 100 mg/L Kanamycin and this concentration was decreased to 50 mg/L during root induction.

Shoots, emerging from axillary meristems of transformed CNs, were subcultured to selection media (Appendix C) at the end of 4 days of co-cultivation period. Selection was applied for a total of 6 weeks with nodal subculture at the third week. During nodal subculture, necrosed or decolorized regions were discarded and only green nodes or shoot tips were subcultured to fresh selection media. After 6 weeks of selection, again only green nodes or shoot tips were subcultured to rooting media (Appendix C). The cultivation on rooting media was performed for 3 weeks.

2.2.2.3. GUS Histochemical Assay and Data Evaluation

For the analysis of putative transgenics, GUS histochemical staining was used at different stages of regeneration and selection. Figure 2.4 summarizes the analysis performed during this study.

Histochemical GUS staining was performed according to the method of Jefferson (1987) in order to monitor the transient gene expression. Four days after transformation the CNs; and nine and sixteen days after transformation only the shoots (Figure 2.4), were stained for transient GUS activity in GUS substrate solution (Appendix G) containing 1 mM chromogenic substrate X-GlcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid).

All CNs and shoots were assayed by incubating them at 37ºC for 2 days in GUS substrate solution. At the end of the incubation period, explants were transferred to GUS fixative solution (Appendix G). After a minimum of 4 hours in fixative, the solution was replaced with 50 % ethyl alcohol for decolorization of explants. After 15 minutes in 50 % ethyl alcohol, explants were transferred to 100 % ethyl alcohol for further decolorization overnight. Then the explants were transferred to GUS fixative solution for preservation for several months. Finally GUS expressing regions on explants were examined under microscope and photographed.

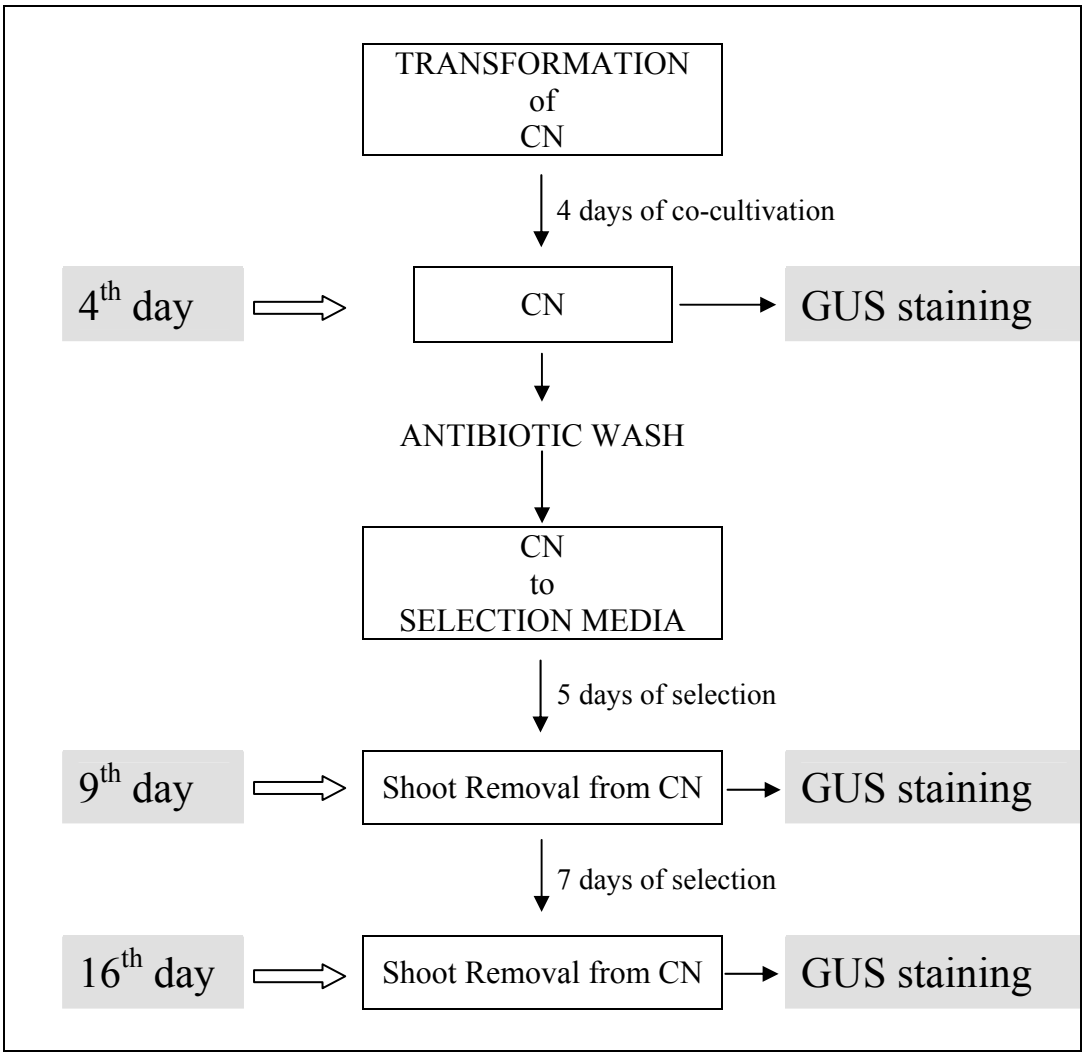


Figure 2.4. Histochemical GUS analysis performed to optimize transformation procedure. See Appendix C for selection media and antibiotic wash. (CN: cotyledonary node).

Results of histochemical GUS staining, that is performed four days after transformation, were recorded as percent of CN explants exhibiting GUS activity and as number of shoots exhibiting GUS activity per CN. On the other hand, results of GUS staining performed nine and sixteen days after transformation, were recorded as GUS expressing area relative to the total surface area of tissues.

This value of relative GUS positive area is measured by image analysis system (Zeiss® KS300).

2.2.3. Statistical Analysis

All of the statistical analyses were carried out by using Minitab 13.0 software. Means and standard error of means (SEM) were calculated by using this software. One-way analysis of variance (ANOVA) was used to detect variances in means and was used to investigate the relationship between response variables.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Tissue Culture Studies

Optimization of a selection and regeneration system for shoots, emerging from cotyledonary nodes (CN) of chickpea cultivar Gökçe, was performed in tissue culture part of this study.

CNs, isolated from germinating seeds, were used as explant throughout both the tissue culture and transformation studies. Main advantage of CN is that it was found to be best responding explant in tissue culture; and therefore, transformation procedures using CN were developed for various legumes such as soybean (Hinchee *et al.*, 1988; Olhoft and Somers, 2001), lentil (Mahmoudian *et al.*, 2002), blackgram (*Vigna mungo* L.) (Saini *et al.*, 2003) and pigeonpea (Thu *et al.*, 2003). Another advantage of CN is that plant transformation starting with CN does not require a continuous culture of the plant material.

In this study, CN explants were isolated from four days old etiolated and *in vitro* germinated chickpea seedlings. They were placed in normal orientation (basal end in medium) into media for shoot induction. Then the shoots emerging from axillary region of CN were removed after 7 to 8 days and subcultured into media for *in vitro* root induction. Root formation occurs in 14 to 15 days. Chickpea regeneration *in vitro* takes about a total of 25 to 28 days (Figure 3.1).

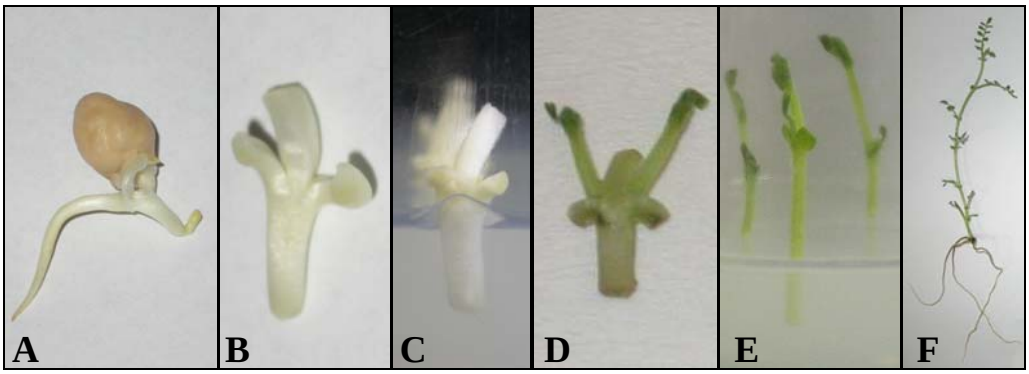


Figure 3.1. Chickpea regeneration *in vitro* via direct organogenesis. Representative figures of (A) a chickpea seedling, (B) CN explant, (C) CN in media, (D) shoots formed on CN, (E) shoots in media, and (F) a regenerated chickpea plant with roots (∼28 days) are displayed.

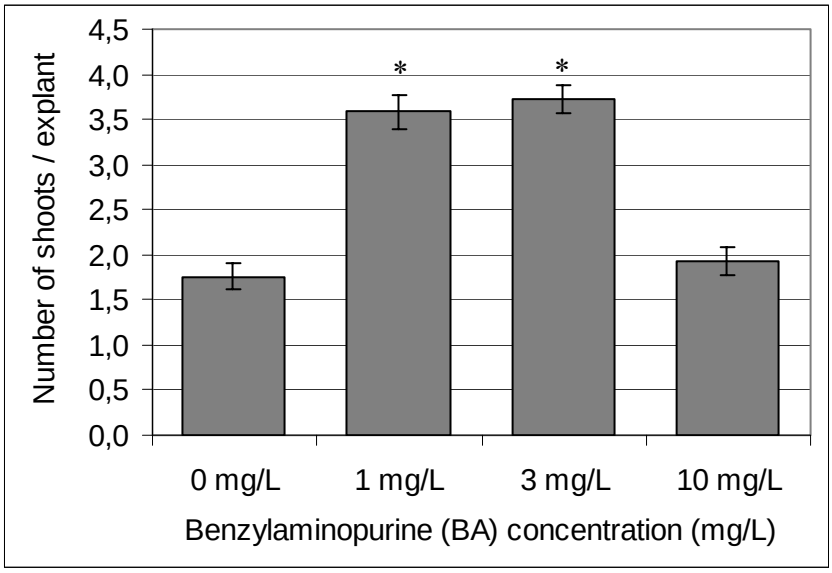
3.1.1. Multiple Shoot Induction via Direct Organogenesis

The CN explants were cultured in BA to perform multiple shoot induction via direct organogenesis from axillary meristems. In chickpea, shoot regeneration is dependent on genotype, explant type and BA concentration (Polisetty *et al.*, 1997); therefore, BA concentration optimization should be performed for a specific genotype. Various concentrations of BA (1 mg/L, 3 mg/L and 10 mg/L) were used in this study to find out the appropriate concentration at which healthy multiple shoots are formed from CN explants of cultivar Gökçe. The explants were cultured for a month and number of shoots per CN was recorded at the end of the culture period. Each set of experiment was carried out with 15 explants for each treatment and results are total of two replicates.

After the report of Polisetty *et al.* (1997) indicating the potential of BA for inducing multiple shoots in chickpea; Krishnamurthy *et al.* (2000) and Polowick *et al.* (2004) used BA for shoot regeneration in chickpea. Gulati *et al.* (2001) also

employed BA to induce shoot formation from lentil CN explants. They used 8.8 μM (∼2 mg/L) BA to regenerate 4 to 5 shoots from explants.

Multiple shoot induction from chickpea CN using BA treatment is represented in Figure 3.2. In our study, number of shoots per CN was increased to 3.59 when CN was cultured on 1 mg/L BA. This value was statistically significant ($p < 0.05$) when compared to growth regulator free MS media, which produced 1.76 shoots per CN (Figure 3.2). Although Kar *et al.* (1996) reported 3 mg/L BA being superior over 1 mg/L BA using chickpea embryo axes as explant, in our study increased BA concentrations (3 mg/L) did not cause a significant increase in shoot number compared to 1 mg/L BA application. Therefore we preferred 1 mg/L BA for multiple shoot induction.



Mean values, SEM and significant values are tabulated in Table H.1 in Appendix H.

Figure 3.2. Multiple shoot induction from chickpea CNs using various BA concentrations. Vertical bars and * indicate SEM (standard error of mean) and significant values ($p < 0.05$) compared to control (0 mg/L), respectively. (n=30 for each treatment).

Even a much higher concentration of BA (10 mg/L) exerted no promotional effect on multiple shoot formation (Figure 3.2). 10 mg/L BA induces formation of callus like structures and few shoots emerge from these structures. A similar observation of callus formation was reported by Thu *et al.* (2003) in pigeonpea cotyledonary nodes. In our study, average number of shoots formed per CN at 10 mg/L BA was found to be 1.93 which is not significantly different from control.

During both tissue culture and transformation studies, 1 mg/L BA was employed to induce shoot regeneration from CN of chickpea.

3.1.2. Rooting of Regenerated Shoots

Whole plant regeneration is completed when the regenerated shoots are induced to form roots. Chickpea shoots emerging from axillary region of CN were removed after 7 to 8 days and subcultured to root induction media. Tewari-Singh *et al.* (2004) and Sarmah *et al.* (2004) employed IBA for rooting of chickpea shoots. In this study IBA was also used for root induction.

Two different concentrations of IBA (0.1 mg/L and 0.5 mg/L) were applied to find out the rooting response of the shoots. At the end of the culture period for one month, number of roots per shoot and the rooting frequency as percent of shoots with roots were recorded. Also the representative photographs of shoots and roots were taken (Figure 3.3). It is found that IBA at a concentration of 0.1 mg/L is appropriate to induce rooting. At 0.1 mg/L IBA, 81.67 % of the shoots rooted with an average of 4.39 roots per shoot (Figure 3.4). Both values for rooting frequency and number of roots per CN are significantly different from rooting at IBA free media. Increasing the IBA concentration (0.5 mg/L) did not further increase the rooting frequency and number of roots per shoot.

Therefore, 0.1 mg/L IBA was employed for root induction from regenerated shoots during tissue culture and transformation studies.

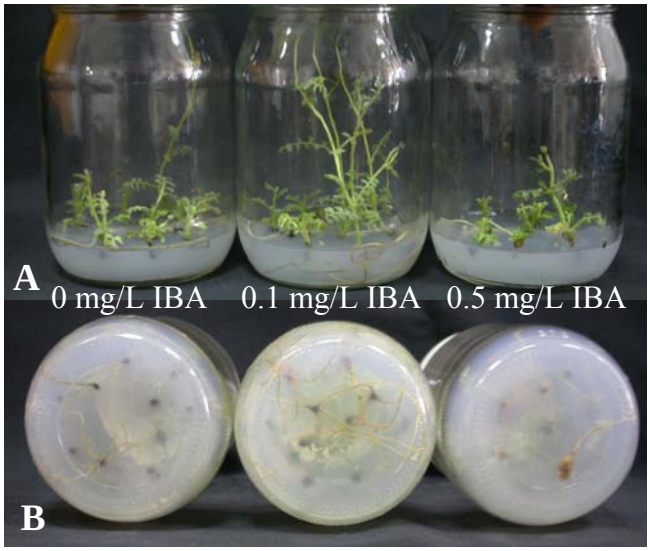
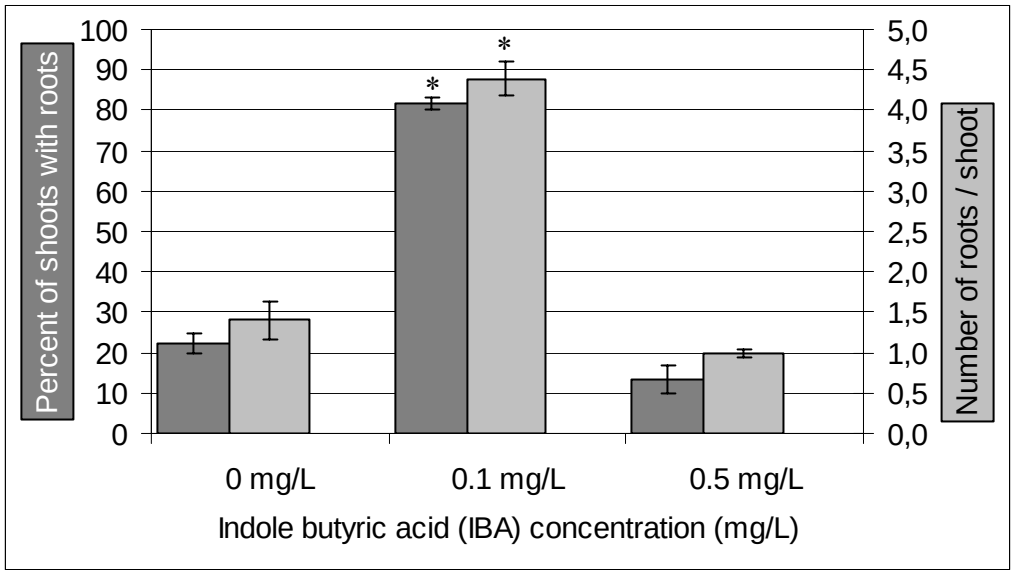


Figure 3.3. Root induction with 0, 0.1 and 0.5 mg/L IBA. (A) Appearance of shoots, (B) appearance of roots after four weeks of culture.



Mean values, SEM and significant values are tabulated in Table H.2 in Appendix H.

Figure 3.4. Root induction from chickpea shoots using two different IBA concentrations. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to control (0 mg/L), respectively. (n=22 for each treatment).

3.1.3. Lethal Dose Determination for Selective Agents

Plant genetic transformation technologies rely upon the selection and recovery of transformed cells or tissues. Selectable marker genes are used to identify the rare putative transgenics that have taken up foreign DNA. Genes encoding antibiotic resistance and herbicide tolerance are widely employed for this purpose in transgenic plant production (Park *et al.*, 1998; Penna *et al.*, 2002; de Vetten *et al.*, 2003; Miki and McHugh, 2004). In this study, chickpea CNs and shoots were subjected to different selective agents to determine their effect on multiple shoot and root induction. For this purpose different concentrations of two antibiotics (Kanamycin, Hygromycin) and two herbicides (PPT, Glyphosate) were used.

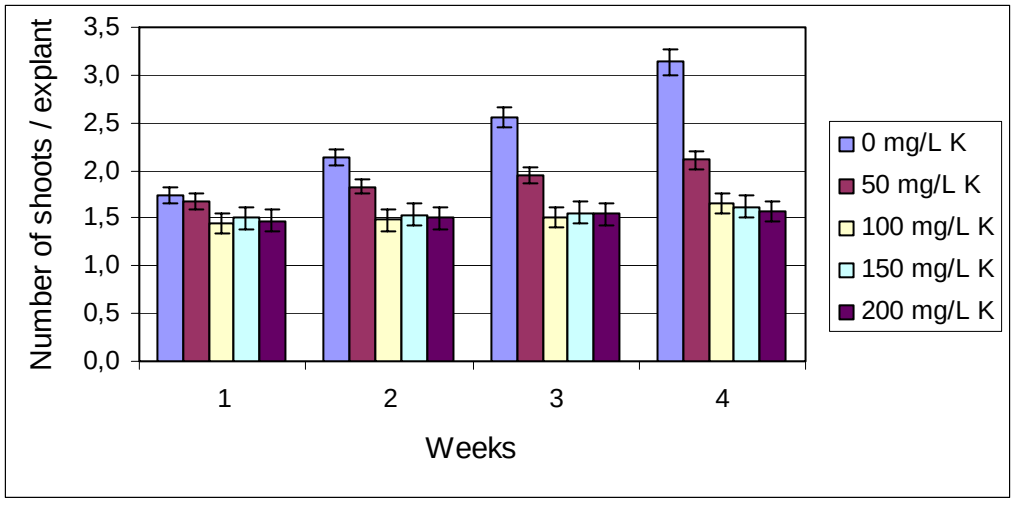
3.1.3.1. Effect of Selective Agents on Multiple Shoot Induction

Optimization of selective agent concentration to be used in gene transfer to chickpea CNs was performed prior to transformation studies. The concentrations used for Kanamycin are 50, 100, 150, and 200 mg/L; for Hygromycin are 10, 20, 30, and 50 mg/L; for PPT are 3, 5, 7, and 10 mg/L; and for Glyphosate are 1, 5, 10, and 25 mg/L.

CNs with selective agents and 1 mg/L BA was cultivated for 4 weeks and each week number of shoots per CN was recorded. At the end of the cultivation period, the explants were photographed. All experiments were carried out together with controls which are selective agent free MS based media containing only 1 mg/L BA. Two independent sets of experiments were performed with 150 explants in each set containing five different treatments (n=60 for each treatment).

Kanamycin is the most widely used antibiotic in the selection of transgenics. Concentration of Kanamycin used in gene transfer to legumes such as pea, peanut, mungbean and *Medicago* species varies around 50 and 100 mg/L (Polowick *et al.*, 2000; Sharma and Anjaiah, 2000; Jaiwal *et al.*, 2001; Ding *et al.*, 2003).

Effect of Kanamycin on multiple shoot induction performed in this study is displayed in Figure 3.5. In this study, 100 mg/L and even 50 mg/L Kanamycin was determined to be appropriate for use in chickpea CN transformation events (Figure 3.5). Explants cultured on Kanamycin free MS media produced 2.56 and 3.13 shoots per CN after 3 and 4 weeks, respectively. However explants cultured on 50 mg/L Kanamycin containing MS media produced 1.94 and 2.11 shoots, which are significantly lower than control, after 3 and 4 weeks, respectively. Even after two weeks of culture under 50 mg/L Kanamycin stress, number of shoots per CN, were significantly ($p < 0.05$) lower when compared to control (Figure 3.5). This result is consistent with the reports of Fontana *et al.* (1993), Kar *et al.* (1997), and Polowick *et al.* (2004), in which 50 mg/L Kanamycin was preferred for transgenic chickpea selection. High concentrations (100 mg/L or more) of Kanamycin reduced multiple shoot induction; however, shoots still regenerated from axillary meristems of CN. Approximately, 1.5 shoots per explant were formed at these high concentrations but these shoots were decolorized or pale yellow in color.



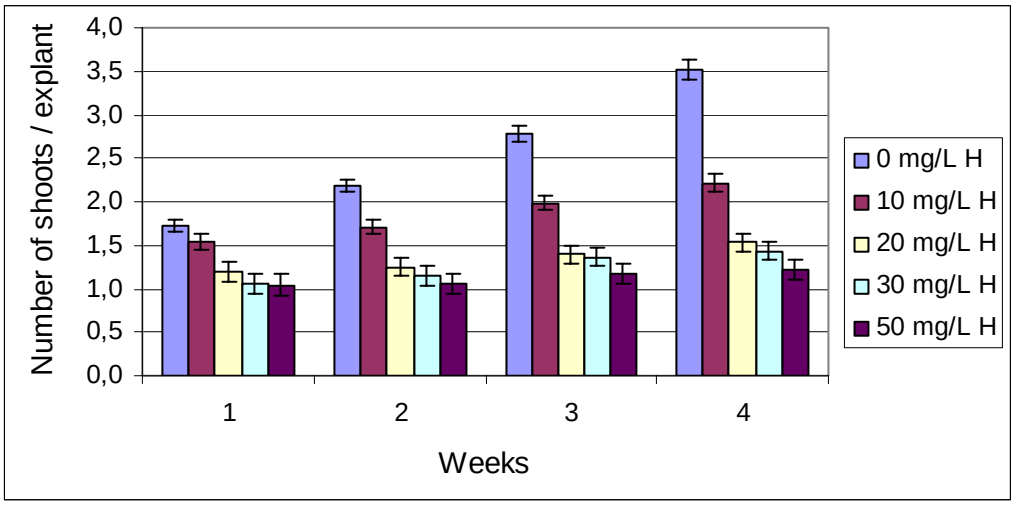
Mean values, SEM and significant values are tabulated in Table H.3 in Appendix H.

Figure 3.5. Effect of Kanamycin (K) on multiple shoot induction. Kanamycin free medium (0 mg/L K) was used as control. Vertical bars indicate SEM. (n=60 for each treatment).

All the shoots regenerated under Kanamycin application were also pale yellow in color. Color loss may be the criteria in transgenic selection but an average of 1.5 shoots per CN may lead to high numbers of escapes after transformation. High concentrations may be employed to decrease the number of escapes. Therefore, 100 mg/L Kanamycin is appropriate for use in chickpea CN transformations. Krishnamurthy *et al.* (2000) preferred 100 mg/L which was also determined to be effective in our study; but Sarmah *et al.* (2004) preferred 200 mg/L Kanamycin in the selection of transgenic chickpea shoots.

Hygromycin is another antibiotic frequently used in plant transformations. The effect of Hygromycin on multiple shoot induction from chickpea CNs, determined in this study, was represented in Figure 3.6. Since concentrations of 10 mg/L or higher Hygromycin were used in the literature for transformation of legumes, different concentrations ranging between 10 to 50 mg/L were investigated in this study. Hygromycin at concentration of 10 mg/L is determined to be inhibiting multiple shoot induction (Figure 3.6). Explants cultured on MS media containing 10 mg/L Hygromycin produced 1.71, 1.98 and 2.22 shoots per CN after 2, 3 and 4 weeks of culture, respectively. These values are significantly ($p < 0.05$) different from shoot numbers produced by control explants. This result is consistent with the report of Olhoft *et al.* (2003) in which 10 mg/L Hygromycin is employed for selection after soybean CN transformation.

In our study, high concentrations (20 mg/L or more) of Hygromycin lead to inhibition of multiple shoot regeneration even after 1 week of culture. The shoots produced at these concentrations were dwarf and decolorized. Color loss and shoot necrosis may provide ease in selection of transgenics during tissue culture therefore, 20 mg/L Hygromycin may be employed in chickpea CN transformation events. Similarly, Livingstone and Birch (1999), and Magbanua *et al.* (2000) employed 20 mg/L Hygromycin for selection in peanut transformation studies.

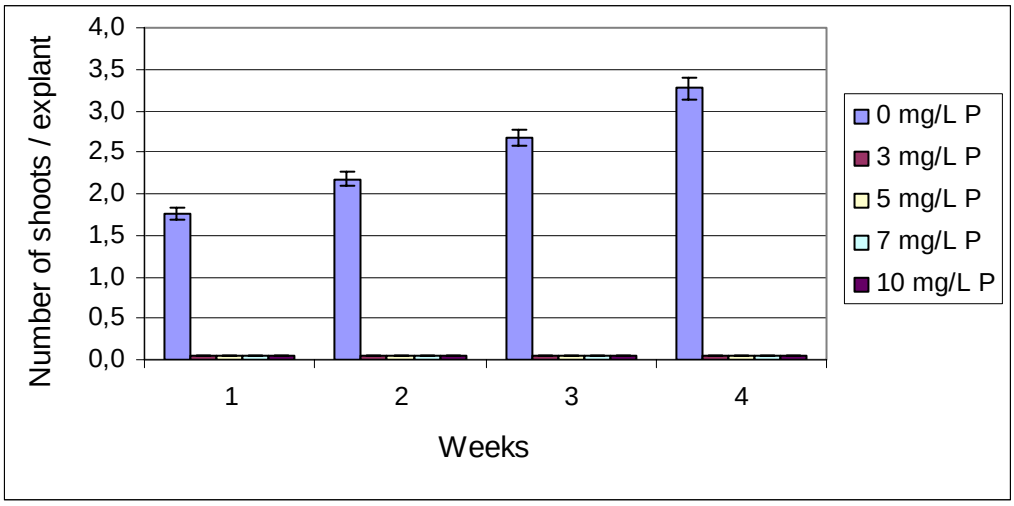


Mean values, SEM and significant values are tabulated in Table H.4 in Appendix H.

Figure 3.6. Effect of Hygromycin (H) on multiple shoot induction. Antibiotic free media (0 mg/L H) were used as control. Vertical bars indicate SEM. (n=60 for each treatment).

PPT is the most widely employed herbicide for the selection of transgenics. It is highly effective and nearly all plants especially legumes lack background activity of PPT tolerance. In the literature different concentrations of PPT were used for selection. For soybean transformation studies, Zhang *et al.* (1999) used 5 mg/L; on the other hand Zeng *et al.* (2004) employed 8 mg/L PPT. Krishnamurthy *et al.* (2000) used 10 mg/L PPT for transgenic chickpea selection.

Effect of PPT on chickpea multiple shoot induction is displayed in Figure 3.7. PPT even at the lowest concentration (3 mg/L) totally inhibited the shoot regeneration; in other words chickpea CNs produced no shoots under the stress of 3 mg/L PPT. Lethal effect of 3 mg/L PPT was observed even after one week of culture (Figure 3.7). The explants with no shoots regenerated on them were also totally decolorized lacking chlorophyll synthesis. This result is consistent with the reports of Tewari-Singh *et al.* (2004), and Senthil *et al.* (2004), in which PPT optimizations for chickpea were performed.



Mean values, SEM and significant values are tabulated in Table H.5 in Appendix H.

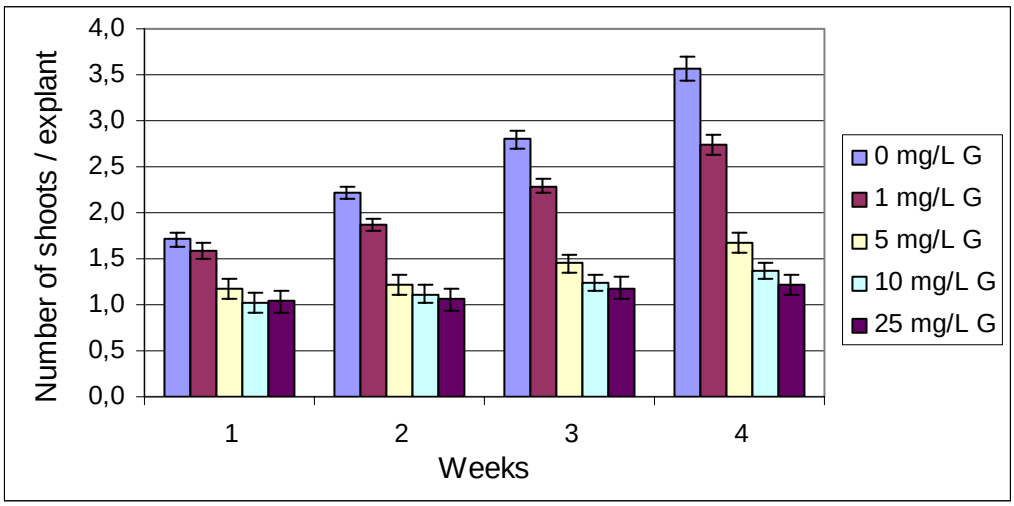
Figure 3.7. Effect of PPT (P) on multiple shoot induction. Herbicide free media (0 mg/L P) were used as control. Vertical bars indicate SEM. (n=60 for each treatment).

Tewari-Singh *et al.* (2004) observed maximum suppression of green shoot development from embryo explants under the stress of 2 mg/L PPT. Senthil *et al.* (2004) applied various concentrations of PPT on shoots derived from chickpea CNs. They concluded that 0.5 mg/L PPT had little effect on shoot growth but 1 or 2.5 mg/L PPT caused shoot necrosis. Therefore, it can easily be concluded that 3 mg/L PPT, determined in this study, can be employed in selection of transgenics after chickpea CN transformations.

Another herbicide used in selection of transgenics is Glyphosate and effect of it on multiple shoot induction from chickpea CNs is displayed in Figure 3.8. Various concentrations of Glyphosate were used for various plants such as 0.1 mM and 2 mM for wheat, 5 mM for maize, and 0.2 mM for tobacco (Hu *et al.*, 2003; Howe *et al.*, 2002; Ye *et al.*, 2001). In our study Glyphosate at concentration of 1 mg/L significantly ($p < 0.05$) decreased the number of shoots regenerated from chickpea CN after two weeks of culture.

CNs produced significantly low number of shoots under 5 mg/L Glyphosate stress even after one week of culture. Shoots regenerated under the stress of high concentrations (5 mg/L or more) of Glyphosate were shorter and less developed compared to ones regenerated on herbicide free MS based media. Such high concentrations of herbicide may suppress the effect of BA therefore, shoot buds were formed but they did not develop into whole adventitious shoots. Glyphosate at concentrations of 5 mg/L can be employed in transformation of chickpea CNs.

Effects of all selective agents on multiple shoot induction from chickpea CNs are also displayed with photographs, taken at the end of 4 weeks of culture, in Figure 3.9 and Figure 3.10.



Mean values, SEM and significant values are tabulated in Table H.6 in Appendix H.

Figure 3.8. Effect of Glyphosate (G) on multiple shoot induction. Herbicide free media (0 mg/L G) were used as control. Vertical bars indicate SEM. (n=60 for each treatment).

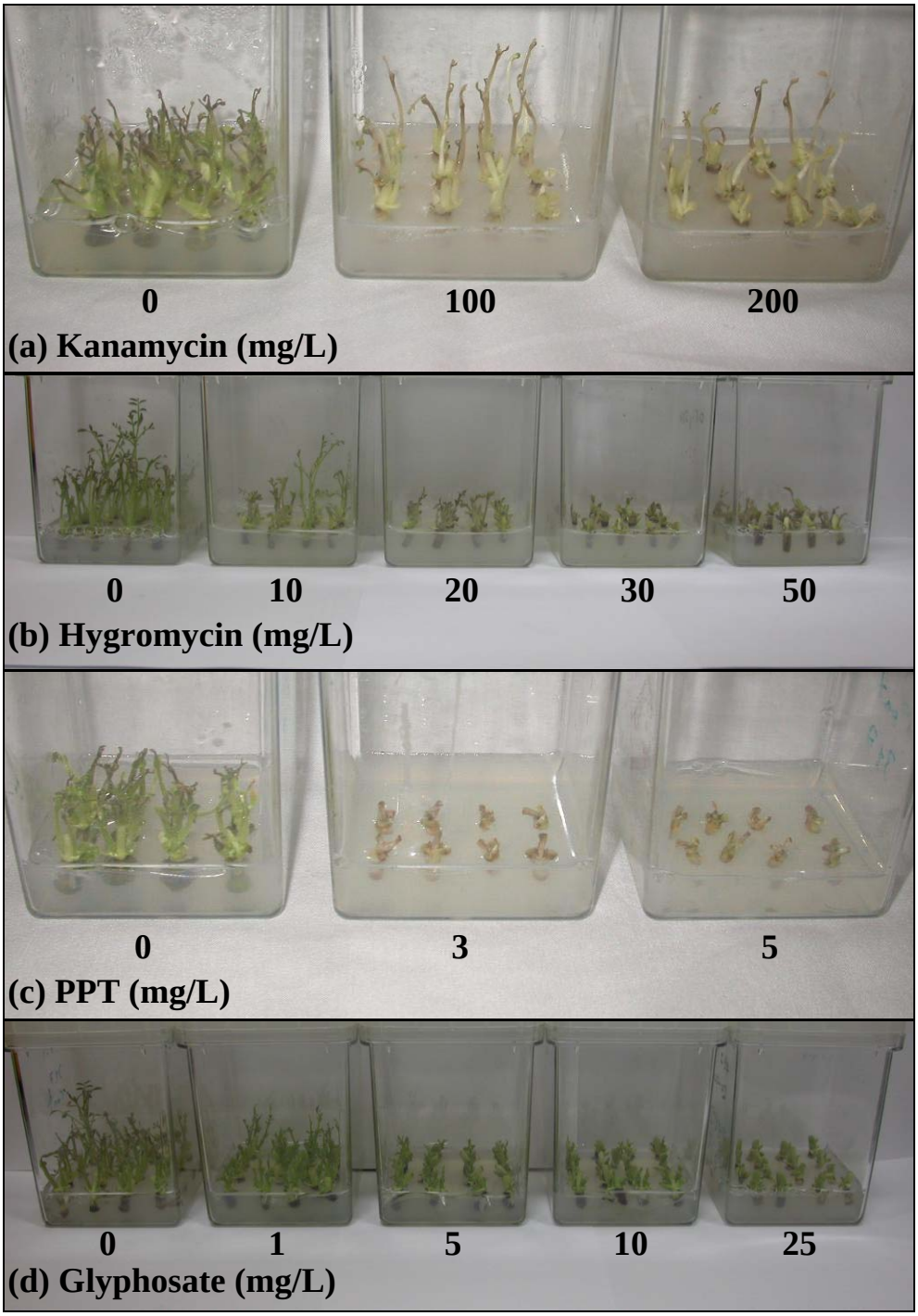


Figure 3.9. Effect of selective agents on multiple shoot induction. Explants were photographed at the end of 4 weeks of culture.

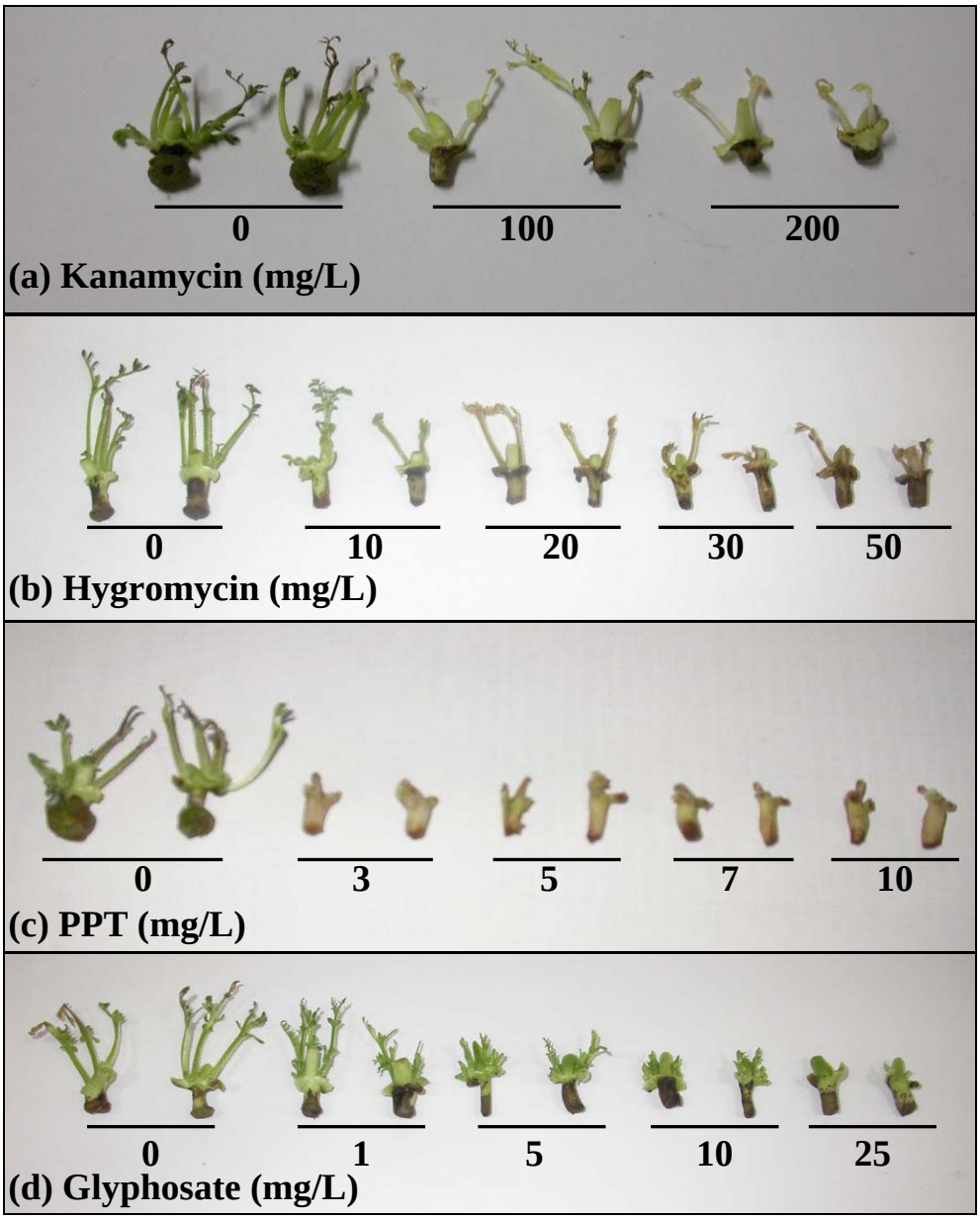


Figure 3.10. Effect of selective agents on multiple shoot induction from chickpea CN. The explants were photographed after 4 weeks of culture.

3.1.3.2. Effect of Selective Agents on Root Induction

During the course of the tissue culture studies on chickpea, effects of selective agents on root induction from regenerated shoots were also investigated. The types and concentrations of antibiotics and herbicides were same as the ones employed for investigation of their effect on multiple shoot induction.

CNs, cultured on MS based media containing 1 mg/L BA, produced shoots within 7 to 8 days. And these regenerated shoots were removed from the explant and subcultured onto MS based media containing 0.1 mg/L IBA for root induction. Selective agents were added to media to find out their effect on rooting of shoots.

The shoots were cultured with selective agents and 0.1 mg/L IBA for 4 weeks and at the end of the culture period, numbers of shoots having roots and numbers of roots per shoot were recorded. Also the explants and jars were photographed. All experiments were carried out with controls which were selective agent free MS based media containing only 0.1 mg/L IBA. Each set was performed with duplicates and a minimum of 10 explants for each treatment (n=20 for each treatment).

The antibiotics used were Kanamycin and Hygromycin. Even the lowest concentrations of Kanamycin (50 mg/L) (Figure 3.11) and Hygromycin (10 mg/L) (Figure 3.12) totally inhibited the root induction. Percent of shoots having roots was around 80 to 85 % and number of roots per shoot was around 4.5 for the explants cultured on antibiotic free media. But both parameters evaluated were zero for all concentrations of Kanamycin and Hygromycin (Figure 3.11 and Figure 3.12). Complete inhibition of rooting under Kanamycin stress is consistent with the report of Estopa *et al.* (2001) in which non-transgenic carnation shoot tips were cultured for root induction with 150 mg/L Kanamycin.

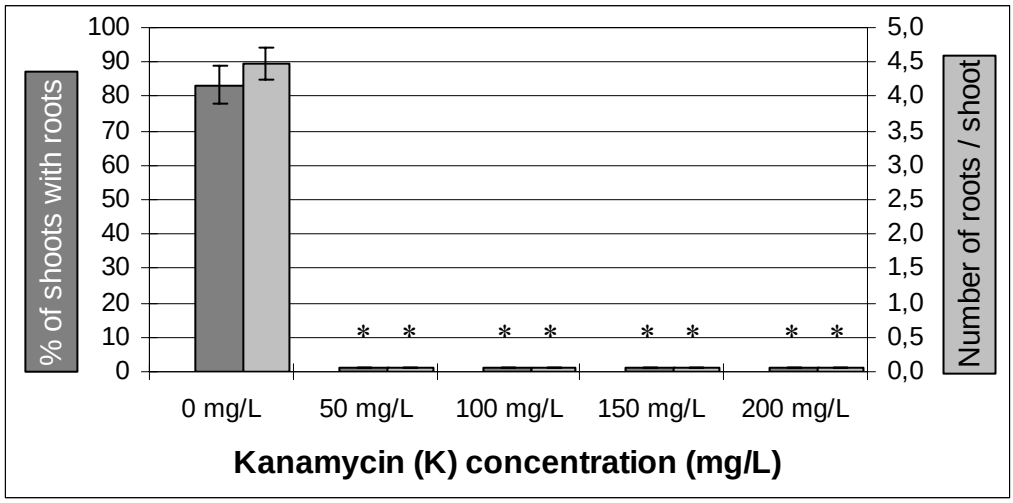


Figure 3.11. Effect of Kanamycin on root induction from regenerated shoots. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to control (0 mg/L), respectively. (n=18 for each treatment).

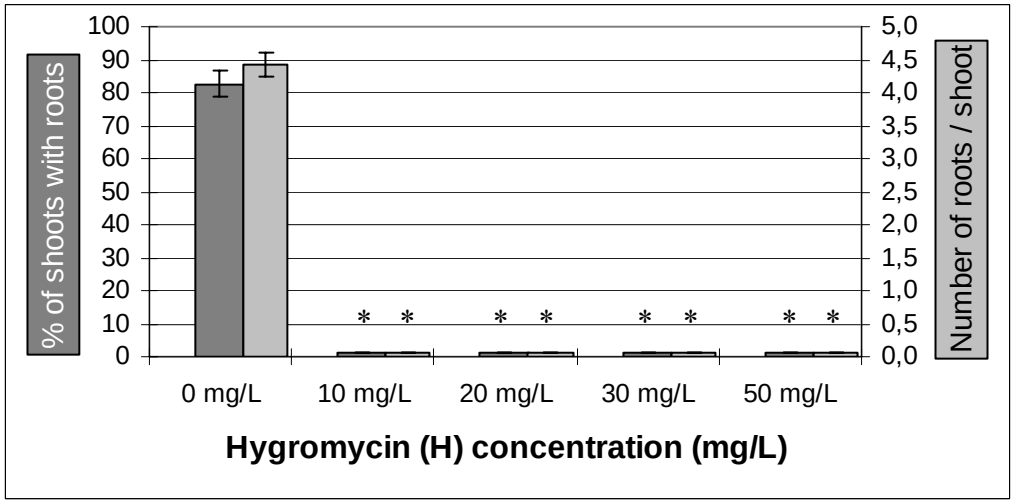


Figure 3.12. Effect of Hygromycin on root induction from regenerated shoots. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to control (0 mg/L), respectively. (n=21 for each treatment).

The shoots after one month of culture in rooting media were also decolorized or pale yellow in color under Kanamycin stress and they were totally brown and necrosed under Hygromycin stress (Figure 3.15 and Figure 3.16). 50 mg/L Kanamycin and 10 mg/L Hygromycin can be employed for rooting of transgenic chickpea shoots after an event of transformation.

The herbicides evaluated for their effect on root induction were PPT and Glyphosate. The lowest concentrations of PPT (3 mg/L) (Figure 3.13) and Glyphosate (1 mg/L) (Figure 3.14) totally inhibited the root induction. Percent of shoots with roots and number of roots per shoot were zero for all concentrations of both herbicides. All PPT concentrations caused total shoot necrosis besides inhibition of rooting after one month of culture (Figure 3.15c and Figure 3.16c). The shoots cultured under Glyphosate stress were dwarf with no root formation even at the lowest concentration. 3 mg/L PPT and 1 mg/L Glyphosate can be employed for rooting of transgenic chickpea shoots after an event of transformation.

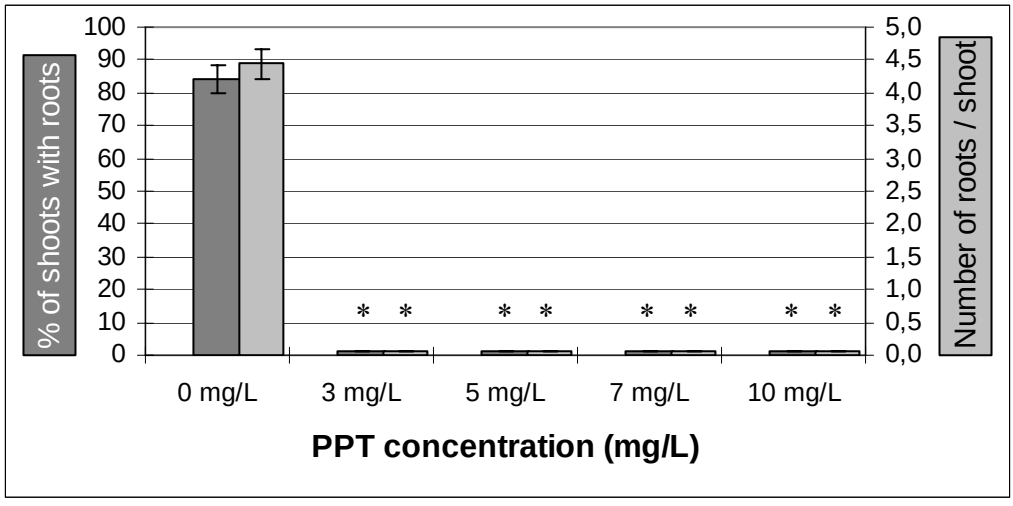


Figure 3.13. Effect of PPT on root induction from regenerated shoots. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to 0 mg/L (control), respectively. (n=18 for each treatment).

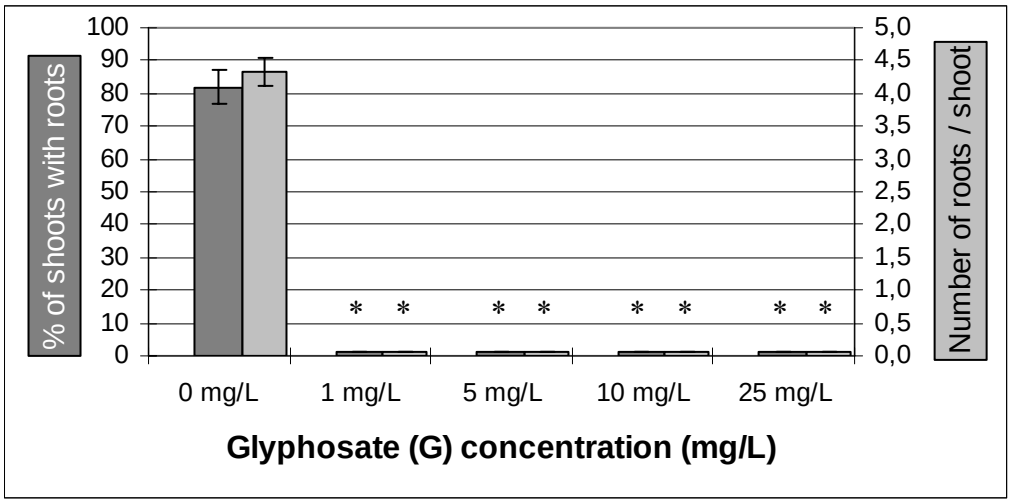


Figure 3.14. Effect of Glyphosate on root induction from regenerated shoots. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to control (0 mg/L), respectively. (n=22 for each treatment).

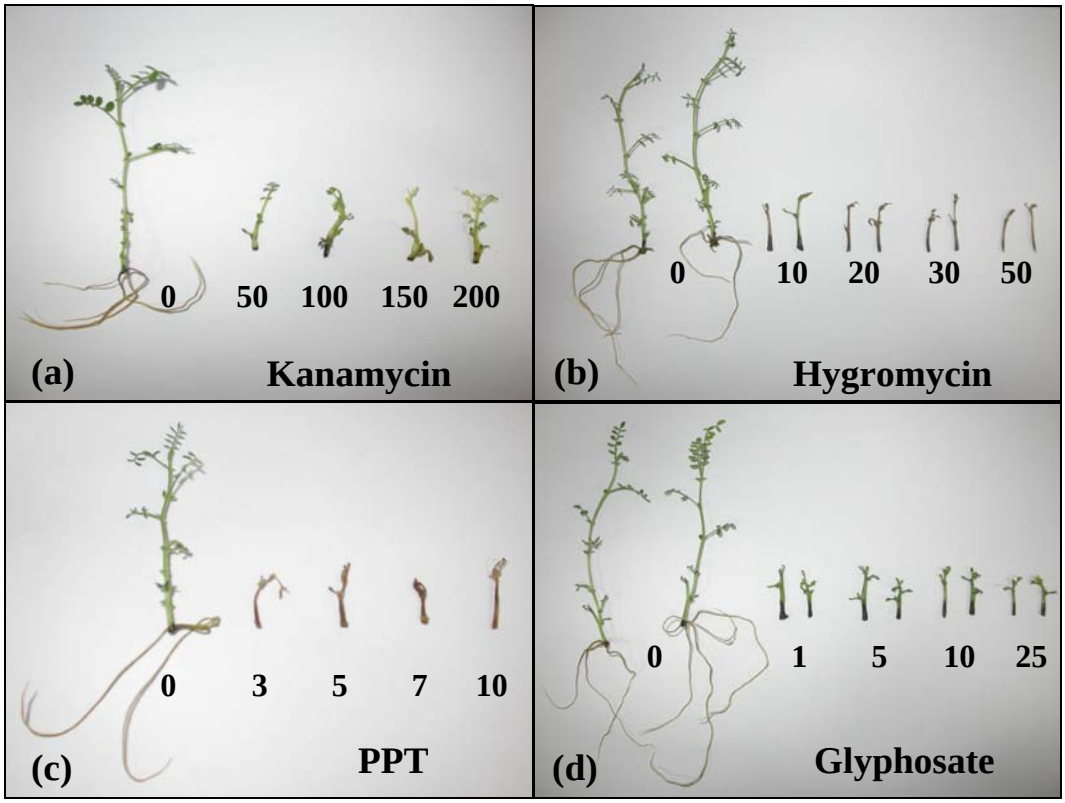


Figure 3.15. Effect of selective agents on root induction from chickpea shoots. The explants were photographed after 4 weeks of culture.

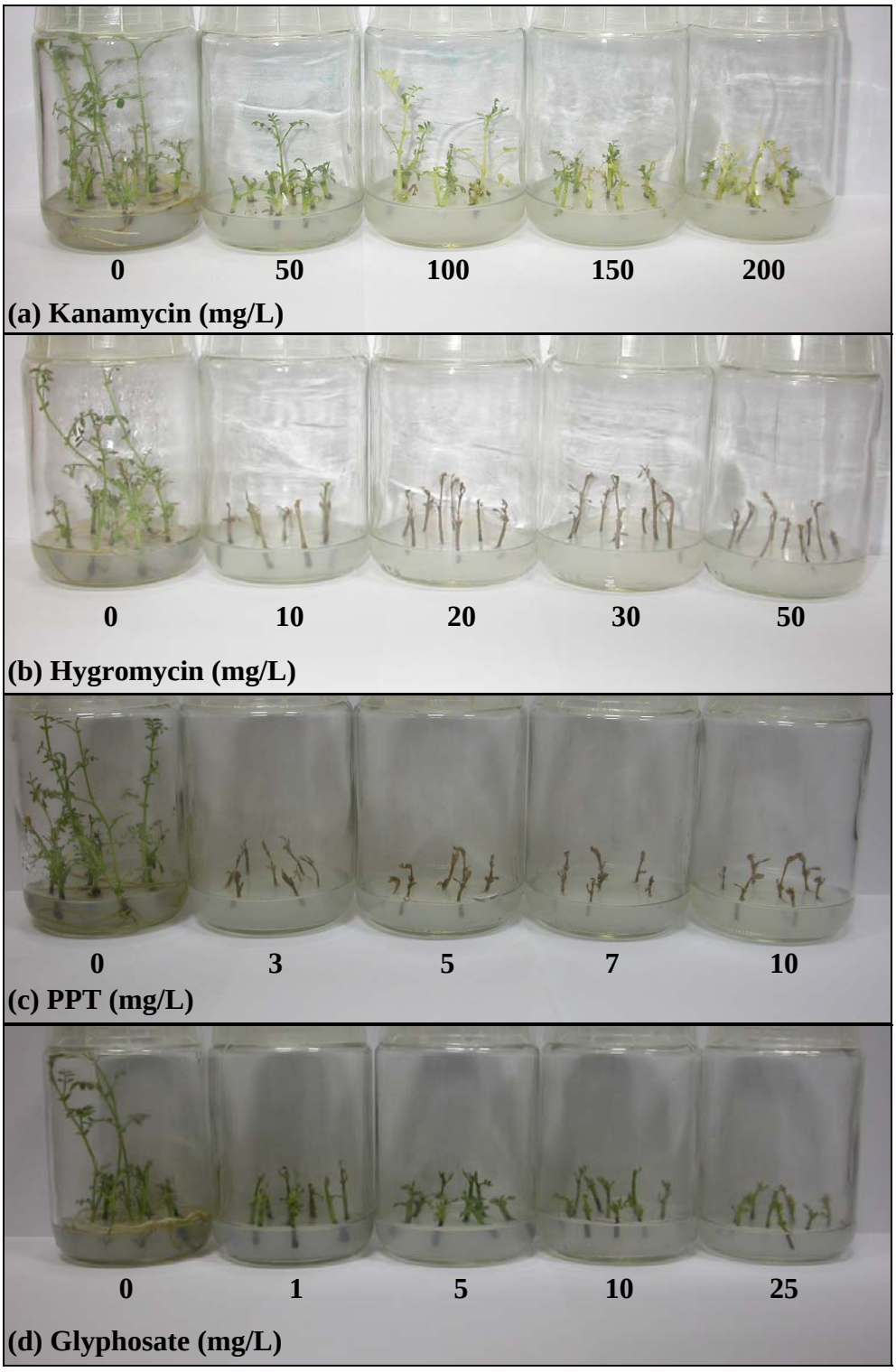


Figure 3.16. Effect of selective agents on root induction. Photographs were taken after 4 weeks of culture. Appearance of roots is displayed in Appendix I.

3.1.4. Effect of Antibiotics on Direct Organogenesis and Rooting

Genetic transformation mediated by *Agrobacterium* requires the use of various antibiotics in the selection and regeneration media. Co-cultivated tissues need to be subcultured several times on a medium containing antibiotics, which can control bacterial growth and does not interfere with regeneration potential of transformed cells or tissues (Nauerby *et al.*, 1997).

High frequency transformation using *Agrobacterium* depends not only on the efficiency of plant regeneration but also on the elimination of bacteria from transformed cells. The continued presence of bacteria interferes with the growth, development, and rooting rates; and even it causes the death of transgenics (Tang *et al.*, 2004). Bacterial presence on putative transgenics may also result in false positives during molecular analyses. Moreover elimination of *Agrobacterium* in transgenic plants is a prerequisite in preventing the possibility of gene release when these plants are transferred to the soil (Barrett *et al.*, 1997).

Various antibiotics are employed for bacterial suppression in plant transformation events. Commonly used antibiotics for elimination of various strains of *Agrobacterium* are Carbenicillin, Cefotaxime, Timentin, and Augmentin (Nauerby *et al.*, 1997; Tang *et al.*, 2000). Possible effects of these antibiotics on regeneration and growth of plant tissues should be performed before *Agrobacterium* mediated transformation. In this study chickpea CNs and shoots were subjected to various concentrations of these antibiotics and combinations of Carbenicillin and Cefotaxime to test their effect on multiple shoot induction and root induction.

3.1.4.1. Effect of Antibiotics on Multiple Shoot Induction

For the evaluation of antibiotic effect on multiple shoot induction, CNs were cultured under the stress of various concentrations of Augmentin (200, 400 and

600 mg/L), Carbenicillin (100, 200 and 300 mg/L), Cefotaxime (200, 400 and 600 mg/L), and Timentin (100, 200 and 300 mg/L).

CNs were cultured together with antibiotics and 1 mg/L BA for one month and every week number of shoots per CN was recorded. At the end of the culture period the explants and jars were photographed. All the experiments were carried out together with controls which are cultured on antibiotic-free MS media containing only 1 mg/L BA. Each set, replicated twice, was performed with duplicates and a minimum of 15 explants for each treatment (n=60 for each treatment).

Augmentin is one of the antibiotics used in elimination of *Agrobacterium* and it is composed of amoxicillin and potassium clavulanate which is a β-lactamase inhibitor. Vergauwe *et al.* (1996) employed 300 mg/L and Akasaka-Kennedy *et al.* (2004) employed 375 mg/L of Augmentin for decontamination of *A. tumefaciens* strain C58C1 in transformation studies. Augmentin at concentrations of 500 mg/L was used in transformation studies of pea and *Medicago truncatula* mediated by *A. tumefaciens* strain EHA105 (Schneider *et al.*, 1999; Kamate *et al.*, 2000).

Effects of Augmentin on regeneration of shoots from chickpea CNs is displayed in Table 3.1. According to the number of shoots per explant, Augmentin is determined to possess no promotional or inhibitory effect on chickpea CNs.

Number of shoots formed under Augmentin application were not significantly ($p < 0.05$) different from control containing no antibiotic. This result is consistent with the report of Vergauwe *et al.* (1996) in which Augmentin is defined to be an efficient antibiotic for elimination of *A. tumefaciens* strain C58C1, in selection of *Artemisia annua* L. with no significant effect on explant.

Table 3.1. Effects of Augmentin (A) on multiple shoot induction. Numbers of shoots per CN were recorded; and below are the mean values ± SEM. Antibiotic free media (0 mg/L A) were used as control. (n=60 for each treatment).

Concentration	Weeks			
(mg/L)	1	2	3	4
0 mg/L A	1.79 ± 0.060[a]	2.26 ± 0.077[a]	2.81 ± 0.077[a]	3.56 ± 0.100[a]
200 mg/L A	1.73 ± 0.070[a]	2.18 ± 0.075[a]	2.82 ± 0.086[a]	3.61 ± 0.108[a]
400 mg/L A	1.77 ± 0.058[a]	2.23 ± 0.069[a]	2.83 ± 0.092[a]	3.60 ± 0.124[a]
600 mg/L A	1.84 ± 0.050[a]	2.25 ± 0.065[a]	2.85 ± 0.095[a]	3.62 ± 0.134[a]

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Another antibiotic frequently used for elimination of bacteria is Carbenicillin and it is an analog of Ampicillin. It is used at various concentrations ranging from 100 to 1000 mg/L. It was reported that Carbenicillin has various positive effects on callus growth and adventitious shoot formation from apple tissues (Yepes and Aldwinckle, 1994; Hammerschlag *et al.*, 1997), from *Antirrhinum majus* (Holford and Newbury, 1992), and from wheat (Mathias and Boyd, 1986). In contrast, negative effects of Carbenicillin on *Arabidopsis thaliana* (Patton and Meinke, 1988), cacao (de Mayolo *et al.*, 2003), rose (Li *et al.*, 2002) and *Vitis* species (Colby and Meredith, 1990) have been reported.

Effects of Carbenicillin at concentrations of 100, 200 and 300 mg/L on chickpea multiple shoot induction from CN is displayed in Table 3.2. At all concentrations, Carbenicillin slightly decreased the number of shoots per explant after 4 weeks of culture. However the decreases were not significant at 2 or 3 weeks of culture (Table 3.2). This significant decrease in shoot induction obtained in our study is in agreement with the findings of de Mayolo *et al.* (2003), and Li *et al.* (2002). de Mayolo *et al.* (2003) found that Carbenicillin (100-300 mg/L) application resulted

in a decrease in the number of somatic embryos per cotyledon in cacao. Li *et al.* (2002) showed that 250 and 500 mg/L Carbenicillin decreased both callus induction from rose leaf explants and somatic embryo formation from callus.

Table 3.2. Effects of Carbenicillin (C) on multiple shoot induction. Numbers of shoots per CN were recorded; and below are the mean values ± SEM. Antibiotic free media (0 mg/L C) were used as control. (n=60 for each treatment).

Concentration	Weeks			
(mg/L)	1	2	3	4
0 mg/L C	1.71 ± 0.065^{a}	2.24 ± 0.079^{a}	2.79 ± 0.077^{a}	3.52 ± 0.075^{a}
100 mg/L C	1.70 ± 0.069^{a}	2.13 ± 0.061^{a}	2.68 ± 0.075^{a}	3.23 ± 0.103^{b}
200 mg/L C	1.67 ± 0.071^{a}	2.10 ± 0.069^{a}	2.63 ± 0.083^{a}	3.15 ± 0.104^{b}
300 mg/L C	1.67 ± 0.068^{a}	2.16 ± 0.060^{a}	2.61 ± 0.078^{a}	3.12 ± 0.094^{b}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Cefotaxime is another antibiotic frequently used for elimination of *Agrobacterium* in plant transformation studies. The employed concentrations range between 100 and 500 mg/L for tomato, *Agapanthus praecox*, and cacao (Ling *et al.*, 1998; Suzuki *et al.*, 2002; de Mayolo *et al.*, 2003). In our study the effect of 200, 400 and 600 mg/L of Cefotaxime on multiple shoot induction from chickpea CN was investigated and displayed in Table 3.3.

According to the number of shoots per CN, Cefotaxime had no effect on chickpea CNs. Numbers of shoots formed under all Cefotaxime concentrations and in the absence of antibiotic showed no significant difference ($p < 0.05$) after 4 weeks of culture. This result is in consistency with the report of Chevreau *et al.* (1997)

stating no effect of 200 mg/L Cefotaxime on bud regeneration from pear leaf explants after one month of culture.

Table 3.3. Effects of Cefotaxime (Cf) on multiple shoot induction. Numbers of shoots per CN were recorded; and below are the mean values ± SEM. Antibiotic free media (0 mg/L Cf) were used as control. (n=60 for each treatment).

Concentration	Weeks			
(mg/L)	1	2	3	4
0 mg/L Cf	1.73 ± 0.074[a]	2.23 ± 0.081[a]	2.73 ± 0.083[a]	3.52 ± 0.119[a]
200 mg/L Cf	1.73 ± 0.077[a]	2.15 ± 0.078[a]	2.73 ± 0.093[a]	3.48 ± 0.133[a]
400 mg/L Cf	1.78 ± 0.065[a]	2.20 ± 0.074[a]	2.75 ± 0.096[a]	3.47 ± 0.132[a]
600 mg/L Cf	1.79 ± 0.060[a]	2.21 ± 0.070[a]	2.79 ± 0.096[a]	3.47 ± 0.130[a]

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Timentin, which is defined as an effective inhibitor of bacterial growth in genetic transformation studies, is composed of Ticarcillin, a β-lactam antibiotic, and clavulanic acid, a β-lactamase inhibitor (Cheng *et al.*, 1998). Various concentrations ranging between 100 and 300 mg/L were used in transformation studies of various plants such as tomato (Ling *et al.*, 1998), *Artemisia annua* (Vergauwe *et al.*, 1996), legume *Astragalus sinicus* (Cho and Widholm, 2002), and pea (Polowick *et al.*, 2000).

The effects of Timentin at concentrations of 100, 200 and 300 mg/L on shoot regeneration of chickpea CNs, performed in this study, are displayed in Table 3.4. According to number of shoots per CN, 300 mg/L Timentin possesses a promotional effect on multiple shoot induction. This promotional effect results in

formation of significantly increased numbers of shoots even after one week of culture. After four weeks of culture all concentrations of antibiotic resulted in significantly high numbers of shoots compared to control. Previously, Estopa *et al.* (2001) reported that 100 mg/L Timentin increased the number of shoots regenerated from carnation leaf explants. This result well correlates with our study in which positive effects of Timentin on multiple shoot induction from chickpea CN is verified.

After transformation mediated by *Agrobacterium*, the explants may also be cultured on media containing combinations of antibiotics. Therefore, in this study effects of antibiotic combinations on multiple shoot induction were also investigated. Since all antibiotics used in this study are β-lactam antibiotics they can be inactivated by β-lactamases produced by bacteria. To test its effects, a β-lactamase inhibitor, Sulbactam, was also added at concentration of 100 mg/L into the media. Carbenicillin is sensitive to β-lactamases whereas Cefotaxime is highly resistant to them (Chevreau *et al.*, 1997). The combinations and concentrations used are summarized in Table 3.5.

Table 3.4. Effects of Timentin (T) on multiple shoot induction. Numbers of shoots per CN were recorded; and below are the mean values ± SEM. Antibiotic free media (0 mg/L T) were used as control. (n=60 for each treatment).

Concentration	Weeks			
(mg/L)	1	2	3	4
0 mg/L T	1.61 ± 0.078^{a}	2.16 ± 0.070^{a}	2.75 ± 0.080^{a}	3.42 ± 0.075^{a}
100 mg/L T	1.71 ± 0.069^{a}	2.23 ± 0.065^{a}	2.98 ± 0.085^{a}	3.81 ± 0.113^{b}
200 mg/L T	1.73 ± 0.060^{ab}	2.27 ± 0.065^{ab}	3.02 ± 0.086^{ab}	3.93 ± 0.111^{b}
300 mg/L T	1.81 ± 0.053^{b}	2.35 ± 0.069^{b}	3.11 ± 0.086^{b}	4.02 ± 0.102^{b}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Table 3.5. Combinations and concentrations of antibiotics and Sulbactam used for their effect on multiple shoot induction and rooting.

	Abbreviations
200 mg/L Carbenicillin + 400 mg/L Cefotaxime	200 C + 400 Cf
200 mg/L Carbenicillin + 400 mg/L Cefotaxime + 100 mg/L Sulbactam	200 C + 400 Cf + 100 S
50 mg/L Carbenicillin + 100 mg/L Cefotaxime + 100 mg/L Sulbactam	50 C + 100 Cf + 100 S

The effect of antibiotic and Sulbactam combinations on multiple shoot induction is displayed in Table 3.6. The numbers of shoots formed under the stress of all combinations of antibiotics did not show any significant difference when compared to controls after 3 weeks of culture. On the other hand, a slight but significant decrease in number of shoots with all combinations was observed after four weeks of culture. This decrease is similar to that observed under the application of Carbenicillin alone. Therefore, it may be concluded that the decrease originates from the inhibitory effect of Carbenicillin. This deduction is in agreement with the report of Li *et al.* (2002) in which 150 mg/L Carbenicillin was found to be exerting the main cause of both somatic embryogenesis and callogenesis inhibition when used in combination with 150 mg/L Cefotaxime in tissue culture of rose leaf explants.

Since numbers of shoots regenerated in the presence and absence of 100 mg/L Sulbactam applied together with 200 mg/L Carbenicillin and 400 mg/L Cefotaxime did not show any significant difference, it can be stated that 100 mg/L Sulbactam does not have any influence on multiple shoot induction. Also since Sulbactam is an effective β-lactamase inhibitor, the concentrations of other antibiotics, Carbenicillin and Cefotaxime can be reduced. The numbers of shoots

regenerated from CN were also not significantly different among the all three combinations employed. Therefore, concentrations of Carbenicillin and Cefotaxime can be reduced when Sulbactam, which has no antimicrobial effect of its own and also has no inhibitory effect on multiple shoot induction, is supplemented in the regeneration media.

Effects of all *Agrobacterium* eliminating antibiotics and their combinations with Sulbactam on multiple shoot induction from chickpea CNs are also displayed with photographs, taken at the end of 4 weeks of culture, in Figure 3.17 and Figure 3.18.

Table 3.6. Effects of antibiotic combinations and Sulbactam on multiple shoot induction. Numbers of shoots per CN were recorded; and below are the mean values ± SEM. Antibiotic free media were used as control. (n=60 for each treatment).

Concentration (mg/L)	Weeks			
	1	2	3	4
Control	1.72 ± 0.064^{a}	2.28 ± 0.077^{a}	2.98 ± 0.090^{a}	3.67 ± 0.093^{a}
200 C + 400 Cf	1.72 ± 0.068^{a}	2.15 ± 0.063^{a}	2.79 ± 0.078^{a}	3.36 ± 0.094^{b}
200 C + 400 Cf + 100 S	1.71 ± 0.069^{a}	2.13 ± 0.062^{a}	2.71 ± 0.084^{a}	3.25 ± 0.095^{b}
50 C + 100 Cf + 100 S	1.72 ± 0.060^{a}	2.18 ± 0.057^{a}	2.75 ± 0.076^{a}	3.23 ± 0.087^{b}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).
Abbreviations are C: Carbenicillin, Cf: Cefotaxime, S: Sulbactam.

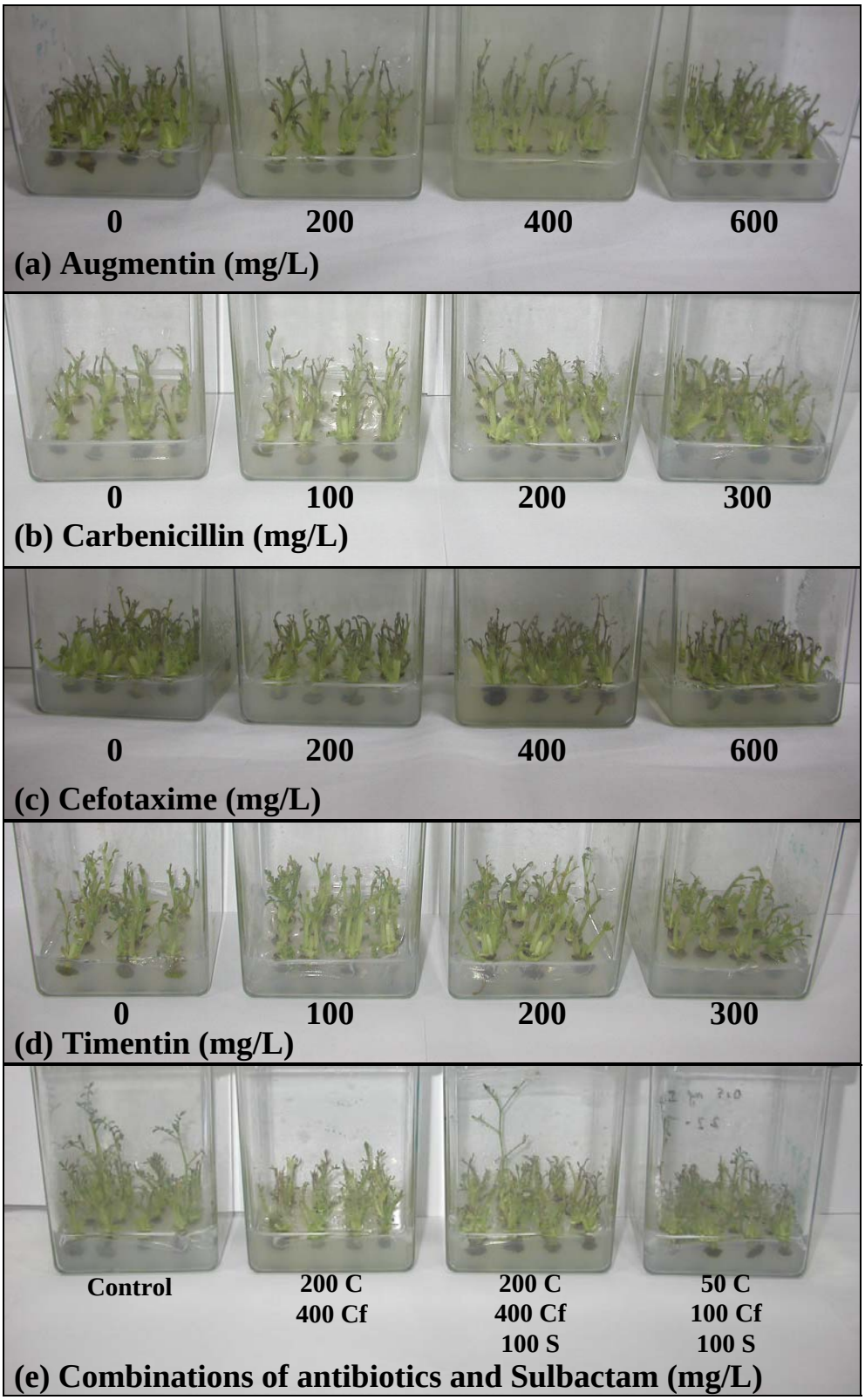


Figure 3.17. Effect of antibiotics on multiple shoot induction.

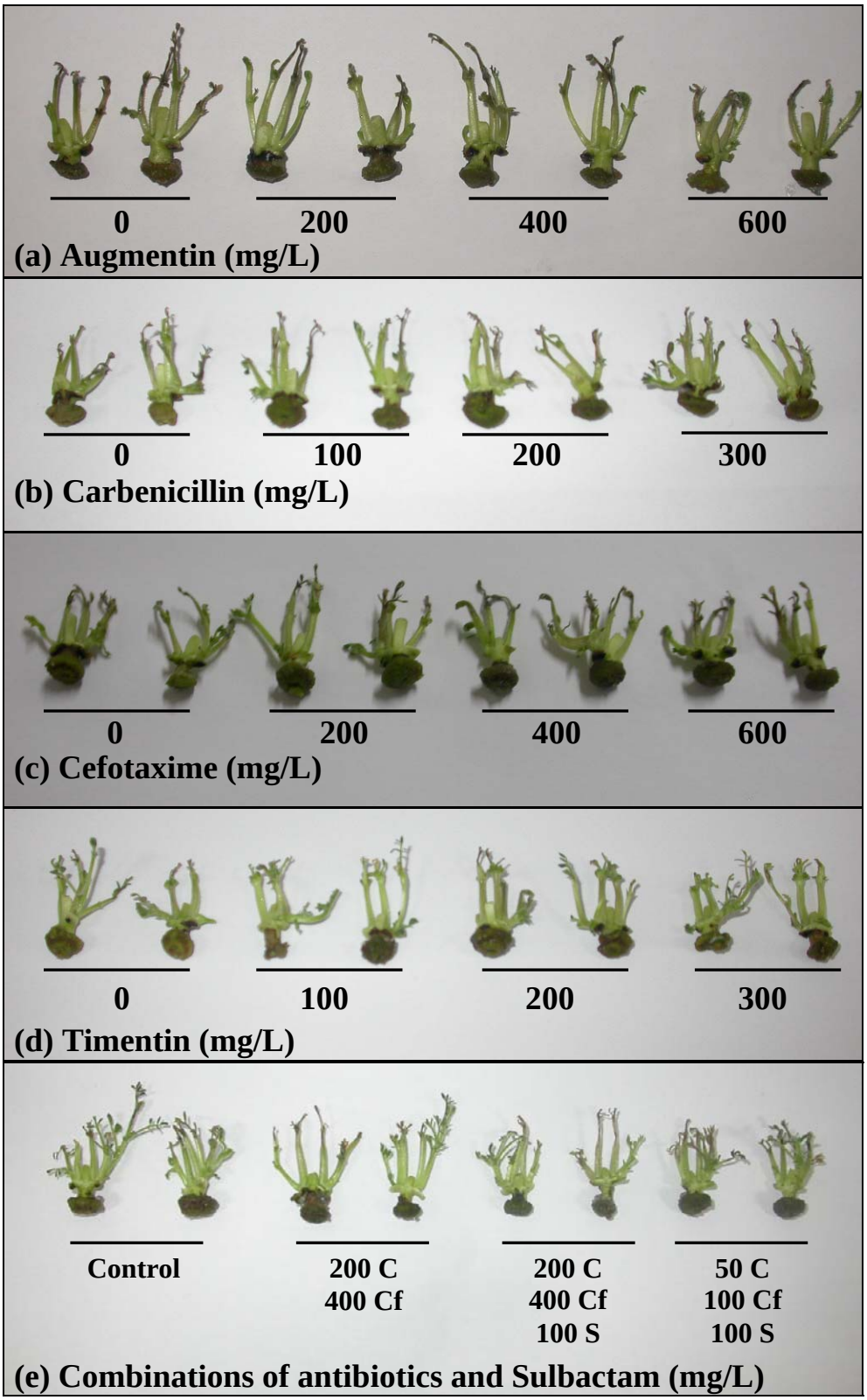


Figure 3.18. Effect of antibiotics on multiple shoot induction from chickpea CN.

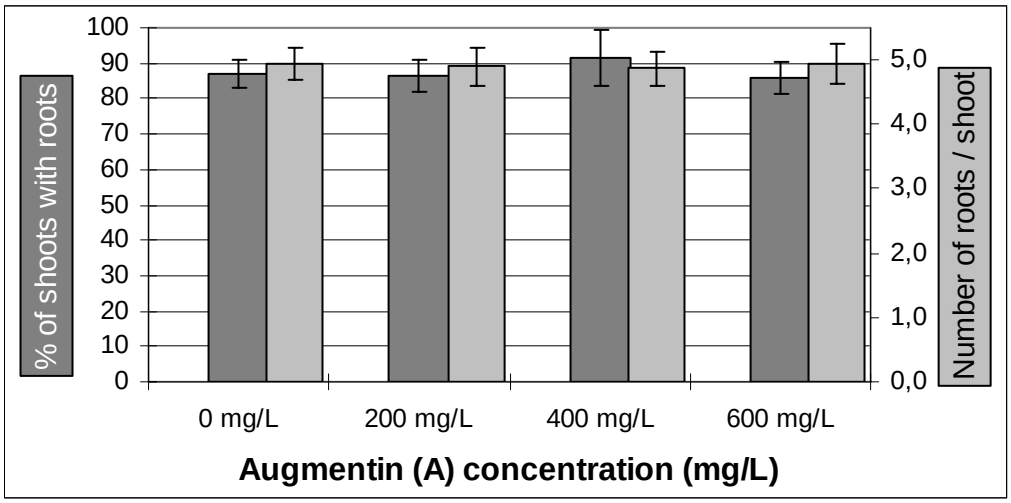
3.1.4.2. Effect of Antibiotics on Root Induction

During the tissue culture studies, effects of *Agrobacterium* elimination antibiotics on root induction from regenerated shoots were also investigated. The types and concentrations of antibiotics were same as the ones employed for investigation of their effect on multiple shoot induction.

The shoots regenerated on CNs within 7 to 8 days were removed from the explant and subcultured onto MS media containing 0.1 mg/L IBA for root induction. The shoots were cultured with antibiotics and 0.1 mg/L IBA for 4 weeks and at the end of the culture period, percent of shoots having roots and numbers of roots per shoot were recorded. The explants and jars were also photographed. All experiments were carried out with controls which were antibiotic free MS media containing only 0.1 mg/L IBA. Each set was performed with duplicates and a minimum of 10 explants for each treatment (n=20 for each treatment).

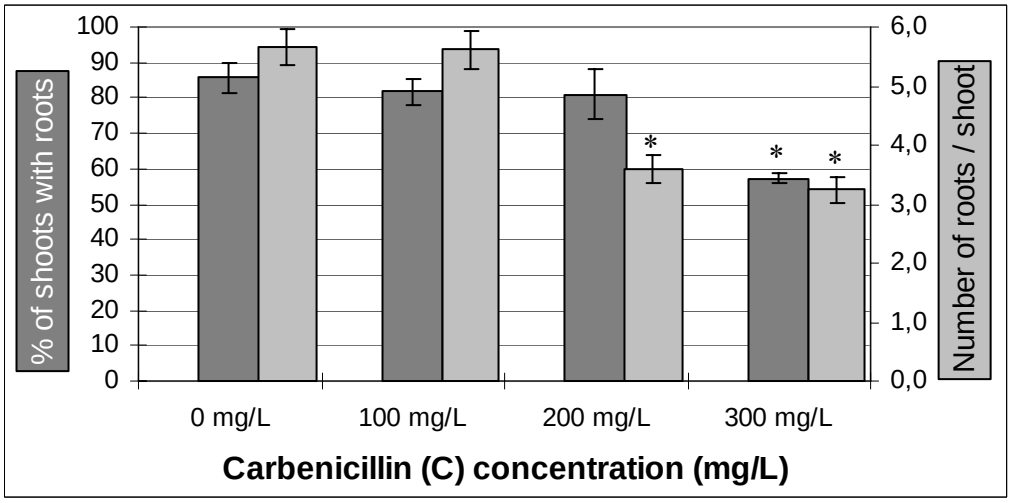
Percent of shoots with roots and number of roots per shoot formed under Augmentin application is displayed in Figure 3.19. According to both parameters evaluated Augmentin at all concentrations exhibited no significant change on root induction.

Similarly Carbenicillin at low concentrations (100 and 200 mg/L) did not cause a significant difference for the number of shoots with roots; however, 300 mg/L Carbenicillin decreased this value to 57 % which is significantly lower than the control (Figure 3.20). On the other hand Carbenicillin at concentrations of 200 and 300 mg/L significantly reduced the average number of roots per shoot to 3.59 and 3.25 respectively. Estopa *et al.* (2001) also reported a decrease in number of roots per shoot when carnation shoot tips are cultured with 500 mg/L Carbenicillin but this decrease was not significantly different from the control.



Mean values, SEM and significant values are tabulated in Table H.7 in Appendix H.

Figure 3.19. Effect of Augmentin on root induction from the shoots. Augmentin free media were used as control (0 mg/L). Vertical bars indicate SEM. (n=22 for each treatment).

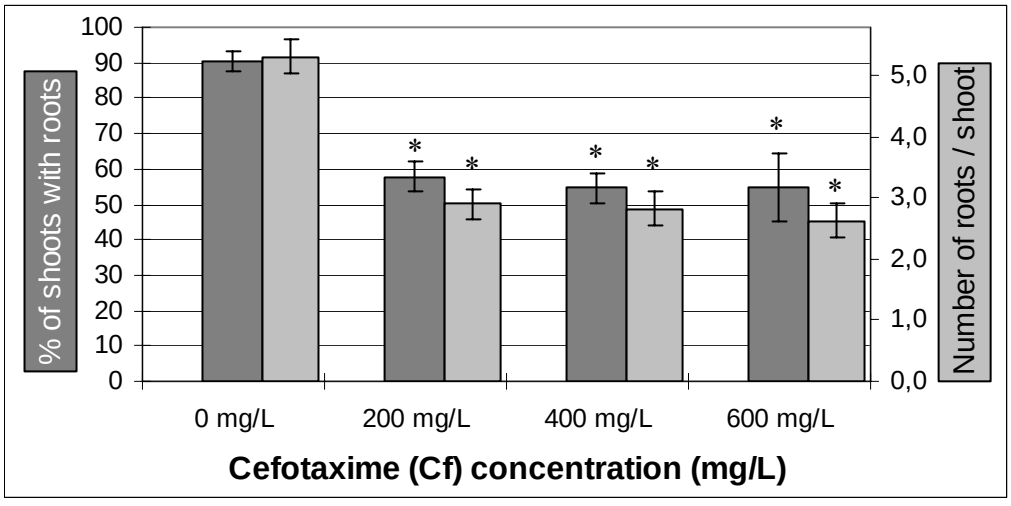


Mean values, SEM and significant values are tabulated in Table H.8 in Appendix H.

Figure 3.20. Effect of Carbenicillin on root induction from the shoots. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to control (0 mg/L), respectively. (n=21 for each treatment).

Cefotaxime, a cephalosporin type antibiotic was used to evaluate of its effects on rooting. All concentrations used in our study reduced the frequency of rooting and number of roots per shoot significantly (Figure 3.21). Cefotaxime at all concentrations resulted in rooting of nearly 55 % of shoots cultured; whereas antibiotic free media resulted in rooting of nearly 90 % of shoots. This negative effect of Cefotaxime on rooting efficiency was also reported by Estopa *et al.* (2001). It significantly reduced the root induction from carnation shoot tips by half. Holford and Newbury (1992) also showed the negative effect of Cefotaxime on number of roots per explant in tissue culture studies of *Antirrhinum majus*.

In addition the number of roots per shoot was decreased significantly under different concentrations of Cefotaxime (Figure 3.21). The decreases in both parameters indicated that Cefotaxime not only inhibited root growth but also inhibited formation of root primordia on chickpea shoots.

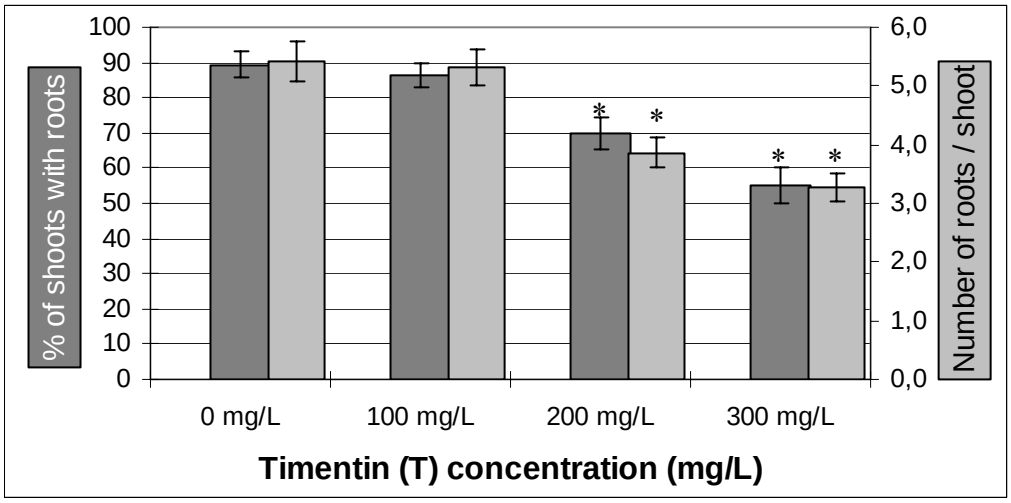


Mean values, SEM and significant values are tabulated in Table H.9 in Appendix H.

Figure 3.21. Effect of Cefotaxime on root induction from the shoots. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to control (0 mg/L), respectively. (n=20 for each treatment).

The regenerated roots in the presence of Cefotaxime were shorter and thicker; and shoots were less developed compared to the ones formed in the control media (Figure 3.23c). These morphological changes are generally observed under abiotic stresses. Therefore, it can be concluded that Cefotaxime may have toxic effects inducing stress response in plant tissues.

Timentin at a concentration of 100 mg/L did not have any influence on root induction in our study (Figure 3.22). Similar to our results, Nauerby *et al.* (1997) stated that 150 mg/L Timentin did not significantly changed the number of rooted shoots which were regenerated from tobacco leaf disc explants. Estopa *et al.* (2001) also reported that 100 mg/L Timentin decreased the root induction of carnation shoots but this decrease was not significant. On the other hand, in our study, Timentin at higher concentrations (200 and 300 mg/L) significantly reduced the percentages of root induction and number of roots per chickpea shoots.



Mean values, SEM and significant values are tabulated in Table H.10 in Appendix H.

Figure 3.22. Effect of Timentin on root induction from the shoots. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to control (0 mg/L), respectively. (n=20 for each treatment).

The effects of all *Agrobacterium* eliminating antibiotics on root induction of chickpea shoots are also displayed with photographs, taken at the end of 4 weeks of culture, in Figure 3.23 and Figure 3.24.

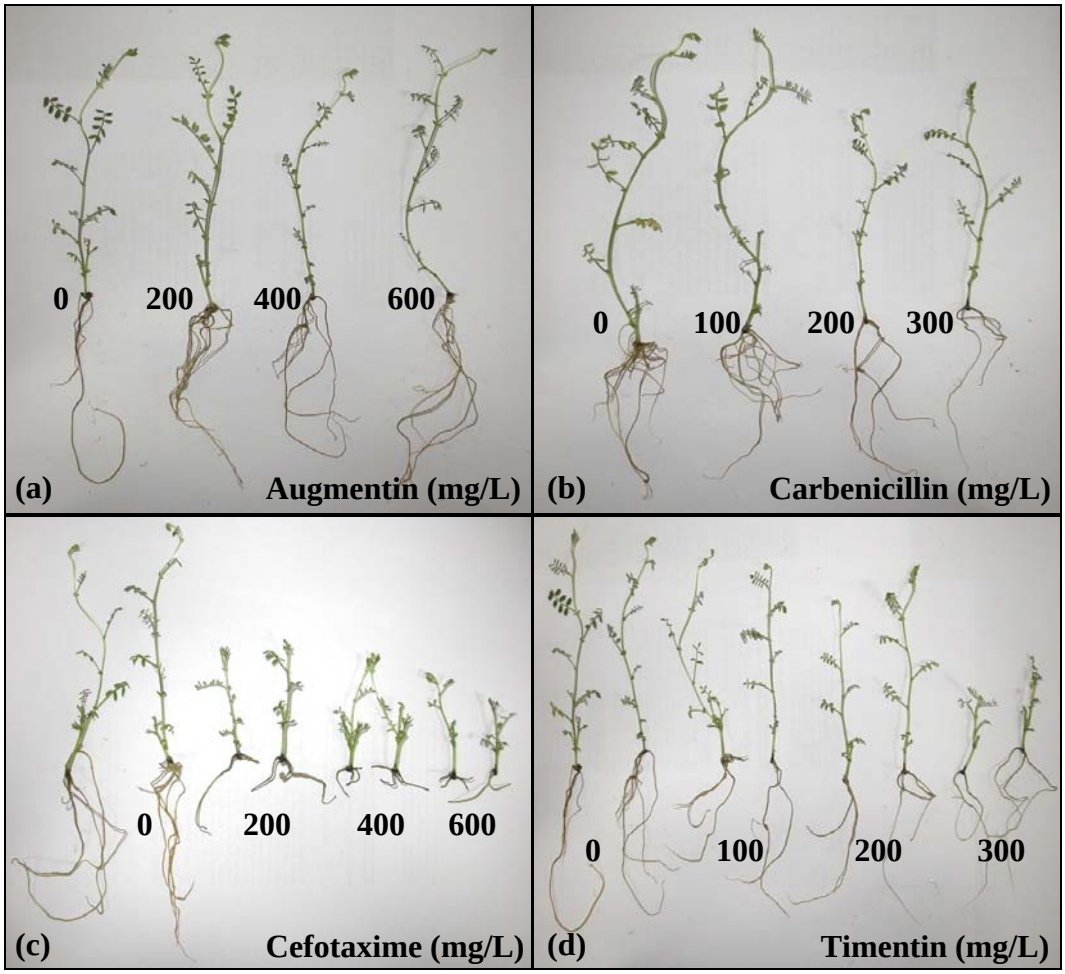


Figure 3.23. Effect of antibiotics on root induction from chickpea shoots. The explants were photographed after 4 weeks of culture.

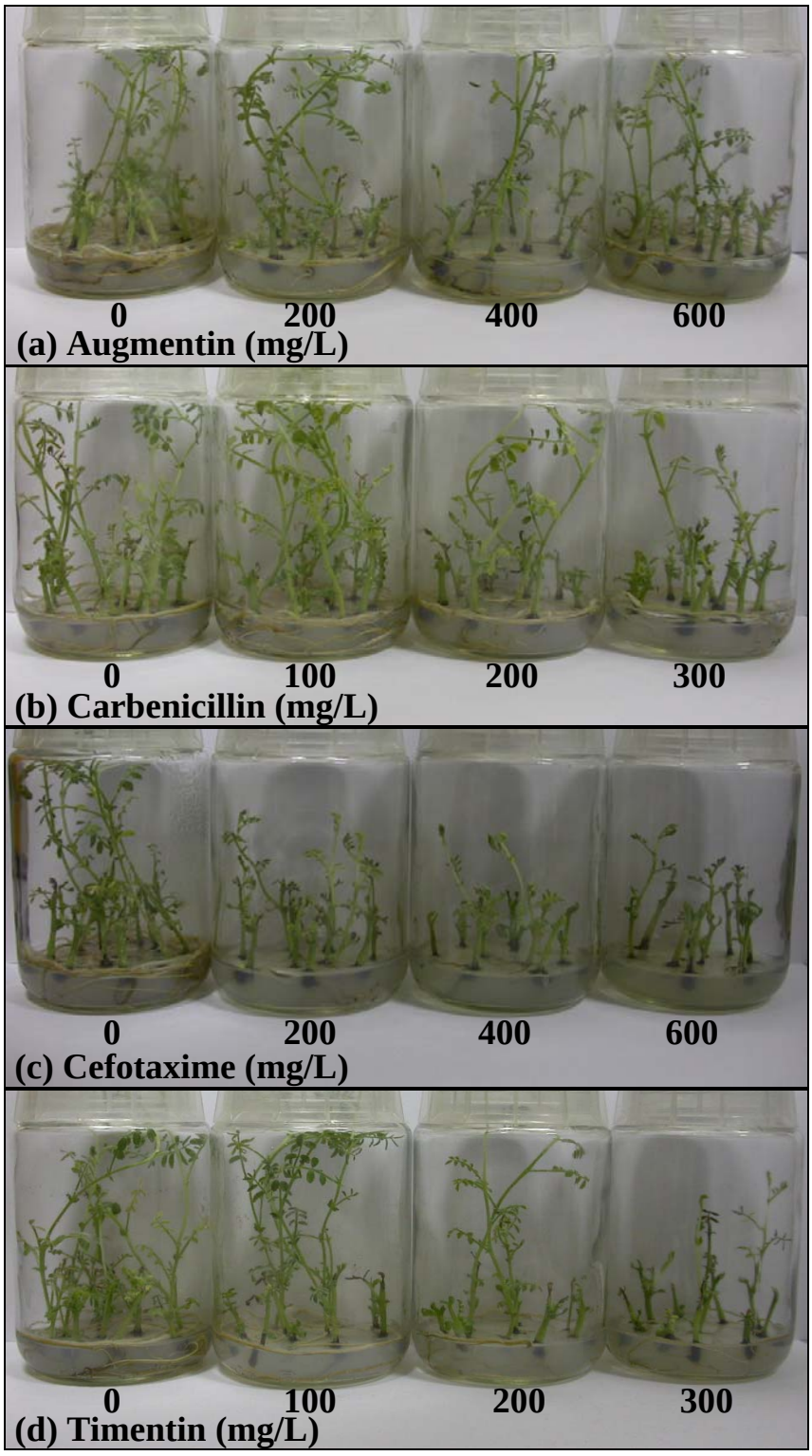
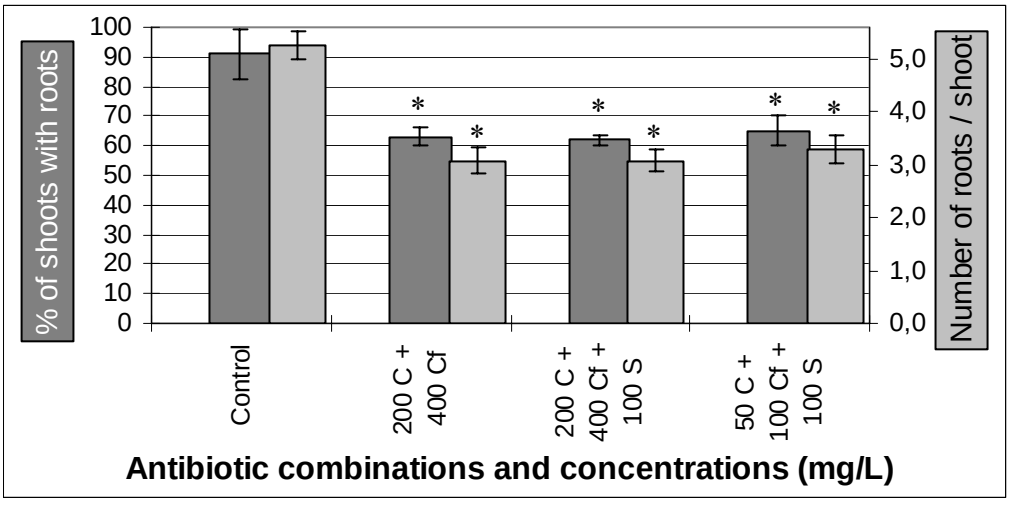


Figure 3.24. Effect of antibiotics on root induction. Photographs were taken at the end of 4 weeks of culture. Appearances of roots are displayed in Appendix J.

The effects of antibiotic and Sulbactam combinations on root induction from chickpea shoots are represented in Figure 3.25. The photographs of shoots and roots are displayed in Figure 3.26. Combinations of antibiotics and Sulbactam used for evaluating their effects on root induction were same as the ones employed for multiple shoot induction.

All combinations reduced the rooting frequency of chickpea shoots and number of roots per shoot significantly. This sharp decrease in root induction is similar to one observed under the stress of Cefotaxime applied alone. Therefore, it can be concluded that the decrease may be originating from the presence of Cefotaxime (Figure 3.25). Any inhibitory effect of Cefotaxime similar to one observed in root induction experiments was not observed in multiple shoot induction. The possible reason for this could be the placement of CN onto media. CNs were inserted into media in this study and emerging shoots were not in touch with the medium escaping from the toxic effects of the antibiotic.



Mean values, SEM and significant values are tabulated in Table H.11 in Appendix H.

Figure 3.25. Effect of antibiotic combinations and Sulbactam on root induction. Vertical bars and * indicate SEM and significant values ($p < 0.05$) compared to control, respectively. (n=20 for each treatment).

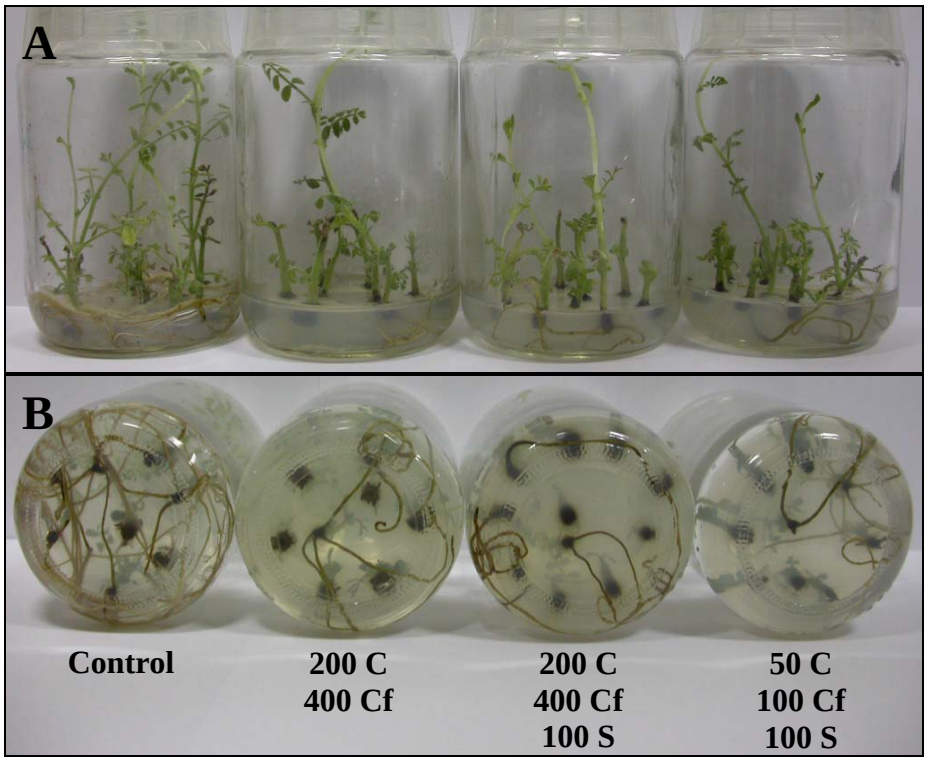


Figure 3.26. Effect of antibiotic combinations and Sulbactam on root induction from chickpea shoots. (A) View of shoots. (B) Appearance of roots.

3.1.5. Effect of Antibiotics on *A. tumefaciens* KYRT1

Bacterial overgrowth after transformation may result in retardations in growth and development of transgenics or may result in tissue death. Therefore, bacterial growth should be inhibited by using effective antibiotics and their effects on bacteria should also be tested. The efficiency of various concentrations and combinations of antibiotics in controlling *A. tumefaciens* strain KYRT1 growth was tested using both an *in vitro* agar diffusion assay and a spectrophotometric method.

3.1.5.1. Antibiotic Sensitivity Testing with Agar Diffusion Method

The effects of antibiotics and Sulbactam on bacterial growth are represented in Figure 3.27 as diameter of growth inhibition zone formed around the filter paper discs. Formerly the discs were sterilized and saturated with solutions of antibiotics containing specified concentrations at μg levels. After the discs were totally dried, they were placed on the surface of agar plates which were previously inoculated with *A. tumefaciens* suspension at 0.8 OD value at 600 nm.

The diameter of inhibition zones are also tabulated in Table H.12 and Table H.13 in Appendix H. The plates were photographed after two days of culture at 28ºC (Figure 3.28, Figure 3.29).

According to the zone diameters, it is found that *A. tumefaciens* strain KYRT1 is resistant to all concentrations of Carbenicillin. This particular strain is selected in YEB medium in the presence of Carbenicillin at concentrations of 100 mg/L (Appendix D). As expected, the strain was found to be resistant to the antibiotic as a result.

Increasing the concentration of Augmentin, Cefotaxime and Timentin when used alone, increased the inhibition zone formed around the discs. Among the three antibiotics; Augmentin produced the narrower zones whereas Cefotaxime produced the wider zones at all concentrations. Although the concentrations of both are equal (200, 400 and 600 μg) and Augmentin contains clavulanic acid, a β-lactamase inhibitor, Cefotaxime is found to be superior over Augmentin in controlling *A. tumefaciens* KYRT1 (Figure 3.28). A similar observation was also reported by de Mayolo *et al.* (2003) that Cefotaxime is superior over Augmentin for elimination of *A. tumefaciens* strain AGL1 using disc diffusion assay.

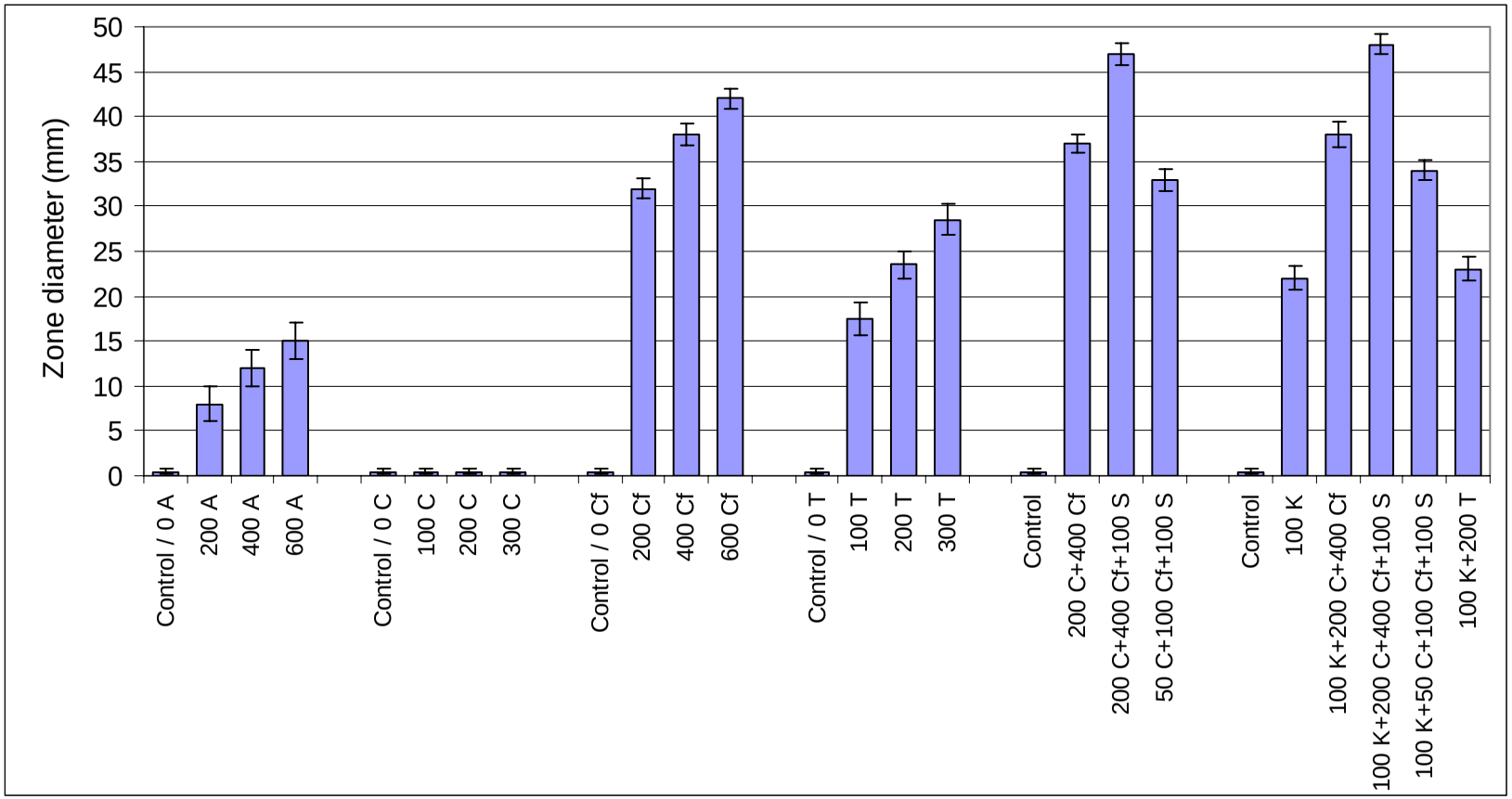


Figure 3.27. Antibiotic sensitivity testing for *A. tumefaciens* strain KYRT1. The effects of various concentrations (μg) and combinations of Augmentin (A), Carbenicillin (C), Cefotaxime (Cf), Timentin (T), Kanamycin (K) and Sulbactam (S) on bacterial growth are represented as diameter of growth inhibition zone in millimeters (mm).

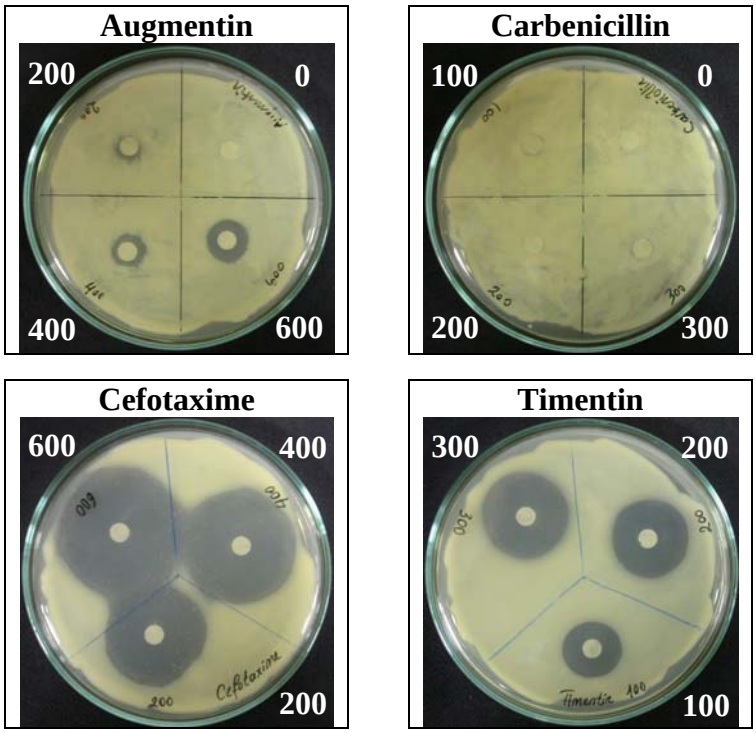
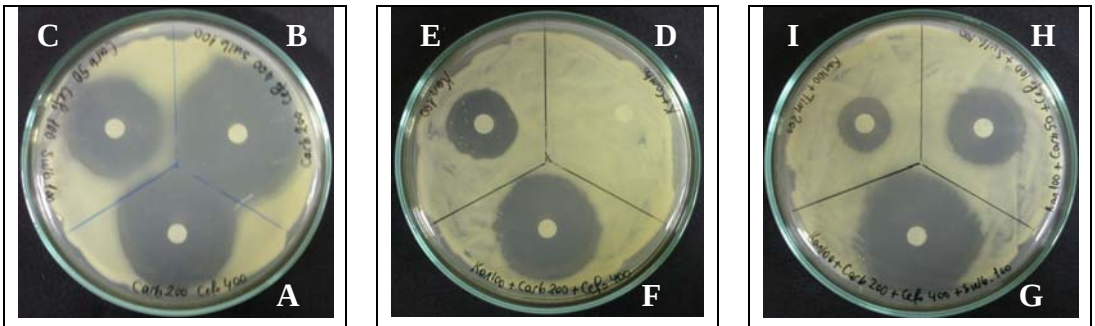


Figure 3.28. Representative plates displaying the antibiotic sensitivity testing. Each disc contains specified concentrations (μg) of specified antibiotic.



A) 200 C+ 400 Cf
B) 200 C+ 400 Cf+ 100 S
C) 50 C+ 100 Cf+ 100 S

D) Control
E) 100 K
F) 100 K+ 200 C+ 400 Cf

G) 100 K+ 200 C+ 400 Cf+ 100 S
H) 100 K+ 50 C+ 100 Cf+ 100 S
I) 100 K+ 200 T

Figure 3.29. Representative plates displaying the antibiotic sensitivity testing with combinations of antibiotics. Each disc contains specified concentrations (μg) and combinations of specified antibiotics.

The effects of combinations of antibiotics and Sulbactam on bacterial growth were also tested using the same assay. Sulbactam presence exhibited an increase in effects of other antibiotics. 100 µg Sulbactam, when applied together with 200 µg Carbenicillin and 400 µg Cefotaxime, displayed the most effective inhibition (Figure 3.27). Also a decrement in concentrations of Carbenicillin to 50 µg and Cefotaxime to 100 µg in the presence of Sulbactam, did not cause significant decreases in zone diameter and such a combination resulted in a zone with a diameter of 33 mm (Figure 3.27). As a conclusion it can be stated that decreased antibiotic concentrations can be employed for elimination of bacteria with the supplement of Sulbactam.

Effects of antibiotic combinations together with 100 µg Kanamycin were also investigated since Kanamycin and antibiotics are used together during selection of transgenics. It was stated in previous sections of this study that 100 mg/L Kanamycin is appropriate for use in selection media. 100 µg Kanamycin, 200 µg Carbenicillin, 400 µg Cefotaxime and 100 µg Sulbactam, when applied together, inhibited the bacteria with the largest inhibition zone (48 ± 1.1 mm) (Figure 3.27).

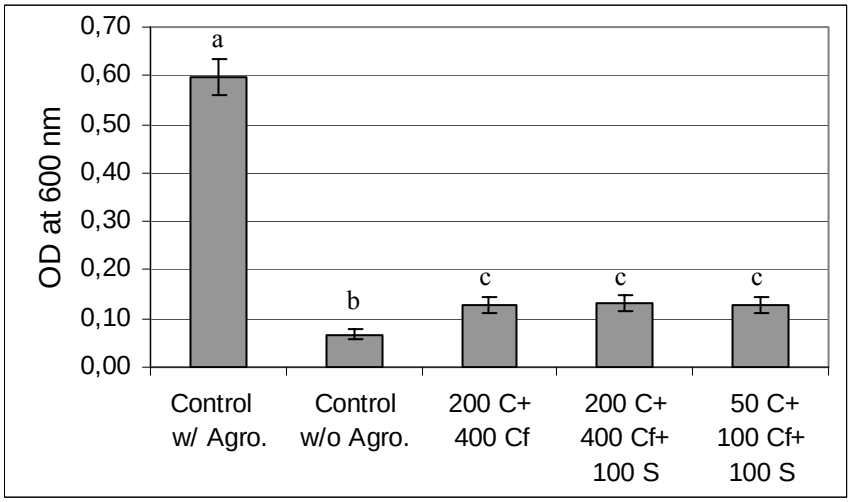
3.1.5.2. Antibiotic Effect on Bacterial Growth

Effects of antibiotics on bacterial growth were also investigated using *in vitro* culture of bacteria together with chickpea CNs to determine the effect of explant presence. The explants, inoculated with *A. tumefaciens* strain KYRT1 for 40 minutes, were washed in sterile distilled water and placed onto media containing various combinations of antibiotics. The combinations were same as the ones used for tissue culture studies and antibiotic sensitivity testing.

The experiment was carried out with double control; one group, in which CNs were inoculated with bacteria and other group, in which CNs were not inoculated and placed onto antibiotic free media. Cultivation was performed for a month and at the end of cultivation period, all the explants were transferred to liquid YEB

medium containing no antibiotic to encourage the multiplication of surviving bacterial cells. At the end of explant incubation for two days in YEB, the OD of the YEB medium was recorded at 600 nm spectrophotometrically to determine the presence of bacteria.

Bacterial density recorded as OD at 600 nm under the suppression of various antibiotic combinations is represented in Figure 3.30. All the combinations of antibiotics significantly decreased the density of bacteria compared to the controls formerly inoculated with bacteria and cultured on antibiotic free media. The explants which were not inoculated with bacteria recorded an OD of 0.067 which probably originates from impurities arising from explants. On the other hand the control explants which were inoculated with bacteria and cultivated on antibiotic free MS media recorded an OD of 0.596 (Figure 3.30).



Mean values, SEM and significant values are tabulated in Table H.14 in Appendix H.

Figure 3.30. Antibiotic effect on bacterial growth determined by a spectrophotometric method. Vertical bars indicate SEM. Values marked with same letter are not significantly different ($p < 0.05$). (n=15 for each treatment).

The OD values recorded by explants cultivated under the stress of antibiotic combinations were around 0.130. These values were significantly higher compared to control CNs (0.067) not inoculated with bacteria. This indicated that there were bacterial cells still surviving. Not all parts of the CNs were in touch with the medium; therefore, bacterial cells on CNs and away from medium may be still surviving. However, numbers of such bacterial cells were very low; leading to low values of OD at 600 nm.

3.2. Transformation Studies

Optimization of *Agrobacterium* mediated transformation system for CNs of chickpea cultivar Gökçe, was performed in transformation part of this study. The procedure, previously reported to be effective in transformation of lentil CNs by Mahmoudian *et al.* (2002), was employed in this study for transformation of chickpea CNs.

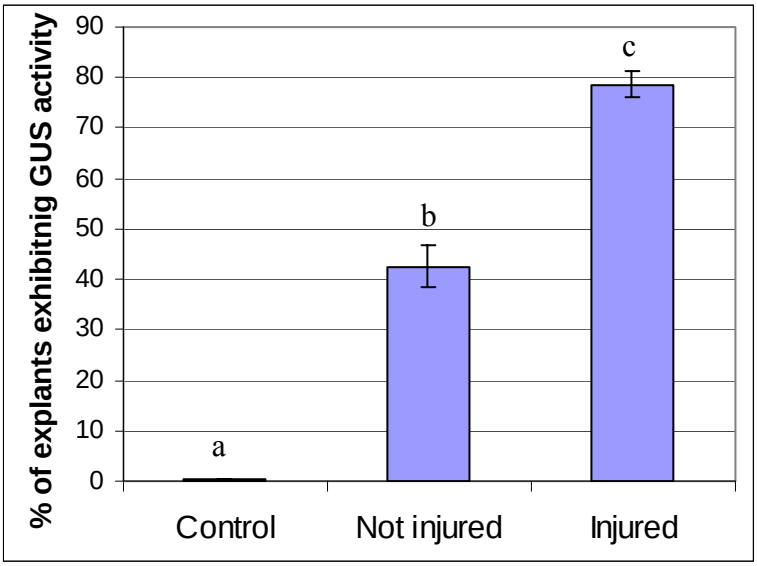
The system was tried to be further improved by mechanical injury of explants prior to transformation, by applying vacuum infiltration during bacterial inoculation of explants and by applying L-Cysteine during co-cultivation period. The effects of each application were investigated using GUS histochemical staining at various stages of regeneration and development.

3.2.1. Effect of Mechanical Injury on Transformation Efficiency

Mechanical injury of CNs was performed using a fine glass needle and by poking 6 to 8 times at each axillary region of cotyledonary petioles where the secondary shoots develop without an intermediary callus phase. For investigation of injury effect on transformation efficiency, transformation was performed both with injured and not injured explants. Each set of experiments was coupled to the control groups which were not inoculated with bacteria.

Effect of mechanical injury represented as percent of explants exhibiting transient GUS activity (*uidA* expression) on the 4th day after transformation is displayed in Figure 3.31. Results are the total of seven independent experiments each performed with at least 25 explants for control and not injured groups and at least 75 explants for injured groups.

Control explants, not injured and not inoculated with bacteria, exhibited no GUS activity whereas 42.6 ± 4.1 percent of explants, not injured but inoculated, exhibited GUS activity. This result indicated the susceptibility of chickpea tissues to *Agrobacterium*. On the other hand 78.6 ± 2.6 percent of injured explants exhibited GUS activity, which was significantly higher than that of not injured explants (42.6 ± 4.1 %). As a result, it can be stated that injury on the axillary region of CN explants significantly increased the transformation efficiency in chickpea according to GUS staining on the 4th day.



Mean values, SEM and significant values are tabulated in Table H.15 in Appendix H.

Figure 3.31. Effect of mechanical injury on transient gene expression on the 4th day after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

Beneficial effect of mechanical injury on gene transfer efficiency to lentil CNs was previously reported by Çelikkol (2002). Our findings well correlate with this study in which *uidA* expression at the injury sites was detected and a number of shoots exhibiting GUS activity were obtained after four weeks of selection. Injury makes the plant cells more vulnerable to *Agrobacterium* by releasing phenolic compounds that attract bacteria to the wound site and function in the induction of virulence genes. Therefore, the reason for occurrences of more *uidA* expression on injured explants may be originating from release of phenolic compounds or from more opportunity to infect more wounded cells.

Representative photographs of control, not injured, and injured explants and shoots stained for GUS activity are displayed in Figure 3.32 and Figure 3.33. The photographs of CNs and shoots clearly showed that the stained tissue parts in not injured explants were generally a single point; however, stained parts of shoots in injured explants were generally composed of several points and areas.

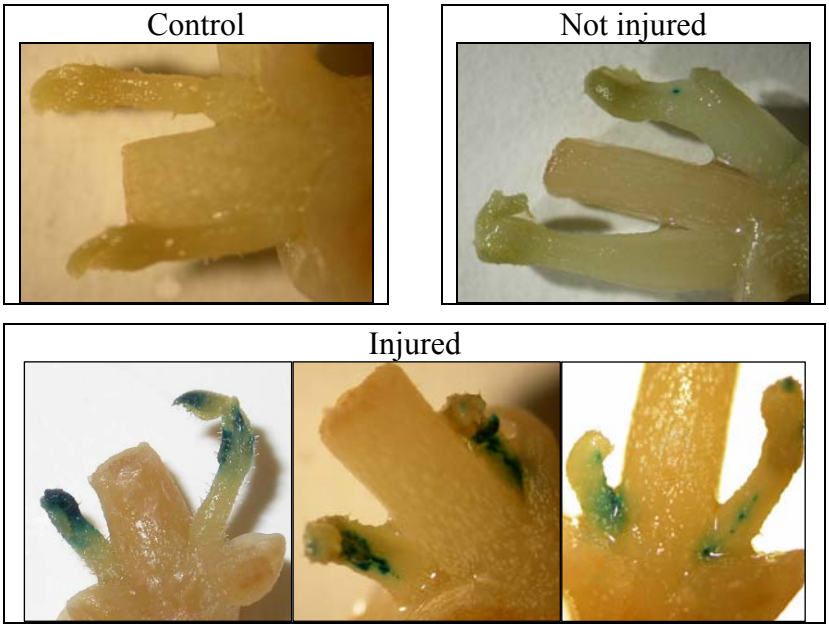


Figure 3.32. Representative photographs of shoots on CNs stained for GUS activity on the 4th day after transformation.

At the end of co-cultivation period for 4 days, the number of shoots and number of GUS expressing shoots per explant were also recorded. Effect of mechanical injury on shoot regeneration and transient gene expression as number of shoots per explant is represented in Figure 3.34.

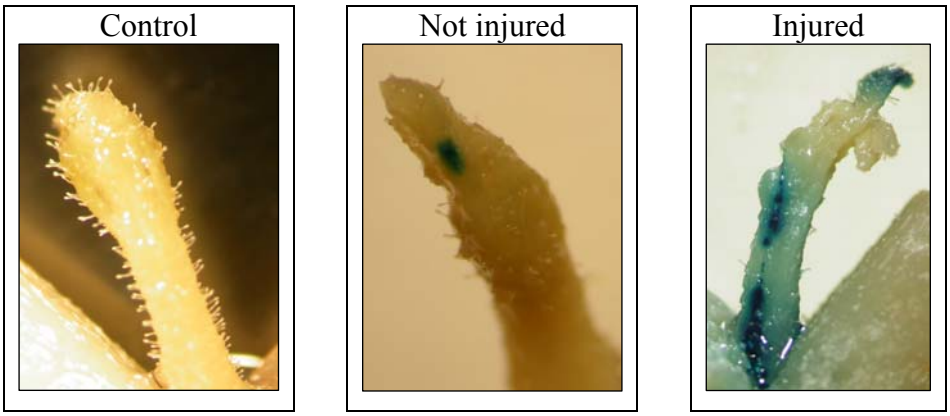
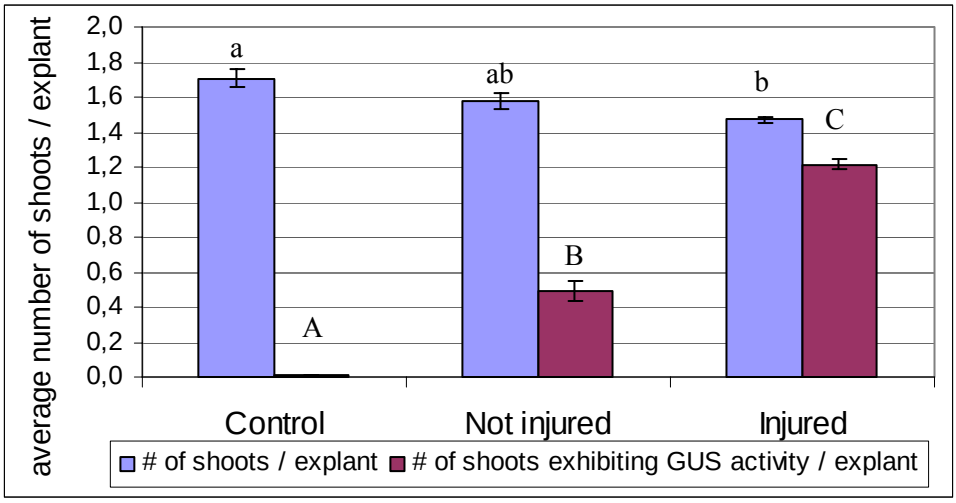


Figure 3.33. Representative photographs of shoots stained for GUS activity on the 4th day after transformation.



Mean values, SEM and significant values are tabulated in Table H.16 in Appendix H.

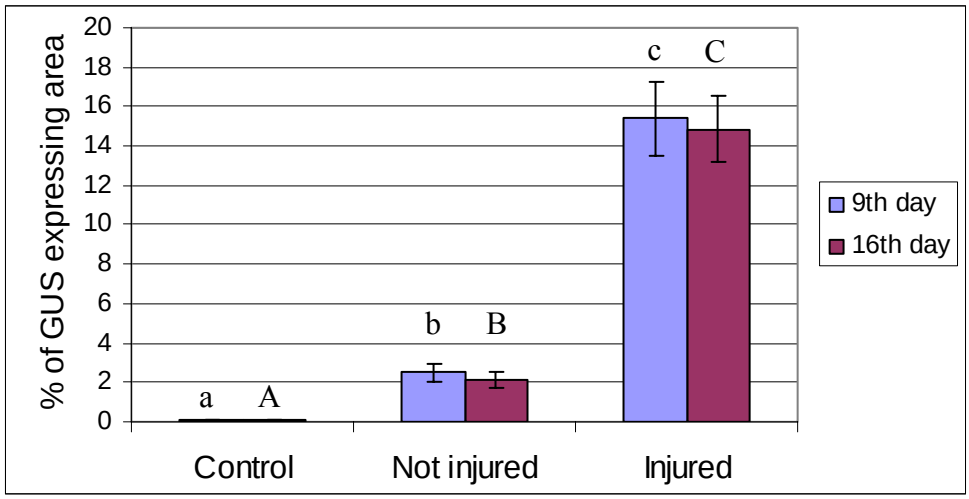
Figure 3.34. Effect of injury on shoot regeneration and transient gene expression after 4 days of co-cultivation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

Inoculation with *Agrobacterium* and mechanical injury decreased the number of shoots regenerated from explants (Figure 3.34). Average number of shoots per explant, when explants were not injured but inoculated, is 1.58 ± 0.05. This value was not significantly different from the controls which produced an average of 1.71 ± 0.05 shoots per explant. However, number of shoots formed per explant was decreased to 1.47 ± 0.02 when explants were both injured and inoculated. This value was significantly different from controls.

When explants were injured, the average number of shoots exhibiting GUS activity per explant was 1.21 ± 0.03 and number of shoots per explant is 1.47 ± 0.02. This result indicated that 82.4 % of the shoots formed per CN were GUS positive after mechanical injury.

Following co-cultivation of CN with bacteria for 4 days, the explants were washed in liquid MS media containing 400 mg/L Cefotaxime and 200 mg/L Carbenicillin. Then the explants were placed onto selection media (Appendix C) containing 100 mg/L Kanamycin, 400 mg/L Cefotaxime and 200 mg/L Carbenicillin. Five and eleven days after subculture onto selection media (nine and sixteen days after transformation), shoots were removed from explants and used for histochemical GUS staining. Analyses of *uidA* gene expression on the 9^{th} and 16^{th} days were performed to investigate the prolonged GUS activity presence in chickpea tissues.

Results of GUS staining, that was performed nine and sixteen days after transformation, were recorded as GUS expressing area relative to the total surface area of tissues. This value of relative GUS positive area was measured by the software Zeiss® KS300. Relative GUS expressing area for injured and not injured explants is displayed in Figure 3.35 as percent of GUS expressing area. Also the representative photographs of shoots after 9 and 16 days after transformation are displayed in Figure 3.36a and Figure 3.36b, respectively.



Mean values, SEM and significant values are tabulated in Table H.17 in Appendix H.

Figure 3.35. Effect of injury on gene expression on the 9th and 16th day after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

Average GUS expressing areas relative to total surface area of shoots emerging from injured explants were 15.4 % and 14.9 % after 9 and 16 days, respectively. These values were significantly ($p < 0.05$) higher than the values, which were 2.5 % and 2.1 %, recorded for shoots emerging from not injured explants (Figure 3.35). Chickpea has intensive hairy structures on leaves and shoots. These structures may inhibit the *Agrobacterium* to reach and come in close interaction with plant cells. Besides inducing phenolic compound release, mechanical injury may also provides an opportunity to bacterial cells to invade injured plant cells.

Histochemical GUS staining analyses on 9th and 16th days revealed that expression of *uidA* gene was still continuing in infected cells or tissue portions. However the relative GUS expressing areas recorded for regenerated shoots (Figure 3.35) and photographs of these shoots (Figure 3.36) indicate that the shoots are chimeric. Regeneration of putative transgenics that are chimeric is the main disadvantage of

direct organogenesis. On the other hand GUS expressing areas in these chimeras can be increased by various modifications of the transformation procedure.

Overall, these results demonstrated that injury of axillary region of CN increased the number of cells infected at this specific region. However, the relative GUS expressing regions were still smaller in area compared to total surface area of shoots.

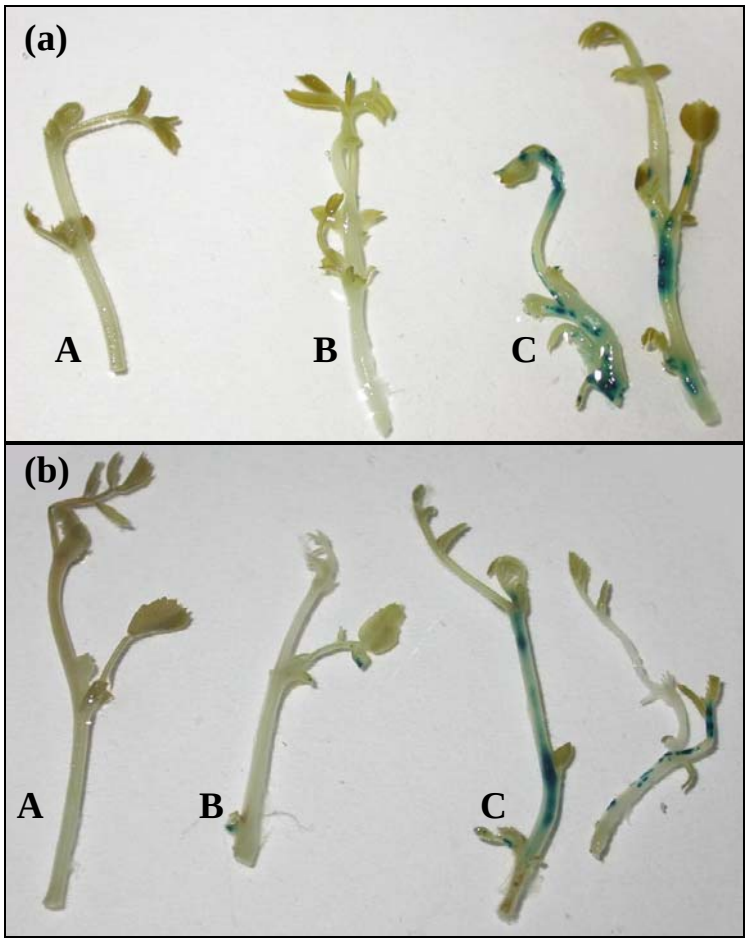


Figure 3.36. Representative shoots stained for GUS activity on the (a) 9th and (b) 16th day after transformation. A) Control, B) Not injured, C) Injured.

3.2.2. Effect of Vacuum Infiltration on Transformation Efficiency

Vacuum infiltration is an effective way of increasing the exposure of bacteria to the plant cells. Reports of Kapila *et al.* (1997) and Mahmoudian *et al.* (2002) demonstrated that vacuum infiltration of *A. tumefaciens* suspensions containing bean and lentil explants resulted in high levels of transient gene expressions. In our study vacuum infiltration was also employed for improvement of the transformation efficiency.

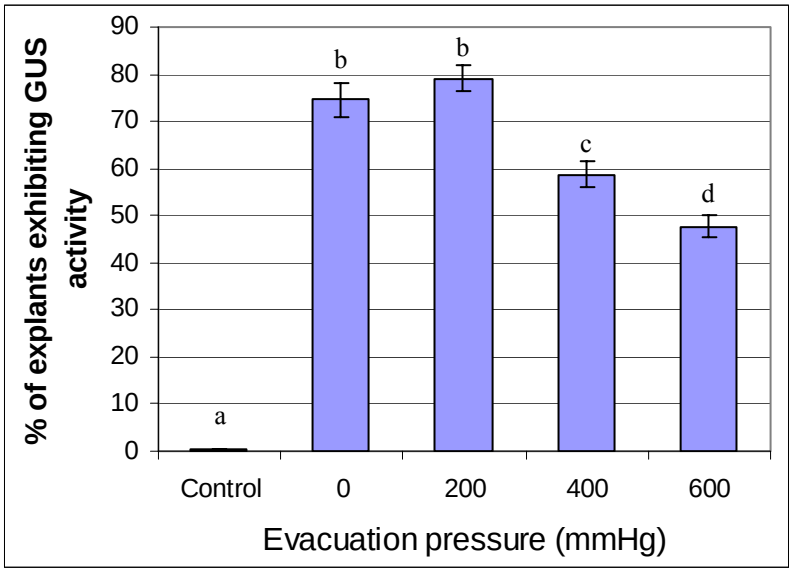
Infiltration was performed for 40 minutes, at evacuation pressures of 200, 400 and 600 mmHg, during inoculation of CN explants with bacteria, using a vacuum chamber. The experiments were coupled to control groups, which were not inoculated and not infiltrated with bacteria. All explants other than control were injured mechanically since injury was found to be increasing transient gene expression. The second control group (0 mmHg), which was inoculated but not infiltrated, was used as control for effect of vacuum infiltration.

Effect of infiltration represented as percent of explants exhibiting transient GUS activity on the 4^{th} day after transformation is displayed in Figure 3.37. All control explants exhibited no GUS activity. On the other hand, percentage of explants exhibiting GUS activity was increased when the explants were infiltrated at 200 mmHg (79.1 ± 2.8 %) compared to no vacuum applied explants (74.5 ± 3.6 %). However, this increase was not significant.

In our study, infiltration at 400 and 600 mmHg decreased the transient GUS expression levels in chickpea CNs significantly (Figure 3.37). When evacuation pressures of 400 and 600 mmHg were applied; 58.6 ± 2.8 % and 47.7 ± 2.4 % of explants exhibited GUS activity, respectively. A similar observation of decreased gene expression at high evacuation pressures was also done by Mahmoudian *et al.* (2002). The researchers reported that lentil CN explants, infiltrated at 600 mmHg for 30 minutes, yielded decreased levels of GUS expression.

Representative photographs of control, not infiltrated, and infiltrated shoots stained for GUS activity are displayed in Figure 3.38. Staining patterns show that in the absence of infiltration, when explants are only injured, stained areas are generally concentrated around wound or infection sites, however after the application of infiltration, such sites are not observed. The reason for such patterns probably results from penetration of bacteria to inner parts of the tissues during vacuum infiltration.

With the application of infiltration, possibly not only the cells on the surface of shoot primordia but also the ones located behind are infected. Penetration of bacteria to inner parts of plant tissue can be both beneficial and harmful. It can be beneficial because it increases the number of transformed cells. On the other hand it can be harmful because it may cause the death of plant cells.



Mean values, SEM and significant values are tabulated in Table H.18 in Appendix H.

Figure 3.37. Effect of vacuum infiltration on transient gene expression on the 4th day after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

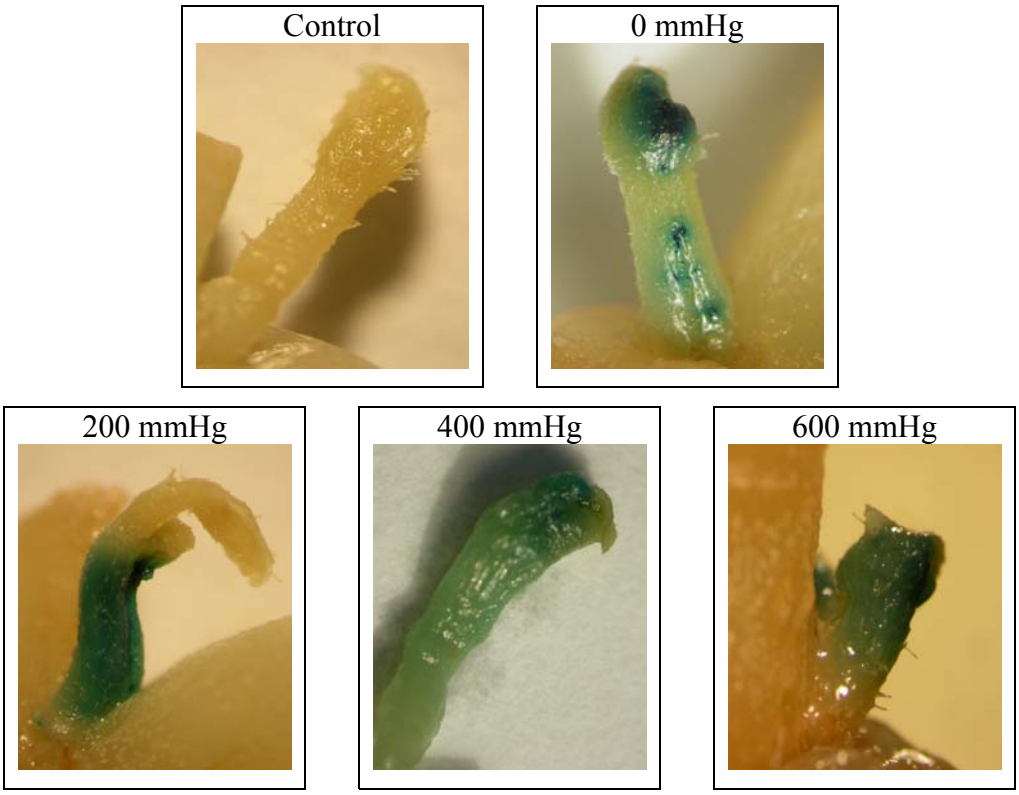
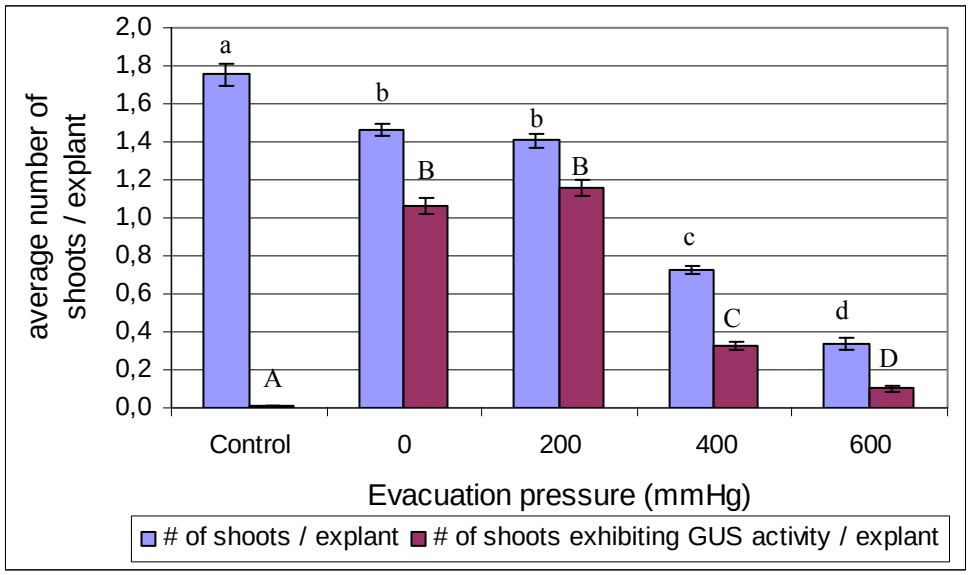


Figure 3.38. Representative photographs of shoots stained for GUS activity on the 4th day after transformation.

On the 4th day after transformation, the number of shoots and number of GUS expressing shoots per explant were also recorded. Effect of infiltration on shoot regeneration and transient gene expression as number of shoots per explant is represented in Figure 3.39. At high evacuation pressures (400 and 600 mmHg) number of shoots regenerated on CN significantly decreases.

This decrease is also displayed in Figure 3.40 showing representative shoots on the 4th day after application of infiltration. The explants infiltrated at 400 mmHg formed 0.73 and ones infiltrated at 600 mmHg formed 0.34 shoots per CN. These sharp reductions in shoot number probably stems from the detrimental effects of vacuum or bacterial penetration derived by vacuum infiltration.



Mean values, SEM and significant values are tabulated in Table H.19 in Appendix H.

Figure 3.39. Effect of infiltration on shoot regeneration and transient gene expression after 4 days of co-cultivation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

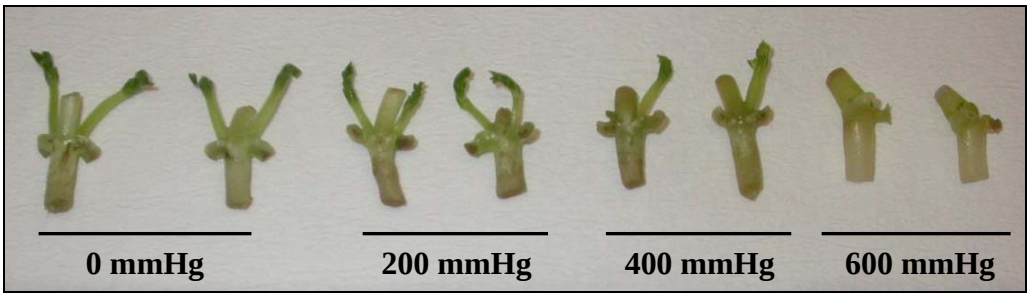


Figure 3.40. Representative shoots displaying the effect of infiltration on shoot regeneration.

No vacuum applied control explants (0 mmHg) and 200 mmHg applied explants produced significantly low numbers of shoots per CN (Figure 3.39). These significant decreases compared to control explants which were not infiltrated and not inoculated probably originate from the negative effects of mechanical injury

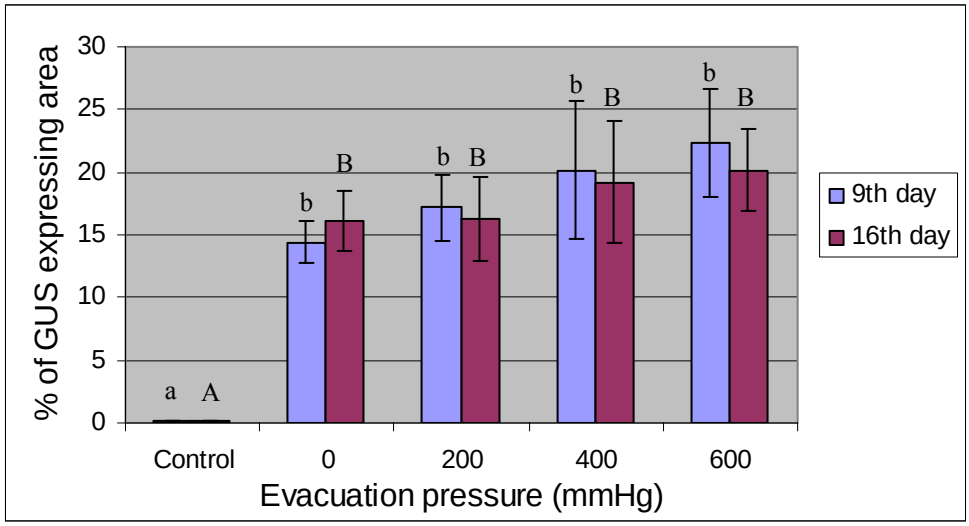
applied prior to infiltration. The number of shoots regenerated per CN was also decreased at 200 mmHg compared to no vacuum applied control; however this decrease was not significant.

Since low numbers of shoots were regenerated at high evacuation pressures, the numbers of GUS positive shoots were also low. Average number of GUS positive shoots per CN obtained at 400 mmHg and 600 mmHg were 0.33 and 0.11, respectively. According to the ratio of GUS positive shoot numbers per CN to shoots regenerated per CN, the most effective evacuation pressure was determined to be 200 mmHg (Figure 3.39).

Analyses of *uidA* gene expression via histochemical staining on 9^{th} and 16^{th} days after transformation were also performed to investigate the effect of infiltration on prolonged GUS expression. Results of GUS staining were recorded as GUS expressing area relative to the total surface area of tissues and they are represented in Figure 3.41. Also the representative photographs of shoots on the 16^{th} day after transformation are displayed in Figure 3.42.

All evacuation pressures applied, increased the relative GUS expressing area on chickpea shoots compared to no vacuum applied control. However none of the infiltration applications (200, 400 and 600 mmHg pressures) caused significant increases on both 9^{th} and 16^{th} days. On the other hand all the shoots regenerated were chimeras and GUS expressing regions were generally the basal portions of shoots (Figure 3.42). Shoots regenerated from explants, which were infiltrated at 600 mmHg, exhibited the most intense GUS expressing regions but these shoots were smaller and less developed. Besides being small, these shoots showed great variations in relative GUS expressing areas.

As a result of all these findings, it can be stated that vacuum infiltration increases the transformation efficiency; and 200 mmHg evacuation pressure is appropriate to improve gene transfer without affecting the shoot regeneration capacity of CNs.



Mean values, SEM and significant values are tabulated in Table H.20 in Appendix H.

Figure 3.41. Effect of infiltration on gene expression on the 9th and 16th day after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

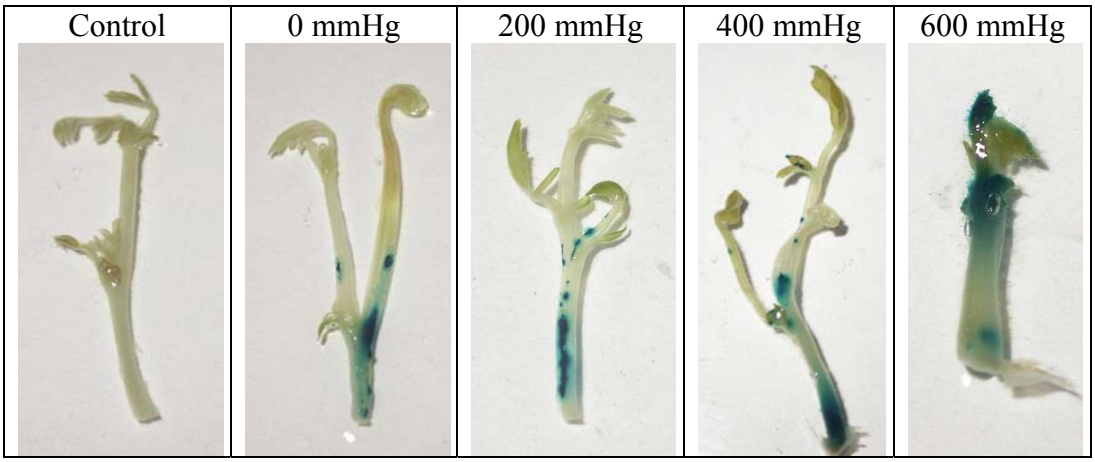


Figure 3.42. Representative shoots stained for GUS activity on the 16th day after transformation.

3.2.3. Effect of L-Cysteine on Transformation Efficiency

In *Agrobacterium* mediated transformation studies of rice and soybean, use of antinecrotic compounds resulted in a reduction in browning and necrosis of the plant tissues; together with increase in transformation efficiencies (Enriquez-Obregon *et al.*, 1999; Olhoft and Somers, 2001). In our study L-cysteine was also employed for improvement of the transformation efficiency of chickpea CN.

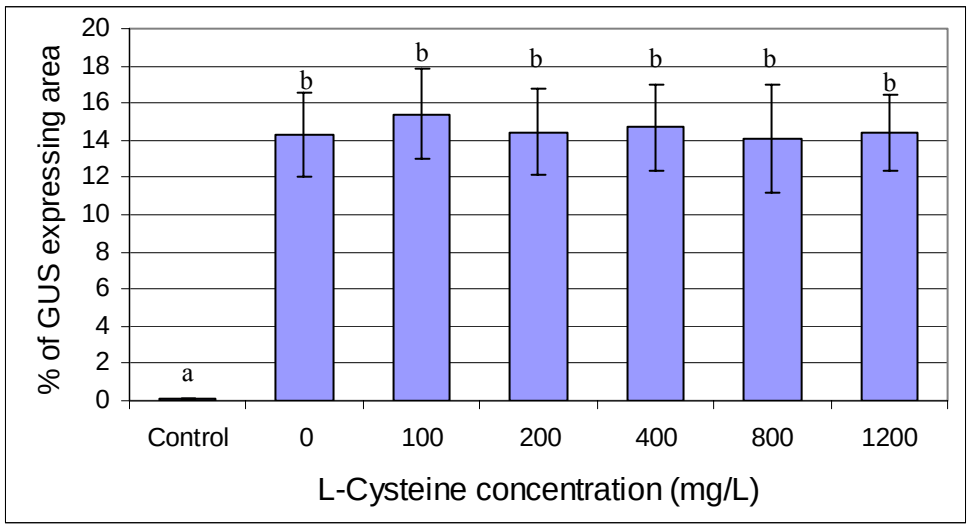
L-cysteine was added to the solid co-cultivation media at various concentrations (100, 200, 400, 800 and 1200 mg/L) to test its effects on gene transfer. Not inoculated CN explants were used as control and L-cysteine lacking co-cultivation media (0 mg/L) were used as control for L-cysteine effect. The explants co-cultivated with bacteria for 4 days together with L-cysteine were rinsed in liquid MS media containing antibiotics to perform antibiotic wash (Appendix C). Then the explants were transferred to selection media. Sixteen days after transformation the shoots were stained for GUS activity and relative GUS expressing areas were determined.

Results of GUS histochemical staining recorded as percent GUS expressing area were displayed in Figure 3.43. All concentrations of L-cysteine application did not cause any change in percentage of GUS expressing area which fluctuated between 14 and 15.5 % both in the presence and absence of L-cysteine. An increase in GUS exhibiting area was observed at 100 mg/L L-cysteine application however this increase was not significantly different from control explants cultured on L-cysteine free media.

Olhoft and Somers (2001) observed decreased necrosis and increased T-DNA transfer in soybean cotyledonary node axillary meristem cells. The controversy between findings of our study and the report of Olhoft and Somers (2001) probably results from the explant orientation on media. The researchers placed the explants adaxial side down on filter papers on top of media. So the axis of

cotyledons faces down on media. On the contrary, in our study the explants were inserted into media in normal orientation, therefore, the shoot primordia are not in touch with the medium containing L-cysteine.

The reason for observation of non-significant GUS expression levels with or without L-cysteine application in our study probably arises from absence of encounter of shoot primordia and L-cysteine. In order to improve transformation efficiency with L-cysteine, the explants may be laid down on co-cultivation media containing L-cysteine or antinecrotic treatment may be applied prior to transformation or prior to co-cultivation.



Mean values, SEM and significant values are tabulated in Table H.21 in Appendix H.

Figure 3.43. Effect of L-cysteine on gene expression on the 16th day after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

Although stable transformants of chickpea have not been recovered yet; employed procedure of transformation is highly promising for obtaining transgenic chickpea plants. The procedure reported by Mahmoudian *et al.* (2002) was improved in this study by mechanical injury and vacuum infiltration at 200 mmHg evacuation pressures applied for 40 minutes. Satisfactory transient GUS expression frequencies were obtained using a Turkish chickpea cultivar Gökçe. GUS histochemical staining assays performed on the 16^{th} day of transformation provides preliminary proofs of stable transformation.

CHAPTER IV

CONCLUSION

In this study, selection conditions and *Agrobacterium* mediated transformation of Turkish chickpea cultivar Gökçe were optimized. In the selection system optimization part of the study, effects of selective agents and *Agrobacterium* eliminating antibiotics on plant regeneration and bacterial decontamination were investigated.

According to the optimizations performed for shoot and root induction, 1 mg/L BA and 0.1 mg/L IBA were employed for shoot regeneration from CN and root induction from shoots, respectively.

In lethal dose determination for selective agents, it was determined that 100 mg/L Kanamycin, 20 mg/L Hygromycin, 3 mg/L PPT and 5 mg/L Glyphosate could be used in selection of chickpea transformants depending on the plant selection marker present in T-DNA.

The lowest concentrations of Kanamycin (50 mg/L), Hygromycin (10 mg/L), PPT (3 mg/L) and Glyphosate (1 mg/L) totally inhibited root induction from regenerated chickpea shoots. Therefore these concentrations can be employed in rooting of putative transgenics.

Among the *Agrobacterium* elimination antibiotics, all concentrations of Augmentin and Cefotaxime were determined to possess no effect on shoot regeneration. On the other hand, Carbenicillin slightly decreased and Timentin slightly increased the number of shoots per explant after four weeks of culture.

In the investigation of antibiotic effect on root induction, it is determined that Augmentin has no effect on rooting capacities of chickpea shoots. However, Cefotaxime at all concentrations significantly decreased root induction via inhibiting both root growth and root primordia formation. On the other hand only high concentrations of Carbenicillin (300 mg/L) and Timentin (200 mg/L) significantly decreased rooting of regenerated shoots.

Combinations of antibiotics and Sulbactam resulted in significant decreases in both multiple shoot and root induction. The decrease in multiple shoot induction probably originates from Carbenicillin and the decrease in root induction probably originates from Cefotaxime.

Disc diffusion assay was conducted for investigation of the effects of antibiotics on bacterial growth. According to the diameters of growth inhibition zones it is found that Carbenicillin has no inhibitory effect on *A. tumefaciens* strain KYRT1. Augmentin is the least and Cefotaxime is the most effective antibiotics when applied alone. Among the combinations employed 100 µg Sulbactam, when applied together with 200 or 50 µg Carbenicillin and 400 or 100 µg Cefotaxime, displayed effective inhibitions of bacterial growth. Therefore decreased antibiotic concentrations can be employed for elimination of bacteria with the supplement of Sulbactam.

According to cumulative results of antibiotic effects on plant regeneration and bacterial decontamination; it is concluded that combination of Cefotaxime and Sulbactam may be employed in selection media after an event of *Agrobacterium* mediated transformation of chickpea CN. It is also concluded that Timentin

together with Sulbactam may be employed in rooting of putative chickpea transformants.

Besides optimization of selection conditions, *Agrobacterium* mediated transformation procedure for CN explants of Gökçe was also optimized by monitoring transient *uidA* expression on 4^{th}, 9^{th}, and 16^{th} days.

One of the parameters tested for improvement of procedure was mechanical injury of axillary region of CN explants. According to GUS histochemical staining assays it was concluded that injury significantly increased the transformation efficiency. Injury of explants prior to inoculation resulted in two fold increase in percent of explants infected and seven fold increase in relative GUS expressing area on a regenerated shoot.

Vacuum infiltration of CN explants with bacteria, increased the transformation efficiency. High evacuation pressures (400 and 600 mmHg) significantly decreased shoot regeneration. Evacuation pressure of 200 mmHg applied for 40 minutes was appropriate to improve gene transfer without affecting the shoot regeneration capacity of CNs.

Incorporation of L-cysteine to cocultivation media did not cause any significant improvement in percentage of GUS expressing area on 16^{th} day after transformation.

Employed procedure of transformation, which is highly promising for obtaining transgenic chickpea plants, was improved in this study by mechanical injury and vacuum infiltration. According to cumulative results of transformation studies, transformation efficiency and percentage of GUS expressing areas on shoots were significantly enhanced by application of mechanical injury and infiltration at 200 mmHg.

Optimization of selection and transformation procedures performed in this study, greatly contributes to the improvement of locally cultivated Turkish chickpea cv. Gökçe. Employing these procedures, CN explants of chickpea can be used to transfer agronomically important genes like fungal resistance or abiotic stress tolerance genes.

APPENDIX A

COMPARISON OF MAJOR CHICKPEA PRODUCING COUNTRIES

Table A.1. Chickpea production (Mt) of major chickpea growing countries (FAOSTAT, 2004).

Chickpea Production (Mt)	**Year**					
	1990	**1995**	**2000**	**2001**	**2002**	**2003**
Australia	190,268	286,909	162,000	258,000	136,000	199,000
Canada	0	1,000	387,500	455,000	156,500	67,600
Ethiopia	ND	124,507	164,627	175,734	180,410	180,410
India	4,217,300	6,435,500	5,120,000	3,855,400	5,473,000	4,130,000
Mexico	180,147	167,244	233,809	326,119	235,053	240,000
Pakistan	561,900	558,500	564,500	397,000	362,100	671,700
Spain	52,100	31,000	55,512	56,949	72,500	64,500
Turkey	860,000	730,000	548,000	535,000	650,000	600,000

Table A.2. Chickpea yield (Hg/Ha) of major chickpea growing countries (FAOSTAT, 2004).

Chickpea Yield (Hg/Ha)	**Year**					
	1990	**1995**	**2000**	**2001**	**2002**	**2003**
Australia	10,724	13,257	6,183	13,231	6,766	9,171
Canada	0	12,987	13,683	9,735	10,182	10,781
Ethiopia	ND	6,960	8,909	8,293	9,214	9,214
India	6,518	8,532	8,330	7,435	8,530	7,283
Mexico	13,387	15,308	17,331	16,770	15,953	16,000
Pakistan	5,427	5,247	5,809	4,387	3,877	4,001
Spain	8,376	2,975	7,037	6,905	8,211	8,259
Turkey	9,795	9,799	8,616	8,295	9,701	9,231

Table A.3. Chickpea harvested area (Ha) of major chickpea growing countries (FAOSTAT, 2004).

Chickpea Area Harv (Ha)	**Year**					
	1990	**1995**	**2000**	**2001**	**2002**	**2003**
Australia	177,421	216,415	262,000	195,000	201,000	217,000
Canada	0	770	283,200	467,400	153,700	62,700
Ethiopia	ND	178,890	184,790	211,910	195,800	195,800
India	6,470,500	7,543,000	6,146,300	5,185,300	6,416,200	5,670,800
Mexico	134,565	109,253	134,909	194,464	147,337	150,000
Pakistan	1,035,400	1,064,500	971,800	905,000	933,900	1,679,000
Spain	62,200	104,200	78,886	82,479	88,300	78,100
Turkey	877,976	745,000	636,000	645,000	670,000	650,000

Table A.4. Abbreviations for Table A.1, A.2, and A.3.

Ha	Hectare
Hg/Ha	Hectogram per hectare
Mt	Metric ton
ND	No data

APPENDIX B

COMPOSITION OF MURASHIGE AND SKOOG (MS) BASAL MEDIA

Table B.1. Composition of MS basal media (micro, macro elements and vitamins)

COMPONENT	**mg / L**
MACRO ELEMENTS	
$CaCl_2$	332.02
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
MICRO ELEMENTS	
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
Kl	0.83
$MnSO_4.H_2O$	16.90
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
ORGANICS (VITAMINS)	
Glycine	2.00
Myo-inositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thiamine HCl	0.10

APPENDIX C

COMPOSITION AND USE OF PLANT TISSUE CULTURE MEDIA

Table C.1. Composition and usage of plant tissue culture media

MEDIUM	COMPOSITION	USAGE
Seed Germination Medium	half-strength MS micro & macro elements + 1.5 % sucrose + 0.6 % agar; pH 5.6 - 5.8	Germination of surface sterilized chickpea seeds.
Multiple Shoot Induction Medium	MS basal media + 3 % sucrose + 0.8 % agar + 1 mg/L BA; pH 5.6 – 5.8	Induction of multiple shoot formation from CN via direct organogenesis.
Root Induction Medium	MS basal media + 3 % sucrose + 0.8 % agar + 0.1 mg/L IBA; pH 5.6 – 5.8	Induction of roots from regenerated shoots.
MMA	MS micro & macro elements + 2 % sucrose + 10 mM MES + 200 µM acetosyringone; pH 5.6	Resuspension of bacteria; bacterial inoculation of CN explants.
Co-cultivation Medium	MS basal media + 3 % sucrose + 0.8 % agar + 1 mg/L BA; pH 5.6 – 5.8	Co-cultivation of CN explants with bacteria for 4 days.
Antibiotic Wash Medium	MS basal media + 3 % sucrose + 400 mg/L Cefotaxime + 200 mg/L Carbenicillin; pH 5.6 – 5.8	Elimination of bacteria after transformation and co-cultivation.

Table C.1. Continued

MEDIUM	COMPOSITION	USAGE
Selection Medium	MS basal media + 3 % sucrose + 0.8 % agar + 1 mg/L BA + 100 mg/L Kanamycin + 400 mg/L Cefotaxime + 200 mg/L Carbenicillin; pH 5.6 – 5.8	Selection of putative transgenics after an event of transformation.
Rooting Medium	MS basal media + 3 % sucrose + 0.8 % agar + 0.1 mg/L IBA + 50 mg/L Kanamycin + 400 mg/L Cefotaxime + 200 mg/L Carbenicillin; pH 5.6 – 5.8	Root induction from putative transgenic shoots developed from CN after an event of transformation.

APPENDIX D

PLASMID MAP AND SELECTION MARKERS

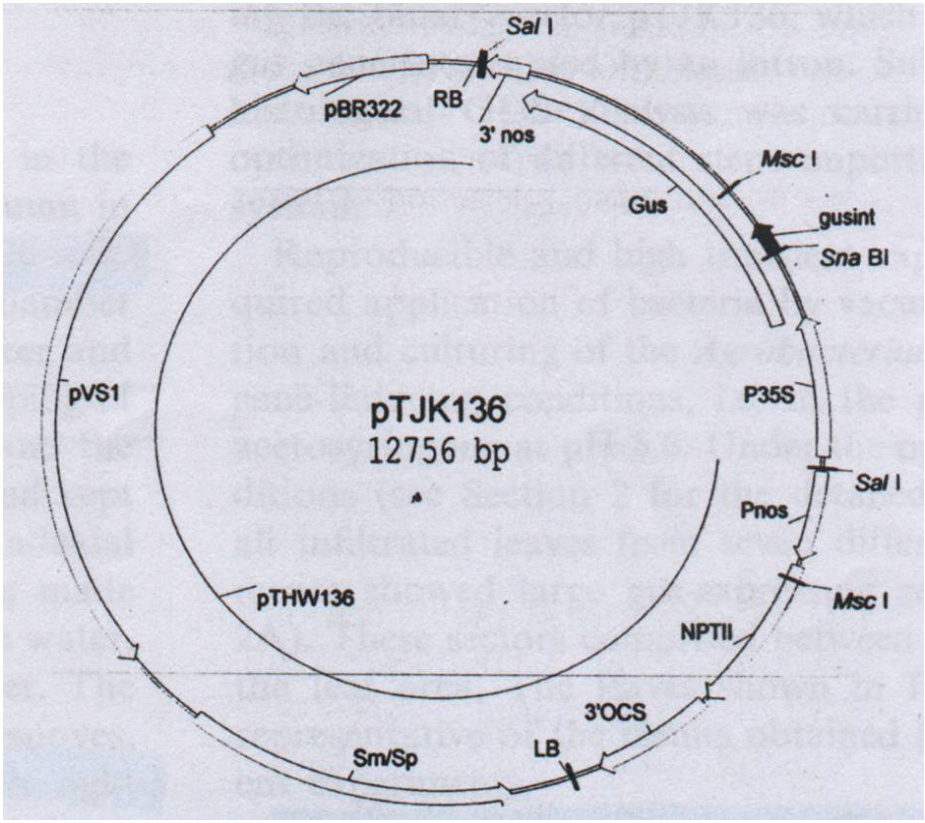


Figure D.1. Map of pTJK136

Table D.1. Selection markers found on bacterial strain and binary plasmid used in this study

Bacterial Strain	Chromosomal/Ti Plasmid Selection Marker
KYRT1	Rif^r (100 mg/L) Carb^r (100 mg/L) Gent^r (40 mg/L)

Plasmid	Bacterial Selection Marker	Plant Selection Marker
pTJK136	Strep^r (300 mg/L) Spect^r (125 mg/L)	*nptII* *uidA*

^{r}: resistance character

Rif (Rifampin), Carb (Carbenicillin), Gent (Gentamicin), Strep (Streptomycin), Spect (Spectinomycin), *nptII* (Gene coding for Neomycin Phospho Transferase II), *uidA* (Gene coding for β-D-glucuronidase)

APPENDIX E

PERMISSION LETTER FOR BINARY VECTOR pTJK136

Laboratorium Genetica
Vakgroep Moleculaire Genetica
K. L. Ledeganckstraat 35
B-9000 Gent, BELGIE
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

APPENDIX F

COMPOSITION OF BACTERIAL CULTURE MEDIA

Table F.1. Composition and usage of bacterial culture media

MEDIUM	COMPOSITION	USAGE
YEB	13.5 g/L nutrient broth + 1 g/L yeast extract + 5 g/L sucrose + 2 mM (0.493 g/L) $MgSO_4.7H_2O$; pH 7.2	Growth of bacteria (initial culture).
YEB+MES	13.5 g/L nutrient broth + 1 g/L yeast extract + 5 g/L sucrose + 2 mM (0.493 g/L) $MgSO_4.7H_2O$ + 10 mM (2.132 g/L) MES + 20 µM acetosyringone; pH 5.6	Growth of bacteria (large scale); induction of *Agrobacterium vir* genes.
MMA	MS micro & macro elements + 2 % sucrose + 10 mM (2.132 g/L) MES + 200 µM acetosyringone; pH 5.6	Resuspension of bacteria; induction of *Agrobacterium vir* genes; bacterial inoculation of CN explants.

APPENDIX G

HISTOCHEMICAL GUS ASSAY SOLUTIONS

GUS Substrate Solution

$NaPO_4$ buffer, pH 7.0	0.1 M
EDTA, pH 7.0	10 mM
Potassium ferricyanide, pH 7.0	0.5 mM
Potassium ferrocyanide, pH 7.0	0.5 mM
X-GlcA	1.0 mM
Triton X-100	0.1 % (v/v)

GUS Fixative Solution

Formaldehyde	10 % (v/v)
Ethanol	20 % (v/v)
Acetic acid	5 % (v/v)
Distilled water	65 % (v/v)

APPENDIX H

TABULATED VALUES OF GRAPHS

Table H.1. Mean values, SEM and significant values for Figure 3.2. (Effect of various BA concentrations on multiple shoot induction).

	0 mg/L BA	1 mg/L BA	3 mg/L BA	10 mg/L BA
Average number of shoots / CN	1.76 ± 0.137^{a}	3.59 ± 0.189^{b}	3.72 ± 0.164^{b}	1.93 ± 0.148^{a}

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.2. Mean values, SEM and significant values for Figure 3.4. (Effect of two different IBA concentrations on root induction).

	0 mg/L IBA	0.1 mg/L IBA	0.5 mg/L IBA
Average percent of shoots with roots	22.50 ± 2.50^{a}	81.67 ± 1.67^{b}	13.33 ± 3.33^{a}
Average number of roots / shoot	1.40 ± 0.245^{a}	4.39 ± 0.216^{b}	1.00 ± 0.050^{a}

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.3. Mean values, SEM and significant values for Figure 3.5. (Effect of Kanamycin (K) on multiple shoot induction).

Concentration (mg/L)	Weeks			
	1	2	3	4
0 mg/L K	1.73 ± 0.083^{a}	2.13 ± 0.078^{a}	2.56 ± 0.097^{a}	3.13 ± 0.135^{a}
50 mg/L K	1.68 ± 0.088^{a}	1.83 ± 0.079^{b}	1.94 ± 0.083^{b}	2.11 ± 0.096^{b}
100 mg/L K	1.45 ± 0.108^{a}	1.48 ± 0.111^{c}	1.52 ± 0.105^{c}	1.66 ± 0.103^{c}
150 mg/L K	1.50 ± 0.115^{a}	1.54 ± 0.111^{c}	1.56 ± 0.115^{c}	1.62 ± 0.110^{c}
200 mg/L K	1.48 ± 0.124^{a}	1.50 ± 0.119^{c}	1.55 ± 0.114^{c}	1.57 ± 0.109^{c}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Table H.4. Mean values, SEM and significant values for Figure 3.6. (Effect of Hygromycin (H) on multiple shoot induction).

Concentration (mg/L)	Weeks			
	1	2	3	4
0 mg/L H	1.72 ± 0.077^{a}	2.19 ± 0.072^{a}	2.78 ± 0.095^{a}	3.52 ± 0.126^{a}
10 mg/L H	1.55 ± 0.093^{a}	1.71 ± 0.081^{b}	1.98 ± 0.080^{b}	2.22 ± 0.099^{b}
20 mg/L H	1.20 ± 0.109^{b}	1.25 ± 0.109^{c}	1.39 ± 0.101^{c}	1.54 ± 0.102^{c}
30 mg/L H	1.06 ± 0.122^{b}	1.15 ± 0.112^{c}	1.36 ± 0.105^{c}	1.43 ± 0.103^{cd}
50 mg/L H	1.04 ± 0.122^{b}	1.06 ± 0.120^{c}	1.18 ± 0.118^{c}	1.22 ± 0.113^{d}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Table H.5. Mean values, SEM and significant values for Figure 3.7. (Effect of PPT (P) on multiple shoot induction).

Concentration (mg/L)	Weeks			
	1	2	3	4
0 mg/L P	1.77 ± 0.071^{a}	2.17 ± 0.081^{a}	2.67 ± 0.102^{a}	3.27 ± 0.135^{a}
3 mg/L P	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
5 mg/L P	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
7 mg/L P	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
10 mg/L P	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Table H.6. Mean values, SEM and significant values for Figure 3.8. (Effect of Glyphosate (G) on multiple shoot induction).

Concentration (mg/L)	Weeks			
	1	2	3	4
0 mg/L G	1.71 ± 0.077^{a}	2.22 ± 0.073^{a}	2.80 ± 0.096^{a}	3.56 ± 0.129^{a}
1 mg/L G	1.59 ± 0.082^{a}	1.86 ± 0.067^{b}	2.29 ± 0.078^{b}	2.74 ± 0.106^{b}
5 mg/L G	1.17 ± 0.108^{b}	1.22 ± 0.107^{c}	1.45 ± 0.099^{c}	1.67 ± 0.103^{c}
10 mg/L G	1.02 ± 0.112^{b}	1.12 ± 0.103^{c}	1.24 ± 0.092^{c}	1.37 ± 0.093^{d}
25 mg/L G	1.04 ± 0.122^{b}	1.06 ± 0.120^{c}	1.18 ± 0.118^{c}	1.22 ± 0.113^{d}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Table H.7. Mean values, SEM and significant values for Figure 3.19. (Effect of Augmentin (A) on root induction from shoots).

	0 mg/L A	200 mg/L A	400 mg/L A	600 mg/L A
Average percent of shoots with roots	86.96±3.79[a]	86.36±4.55[a]	91.67±7.93[a]	85.71±4.49[a]
Average number of roots / shoot	4.95±0.25[a]	4.89±0.30[a]	4.86±0.27[a]	4.94±0.31[a]

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.8. Mean values, SEM and significant values for Figure 3.20. (Effect of Carbenicillin (C) on root induction from shoots).

	0 mg/L C	100 mg/L C	200 mg/L C	300 mg/L C
Average percent of shoots with roots	85.71±4.09[a]	81.82±3.67[a]	80.95±6.94[a]	57.14±1.39[b]
Average number of roots / shoot	5.67±0.31[a]	5.61±0.33[a]	3.59±0.24[b]	3.25±0.22[b]

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.9. Mean values, SEM and significant values for Figure 3.21. (Effect of Cefotaxime (Cf) on root induction from shoots).

	0 mg/L Cf	200 mg/L Cf	400 mg/L Cf	600 mg/L Cf
Average percent of shoots with roots	90.48±2.78[a]	57.89±4.22[b]	54.55±4.17[b]	55.00±9.60[b]
Average number of roots / shoot	5.32±0.28[a]	2.91±0.25[b]	2.83±0.27[b]	2.64±0.28[b]

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.10. Mean values, SEM and significant values for Figure 3.22. (Effect of Timentin (T) on root induction from shoots).

	0 mg/L T	100 mg/L T	200 mg/L T	300 mg/L T
Average percent of shoots with roots	89.47±3.70[a]	86.36±3.33[a]	70.00±4.55[b]	55.00±5.00[c]
Average number of roots / shoot	5.41±0.34[a]	5.32±0.30[a]	3.86±0.25[b]	3.27±0.24[b]

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.11. Mean values, SEM and significant values for Figure 3.25. (Effect of antibiotic combinations and Sulbactam on root induction).

	Control	200 C 400 Cf	200 C 400 Cf 100 S	50 C 100 Cf 100 S
Average percent of shoots with roots	90.91±8.33[a]	63.16±3.33[b]	61.90±1.82[b]	65.00±5.00[b]
Average number of roots / shoot	5.25±0.27[a]	3.08±0.23[b]	3.08±0.21[b]	3.31±0.26[b]

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.12. Diameter of growth inhibition zones formed around filter paper discs containing various concentrations of antibiotics.

Antibiotic	Concentration in disc (μg)	Diameter of growth inhibition zone (mm) Average ± SEM
Augmentin	200	8 ± 1.9
	400	12 ± 2.1
	600	15 ± 2.0
Carbenicillin	100	0 ± 0.0
	200	0 ± 0.0
	300	0 ± 0.0
Cefotaxime	200	32 ± 1.1
	400	38 ± 1.3
	600	42 ± 1.1
Timentin	100	17.5 ± 1.75
	200	23.5 ± 1.5
	300	28.5 ± 1.7

Table H.13. Diameter of growth inhibition zones formed around filter paper discs containing various combinations of antibiotics.

Combinations and concentrations of antibiotics in disc (μg)	Diameter of growth inhibition zone (mm) Average ± SEM
200 C + 400 Cf	37 ± 1.0
200 C + 400 Cf + 100 S	47 ± 1.2
50 C + 100 Cf + 100 S	33 ± 1.2
100 K	22 ± 1.3
100 K + 200 C + 400 Cf	38 ± 1.5
100 K + 200 C + 400 Cf + 100 S	48 ± 1.1
100 K + 50 C + 100 Cf + 100 S	34 ± 1.1
100 K + 200 T	23 ± 1.3

Table H.14. Mean values, SEM and significant values for Figure 3.30. (Antibiotic effect on bacterial growth determined by a spectrophotometric method).

	OD at 600 nm Average ± SEM
Control with *Agrobacterium*	0.596 ± 0.038^{a}
Control without *Agrobacterium*	0.067 ± 0.011^{b}
200 C + 400 Cf	0.128 ± 0.017^{c}
200 C + 400 Cf + 100 S	0.133 ± 0.017^{c}
50 C + 100 Cf + 100 S	0.127 ± 0.016^{c}

Values indicated with same letter are not significantly different ($p < 0.05$).

Table H.15. Mean values, SEM and significant values for Figure 3.31. (Effect of mechanical injury on transient gene expression on the 4th day after transformation).

	Control	Not injured	Injured
Percent of explants exhibiting GUS activity. Average ± SEM	0.00 ± 0.0^{a}	42.57 ± 4.12^{b}	78.59 ± 2.63^{c}

Values indicated with same letter are not significantly different ($p < 0.05$).

Table H.16. Mean values, SEM and significant values for Figure 3.34. (Effect of injury on shoot regeneration and transient gene expression after 4 days of co-cultivation).

	Control	Not injured	Injured
Average number of shoots per explant	1.71 ± 0.05^{a}	1.58 ± 0.05^{ab}	1.47 ± 0.02^{b}
Average number of shoots exhibiting GUS activity per explant	0.00 ± 0.0^{a}	0.49 ± 0.06^{b}	1.21 ± 0.03^{c}

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.17. Mean values, SEM and significant values for Figure 3.35. (Effect of injury on gene expression on the 9th and 16th days after transformation).

		Control	Not injured	Injured
Percent of GUS expressing area	9th day	0.00 ± 0.0^{a}	2.49 ± 0.43^{b}	15.40 ± 1.88^{c}
	16th day	0.00 ± 0.0^{a}	2.11 ± 0.42^{b}	14.85 ± 1.68^{c}

Values in the same row indicated with same letter are not significantly different ($p < 0.05$).

Table H.18. Mean values, SEM and significant values for Figure 3.37. (Effect of vacuum infiltration on transient gene expression on the 4th day after transformation).

Evacuation pressure (mmHg)	Percent of explants exhibiting GUS activity
Control	0.00 ± 0.0^{a}
0	74.54 ± 3.60^{b}
200	79.07 ± 2.79^{b}
400	58.64 ± 2.75^{c}
600	47.67 ± 2.38^{d}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Table H.19. Mean values, SEM and significant values for Figure 3.39. (Effect of infiltration on shoot regeneration and transient gene expression after 4 days of co-cultivation).

Evacuation pressure (mmHg)	Average number of shoots per explant	Average number of shoots exhibiting GUS activity per explant
Control	1.76 ± 0.06^{a}	0.00 ± 0.00^{a}
0	1.46 ± 0.03^{b}	1.06 ± 0.04^{b}
200	1.41 ± 0.03^{b}	1.16 ± 0.04^{b}
400	0.73 ± 0.02^{c}	0.33 ± 0.02^{c}
600	0.34 ± 0.03^{d}	0.10 ± 0.02^{d}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Table H.20. Mean values, SEM and significant values for Figure 3.41. (Effect of infiltration on gene expression on the 9th and 16th days after transformation).

	Percent of GUS expressing area	
Evacuation pressure (mmHg)	9th day	16th day
Control	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}
0	14.43 ± 1.66^{b}	16.14 ± 2.36^{b}
200	17.19 ± 2.60^{b}	16.29 ± 3.36^{b}
400	20.18 ± 5.45^{b}	19.20 ± 4.82^{b}
600	22.32 ± 4.36^{b}	20.16 ± 3.30^{b}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

Table H.21. Mean values, SEM and significant values for Figure 3.43. (Effect of L-cysteine on gene expression on the 16th day after transformation).

L-cysteine concentration (mg/L)	Percent of GUS expressing area at the 16th day
Control	0.00 ± 0.0^{a}
0	14.32 ± 2.26^{b}
100	15.42 ± 2.46^{b}
200	14.46 ± 2.31^{b}
400	14.68 ± 2.31^{b}
800	14.11 ± 2.90^{b}
1200	14.45 ± 2.03^{b}

Values in the same column indicated with same letter are not significantly different ($p < 0.05$).

APPENDIX I

APPEARANCE OF ROOTS DISPLAYING SELECTIVE AGENT EFFECT ON ROOT INDUCTION

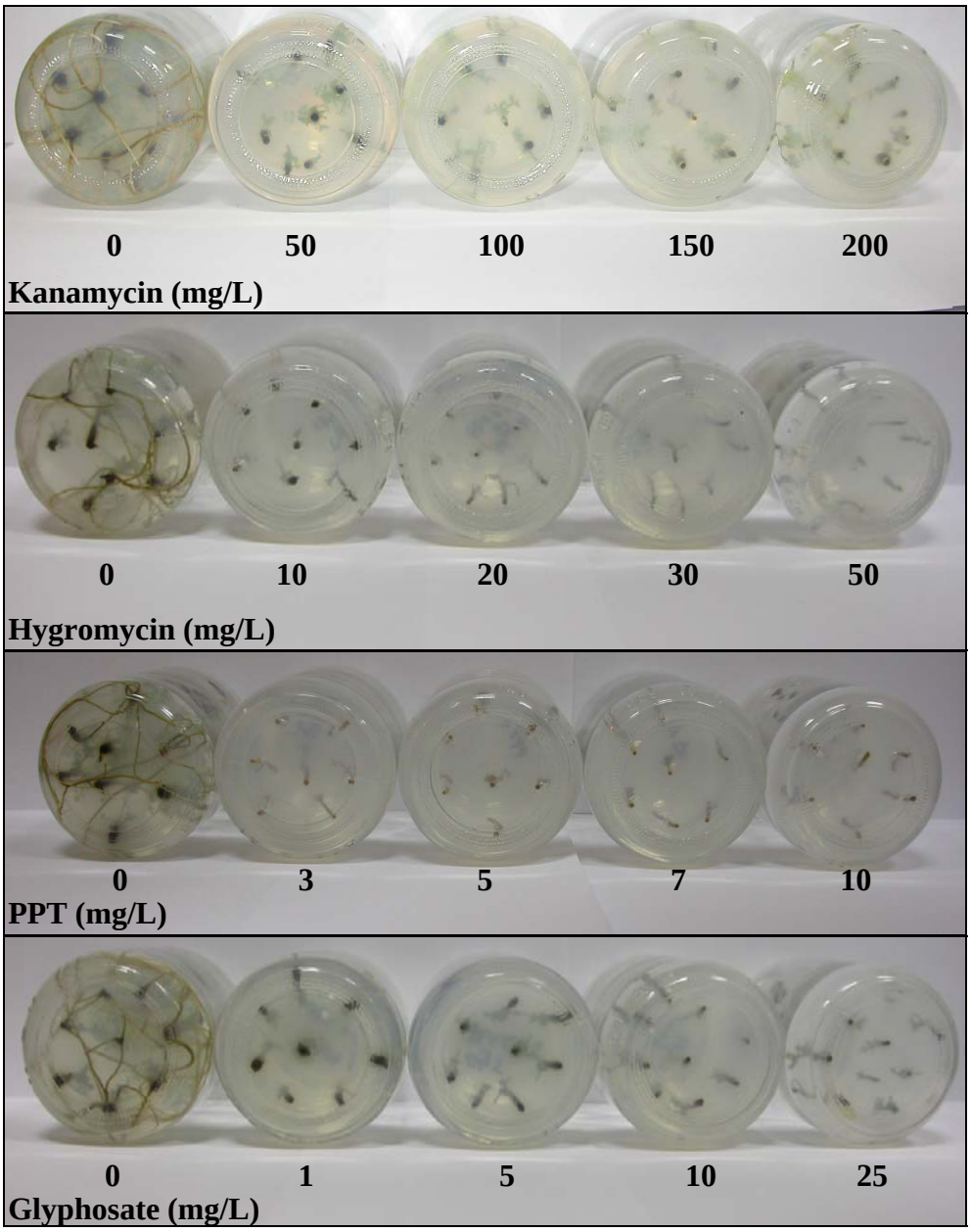


Figure I.1. Appearance of roots displaying the effect of selective agents on root induction from regenerated shoots.

APPENDIX J

APPEARANCE OF ROOTS DISPLAYING ANTIBIOTIC EFFECT ON ROOT INDUCTION

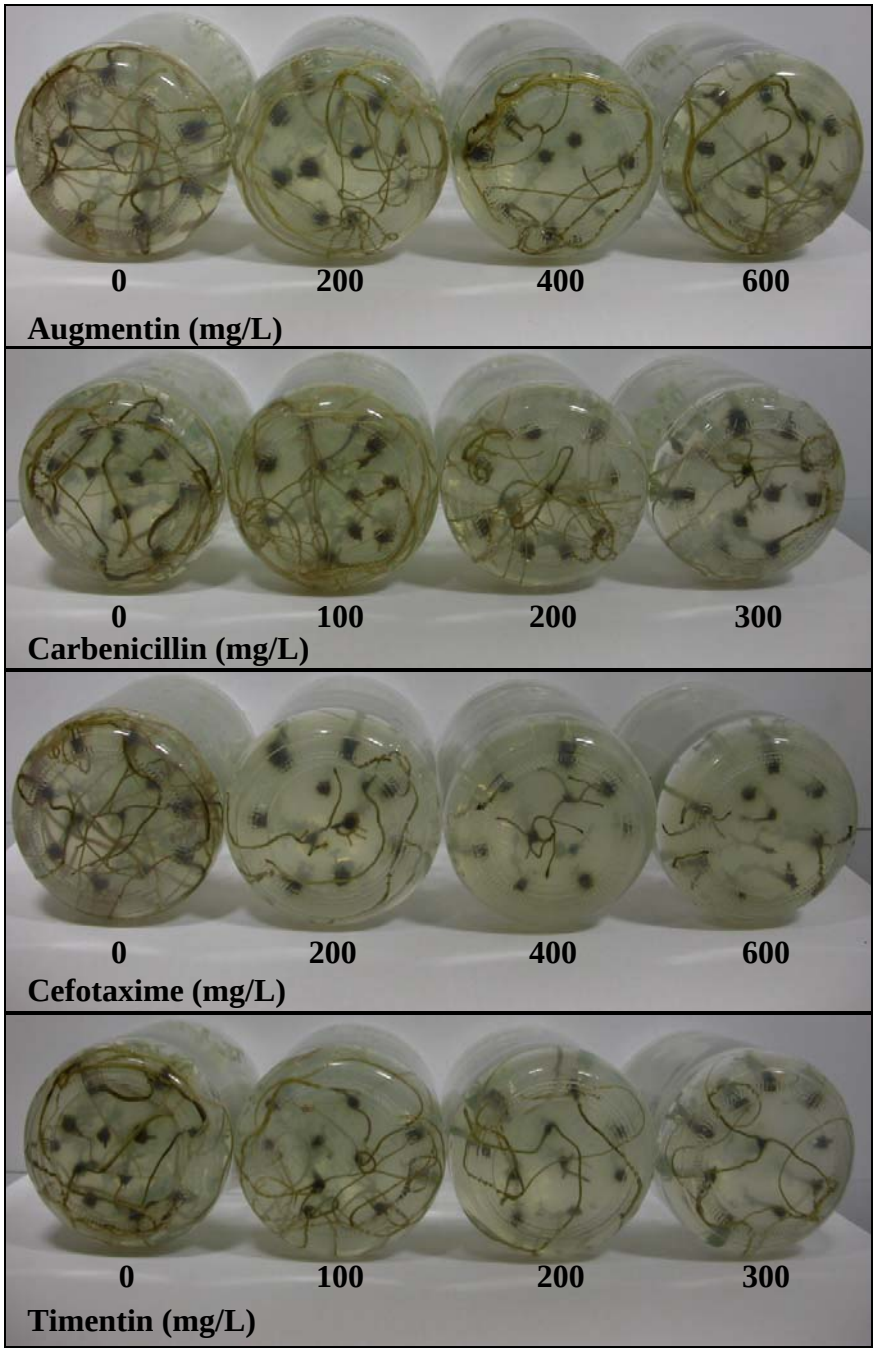


Figure J.1. Appearance of roots displaying the effect of antibiotics on rooting.

APPENDIX K

CHEMICAL STRUCTURES OF ANTIBIOTICS

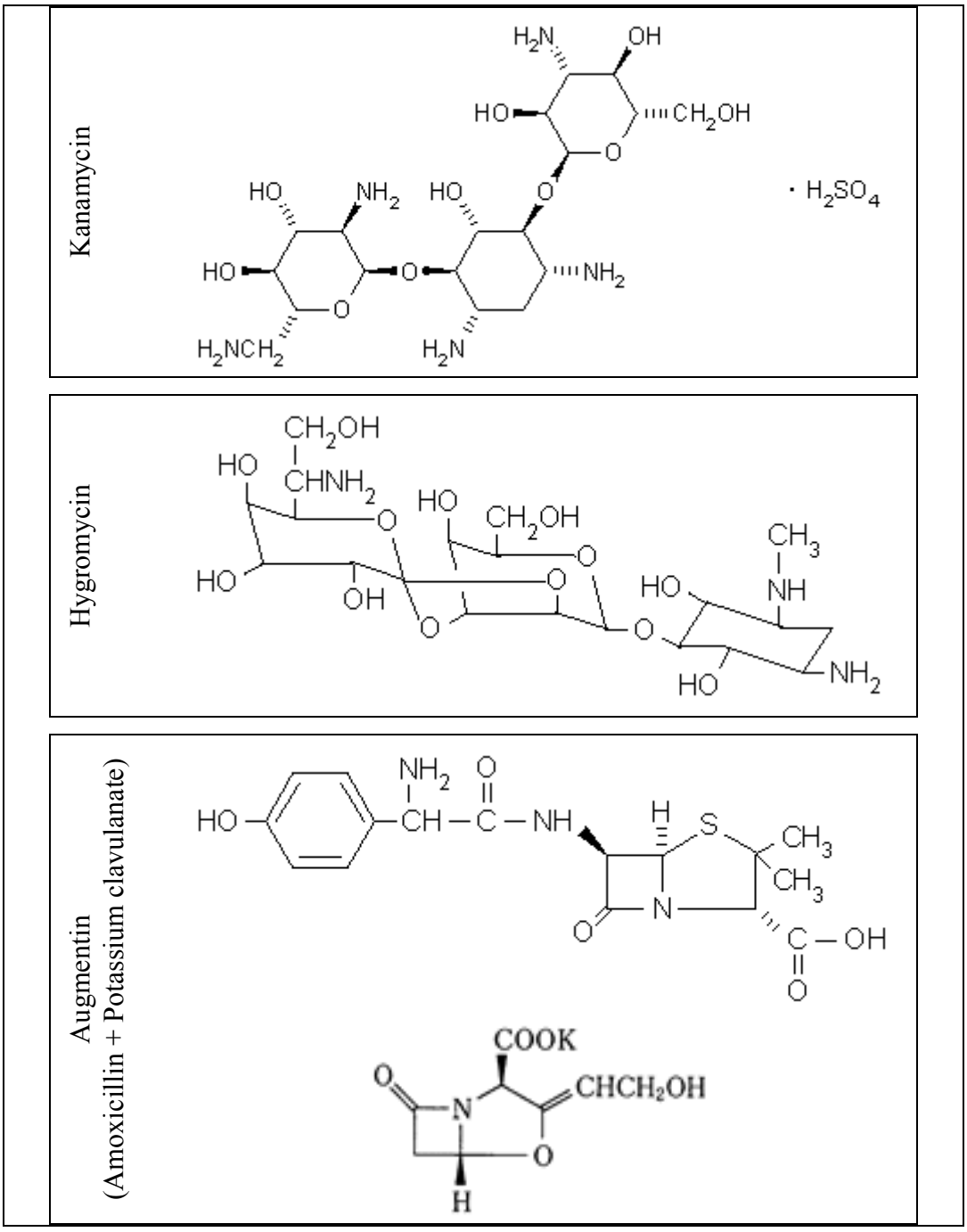
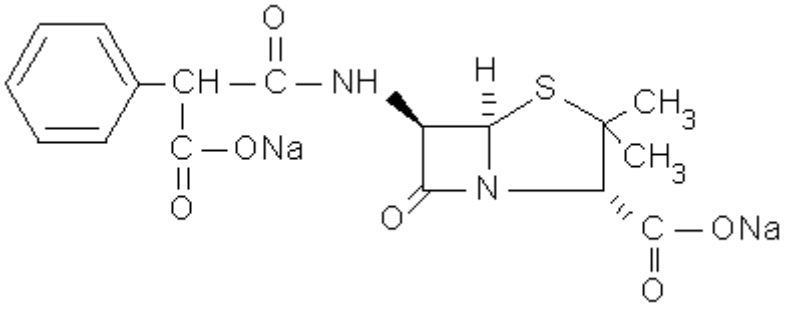
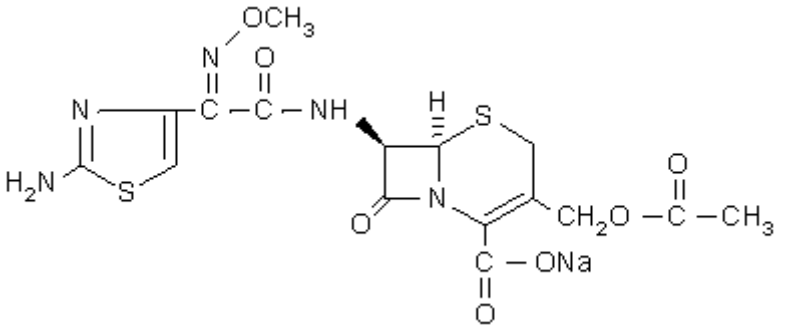


Figure K.1. Open chemical formula of antibiotics.

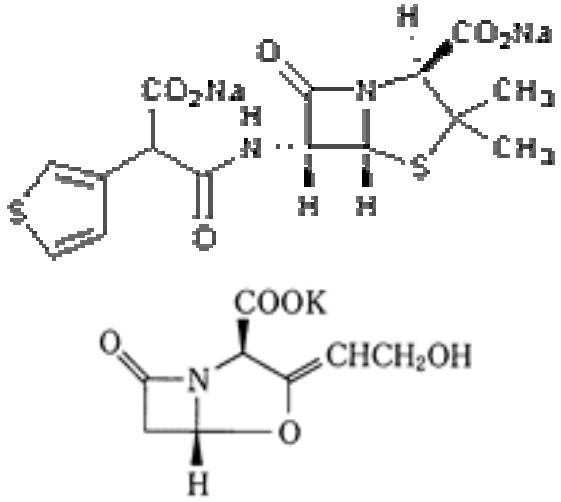
Carbenicillin



Cefotaxime



Timentin
(Ticarcillin + Potassium clavulanate)



Sulbactam

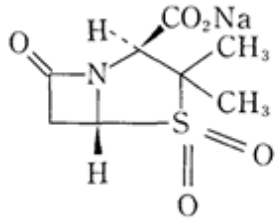


Figure K.1. continued.

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