OPTIMIZATION OF REGENERATION AND *AGROBACTERIUM* MEDIATED TRANSFORMATION OF SUGAR BEET (*Beta vulgaris* L.)

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

SEPTEMBER 2005

Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

OPTIMIZATION OF REGENERATION AND *AGROBACTERIUM* MEDIATED TRANSFORMATION OF SUGAR BEET (*Beta vulgaris* L.)

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September 2005, 130 pages

In this study, optimization of a transformation and regeneration system via indirect and direct organogenesis in cotyledon, hypocotyl, petiole, leaf and shoot base tissues of sugar beet (*Beta vulgaris* L. cv. ELK 345 and 1195) was investigated. Two different germination, three different callus induction and shoot induction medium was used for indirect organogenesis of sugar beet cultivar ELK 345. Except cotyledon, other explants (hypocotyl, petiole and leaf) produced callus. However no shoot development was observed from callus of these explants. Shoot base tissue of sugar beet cultivar 1195 was employed for direct organogenesis. Shoot development was achieved via direct organogenesis using 0.1 mg/L IBA and 0.25 mg/L BA. Root development and high acclimatization rate were accomplished from shoot base tissue.

Different concentrations of kanamycin and PPT were applied to leaf blade explants to find out optimum dose for selection of transformants. Kanamycin at 150 mg/L and PPT at 3 mg/L totally inhibited shoot development from leaf blades.

Moreover, an *Agrobacterium* mediated transformation procedure for leaf explants of ELK 345 was also optimized by monitoring transient *uidA* expression 3rd days after transformation. Effects of different parameters (vacuum infiltration, bacterial growth medium, inoculation time with bacteria, *Agrobacterium* strains and L-cysteine application in co-cultivation medium) were investigated to improve transformation procedure. Vacuum infiltration and *Agrobacterium* strains were significantly improved transformation procedure. Percentage of GUS expressing areas on leaves increased three folds from the beginning of the study.

Keywords: Sugar beet, indirect organogenesis, direct organogenesis, shoot base, *Agrobacterium tumefaciens*, GUS, transient gene expression.

ÖZ

ŞEKER PANCARINDA (*Beta vulgaris* L.) REJENERASYON VE AGROBAKTERİYE DAYALI TRANSFORMASYONUN OPTİMİZASYONU

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Eylül 2005, 130 sayfa

Bu çalışmada şeker pancarı (*Beta vulgaris* L. cv. ELK 345 and 1195) kotiledon, hipokotil, yaprak sapı, yaprak ve sürgün ucu dokularının transformasyonu ve indirekt ve direkt organogenesis yolu ile rejenerasyonu incelenmiştir. Şeker pancarı çeşidi ELK 345' in indirekt rejenerasyonu için iki farklı çimlendirme, üç farklı kallus oluşturma ve sürgün oluşturma besiyerleri kullanılmıştır. Kotiledon dışında diğer eksplantlar (hipokotil, yaprak sapı ve yaprak) kallus oluşturmuştur. Fakat bu ekplantların kalluslarından sürgün gelişimi gözlenmemiştir. Direkt organogenesis için şeker pancarı çeşidi 1195' in sürgün ucu dokusu kullanılmıştır. 0.1 mg/L IBA and 0.25 mg/L BA kullanılarak direct organogenesis yolu ile sürgün gelişimi başarılmıştır. Sürgün ucu dokusundan kök gelişimi ve yüksek oranda iklimlendirme başarıyla sonuçlandırılmıştır.

Transformantların seçimi için gereken ideal dozu bulmak için tüm yaprak eksplantlarına farklı konsantrasyonda kanamisin ve PPT uygulanmıştır. 150 mg/L kanamisin ve 3 mg/L PPT tüm yapraktan sürgün gelişimini tamamen durdurmuştur.

Ayrıca ELK 345 yaprak eksplantları için Agrobacterium' a dayalı transformasyon prosedürü, transformasyonu takip eden 3. günde uidA geni geçici ifadesi izlenerek optimize edilmiştir. Transformasyon prosedürünü geliştirmek için, farklı parametrelerin (vakum infiltrasyonu, bakteri büyütme besiyerleri, bakteri ile birlikte inokülasyon zamanı, Agrobacterium çeşitleri ve ko-kultivasyon besiyeri içine L-sistein uygulanması) etkileri incelenmiştir. Vakum infiltrasyonu ve Agrobacterium çesitleri transformasyon prosedürünü önemli bir şekilde geliştirmiştir. Çalışmanın başından itibaren, yaprak üzerindeki % GUS ifade bölgeleri üç kat arttırılmıştır.

Anahtar Kelimeler: Şeker pancarı, indirekt organogenesis, direct organogenesis, sürgün ucu, *Agrobacterium tumefaciens*, GUS, geçici gen ifadesi.

To BALOĞLU family

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Prof. Dr. Meral Yücel for her guidance, suggestions and criticisim in the preparation of this thesis.

I would also like to express my deepest gratitude my co-supervisor Prof. Dr. Hüseyin Avni Öktem for his scientific support, encouragement, suggestions and unfailing interest during the research.

I would like to thank my thesis examining committee, Prof. Dr. Ekrem Gürel, Assoc. Prof. Dr. Sertaç Önde, Assist. Prof. Dr. Füsün Eyidoğan for their suggestions, criticisims and advices.

I would also like to thank Dr. Songül Gürel, Director of Plant Breeding Department of Sugar Institute, for sharing her deepest knowledge about sugar beet and her suggestions.

I would like to give special thanks to Hamdi Kamçı for his advices in experiments and encouragement; Gülsüm Kalemtaş for always being friendly, patient and helpful; Musa Kavas for his support and help; M.Tufan Öz for sharing his knowledge and experience with me; Ufuk Çelikkol Akçay for her guidance and advices in experiments and for her kindness.

I am thankful to my lab-mates Feyza Selçuk, İrem Karamollaoğlu, Ebru Bandeoğlu, Betul Deçani, Tahir Bayraç, Taner Tuncer, Simin Tansı, Didem Demirbaş, Özgür Çakıcı, Beray Gençsoy, Serpil Apaydın, Ceyhun Kayıhan and Nilüfer Avşar for their friendships and supports.

I would like to thank Börekci family for their support and being interested in my opinion.

I would like to give special thanks to my parents, Gülsüm and Abdullah Baloğlu, to my sister Tuğba Baloğlu for their emotional support and sincere wishes throughout my study.

I express my deepest love to my wife, Pınar Baloğlu, for her encouragement, motivation, valuable suggestions and comments and always being there whenever I needed.

This work is supported by the research fund: BAP-08-11-DPT2002K120510.

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2,4-D	2,4-dichlorophenoxyacetic acid
Acetosyringone	3',5'-Dimethoxy-4-Hydroxyacetophenone
ANOVA	Analysis of variance
BA	Benzylaminopurine
CaMV35S	Cauliflower Mosaic Virus 35S Promoter
cv	cultivated variety
GUS	β-glucuronidase
IBA	Indole-3-butyric acid
MES	2-[N-Morpholino] ethanesulfonic acid
MS	Murashige and Skoog
NAA	Naptalen acetic acid
npt-II	Neomycin phosphotransferase II
OD	Optical Density
PGR	Plant Growth Regulators
PPM	Plant Preservation Mixture
PPT	Phosphinotricin
SEM	Standard Error of Mean
T-DNA	Transferred DNA
TDZ	Thidiazurone
TIBA	2,3,5-Triiodobenzoic acid
Ti	Tumor inducing
uidA (gusA)	Gene coding for β-D-glucuronidase
X-Gluc	5-bromo-4-chloro-3-indolyl glucoronide
YEB	Yeast Extract Broth

CHAPTER I

INTRODUCTION

1.1. General Description About the Sugar Beet Plant

Sugar beet (*Beta vulgaris* L.) belongs to the family *Chenopodiaceae* in plant systematics. This family includes approximately 1400 species divided into 105 genera (Watson and Dallwitz, 1992). Members of this family are dicotyledonous and usually herbaceous in nature. Economically important species in this family include sugar beet, fodder beet (mangolds), red table beet, Swiss chard/leaf beet (all *Beta vulgaris*), and spinach (*Spinacia oleracea*).

The center of origin of beet (*Beta*) is believed to be the Middle East, near the Tigris and Euphrates Rivers. It is thought that wild beets spread west into the Mediterranean and North along the Atlantic sea coast. Cultivated sugar beet is likely to have originated from wild maritime beet (*B. Vulgaris* subsp. *maritima*) Repeated selection and breeding have raised the sugar content to its present level (Cooke and Scott, 1993).

Historically, sugar has been used for main component of human diet for thousands of years. The first recorded utilization of beets is from the Middle East. Records dating to the 12^{th} century contain the earliest descriptions of sugar beets as plants with swollen roots (Toxopeus, 1984). It was not until the late 18^{th} century, that German scientists began to breed beets to increase the sugar content of their roots (American Sugar beet Growers Association, 1998).

1.1.1. Sugar Beet As a Source of Sucrose

Sugar beet (*Beta vulgaris* L.) is the most important sugar producing crop in Europe and other temperature regions of the world. About 40 % of the total sugar in the world market is produced from sugarbeet (Atanassova, 1986). Although sugar beet has large economic value and investigations have been carried out for long periods, plant remained a recalcitrant species, particularly with respect to tissue culture and genetic transformation.

1.1.2. Growth Habits

Sugar beet is normally a biennial species. That is it completes its life cycle in two years. During the first growing season, the vegetative stage, a fleshy swollen tap root, in which much of the sucrose is accumulated, develops. Figure 1.1 indicates stages of growth from germination to mature sugar beet plant. During the second growing season, the reproductive stage, the stem arise from the root. Typically sugar beet root crops are planted in the spring and harvested in the autumn of the same year. For seed production, however, vernalisation is required. After vernalisation, a flowering stalk elongates and then flowering and seed development take place. Sucrose is lost from the storage root of this flowering plant. So, harvesting efficiency and sugar yield are decreased.

1.1.3. Morphological and Physiological Characters of Sugar Beet

The stem remains very short in the first year and forms the crown of the plant, from which arise numerous large glabrous dark-green leaves, ovate in shape. The large leaves are settled on the crown. The leaf blade has a smooth surface. The petiole of this plant contains both large and small vascular bundles. Sugar beet plants have white roots of conical shape, growing deep into the soil with only the crown exposed (Figure 1.2). Flowers of sugar beet plant are small. They are directly attached to the stem. They occur singly or in clusters depending

on monogerm or multigerm. Sugar beets produce a perfect flower consisting of a tricarpelate pistil surrounded by five stamens and a perianth of five narrow sepals.

Figure 1.1. A photograph showing stages of growth from germination to mature sugar beet.

Sugar beet grows well in a variety of soils, growing best in a deep, friable well-drained soil abundant with organic matter, but poorly on clay. The beets grow best on soils with a pH of 6.0 to 8.0. Some salinity may be tolerated after the seedling stage. Beets are notable for their tolerance to manganese toxicity. (Cattanach, Dexter and Oplinger, 1991). Also the sugar content in the root is affected by nitrogen availability Too little nitrogen in the soil results in poor leaf canopies and too much nitrogen lead to reduced sugar contents. To optimize

sucrose storage in the roots, plants should exhaust the available nitrogen supply 4-6 weeks prior to harvest. In addition to this, sowing date is quite crucial, early sowing gives better sugar yields due to increased water availability earlier in the season, but sowing too early leads to a high population of bolters.

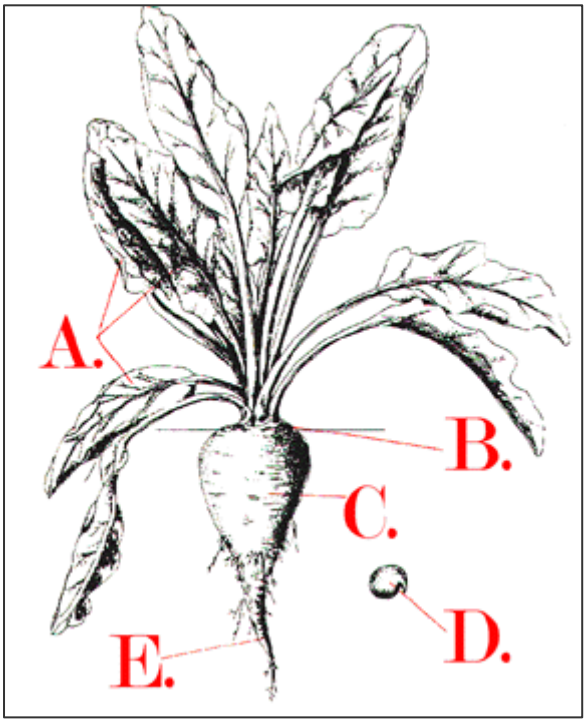


Figure 1.2. Parts of the sugar beet plant. **A.**Leaves **B.**Crown **C.**Sugar Beet **D.**Seed **E.**Tap Root

1.1.4. Biochemical Composition of a Sugar Beet Root

The sugar beet root is mainly composed of water (75.9 %). The solids of the root are made up of 18.0 % sugar, 5.5 % pulp and 2.6 % non-sugars. Highest sugar concentration is associated with phloem of vascular rings. In addition to

this, roots with numerous narrow rings usually have the highest sugar content (Bichsel, 1987).

1.1.5. Nutritional Value

Sugar beet is quite a nutritional plant in terms of all parts. The root and leaves of the sugar beet contain protein, carbohydrates as well as vitamins including β-carotene, thiamine, riboflavin, niacin and ascorbic acid. The root also contains various amino acids; leucine, tryptophane, valine, alanine, phenylalanine, tyrosine, glutamine, glutamic acid (Duke and Atchley, 1984). Therefore, these parts are used in folk medicine.

Even though the leaves of sugar beet plant are considered to be highly nutritious, they contain antinutritional factors such as oxalic acid, dangerous levels of HCN, nitrates and nitrites that cause poisoning. However, cooking overcome these adverse effects.

1.1.6. Close Relatives of Sugar Beet

Beta vulgaris is a member of the *Chenopodiaceae* and like many others in the family is a halophyte. It is a highly variable species containing four main groups of agricultural significance: leaf beets (such as Swiss chard), garden beets (such as beetroot), fodder beets (including mangolds) and sugar beet. All groups have a diploid chromosome number of 18, although most current European sugar beet varieties are triploid hybrids of diploid, male-sterile females and tetraploid pollinators (Elliot and Wetson, 1993).

1.1.7. Sugar Beet Production in the World and Turkey

After processing of sugar beet root, crystalline sucrose is chiefly recovered. 100 kg fresh sugar beet can give 12 – 15 kg sucrose, 4.5 kg dried pulp and 3.5 kg molasses. Sugar is a carbohydrate that contributes significantly to the

flavour, aroma, texture, colour and body of a variety of foods. In addition to processing pure sugar, sugar factories also produce a by-product known as dried sugar beet pulp. This pulp is used as feed for cattle and sheep, and is produced and shipped in pressed plain dried, molasses dried, and pelleted forms. Another important by-product is sugar beet molasses, a viscous liquid containing about 48 % saccharose, which cannot be economically crystallized. Sugar beet molasses is used for production of yeast, chemicals, pharmaceuticals, as well as in the production of mixed cattle feeds.

Currently, sugar beet is the major sugar crop grown in temperate regions of the world. The major sugar beet producer and exporters are the EU, France, USA, Germany, Russia Federation and Turkey (Figure 1.3). The major sugar beet importing contries include USA, China, Russia, Mexico, Pakistan, Indonesia and Japan .

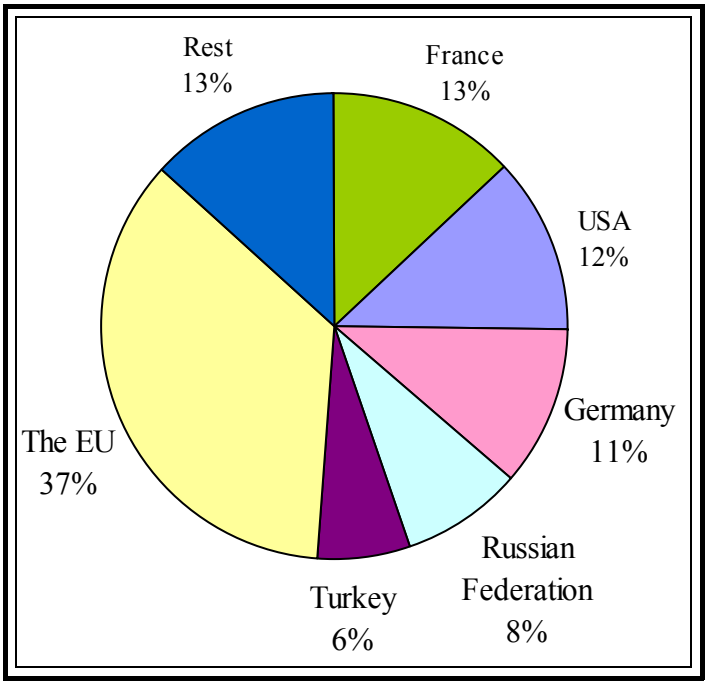


Figure 1.3. Major sugar beet producing countries and their percentages of world production in 2004 (FAOSTAT, 2005).

In 2004 world sugar beet production was nearly two hundred fifty million tons. Over the last five years, yield in world sugar beet production was ranged between 405,000 and 424,000 hectograms per hectare. In 2000 to 2004, world sugar beet production was around 229 to 256 million metric tones (Table 1.1).

Turkey was the fifth country in 2004 in sugar beet production. In 2004 sugar beet production was 13.965.000 metric tons with a yield of 423.182 hectograms per hectare in Turkey (Table 1.2). Sugar beet cultivated area in Turkey in 2004 was 330.000 hectars. Although annual yield has not changed significantly, cultivated area has drastically decreased between 1990 to 2004, probably because of contamination of soil by nematodes.

Table 1.1. Sugar beet production in the world (Ha: Hectare; Hg/Ha: Hectogram per hectare; Mt: Metric tons) (FAOSTAT, 2005)

Years	Area Harvested (Ha)	Yield (Hg/Ha)	Production (Mt)
1990	8,657,447	357,134	309,186,724
1995	7,858,752	336,880	264,745,685
2000	5,993,844	410,784	246,217,265
2001	6,002,610	381,833	229,199,298
2002	6,035,638	424,883	256,444,199
2003	5,738,048	405,791	232,844,818
2004	5,843,636	407,037	237,857,862

Table 1.2. Sugar beet production in Turkey (Ha: Hectare; Hg/Ha: Hectogram per hectare; Mt: Metric tons) (FAOSTAT, 2005)

Years	Area Harvested (Ha)	Yield (Hg/Ha)	Production (Mt)
1990	377,543	370,441	13,985,741
1995	312,251	357,744	11,170,600
2000	410,023	459,023	18,821,000
2001	358,763	352,113	12,632,520
2002	372,468	443,613	16,523,166
2003	314,000	402,003	12,622,900
2004	330,000	423,182	13,965,000

1.1.8. Diseases and Pests of Sugar Beet and Their Control

Diseases have played an extremely important role in the case of production of sugar beet. Many diseases caused by viruses, fungi, bacteria, insects and nematode may reduce yield of sugar beet root severely.

Virus diseases have the most detrimental effect on sugar beet cultivation. The most serious virus disease is Rhizomania disease. The viral agent causing the disease is beet necrotic yellow vein virus (Tamada, 1975). Canova (1966) named the disease rizomania or root madness which means the abnormal proliferation of dark and necrotic lateral roots. So, the root of the plant becomes small and production of white sugar from this infested root decreases. In many cases, root of plant was so affected that, cultivation have to be abandoned. In order to control this harmful disease, transplanting technique (in which sugar beet seedlings grown in strelised soil in pots are mechanically transplanted in the field), biological and chemical treatment can be carried out. In addition to this hazardous virus, there are many viruses which are responsible for several disease problems of sugar beet. Important viral diseases of sugar beet are given in Table 1.3.

Table 1.3. Important viruses that cause yield loss for sugar beet.

Beet western yellows
Beet yellows
Beet yellow stunt
Lettuce infectious yellows
Beet curly top
Beet mosaic
Beet cryptic
Beet distortion mosaic

Sugar beet crops are also affected by several fungal diseases. Because of survival of fungi in the soil for long periods, rotation with other crops is of little value as a control measure. Development of resistant cultivars are required to control destructive fungal disease (Duffus and Ruppel, 1993). Important major fungal diseases of sugar beet are indicated in Table 1.4.

Table 1.4. Important fungal diseases of sugar beet crop.

Diseases	**Fungi species**
Seedling disease and root rot	*Aphanomyces cochlioides*
Leaf spot	*Cercospora beticola, Ramularia beticola*
Downy mildew	*Peronospora farinosa*
Seedling damping-off, leaf spot, preharvest and postharvest root rot	*Phoma betae, Pythium aphanidermatum, P. ultimum, P. debaryanum, P. sylvaticum*
Powdery mildew	*Erysiphe polygoni*
Root and crown rot	*Rhizoctonia solani*

Moreover, bacterial diseases of sugar beet are common but, the exception of bacterial vascular necrosis and rot, they cause little damage. So, they are not economically significant in sugar beet. Furthermore, a number of pests including cutworms, wireworms, flea beetles, grasshoppers, sugar beet root aphid, beet webworm and beet leaf miner can attack the developing plant. Every commercial beet crop is host to some of these pests during its growth. In order to minimize the extent of yield loss resulting from pest attack, pesticides have been used. However, there has been a great deal of public concern about of the hazardous effects of pesticides. So, alternative methods including pest-resistant sugar beet lines, biological control techniques and ways of improving the existing methods for crop protection against pests have also been investigated (Cooke, 1993).

The sugar beet nematode, major parasite of sugar beets, causes serious halt and yield reductions wherever sugar beets are grown. The most destructive sugar beet nematode is sugar-beet cyst nematode, *Heterodera schachtii,* which brings about disease resulting in a reduced size of roots and a dense system of secondary roots called hairy roots.

The primary control for sugar beet cyst nematode and diseases affecting sugar beet is crop rotation. However, this is not a certain solution because of already contamination of soil by nematodes. Instead of crop rotation, biotechnological techniques can be utilized because wild relatives of beet have desirable characters such as disease and pest resistance. $Hs1^{pro1}$, nematode resistance gene, which is isolated from wild beet can be given as an example for these characters. If these desirable characters are transferred into cultivated sugar beet plant through biotechnological techniques, resistant sugar beet lines can be obtained in a short period compared with classical breeding techniques. So, nematode cannot affect this valuable plant. Other consequential nematode diseases of sugar beet are shown in Table 1.5.

Table 1.5. Important nematode diseases of sugar beet.

Diseases	Nematode species
Wounding parenchymatous tissue in stems and bulbs	*Ditylenchus dipsaci* (Stem nematode)
Root galling	*Nacobbus aberrans* (False root knot nematode)
Formation of galls on lateral roots	*Meloidogyne spp*. (Root-knot nematode)
Aggregate round the tips of young roots	*Trichodorus spp.* and *Paratichodorus spp*. (Stubby root nematode)

1.2. Plant Tissue Culture Techniques

Plant tissue culture is a technique, which provide producing of whole plant from the different parts of plant in artificial medium aseptically. The type of plant parts used in tissue culture can be cells (meristematic cells, suspension or callus cells), organs (meristem, shoot tip, root and anther) and nearly all types of tissue. Actually plant biotechnology relies on tissue culture techniques. After delivering a foreign gene into a target plant genome, whole plant should be regenerated from these transformed cells. Therefore, plant tissue culture is the foundation and in most cases the bottle-neck step for plant biotechnology. Moreover, tissue culture allows breeders to improve existing species.

Two concepts, plasticity and totipotency, are central to understanding plant cell culture and regeneration. Plants, due to their sessile nature and long life span, have developed a greater ability to endure extreme conditions and predation than have animals. This is called plasticity which allows plants to change their metabolism, growth and development to best suit their enviroment. Plasticity

allows one type of tissue or organ to be initiated from another type. So, entire plant can be regenerated. Plants have a capacity to develop into whole plants or plant organs *in vitro* when given the correct conditions. This maintenance of genetic potential is called totipotency. Totipotency implies that all the information necessary for growth and reproduction of the organism is contained in every cell. In practical terms, identifying the culture conditions and stimuli required to manifest this totipotency is extremely important.

Culture media is the most important part of plant tissue culture. A successful plant tissue culture system largely relies on a right culture medium formulation. Generally plant tissue culture media contains inorganic elements, organic compounds and a support matrix. Culture media provides the cultures the necessary inorganic nutrients that are usually available from soil and also provides the cultures the essential organic compounds such as vitamins, amino acids and carbon source, which are usually produced in plants. Another important function of a culture medium is creating a necessary enviroment for plants to develop. Solid media, functioning like the soil, enables a physical support for the cultures.

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

1) Macroelements (or macronutrients);
2) Microelements (or micronutrients);

Macroelements consist of elements in large supply for plant growth and development. Calsium, magnesium, nitrogen, phosphorus, potassium, sulfur and iron are macroelements. These elements generally comprise at least 0,1 % of dry weight of plants (George, 1993). The nitrogen is the most commonly used element in tissue culture media in forms of nitrate ion (NO_{3-} oxidized) and ammonium ion (NH_{4+} reduced). Calsium has a important funtion for plant growth and development in terms of functioning as a cofactor with many enzymes. Calsium

utilized in plant tissue culture is mostly in the forms of calsium chloride and calsium nitrate.

Microelements are used in trace amounts for plant growth and development. Boron, cobalt, copper, iodine, manganese, molybdenum and zinc are regarded as a microelements.If they are used at higher concentrations, it may be toxic for plant.

Organic compounds consist of four important components. They are added in large quantities to culture media.

1) Sugar
2) Vitamins
3) Amino acids
4) Complex organic compounds

Sugars serve as an enegy source for plant culture. The most commonly used sugar in plant culture media is sucrose because of cheapness, easy availability and readily assimilation. Also, other sugars such as glucose, maltose, galactose, sorbitol and starch can be used in tissue culture media for different purposes.

Vitamins are required for carbohydrate metabolism, the biosynthesis of some amino acids and have a catalytic activity on enzyme reactions. Only two vitamins, thiamine (vitamin B1) and myoinositol (considered B vitamin) are considered essential for the culture of plant cells *in vitro*. However, other vitamins are often added to plant cell culture media.

Amino acids are also prevalently used in the organic supplement. Glycine is the most frequently used in all amino acids but in many cases its inclusion is not essential. Amino acids provide a source of reduced nitrogen.

Complex organic compounds such as coconut milk or juice, yeast extract, fruit juices and fruit pulps are used in some medium formulas. They are responsible for the improved growth of the culture.

Media for plant cell culture *in vitro* can be used in either liquid or solid forms, depending on the type of culture being grown. Support matrix is often required to keep explants from being submerged in the medium. It also provides a physical support for the growing plant in the medium. The support matrix are formed by solidification of a gelling agent, such as agar, agarose or phytagel. The selection of a gelling agent is often empirical. Agar, which mainly used as a gelling agent in plant tissue culture, does not react with medium components. It is a mixture of polysaccharides derived from red algae. Agarose is extracted from agar. Agarose has higher gel strength than agar. Phytagel produced by bacterium *Pseudomonas elodea* is clear gelling agent so detection of contamination is easier than agar.

1.2.1 Plant Growth Regulators

Generally, two important plant growth regulators are used for regeneration studies in plant tissue culture.

1) Auxins
2) Cytokinins

Auxins

The name of the auxins comes from Greek word auxein, which means to increase or augment. Auxins are synthesized in the stem and root apices and transported through the plant axis. Auxins stimulate cell elongation and influence a host of other developmental responses, such as root initiation, vascular differentiation, apical dominance and the development of auxiliary buds.

While IAA (indole-3-acetic acid) and IBA (Indole-3-butyric acid) are the natural auxins, NAA (1-naphthylacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), Dicamba (2-methoxy-3,6-dichlorobenzoic acid) and Picloram (4-amino-2,5,6-trichloropicolinic acid) are synthetic auxin-like growth regulators.

Cytokinins

Cytokinins are adenine-like compounds. They are mainly produced in young organs and transported through the xylem. Cytokinins stimulate cell division and induce shoot bud formation in tissue culture. Cytokinins prevent embryogenesis and root induction. Frequently used cytokinins in tissue culture include zeatin (4-hydroxy-3-methyl-trans-2-butenylaminopurine), 2iP[N6-(2-isopentyl)adenine], kinetin (6 furfurylaminopurine), BAP (6-benzylaminopurine) and Thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea).

1.2.2. Organogenesis

The developmental process in which shoots and roots have been produced from a cell or cell groups is called organogenesis. During organogenesis, several events including development of compotence in cells, determination of cells for organogenesis and morphological differentiation take place. During the competency, explant tissue goes through a dedifferentiation process. After competent cells developed, these cells should be determined for organogenesis development or callus with the help of *in vitro* culture conditions. The growth regulators have great importance for this stage to be achieved. Once the determined cells are formed, the direction of organogenesis will be clear and there will be no modification in development of organ type.

Organogenesis is divided into two categories, direct and indirect organogenesis. In the case of direct organogenesis, shoot or root formation take place without an intermediary callus stage. However, in the case of indirect

organogenesis, before development of meristematic centers (shoot or root) callus formation occurs. This means that, after initiation of callus phase, shoot or root development take place and then planlet is obtained. For genetic transformation studies, utilization of indirect organogensis is more effective than direct organogenesis in order to prevent chimerism. If the plant regeneration and transformation are carried out by indirect organogenesis, selection of transformed cells will be easy because callus cells are undifferentiated cells. So, in the selective conditions, only transformed cells can survive, others can not. If the direct organogenesis is used for genetic transformation, some of the cells are transformed, whereas others are not transformed. So, chimeric plant can be obtained and this is undesirable situation for transformation of plants.

1.3. Gene Transfer Techniques

There are many methods that allow us to introduce foreign genes into plants. The main aims of producing transgenic plants are stable gene expression and transmission of the foreign genes from generation to generation. In other words in order for the gene transfer to be successful, the modification has to be inheritable and the seed produced has to contain the modification. Some specific characters such as resistance to disease, abiotic and biotic stresses and herbicides, enhance food and yield quality or produce pharmaceuticals can be gained by the aid of these foreign genes into plants.

Gene transfer systems can be divided into two types:

1) vector-mediated (indirect), in which another organism is used to affect the transfer and/or integration.
2) direct gene transfer, in which naked DNA is introduced into cells via any physical and/or chemical treatment;

Vector-mediated transformation relies almost exclusively on the use of the soil bacteria, *Agrobacterium tumefaciens* and *A. rhizogenes* as vectors. In direct gene transfer any piece of DNA may be transferred without using specialized vectors. Several direct gene transfer methods have been developed to transform plant species such as PEG, electroporation, liposome and microprojectile bombardment. The most promising procedure is microprojectile bombardment. This process involves high velocity acceleration of microprojectiles carrying foreign DNA, its penetration through the cell wall and membrane by microprojectile, and its delivery into plant cells.

1.3.1. *Agrobacterium* Mediated Gene Transfer

Plant transformation mediated by *Agrobacterium* has become the most frequently used method for the introduction of foreign genes into dicotyledonous plant cells. *Agrobacterium* is a gram-negative soil bacteria related to *Rhizobia.* There are economically three important species of *Agrobacterium*. *Agrobacterium rubi* causes small galls on a few dicots. A closely related disease called hairy root is caused by *Agrobacterium rhizogenes*.

Agrobacterium tumefaciens causes plant tumors commonly seen near the junction of the root and the stem, deriving from it the name of crown gall disease. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors. It naturally inserts its genes into plants and uses the machinery of plants to express those genes in the form of compounds that the bacterium uses as nutrients. The disease afflicts a great range of dicotyledonous plants, which constitute one of the major groups of flowering plants.

Agrobacterium transfers a discrete portion of its DNA (T-DNA) into the nuclear genome of the host plant. T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and

cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines. The T-DNA is located on a large plasmid called Ti (tumor-inducing)-plasmid, which also contains other functional parts for virulence (*vir*), conjugation (*con*) and the origin of its own replication (*ori*). In the natural infection by wild type bacteria, the T-DNA and the *vir* genes are essential for inducing plant tumors. The *vir* region is about 30 kb and encodes at least 10 operons (*virA virJ*) whose products are vital to T-DNA processing and transfer. Any genes located in the T-DNA region in principle can be transferred, but they themselves are dispensable for this process. Only the 25- bp direct repeats at the right and the left borders are necessary, of which 14 base pairs are completely conserved and cluster as two separate groups (Wei *et al*., 2000).

Deletion of the oncogenic genes from the T-DNA region of the Ti plasmid does not impede the ability of bacteria to transfer this DNA but does prevent the formation of tumors (Hellens and Mullineaux, 2000). If the native genes are removed from T-DNA, Ti plasmids and their *Agrobacterium* strains are called disarmed. Genes of interest can be introduced into plants by linking them to the disarmed T-DNA region via recombination (integration vector) or by cloning them between the border repeat present in an independent replicon (binary vector). These interested genes include selectable or scorable markers in plant, multiple cloning sites for integration of various genes and origins of replication. Plant cells transformed with such disarmed T-DNA behave like untransformed cells in the regeneration protocol. Therefore, by using such disarmed *Agrobacterium* strains it is possible to obtain normal appearing, fertile transgenic plants.

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 establihed rapid and reproducible procedures, which are further extended to other species.

In order to improve the efficiency of transformation with *Agrobacterium*, many different techniques have been used. They are focused on attempts to make possible penetration of bacteria into plant cells.

Low molecular weight phenolic compounds released from wounded plant cells attract the *Agrobacterium* and induce the *vir* genes. Intermediates of lignin synthesis or phenolic compound precursors such as acetosyringone (AS) are chemo attractants at very low concentrations but they are *vir* gene inducer at high concentrations. Other groups of phenolic compounds such as; hydroxycinnamides are known to act as *vir* gene inducers (Sangwan *et al*., 2002). Moreover, opines and flavnoid compounds may be involved in *vir* gene induction (Zerback *et al*., 1989). Wounding of explants, addition of phenolic compounds to the bacterial growth media, inoculation media or co-cultivation media may trigger the *vir* gene induction so result in increasing transformation efficiency. Tingay *et al*., (1997) used non-super virulent strain and reported successful transformation of barley. A phenolic compound 'acetosyringone' which is known to induce expression of virulence (vir) genes located on the Ti-plasmid, played a major role in the success of transformation.

Cheng and co-workers (1997) also reported the importance of acetosyringone in successful transformation of wheat and showed that efficiency of T-DNA delivery into the target was significantly decreased in the absence of acetosyringone. Guo *et al*., (1998) investigated various factors and reported that acetosyringone and *Agrobacterium* strain were vital for achieving high frequency of transient GUS expression in transformed tissue of wheat.

In addition to acetosyringone, utilization of vacuum infiltration also increase the transformation efficiency. Mahmoudian *et al*., (1997) indicated that effect of different evacuation pressure on lentil cotyledonary nodes. Compared to non-infiltrated explants, infiltrated ones yielded higher amount of GUS gene

expression. Vacuum infiltration provides bacteria for easy penetration into plant cells.

Addition of anti-necrotic compounds which reduce browing and necrosis of the plant tissues undergoing co-cultivation with *Agrobacterium* also increases the transformation efficiency. Olhoft and Somers (2001) demonstrated that addition of L-cysteine, anti-necrotic compound, to co-cultivation medium reduced necrosis and increased T-DNA transfer in soybean. Olhoft *et al*., (2001) pointed out that anti-necrotic compounds have a capacity to increase *Agrobacterium* infection to plant tissue and to increase the frequency of infected cells that remain viable and become transformed.

Great progress has been made in recent years in studies on *Agrobacterium* mediated transformation. *Agrobacterium*-based transformation systems has been used for wide range of plant species including both monocotyledons and dicotyledons for crop improvement practice.

1.3.2. Direct Gene Transfer Systems

The fact that only certain types of plants are naturally susceptible to infection with the host bacterial organism initially limited the usefulness of the Ti plasmid as a cloning vector. In nature, *A. tumefaciens* infects only dicotyledons, plants with two embryonic leaves. Unfortunately, many important crop plants, including corn, rice, and wheat, are monocotyledons - plants with only one embryonic leaf - and thus could not be easily transfected using this bacterium. So, in order to transfer valuable genes into recalcitrant monocotyledon plants, there are direct gene transfer systems including microprojectile bombardment, microinjection, electroporation and chemical treatments (PEG and Liposomes).

Microprojectile Bombardment

The method of genetic bombardment has demonstrated its broad utility and appears to be effective for all plant species tested so far. The method has created opportunities to transform many important crop species which have been difficult to transform using other methods. There have recently been reports about foreign genes delivered and expressed in both dicots and monocots, including economically important crop species such as soybean, wheat, maize, rice, etc. Theoretically any type of cell or tissue can be used as a target for gene transfer. Embryogenic and meristematic tissues have proven to be transformable and are able to regenerate transgenic plants.

Particle bombardment method which is one of the technologies for introducing foreign genes into cells was developed by John Sanford and his co-workers (Klein *et al*., 1987 and Sanford, 1990) at Cornell University in the United States. This technique involves accelerating DNA-coated particles (microprojectiles) directly into intact tissues or cells. The research was conducted with a view to avoiding the host-range restrictions of *Agrobacterium tumefaciens*, and the regeneration problems of protoplast transformation.

As it is described, to transfer the gene DNA-coated particles should be accelerated. This can be done by a number of mechanisms. The basic system employs a macroprojectile (or macrocarrier), a mechanism for accelerating the macroprojectile, and a means of stopping the macroprojectile. The DNA-coated particles (generally gold or tungsten powders) are placed as a suspension in a small aqueous volume, on the front end of a bullet like plastic macroprojectile. In the first system, the macroprojectile is accelerated to a velocity by a gun powder charge. Upon impact with a plastic stopping plate at the end of the acceleration tube, the macroprojectile extrudes through a small orifice. This extrusion further accelerates the microprojectiles (DNA-coated particles).

Although the gunpowder model was found to be successful for genetic transformation of various plant species in several laboratories, lack of control over the power of the bombardment as well as physical damage to target cells limited the number of stable transformations (Kikkert, 1993).

Another mechanism for the acceleration of microprojectiles is the use of compressed helium. The PDS –1000/He (Bio-Rad) uses a shock wave generated by the sudden release of compressed helium to accelerate a thin plastic sheet into a metal screen and DNA-coated particles are sent onto the sample found in the chamber. Compared to the gunpowder device, it is cleaner and safer, allows better control over bombardment parameter, distributes microcarriers more uniformly over target cells, is more gentle to target cells, is more consistent from bombardment to bombardment, and yields several fold more transformations in the species tested (Kikkert, 1993).

Electroporation

Application of high-voltage electrical pulses to protoplast suspensions increases the permeability of plasma membrane to DNA. Above a critical field strength membrane breaks down, but below this a transient increase in membrane permeability can be induced. Because it has been suggested that this process results in transient pore formation in the membrane, the process has been termed electroporation. Electroporation therefore requires a balance between conditions that increase membrane permeability and conditions which result in mambrane breakdown and loss of protoplast viability.

Transient expression of introduced genes has been found to depend not only on the physical parameters of electroporation, such as plasmid concentration, electrical conditions, protoplast size and density, but also on physiological properties of the protoplasts. Low field strengths and long pulse durations are generally considered to give high rates of transient expression, while high field

strengths and short pulses give higher rates of stable integration Optimized PEG-mediated transformation methods are now considered to be more reliable and efficient than electroporation for direct DNA transfer to protoplasts, although electroporation may still be the method of choice of systems sensitive to high PEG concentrations (Gatehouse, Hilder and Boulter 1992).

1.4. Tissue Culture Studies in Sugar Beet

Tissue culture of sugar beet has been studied for about 30 years (Butenko *et al*., 1972). However, despite the large economic value of the crop and the rather long period of investigations, sugar beet remained a recalcitrant species. Different types of sugar beet tissues produce callus when they are put on media containing plant growth regulators. However, regeneration of this rebellious plant is both infrequent and unpredictable.

Callus can easily be obtained from many parts of the sugar beet plant, including seedling tissues (hypocotyl and cotyledons), leaves, petioles, roots inflorescence flower stalks, anthers, embryos and seeds when cultured on media containing cytokinin alone or in combination with a low concentration of auxin (Margara 1970; Welander 1976; Saunders and Doley 1986; Krens and Jamar 1989; Catlin 1990). Genotype and tissue age are also important related with callus production and subsequent organ formation. Some genotypes have higher capacity for organogenesis than others, and young tissues being more responsive than older tissues (Bhat *et al*., 1985; Keimer 1985; Mikami *et al*., 1989).Two types of callus have frequently been described; (i) white and friable callus consisting of large cells which often have capacity for forming organs (Saunders and Daub 1984; Nakashima *et al*., 1988; Ritchie *et al*., 1989; Konwar and Coutts 1990) and (ii) green and compact non-organogenic callus of small cells which is not capable of forming organs, (Tetu *et al*., 1987; Ritchie *et al*., 1989 and Gürel 1993).

Indirect organogenesis i.e. formation of adventitious shoots or roots from callus was firstly achieved by two groups, Hooker and Nabors (1977) and De Greef and Jacobs (1979). A cytokinin, usually BA, and an auxin, mostly IAA, NAA and 2,4-D, were used to induce callus formation in their studies. After callus formation took place, in order to develop shoot from callus a lower auxin to cytokinin ratio was employed.

Freytag *et al*., (1988) revealed some regeneration from tissue explants but there was a high degree of variability in the regeneration frequency from different explants of different genotypes. The use of BA and IBA has promoted regeneration at genotype dependent frequencies of 55–70% in six North American varieties.

Although protoplast studies on sugar beet have been carried out by numerous investigators, there are limited reports related with plant regeneration from sugar beet protoplasts. This is because plant regeneration from sugarbeet protoplasts is still a genotype-dependent process and is far away from being a routine procedure (Snyder *et al*., 1999).

Hall *et al*., (1997) reported a highly efficient system of protoplast regeneration based on the use of stomatal guard cell protoplasts. The Waring blender method applied by these authors allowed them to obtain a high content of guard cell protoplasts (70–90%). Although protoplast-derived colonies could be obtained in this investigation, the plating efficiencies remained low (about 1% or less) in comparison with division frequencies (over 50%).

Kulshreshtha and Coutts (1997), developed a protocol indicating direct somatic embryogenesis of zygotic cotyledons from mature sugar beet embryos. Explants were cultured on MS medium supplemented with different combinations of 2,4-D, NAA, BAP and TIBA. Within 4 weeks of culture, proliferation of somatic embryos was observed on embryo proliferation medium,

which contained MS medium supplemented with BAP and NAA. They succeeded in high frequencies of plant regeneration in excess of 90 %.

The effect of different BA concentrations and temperature treatments on plant regeneration from petiole is reported Grieve *et al*., (1997). Shoots regenerated from the cut ends and from the adaxial surface of petiole sections within 10 days. At 30°C and 0.5 mg/L BA promoted higher percentage shoot regeneration. Percentage regeneration from petiole sections ranged from 10 to 53 %.

Rady (1997) revealed *in vitro* shoot propagation from excised shoot tips. In this study, four different shoot multiplication and five different root formation medium were utilized. The highest number of shoots occurred when shoot tips were grown on MS medium with 0.25 mg/L NAA and 1.0 mg/L BA. The highest number of root was obtained when 3.0 mg,/L IBA and 0.02 mg/L 2ip were incorporated into medium.

Gürel and Gürel (1998) reported a method for plant regeneration from unfertilized ovaries of sugar beet. Ovary explants were cultured on MS medium containing 2.0 mg/L BA and kept in darkness. Callus obtained from ovary was cultured to induce shoot formation and root induction was achieved using 2.0 mg/L NAA and 2.0 mg/L $AgNO_3$ (silver nitrate).

In another study of ovule culture in sugar beet was performed by Gürel *et al*., (2000). In this study, they examined the effects of cold pretreatment of unopened flower buds and the addition of charcoal or silver nitrate ($AgNO_3$) to the culture medium on the production of haploid plants from cultured ovules. They obtained that both cold pretreatment and the addition of charcoal increased the frequency of embryo formation, whereas $AgNO_3$ reduced or completely inhibited it.

Moghaddam *et al*., (2000) assessed the effect of *in planta* TIBA and L-proline on *in vitro* seedlings and cell culture of sugar beet. They have used different concentrations of TIBA for germination and different dose of L-proline for somatic embryo induction. The utilization of *in planta* TIBA and L-proline combination in the culture procedure resulted in a considerable number of embryos.

Gürel S. (2000) investigated the effects of BA and KIN in combination with NAA for indirect shoot regeneration from seedling explants of sugar beet. Hypocotyl, cotyledon, petiole and leaf of sugar beet were used as explants sources. They achieved shoot development from the pre-treated callus and they have 90 % success in rooting of regenerated shoots in 3.0 mg/L IBA.

Also in another study, Gürel *et al*., (2002) described a method related with plant regeneration from cell suspension culture. In this study, using different concentrations and combinations of BA and 2,4-D the growth patterns of cell suspension cultures were examined. Medium containing 0.25 mg/L BA and 0.25 mg/L 2,4-D induced higher rates of cell division. They have achieved 50 % shoot formation from suspension-derived callus. Regenerated shoots formed root in a medium containing 3.0 mg/L IBA.

Direct organogenesis without an intervening callus phase is reported by Gürel *et al*., (2003). They inspected the effect of *in planta* TDZ treatment on adventitious shoot regeneration from petiole explants. They obtained 100 % seed sterility and 75.6 % seed germination rate. Three germination medium containing 1, 3, and 5 mg/L TDZ, two different regeneration and rooting medium were used. They have achieved a 1.67 shoots per explant and 42.6 % of the regenerated shoots developed roots on medium containing 3 mg/L NAA.

Similar study related with direct organogenesis was performed by Gürel *et al*., (2003). In this case, they examined the effect of pretreating seedlings with

BAP on direct shoot regeneration from petiole explants. Seeds were germinated on medium supplemented with 1, 3, and 5 mg/L BAP. However when it is compared to TDZ pretreatment, in this study only 1 shoot per explant was obtained. Also root formation rate from regenerated shoots decreased to 21.1 %.

Finally in 2003, Dovzhenko and Koop succeeded in protoplast regeneration from friable callus of hypocotyl explants. This represents the first report on callus protoplast to plant regeneration in sugar beet. In this study, they demonstrated that regeneration efficiency from hypocotyl-derived callus protoplast varied from 0% to 38%. Unlike hypocotyl callus protoplast, root callus protoplast was not able to regenerate shoots.

1.5. Transformation Studies in Sugar Beet

Sugar, as sucrose is almost invariably called, has been a valued component of the human diet for thousands of years. For the great majority of that time, sucrose has been obtained from two sources namely, sugar cane and sugar beet. Although sugar production from sugar cane is higher than sugar beet, the sugar cane crop has been restricted to tropical and subtropical regions. On the other hand, the sugar beet crop has spread around world, and it is now grown in all of the populated continents. In the light of these facts, sugar beet is receiving much more attention than sugar cane in terms of both regeneration and transformation. Several methods that were utilized for the introduction of foreing genes into sugar beet reveal the fact that a single technique is not optimal for the transformation of sugar beet, because sugar beets have proven to be highly recalcitrant to transformation until recently.

Lindsey and Jones (1987) show stable transformation of sugar beet protoplasts by the use of direct gene transfer system. However, the production of transgenic plants by this method has limited due to technical difficulties in regenerating from protoplasts. Therefore they have obtained limited success.

Lindsey and Gallois (1989) descirbe a method for gene transfer to morphogenetic tissues of sugar beet using *A.tumefaciens* and for the subsequent regeneration of transgenic plants, avoiding a callus phase. Shoot base tissue slices were inoculated by immersion in a liquid bacterial suspension, induced with BA to produce shoot, and selected by kanamycin antibiotic resistance. Shoots were transferred to root induction medium containing NAA and kanamycin. Although plants have been regenerated under the conditions of antibiotic selection, only a low percentage (approximately 30 %) of resistant shoots exhibited screenable gene activity in their study. Little success was obtained in regenerating shoots from leaf tissues.

Fry *et al*., (1989) demostrated the transformation and regeneration of sugar beet cotyledon explants inoculated with *Agrobacterium tumefaciens* harboring exogenous nucleic acid sequences.

Joersbo and Brunstedt (1990) used sugar beet and tobacco as explant sources. The technique applied was mild sonication and they have used protoplasts of both explant types and CAT as a reporter gene. They concluded that mild sonication has been more efficient method for obtaining transient expression in sugar beet protoplasts than electroporation. Utilization of sonication, the transient gene expression in sugar beet was increased 7-15 fold compared to electroporation.

D'Halluin *et al*., (1992) have developed an *Agrobacterium*-mediated transformation procedure for sugar beet. They used a friable type of callus as the starting material, and combinations of different chimeric gene constructs, consisting of antibiotic and herbicide resistance genes. They also performed field tests on transformed plants. They secured the shoots that resistant to PPT in ratio of 30%. In order to indentify *bar* gene in plants, they also performed southern blot hybridization.

Jacq *et al*., (1993) report the effects of genotype, acetosyringone, preculture and coculture duration on the *Agrobacterium*-mediated transformation of sugar beet. Hypocotyl and cotyledon explants were excised from seedling and co-cultured with *Agrobacteria*. Transformants were quantitated by histochemical and fluorometric GUS assays, however transformed plants were not recovered.

Konwar (1994) also showed that shoot bases can be infected with *A. tumefaciens* to produce transgenic plants. Using the method of Konwar (1994), Mannerlof *et al*., (1997) generated glyphosate-tolerant sugar beets.

In a study of Mahn *et al*., (1995) the apices of sugar beet seedlings were used as targets for particle bombardment to study the penetration of particles into the apex, the transient expression of marker genes and the viability of cells after the bombardment.

Hall *et al*., (1996) describe a method of generating transgenic sugar beet plants from protoplasts obtained from stomal guard cells. A polyethylene glycol (PEG) mediated gene transfer was performed on protoplasts stomal guard cells. Selection of transformants were achieved by testing their resistance to bialaphos. To achieve high transformation frequencies, they enriched the protoplasts for a totipotent cell type from stomatal guard cells. Stable transformation efficiencies for protoplasts were between 1.2 to 5.2 x 10^{-4}. Protoplasts were regenerated into calli and plants. This method was later used to produce transgenic sugar beets accumulating high levels of fructan (Sevenier *et al*., 1998).

Joersbo *et al*., (1998) described a new selection method based on mannose selection which is shown to be particularly useful for the transformation of a recalcitrant species like sugar beet. The selection system is based on the *Escherichia coli* phosphomannose isomerase (PMI) gene as selectable gene and mannose as selective agent. They increased transformation frequencies about 10-

fold higher than for kanamycin selection and obtained at low selection pressures (1.0–1.5 g/l mannose) where 20–30% of the explants produced shoots.

Kifle *et al*., (1999) reported a transformation protocol based on co-cultivation with two *Agrobacterium* strains, *Agrobacterium tumefaciens* LBA4404 and *A. rhizogenes*, which markedly increased the induction of sugar beet hairy roots expressing foreign genes. To determine stable expression of foreign genes in hairy roots, the nematode resistance gene $Hs1^{pro-1}$ was used as a reporter gene. However, foreign gene is not heritable because sugar beet is biennial plant species and vernelization is required for seed production.

Önde *et al*., (2000) transferred of a β-Glucuronidase reporter gene to sugar beet (*Beta vulgaris* L.) callus and leaf explants via microprojectile bombardment. They tested various rupture disk pressures and sample plate distances. They indicated the superiority of leaf explants over the callus structures as targets. They also found that the sample plate distances affected the distribution pattern of the particles and the cells expressing the GUS reporter gene were noted to be aggregated in short distances whereas longer distance shots yielded better distribution of transformed cells.

Menzel *et al*., (2003) revealed plastidic PHB polymer accumulation in sugar beet roots. Three genes from *Ralstonia eutropha* necessary for poly (3-hydroxybutyrate) (PHB) synthesis were introduced into the hairy roots of sugar beet. Accumulation of PHB polymer in sugar beet root leucoplasts was confirmed by transmission electron microscopy.

The most recent study on sugar beet transformation is based upon *Agrobacterium*-mediated gene transfer to shoot base of sugar beet (Hisano *et al*., 2004). They analyzed the frequency of regeneration from shoot bases of sugar beet. In their study genomic DNA analysis and ß-glucuronidase reporter assays showed that the transgene was inherited and expressed in subsequent generations.

They also reported that the transformation method using shoot bases does not involve a detectable callus phase prior to regeneration, suggesting that the possibility of somaclonal variation is minimized.

1.6. Aim of the Study

Although there are various regeneration and transformation protocols for sugar beet, they are still far from routine. In this study, we aim to optimize a regeneration and *Agrobacterium* mediated transformation procedure for two sugar beet cultivars.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

The sugar beet cultivars ELK 315 and 1195 that are commonly cultivated in Turkey was used in the experiments. Seeds of these breeding lines were obtained from the Sugar Institute, in Ankara.

2.1.2. Plant Tissue Culture Media

MS (Murashige and Skoog 1962) basal medium supplemented with sucrose and agar or phytagel was used throughout the study (Compositions of media are given in Appendix A). Different combinations and concentrations of plant growth regulators including dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (BA), α-Naphthalene acetic acid (NAA), Thidiazuron (TDZ), IBA (Indole-3-butyric acid) and TIBA (2,3,5-Triiodobenzoic acid) were used. Kanamycin, PPT and cefotaxime were utilized in the medium for selection of transformants and removal of *Agrobacterium tumefaciens*. Firstly the basal media was dissolved in distilled water. Then the pH of the media was adjusted to 5.8 with NaOH and HCl before adding agar or phtygel and autoclaved at 120 ^{o}C for 20 minutes. Plant growth regulators and antibiotics were filter-sterilized by the aid of 0.2 μm pore sized filters and added to the cooled medium prior to dispersing.

2.1.3. Bacterial Strains and Plasmid

The *Agrobacterium tumefaciens* strains, EHA105, GV2260 and LBA4404 were used throughout this study (Table 2.1). *Agrobacterium* strains are different from each other in terms of opine catabolism (Hellens and Mullineaux 2000).

Table 2.1. *Agrobacterium* strains are grouped according to the opine catabolism and chromosomal background.

Agrobacterium strains	Chromosomal Background	Marker Gene	Opine Catabolism
EHA105	C58	rif	Succinamopine
LBA4404	TiAch5	rif	Octopine
GV2260	C58	rif	Octopine

Binary plasmid pGUSINT is a derivative of pBI121 (Jefferson *et al*., 1987) and carries neomycinphosphotransferase-II (*npt*-II) gene conferring kanamycin resistance trait for both plant and bacteria and an intron containing *uidA* (GUS) gene for plant selection. T-DNA region of pGUSINT is given in Appendix B.

Agrobacterium tumefaciens EHA105 strain was obtained from MOGEN. The binary vector pGUSINT was kindly donated by Dr.Willmitzer. The permission letters are given in Appendix C.

2.1.4. Bacterial Culture Media

Yeast extract broth (YEB), YEB + MES (2-[N-Morpholino] ethanesulfonic acid) and MG/L (Wu *et al*., 2003) were used for growth of *Agrobacterium* cultures in the plant transformation experiments. Depending on bacterial strain and bacterial selection marker on binary vector, it was supplemented with appropriate antibiotics and acetosyringone (3',5'-Dimethoxy-4-Hydroxyacetophenone). The antibiotic requirements for each strain and binary plasmid were given in Appendix D. Different inoculation and co-cultivation medium were used depending on bacteria culture medium.

2.1.5. Other Materials

Antibiotics (rifampicin, ampicilin, streptomycin, cefotaxim and kanamycin) GUS histochemical substrate which is abbreviated as X-Gluc (5-bromo-4-chloro-3-indolyl glucoronide), acetocyringone and all other chemicals used in solutions were supplied from Merck, Sigma, Aldrich, Difco and Applichem chemical companies.

2.2. Methods

2.2.1. Tissue Culture Studies

2.2.1.1. Seed Surface Sterlization and Germination

Seeds of sugar beet have rough surface when compared to other seeds of plants. In order to find an effective method related with seed surface sterilization, various experiments were conducted. Among the other methods, two methods were found to be effective.

In the first protocol, seeds were washed under the tap water until all of the dust was removed. 10% Captan, which is a funguside, was used to remove fungi that might be present on the seeds. Seeds were kept in this funguside solution for two hours at room temperature by continuous stirring. After this period, seeds were washed with sterile disteled water and exposed to 70% ethanol for 5 minutes. Then they were treated with 10 % sodium hypochlorite for 30 minutes. They were dried out and stored at 4°C for two or more days. Then these sterile seeds were again surface sterilized with 70 % ethanol for 5 minutes and then 10 % sodium hypochlorite for 30 minutes. Finally they were dried out and placed on MS basal medium supplemented with sucrose and agar.

In the second protocol which was more effective than first one, the seeds of the sugar beet were surface sterilized by immersion in 70 % ethanol for 5 minutes and then in 80 % sodium hypochlorite for one hour by continuous stirring with magnetic stirrer. After three rinse in the sterile distilled water, they were kept in sterile distilled water overnight in the dark at 23°C for imbibition. Imbibed seeds were rinsed in 5 % PPM^{TM} solution (Plant Preservation Mixture, Plant Cell Technology Inc. WA, USA) for 10 minutes. After sterilization, seeds were cultured on MS medium containing 3 % sucrose and 0.8 % agar. Seeds were germinated at 24±2°C under light with a 16/8 hour (light/dark) photoperiod. Explants (cotyledon, hypocotyl, shoot base, petiole and leaves) from these germinated plantlets were used in both regeneration and transformation studies.

2.2.1.2. Establishment of Stock Material for Culture Studies

In order to carry out both regeneration and transformation experiments, subculture of sugar beet should be optimized. Roots of the 8-9 days old germinated plantlets were removed. This part of the plant was refered to as shoot base. Then these explants were placed on MS medium supplemented with sucrose and phytagel. For further experiments, shoot bases were used as explants source

containing hypocotyl, cotyledon, petiole and leaves. Figure 2.1 shows the preparation of the shoot base.

2.2.1.3. Indirect Organogenesis

For regeneration of sugar beet via indirect organogenesis, different explant types including cotyledons, hypocotyles, petioles and leaves were cultured on a basal medium composed of MS salts, 3 % sucrose, and 0.8 % agar supplemented with growth regulators as described in Table 2.2. The pH of the medium used in experiments was adjusted to 5.6 with NaOH and HCl prior to autoclaving at 121°C for 20 minutes. Plant growth regulators were fitler-sterilized by using 0.2 μm pore sized filters and added to the cooled medium prior to dispersing.

For indirect organogenesis of sugar beet, two different germination medium and three different callus induction and shoot regeneration medium were prepared. After seed germination occured in two different germination medium, explants including cotyledons and hypocotyl were transfered into three different callus induction medium. Following development of leaves and petiole, they were also placed on callus induction medium. For induction of callus, explants were kept in these medium at 24±2°C in the dark for 3 weeks. At the end of third week developed callus was transfered into different shoot regeneration medium.

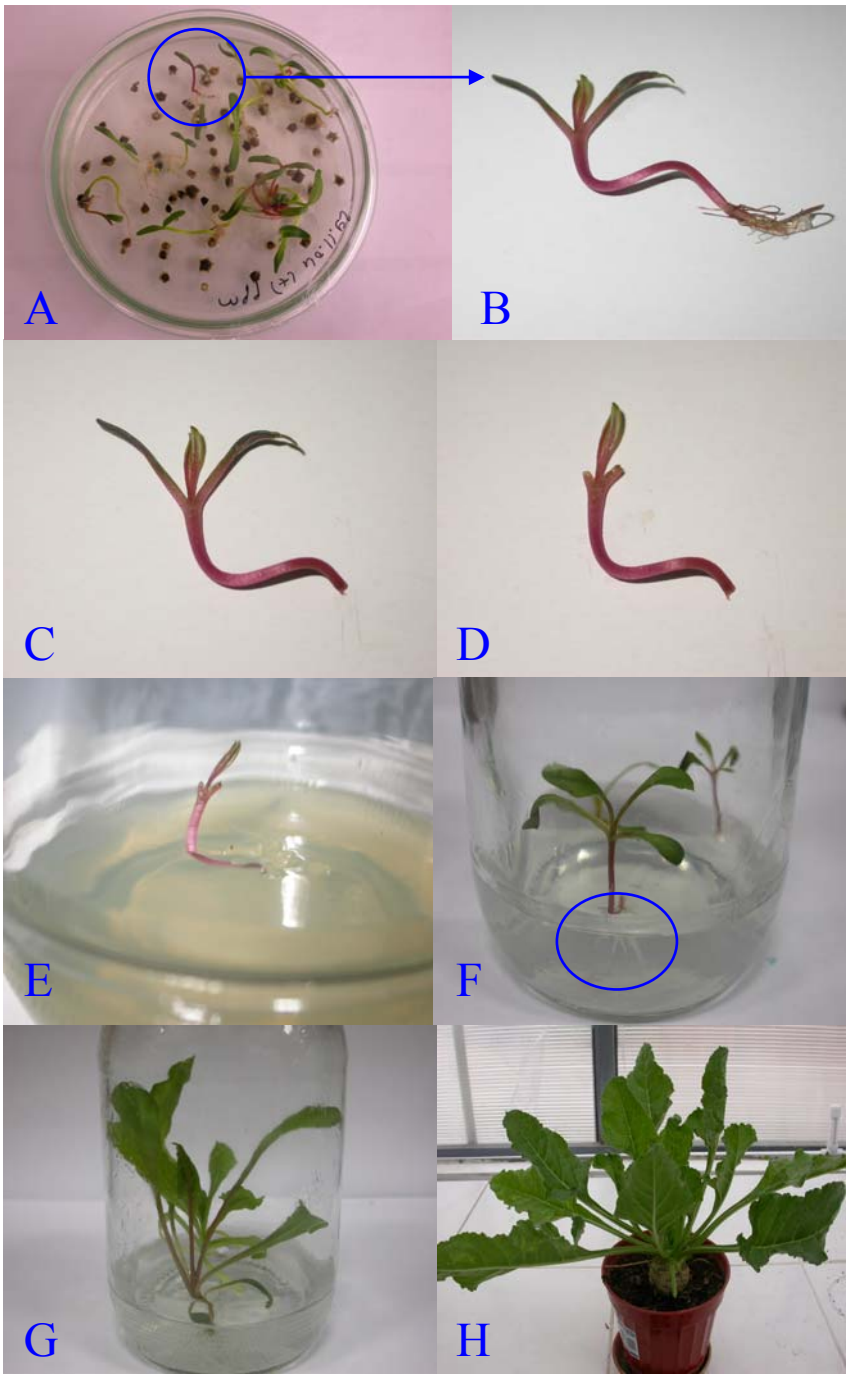


Figure 2.1. Preparation of the shoot base. A) Seeds of the sugar beet were germinated. B) Plantlet was removed from petri plate. C) Root of the plant was cut and removed and shoot base was obtained. D) For regeneration, hypocotyl and/or cotyledon was cut from shoot base. E) Remainder of the plantlet was placed on MS basal medium including sucrose and phytagel. F-G) Root and leaf formation took place and plant could be employed for regeneration and transformation experiments.H) Plantlet was transferred into pots containing soil and root formation was observed.

Each growth regulator concentration was tested in 3 sets of 5 plates each containing 10 explants.

Table 2.2. Growth regulator combinations and concentrations used for indirect organogenesis. PGR: Plant Growth Regulators

TISSUE TYPES	GERMINATION MEDIUM	CALLUS INDUCTION MEDIUM	SHOOT REGENERATION MEDIUM
Cotyledons hypocotyles petioles leaves	Control(No PGR)	Control (No PGR)	Control (No PGR)
			1.0 mg/L BA
			2.0 mg/L TDZ
		0.1 mg/L 2-4-D 1.0 mg/L BA	Control (No PGR)
			1.0 mg/L BA
			2.0 mg/L TDZ
		2.0 mg/L TDZ	Control (No PGR)
			1.0 mg/L BA
			2.0 mg/L TDZ
	3.0 mg/L TIBA	Control (No PGR)	Control (No PGR)
			1.0 mg/L BA
			2.0 mg/L TDZ
		0.1 mg/L 2-4-D 1.0 mg/L BA	Control (No PGR)
			1.0 mg/L BA
			2.0 mg/L TDZ
		2.0 mg/L TDZ	Control (No PGR)
			1.0 mg/L BA
			2.0 mg/L TDZ

2.2.1.4. Direct Organogenesis

For sugar beet regeneration via direct organogenesis, two different methods were employed. In the first method cotyledon, hypocotyl, petiole and leaf explants were used. Firstly, seed germination was achieved in two different germination medium. Then each explant was cultured on shoot regeneration medium. Growth regulator combinations and concentrations used in shoot regeneration were given in Table 2.3. For shoot regeneration, explants were kept in these medium at 24±2°C under light with a 16/8 hour (light/dark) photoperiod for 4 weeks in culture room. Each growth regulator concentration was tested in 3 sets of 5 plates each containing 10 explants.

Table 2.3. Growth regulator combinations and concentrations used for first direct organogenesis method

TISSUE TYPES	GERMINATION MEDIUM	SHOOT REGENERATION MEDIUM
Cotyledons hypocotyles petioles leaves	Control (No PGR)	Control (No PGR)
		0.5 mg/L TDZ
		1.0 mg/L TDZ
		2.0 mg/L TDZ
	0.5 mg/L BA 0.1 mg/L NAA	Control (No PGR)
		0.5 mg/L TDZ
		1.0 mg/L TDZ
		2.0 mg/L TDZ

In the second method, only leaves of sugar beet were used as an explant source. In this direct regeneration method, germination occurred after 2–3 weeks. After the cotyledons emerged, the hypocotyls were cut at the base and transferred onto shoot formation medium to induce leaf growth. Then, leaf blades were cut from young plants and placed on shoot formation medium. Shoots were regenerated from the veins of the leaf blades. The shoots were removed and placed on growth medium. Finally, regenerated plantlets were transfered onto rooting medium. For this direct regeneration method, growth regulator combinations and concentrations were presented in Table 2.4. For shoot regeneration, explants were kept in these medium at 24±2°C under light with a 16/8 hour (light/dark) photoperiod for 3-6 weeks in culture room.

2.2.1.5. Lethal Dose Determination for Selective Agents

Selective agents were used to select the transformants after an event of transformation. Generally used ones in plant transformations are antibiotics and herbicides. In this study, leaves of sugar beet were exposed to selective agents to determine their effect on direct shoot formation and to find out the lethal dose that can be used during transformation studies of sugar beet. Different combinations of kanamycin and PPT were employed for this purpose. Explants were cultured on medium containing selective agents and 0.1 IBA mg/L, 0.25 BA mg/L for 4 weeks. Number of shoot formation per leaf was recorded and photographed.

2.2.1.6. Rooting

Leaf blades on MS basal media enriched with 0.1 mg/L IBA and 0.25 mg/L BA produced shoots. These shoots were removed from the explant and subcultured to MS basal media supplemented with sucrose, phytagel and 1.0 mg/L IBA. The shoots were cultured at 24±2°C under light with a 16/8 hour (light/dark) photoperiod in culture room. In 4-6 weeks plantlets matured and became ready to be transfered in to the soil.

2.2.1.7. Acclimatization

For acclimatization of sugar beet plant, mature plants with root and phytgel were taken into soil. Pots were placed into containers with water, and covered with the transparent plastic bags to avoid desiccation of the plantlets. Plastic bags were removed at 2 days. The plants were maintained in the greenhouse.

Table 2.4. Growth regulator combinations and concentrations used for second direct organogenesis method.

MEDIUM TYPES	PLANT GROWTH REGULATORS (mg/L)	
	IBA	BA
Shoot Formation Medium	0.1	0.25
Growth Medium	0.1	0.25
Rooting Medium	1.0	

2.2.2. Transformation Studies

In transformation studies *Agrobacterium* mediated gene transfer method was performed. Leaf disks and leaf blades of sugar beet were used as explant sources in transformation studies.

2.2.2.1. Preparation of *Agrobacterium* Cells

A single colony of *A. tumefaciens* strains were grown overnight at 28±1°C with 180-200 rpm shaking incubator in 5 ml liquid YEB medium supplemented with appropriate antibiotics. Then 500 ml of liquid medium were inoculated with 100 µL of this overnight grown initial culture. The bacterial culture was grown

overnight at 28±1°C at 180-200 rpm till OD_{600} reaches to 0.8. Then the culture was centrifuged at 1500 g for 15 minutes at 4°C. The pellet was resuspended with inoculation medium (Table 2.5) to final OD_{600} of 2.4. Finally the bacterial suspension was incubated at 24±2°C under dark condition for 1 hour and then used for transformation of explants (Çelikkol, 2002).

2.2.2.2 *Agrobacterium* Mediated Transformation of Leaf Disks

For transformation of leaf disks, 10-15 days old leaves of sugar beet were cut into small pieces from stock material. Then they were vacuum infiltrated at different pressure including 0, 200, 400 and 600 mmHg for 10 minutes. At the end of this period, they were directly placed on MS basal medium. Explants were pre-cultured on co-cultivation medium. Transient GUS expression was determined after 3 days.

After the most appropirate pressure was determined, in this case effect of different bacterial growth medium on transformation efficiency was demostrated. Bacterial growth and inoculation medium are indicated in Table 2.5. Then bacterial culture grown in these medium were inoculated with leaf disk for 10 minutes at 400 mmHg vacuum pressure. Lastly, they were directly transferred in to different co-cultivation medium which are described as Table 2.6. After 3 days, transient GUS expression was performed.

After the most suitable bacterial media was decided, effect of inoculation time on transformation efficiency was investigated. Leaf disks were inoculated with bacterial culture for different periods including 10, 20, 40 and 60 minutes at 400 mmHg vacuum pressure. At the end of these periods, explants were cultured on co-cultivation medium. Three days later, transient GUS expression was determined.

Table 2.5. Bacterial growth and their inoculation medium.

Bacterial Growth Medium	Composition (for 1L)	Inoculation Medium	Composition (for 1L)
YEB	Nutrient broth 13.5 g Yeast extract 1 g Sucrose 5 g $MgSO_4.7(H_2O)$ 2 mM (0.493 g) pH: 7.2	MMA	Sucrose 20 g MS salts 4.3 g MES 1.95 g pH: 5.6
YEB+MES	Nutrient broth 13.5 g Yeast extract 1 g Sucrose 5 g $MgSO_4.7(H_2O)$ 2 mM (0.493 g) MES10 mM (2.132 g) Acetosyringone 20 µM pH: 5.6	MMA	Sucrose 20 g MS salts 4.3 g MES 1.95 g pH: 5.6
MG/L	Yeast extract 2.5 g Mannitol 5 g Glutamic Acid 1 g KH_2PO_4 0.25 g NaCl 0.25 g Tryptone 5 g $MgSO_4.7(H_2O)$ 0.1 g Biotin 1µg pH: 7.0	Inoculation Media for MG/L	MS salts 4.3 g Glutamine 500mg Casein hydrolysate 100mg MES 1.95 g Glucose 10 g Maltose 40 g pH: 5.8 After autoclaving Picloram 2.2 mg 2,4-D 2 mg Acetosyringone 200 µM Ascorbic acid 100 mg

Table 2.6. Co-cultivation medium for different bacterial growth medium.

Co-cultivation Medium	Composition (for 1L)
MS Medium for YEB	MS salts 4.3 g Sucrose 30 g Plant Agar 4 g pH: 5.8
MMD for YEB+MES and MG/L	MS salts 4.3 g MES 1.95 g Sucrose 15 g Phytagel 0.28 g After autoclaving 2,4-D 1 mg Acetosyringone 200 μM Ascorbic acid 100 mg pH: 5.6

For determination of bacterial strains on transformation efficiency, different *Agrobacterium* strains including EHA105, GV2260 and LBA4404 were employed for transformation of leaf disks. Leaf disks were inoculated with these bacterial strains at 400 mmHg vacuum pressure for 20 minutes. Then they were cultured on co-cultivation medium. Transient GUS expression was determined on explants after 3 days.

The last parameter was effect of L-cysteine on transformation efficiency. For this purpose, various concentrations (100, 200, 400, 800 and 1200 mg/L) of L-cysteine were added to the co-cultivation medium. Leaf disks were taken in to the *Agrobacterium* suspension and co-cultivated at 400 mmHg vacuum pressure for 20 minutes. Then explants were transferred into co-cultivation medium which contains different concentrations of L-cysteine. Explants were maintained in co-cultivation medium for 3 days. At the end of 3 days, GUS histochemical assay was performed.

Each parameter was tested in 6 sets of 2 plates each containing 20 explants. Figure 2.2 summarizes the parameters of transformation studies.

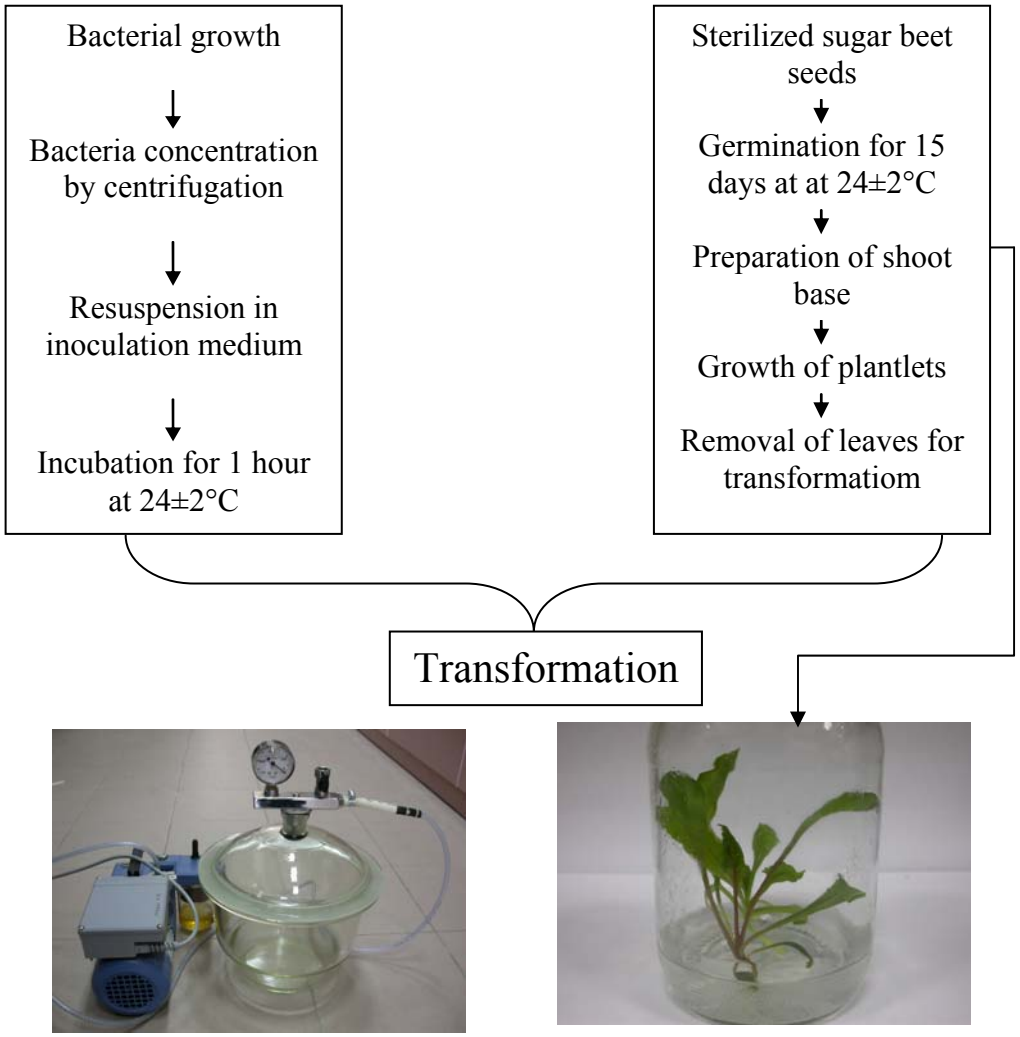


Figure 2.2. Treatments performed throughout this study.

2.2.2.3 *Agrobacterium* Mediated Transformation of Leaf Blades

A method based on the procedure of Hisano *et al*., (2004) was employed with slight modification. Firstly seeds were germinated. After the cotyledons emerged, the hypocotyls were cut at the base and transferred onto shoot formation medium to induce leaf growth. Then, leaf blades were cut from young plants and placed on shoot formation medium. Shoots were regenerated from the veins of the leaf blades. The shoots were removed, and the remainder of the leaf blades, on which the shoot bases were emerged, was used as explants for transformation. Media used for transformation are listed in Table 2.7.

Table 2.7. Medium used for transformation of leaf blades.

Medium Types	Plant Growth Regulators (mg/L)		Antibiotics (mg/L)		
	IBA	BA	Cefotaxime	Kanamycin	Acetosyringone (μM)
Shoot-formation Medium	0.1	0.25			
Co-cultivation Medium					20
Washing Medium	0.1	0.25	1.000		
Selection Medium	0.1	0.25	500	150	
Growth Medium	0.1	0.25	500	150	
Rooting Medium		1.0	500		

Bacterial culture was prepared as described in preparation of *Agrobacterium* cells part. The explants, on which shoot bases were emerged, were immersed in the *Agrobacterium* culture for 10 min, and excess liquid was removed by placing the explants on sterilized filter paper. Samples were transferred to co-cultivation medium supplemented with 4 mg/L acetosyringone and cultured for 3 days. The explants were rinsed with washing medium to remove *Agrobacterium* from the surface and then transferred to selection medium. After 3 weeks, explants from which shoots were regenerated were transferred to growth medium. When shoots grew 2–3 cm, they were cut and transferred to root-formation medium. After roots were generated, the plants were transferred to soil and acclimatized as described previously.

2.2.3.Analysis of Transformants

Histochemical GUS staining was performed to monitor the transient gene expression.

2.2.3.1. GUS Histochemical Assay

GUS histochemical staining was performed according to Jefferson (1987) to indicate transient gene expression. Three days after transformation leaf disks were stained in GUS substrate solution. Leaf disks were incubated at 37°C for 2 days in GUS substrate solution. At the end of the incubation time, explants were transferred to fixative solution for 4 hours. Then, they were transferred to 50 % ethyl alcohol for decolorization. After 15 minutes in 50 % ethyl alcohol, leaf disks were kept in 100 % ethyl alcohol overnight for further decolorization. Explants were transferred to GUS fixative solution for preservation for several months. Finally GUS expressing regions on explants were examined under microscope. Composition of GUS substrate solution and fixative solution were given in Appendix E.

2.2.3.2. Image Analysis System

After GUS histochemical staining was performed, for each treatment, leaf disks were photographed and analyzed by image analysis system (Zeiss® KS3000 in METU Central Laboratory). Percentage of GUS staining area for each leaf disks was calculated.

2.2.4. Statistical Analysis

Minitab 13.0 software was used to for all of the statistical analyses. 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 terms of GUS expression units on explants which were exposed to different experimental treatments.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Tissue Culture Studies

In order to establish a successful regeneration and selection system, four different parts of shoot base of sugar beet cultivars ELK 345 and 1195 were used in tissue culture part of this study (Figure 3.1).

Shoot base isolated from germinated seedling was used as explant throughout both the regeneration and transformation studies. Main advantages of using shoot base are that shoot base are prepared by a simple procedure and transformation does not involve the callus phase. Therefore, transformation procedures using shoot base were performed by various researchers Lindsey and Gallois (1989), Konwar (1994), Mannerlof *et al*., (1997) and Hisano *et al*., (2004).

3.1.1. Seed Surface Sterilization

Surface sterilization of sugar beet seeds is often difficult due to the rough surface of seeds. Figure 3.2 shows seeds of two different cultivar which were used in this study. So, contamination of seeds is inevitable because of the availability of fungus and bacteria. The use of the previously published protocols (Tetu *et al*., 1987; Fregtag *et al*., 1988; Ritchie *et al*.,1989 and Zhong *et al*., 1993) failed to obtain successful sterilization with our material since the treatments were toxic to the plant material.

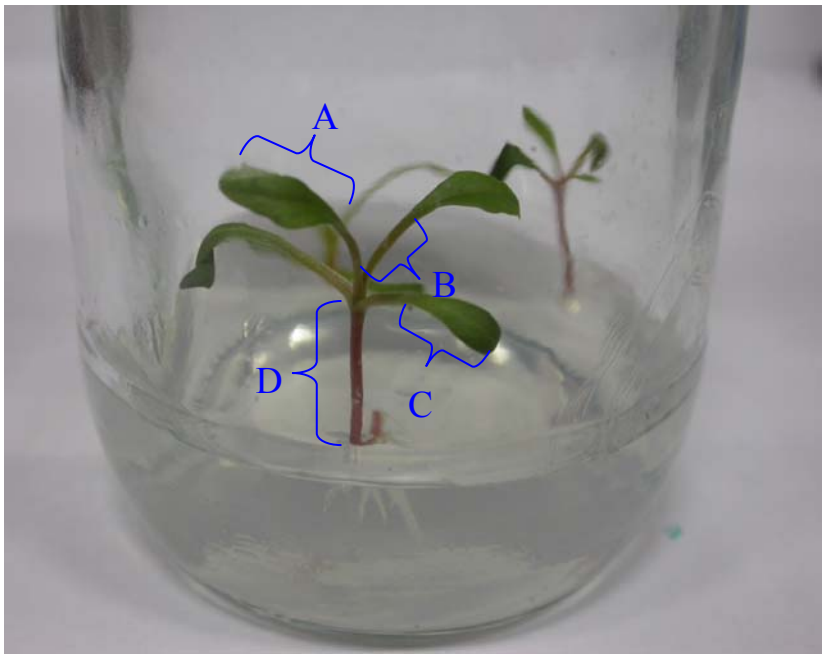
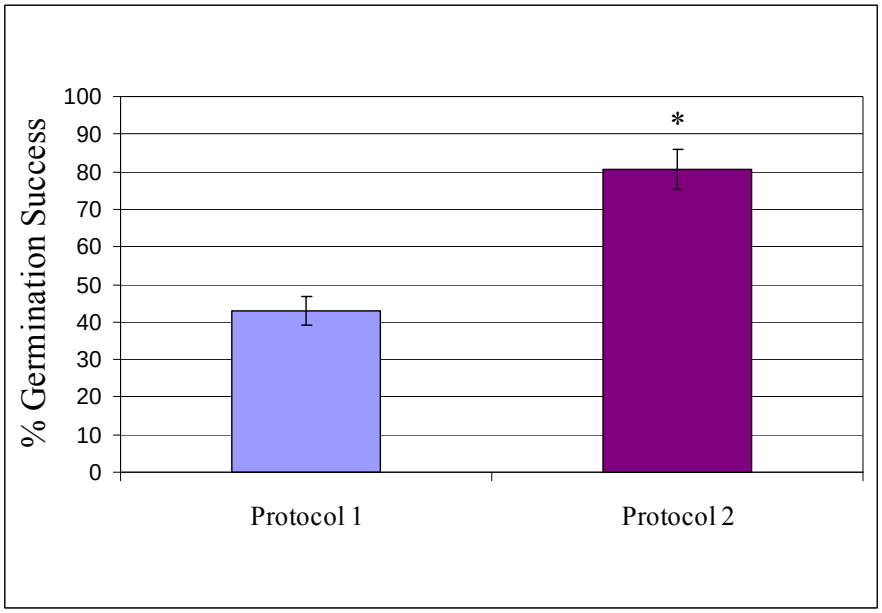


Figure 3.1. Explant material that was used in the experiments. **(A)** Leaf, **(B)** Petiole, **(C)** Cotyledon, **(D)** Hypocotyl

Figure 3.2. Sugar beet seeds **(A)** Seeds of sugar beet cultivar ELK 345
(B) Seeds of sugar beet cultivar 1195

Babaoğlu and Yorgancılar (2000), Crompton and Koch (2001), George and Tripepi (2001) used PPM^{TM} (Plant Preservation Mixture, Plant Cell Technology Inc. WA, USA) for sterilization. Gürel *et al*., (2003) first developed an effective seed surface sterilization method using different concentrations and combinations of Domestos and PPM^{TM}. In the light of these reports we established two protocols for seed sterilization. The difference between two protocols was that two different fungisides, Captan and PPM, were employed.

Seed surface sterilization of sugar beet using different protocols is represented in Figure 3.3. In our study, utilization of PPM in Protocol 2 increased the seed germination success. We achieved 80.6 % germination success using Protocol 2 when compared to Protocol 1 which enabled 43 % germination success. Differences between two protocols were analyzed by One-way ANOVA test. Protocol 2 was significantly better ($p< 0.05$) than Protocol 1.



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

Figure 3.3. Seed germination success by using different sterilization protocols. Vertical bars and * show SEM (standard error of mean) and significant values ($p< 0.05$, n=150 for each treatment), respectively.

PPM containing isothiazolone biocides is a relatively new, broad-spectrum agent. It is employed both for surface sterilization and also included in the culture medium to eliminate the internal contaminants that may be present in explants. So, utilization of PPM has been increasingly used in tissue cultures of many species including salad burnet (Babaoğlu and Yorgancılar, 2000) and sugar beet (Gürel *et al*., 2003). Babaoğlu and Yorgancılar (2000) reported that the use of PPM was effective in controlling contamination without impairing the shoot regeneration from petiole and hypocotyls explants of salad burnet. Moreover, Gürel *et al*., (2003) revealed high rate of seed sterilization without reducing the germination efficiency of sugar beet seeds using PPM. Our results are consistent with their findings that PPM increased the rate of seed sterilization without being harmful to the embryo.

3.1.2. Callus Induction Studies for Indirect Organogenesis

In this part of the study, two different germination medium were employed to demonstrate the effect of pretreatment of seedling with TIBA on indirect shoot regeneration from cotyledons, hypocotyles, petioles and leaves. To achieve callus induction from these explants, three different medium were used. Lastly, the callus obtained from these explants were cultured on three different shoot regeneration medium.

Generally, two types of callus were obtained. White and friable callus which is capable of forming organs, and green and compact callus which does not have organogenic capacity, are shown in Figure 3.4. Friable callus was composed of large and translucent cells while compact callus consisted of small and green cells.

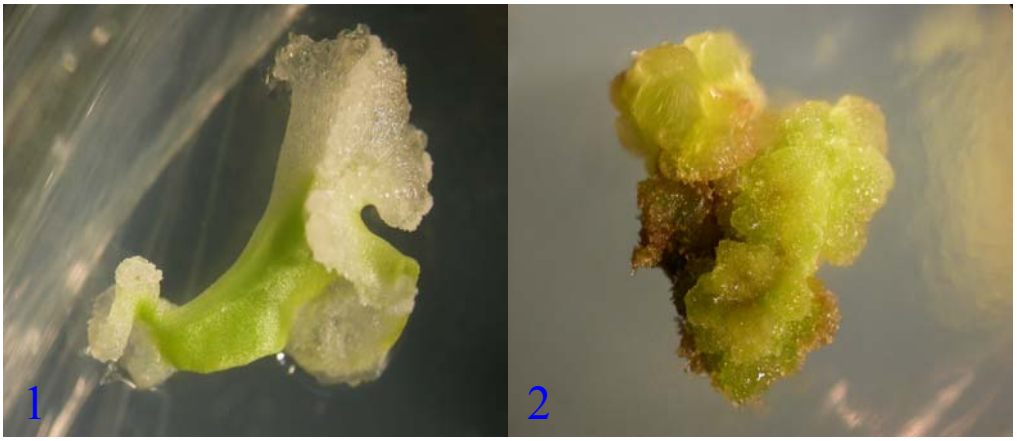


Figure 3.4. Two types of callus obtained from different parts of sugar beet.

(1) White and friable callus derived from leaf explants of ELK 345 breeding line cultured on medium containing 0.1 mg/L 2,4-D and 1.0 mg/L BA.

(2) Green and compact callus derived from cotyledon explants of ELK 345 breeding line cultured on medium containing 0.1 mg/L 2,4-D and 1.0 mg/L BA.

Seeds that were grown on medium containing 3 mg/L TIBA produced smaller seedlings as compared to those of the control medium and showed curved leaves. Cotyledon taken from medium containing TIBA and hormone-free medium produced callus in two different callus induction medium. However, in hormone-free medium as a control, no callus formation was observed and root development occurred on the control medium. Figure 3.5 shows the root development in the control medium. Medium containing 2,4-D - BA and only TDZ induced callus formation. Although callus formation was achieved from cotyledon, no shoot development was observed due to formation of compact callus which is not capable of forming organs. Figure 3.6 indicates compact callus formation on medium containing 2,4-D – BA and only TDZ.

Figure 3.5. Root formation from cotyledon of cultivar ELK 345 on control medium. Explants were photographed after 4 weeks.

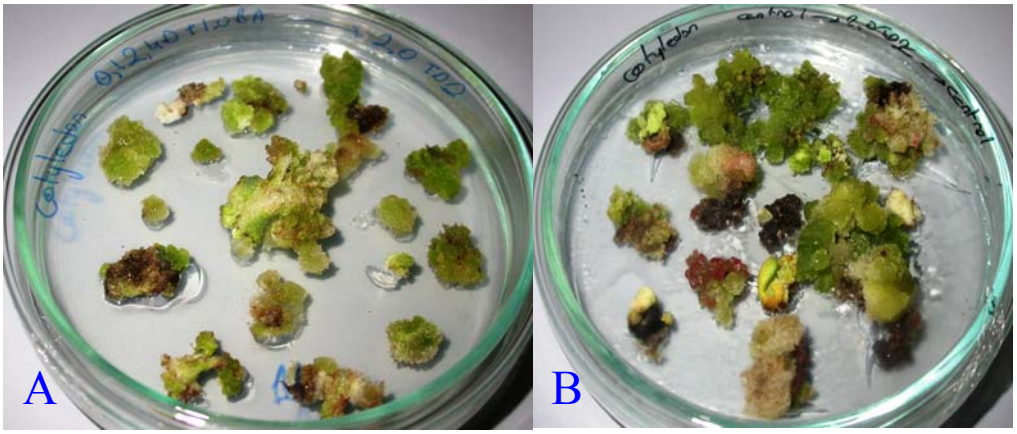


Figure 3.6. Compact callus development from cotyledon on callus induction medium and shoot regeneration medium. Callus tissue were photographed after (A): 3 weeks (B): 6 weeks.

TIBA is an inhibitor of auxin transport and has been included in some cultures, but a report related with effect of TIBA on callus formation and organ induction is conflicting. The growth of callus from cotyledons was inhibited by TIBA (Miedema *et al*., 1980). However, our result was opposite to this investigation because we obtained callus from cotyledon which were incubated in TIBA containing medium and control medium. Also Moghaddam *et al*., (2000) used TIBA in germination medium and obtained callus from leaf. Moreover, the positive effect of TIBA treatment on organogenesis has already been mentioned by Hooker and Nabors (1977), Jacq *et al*., (1992, 1993) and Kulshreshtha and Coutts (1997). Additionally, Roussy *et al*., (1996) obtained a high regeneration capacity in cultured explants prepared from *in planta* TIBA treated green house plants.

In earlier reports (Krens *et al*., 1990; Pedersen *et al*., 1993; Lenzner *et al*., 1995), shoot regeneration from compact callus was never described. So, our result are similar with their findings because we also obtained compact callus formation from cotyledon and no shoot development was observed from this type of callus. However, Catlin (1990) obtained callus and plantlet from cotyledons on medium containing 0.2 mg/L BA only. Gürel (2000) also obtained friable callus without shoot regeneration.

Hypocotyl explants were also used for indirect regeneration studies. For callus induction, hypocotyl explants were taken from 10-12 days-old seedlings. Hypocotyl explants gave similar result to that of cotyledon explants in terms of root formation on control medium. Contrary to cotyledon, hypocotyl produced friable callus on medium containing 2,4-D - BA and only TDZ (Figure 3.7). Friable callus derived from hypocotyl were placed on three different shoot regeneration medium. i) Control medium, ii) medium containing TDZ, iii) medium containing BA did not induce shoot development. After 4 weeks, size of callus increased when compared to beginning level. Then type of callus changed and became compact callus. (Figure 3.8).

Figure 3.7. (A) Root and (B) friable callus formation from hypocotyl of cultivar ELK 345. Callus tissue were photographed after 3 weeks.

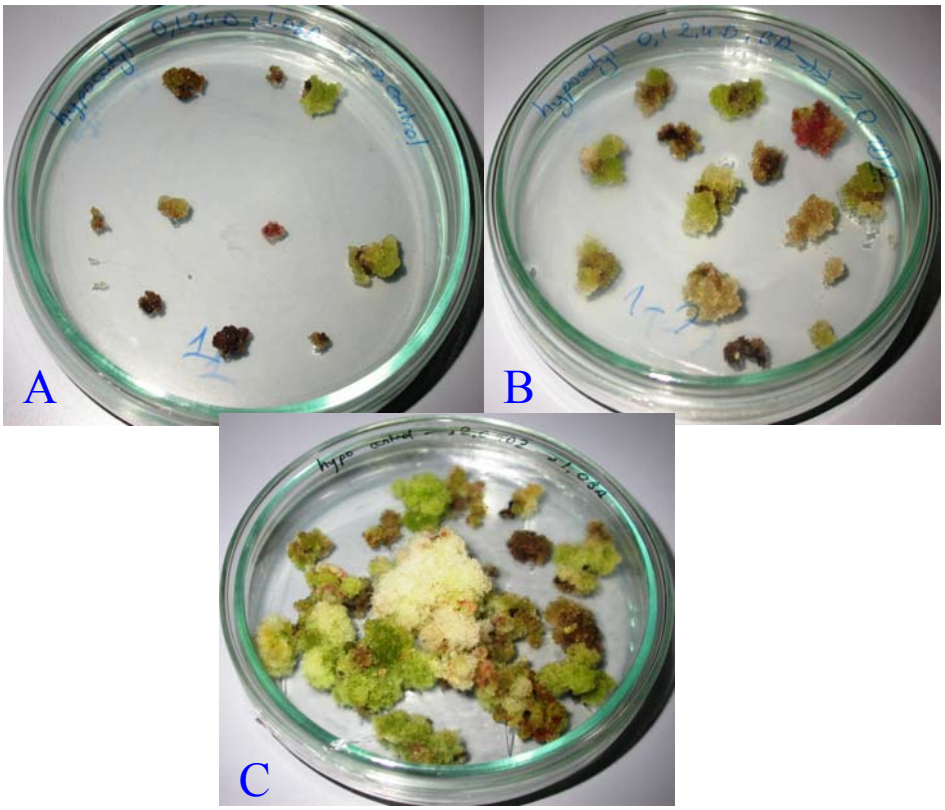


Figure 3.8. Friable callus placed on three different shoot regeneration medium. Callus tissue were photographed after (A): 3 weeks (B): 6 weeks (C): 12 weeks.

Organogenic callus from hypocotyl explants of sugar beet was initiated at concentrations of 0.3 mg/L BA and 0.1 mg/L NAA (Jacq *et al*., 1992). Also Gürel *et al*., (2001) reported that increasing BA concentrations in the culture medium augmented callus development from hypocotyl. In their study shoot development from callus derived from hypocotyl was not observed. Doley and Saunders (1989) pointed out that the explants on hormone-free medium remained small and became brown sooner. For control medium, our results resemble their findings. We observed that callus cultured on control medium became small and brown.

Petiole was also employed to regenerate whole sugar beet plant. For callus induction, petiole explants were taken from 22-25 day-old seedlings. Petiole explants gave the smallest callus compared to other parts of sugar beet (Figure 3.9). Similarly petiole produced friable callus in medium containing 2,4-D - BA and only TDZ. Callus obtained from petiole explants was placed on three different shoot induction medium. However, shoot development from callus of petiole was not observed owing to formation of compact, brown and small callus (Figure 3.10).

Gürel (2000) also obtained friable callus from petiole. However, in their study shoot formation per explant was very low. Our results were comparable with their findings. Krens and Jamar (1989) obtained shoots from the callus formed on the cut edges of petiole and not directly on unwounded areas of petiole.

Sugar beet leaves were also used for indirect shoot regeneration. Leaf explants produced considerably more callus than other explant types. Callus was cultured on TDZ and BA medium as shoot regeneration medium. After callus formation occured, the size of leaf explants increased on shoot regeneration medium. Due to compact callus formation, no shoot regeneration was observed (Figure 3.11).

Figure 3.9. Smallest callus derived from petiole of cultivar ELK 345.

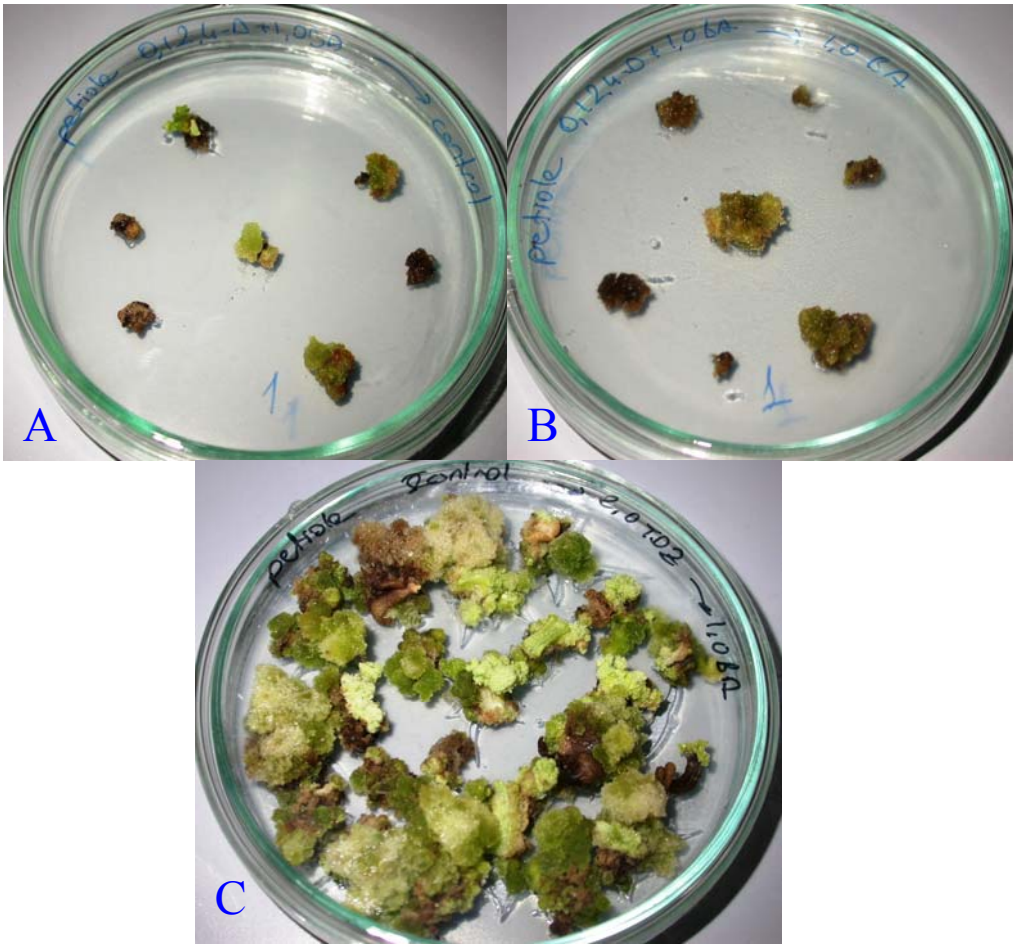


Figure 3.10. Compact callus formation on shoot regeneration medium. Callus tissue were photographed after (A): 3 weeks (B): 6 weeks (C): 12 weeks.

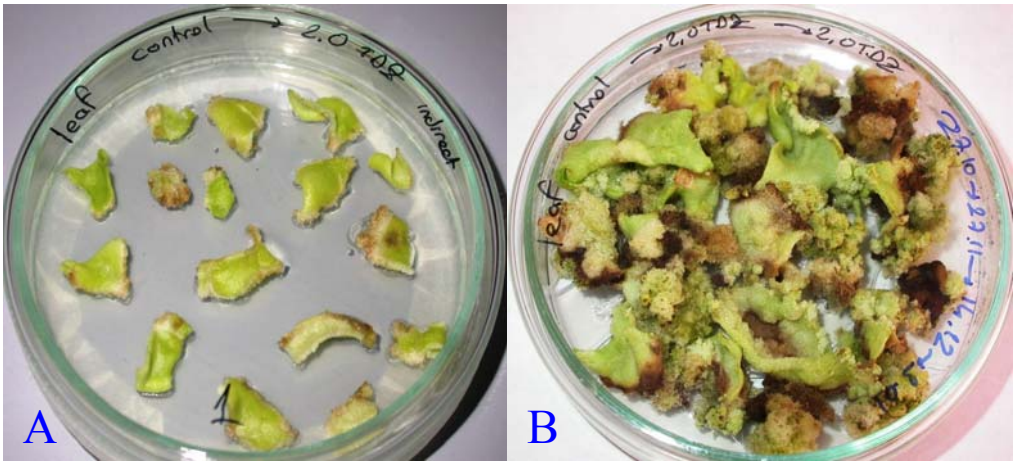


Figure 3.11. Leaf explants of cultivar ELK 345 placed on callus induction and shoot regeneration medium. Leaf tissue were photographed after (A): 2 weeks (B): 8 weeks.

Leaves of sugar beet was commonly used as an explant type for indirect regeneration studies. Coumans *et al*., (1982) reported that an auxin-dependent callus was initiated from leaf pieces of sugar beet on medium supplemented with 1.0 mg/L IAA and 0.1 mg/L KIN. Callus initiation from leaf pieces was also achieved by Gürel (2000). Means of shoot formation per explant was low in their study.

There were differences between different types of explants in their ability to form callus. In our experiments, apart from cothyledon all explant types produced friable callus. However, in shoot regeneration medium callus of all explants became compact callus and no shoot development was observed (Table 3.1). All explant types produce roots on the control medium. These results indicate that the source of explant is an important factor for indirect organogenesis of sugar beet. This may also suggest that differences in endogenous hormone levels or in sensitivity to them might vary between organs.

Table 3.1. Overall results of indirect organogenesis of sugar beet

Explant Types	**Friable callus formation**	**Shoot regeneration**
Cotyledon	Not obtained	Not obtained
Hipocotyl	Obtained	Not obtained
Petiole	Obtained	Not obtained
Leaf	Obtained	Not obtained

In sugar beet, adventitious bud differentiaiton can be induced in leaves and petioles. However, using an auxin and a cytokinin failed to produce shoot buds from callus of these explants. This may be because of the effect of the auxin utilized for callus induction or owing to callus arising from non-component cells (Bhat *et al*., 1985). Although indirect organogenesis is preferable for transformation studies, we focused on direct shoot regeneration for both tissue culture and transformation studies since no shoot development from callus of any parts of sugar beet was achieved.

3.1.3. Direct Organogenesis

In this part of the study, results of the first method related with direct organogenesis were exhibited. According to the first method, seeds were again germinated in two different germination medium. Then same explant types which were used for indirect organogenesis were placed on shoot regeneration medium containing different concentrations of TDZ.

Seeds that were grown on medium containing 0.5 mg/L BA 0.1 mg/L NAA showed small seedlings as compared to those of the control medium and produced small cotyledon and hypocotyl. Although direct

regeneration system was tried to be optimized in this part, all explant types including cotyledon, hypocotyl, petiole and leaf produced callus. After subculture of these explants, the size of the callus increased. Necrosis of the callus took place. Instead of direct shoot regeneration using different concentrations of TDZ, callus formation was observed.

Cotyledon taken from medium containing BA and NAA was so small that different combinations of TDZ treatment did not reveal shoot development. All explants produced small amount of callus (Figure 3.12).

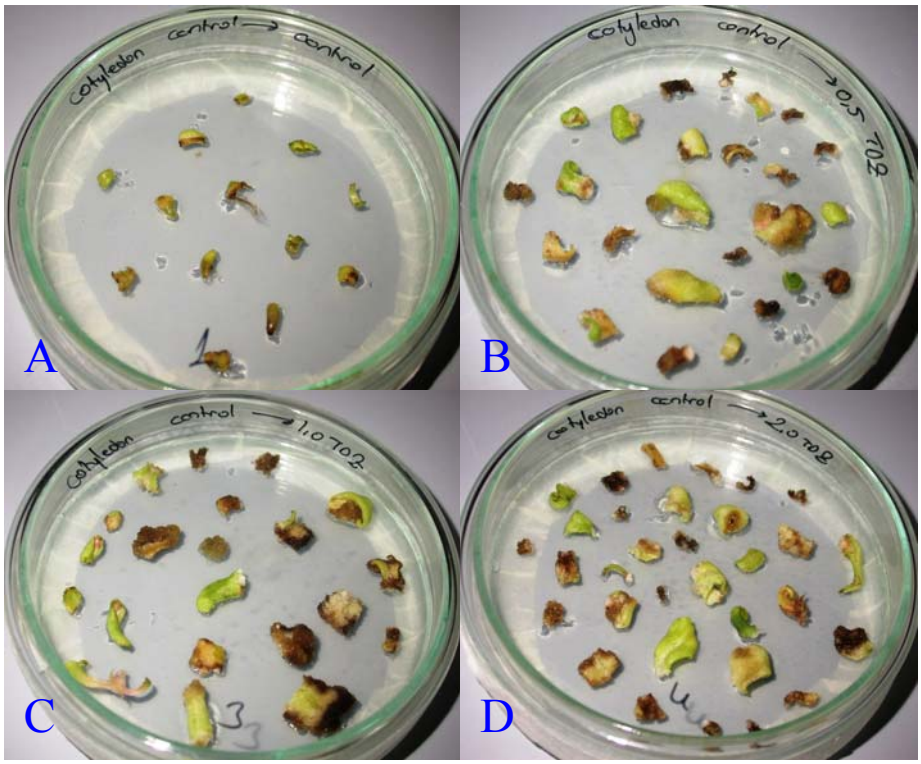


Figure 3.12. Small cotyledon explants of cultivar ELK 345 which formed callus in all concentrations of TDZ treatment. Explants were photographed after 4 weeks. (A): Control (B): 0.5 mg/L TDZ (C): 1.0 mg/L TDZ (D): 2.0 mg/L TDZ.

Like cotyledon, hypocotyl germinated in medium containing BA and NAA had small size. Callus development from these hypocotyl explants was observed. After two or three weeks, their size decreased and necrosis began (Figure 3.13). Gürel (2000) tried to optimize direct shoot regeneration from cotyledon and hypocotyl. However researcher did not achieve direct shoot formation from these explants. Growth regulators as well as explant sources influenced the rate of shoot formation.

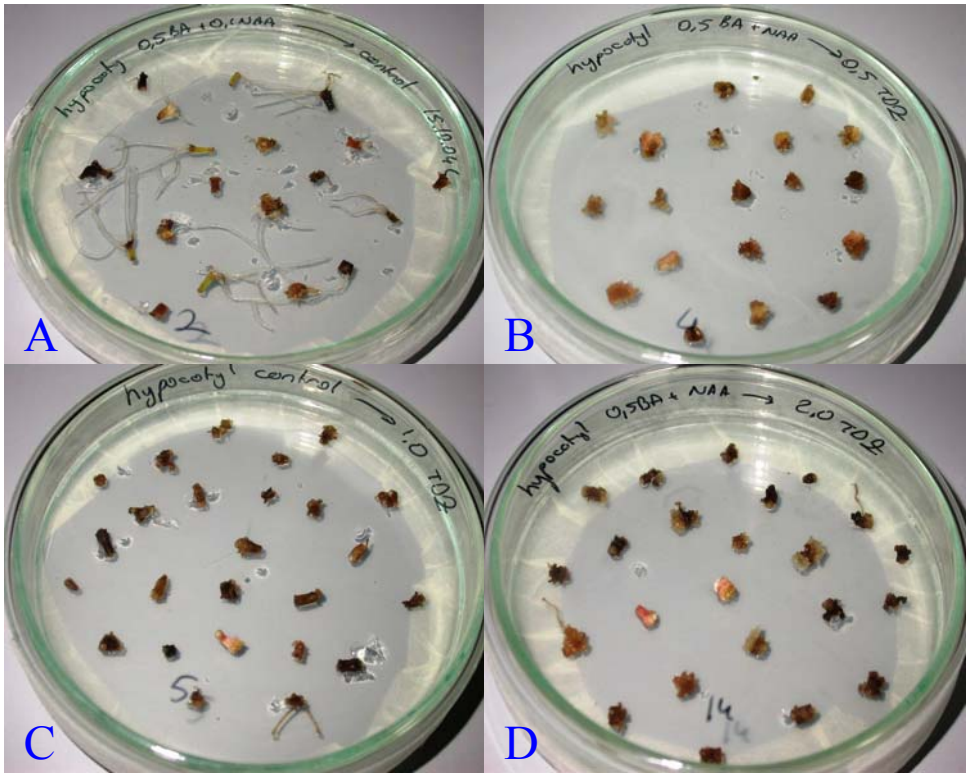


Figure 3.13. Necrose formation occured when hypocotyl explants of cultivar ELK 345 were placed on shoot regeneration medium. Explants were photographed after 3 weeks. (A): Control (B): 0.5 mg/L TDZ (C): 1.0 mg/L TDZ (D): 2.0 mg/L TDZ.

Direct shoot development from petiole explants was also examined. Petiole explants were excised from the seedlings that was grown on medium containing 0.5 mg/L BA, 0,1 mg/L NAA and hormone-free medium for four weeks and placed on medium supplemented with different concentration of TDZ. Instead of shoot development, petiole explants also produced callus on medium containing 0.5, 1.0 and 2.0 mg/L TDZ (Figure 3.14). There was no direct shoot development and callus formation, when petiole explants were placed on hormone-free medium. Utilization of different TDZ concentrations was not effective, either, for shoot regeneration from petiole.

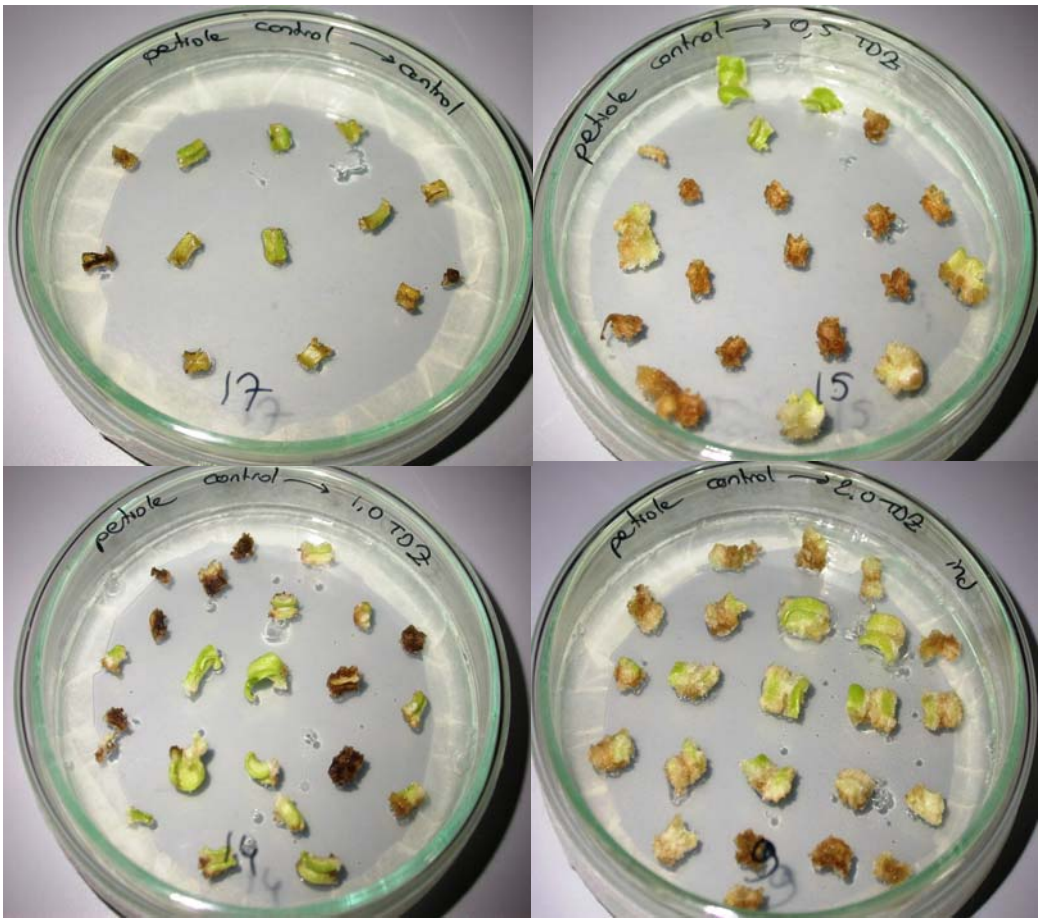


Figure 3.14. Petiole explants of cultivar ELK 345 cultured on shoot induction medium. Petiole explants were photographed after 4 weeks.

Previous studies indicated that petiole of sugar beet has been commonly used for regeneration studies due to its high regeneartion capacity. A one-step method for regenerating shoots from petiole explants on medium containing low concentrations of NAA and BA was previously described by Saunders and Doley (1986) and Freytag *et al*., (1988). Freytag *et al*., (1988) obtained high frequencies of shoot formation from petiole and many of their regenerants originated from silent meristem or pre-determined cells within the explant tissue. Zhong *et al*., (1993) achieved 23.3 % shoot formation per explant from petiole taken from donor plants of two sugar beet cultivars pretreated with 0.5 mg/L BA. They also observed no shoot development from petiloe which was grown on hormone free medium. It was also shown, in a recent study (Zhang *et al*., 2001), that no shoots developed from petiole or lamina explants of sugar beet seedlings that were precultured on hormone-free medium when the explants were subsequently cultured on a regeneration medium containing 1, 2 or 4 mg/L BA. Gürel *et al*., (2003) also inspected the effect of pretreating seedlings with TDZ on direct shoot regeneration from petiole explants of sugar beet. In another study, Gürel *et al*., (2003) examined the effect of pretreating seedlings with BA on direct shoot regeneration from petiole explants of sugar beet. However, in both studies shoot regeneration rate were low when compared to other studies. They obtained 1.67 and 0.97 shoots per explants, respectively.

Lastly, shoot regeneration from leaf of sugar beet was examined. Leaves were cultured on three shoot regeneration medium containing 0.5, 1.0 and 2.0 mg/L concentrations of TDZ. All TDZ treatment produced callus. After two weeks, the size of the leaf explants increased and then compact callus formation occured. No shoot development was achieved from leaf explants (Figure 3.15).

Shoot formation from leaf explants of sugar beet was also reported by Mikami *et al*., (1989), Owens and Eberts (1992) and Munthali *et al*., (1996). Also, the effects of pretreating leaf explants on the subsequent shoot regeneration were examined in sugar beet (Zhong *et al*., 1993 and Krens *et al*., 1996).

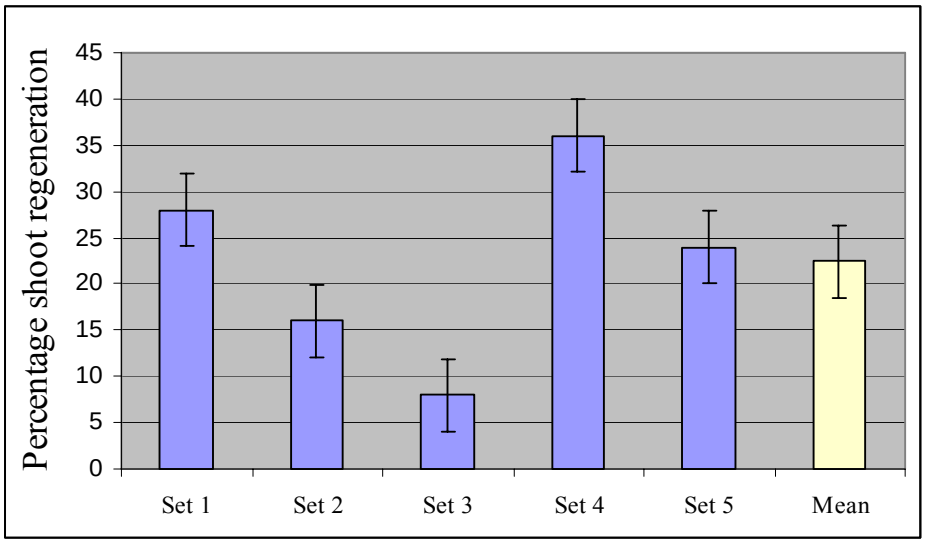
Figure 3.15. Callus formation from leaf explants of cultivar ELK 345 on shoot regeneration medium. Leaf explants were photographed after 4 weeks.

In our experiments, all explant types including cotyledon, hypocotyl, petiole and leaf produced callus instead of shoot in shoot regeneration medium. Although direct shoot regeneration from petiole and leaf was accomplished (Saunders and Doley 1986; Freytag *et al*., 1988; Zhong *et al*., 1993; Zhang *et al*., 2001 and Gürel *et al*., 2003) genotypic variation influences regeneration of sugar beet. Previous studies indicate that some genotypes of sugar beet are more amenable to tissue culture than others (Jacq *et al*., 1992; Zhong *et al*., 1993; Gürel, 1997; Saunders and Tasai, 1999). An alternative method might be to screen out material with low organogenic or embryogenic potential and to use only those, which regenerate readily *in vitro*.

3.1.4. Multiple Shoot Induction via Direct Organogenesis

In this part of the study, results of the second method related with direct organogenesis of leaf were demonstrated. The shoot base explants were cultured on IBA and BA to induce leaf growth. Then leaf blades were placed on shoot regeneration medium to perform multiple shoot induction. Shoots were regenerated from the veins of the leaf blades. The shoots were removed and placed on growth medium. Finally, regenerated plantlets were transfered into rooting medium (Figure 3.16).

We examined the percentage of regeneration from shoot base preparations in sugar beet cultivar 1195. Each leaf blade was considered as one individual explant. Shoot regeneration from this individual explant was counted. Five different sets of experiments were designed. We achieved maximum shoot regeneration in the set four in terms of 36 % whereas minimum shoot regeneration was obtained in set three (8 %) (Figure 3.17).



Mean values and SEM are tabulated in Table F.2 in Appendix F.

Figure 3.17. Multiple shoot induction from sugar beet leaf blades using IBA and BA.Vertical bars indicate SEM (standard error of mean, n=50 for each treatment).

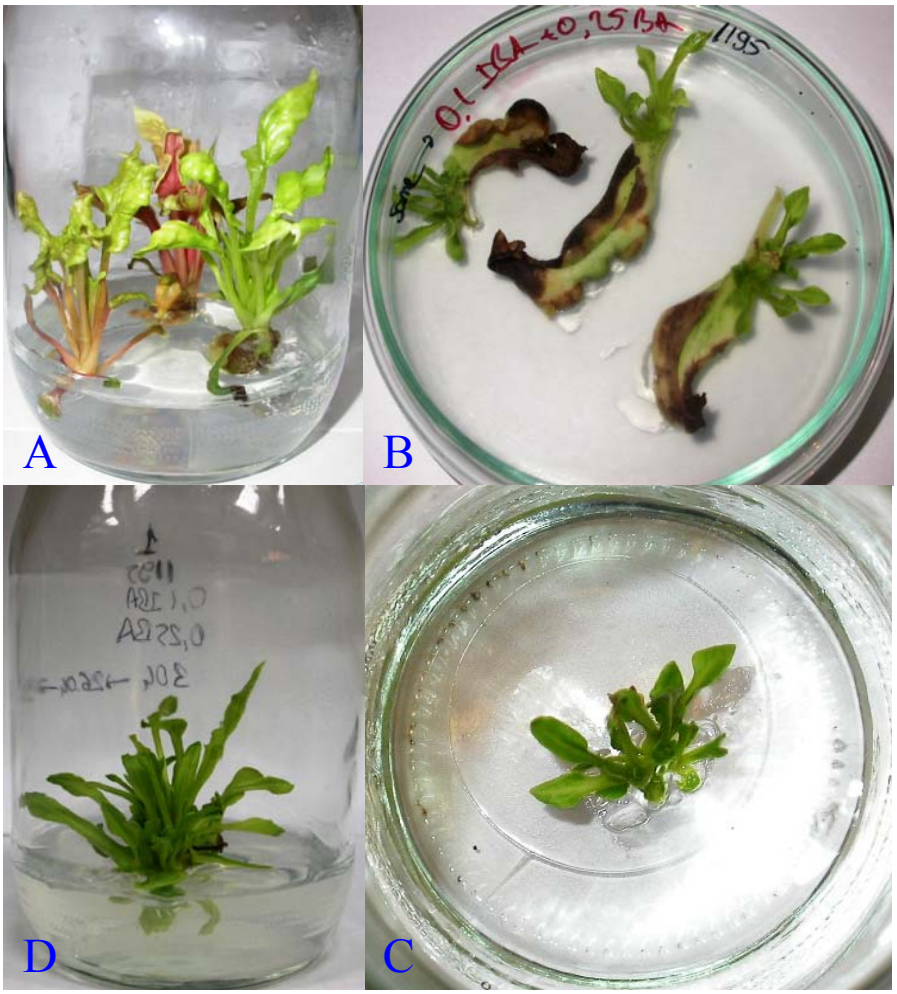


Figure 3.16. Sugar beet regeneration via direct organogenesis. Representative figures of (A) shoot base in media supplemented with 0.1 mg/L IBA and 0.25 mg/L BA after 2 weeks (B) shoot regeneration from veins of leaf blade 2 weeks after transferred to medium (C) shoots in growth media after 2 weeks (D) a regenerated sugar beet plant with roots (4 weeks) are displayed.

In our study, average percentage of shoot regeneration per leaf blade was 22.4 %. A similar study of shoot regeneration from shoot base was performed by Hisano *et al*., (2004). They examined the frequency of regeneration from shoot base preparations in seven accessions of sugar beet and two accessions of *B. maritima*.

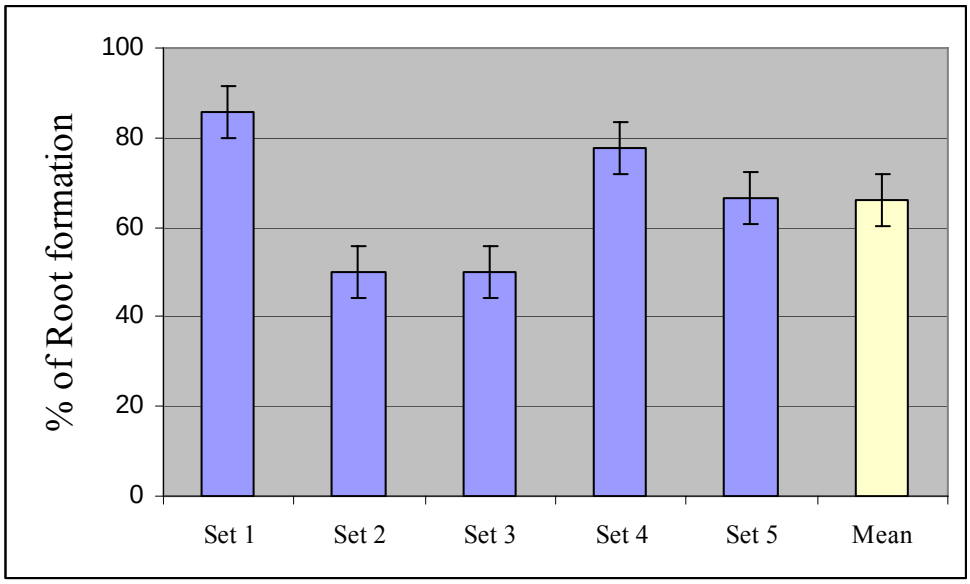
In their study, 80 % shoot regeneration from *B.martima* and 48 % shoot regeneration from *B. vulgaris* were achieved, respectively. Although we used same hormone combinations for shoot formation, they obtained high shoot regeneration rate when compared to our results. This is most probably because of genotypic variation of used sugar beet lines. Genotypic variation is a serious problem in experimental work for sugar beet. In order to eliminate this problem, different regimes of plant growth regulator treatments, combinations of pretreatment and regeneration protocols have to be optimized.

3.1.5. Rooting of Regenerated Shoots

Sugar beet (cultivar 1195) shoots emerging from leaf blades were removed after 15 to 20 days and subcultured to root induction medium containing IBA. IBA was used by Hisano *et al*., (2004) and Gürel (2000) for root induction of regenerated sugar beet shoots. In this study, 1.0 mg/L IBA was also employed for root initiation.

Results of root formation from regenerated shoots were given as percent in Figure 3.18. According to our results, 66 % of the regenerated shoots developed roots. We obtained maximum root formation from regenerated shoots in set one as 85.7 % whereas minimum root development was observed in set two and three as 50 % (Figure 3.19).

Shoot regeneration from petiole and then root development from regenerated shoots was reported by Gürel *et al*., (2003). They obtained 42.6 % of root formation from regenerated shoots using 3 mg/L NAA. Compared to our result, we achieved 66 % root development from regenerated shoots. This difference may come from the utilization of different sugar beet lines and difference in regeneration procedure.



Mean values and SEM are tabulated in Table F.3 in Appendix F.

Figure 3.18. Root induction from regenerated shoots using IBA. Vertical bars indicate SEM.

Figure 3.19. Root induction with 1.0 mg/L IBA. (A) Appearance of shoots and (B) appearance of roots. Plantlets were photographed after 3 months.

Also shoot bases which were used both for regeneration and transformation were subcultured to hormone-free medium for root induction. In order to induce root formation, two different support matrix, plant agar and phytagel, were used. Hormone-free medium supplemented with phytagel was more effective in root formation than the medium supplemented with plant agar. At the end of the culture period of two weeks, root formation was observed in medium containing phytagel while at the end of the six weeks, root initiation was monitored in medium containing plant agar (Figure 3.20).

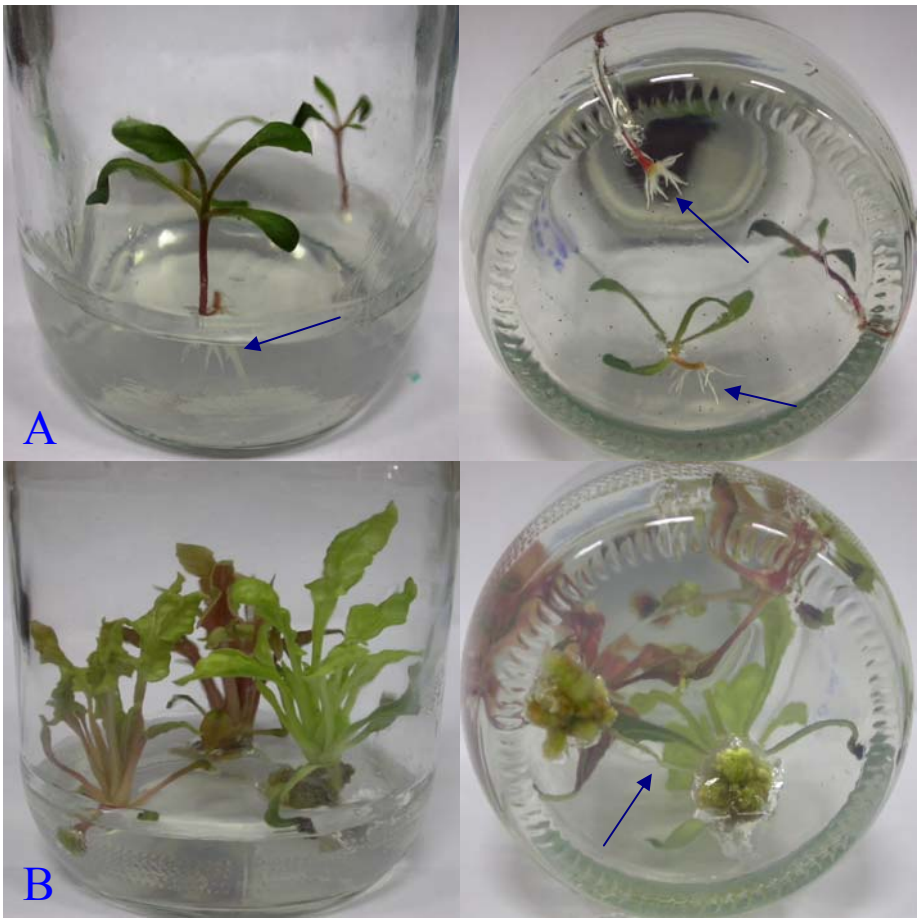
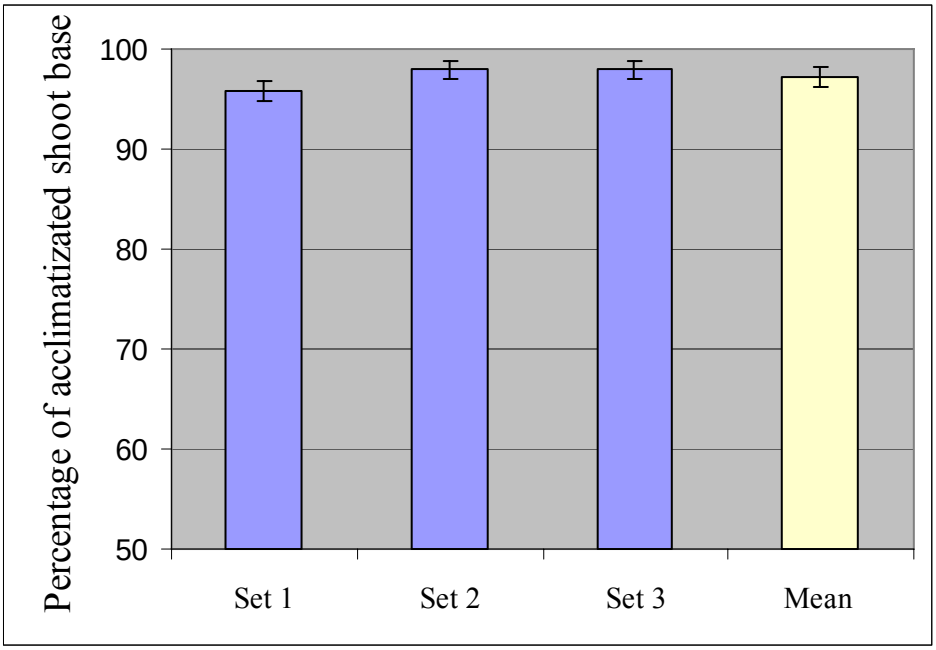


Figure 3.20. Root induction with different support matrix. (A) Appearance of shoots and roots of shoot bases medium containing phytagel after two weeks of culture (B) appearance of shoots and roots of shoot bases medium supplemented with plant agar after six weeks of culture.

Acclimatization of rooted shoot bases was performed successfully. Root formation was efficient when phytagel was used as a support matrix. 97 % acclimatization rate was achieved (Figure 3.21). Shoot bases with phytagel were transferred into pots containing soil. The pots were covered with plastic bag for 2 or 3 days. At the end of this period, plastic bag was removed. Plantlets continued their development and tap root formation was observed.



Mean values and SEM are tabulated in Table F.4 in Appendix F.

Figure 3.21. Acclimatization of rooted shoot base. Vertical bars indicate SEM (standard error of mean, n=50 for each set of experiments).

Figure 3.22. shows whole regeneration from shoot base to mature sugar beet plant (cultivar 1195) with tap root. This process completed in four months. In this figure, two plants were grown in same pot. So, this provides easy regeneration system for sugar beet in a short period.

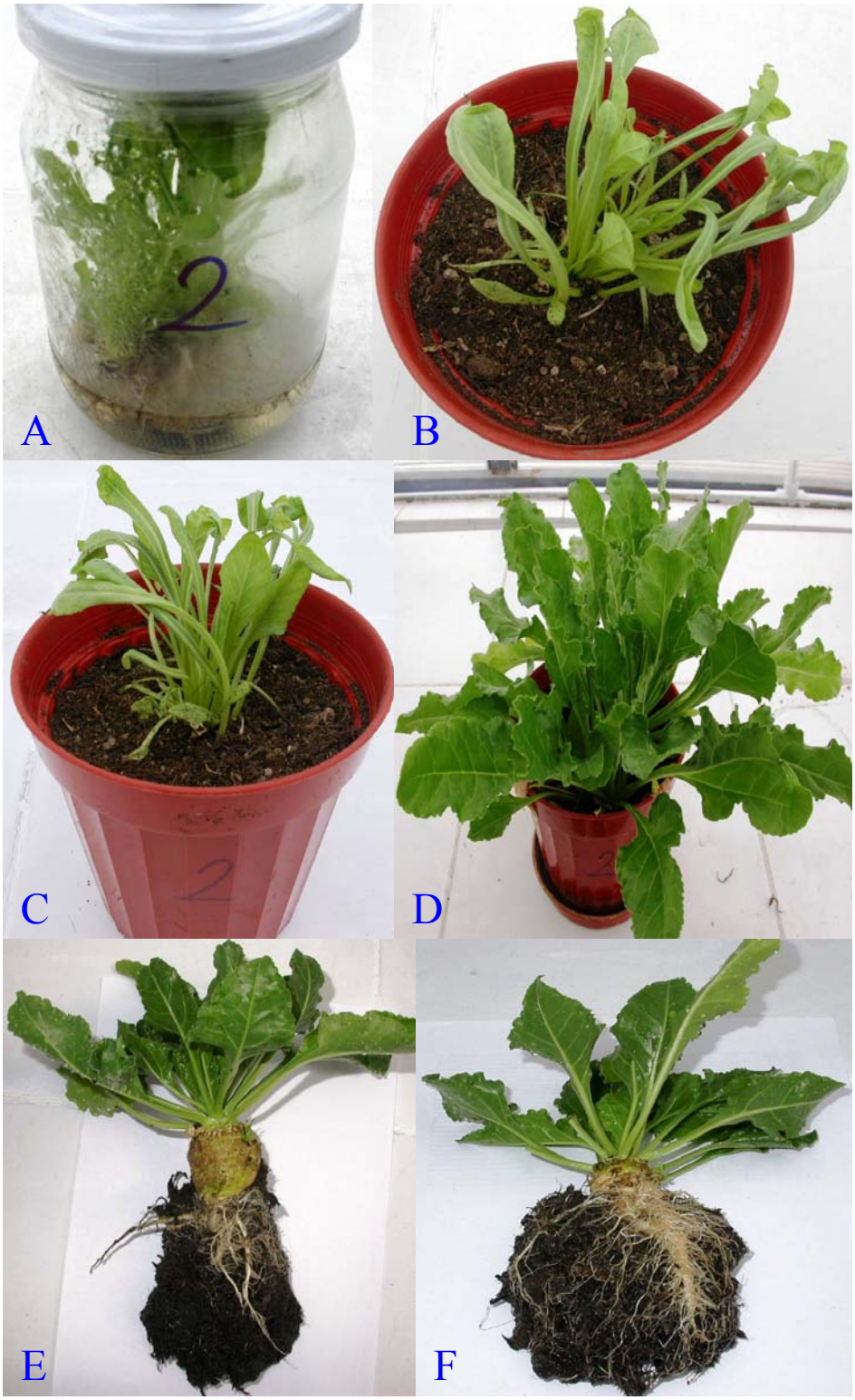


Figure 3.22. Acclimatization of platlets that were driven from shoot base of sugar beet. (A) Rooted shoot base in the jar (B) Appearance of shoots from upper position (C) Plantlets in soil at their four weeks (D) Appearance of shoots from upper position at their twelve weeks (E-F) Tap root formation.

To our knowledge, this is the first study exhibiting an easy acclimatization procedure for sugar beet in a short period. Rady (1997) reported micropropogation of sugar beet from the excised shoot tips. However, this procedure includes using plant growth regulators for shoot multiplication and root formation. When compared to our method, no plant growth regulators were required for shoot multiplication and root initiation. Therefore, application of our method was easier than the other one. Another advantage of our method is that regenerated plants showed no morphological differences from those grown naturally due to growing in hormone-free medium supplemented with only phytagel. The use of multiple clone lines, each one coming from one seed, enable the maintenance of variability through micropropagation. By using shoot base as a starting material, mature sugar beet plants can easily be obtained in three or four months.

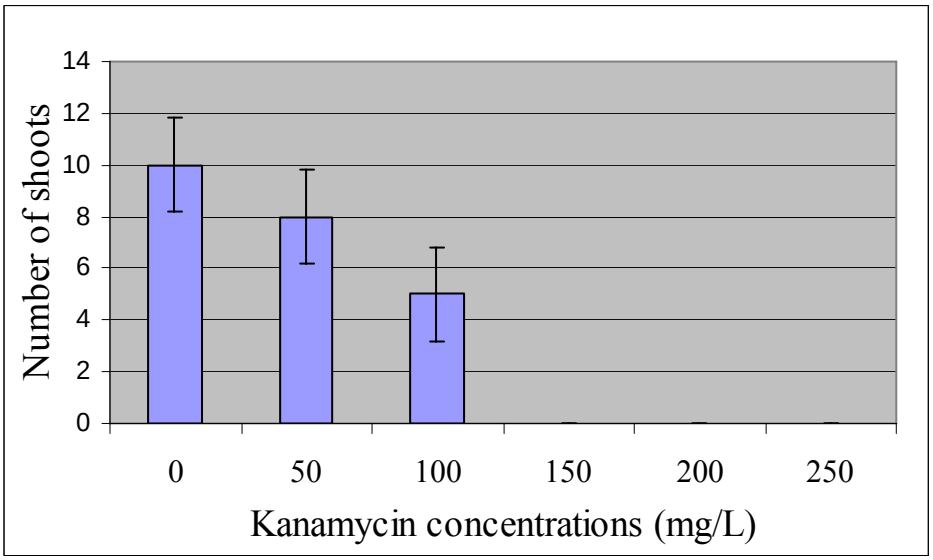
3.1.6. Lethal Dose Determination for Selective Agents

In order to select and recover the transformed cells or tissues from non-transformed ones, selectable marker genes including antibiotic resistance and herbicide tolerance are widely used (Miki and McHugh, 2004). They allow transformed cells expressing themselves to be selected over non-transformed cells. In this study, leaf blades from which shoot regeneration occured were exposed to different selective agents. For this purpose different concentrations of kanamycin and Phosphinotricin (PPT) were employed.

The concentrations used for kanamycin and PPT were 50, 100, 150, 200, 250 mg/L and 1, 3, 5, 10 mg/L, respectively. All experiments were carried out together with controls which are selective agent free medium containing 0.1 mg/ L IBA and 0.25 mg/L BA. Three indipendent sets of experiments were performed.

Effect of kanamycin on shoot development from leaf blades is displayed in Figure 3.23. Explants produced shoots when they were cultured on kanamycin

free medium and MS media supplemented with 50 and 100 mg/L kanamycin. However there was no shoot development when explants were cultured on 150, 200 and 250 mg/L kanamycin. High concentrations (150 mg/L or more) of kanamycin inhibited shoot regeneration from leaf blades.

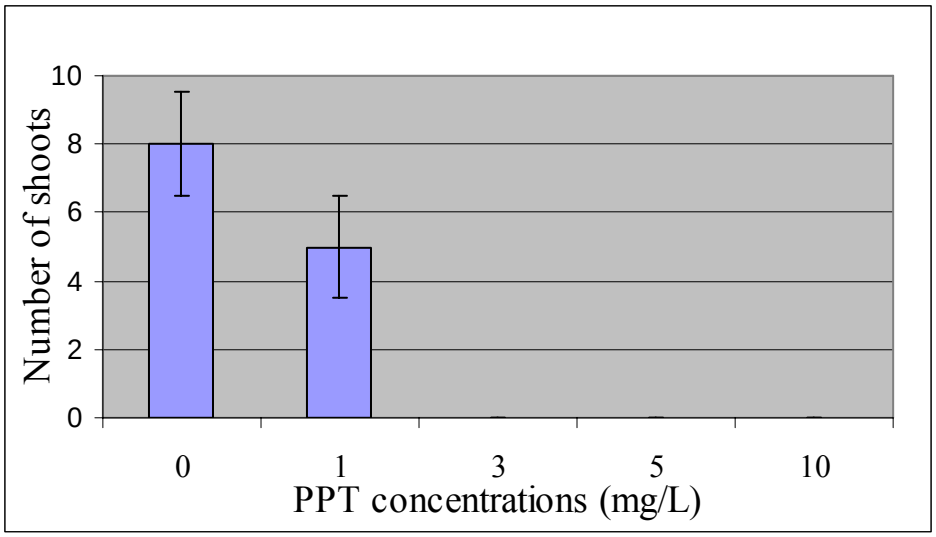


Mean values and SEM are tabulated in Table F.5 in Appendix F.

Figure 3.23. Effect of kanamycin on shoot development. Kanamycin free medium (0 mg/L) was used as control. Vertical bars indicate SEM (n=15 for each treatment).

Color loss and necrosis was observed due to high concentrations (150 mg/L or more) of kanamycin. As a result of all these findings, it can be stated that regeneration from non-transformed cells could be efficiently suppressed on medium containing 150 mg/L kanamycin or more. This result is consistent with the report of Hisano *et al*., (2004), in which 150 mg/L kanamycin was preferred for transgenic sugar beet selection. Therefore, 150 mg/L kanamycin is appropriate for use in sugar beet transformation.

PPT was another selection agent used in this study. The effect of PPT on shoot regeneration from leaf blades is represented in Figure 3.24. Shoot regeneration was observed when explants were cultured on PPT free medium or medium containing 1 mg/L PPT. However PPT at concentrations above 3 mg/L totally inhibited the shoot regeneration.



Mean values and SEM are tabulated in Table F.6 in Appendix F.

Figure 3.24. Effect of PPT on shoot development. PPT free medium (0 mg/L) was used as control. Vertical bars indicate SEM (n=15 for each treatment).

Like effect of kanamycin, color loss and necrosis were observed due to high concentrations (3 mg/L) of PPT (Figure 3.25). According to the results of PPT effects on shoot regeneration, it is conculuded that 3 mg/L or more may be employed in selection media after event of *Agrobacterium* mediated transformation of sugar beet. This result is similar with the study of Öz (2005), in which PPT optimizations for chickpea were performed.

Figure 3.25. Effect of different PPT concentrations on shoot regeneration from leaf blades. The explants were photographed after 4 weeks of culture. Arrows indicate newly established shoots.

3.2. Transformation Studies on Leaf Disks

In transformation part of this study, optimization of *Agrobacterium* mediated transformation system for leaves of sugar beet cultivar ELK 345 was performed. Different parameters were studied. These include: vacuum infiltration during bacterial inoculation of explants, different bacterial growth medium, different inoculation time with bacteria, different bacteria strains and application of L-Cysteine during co-cultivation period. The effect of each application was investigated by using GUS histochemical staining assay (Jefferson, 1987) after co-cultivation period of 3 days. Qauntitative analysis of histochemically stained leaves (blue sectors) was performed to determine the effect of each application on transformation efficiency.

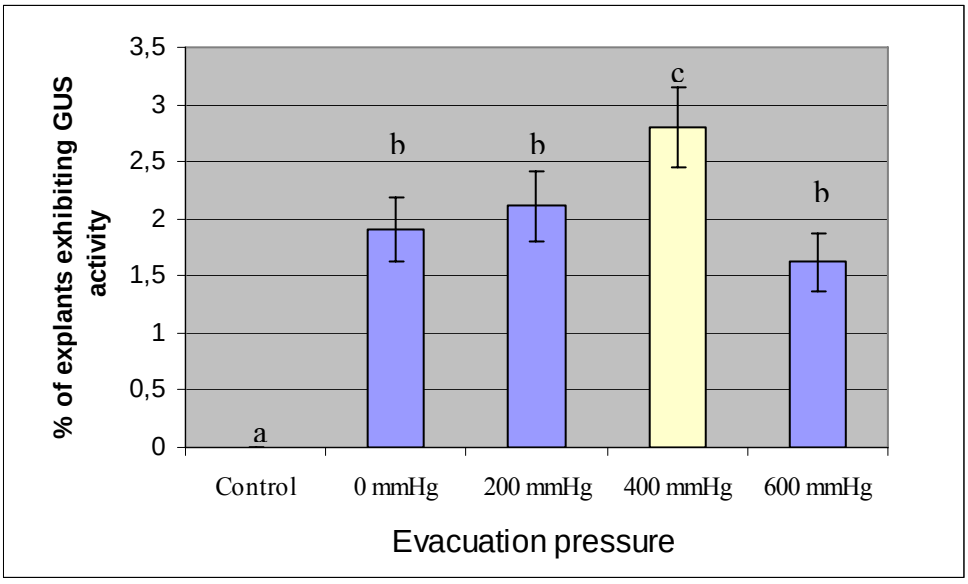
3.2.1. Effect of Vacuum Infiltration

Vacuum infiltration is an effective way of promoting close contact between bacterium and host plant cell. Physically, vacuum generates a negative atmospheric pressure that cause the air spaces between the cells in the plant tissue to decrease. The use of *Agrobacterium* mediated transformation assisted by vacuum infiltration was first reported in 1993 (Bechtold *et al*.,) for transforming *Arabidopsis* and since then many improvements have been made. Also Mahmoudian *et al*., (2002) demonstrated that vacuum infiltration of *A. tumefaciens* suspensions containing lentil explants resulted in high levels of transient gene expressions. In this study, vacuum infiltration was also appplied to improve transformation efficiency.

In order to indicate effect of vacuum infiltration on transformation efficiency 200, 400 and 600 mmHg evacuation pressures were applied to leaves of sugar beet for 10 minutes. The experiments were coupled to control groups, which were not inoculated with bacteria and not infiltrated. The first control group was composed of explants that were not inoculated with bacteria.

The second control group (0 mmHg), which was inoculated but not infiltrated, was used as control to evaluate the effect of vacuum infiltration. All explants including control groups were injured with a blade for the bacteria to easily penetrate into plant cells.

Effect of infiltration represented as percent of explants exhibibting GUS activity on 3rd day after transformation is shown in Figure 3.26. In the control group, no GUS activity was obsreved. On the other hand , percentage of explants exhibiting GUS activity was increased when the explants were infiltrated at 400 mmHg (2.8 ± 0.3) compared to no vacuum applied explants (1.9 ± 0.2). As a result, it can be stated that application of 400 mmHg evacuation pressure significantly increased the transformation efficiency in leaves of sugar beet according to GUS staining on the third day of co-cultivation.



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

Figure 3.26. Effect of vacuum infiltration on transient gene expression 3 days after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p > 0.05$).

In our study, infiltration at 600 mmHg significantly reduced the transient GUS expression levels in leaves of sugar beet. When leaves were exposed to 600 mmHg, 1.6 ± 0.2 of explants exhibited GUS activity. A similar observation of decreased gene expression at high evacuation pressure was also reported by Öz (2005) in chickpea. In his study, cotyledonary node of chickpea was infiltrated at 600 mmHg. This evacuation pressure decreased the transient GUS expression levels in chickpea.

Representative photographs of control, non-infiltrated and infiltrated leaves stained for GUS activity and their qauntification are given in Figure 3.27. Staining patterns showed that without vacuum infiltration (0 mmHg), stained areas were generally concentrated around wounded sites. However upon infiltration, penetration of bacteria to inner parts of the tissues was observed due to decrease of air spaces between the cells of plant tissue. Therefore, application of vacuum infiltration provides easy penetration for bacteria into plant tissues. Although infiltration increases GUS stained area in plant tissues, utilization of this technique can be harmful due to possible reduction in regeneration potential of plant cells. On the other hand, this application can be benefical for increasing the number of transformed cells.

As a result of all these findings, it can be concluded that vacuum infiltration increases the transformation efficiency; and 400 mmHg evacuation pressure is appropriate pressure to improve gene transfer. Therefore, for determination of other application on transformation efficiency, 400 mmHg evacuation pressure was applied to leaves throughout this study.

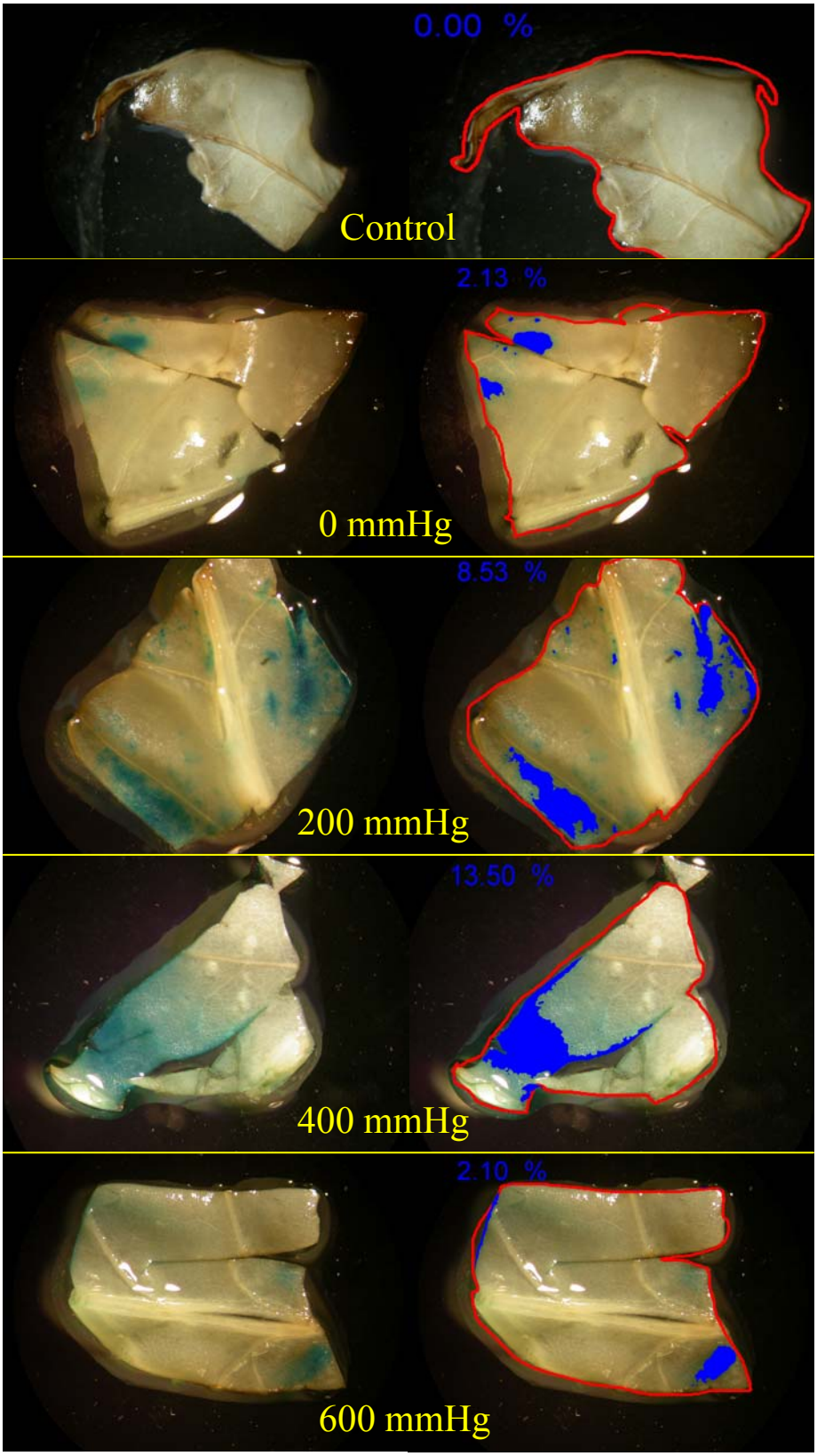


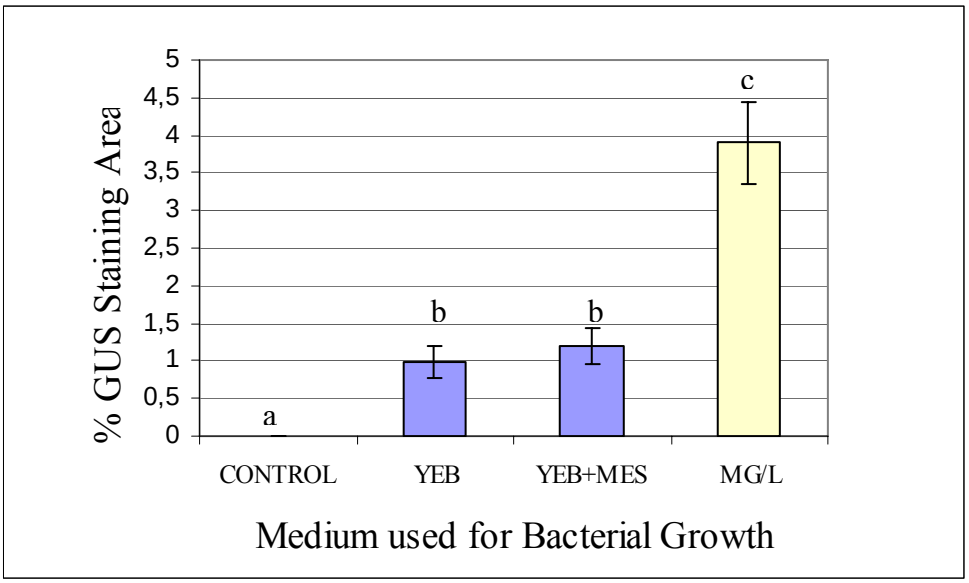
Figure 3.27. Representative photographs of leaves and their qauntification for vacuum infiltration.

3.2.2. Effect of Bacterial Growth Medium

In this study YEB, YEB+MES and MG/L medium was used for bacterial growth to examine effect of transformation efficiency. YEB and YEB+MES medium are similar to each other. The only difference between two media is that YEB+MES media contains MES which provides asidic environment for bacterial growth. Besides, after centrifugation MMA medium was employed for resuspension of bacteria for both media. However MG/L medium, (Tingay *et al*., 1997) which is frequently used for monocot transformation, is completely different from others. MG/L medium contains mannitol, glutamic acid, biotin and various salts including NaCl and KH_2PO_4. Its inoculation medium is also different from MMA. In our study, three different bacterial media were employed for improvement of the transformation efficiency. To our knowledge, this is the first study related with the use of various bacterial media for transformation of sugar beet. *Agrobacterium tumefaciens* strain EHA105 was used in this experiment.

Bacterial culture grown in these media were inoculated with leaf disks for 10 minutes at 400 mmHg evacuation pressure. As a control group, explants were not inoculated with bacteria.

Results of GUS histochemical staining described as percent GUS expressing area are displayed in Figure 3.28. Control explants, not inoculated with bacteria, exhibited no GUS activity. When YEB and YEB+MES media were used for transformation; 1.0 ± 0.2 and 1.2 ± 0.2 % of explants exhibited GUS activity, respectively. On the other hand when the MG/L medium was employed for transformation, 3.9 ± 0.5 percent of explants exhibited GUS activity, which was significantly higher than others. In the light of these results, it can be stated that utilization of MG/L medium for bacterial growth significantly increased the transformation efficiency in sugar beet according to GUS staining.



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

Figure 3.28. Effect of bacterial growth medium on transient gene expression on the 3rd day after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p > 0.05$).

Representative photographs of control and leaf explants which were transformed with bacteria grown in different medium stained for GUS activity and their qauntification are indicated in Figure 3.29. Stained shoots clearly showed that the stained plant tissues were significantly increased when the transformation was carried out by using MG/L medium. Explanation of this situation may be based on composition of MG/L medium.

As a conclusion when bacteria was grown in MG/L medium for transformation of leaf disks, GUS stained area on leaf disks was meaningfully augmented compared to bacteria grown in YEB and YEB+MES medium. In order to determine other application on transformation efficiency, bacteria was grown in MG/L medium in the rest of the experiments.

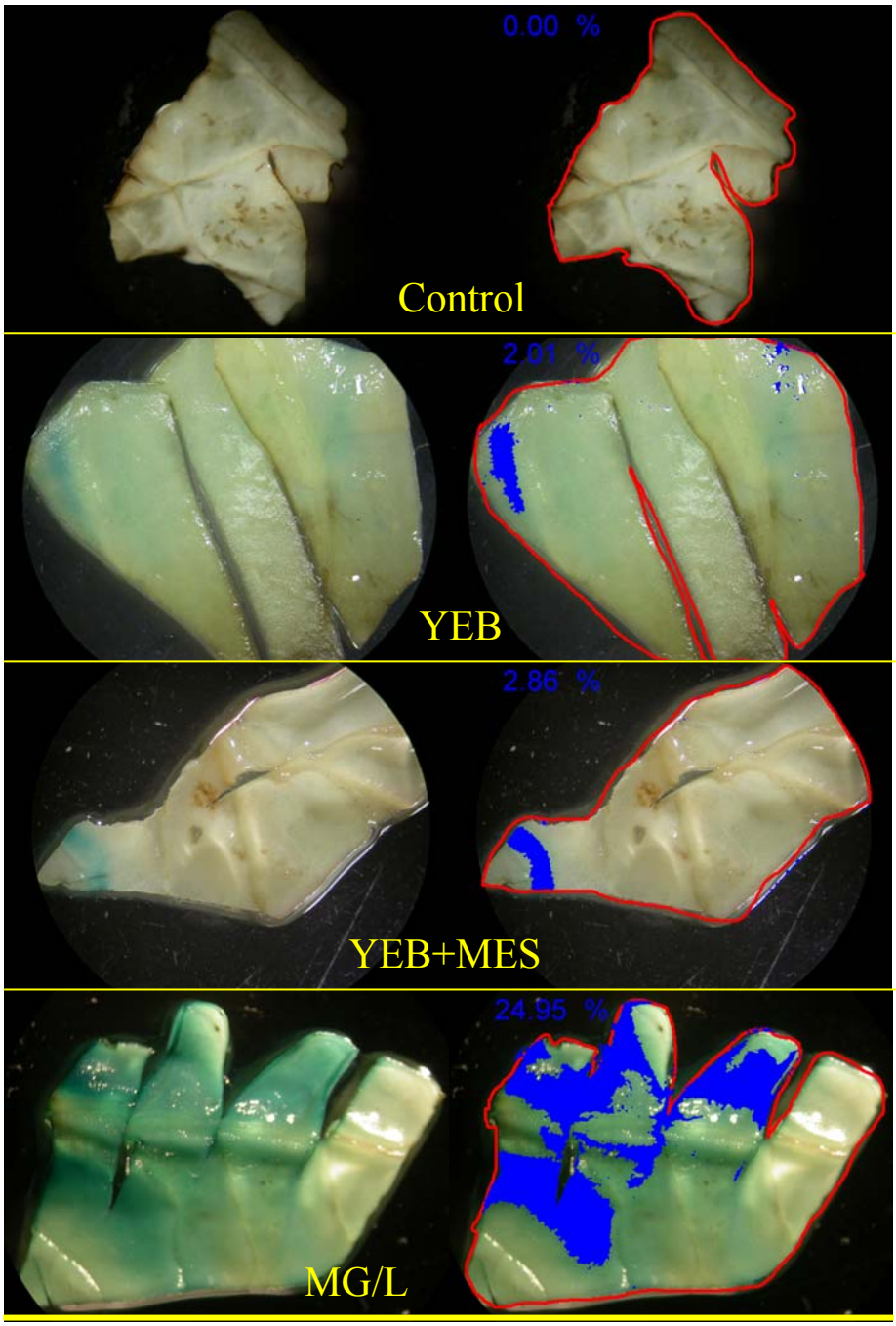
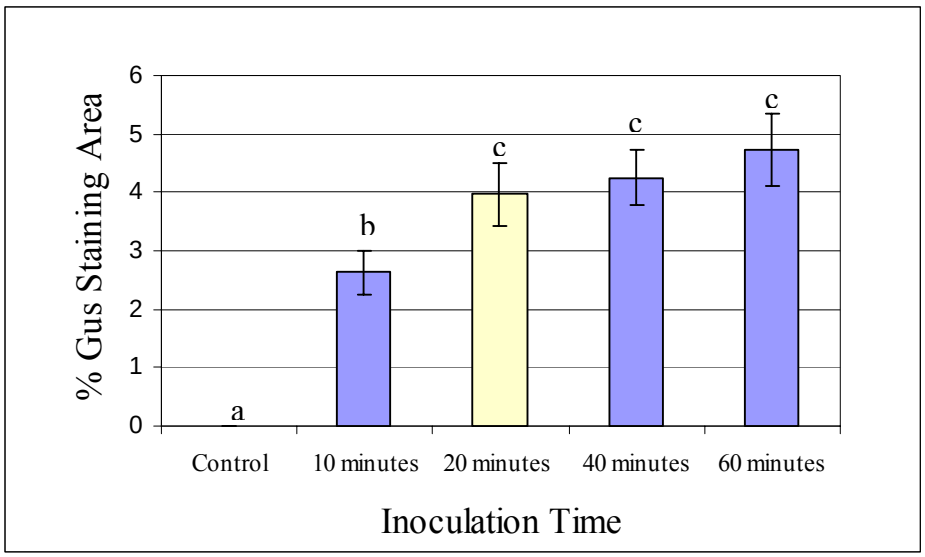


Figure 3.29. Representative photographs of leaves and their qauntification for different bacterial growth medium.

3.2.3. Effect of Inoculation Time

In our study, for investigation of inoculation time on transformation efficiency leaf disks were inoculated with bacterial culture grown in MG/L medium for different periods of time (10, 20, 40 and 60 minutes) at 400 mmHg evacuation pressure. Non-inoculated leaf disks formed the control group.

Effect of bacterial inoculation time characterized as percent of explants showing transient GUS activity on the 3rd day after transformation is displayed in Figure 3.30. All control explants showed no GUS activity. GUS activity was proportionally increased, when the inoculation time with bacteria was increased. When the explants were inoculated with bacteria for 10 minutes, 2.6 ± 0.3 % of explants exhibited GUS activity. Application of 10 minutes inoculation time was significantly lower than other ones. On the other hand, application of 20, 40 and 60 minutes with bacteria did not cause any significant change in percentage of GUS expressing area which fluctuated between 3.9 and 4.7 %.



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

Figure 3.30. Effect of inoculation time with bacteria on transient gene expression on the 3rd day after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

Representative photographs of control and leaf disks which were co-cultivated with bacteria for different inoculation time stained for GUS activity and their qauntification analysis are demonstrated in Figure 3.31. Statistically, there were no significant differences between 20, 40 and 60 minutes incubation periods. However, it is viewed in Figure 3.28 that 20 minutes inoculation time with bacteria gave the highest GUS activity when compared to others. Moreover in order to prevent death of plant cells due to long incubation time, 20 minutes inoculation time was used in the rest of experiments. Although leaves of potato were co-cultivated with bacteria for 2 days (Tansı 2002) or immature embryos of wheat immersed in bacteria suspension for 3 hours (Wu *et al*., 2003), regeneration of sugar beet explants may be influenced with such a long inoculation period for further studies. In contrast to other plants, explants of sugar beet was generally incubated with bacteria for short period. Hisano *et al*., (2004) reported that leaf blades of sugar beet were immersed in bacteria culture for 1 minute. Therefore, we also selected the lowest incubation period.

Application of different incubation periods increased overall GUS staining activity. When the vacuum infiltration results were examined, percent of GUS activity was relatively increased. At the begining of the study, 2.8 ± 0.3 % of explants exhibited GUS activity whereas 4.7 ± 0.6 % of explants exhibited GUS activity after the this application.

As a result of all these findings, it can be stated that 20 minutes inoculation period resulted in two fold increase in the transformation effficiency. Therefore, for further studies co-cultivation period with bacteria and leaf disks was determined to be 20 minutes.

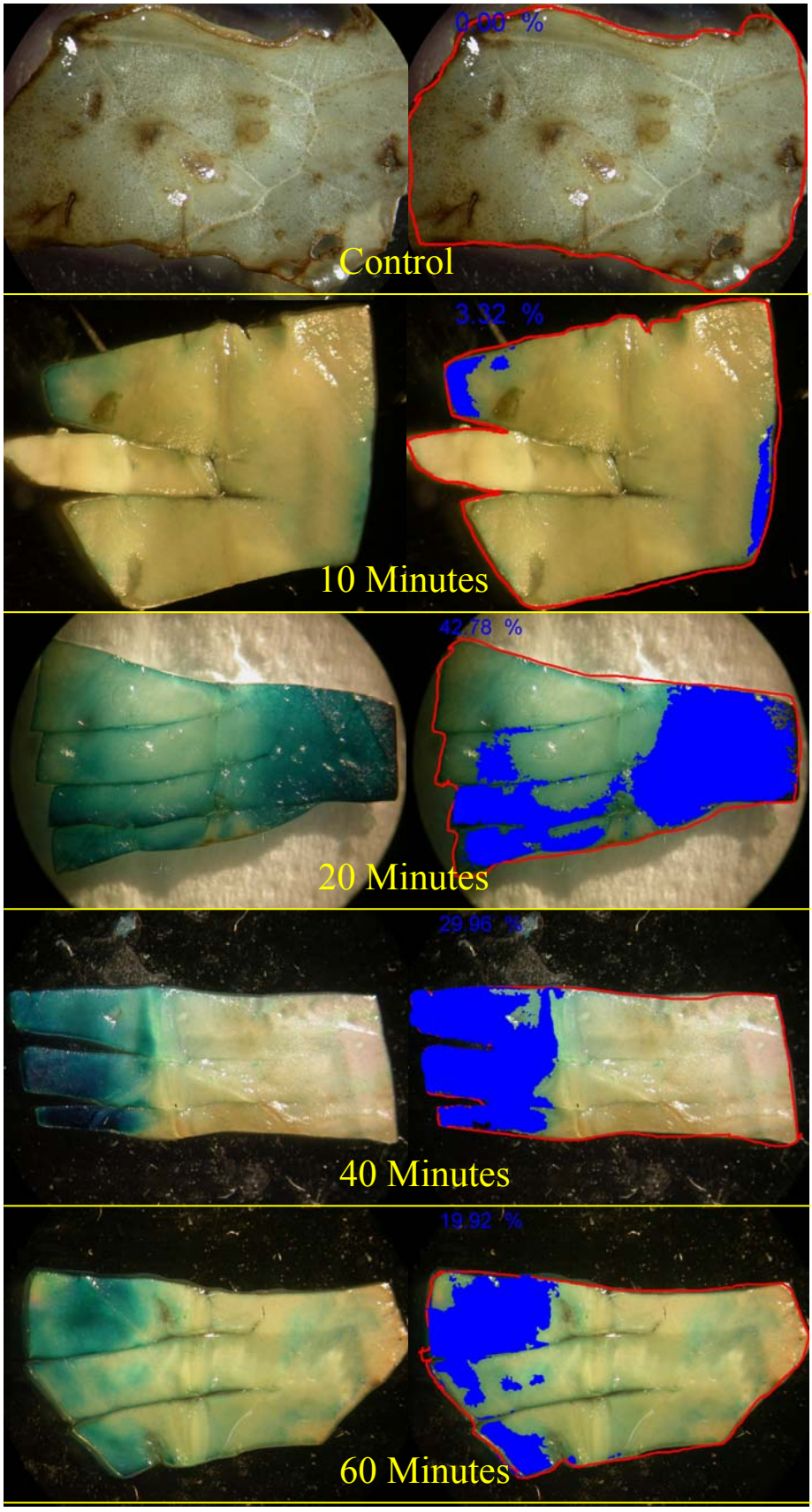


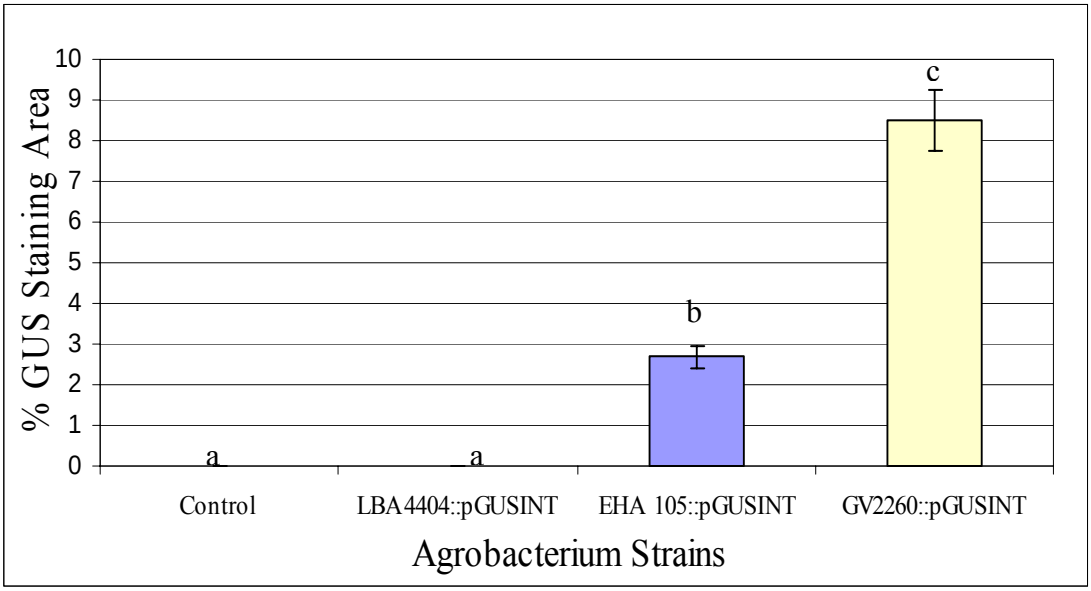
Figure 3.31. Representative photographs of leaves and their quantification for inoculation time.

3.2.4. Effect of Different Bacterial Strains

Evaluation of the influence of different *Agrobacterium* strains on transient GUS gene expression of leaf disks was the objective of this part. Throughout the study, efficiencies of three strains EHA105, GV2260 and LBA4404 were compared. Each strain carried the same binary plasmid, pGUSINT. Strains of *Agrobacterium* are defined by their chromosomal background. Chromosomal background of EHA 105 and GV2260 is C58. The C58 chromosomal background has proved to be popular for plant transformation especially for cereals. However choromosomal background of LBA4404 is TiAch5 (Hellens and Mullineaux 2000).

Leaf disks were inoculated with diferrent strains grown in MG/L medium. Infiltration was performed for 20 minutes, at evacuation pressure 400 mmHg. As a control group, leaf disks were directly placed on co-cultivation medium without bacterial treatment.

Effect of bacterial strains described as percent of explants showing transient GUS activity on the third day after transformation is given in Figure 3.32. Control explants did not exhibit GUS activity. Also leaf disks which were inoculated with LBA4404::pGUSINT showed no GUS activity due to necrosis of explants after the first day of transformation (Figure 3.33). When the transformation was performed using GV2260::pGUSINT, percentage of explants exhibiting GUS activity was 8.5 ± 0.7. This was the highest value in terms of percentage of GUS staining area per leaf compared to other applications and demonstrated a significant ($p<0.05$) enhancement in transformation efficiency when compared to other experimental sets.



Mean values, SEM and significant values are tabulated in Table F.13 in Appendix F.

Figure 3.32. Effect of *Agrobacterium* strains on transient gene expression 3 days after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different (p> 0.05).

Figure 3.33. Necrose formation after using *Agrobacterium* strain LBA4404.

From the comparison of efficiency of strains in Figure 3.34, it was clearly seen that the highest percentage of blue stained GUS expression area was observed upon inoculation of explants with GV2260. Effect of different *Agrobacterium* strains on gene transfer efficiency to lentil was previously reported by Çelikkol (2002). Researcher investigated the effect of different *Agrobacterium* strains and binary plasmids on transient GUS gene expression of lentil explants. LBA4404, EHA105, C58C1, KYRT1 and two binary plasmids, pGUSINT and pTJK136 were used for transformation of lentil. Our findings are consistent with this study, in which low gene expression frequency was observed by using EHA 105. Also, no GUS gene expression was observed when LBA4404 was employed for transformation. Also Krishnamurty *et al*., (2000) carried out *Agrobacterium* mediated transformation of chickpea using *Agrobacterium* strains C58C1, GV2260 and EHA 101.

In conclusion, it appears that the use of the *Agrobacterium* strain GV2260 has a significant influence on transformation efficiency. Therefore, other two strains carrying pGUSINT plasmid were excluded from further use in transformation experiments.

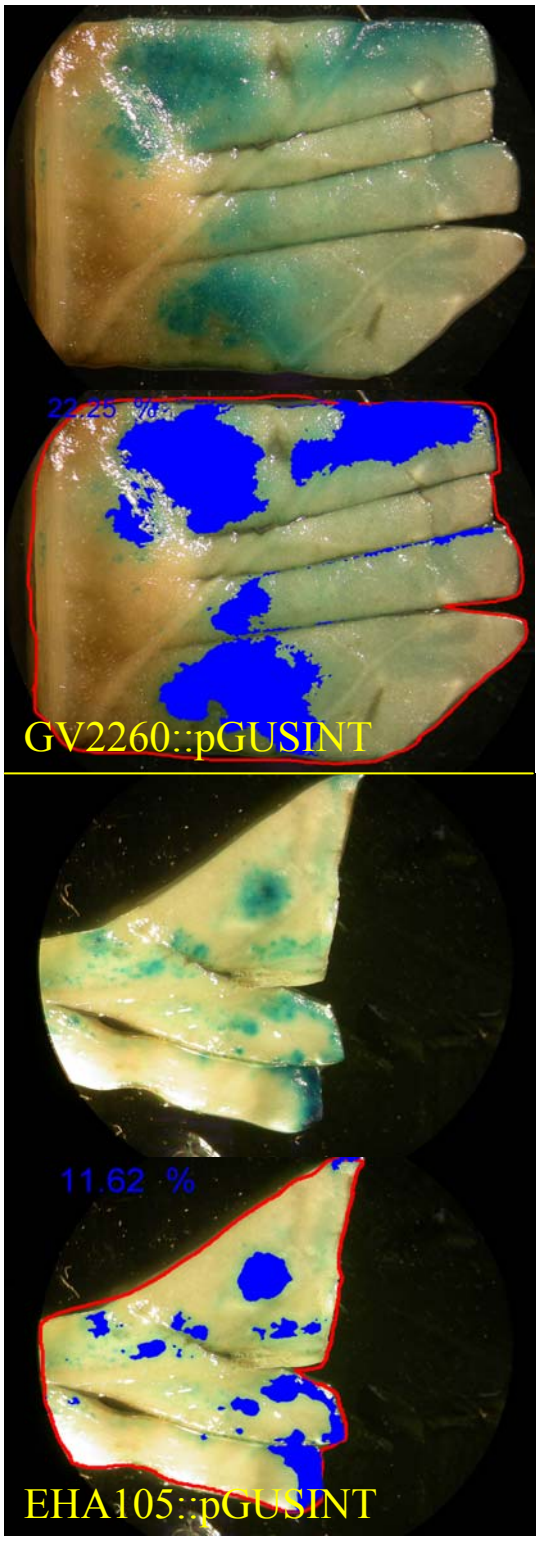


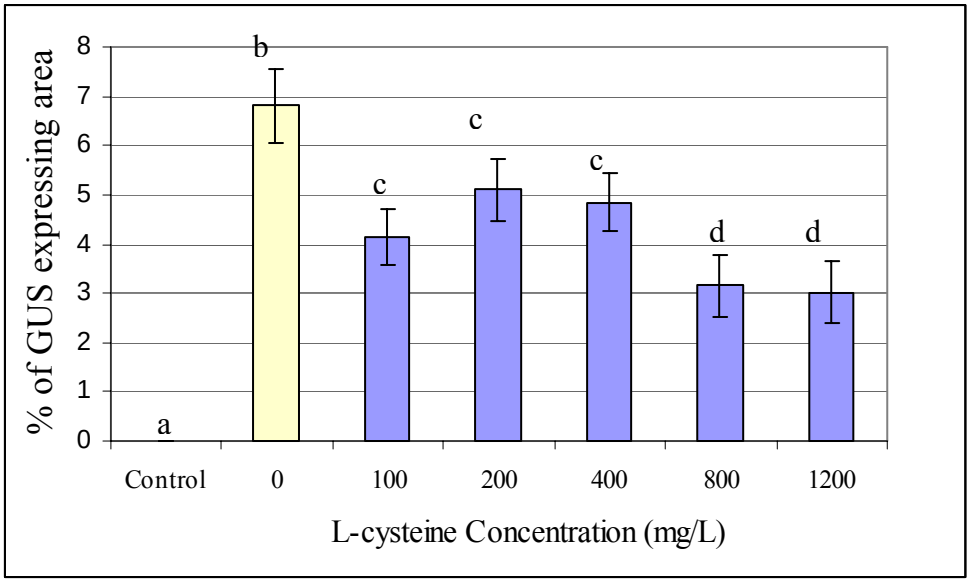
Figure 3.34. Representative photographs of leaves and their qauntification for *Agrobacterium* strains.

3.2.5. Effect of L-Cysteine Application

In *Agrobacterium* mediated transformation studies of rice and soybean, browning and necrosis of the plant tissues were observed due to response to wounding. The use of the antinecrotic compounds also resulted in increase in transformation efficiency (Enriquez-Obregon *et al*., 1999; Olhoft and Somers, 2001). In our study, we also used L-cysteine for enhancement of transformation efficiency of leaf disks.

The solid co-cultivation medium supplemented with different concentrations of L-cysteine (100, 200, 400, 800 and 1200 mg/L) were prepared to examine their effects on *uidA* (GUS) gene expression. Non-inoculated leaf disks were used as control and L-cysteine lacking co-cultivation media (0 mg/L) were used as control for L-cysteine effect. Transformation was performed using *Agrobacterium* strain GV2260 for 20 minutes at 400 mmHg evacuation pressure.

Results of GUS histochemical staining recorded as percent GUS gene expression area are indicated in Figure 3.35. The use of the L-cysteine lacking co-cultivation medium (0 mg/L) significantly rised percentage of GUS staining area on leaf disks compared to medium containing different concentrations of L-cysteine. 6.8 ± 0.7 percent of explant exhibited GUS activity when explants were cultured on L-cysteine free media. On the other hand, all concentrations of L-cysteine application reduced the percentage of GUS expressing area, which ranged from 3.0 to 5.1 % in the presence of L-cysteine. Moreover, a decline in GUS staining area was observed correspondingly when concentration of L-cysteine was increased after the 200 mg/L L-cysteine application.



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

Figure 3.35. Effect of different concentrations of L-cysteine application on transient gene expression on the 3rd day after transformation. Vertical bars indicate SEM. The values marked with same letter are not significantly different ($p < 0.05$).

Figure 3.36 and Figure 3.37 showed effect of various concentrations of L-cysteine application. Effect of various concentrations of L-cysteine on chickpea cotyledonary node was previously reported by Öz (2005). Our findings correlate with this study in which L-cysteine application did not cause any increase in GUS expression area of chickpea cothledonary node.

Although L-cysteine is generally used for both improvement of transformation efficiency and antinecrotic treatments, in our study L-cysteine usage did not cause any change in terms of GUS activity.

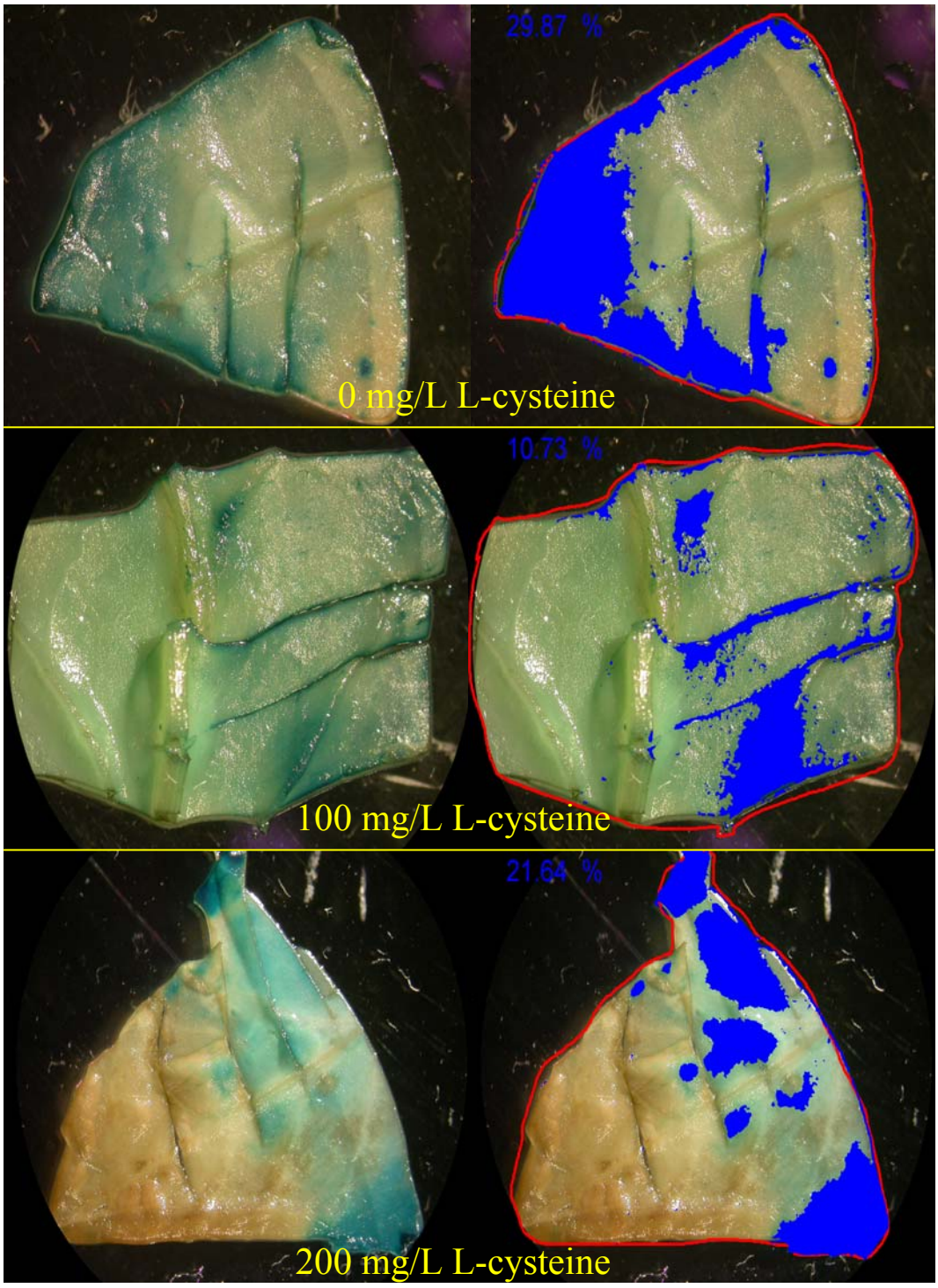


Figure 3.36. Representative photographs of leaves and their qauntification at 0, 100 and 200 mg/L L-cysteine application.

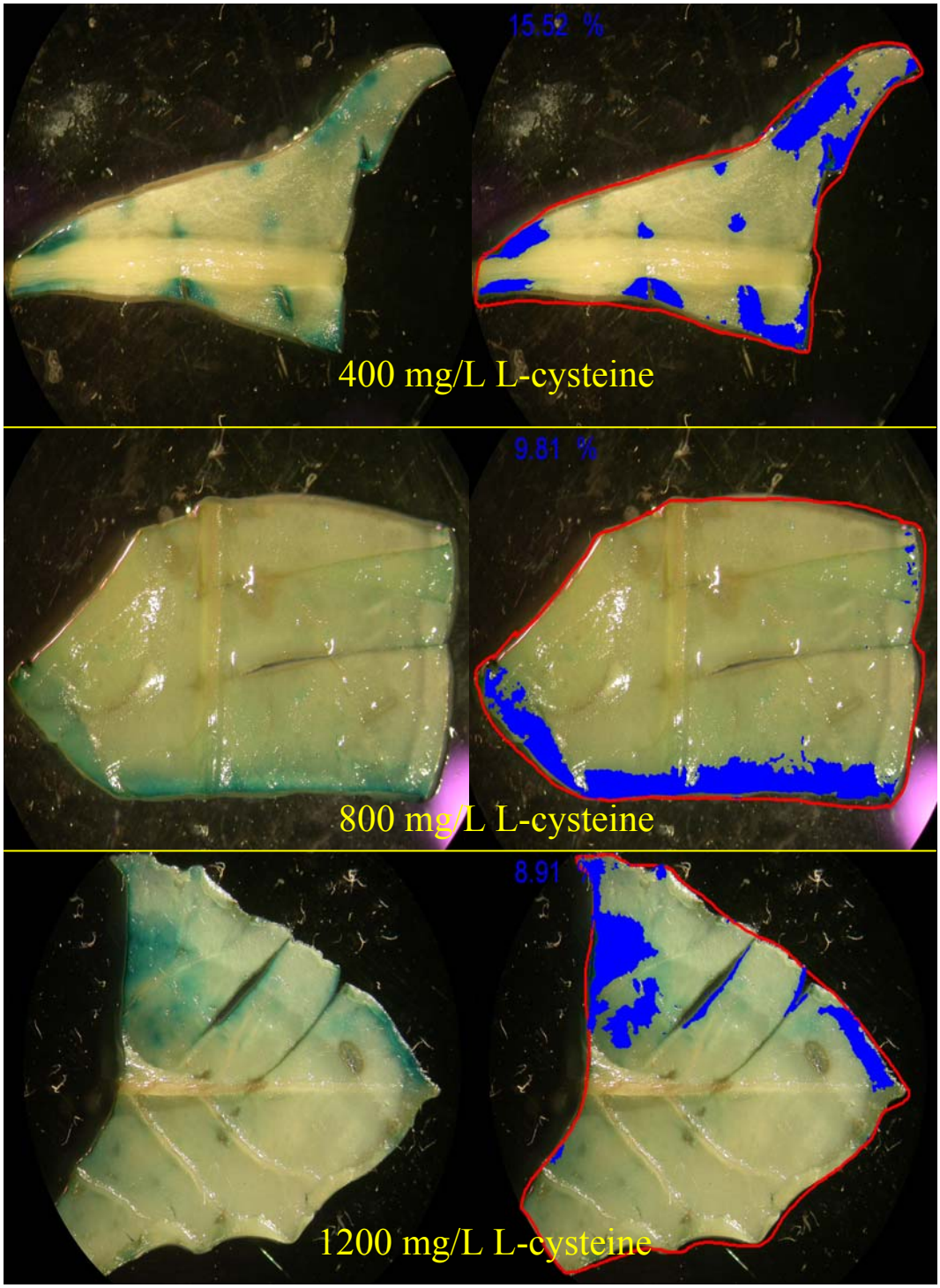


Figure 3.37. Representative photographs of leaves and their qauntification at 400, 800 and 1200 mg/L L-cysteine application.

As a result of all these findings, it can be stated that different parameters including vacuum infiltration, bacteria growth media, inoculation time with bacteria, *Agrobacterium* strains and L-cysteine application were tried to increase transformation efficiency of sugar beet leaf disks. From the begining of the study, percentage of GUS expressing area increased to three folds. At the beginning of the study 2.8 % of GUS activity was obtained. However, at the end of the study, approximately 8.5 % of GUS activity was achieved. Using different *Agrobacterium* strains provided significant increase for transient GUS expression levels. Except for L-cysteine application, other procedures also increased GUS activity.

3.2.6. Transformation Studies on Leaf Blades

Based on the report that shoot bases are amenable to both *Agrobacterium*-mediated transformation and regeneration (Lindsey and Gallois 1989), we attempted to optimize a sugarbeet transformation system using these tissues. We prepared shoot-base tissue using a simple method suitable for the production of a large number of transformed plants.

Transformation was carried out using *Agrobacterium* strain EHA105 harboring the plasmid pGUSINT, which contains a kanamycin resistance gene and a GUS reporter gene. As a plant material, leaf blades of sugar beet cultivar of 1195 were used. To determine the amount of kanamycin suitable for selection of transformed cells 50, 100, 150, 200 and 250 mg/l kanamycin was added to the shoot formation medium, and regeneration from nontransformed explants was tested prior to the screening of transformed cells. Figure 3.38 shows the effect of different concentrations of kanamycin on shooot regeneration from leaf blades. Leaf blades produced shoots in medium containing 50 and 100 mg/L kanamycin whereas no shoot development was observed when the explants were cultured on medium containing 150, 200 and 250 mg/L kanamycin. Shoots were developed in kanamycin free media as a control.

Using 150 mg/L kanamycin selection condition, we analyzed the formation of shoots after infection of shoot bases with *Agrobacterium* containing a vector with the kanamycin resistance gene in the T-DNA region. Shoots were obtained under this selection condition (Figure 3.39). Then shoots were transferred into growth and root induction medium, respectively. GUS histochemical assay was performed to indicate the presence of the inserted gene on leaves of transformed plants. Although after transformation all of the regenerated shoots were able to survive on medium containing 150 mg/L kanamycin, no GUS activity was observed from these kanamycin resistant shoots.

Figure 3.38. Effect of different kanamycin concentrations (50, 100, 150, 200 and 250 mg/L kanamycin) on shoot regeneration from leaf blades. The explants were photographed after 4 weeks of culture. Arrows indicate newly established shoots.

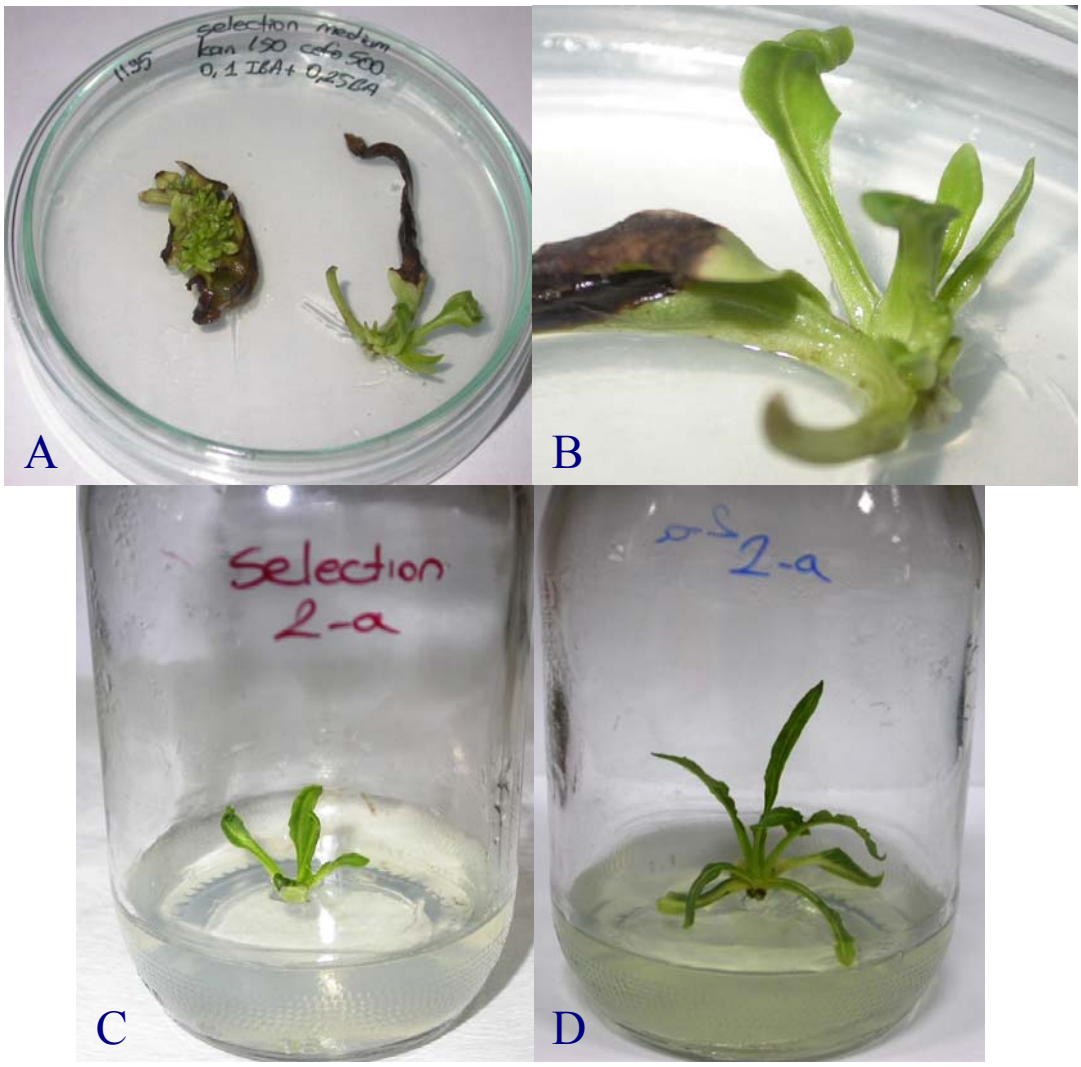


Figure 3.39. Response of transformed explants in selective medium. (A-B) indicate shoot formation after 2 weeks of transformation. (C-D) shoot development in the jars after 4 weeks.

The reason of this unsuccessful transformation may be that bacteria infection was not achieved. This means that bacteria were not able to penetrate into plant cells. Therefore, no GUS activity was obtained from the leaves of these plants. Moreover another explanation might be the low selective agent concentration (150 mg/L kanamycin) that was used in these experiments.

The first transformation study indicates that the use of 150 mg/L kanamycin was not effective for selection of transformed cells. So, subsequent transformation studies were carried out using 200 mg/L kanamycin selection, together with vacuum infiltration for 20 minutes, at evacuation pressure of 400 mmHg.

Two independent experiments were designed to examine both effect of vacuum infiltration and kanamycin selection on transformation efficiency. In the first experiment, leaf blades were transformed with bacteria for 20 minutes and then cultured on co-cultivation medium supplemented with acetosyringone. Three days after transformation, the explants were cultured on selection medium containing 200 mg/L kanamycin (Figure 3.40).

Although transformed plantlets, which were vacuum infiltrated and non-infiltrated, were able to survive on medium containing 200 mg/L kanamycin, no GUS activity were observed from these kanamycin resistant plantlets (Figure 3.41). Transformation of leaf blades does not involve a detectable callus phase prior to regeneration, suggesting that the possibility of somaclonal variation is minimized. In the light of these facts, it is claimed that this procedure has a potential to produce uniform transgenic plants at a high frequency. Therefore, further experiments are required to optimize transformation in leaf blade explants.

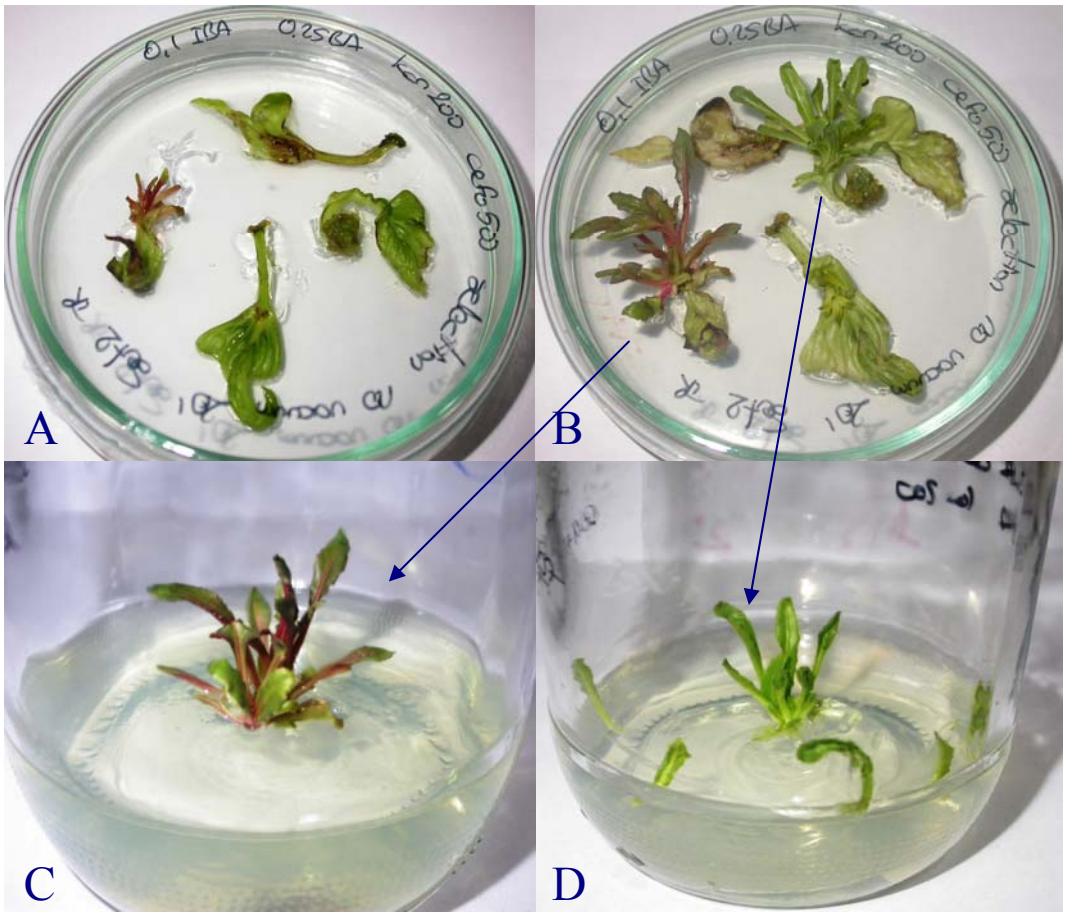


Figure 3.40. Transformed plants without vacuum infiltration. (A) shows the shoot formation after 1 week transformation. (B) Development of shoots. (C-D) indicates two different transformed plants.

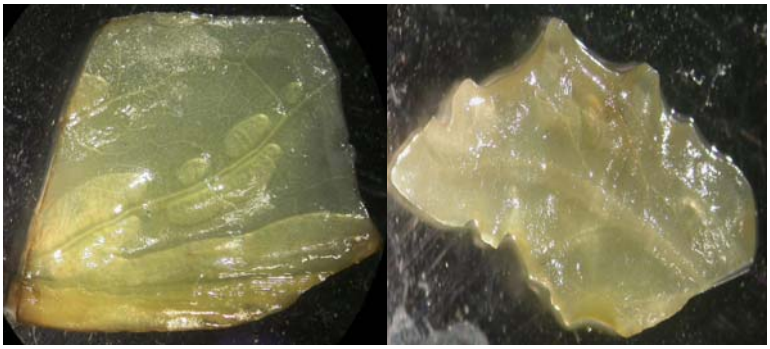


Figure 3.41. GUS photos of leaf of (A): vacuum infiltrated (B): non-infiltrated

CHAPTER IV

CONCLUSION

In this study, regeneration and *Agrobacterium* mediated transformation of Turkish sugar beet cultivars ELK 345 and 1195 were intended to be optimized. In the regeneration part of the study, although all explant types including hypocotyl, cotyledon, petiole and leaf produced callus, no shoot regeneration was obtained from these explants due to compact callus formation. On the other hand, multiple shoot induction was achieved via direct organogenesis using 0.1 mg/L IBA and 0.25 mg/L BA. 22.4 % of explants produced shoots. Rooting of plantlets was also successful. 66 % of regenerated shoots developed root on medium containing 1.0 mg/L IBA. High acclimatization rate (97 %) was accomplished from shoot base explants.

In the transformation part of the study, effect of five parameters were investigated on transient GUS expression of leaf explants of sugar beet cultivar ELK 345. Transient *uidA* expression was monitored 3 days after transformation. Percentage of GUS activity was calculated using image analysis system (Zeiss® KS300).

One of the parameters tested for improvement of procedure was vacuum infiltration. Vacuum infiltration increased transformation efficiency. 400 mmHg was found as optimum evacuation pressure for leaves. High evacuation pressure (600 mmHg) decreased GUS activity.

The use of the different bacteria growth medium was another parameter to enhance GUS activity. According to GUS histochemical assays it was concluded that utilization of MG/L media significantly increased transformation efficiency. YEB and YEB+MES medium did not cause any change in percentage of GUS expressing area.

Different inoculation period with bacteria was also tested. Although GUS activity was proportionally increased when the inoculation time was extended, long inoculation period with bacteria (40 and 60 minutes) may influence the regeneration of sugar beet. Infection of leaves for 20 minutes was appropriate to improve gene transfer without affecting shoot regeneration.

Effect of strain difference on transformation efficiency was very prominent. Application of different *Agrobacterium* strains gave the best result in terms of overall GUS activity when compared to other parameters. *Agrobacterium* strain GV2260::pGUSINT significantly enhanced GUS activity upto 8.5 %. EHA105::pGUSINT did not bring about any change in percentage of GUS expressing area. Also LBA4404::pGUSINT damaged transformed leaves, so no GUS activity was observed.

Utilization of L-cysteine in co-cultivation medium did not improve transient GUS expression. The usage of higher concentration of L-cysteine (800 and 1200 mg/L) reduce the transient GUS expression.

Preliminary studies on transformation of leaf blade explants holds great promise for transformation experiments. However, further experiments are necessary to optimize transformation in leaf blade explants.

Vacuum infiltration and *Agrobacterium* strain were significantly improved transformation procedure, which is highly promising for obtaining transgenic sugar beet plants. According to cumulative results of transformation studies, percentage of GUS expressing areas on leaves were raised three folds from the beginning of the study.

Regeneration and transformation of locally cultivated Turkish sugar beet cv. ELK 345 and 1195 were attempted to optimize. These features presumably contribute to the production of transgenic sugar beet plants which can carry fungal and nematode resistance or abiotic stress tolerance genes.

REFERENCES

American Sugar Beet Growers Association 1998, last updated February 1998, http://members.aol.com/asga/sugarbeet.htm.

Atanassova A.I., 1986 Sugarbeet. In: Evans D.A., Sharp W.R., Ammirato P.V., (eds) "Handbook of plant cell culture". Volume 4, pp 652–680. MacMillan New York, USA:

Babaoglu M., Yorgancilar M., 2000 "TDZ-specific plant regeneration in salad burnet." Plant Cell Tissue and Organ Culture, 63: 31-34.

Bechtold N., Ellis J., Pelletier G., 1993 "In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants." C. R. Acad. Sci. Paris, Life Sci., 316: 1194–1199.

Bhat S.R., Ford-Lyod B.V., Callow J.A., 1985 "Isolation of protoplasts and regeneration of callus from suspension cultures of cultivared beets." Plant Cell Rep., 4: 348-350.

Bichsel S.E., 1987 "An overview of the U.S. sugar beet industry". Proceedings of the Symposium on the chemistry and processing of sugar beet. Denver, Colorado. 108 pp.

Butenko R.G., Atanassov A.I., Urmantseva V.V., 1972 "Some feature of sugarbeet tissue cultures." Phytomorphology, 22: 140–143.

Canova A., 1966 “Si studia la rizomanie della bietola. Informatore Fitopatologico,” Fitopatologico 16: 235-239

Catlin D.W., 1990 “The effect of antibiotics on the inhibition of callus induction and plant regeneration from cotyledons of sugar beet (*Beta vulgaris* L.).” Plant Cell Rep., 9: 285-288.

Cattanach A.W., Dexter A.G., Oplinger E.S., 1991 “Sugarbeets Alternative Fields Crop Manual”. University of Wisconsin Cooperative Extension Service, the University of Minnesota Extension Service and the Center for Alternative Plant and Animal Products.

Cheng M., Fry J.E., Pang S., Zhou H., Hironaka C.M., Duncan D.R., Conner T.W., Wan Y., 1997 “Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*.” Plant Physiology, 115: 971-980.

Cooke D.A., 1993 “Pests”. In: Cooke DA and Scott RK (eds), The Sugar Beet Crop, pp.428-483, Chapman and Hall.

Cooke D.A., Scott R.K., 1993 “The Sugar Beet Crop.” Chapman and Hall, Publishers. 675 pp.

Coumans M., Coumans-Gilles M.F., Menard D., Kevers C., Ceulemans E., 1982 “Micropropagation of sugar beet: Possible ways.” Proc. 5th Intl. Cong. Plant Tissue and Cell Culture, Tokyo.

Crompton M.E., Koch J.M., 2001 “Influence of plant preservative mixture (PPM^{TM}) on adventitious organogenesis in melon, petunia and tobacco.” In Vitro Cellular and Developmental Biology-Plant, 37: 259-261.

Çelikkol U., 2002 “Optimization of an integrated bombardment and *Agrobacterium* infiltration based transformation system for lentil.”MSc. Thesis submitted to Graduate School of Natural and Applied Sciences of Middle East Technical University, Ankara.

D’Halluin K., Bossut M., Bonne E., Mazur B., Leemans J., Botterman J., 1992 “Transformation of sugar beet (*Beta vulgaris L*) and evaluation of herbicide resistance in transgenic plants .” Bio/Technology., 10: 309-314.

De Greef W., Jacobs M., 1979 “*In vitro* culture of sugar beet: Description of a cell line with high regeneration capacity.” Plant Sci. Lett., 17: 55-61.

Doley W.P., Saunders J.W., 1989 “Hormone-free medium will support callus production and subsequent shoot regeneration from whole plant leaf explants in some sugarbeet (*Beta vulgaris* L.) populations.” Plant Cell Rep., 8: 222-225.

Dovzhenko A., Koop H.U., 2003 “Sugarbeet (*Beta vulgaris* L.): shoot regeneration from callus and callus protoplasts.” Planta, 217: 374–381.

Duffus J.E., Ruppel E.G., 1993 “Diseases” . In: Cooke DA and Scott RK (eds), The Sugar Beet Crop, pp.347-427, Chapman and Hall.

Duke J.A., Atchley A.A., 1984 Proximate analysis. In: Christie, B.R. (ed.), “The handbook of plant science in agriculture”. CRC Press, Inc., Boca Raton, FL.

Elliot M.C., Wetson G.D., 1993 “Biology and Physiology of the Sugar Beet Plant”. In: Cooke DA and Scott RK (eds), The Sugar Beet Crop, pp.37-66, Chapman and Hall.

Enriquez-Obregon G.A., Prieto-Samsonov D.L., de la Riva G.A., Perez M., Selman-Housein G., Vazquez-Padron R.I., 1999 "*Agrobacterium* mediated Japonica rice transformation: a procedure assisted by an antinecrotic treatment." Plant Cell Tissue and Organ Culture, 59: 159-168.

FAOSTAT Food and Agriculture Organization of the United Nations (FAO) Statistical Databases 2005, last updated 24 June 2005, www.fao.org.

Freytag A.H., Anand S.C., Rao-Arelli A.P., Owens L.D., 1988 "An improved medium for adventitious shoot formation and callus induction in *Beta vulgaris in vitro* varieties." Plant Cell Rep., 7: 30–34.

Fry J.E., Horsch R.B., Hofmann N.L., Eichholtz D., Rogers S.G., Fraley R.T., 1989 "Gordon Research Conference on Plant Cell and Tissue Culture," June 11-16, 1989 Poster.

Gatehouse, A.M.R., Hilder, V.A., Boulter, D., 1992, "Plant genetic Manipulation of Crop Plants," CAB International, Wallinford, pp. 7-37.

George E.F., 1993 "Plant Propogation by Tissue Culture Part I. The Technology" Second Edition, pp. 574, Exegetics Ltd., England.

George M.W., Tripepi R.R., 2001 "Plant Preservative Mixture^{TM} can affect shoot regeneration from leaf explants of chrysanthemum, European birch and rhododendron." Hortscience, 36: 768-769.

Grieve T.M., Gartland K.M.A., Elliott M.C., 1997 " Micropropagation of commercially important sugar beet cultivars." Plant Growth Regulation, 21**:** 15–18.

Guo G.Q., Maiwald F., Lorenzen P., Steinbiss H.H., 1998 “Factors influencing T-DNA transfer into wheat and barley cells by *Agrobacterium tumefaciens* ,” Cereal Res. Commun., 26: 15-22.

Gürel E., 1997 “Callus and root development from leaf explants of sugar beet (*Beta vulgaris* L): Variability between cultivars, plants and organs.” Turkish J Bot., 21: 131-136.

Gürel E., Gürel S., 1998 “Plant regeneration from unfertilized ovaries of sugar beet (*Beta vulgaris* L.) cultured *in vitro*.” Tr. J. of Botany., 22: 233-238.

Gürel S., 2000 “*In vitro* growth and development of sugar beet (*Beta vulgaris* L.) from callus, ovule, suspension cells and protoplasts.” PhD. Thesis submitted to Graduate School of Natural and Applied Sciences of Middle East Technical University, Ankara

Gürel S., Gürel E., Kaya Z., 2000 “Doubled haploid plant production from unpollinated ovules of sugar beet (*Beta vulgaris* L.).” Plant Cell Reports, 19: 1155-1159.

Gürel S., Gürel E., Kaya Z., 2002 “Establishment of cell suspension cultures and plant regeneration in sugar beet (*Beta vulgaris* L.).” Tr. J. of Botany., 26: 197-205.

Gürel S., Topal E., Gürel E., 2003 “The effect of pretreating seedlings with TDZ on direct shoot regeneration from petiole explants of sugar beet (*Beta vulgaris* L.).” Asia Pacific J. of Molecular Biology and Biotechnology., 11 (1): 57-62.

Gürel S., Topal E., Gürel E., 2003 “The effect of pretreating seedlings with BAP on direct shoot regeneration from petiole explants of sugar beet (*Beta vulgaris* L.).” Biotechnol.& Biotechnol. Eq., 17: 89-96.

Hall R.D., Riksen-Bruinsma T., Weyens G., Lefebvre M., Dunwell J.M., van Tunnen A., Krens F.A., 1997 "Sugar beet guard cell protoplasts demonstrate a remarkable capacity for cell division enabling applications in stomatal physiology and molecular breeding." J Exp Bot., 48: 255–263.

Hall R.D., Riksen-Bruinsma T., Weyens G.J., Rosquin I.J., Denys P.N., Evans I.J., Lathouwers J.E., Lefebvre M.P., Dunwell J.M., van Tunen A., Krens F.A., 1996 "A high efficiency technique for the generation of transgenic sugar beets from stomatal guard cells." Nat Biotechnol., 14: 1133–1138.

Hellens R., Mullineaux P., 2000 "A guide to Agrobacterium binary Ti vectors." Trends in Plant Science, 5: 446-451.

Hisano H., Kimoto Y., Hayakawa H., Takeichi J., Domae T., Hashimoto R., Abe J., Asano S., Kanazawa A., Shimamoto Y., 2004 "High frequency *Agrobacterium*-mediated transformation and plant regeneration via direct shoot formation from leaf explants in *Beta vulgaris* and *Beta maritima*." Plant Cell Rep., 22: 910–918.

Hooker M.P., Nabors M.W., 1977 "Callus initiation, growth and organogenesis in sugar beet (*Beta vulgaris* L.)." Z. Pflanzenphysiol., 84: 237-246.

Horsch R.B., Fry J., Hoffmann N., Eichholtz D., Rogers S., Fraley R.T., 1985 "A simple and general method for transferring genes into plants." Science, 227: 1229-1231.

Jacq B., Lesobre O., Sangwan R.S., Sangwan-Norreel B.S., 1993 "Factors influencing T-DNA transfer in *Agrobacterium*-mediated transformation of sugar beet." Plant Cell Rep., 12: 621-624.

Jacq B., Tetu T., Sangwan R.S., de Laat A., Sangwan- Norreel B.S., 1992 "Plant regeneration from sugar beet (*Beta vulgaris* L.) hypocotyls cultured *in vitro* and flow cytometric nuclear DNA analysis of regenerants." Plant Cell Rep., 11: 329–333.

Jefferson R.A., 1987 "Assaying chimeric genes in plants: The GUS gene fusion system." Plant Molecular Biological Reports, 5: 387-405.

Jefferson R.A., Kavanagh T.A., Bevan M.W., 1987 "GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants." EMBO., 6: 3901-3907.

Joersbo M., Brunstedt J., 1990 "Direct gene transfer to plant protoplasts by mild sonication." Plant Cell Rep. 9: 207-210.

Joersbo M., Donaldson I., Kreiberg J., Petersen S.G., Brunstedt J., Okkels F.T., 1998 "Analysis of mannose selection used for transformation of sugar beet." Molecular Breeding, 4**:** 111–117.

Keimer B., 1985 "*In vitro* vegetative multiplication of sugar beet (*Beta vulgaris* L.)." Z. Naturforsch., 28: 737-741.

Kifle S., Shao M., Jung C., Cai D., 1999 "An improved transformation protocol for studying gene expression in hairy roots of sugar beet (*Beta vulgaris* L.)." Plant Cell Reports, 18: 514–519.

Kikkert J.R., 1993 "The biolistic PDS-1000/He device." Plant Cell Tissue Organ Culture, 33: 221-226.

Klein T.M., Wolf E.D., Wu R., Sanford J.C., 1987 "High-velocity microprojectiles for delivering nucleic acids into living cells." Nature, 327: 70-72.

Konwar B.K., 1994 "*Agrobacterium tumefaciens*-mediated genetic transformation of sugar beet (*Beta vulgaris L.*)." J Plant Biochem Biotech., 3: 37–41.

Konwar B.K., Coutts R.H.R., 1990 "Rapid regeneration of sugar beet (*Beta vulgaris* L.) plants from *in vitro* cultures." In: Nijkamp HJI, Van Der Plas LHW and Van Aartrijk J (eds) Progress in Plant Cellular and Molecular Biology, pp. 114-118, Kluwer Academic Publishers, The Netherlands.

Krens F.A., Jamar D., 1989 "The role of explant source and culture conditions on callus induction and shoot regeneration in sugar beet (*Beta vulgaris* L.)." J. Plant Physiol., 13(6): 651-655.

Krens F.A., Jamar D., Rouwendal G.J.A., Hall R.D., 1990 "Transfer of cytoplasm from new *Beta* CMS sources to sugar beet by asymmetric fusion. I. Shoot regeneration from mesophyll protoplasts and characterization of regenerated plants." Theor. Appl. Genet., 79: 390-396.

Krens F.A., Trifonova A., Keizer L.C.P., Hall R.D., 1996 "The effect of exogenously-applied phytohormones on gene transfer efficiency in sugarbeet (*Beta vulgaris* L.)." Plant Sci., 116: 97–106.

Krishnamurthy K.V., Suhasini K., Sagare A.P., Meixner M., de Kathen A., Pickardt T., Schieder O., 2000 "Agrobacterium mediated transformation of chickpea (*Cicer arietinum* L.) embryo axes." Plant Cell Reports, 19: 235-240.

Kulshreshtha S., Coutts R.H.A., 1997 "Direct somatic embryogenesis and plant regeneration from mature sugar beet (*Beta vulgaris* L.) zygotic cotyledons." Plant Growth Regulation, 22: 87–92.

Lenzler S., Zoglauer K., Schieder O., 1995 "Plant regeneration from protoplasts of sugar beet (*Beta vulgaris*)." Physiol. Planta., 94: 342-350.

Lindsey K., Gallois P., 1989 “Transformation of sugar beet (*Beta vulgaris*) by *Agrobacterium tumefaciens*.” J. Exp. Bot., 226: 529-536.

Lindsey K., Jones M.G.K., 1987 “Transient gene expression in electroporated protoplast and intact cells of sugar beet.” Plant Molecular Biology, 10: 43-52.

Mahmoudian M., Yücel M., Öktem H.A., 2002 “Transformation of lentil (Lens culinaris M.) cotyledonary nodes via vacuum infiltration of *Agrobacterium tumefaciens*.” Plant Molecular Biology Reporter, 20: 251-257.

Mahn A., Matzk A., Sautter C., Schiemann J., 1995 “Transient gene expression in shoot apical meristems of sugar beet seedlings after particle bombardment.” J. Exp. Bot., 46: 1625-1628.

Mannerlof M., Tuvesson S., Steen P., Tenning P., 1997 “Transgenic sugar beet tolerant to glyphosate.” Euphytica, 94: 83–91.

Margara J., 1970 “Neoformation de bourgeons *in vitro* chez la Betterave sucriere (*Beta vulgaris* L.).” C.R. Acad. Sci., D270: 689-701.

Menzel G., Harloff H.J., Jung C., 2003 “ Expression of bacterial poly (3-hydroxybutyrate) synthesis genes in hairy roots of sugar beet *(Beta vulgaris* L.).” Appl Microbiol Biotechnol., 60: 571–576.

Miedema P., Groot P.J., Zuidgeest J.H.M.,1980 “Vegetative propagation of *Beta vulgaris* L. by leaf cuttings.” Euphytica, 29: 425-432.

Mikami T., Sudoh R., Nagao and Kinoshita T., 1989 “Genotypic variation in the *in vitro* morphogenesis from leaf explants of *Beta vulgaris* L. and *Beta maritima* L.” Euphytica, 40: 271-273.

Miki B., McHugh S., 2004 “Selectable marker genes in transgenic plants: applications, alternatives and biosafety.” Journal of Biotechnology, 107: 193-232.

Moghaddam B.E., Mesbah M., Yavari N., 2000 “The effect of *in planta* TIBA and proline treatment on somatic embryogenesis of sugar beet (*Beta vulgaris* L.)” Euphytica, 112**:** 151–156.

Munthali M.T., Newbury H.J., Ford-Lloyd B.V., 1996 “The detection of somoclonal variants of beet using RAPD.” Plant Cell Rep., 15: 474-478.

Murashige T., Skoog F., 1962 “A revised medium for rapid growth and bio assays with tobacco tissue cultures.” Physiologia Plantarum, 15: 473-497.

Nakashima H., Tabayashi N., Abe J., Shimamoto Y., 1988 “Tissue culture of sugar beet: Effect of genotypes on callus induction from seeds, callus proliferation and regeneration from callus.” Proc. Japan. Soc. Sugar Beet Technol., 30: 113-117.

Olhoft P.M., Lin K., Galbraith J., Nielsen N.C., Somers D.A., 2001 “The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary node cells.” Plant Cell Rep., 20: 731-737.

Olhoft P.M., Somers D.A., 2001 “L-Cysteine increase *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells.” Plant Cell Rep., 20: 706-711.

Owens L.D., Eberts D.R.,1992 “Sugar beet leaf disc culture: an improved procedure for inducing morphogenesis.” Plant Cell Tissue and Organ Culture, 31: 195-201.

Önde S., Birsin M., Yıldız M., Sancak C., Özgen M., 2000 " Transfer of a β-Glucuronidase Reporter Gene to Sugarbeet (*Beta vulgaris* L.) Via Microprojectile Bombardment." Turk. J. Agric. For., 24 : 487–490.

Öz M., 2005 "Optimization of selection conditions and *Agrobacterium* mediated transformation of chickpea (*Cicer arietinum* L.)." MSc. Thesis submitted to Graduate School of Natural and Applied Sciences of Middle East Technical University, Ankara.

Pedersen C., Hall R.D., Krens F.A., 1993 "Petioles as the tissue source for isolation and culture of *Beta vulgaris* and *Beta martima* protoplasts." Plant Sci., 95: 89-97.

Rady M.R., 1997 " *In vitro* multiplication of *Beta vulgaris* L. throughout excised shoot tips." Biologia Plantarum, 40 (4): 515-522.

Ritchie G.A., Short K.C., Davey M.R., 1989 "*In vitro* shoot regeneration from callus, leaf axils and petioles of sugar beet (*Beta vulgaris* L.)." J. Exp. Bot., 40 (211): 277-283.

Roussy I.F., Dubois R.S., Sangwan B.S., Sangwan-Norreel B.S., 1996 "*In planta* 2,3,5 triiodobenzoic acid treatment promotes high frequency and routine *in vitro* regeneration of sugar beet (*Beta vulgaris* L.). Plants." Plant Cell Rep, 16: 142–146.

Sanford J.C., 1990 "Biolistic plant transformation." *Physiologica Plantarum*, 79: 206-209.

Sangwan R.S., Joubert P., Beaupere D., Lelievre P., Wadouachi A., Sangwan Norrell B.S., 2002 “Effects of phenolic compounds on *Agrobacterium vir* genes and gene transfer induction- a plausible molecular mechanism of phenol binding protein activation.” Plant Science, 162: 733-743.

Saunders J.W., Daub M.E., 1984 “Shoot regeneration from hormone-autonomous callus from shoot cultures of several sugar beet (*Beta vulgaris* L.) genotypes.” Plant Sci.Lett., 34: 219-223.

Saunders J.W., Doley W.P., 1986 “One step shoot regeneration from callus of whole plant leaf explants of sugar beet lines and somoclonal variant for *in vitro* behaviour.” J. Plant Physiol., 124: 473-479.

Saunders J.W., Tasai C.J., 1999 “Production of somatic embryos and shoots from sugar beet callus: effects of abscisic acid, other growth regulators, nitrogen source, sucrose concentration and genotype.” In Vitro Cellular and Developmental Biology-Plant, 35: 18-24.

Sevenier R., Hall R.D., van der Meer I.M., Hakkert H.J.C., van Tunen A.J., Koops A.J., 1998 “High level fructan accumulation in a transgenic sugar beet.” Nat Biotechnol., 16: 843–846.

Snyder G.W., Ingersoll J.C., Smigocki A.C., Owens L.D., 1999 “Introduction of pathogen defence genes and a cytokinin biosynthesis gene into sugarbeet (Beta vulgaris L.) by *Agrobacterium* or particle bombardment.” Plant Cell Rep., 18: 829–834.

Tamada T., 1975 “Beet Necrotic Yellow Vein Virus.” CMI/ AAB Desriptions of Plant Viruses,144, 4 pp.

Tansı M.S., 2002 “Genetic transformation of potato with P5CS gene and evaluation of salt resistance in transgenic plants.” MSc. Thesis submitted to Graduate School of Natural and Applied Sciences of Middle East Technical University, Ankara.

Tetu T., Sangwan R.S., Sangwan-Norreel B.S., 1987 “Hormonal control of organogenesis and somatic embryogenesis in *Beta vulgaris* L callus.” J. Exp. Bot., 38 (188): 506-517.

Tingay S., McElroy D., Kalla R., Fieg S., Wang M., Thornton S., Brettell R., 1997 “*Agrobacterium tumefaciens*-mediated barley transformation.” Plant J., 11: 1369–1376.

Toxopeus H., 1984 “De historie van de Europese kruisbloemige en bietegewassen”. Bedrijfsontwikkeling, 15: 537-542.

Watson L., Dallwitz M.J., 1992 “The Families of Flowering Plants: Descriptions, Illustrations, Identification, and Information Retrieval,” last updated 15 October 1998, http://biodiversity.uno.edu/delta/.

Wei L., Guangqin G., Guochang Z., 2000 “*Agrobacterium*-mediated transformation: state of the art and future prospect.” *Chinese Science Bulletin*, Vol. 45 No:17

Welander T., 1976 “Effects of nitrogen, sucrose, IAA and kinetin on explants of *Beta vulgaris* L grown *in vitro*.” Physiologia Planta., 36: 7-10.

Wu H., Sparks C., Amoah B., Jones H.D., 2003 “Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat.” Plant Cell Rep., 21: 659–668.

Zerback R., Dressler K., Hess D., 1989 “Flavonoid compounds from polen and stigma of *Petunia hybrida*: inducers of the vir region of the *Agrobacterium tumefaciens* Ti plasmid.” Plant Science, 62: 83-91.

Zhang C.I., Chen D.F., Elliott M.C., 2001 “Thidiazuron-induced organogenesis and somatic embryogenesis in sugar beet (*Beta vulgaris*).” In Vitro Cellular and Developmental Biology-Plant, 37: 305-310.

Zhong Z., Smith H.G., Thomas T.H., 1993 “*In vitro* culture of petioles and intact leaves of sugar beet (*Beta vulgaris* L.).” Plant Growth Regulation, 12: 59-66.

APPENDIX A

COMPOSITIONS OF MS BASAL MEDIUM

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

From DUCHEFA Plant Cell Cultures Catalogue	Murashige& Skoog
MICRO ELEMENTS	mg/L
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
KI	0.83
$MnSO_4.H_2O$	16.90
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
MACRO ELEMENTS	
$CaCl_2$	332.02
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NaH_2PO_4	----
$(NH_4)_2SO_4$	----
NH_4NO_3	1650.00
VITAMINS	
Glycine	2.00
myo-Inositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thiamine HCl	0.10

APPENDIX B

T-DNA REGION of pGUSINT

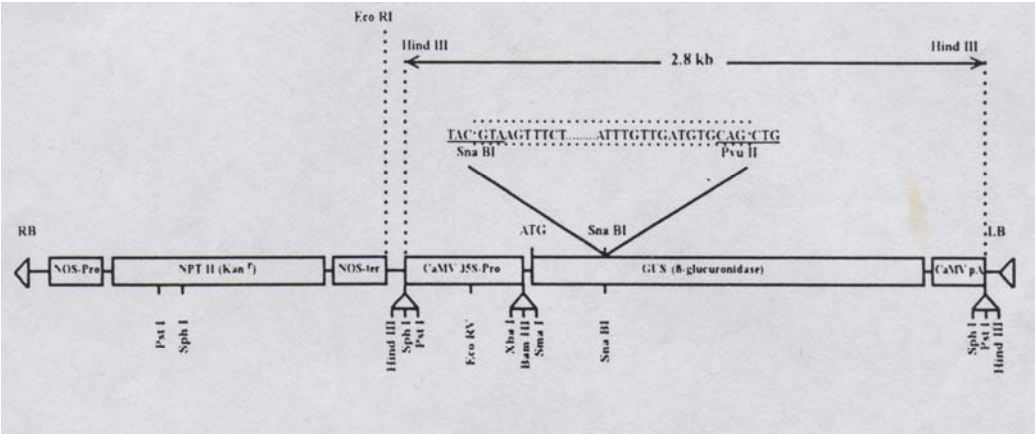


Figure B.1. Map of pGUSINT

APPENDIX C

PERMISSION LETTERS FOR pGUSINT and *AGROBACTERUIM* STRAIN EHA105

Institut für Genbiologische Forschung Berlin GmbH
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Dr. Hüseyin Avni Öktem
Dept. of Biology
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Telefax (030) 83 00 07 - 36
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Bearbeiter Wi/kr

Berlin, den 24.08.1994

Dear Dr. Öktem,

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

HindIII - sphI - PstI - HincII - 35S promoter - XbaI - BamHI - SmaI - GUS- gene -

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

Good luck!

Yours sincerely,

Prof. Dr. L. Willmitzer

MOGEN
The agricultural
bio-engineers

Hüseyin Avni Öktem, Ph.D.,
Department of Biology,
Middle East Technical University,
06531 Ankara,
TURKEY.

Dear Dr. Hüseyin Avni Öktem,

Enclosed you find the Agrobacterium strain EHA105.

A. tum strain EHA105 is grown at 29°C on LB-medium containing 20µg/ml Rifampicin.

Excuses for the delay in sending you the plasmids.

In case of questions don't hesitate to contact me at any time.

Success with your research.

Sincerely yours,

Theo C. Verwoerd.

APPENDIX D

SELECTION MARKERS FOUND ON BACTERIAL STRAINS AND BINARY PLASMID

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

Bacterial Strain	Chromosomal/ Ti Plasmid Selection Marker
EHA105	Rif (20 mg/L)
LBA4404	Strep (100 mg/L)
GV2260	Rif (20 mg/L)

Plasmid	Bacterial Selection Marker	Plant Selection Marker
pGUSINT	Kan (50mg/L)	Kan (*npt*II gene) *uid-a* gene

Rif (Rifampicin), Kan (Kanamycin), Strep (Streptomycin).

APPENDIX E

HISTOCHEMICAL GUS ASSAY SOLUTIONS

GUS Substrate Solution

$NaPO_4$ Buffer, pH 7.0	0.1 M
EDTA, pH 7.0	10 mM
K-ferricyanide, pH 7.0	0.5 mM
K-ferrocyanide, pH 7.0	0.5 mM
X-Glucoronide (dissolved in dimethyl formamide)	1 mM
Triton X-100	10% v/v

GUS Fixative Solution

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

APPENDIX F

TABULATED VALUES OF GRAPHS

Table F.1. Mean values, SEM and significant values for Figure 3.3. (Seed germination success after using different sterilization protocols).

	Protocol 1	Protocol 2
Average percent of germination success	43 ± 3.75 [a]	80.6 ± 5.48 [b]

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

Table F.2. Mean values and SEM for Figure 3.17. (Multiple shoot induction from sugar beet leaf blades using IBA and BA.)

	Set 1	Set 2	Set 3	Set 4	Set 5	Mean
Average percent of shoot regeneration	28±3.95	16±3.95	8±3.95	36±3.95	24±3.95	22±3.95

Table F.3. Mean values and SEM for Figure 3.18. (Root induction from regenerated shoots using IBA).

	Set 1	Set 2	Set 3	Set 4	Set 5	Mean
Average percent of root formation	85.7±5.9	50±5.9	50±5.9	77.7±5.9	66.6±5.9	66±5.89

Table F.4. Mean values and SEM for Figure 3.21. (Acclimatization of rooted shoot base).

	Set 1	Set 2	Set 3	Mean
Average percent of acclimatization of rooted shoot base	95.8 ± 0.99	97.9 ± 0.99	97.9±0.99	97.2 ± 0.99

Table F.5. Mean values and SEM for Figure 3.23. (Effect of kanamycin (K) on shoot development).

	0 mg/L K	50 mg/L K	100 mg/L	150 mg/L K	200 mg/L K	250 mg/L K
Number of shoot regeneration	10±1.83	8±1.83	5±1.83	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table F.6. Mean values and SEM for Figure 3.24. (Effect of PPT (P) on shoot development).

	0 mg/L P	1 mg/L P	3 mg/L	5 mg/L P	10 mg/L P
Number of shoot regeneration	8 ± 1.52	5 ± 1.52	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table F.7. Mean values, SEM and significant values for Figure 3.26. (Effect of vacuum infiltration on transient gene expression 3 days after transformation).

Evacuation pressure (mmHg)	Percent of explants exhibiting GUS activity
Control	0.0 ± 0.0 [a]
0	1.90 ± 0.27 [b]
200	2.11 ± 0.30 [b]
400	2.80 ± 0.35 [c]
600	1.62 ± 0.24 [b]

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

Table F.8. One-way ANOVA (stack) test of percentage of explants exhibiting GUS activity applying different evacuation pressure.

Level	N	Mean	StDev	------+---------+---------+---------+
0 mmHg	181	1,905	3,704	(-------*--------)
200 mmHg	190	2,114	4,230	(-------*--------)
400 mmHg	205	2,803	5,030	(-------*-------)
600 mmHg	198	1,620	3,484	(-------*-------)
				------+---------+---------+---------+
Pooled StDev =		4,172		1,40 2,10 2,80 3,50

Source	DF	SS	MS	F	**P**
C2	3	153,6	51,2	2,94	**0,032**
Error	770	13403,4	17,4		
Total	773	13557,0			

Table F.9. Mean values, SEM and significant values for Figure 3.28. (Effect of bacterial growth medium on transient gene expression on the 3rd day after transformation).

Bacterial growth medium	Percent of explants exhibiting GUS activity
Control	0.0 ± 0.0 [a]
YEB	0.98 ± 0.21 [b]
YEB+MES	1.20 ± 0.24 [b]
MG/L	3.90 ± 0.54 [c]

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

Table F.10. One-way ANOVA (stack) test of percentage of explants exhibiting GUS activity using different bacterial growth medium. P value is 0 indicated the highly significant difference (Confidence intervals, 95 %).

Level	N	Mean	StDev	--------+---------+---------+--------
MG/L	83	3,903	4,976	(-----*----)
YEB	101	0,983	2,127	(----*-----)
YEB+MES	107	1,209	2,484	(----*----)
				--------+---------+---------+--------
Pooled StDev =		3,300		1,2 2,4 3,6

Source	DF	SS	MS	F	**P**
C6	2	469,1	234,5	21,53	**0,000**
Error	288	3137,2	10,9		
Total	290	3606,3			

Table F.11. Mean values, SEM and significant values for Figure 3.30. (Effect of inoculation time with bacteria on transient gene expression on the 3rd day after transformation).

Inoculation time with bacteria (minutes)	Percent of explants exhibiting GUS activity
Control	0.0 ± 0.0 [a]
10	2.63 ± 0.37 [b]
20	3.96 ± 0.54 [c]
40	4.24 ± 0.47 [c]
60	4.73 ± 0.61 [c]

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

Table F.12. One-way ANOVA (stack) test of percentage of explants exhibiting GUS activity applying different inoculation time with bacteria.

Level	N	Mean	StDev	------+---------+---------+---------
10 min	101	2,636	3,797	(-------*-------)
20 min	85	3,962	4,980	(--------*--------)
40 min	108	4,249	4,891	(------*-------)
60 min	95	4,732	5,946	(-------*--------)
				------+---------+---------+---------
Pooled StDev =		4,943		2,4 3,6 4,8

Source	DF	SS	MS	F	P
C6	3	240,5	80,2	3,28	**0,021**
Error	385	9407,1	24,4		
Total	388	9647,6			

Table F.13. Mean values, SEM and significant values for Figure 3.32. (Effect of *Agrobacterium* strains on transient gene expression 3 days after transformation).

Agrobacterium strains carrying pGUSINT plasmid	Percent of explants exhibiting GUS activity
Control	0.0 ± 0.0 [a]
LBA4404	0.0 ± 0.0 [a]
EHA105	2.67 ± 0.25 [b]
GV2260	8.49 ± 0.73 [c]

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

Table F.14. One-way ANOVA (stack) test of percentage of explants exhibiting GUS activity using different *Agrobacterium* strains. P value is 0 indicated the highly significant difference (Confidence intervals, 95 %).

Level	N	Mean	StDev	-----+---------+---------+---------+-
EHA	79	2,679	2,306	(----*----)
GV	105	8,496	7,501	(----*----)
				-----+---------+---------+---------+-
Pooled StDev =		5,868		2,5 5,0 7,5 10,0

Source	DF	SS	MS	F	P
C2	1	1525,6	1525,6	44,31	**0,000**
Error	182	6266,7	34,4		
Total	183	7792,2			

Table F.15. Mean values, SEM and significant values for Figure 3.35. (Effect of different concentrations of L-cysteine application on transient gene expression on the 3rd day after transformation).

L-cysteine concentration (mg/L)	Percent of explants exhibiting GUS activity
Control	0.0 ± 0.0 [a]
0	6.81 ± 0.75 [b]
100	4.14 ± 0.56 [c]
200	5.10 ± 0.63 [c]
400	4.84 ± 0.59 [c]
800	3.14 ± 0.63 [d]
1200	3.02 ± 0.63 [d]

Table F.16. One-way ANOVA (stack) test of percentage of explants exhibiting GUS activity applying different L-cysteine concentration in co-cultivation medium. P value is 0 indicated the highly significant difference (Confidence intervals, 95 %).

Level	N	Mean	StDev	--+---------+---------+---------+----
0 mg/L	74	6,819	6,464	(-----*-----)
100 mg/L	73	4,146	4,782	(-----*-----)
1200 mg/	68	3,022	5,267	(-----*------)
200 mg/L	74	5,103	5,431	(------*-----)
400 mg/L	71	4,840	5,018	(-----*------)
800 mg/L	70	3,148	5,300	(------*-----)
				--+---------+---------+---------+----
Pooled StDev =		5,410		2,0 4,0 6,0 8,0

Source	DF	SS	MS	F	P
C10	5	717,8	143,6	4,91	**0,000**
Error	424	12408,9	29,3		
Total	429	13126,7			