# THE EFFECT OF PLOIDY LEVEL ON PLANT REGENERATION IN SUGAR BEET (*Beta vulgaris* L.)

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# ABSTRACT

THE EFFECT OF PLOIDY LEVEL ON PLANT REGENERATION
IN SUGAR BEET (*Beta vulgaris* L.)

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Three different genotypes of sugar beet (diploid, triploid and tetraploid); 4 varieties from diploid and triploid genotypes Soraya (KWS8123) and Leila (diploid), Visa (H68121) and Kassandra (triploid) and 2 lines from tetraploid genotype ÇBM315 and EA2075 (tetraploid) were used for investigating the effects of ploidy level on plant regeneration.

Within three sugar beet genotypes, with respect to the treatments, triploids or tetraploids were found to respond to treatments significantly different when compared with diploids. The responses of polyploids were superior over the responses of diploids. Moreover, varieties from same genotype responded differently to treatments.

Two types of calli were obtained; one white and friable with regenerative capacity and the other green and compact with no regenerative capacity.

Concentration of sucrose on callus development was observed to be important. High concentration of sucrose (30 g/L) was found to cause discoloration and irresponsiveness of formed calli at callus enlargement and subsequent shoot regeneration stages. Therefore, low concentration (10 g/L) is advised to be used at these stages; although this caused less callus induction.

Although initially used for the prevention of tissue discoloration, L-ascorbic acid inclusion into the medium was found to be positively affecting the regeneration capacity. When used at 20 mg/100 mL concentration, the only two spontaneous shoots from the tetraploid EA2075 line were obtained. Subsequently, these shoots were successfully rooted and whole plants were obtained.

The effect of silver nitrate, in combination with L-ascorbic acid, on the prevention of sugar beet tissue discoloration was investigated. Unfortunately, the symptoms of discoloration did not diminish. Moreover, callus formation was reduced and the subsequent shoot recovery could not be achieved.

Since a total of 3456 explants were used during this study, and only 2 whole plants were regenerated, the efficiency of plant recovery was calculated as a rather low value of 0.058 %.

**Keywords:** Ploidy level, sugar beet, callus induction, tissue discoloration, L-ascorbic acid, silver nitrate

# ÖZ


ŞEKER PANCARINDA (*Beta vulgaris* L.) PLOİDİ SEVİYESİNİN
BİTKİ REJENERASYONUNA ETKİSİ

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Tez yöneticisi: Doç. Dr. Sertaç Önde
Eylül 2006, 92 sayfa

Üç farklı şeker pancarı genotipi (diploid, triploid and tetraploid); 4 varyete diploid ve triploid genotiplerden Soraya (KWS8123) ve Leila (diploid), Visa (H68121) ve Kassandra (triploid) ve 2 hat tetraploid genotipten ÇBM315 and EA2075 (tetraploid) kullanılarak şeker pancarında farklı ploidi seviyelerinin rejenerasyon üzerindeki etkisi incelenmiştir.

Kullanılan bu üç genotip içinde triploid ya da tetraploidler, yapılan uygulamalara bağlı olarak, diploidlere oranla daha farklı tepkiler göstermişlerdir. Polyploid genotiplerin tepkileri, diploid genotip tepkilerine oranla daha olumlu olmuştur. Ayrıca ayni genotip içindeki varyetelerin tepkilerinde farklılıklar göze çarpmıştır.

İki farklı kallus tipi elde edilmiştir: Biri rejenerasyon kapasitesine sahip beyaz ve yumuşak kallus, diğeri rejenerasyon kapasitesine sahip olmayan yeşil ve sert kallus.

Yapılan uygulamalarda sükroz konsantrasyonunun kallus gelişiminde etkili olma ihtimali gözlemlenmiştir. Yüksek sükroz dozu (30 g/L) kalluslarda kararma ve ileri safhalarda sürgün rejenerasyonunda tepkisizliğe neden olmuştur. Daha az miktarda kallus oluşturmasına rağmen, bu aşamalarda düşük sükroz konsantrasyonu (10 g/L) önerilmektedir.

Başlangıçta doku kararmasını engellemek için kullanılmış olsa da, L-askorbik asit kullanımının rejenerasyon kapasitesi üzerinde arttırıcı etkisi saptanmıştır. Tüm çalışma boyunca elde edilen 2 sürgün hat EA2075 (tetraploid)'den elde edilmiştir ve 20 mg/100 mL L-askorbik asit dozu içeren uygulamada oluşmuştur. Bu sürgünler daha sonra başarılı bir şekilde köklendirilmiş ve sağlıklı bitkiler haline getirilmiştir.

Gümüş nitratın L-askorbik asitle kombine olarak şeker pancarı doku kararmasında etkisi araştırılmıştır. Doku kararması semptomlarında azalma olmamasına ek olarak, kallus indüksiyonunda da düşme görülmesinin yanısıra sürgün de elde edilememiştir.

Tüm çalışma boyunca toplam 3456 adet explant kullanılmış ve sadece 2 adet bitki rejenere edilmiş olduğu için bitki eldesi verimi oldukça düşük bir değer olan % 0.058 olarak hesaplanmıştır.

**Anahtar kelimeler:** Ploidi seviyesi, şeker pancarı, kallus indüksiyonu, doku kararması, L-askorbik asit, gümüş nitrat

**To my beloved Roshanak,**

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| | |
|---|---|
| $AgNO_3$ | Silver nitrate |
| ANOVA | Analysis of variance |
| BAP | 6-benzylaminopurine |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| GLM | General linear model |
| HCL | Hydrogen chloride |
| IBA | Indole-3-butyric acid |
| KIN | Kinetin |
| MS | Murashige and Skoog basal salts |
| NAA | Naphthaleneacetic acid |
| NaOH | Sodium hydroxide |
| PGR | Plant growth regulator |
| Ssp. / Subsp. | Subspecies |
| TIBA | 2,3,5-triiodobenzoic acid |

# CHAPTER I

# INTRODUCTION

## 1.1 The Sugar Beet Plant

Beet was a well-established vegetable in "classical" ancient Greece and Rome. The first known description of beets are of foliage beets (or chards) by Aristotle (c. 350 BC), who described a red chard, and Theophrastus (c. 300 BC) who recognized two different beets, white and black, the colors referring to light and dark green appearance of the leaves. The use of roots of beet is referred to for both culinary and medicinal purposes by Roman writers (Ford-Lloyd and Williams, 1975).

In 1747, when the pharmacist Markgraf found that the sweet substance in beets was sucrose, efforts to extract sugar from beets began. At this time the sucrose content was 6.2 %. A student of Markgraf built the first factory extracted the sugar from beet in 1801 (Campbell, 1976; OECD, 1993) and produced the first variety "White Silesian". In the following 70 years, selection produced beet varieties with sugar contents of 16 %. Today's sugar beet has a sugar content of 18-20 %. In 1925, the global production of beet sugar represented 50 % of the cane sugar production. By 1982, 30 % of all sugar produced was from sugar beets.

### 1.1.1 Sugar Beet Taxonomy

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*) belongs to the family *Chenopodiaceae* and the genus *Beta*. *B. vulgaris* comprises several cultivated forms of

*B. vulgaris* subsp. *vulgaris*. Cultivars include leaf beet (var. *cicla*) and beetroot (root beet USA). The genus *Beta* is divided into four sections shown in Table 1.1 (Ford-Lloyd and Williams, 1975; Campbell, 1976; Tranzschel, 1927 and Ulbrich, 1934):

**Table 1.1:** Classification of the *Beta* species

| Species Name | Chromosome number |
|---|---|
| **Section I:** *Beta* Tranzschel | |
| *B. vulgaris* L. ssp. *vulgaris* | 18 |
| *B. vulgaris* L. ssp. *maritima* (L.) Arcang | 18 |
| *B. vulgaris* L. ssp. *adanensis* (Pam.) Ford-Lloyd and Williams | 18 |
| *B. vulgaris* L. ssp. *trojana* (Pam.)* | 18 |
| *B. patula* Ait. | 18 |
| *B. macrocarpa* Guss. | 18, 36 |
| **Section II:** *Corollinae* Ulbrich | |
| *B. macrorhiza* Stev. | 18 |
| *B. corolliflora* Zoss | 36 |
| *B. lomatogona* F. et M. | 18, 36 |
| *B. intermedia* Bunge | 36, 45 |
| *B. trigyna* W. et K. | 36, 54 |
| **Section III:** *Nanae* Ulbrich | |
| *B. nana* Boiss. et Heldr. | 18 |
| **Section IV:** *Procumbentes* Ulbrich | |
| *B. procumbens* Chr. Sm. | 18 |
| *B. webbiana* Moq. | 18 |
| *B. patellaris* Moq. | 18, 36 |

* According to Davis (1967)

Sugar beet is cultivated world-wide (Table 1.2), but primarily in warm and temperate climates with little precipitation. There is an increase in cultivation in subtropical regions (Brouwer et al., 1976). The largest areas of cultivated sugar beet are in the USA, CIS (Commonwealth of Independent States, formerly the USSR), the EU and in China (FAO Yearbook, 1992).

**Table 1.2:** Global distribution of the wild species of the genus *Beta* (Mansfeld, 1986)

| **Species** | **Subspecies** | **Distribution** |
|---|---|---|
| *Beta vulgaris* | *maritima* | Mediterranean, Canary Islands, Near East, Madeira, European Atlantic coast to India, North sea, Middle and South America |
| *Beta vulgaris* | *adanensis* | Mediterranean, Canary Islands, Near East, Madeira, European Atlantic coast to India, North sea, Middle and South America |
| *Beta vulgaris* | *trojana** | Mediterranean, Near East |
| *Beta macrocarpa* | | India |
| *Beta patula* | | Mediterranean, Western Europe, North-west Africa |
| *Beta vulgaris* | *adanensi* | Mediterranean, Western Europe, North-west Africa |
| *Beta intermedia* | | Asia Minor to Hungary, Persia |
| *Beta corolliflora* | | Asia Minor, Caucasus, Black sea coast |
| *Beta macrorhiza* | | Asia Minor, Caucasus, Black sea coast |
| *Beta trigyna* | | Asia Minor, Caucasus, Black sea coast |
| *Beta patellaris* | | North-west African coast and Islands, southern Spain |
| *Beta procumbens* | | Canary and Cape Verde Islands, North-west African coast |
| *Beta webbiana* | | Canary and Cape Verde Islands, North-west African coast |

* According to Davis (1967)

### 1.1.2 Sugar Beet Uses

Sugar beet is used for the production of sugar. By-products of sugar production as pulp, molasses, fiber etc. are used as feed.

When sugar beet is grown in areas of livestock production, leaves of the plant may also be used for fodder (Figure 1.1). More recently, sugar beet has been used for molasses production. Molasses are used for alcohol production and in other forms of fermentation (penicillin production, etc.).

### 1.1.3 Morphology

A glabrous or slightly hairy annual, biennial or perennial of very varied habit, from 30 to 120 cm (or even 200 cm) in height. The root is stout, sometimes conspicuously swollen forming a beet together with the hypocotyl, and sometimes forming a branched taproot (as in spp. *maritima*). Stems are decumbent, ascending or erect, and more or less branched. Leaves are very varied in size, shape and color-often dark green or reddish and rather shiny, frequently forming a radicle rosette. Inflorescences are usually large and more or less branched. The flowers are hermaphrodite arranged in small cymes (Clapham *et al*., 1962; Højland and Pederson, 1994).

Cultivated forms of sugar beet are essentially biennial and are grown for the swollen roots that develop at the end of the first growing season. Sugar beet is biennial and requires a period of vernalization at the end of the first year before they can flower, although a small proportion of plants flower in their first year and are able to set seeds that persist in the soil. This phenomenon is known as “bolting”. A possible source of annual weed beets is the pollination of seed crops by contaminating pollen from annual wild beets (Longden, 1976; Evans and Weir, 1981).

**Figure 1.1:** Field of sugar beet plants (Ian Britton website-available at http://www.freefoto.com, Last access date: September 14, 2006)

*Beta vulgaris* ssp. *vulgaris* is customarily divided into two types: fodder beet and sugar beet. Some authors refer to sugar beet as var. *saccharifera*, however the distinction is not clear. The obvious morphological difference is that the beet in fodder beet is formed primarily by the hypocotyl, whereas in the sugar beet a considerable part of the beet is formed by the root (Figure 1.2). This results in a higher dry matter content in sugar beet, and also the beet itself is placed deeper in the soil. A variety of beet is, as a rule, only accepted as a sugar beet if the dry matter content is 20 % or more and the beet is white (Højland and Pederson, 1994).

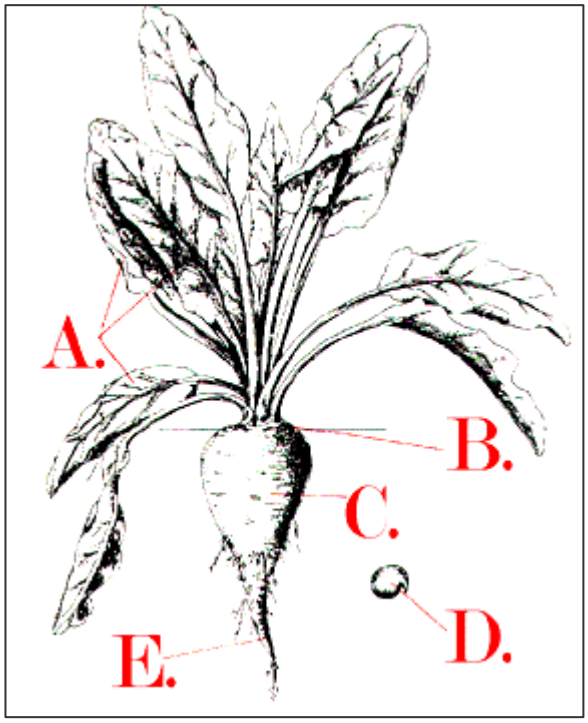


**Figure 1.2:** Parts of the sugar beet plant.

**A.** Leaves, **B.** Crown, **C.** Sugar Beet, **D.** Seed, **E.** Tap Root (Nebraska Agriculture in the Classroom web page-available at http://www.nefb.org/ag-ed/beets.html, Last access date: September 14, 2006)

The wild *Beta vulgaris* ssp. *maritima* is hardy, has thin, multi-stemmed roots, and low-lying stalks in a rosette-like array.

### 1.1.4. Genetic Characteristics (Ploidy Number)

The genus *Beta* exists in diploid, tetraploid and hexaploid forms with a chromosome number of x=9 (Walter, 1963) (see also Table 1.1). All wild and cultivated *Beta* species are capable of hybridizing, and wild beet species represent a valuable gene reservoir and are frequently used in variety breeding programs.

Most of the sugar beets grown since 1970's are triploid hybrids, although actually the diploid varieties represent 50 % in France. Triploid hybrids are produced by crossing a tetraploid male parent, onto a diploid male-sterile plant, used as the female parent. The resulting plants are usually doubly sterile because of chromosome imbalance and cytoplasmically inherited male sterility in the same plant. However, small proportions of plants do produce aneuploid pollen, which will give fertile progeny when used to pollinate the diploid male-sterile plants.

The goal of breeding programs are to develop sugar beet varieties with higher root yield and higher sugar content, better extraction yield (juicy purity), higher seed germination percentages; lower tendency to "bolt"; physical attributes of the root well adapted to mechanical harvesting; higher resistance to leaf diseases; and, higher root dry matter content (especially for fodder beet).

### 1.1.5 Survival Strategies

Sugar beet possesses long-lived dormant seeds that can become volunteer weeds in sugar beet fields (Højland and Pederson, 1994). They tend to germinate in field 1-3 days later than planted sugar beet seeds (Højland and Pederson, 1994). Sugar beet seeds may remain in the soil for ten years or more and still retain some germination

capacity (OECD, 1993; Brouwer *et al*., 1976; Lysgaard, 1991). It is generally accepted that six-year-old multigerm and four-year-old multigerm sugar beet seeds exhibit the same germination level of 70 %. Eight-year-old sugar beet seeds have been shown to germinate at a level of 59 % in laboratory conditions. These germination percentages depend on the quality of the seeds and on the conditions of germination. Thus *Beta vulgaris* has the ability to regenerate a viable seed bank (Højland and Pederson, 1994). The seed-balls of *Beta* are resistant to salt water, and ocean currents can move propagules over relatively long distances. Above the high water line, strong winds distribute them over the shoreline, and sometimes even inland (Smart, 1992).

Since commercial sugar producing sugar beet is biennial and is harvested during the first year whilst still in the vegetative phase, sexual reproductive organs (floral parts) never develop. Varieties that tend to bolt in the first year of growth pose some problems and much effort has gone into developing currently cultivated varieties that limit bolting. When *Beta vulgaris* is planted for seed production, some seeds may remain on the field after harvesting the seed crop. Agricultural practices tend to limit those shoots.

### 1.2 Sugar Beet Production in the World and Turkey

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).

Currently, sugar beet is the major sugar crop grown in temperate regions of the world. The major sugar beet producers and exporters are the EU, France, USA, Germany,

Russia Federation and Turkey (Figure 1.3). The major sugar beet importing countries include USA, China, Russia, Mexico, Pakistan, Indonesia and Japan.

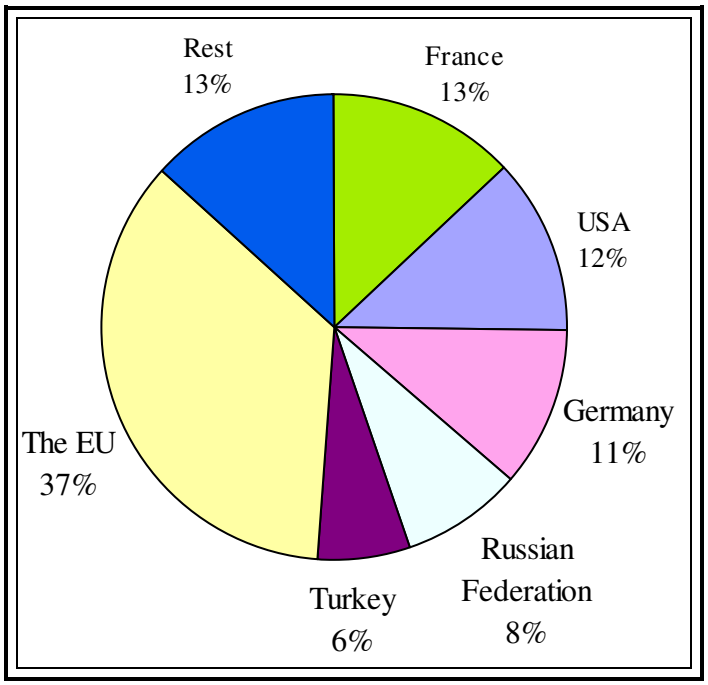


**Figure 1.3:** Major sugar beet producing countries and their production percentages in 2004 (FAOSTAT, 2005)

Between 2000 to 2004 world sugar beet production was nearly 250 million metric tons (Table 1.3). Over these years, yield in world sugar beet production ranged between 381,000 to 424,000 hectograms per hectare.

**Table 1.3:** 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 |

Turkey was the fifth country in 2004 in sugar beet production (Table 1.4).

**Table 1.4:** 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 |

## 1.3 Sugar Beet Tissue Culture Studies

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 remains as a "recalcitrant" species.

The ultimate aim in the development of *in vitro* plant cell/tissue culture systems is to be able to regenerate whole plants from utilized explant materials. For the regeneration of whole plants 3 factors play a crucial role and therefore must be considered carefully: i) effects of genotype, ii) effects of explant material, iii) effects of culture conditions (including medium and environment) (Maes *et al*., 1996).

In sugar beet (*Beta vulgaris* L.), successful plant regeneration was reported from various explants including hypocotyls, young leaves, embryos, inflorescences, shoot apices, anthers (reviewed in Atanassov, 1986; Hussey and Hepher, 1978; Miedema, 1982; Saunders and Doley, 1986; Tétu et al., 1987) and from flower buds (Margara, 1970; Miedema *et al*., 1980). In a report the most important factor for shoot formation appeared to be the origin of the explant. For the first time a comparison was presented between different organs as a source of explants. Petioles provided the best results (Krens and Jamar, 1989). Petioles from *in vitro* sugar beet shoots are known to have a high morphogenic capacity (Saunders and Shin, 1986; Tétu *et al*., 1987; Detrez *et al*., 1988, 1989; Freytag *et al*., 1988).

Attempts to regenerate plants *in vitro* have met with only limited success and have been hampered by the erratic behavior of cultured material (Steen et al., 1986; Doley and Saunders, 1989; Gürel, 1991; Gürel and Wren, 1995a; Gürel and Wren, 1995b). This has led to a study of the problem of variability by comparing the response shown by explants taken from different varieties, from different plants of the same variety

and from different leaves on the same plant. The nature of variability, when leaf explants of sugar beet (*Beta vulgaris* L.) were cultured *in vitro*, was investigated at different levels of classification (i.e. variety, plant and organ). Large differences in rooting capacity were observed when 10 commercial varieties were compared. Significant inter-plant and inter-leaf (intra-plant) variability was found to exist within a variety but explants taken from different positions within the leaf behaved similarly (Gürel, 1997).

### 1.3.1 Direct and Indirect Organogenesis

Efficient direct organogenesis from petiole explants was developed for large scale multiplication (Detrez *et al*., 1988). This system also showed a low frequency of somaclonal variation (Detrez *et al*., 1989). On the other hand, the frequency of indirect regeneration via a callus phase is low, unpredictable and is limited to certain genotypes only (Saunders and Doley, 1986; Ritchie *et al*., 1989).

Shoot regeneration from sugar beet (*Beta vulgaris* L.) callus was reported by several researchers (Butenko *et al*., 1972; De Greef and Jacobs, 1979; Enomoto and Ohyama, 1985; Hooker and Nabors, 1977; Margara, 1977; Mohammad and Collin, 1979; Slavova, 1981). The frequency of shoot production was influenced by the cultivar and pre-treatment of the donor cultures, in particular, the time after transfer to culture, the temperature, and the BAP (6-benzylaminopurine) concentration of the medium (Ritchie *et al*., 1989).

Callus can be obtained from many parts of the sugar beet plant, including seedling tissues (hypocotyl and cotyledons), leaves, petioles, roots, 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). Two types of calli 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, (Tétu *et al*., 1987; Ritchie *et al*., 1989 and Gürel 1993).

Tétu *et al.* (1987) indicated that the organogenic potentiality of sugar beet callus could be regulated and buds could be obtained in high percentages, contrary to the earlier studies in which only infrequent bud formation had been obtained from the callus (Margara, 1970; Butenko *et al.*, 1972; Hooker and Nabors, 1977). In order to obtain organogenic calli, growth hormones such as BAP and Zeatin as well as TIBA (2,3,5-triiodobenzoic acid) were used. TIBA is a known inhibitor of the transport of endogenous auxin. Since it reacted at a low level, the optimal concentration found was 1.0 mg/L. The green and friable calli obtained on MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg/L NAA (naphthaleneacetic acid) and 5.0 mg/L BAP were subcultured on different media. Raising the concentration of cytokinin (BAP or Zeatin) increased in each case the number of buds per callus. However, a relatively high level of BAP (3.0 mg/L) induced bud vitrification. The best results were obtained, invariably, with either of the basal media used (Murashige-Skoog (MS) basal medium (Murashige and Skoog, 1962) or PGo medium (De Greef and Jacobs, 1979)) having 1.0 or 3.0 mg/L of Zeatin with TIBA (1.0 mg/L). The best results were obtained from shoot tip, flower bud and petiole (Tétu *et al*., 1987).

Two reports (De Greef and Jacobs, 1979; Saunders and Daub, 1984) described shoot regeneration from habituated (hormone-autonomous) callus, that was, not requiring growth regulators for growth. De Greef and Jacobs (1979) described a single hormone

autonomous, continuously regenerating cell line that was isolated fortuitously and could not be obtained again. Saunders and Daub (1984) reported that hormone-autonomous callus could repeatedly be induced directly from shoot cultures of several genotypes. Two of the genotypes were capable of shoot regeneration from this callus. Subsequent work (Saunders, 1982) revealed that morphologically uniform hormone-autonomous callus could be induced directly on 5 to 10 % of the shoot culture petiole explants cultured on MS medium without growth regulators added.

The effects of pre-culture treatment, BAP and sucrose concentrations, and cultivar on shoot regeneration using petioles and intact leaves as explants were investigated. The shoot regeneration rate was affected by BAP concentration in the medium, by cultivar and particularly by the pre-treatment of donor plants in culture (Zhong *et al*., 1993). Petioles were transferred to MS media supplemented with either 1.0 mg/L BAP or 1.0 mg/L KIN to compare the effect of BAP and KIN on adventitious shoot regeneration. The results indicated that BAP was much more active than KIN and that the optimum BAP concentrations were between 0.5 and 1.0 mg/L. According to Lindsey and Gallois (1990), leaf squares, petioles and shoot base tissue slices from genotypes of sugar beet were cultured on a range of media to determine the best combination of tissue and medium for shoot regeneration. The highest numbers of shoots were obtained on medium containing 1.0 mg/L BAP.

The frequency of callus formation and plant regeneration in sugar beet depends on the types of explants: cotyledons (Hooker and Nabors, 1977; Jacq *et al.*, 1993), hypocotyls (Krens and Jamar, 1989; Jacq *et al.*, 1992) or leaves (De Greef and Jacobs, 1979; Owens and Eberts, 1992). A protocol for efficient regeneration from cotyledon explants was described and the nuclear DNA levels (ploidy) of the organogenic calli

and the regenerated plants were analyzed. High yield of organogenic calli from cotyledon explants that consistently gave regeneration of stable true-to-type sugar beet plants were obtained (Jacq *et al*., 1993). Both organic supplements and phytohormones were required to initiate organogenic calli in sugar beet cultures. The initiated calli were then transferred onto fresh medium supplemented with 1.0 mg/L of BAP in order to promote stronger shoot differentiation.

### 1.3.2 Somatic Embryogenesis

Tétu *et al.* (1987) reported that somatic embryos were formed only in the calli derived from petiole explants and multiple hormonal sequences were necessary for the induction and development of these somatic embryos. However, Freytag *et al*. (1988) demonstrated that this was not necessary when callus was incubated on MS medium supplemented with 10 vitamins and 6 amino acids.

Previously, Tenning *et al*. (1992) described direct somatic embryogenesis with immature sugar beet embryo explants. Afterwards, a simple strategy for direct somatic embryogenesis using mature zygotic sugar beet embryos was reported (Kulshreshtha and Coutts, 1997). Explants were sequentially cultured on modified MS media supplemented with different combinations of 2,4-D (2,4-dichlorophenoxyacetic acid), NAA, BAP and TIBA. Somatic embryogenesis was induced within 4 weeks of culture on embryogenesis induction medium which contained MS medium supplemented with BAP and TIBA. Proliferation of somatic embryos was observed on embryo proliferation medium, which contained MS medium supplemented with BAP and NAA within 4 weeks of culture. Plants were regenerated on hormone free ½ strength MS medium containing a low sucrose concentration. With some sugar beet lines high frequencies of plant regeneration in excess of 90 % were observed. The incorporation of TIBA in the media was essential for successful regeneration (Kulshreshtha and Coutts, 1997).

### 1.3.3 Prevention of Callus and Explant Discoloration Studies

One of the major problems related to especially, sugar beet tissue culture studies is the fast discoloration (blackening) of explants as well as the formed calli. This discoloration majorly manifests itself with characteristic necrosis-like appearance and characteristic black color. It has been reported that blackening is caused by the oxidation of phenolic compounds by several enzymes such as polyphenoloxidase (PPO) and peroxidase (Lee *et al*., 1990 and Amiot *et al*., 1992). To relieve tissue blackening caused by certain enzymes, it was reported that some chemicals such as activated charcoal, ascorbic acid, citric acid and sodium chloride must be added into basal medium (Pizzocaro *et al*., 1993).

Blackening might be relieved when sterilization conditions are softened (personal observation). However, this brings about the risk of infection in later stages of tissue culture. Adjustments in medium such as reducing sugar content and adding activated charcoal were mentioned to be effective in prevention of blackening (Yıldız *et al*., 1997 and Pizzocaro *et al.,* 1993). However, according to our personal experience, reducing the sugar content also resulted with slow growth of callus.

Since tissue discoloration and necrosis are the first signs of ethylene build-up in plants, various researchers attacked this issue by employing ethylene inhibitors in their studies. Silver ion is a potent inhibitor of ethylene action (Bayer, 1976) and has been found to enhance shoot production in plant systems (Hyde and Philips, 1996; Bias *et al*., 2000; Reddy *et al*., 2001). Ozden-Tokatli *et al*. (2005) reported that ethylene can play a negative regulatory role in micropropagation of pistachio and silver nitrate ($AgNO_3$) seems to be effective in counteracting this regulation. $AgNO_3$-containing media completely inhibited browning in pistachio tissues. Inhibition of

tissue browning was also reported in mangosteen leaf cultures with the use of silver nitrate in the media (Goh *et al*., 1997). However, $AgNO_3$ reduced callus production in pistachio nodal cultures, indicating the possible role of ethylene on basal callus formation of pistachio shoots (Ozden-Tokatli *et al*., 2005). Moreover, it was reported that ethylene could cause considerable callus growth at high concentrations in mangosteen leaf explants (Goh *et al*., 1997). The same concomitant inhibition of callus growth also occurred in cassava (Zhang *et al*., 2001) with the addition of silver nitrate to the medium.

According to Gürel (2000), for haploid embryo induction sugar beet lines were cultured on MS medium containing 2.0 mg/L BAP in combination with 2.5 or 5.0 mg/L $AgNO_3$, or with 0.5 % charcoal. The inclusion of $AgNO_3$ in the culture medium either decreased or completely inhibited embryo formation from ovules. The addition of 0.5 % activated charcoal to the culture medium increased the rate of haploid embryo formation.

## 1.4 Objectives of the Study

In this study, three different genotypes of sugar beet (diploid, triploid and tetraploid); 4 varieties from diploid and triploid genotypes Soraya (KWS8123) and Leila (diploid), Visa (H68121) and Kassandra (triploid), and 2 lines from tetraploid genotype ÇBM315 and EA2075 (tetraploid) were used to achieve the following objectives:

- To understand the effects of tissue culture parameters, which are critical for callus formation and subsequent regeneration, at genotype as well as at variety and line levels

- To determine the most effective tissue culture parameters on most responsive genotypes as well as varieties and lines by comparing results with statistical analysis

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Plant Material

In this study three genotypes and four varieties from diploid and triploid genotypes and two lines from tetraploid genotype were used. Description of the plant material is given in Table 2.1.

**Table 2.1:** Description of the plant material

| Ploidy Level | Varieties |
|---|---|
| Diploid | Soraya (KWS8123) and Leila |
| Triploid | Visa (H68121) and Kassandra |
| | |
| Ploidy Level | Lines |
| Tetraploid | ÇBM315 and EA2075 |

The seeds were kindly provided by Dr. Songül Gürel, Turkish Sugar Factories, Sugar Institute, Etimesgut, Ankara.

### 2.1.2 Chemicals

All chemicals were tissue culture tested. They were purchased from Duchefa Biochemie B.V., 2003 RV Haarlem, The Netherlands; Carlo Erba Reagenti SpA, Strada, Rivoltana, Rodano (MI) and Sigma-Aldrich Chemical Company, Gilingham, Dorset, UK.

Murashige and Skoog (MS) medium including micro and macro elements and vitamins (Duchefa Prod. No: M0222) was used in this study. The composition of this medium is given in Appendix A.1 and A.2.

Plant growth regulators BAP (Sigma Cat. No: B-3408), NAA (Sigma Cat. No: N-0640), Kinetin (Sigma Cat. No: K-0753), 2,4-D (Sigma Cat. No: D-4517), IBA (Sigma Cat. No: I-5386), TIBA (Duchefa Prod. No: T0929) and Zeatin (Duchefa Prod. No: Z0917) were used as supplements to MS media.

L-ascorbic acid (Sigma Cat. No: A-7506), $AgNO_3$ (Sigma Cat. No: S-7276), activated charcoal (Sigma Cat. No: C-3790), sucrose (Carlo Erba Code No: 477187), plant agar (Duchefa Prod. No: P1001) and gelrite (Duchefa Prod. No: G1101) were used as media supplements for various purposes throughout this study.

Zephran (İlsan İlaç Sanayii ve Tic. A. Ş., Gebze, Kocaeli) and %97 ethanol (Botafarma Laboratuarı, Kimyasal Maddeler ve Tıbbi Ürünler A. Ş., Anıttepe, Ankara) were used for sterilization.

### 2.1.3 Glassware

Standard 10 cm glass petri plates (Labor İldam) and baby jars (Sigma Cat. No: V-8630) with autoclavable caps (Sigma Cat. No: B-8648) were used throughout the study. Forceps, scalpels and razor blades (Hecos, No: 10 and No: 21) were obtained from local commercial sources.

### 2.1.4 Tissue Culture Instruments

Holten Laminar TL2472 (Alerot, Denmark) supported with UV sterilization lamp (Philips TUV 30W G30T8 UV Longlife, Holland) and Bassaire 04HB Laminar (Southampton, UK) flowhoods were used during aseptic transfer of culture materials. Ika-Werke shaker (Type: RS/OS 10 basic, Staufen, Germany) was used throughout surface sterilization of seeds.

Growth room was illuminated by cool white fluorescent lamps 36W/33 x 2/shelf (Philips TLD, Poland) and equipped with timer programmed 16h light/8h dark regime. Incubation temperature of the growth room was adjusted to 24±1 $^{0}$C.

### 2.1.5 Photography

All photography was performed with HP FCLSD-0411 Digital Camera with 4.1 megapixel sensor resolution and Sony Cyber-shot DSC-T3/S Digital Camera with 5.1 megapixel sensor resolution.

## 2.2 Methods

### 2.2.1 Surface Sterilization of Sugar Beet Seeds

In this study two different surface sterilization protocols were used for monogerm varieties and multigerm lines (Table 2.2).

**Table 2.2:** Distinction of monogerm varieties and multigerm lines

| | |
|---|---|
| Monogerm varieties | Soraya (KWS8123) and Leila (Diploid)<br>Visa (H68121) and Kassandra (Triploid) |
| Multigerm lines | ÇBM315 and EA2075 (Tetraploid) |

Monogerm seeds were surface sterilized with commercial bleach (ACE containing 5-6 % NaOCl) without any dilution for 5 hours under continuous stirring (150 rpm) and rinsed three times in sterile distilled water. The seeds then were transferred to sterile distilled water and further stirred for 20 hours at 150 rpm to remove all bleach solution and again rinsed three times in sterile distilled water before transferring to germination medium.

Multigerm seeds were surface sterilized with commercial bleach (ACE containing 5-6 % NaOCl) without any dilution for 18 hours under continuous stirring (150 rpm) and rinsed three times in sterile distilled water. The seeds then were transferred to sterile distilled water and further stirred for 6 hours at 150 rpm to remove all bleach solution and again rinsed three times in sterile distilled water before transferring to

germination medium. The original version of the protocol for multigerm seeds was reported by Yıldız *et al*. (1997). The protocol for monogerm seeds was developed by altering incubation times.

### 2.2.2 Sterilization of Media and Glassware

All tissue culture media were sterilized by autoclaving at 121 $^0$C under the pressure of 1.1 kg/cm$^2$, for 20 minutes. Baby jars with autoclavable caps were sterilized by autoclaving for 20 minutes. All petri plates, baby jars with a larger size, forceps and scalpels were heat sterilized at 180 $^0$C for two hours in a dry oven. Inside the laminar flowhood all tissue culture media were aseptically dispensed into the sterile petri plates and baby jars.

### 2.2.3 Transfer Conditions

All transfers and manipulations of the explants and culture material were performed in a sterile environment provided by a laminar flowhood. Panels of the cabinet and working surface were first cleaned up with 1 % (v/v) zephran, subsequently disinfected with UV light for 15 minutes. Then the unit was sterilized with 70 % ethanol and turned on 30 minutes before use.

### 2.2.4 Germination of Seeds

Surface sterilized seeds were incubated in pre-sterilized baby jars containing germination medium (Appendix B.1) in growth room with a temperature of 24±1 $^0$C and 16h light/8h dark regime (Rady and Ali, 1999).

### 2.2.5 Source of Explant

Monogerm seedlings grew to a length of 8-10 cm in 5 weeks (Figure 2.1) and multigerm seedlings in 10 weeks (Figure 2.2). Petioles of these aseptically grown plants were used as explant source in this study. Young and green seedlings, possible to be excised in at least three 0.5-1.0 cm long petioles, were selected.

**Figure 2.1:** Monogerm sugar beet seedlings

**Figure 2.2:** Multigerm sugar beet seedlings

### 2.2.6 Preparation of Tissue Culture Media and Culture Conditions

Experiments were composed of four sets. Each set included various media treatments for callus induction and shoot regeneration.

For each of the callus induction treatments, 3 petri plates each containing 8 explants from each sugar beet variety and line were prepared. All petri plates were incubated in

growth room for 10 weeks. The petioles were subjected to subculturing after 5-week period. After the completion of 10-week incubation period, data on the frequency and weight of calli produced by each sugar beet variety and line and each treatment were recorded. Calli were excised and transferred into the same fresh callus induction media to enlarge for further 4 weeks. At this stage, sucrose concentration of all media was fixed at 10 g/L.

For shoot regeneration studies (except Experimental Set 2), every callus that has been formed, were divided into parts and transferred into petri plates containing shoot regeneration media. These cultures were incubated for 12 weeks under conditions described for callus cultures, while for every 4 weeks the cultures were refreshed.

All media were solidified with 8.0 g/L agar. The pH of the media was adjusted to 5.7-5.8 by the addition of NaOH or HCl dropwise.

The details of the four experimental sets are given below;

#### 2.2.6.1 Experimental Set 1 (The Effect of Plant Growth Regulator Combinations and Sucrose Concentration)

For callus induction, 8 different treatments were prepared by using MS medium including micro and macro elements and vitamins and supplemented with the plant growth regulator combinations and sucrose as shown in Table 2.3.

**Table 2.3:** Experimental Set 1 callus induction treatments and supplementary material to MS media

| Treatments | [cytokinin] (1.0 mg/L) | [auxin] (0.25 mg/L) | [sucrose] (g/L) |
|---|---|---|---|
| 1 | BAP | NAA | 10 |
| 2 | BAP | 2,4-D | 10 |
| 3 | KIN | NAA | 10 |
| 4 | KIN | 2,4-D | 10 |
| 5 | BAP | NAA | 30 |
| 6 | BAP | 2,4-D | 30 |
| 7 | KIN | NAA | 30 |
| 8 | KIN | 2,4-D | 30 |

For shoot regeneration, 3 different treatments were planned by using MS medium including micro and macro elements and vitamins and supplemented with two different concentrations of the plant growth regulator BAP (third treatment in combination with TIBA) as shown in Table 2.4. All media included 10 g/L sucrose.

**Table 2.4:** Experimental Set 1 shoot regeneration treatments and supplementary material to MS media

| Treatments | [plant growth regulator] (mg/L) |
|---|---|
| 1 | 0.5 BAP |
| 2 | 1.0 BAP |
| 3 | 1.0 BAP + 1.0 TIBA |

### 2.2.6.2 Experimental Set 2 (The Effect of L-ascorbic Acid)

While Experimental Set 1 was dealing with callus induction, we constructed this set. The combination of the growth regulators were determined from a preliminary experiment that we had performed even before Experimental Set 1 (data not shown). For callus induction 2 different treatments, containing 10 and 20 mg/100 mL L-ascorbic acid, were tested by using MS medium including micro and macro elements and vitamins supplemented with BAP (1.0 mg/L) + NAA (0.25 mg/L ) and 10 g/L sucrose as shown in Table 2.5.

**Table 2.5:** Experimental Set 2 callus induction treatments and supplementary material to MS media

| Treatments | [L-ascorbic acid] (mg/100 mL) |
|---|---|
| 1 | 10 |
| 2 | 20 |

Due to spontaneous shoot regeneration (details given in Chapter III, Results & Discussion), no additional shoot regeneration study was performed for this set.

Shoots at least 2 cm long (Tétu *et al*., 1987) were transferred to baby jars containing rooting medium (Appendix B.2) in growth room with a temperature of 24±1 $^{0}$C and 16h light/8h dark regime.

For root induction two methods were applied. According to the first method, shoots with vitrified appearance were incubated in MS medium supplemented with 10 g/L sucrose without any plant growth regulator (Roussy *et al*., 1996) for 1-2 weeks. When the symptoms of vitrification were diminished, the shoots were transferred to root induction medium (MS medium supplemented with 3.0 mg/L IBA and 30 g/L sucrose) (Gürel *et al*., 2002). According to the second method, shoots with no vitrified appearance were directly transferred to root induction medium (Gürel *et al*., 2002). Rooting medium was refreshed every 4 weeks.

Shoots grown to at least 8 cm long with strong and dense roots were transferred to pots containing garden soil. In order to attain high humidity (acclimatization), the potted tissue culture plants were covered by plastic bags and incubated in growth room.

#### 2.2.6.3 Experimental Set 3 (The Combined Effect of L-ascorbic acid and Silver Nitrate)

For callus induction, 12 different treatments were planned by using MS medium including micro and macro elements and vitamins and supplemented with the plant growth regulator combinations and sucrose as shown in Table 2.6. In this set, the results of Experimental Set 2 were considered and 20 mg/100 mL L-ascorbic acid was added to each medium composition. Also, the probable effect of silver nitrate ($AgNO_3$) on the prevention of tissue discoloration was investigated by adding silver nitrate at a fixed concentration of 2.5 mg/mL (Gürel, 2000).

**Table 2.6:** Experimental Set 3 callus induction treatments and supplementary material to MS media

| Treatments | [cytokinin] (mg/L) | [auxin] (mg/L) | [sucrose] (g/L) |
|---|---|---|---|
| 1 | 1.0 BAP | 0.25 NAA | 10 |
| 2 | 1.0 BAP | 0.25 2,4-D | 10 |
| 3 | 1.0 KIN | 0.25 NAA | 10 |
| 4 | 1.0 KIN | 0.25 2,4-D | 10 |
| 5 | 2.0 BAP | 0.25 NAA | 10 |
| 6 | 1.0 KIN | 1.0 2,4-D | 10 |
| 7 | 1.0 BAP | 0.25 NAA | 30 |
| 8 | 1.0 BAP | 0.25 2,4-D | 30 |
| 9 | 1.0 KIN | 0.25 NAA | 30 |
| 10 | 1.0 KIN | 0.25 2,4-D | 30 |
| 11 | 2.0 BAP | 0.25 NAA | 30 |
| 12 | 1.0 KIN | 1.0 2,4-D | 30 |

For shoot regeneration, 3 different treatments were planned by using MS medium including micro and macro elements and vitamins and supplemented with the plant growth regulator BAP (third treatment in combination with TIBA) as shown in Table 2.7. All media included 10 g/L sucrose.

**Table 2.7:** Experimental Set 3 shoot regeneration treatments and supplementary material to MS media

| Treatments | [plant growth regulator] (mg/L) |
|---|---|
| 1 | - (Hormone Free) |
| 2 | 1.0 BAP |
| 3 | 1.0 Zeatin + 1.0 TIBA |

### 2.2.6.4 Experimental Set 4 (The Effect of Zeatin on Callus Induction and Cold Treatment on Shoot Regeneration)

For callus induction, 2 different treatments were planned by using MS medium including micro and macro elements and vitamins and supplemented with the plant growth regulator combinations as shown in Table 2.8. All media included 10 g/L sucrose.

**Table 2.8:** Experimental Set 4 callus induction treatments and supplementary material to MS media

| Treatments | [plant growth regulator] (mg/L) |
|---|---|
| 1 | 1.0 Zeatin + 0.25 2,4-D + 1.0 TIBA |
| 2 | 1.0 BAP + 0.25 NAA |

Although treatment 1 resulted with callus growth, the proceeding 4 weeks of callus enlargement phase at the same media, prior to shoot regeneration, caused the formed calli to blacken and die.

For treatment 2, the proposed "cold treatment" (Gürel, 2000) for shoot regeneration was investigated. The formed calli were transferred to MS medium supplemented with BAP (1.0 mg/L) and 10 g/L sucrose and incubated at 4 $^{0}$C in dark for 2 weeks. Next, cold-treated calli were transferred to MS medium supplemented with BAP (2.0 mg/L) and 10 g/L sucrose and incubated in growth room for 4 weeks. However, calli blackened and died.

### 2.2.6.5 Supplementary Experiments

Although no callus induction was observed, the following parameters were also considered and tested:

For callus induction, 4 different treatments were planned by using MS medium including micro and macro elements and vitamins and supplemented with the plant growth regulator combinations as shown in Table 2.9. All media included 10 g/L sucrose.

**Table 2.9:** Callus induction treatments and supplementary material to MS media

| Treatments | [plant growth regulator] (mg/L) |
|---|---|
| 1 | - (Hormone Free) |
| 2 | 1.0 BAP |
| 3 | 1.0 KIN |
| 4 | 1.0 NAA + 5.0 BAP |

Since callus could not be obtained, shoot regeneration studies were not performed.

### 2.2.7 Data Collection and Statistical Analysis

Data on the frequency and weight of calli produced by each sugar beet genotype and variety and line were collected by weighing the individual calli, and frequencies were

calculated by comparing the initial explant number and the number of explants showing callus development. The mean and standard error (SE) values were calculated for each treatment.

Multivariate analysis of variance (ANOVA) with General Linear Model (GLM) was used to examine the effects of multiple factors (genotypes, varieties, lines and treatments) on response variables (callus frequency and callus weight) by using the SAS statistical package (SAS Inst., 1988).

The differences between the treatment means were analyzed by using Duncan's Multiple Range Tests at genotype as well as variety and line levels by using MSTAT-C program (MSTAT-C., 1989).

# CHAPTER III

# RESULTS & DISCUSSION

## 3.1 Surface Sterilization Optimization

Sugar beet has a notorious seed structure with multi-surfaces (Figure 3.1), which makes initiating "sterile" tissue culture studies difficult. Therefore, lengthy surface sterilization protocols were applied. Previous workers emphasized the difficulties encountered to produce aseptic cultures of sugar beet from seeds and seedlings (Hussey and Hepher, 1978). Moreover, previous reports indicated only 40-50 % yield (Bannikova *et al*., 1995). Different variants of aseptic treatment of the sugar beet seeds are known (Il'enko, 1983; Van Geyt and Jacobs, 1985; Tétu *et al*., 1988; Krens and Jamar, 1989 and Bannikova *et al*., 1995). However, Kolodyazhnaya and Deineko (2002) reported that in their study, these variants gave no desirable effects: the proportion of disinfected germlings did not exceed 10 %. They developed a new scheme of disinfection with a longer treatment of seeds, which gave a practically 100 % yield of the seed materials free from bacterial and fungal infections. Ritchie *et al*. (1989) observed considerable cultivar variation in seed sterilization of sugar beet. However, it was reported that sterilization after seed coat removal was effective and practical (Zhong *et al*., 1993; Freytag *et al*., 1988).

Surface sterilization protocols used in this study revealed results (Table 3.1) that were satisfactory enough for obtaining necessary number of explants to initiate experimental sets.


**Figure 3.1:** Sugar beet monogerm (left) and multigerm (right) seeds

**Table 3.1:** Surface sterilization protocols of monogerm and multigerm seeds

| | exposure to bleach (hour) | exposure to water (hour) | germination (%) | week |
|---|---|---|---|---|
| Monogerm seed | 5 | 20 | 81 | 6th |
| Polygerm seed | 18 | 6 | 56 | 6th |

### 3.2 Explant Response

Petiole explants were transferred to callus induction media. Petioles had been chosen as explant source in previous studies (Saunders, 1982; Saunders and Shin, 1986; Tétu *et al*., 1987; Detrez *et al*., 1988; Krens and Jamar, 1989; Ritchie *et al*., 1989; Sullivan *et al*., 1993; Zhong *et al*., 1993 and Grieve *et al*., 1997). According to Krens and Jamar (1989), the most important factor for shoot formation appeared to be the origin of the explant. For the first time a comparison was presented between different organs as sources of explants. Petioles provided the best results. Another study also showed that the bud-inducing capacity was determined by the origin of the explants. The best results were obtained from shoot tip, flower bud and petiole (Tétu *et al*., 1987).

Two types of calli-one being white and friable with regenerative capacity (Figure 3.2) and the other being green and compact without any regenerative capacity-were derived (Figure 3.3) in this study. The production of white and friable callus from various sugar beet explants was also reported by others (Saunders and Daub, 1984; Nakashima *et al*., 1988; Ritchie *et al*., 1989; Catlin, 1990; Konwar and Coutts, 1990; Shimamoto *et al*., 1993; Zhong *et al*., 1993 and Baloğlu, 2005). Only white and friable callus was capable of shoot development, a fact reported earlier (Saunders and Daub, 1984; Ritchie *et al*., 1989 and Gürel, 2000). This callus developed mostly from brown tissue shown in Figure 3.2. Same observation was reported by Ritchie *et al*. (1989).

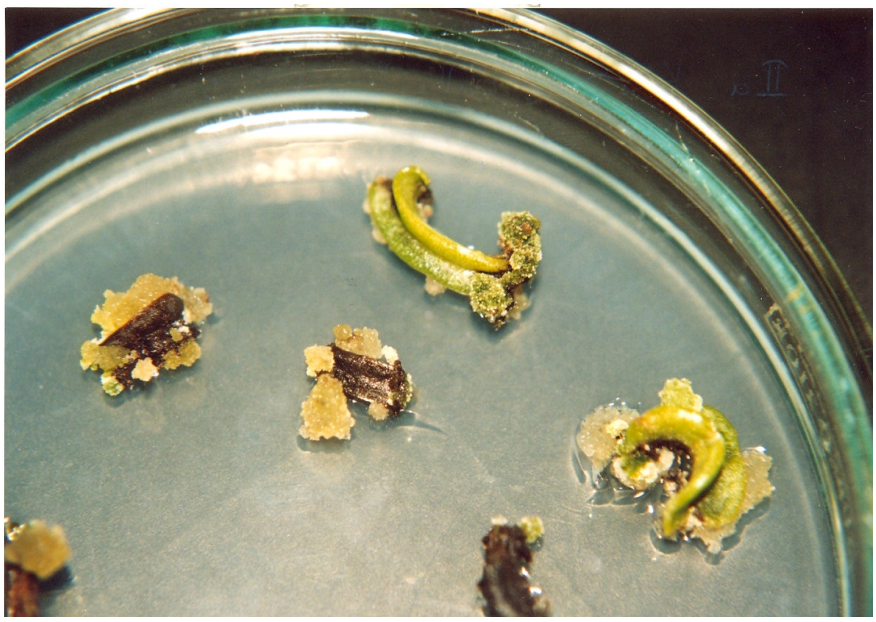

**Figure 3.2:** White and friable calli

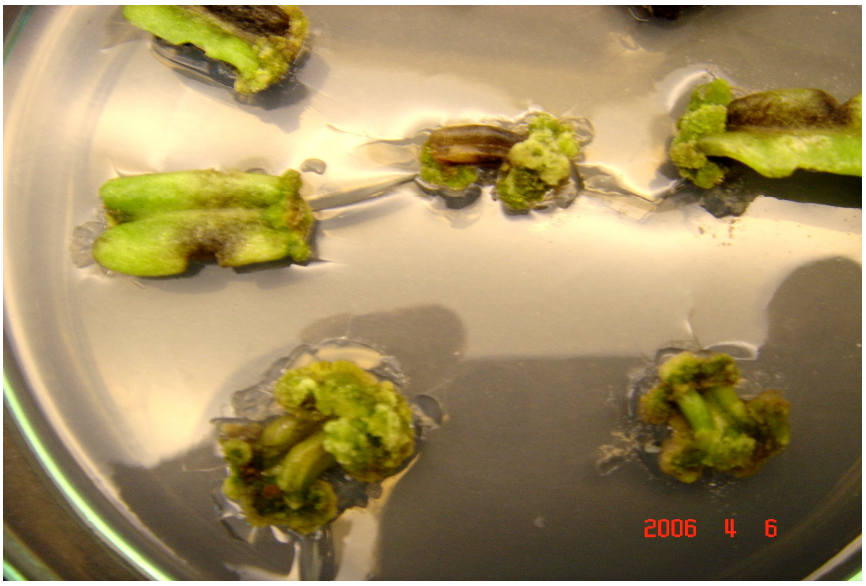


**Figure 3.3:** Green and compact calli

### 3.3 Experimental Sets

For investigating the tissue culture responses of 3 different genotypes (diploid, triploid and tetraploid), 4 different varieties (2 varieties for each of diploid and triploid genotypes, given in Table 3.2) and 2 lines (for tetraploid genotype, given in Table 3.2) from these sugar beet genotypes were used. Experimental sets were designed with triplicates.

**Table 3.2:** The genotypes and varieties and lines used in conjunction with experimental sets (Numbers are given to genotypes and varieties and lines for easing the evaluation of Duncan's Multiple Range Tests.)

| Genotype | Varieties |
|---|---|
| Diploid 1 | Variety 1: Soraya (KWS8123) |
| | Variety 2: Leila |
| | |
| Genotype | Varieties |
| Triploid 2 | Variety 3: Visa (H68121) |
| | Variety 4: Kassandra |
| | |
| Genotype | Lines |
| Tetraploid 3 | Line 5: ÇBM315 |
| | Line 6: EA2075 |

Only white and friable calli with regenerative capacity were used for data collection (Figure 3.2). Statistical analysis was conducted based on white and friable calli frequencies and weights.

### 3.3.1 Experimental Set 1

Experimental Set 1 aimed to test the effect of plant growth regulator combinations and sucrose concentration on different genotypes as well as varieties and lines with respect to callus induction. For callus induction, 8 different treatments were prepared by using MS medium including micro and macro elements and vitamins supplemented with 2 different cytokinin-type growth regulators (BAP and KIN) at 1.0 mg/L concentration, 2 different auxin-type growth regulators (NAA and 2,4-D) at 0.25 mg/L concentration and 2 different concentrations of sucrose (10 and 30 g/L) as summarized in Table 3.3.

**Table 3.3:** Description of Experimental Set 1

| Treatments* | [cytokinins] (1.0 mg/L) | [auxins] (0.25 mg/L) | [sucrose] (g/L) |
|---|---|---|---|
| 1 | BAP | NAA | 10 |
| 2 | BAP | 2,4-D | 10 |
| 3 | KIN | NAA | 10 |
| 4 | KIN | 2,4-D | 10 |
| 5 | BAP | NAA | 30 |
| 6 | BAP | 2,4-D | 30 |
| 7 | KIN | NAA | 30 |
| 8 | KIN | 2,4-D | 30 |

* Numbers are given to treatments for easing the evaluation of Duncan's Multiple Range Tests.

The data obtained from these treatments (callus frequency and callus weight) were subjected to Multivariate Analysis of Variance (ANOVA) with General Linear Model (GLM) by using SAS package.

The ANOVA results for callus frequency and callus weight values obtained from Experimental Set 1 treatments are given in Tables 3.3.1 and 3.3.2, respectively.

**Table 3.3.1:** Results of ANOVA for Experimental Set 1 (callus frequency)

Dependent Variable: **calf (callus frequency)**

| **Source** | **DF** | **Mean Square** |
|---|---|---|
| rep | 2 | 1237.05757 |
| trt | 7 | 4235.26174 |
| gen(trt) | 16 | 778.86507** |
| vari(trt) | 24 | 984.25354** |
| Error | 94 | 164.28253 |

** Significant at P<0.01

According to the table above, ANOVA results revealed significant effects of responses of genotypes as well as varieties and lines on callus induction frequency with respect to treatments (at P<0.01).

**Table 3.3.2:** Results of ANOVA for Experimental Set 1 (callus weight)

Dependent Variable: **calw (callus weight)**

| **Source** | **DF** | **Mean Square** |
|---|---|---|
| rep | 2 | 0.03642134 |
| trt | 7 | 0.03898317 |
| gen(trt) | 16 | 0.03154434** |
| vari(trt) | 24 | 0.03275474** |
| Error | 94 | 0.00963368 |

** Significant at P<0.01

According to the table above, ANOVA results revealed a similar significant effects of responses of genotypes as well as varieties and lines on callus weight with respect to treatments (at P<0.01).

In addition to ANOVA, Duncan's Multiple Range Tests (at 0.05 significance level) were also performed to investigate which treatment revealed a significantly different effect on callus frequency and callus weight at genotype as well as variety and line levels. The results of these tests for Experimental Set 1 at genotype level are given in Table 3.3.2.

**Table 3.3.3:** Results of Duncan's Multiple Range Tests for the means of genotype callus frequency and callus weight values (Experimental Set 1)

| Genotype* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 1 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 2 | 1 | 6 | 3.12 ± 2.13 g | 0.0892 ± 0.0609 bcd |
| 3 | 1 | 6 | 39.58 ± 7.51 bc | 0.0765 ± 0.0249 bcd |
| 1 | 2 | 6 | 4.17 ± 2.63 g | 0.0942 ± 0.0801 bcd |
| 2 | 2 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 3 | 2 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 1 | 3 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 2 | 3 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 3 | 3 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 1 | 4 | 6 | 31.25 ± 9.55 cd | 0.0638 ± 0.0228 bcd |
| 2 | 4 | 6 | 35.42 ± 8.17 bcd | 0.0305 ± 0.0069 cd |
| 3 | 4 | 6 | 47.92 ± 14.93 ab | 0.0733 ± 0.0262 bcd |
| 1 | 5 | 6 | 14.58 ± 12.25 efg | 0.0130 ± 0.0083 cd |
| 2 | 5 | 6 | 21.87 ± 7.87 def | 0.0663 ± 0.0301 bcd |
| 3 | 5 | 6 | 9.37 ± 3.87 fg | 0.0122 ± 0.0048 cd |
| 1 | 6 | 6 | 20.83 ± 11.93 def | 0.1123 ± 0.0845 bc |
| 2 | 6 | 6 | 27.08 ± 9.36 cde | 0.1685 ± 0.1021 b |
| 3 | 6 | 6 | 26.03 ± 11.46 cde | 0.0312 ± 0.0181 cd |
| 1 | 7 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 2 | 7 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 3 | 7 | 6 | 0.00 ± 0.00 g | 0.0000 ± 0.0000 d |
| 1 | 8 | 6 | 20.83 ± 9.50 def | 0.0315 ± 0.0176 cd |
| 2 | 8 | 6 | 25.00 ± 7.22 cde | 0.0275 ± 0.0109 cd |
| **3** | **8** | **6** | **58.33 ± 14.31 a** | **0.3322 ± 0.1657 a** |

* For numbers refer to Table 3.2 and 3.3.
[1] Same letters show statistical insignificance at 0.05 level.
[1] Bold scripts display the statistically significant PGR combination.

According to Table 3.3.3, genotype 3 (tetraploid) produced significantly higher callus frequency as well as callus weight values compared to the other two genotypes when treatment 8 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and 30 g/L sucrose) was employed. In agreement with our findings, callus induction has been reported on media composed of a combination of a cytokinin and an auxin (Van Geyt and Jacobs, 1985; Saunders and Shin, 1986; Freytag *et al*., 1988 and Gürel, 2000). Auxin-type growth regulator 2,4-D was more effective for callus induction than NAA and this is in agreement with other's findings (Hooker and Nabors, 1977 and Gürel, 2000). Gürel (2000) reported that in comparison of two types of cytokinin-type growth regulators, BAP was clearly more effective than KIN, especially when it was used at 1.0 mg/L in combination with 0.5 or 1.0 mg/L 2,4-D. However, we found KIN in combination with 2,4-D to be more effective in Experimental Set 1.

Callus frequency and callus weight data for Experimental Set 1 were also analyzed by Duncan's Multiple Range Tests at the variety and line levels. The results are given in Table 3.3.4.

**Table 3.3.4:** Results of Duncan's Multiple Range Tests for the means of variety and line callus frequency and callus weight values (Experimental Set 1)

| Variety and Line* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 1 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 2 | 1 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 3 | 1 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 4 | 1 | 3 | 6.23 ± 3.61 hi | 0.1783 ± 0.1032 bcde |
| 5 | 1 | 3 | 29.17 ± 11.04 cdefg | 0.1070 ± 0.0467 bcdef |
| 6 | 1 | 3 | 50.00 ± 7.23 b | 0.0460 ± 0.0012 def |
| 1 | 2 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 2 | 2 | 3 | 8.33 ± 4.17 hi | 0.1883 ± 0.1526 bcd |
| 3 | 2 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 4 | 2 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 5 | 2 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 6 | 2 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 1 | 3 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 2 | 3 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 3 | 3 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 4 | 3 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 5 | 3 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 6 | 3 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 1 | 4 | 3 | 12.50 ± 7.23 ghi | 0.0143 ± 0.0084 f |
| 2 | 4 | 3 | 50.00 ± 7.23 b | 0.1133 ± 0.0087 bcdef |
| 3 | 4 | 3 | 45.83 ± 15.04 bc | 0.0193 ± 0.0065 f |
| 4 | 4 | 3 | 25.00 ± 0.00 defgh | 0.0417 ± 0.0085 def |
| 5 | 4 | 3 | 16.67 ± 8.34 fghi | 0.0227 ± 0.0198 ef |
| **6** | **4** | **3** | **79.17 ± 8.34 a** | 0.1240 ± 0.0217 bcdef |
| 1 | 5 | 3 | 29.17 ± 23.23 cdefg | 0.0260 ± 0.0131 ef |
| 2 | 5 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 3 | 5 | 3 | 6.23 ± 3.61 hi | 0.0070 ± 0.0041 f |
| 4 | 5 | 3 | 37.50 ± 7.23 bcde | 0.1257 ± 0.0318 bcdef |
| 5 | 5 | 3 | 12.50 ± 7.23 ghi | 0.0090 ± 0.0052 f |
| 6 | 5 | 3 | 6.23 ± 3.61 hi | 0.0153 ± 0.0090 f |

**Table 3.3.4 (cont'd)**

| Variety and Line* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 6 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 2 | 6 | 3 | 41.67 ± 16.69 bcd | 0.2247 ± 0.1522 b |
| 3 | 6 | 3 | 37.50 ± 7.23 bcde | 0.1353 ± 0.1020 bcdef |
| 4 | 6 | 3 | 16.67 ± 16.69 fghi | 0.2017 ± 0.2019 bc |
| 5 | 6 | 3 | 33.33 ± 22.07 bcdef | 0.0510 ± 0.0346 cdef |
| 6 | 6 | 3 | 18.73 ± 10.84 efghi | 0.0113 ± 0.0067 f |
| 1 | 7 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 2 | 7 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 3 | 7 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 4 | 7 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 5 | 7 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 6 | 7 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 1 | 8 | 3 | 41.67 ± 4.17 bcd | 0.0630 ± 0.0235 cdef |
| 2 | 8 | 3 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 f |
| 3 | 8 | 3 | 29.17 ± 15.04 cdefg | 0.0227 ± 0.0121 ef |
| 4 | 8 | 3 | 20.83 ± 4.17 efgh | 0.0323 ± 0.0208 def |
| 5 | 8 | 3 | 29.17 ± 11.04 cdefg | 0.0333 ± 0.0007 def |
| **6** | **8** | **3** | **87.50 ± 7.23 a** | **0.6310 ± 0.2196 a** |

* For numbers refer to Table 3.2 and 3.3.
[1] Same letters show statistical insignificance at 0.05 level.
[1] Bold scripts display the statistically significant PGR combinations.

According to Table 3.3.4, line 6 (tetraploid, EA2075) displayed significantly high responses to treatments 4 and 8, when callus frequency parameter was concerned. The only difference between treatment 4 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and **10 g/L sucrose**) and treatment 8 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and **30 g/L sucrose**) was the concentration of sucrose. Therefore at least for this line, the concentration of

sucrose was inconsequential for callus induction from explants and probably served just as a carbon source rather than having any other effect on developmental processes that leads to callus induction. Similarly, according to Zhong *et al*. (1993), increasing the sucrose concentration of the medium from 3 % to 5 % or 8 % had no apparent effect.

As for callus weight, the same line (line 6, tetraploid, EA2075) responded positively only to treatment 8 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and 30 g/L sucrose). However, majority of these calli when incubated for further 4 weeks in their original media, containing 30 g/L sucrose for enlargement prior to shoot regeneration, discoloration and subsequent death occurred. Therefore, starting from the preparation stages (4 weeks) onwards throughout the shoot regeneration stages (12 weeks) the sucrose concentration was reduced to 10 g/L. Yıldız *et al*. (1997) has reported that sucrose content of MS medium could contribute to discoloration problem. In sugar beet crop, sugar is produced in leaves and transferred to roots in field conditions. But in culture conditions, sugar beet explants produce sugar in the form of sucrose and no root storage is possible. Therefore, addition of high amounts of sucrose to MS medium might not be necessary. Explant discoloration was significantly decreased in low sugar contents (Nicoli *et al*., 1991 and Yıldız *et al*., 1997). However, Yıldız *et al*. (1997) also reported that although discoloration was decreased, explant growth was unfortunately reduced.

Several researchers reported that BAP plays a key role in the process of shoot regeneration in sugar beet (Hussey and Hepher, 1978; Coumans-Gilles *et al*., 1981; Saunders, 1982; Freytag *et al*., 1988) but KIN was effective only at higher concentrations (Saunders and Shin, 1986) or was ineffective (Miedema *et al*., 1980; Coumans-Gilles *et al*., 1981). In addition, Zhong *et al*. (1993) confirmed that BAP

was much more active than KIN and that the optimum BAP concentration was between 0.5 and 1.0 mg/L. According to Tétu *et al*. (1987), friable calli obtained were subcultured on MS medium containing 1.0 mg/L BAP and 1.0 mg/L TIBA (a known inhibitor of the transport of endogenous auxin). After 2-3 weeks, greenish patches appeared on calli, which corresponded to the future buds (Table 3.4, treatment 3, Experimental Set 1 and Table 3.8, treatment 3, Experimental Set 3).

The three different shoot regeneration strategies used for Experimental Set 1 are shown in Table 3.4.

**Table 3.4:** Experimental Set 1 shoot regeneration treatments and supplementary material to MS media

| Treatments | [plant growth regulator] (mg/L) | [sucrose] g/L |
|---|---|---|
| 1 | 0.5 BAP | 10 |
| 2 | 1.0 BAP | 10 |
| 3 | 1.0 BAP + 1.0 TIBA | 10 |

Unfortunately, all the employed types and combinations of growth regulators were unsuccessful for shoot regeneration.

The overall results of Experimental Set 1;

- ✓ Responses of genotypes as well as varieties and lines had significant effects on both callus frequency and callus weight values with respect to treatments.

- ✓ Within genotypes, genotype 3 (tetraploid) responded best both for callus frequency and callus weight when treatment 8 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and 30 g/L sucrose) was employed.

- ✓ Within varieties and lines, line 6 (tetraploid, EA2075) responded best for callus frequency parameter regardless of sucrose concentration when the plant growth regulator combination of 1.0 mg/L KIN + 0.25 mg/L 2,4-D was employed.

- ✓ As for callus weight parameter, however, line 6 (tetraploid, EA2075) responded significantly different to 30 g/L sucrose concentration when compared to 10 g/L sucrose concentration with the same combination of plant growth regulators.

- ✓ For pre-shoot regeneration stage as well as through shoot regeneration stage 10 g/L sucrose was determined to be suitable.

- ✓ Unfortunately, the employed types and combinations of growth regulators were unsuccessful for shoot regeneration. Out of 1152 explants (24 explants, 8 treatments, 4 varieties and 2 lines) no shoot production was achieved.

### 3.3.2 Experimental Set 2

Both at the beginning of the Experimental Set 1 and at the shoot regeneration stage, tissue discoloration (Figure 3.4) was commonly observed, and therefore even before the completion of this set and statistical analysis, we constructed Experimental Set 2.

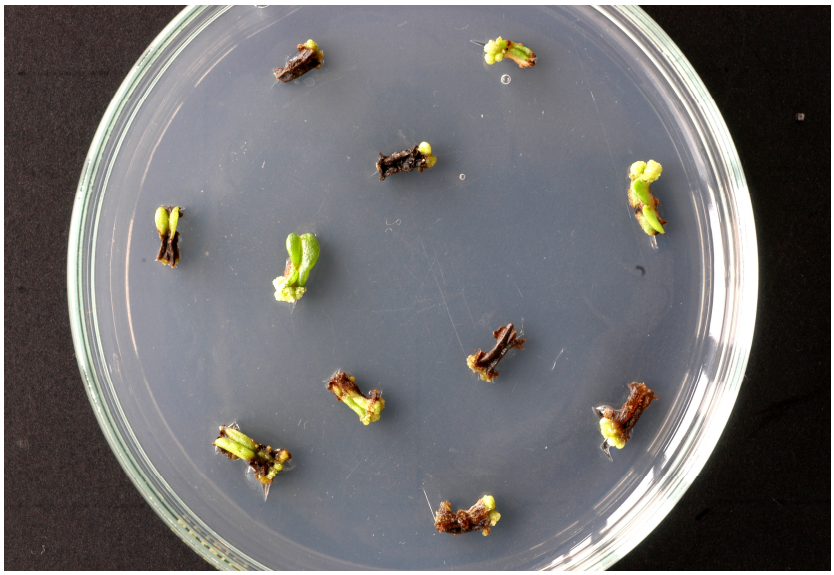

**Figure 3.4:** Tissue discoloration in sugar beet petiole explants

Experimental Set 2 aimed to test the effect of L-ascorbic acid on different genotypes as well as varieties and lines with respect to callus induction and subsequent shoot regeneration. While the media composition was kept constant (1.0 mg/L BAP + 0.25 mg/L NAA and 10 g/L sucrose) according to a study conducted (even before Experimental Set1) in Ankara University, Faculty of Agriculture, Department of Crop

Sciences, L-ascorbic acid treatments were employed due to its strong antioxidant effect. Two concentrations of L-ascorbic acid (10 and 20 mg/100 mL) (Bhagyalakshmi, 1993; Pizzocaro *et al*., 1993) were employed.

**Table 3.5:** Description of Experimental Set 2

| Treatments* | [L-ascorbic acid] (mg/100 mL) |
|---|---|
| 1 | 10 |
| 2 | 20 |

* Numbers are given to treatments for easing the evaluation of Duncan's Multiple Range Tests.

The ANOVA results for callus frequency and callus weight values obtained from Experimental Set 2 treatments are given in Tables 3.5.1 and 3.5.2, respectively.

**Table 3.5.1:** Results of ANOVA for Experimental Set 2 (callus frequency)

Dependent Variable: **calf (callus frequency)**

| **Source** | **DF** | **Mean Square** |
|---|---|---|
| rep | 2 | 52.083333 |
| trt | 1 | 156.250000 |
| gen(trt) | 4 | 693.359375** |
| vari(trt) | 6 | 97.656250* |
| Error | 22 | 30.776515 |

* Significant at P<0.05
** Significant at P<0.01

According to Table 3.5.1, ANOVA results revealed significant effects of responses of genotypes (at P<0.01) as well as varieties and lines (at P<0.05) on callus induction frequency with respect to treatments.

**Table 3.5.2:** Results of ANOVA for Experimental Set 2 (callus weight)

Dependent Variable: **calw (callus weight)**

| **Source** | **DF** | **Mean Square** |
|---|---|---|
| rep | 2 | 0.00000842 |
| trt | 1 | 0.00314534 |
| gen(trt) | 4 | 0.00483972** |
| vari(trt) | 6 | 0.00056631** |
| Error | 22 | 0.00005533 |

** Significant at P<0.01

According to the table above, ANOVA results revealed similar significant effects of responses of genotypes as well as varieties and lines on callus weight with respect to treatments (at P<0.01).

In addition to ANOVA, Duncan's Multiple Range Tests (at 0.05 significance level) were also performed to investigate which treatment revealed a significantly different effect on callus frequency and callus weight at genotype as well as at variety and line levels. The results of these tests for Experimental Set 2 are given in Table 3.5.3.

**Table 3.5.3:** Results of Duncan's Multiple Range Tests for the means of genotype callus frequency and callus weight values (Experimental Set 2)

| Genotype* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 1 | 6 | 0.00 ± 0.00 c | 0.0000 ± 0.0000 c |
| 2 | 1 | 6 | 3.13 ± 2.13 bc | 0.0038 ± 0.0026 b |
| 3 | 1 | 6 | 9.38 ± 6.40 b | 0.0093 ± 0.0063 b |
| 1 | 2 | 6 | 0.00 ± 0.00 c | 0.0000 ± 0.0000 c |
| 2 | 2 | 6 | 0.00 ± 0.00 c | 0.0000 ± 0.0000 c |
| **3** | **2** | **6** | **25.00 ± 0.00 a** | **0.0691 ± 0.0104 a** |

* For numbers refer to Table 3.2 and 3.5.
[1] Same letters show statistical insignificance at 0.05 level.
[1] Bold scripts display the statistically significant PGR combination.

According to the table above, genotype 3 (tetraploid) produced significantly the highest callus frequency as well as callus weight values compared to the other two genotypes when treatment 2 (20 mg/100 mL L-ascorbic acid) was employed.

Callus frequency and callus weight data for Experimental Set 2 were also analyzed by Duncan's Multiple Range Tests at the variety and line levels. The results are given in Table 3.5.4.

**Table 3.5.4:** Results of Duncan's Multiple Range Tests for the means of variety and line callus frequency and callus weight values (Experimental Set 2)

| Variety and Line* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 1 | 3 | 0.00 ± 0.00 b | 0.0000 ± 0.0000 d |
| 2 | 1 | 3 | 0.00 ± 0.00 b | 0.0000 ± 0.0000 d |
| 3 | 1 | 3 | 0.00 ± 0.00 b | 0.0000 ± 0.0000 d |
| 4 | 1 | 3 | 6.25 ± 3.61 b | 0.0075 ± 0.0044 cd |
| 5 | 1 | 3 | 0.00 ± 0.00 b | 0.0000 ± 0.0000 d |
| **6** | **1** | **3** | **18.75 ± 10.84 a** | 0.0185 ± 0.0107 c |
| 1 | 2 | 3 | 0.00 ± 0.00 b | 0.0000 ± 0.0000 d |
| 2 | 2 | 3 | 0.00 ± 0.00 b | 0.0000 ± 0.0000 d |
| 3 | 2 | 3 | 0.00 ± 0.00 b | 0.0000 ± 0.0000 d |
| 4 | 2 | 3 | 0.00 ± 0.00 b | 0.0000 ± 0.0000 d |
| **5** | **2** | **3** | **25.00 ± 0.00 a** | 0.0475 ± 0.0008 b |
| **6** | **2** | **3** | **25.00 ± 0.00 a** | **0.0907 ± 0.0085 a** |

* For numbers refer to Table 3.2 and 3.5.
[1] Same letters show statistical insignificance at 0.05 level.
[1] Bold scripts display the statistically significant PGR combinations.

Interesting results are present in Table 3.5.4. To start with, line 6 (tetraploid, EA2075) produced significantly high callus frequency responses to both treatments, while line 5 (tetraploid, ÇBM315) produced significantly high callus frequency response only to treatment 2 (20 mg/100 mL L-ascorbic acid). One has to notice that both of these lines belong to genotype 3 (tetraploid), which also behaved similarly, as can be seen in Table 3.5.3. Similarly, Kolodyazhnaya and Deineko (2002) reported that there was a relationship between the sugar beet genotype and capacity of callus formation and organogenesis.

As for callus weight, line 6 (tetraploid, EA2075) produced significantly high callus weight response to treatment 2 (20 mg/100 mL L-ascorbic acid).

These calli, following the data collection, were further incubated in fresh same medium for 4 weeks for callus enlargement purposes prior to shoot regeneration. However, at the second week of this incubation, two spontaneous shoots appeared from line 6 (tetraploid, EA2075). In agreement with this finding, Jacq *et al*. (1993) found variation among sugar beet lines in response to callus induction and subsequent plant regeneration. Similarly, the cultivar can significantly affect adventitious shoot regeneration from both callus and petioles (Ritchie *et al*., 1989). Our study confirmed the work of Freytag *et al*. (1988) and Jacq *et al*. (1993), who reported that both organic supplements and phytohormones were required to initiate organogenic calli in sugar beet cultures.

No further shoot regeneration treatments were applied to the rest of the formed calli. At the end of the 12-week period, however, no further shoots were obtained (Table 3.6).

From these shoots, the one being vitrified in appearance (Figure 3.5) was incubated in MS medium supplemented with 10 g/L sucrose without any plant growth regulator (Roussy *et al*., 1996) for 1-2 weeks. When the symptoms of vitrification were diminished, this shoot was transferred to root induction medium (MS medium supplemented with 3.0 mg/L IBA and 30 g/L sucrose).

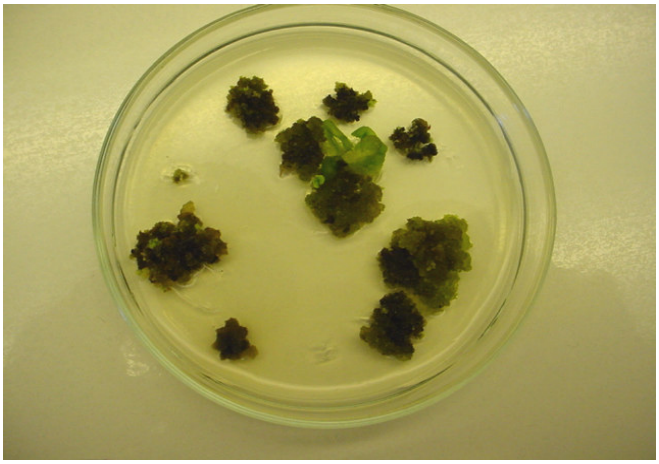

**Figure 3.5:** Vitrified shoot obtained from line 6 (tetraploid, EA2075)

The other shoot which had non-vitrified appearance (Figure 3.6) was directly transferred to root induction medium (Gürel *et al*., 2002). Rooting media were refreshed every 4 weeks.

**Figure 3.6:** Non-vitrified shoots obtained from line 6 (tetraploid, EA2075)

Shoots grown to at least 8 cm long (Figure 3.7) with strong and dense roots (Figure 3.8) were transferred to pots containing garden soil (Figure 3.9). In order to attain high humidity (acclimatization), the potted tissue culture plants were covered by plastic bags and incubated in growth room.

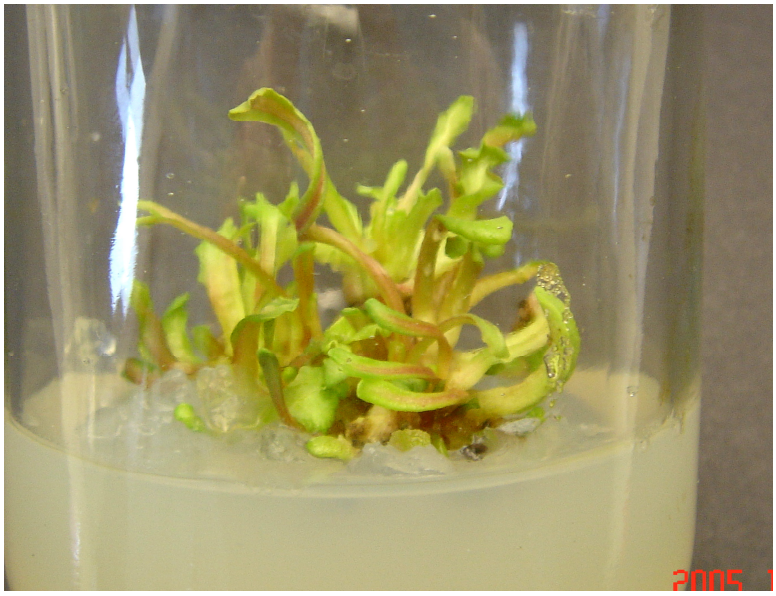

**Figure 3.7:** Rooted shoots of line 6 (tetraploid, EA2075)


**Figure 3.8:** Roots of line 6 (tetraploid, EA2075)


**Figure 3.9:** Potted plantlets of line 6 (tetraploid, EA2075)

The ratios and percentages of shoot production and root production in Experimental Set 2 are given in Table 3.6.

**Table 3.6**: Shooting and rooting for Experimental Set 2 at variety and line levels

| **Variety and line*** | **Treatment*** | **N** | **Shooting (%)** | **Rooting (%)** |
|---|---|---|---|---|
| 1 | 1 | 3 | 0.00 | 0.00 |
| 2 | 1 | 3 | 0.00 | 0.00 |
| 3 | 1 | 3 | 0.00 | 0.00 |
| 4 | 1 | 3 | 0.00 | 0.00 |
| 5 | 1 | 3 | 0.00 | 0.00 |
| 6 | 1 | 3 | 0.00 | 0.00 |
| 1 | 2 | 3 | 0.00 | 0.00 |
| 2 | 2 | 3 | 0.00 | 0.00 |
| 3 | 2 | 3 | 0.00 | 0.00 |
| 4 | 2 | 3 | 0.00 | 0.00 |
| 5 | 2 | 3 | 0.00 | 0.00 |
| **6** | **2** | **3** | **2 / 24 (8.33)** | **2 / 2 (100)** |

* For numbers refer to Table 3.2 and 3.5.

According to the table above, shoot production was observed only when treatment 2 (20 mg/100 mL L-ascorbic acid) was employed (Figure 3.5). Only line 6 (tetraploid, EA2075) produced shoots with a ratio of 2/24 (for each treatment, 24 explants from each variety and line), which equals 8.33 %. Since both shoots later formed roots, rooting ratio was 2/2, which equals 100 %.

The overall results of Experimental Set 2;

- ✓ Responses of genotypes as well as varieties and lines had significant effects on both callus frequency and callus weight values with respect to treatments.

- ✓ Within genotypes, genotype 3 (tetraploid) produced significantly the highest callus frequency as well as callus weight values when treatment 2 (20 mg/100 mL L-ascorbic acid) was employed.

- ✓ Within varieties and lines, line 6 (tetraploid, EA2075) produced significantly high callus frequency values regardless of sucrose concentration, whereas line 5 (tetraploid, ÇBM315) only produced high and statistically equally significant callus frequency value when 10 mg/100 mL L-ascorbic acid was employed.

- ✓ Line 6 (tetraploid, EA2075) produced the 2 shoots out of 24 explants, when treatment 2 (20 mg/100 mL L-ascorbic acid) was employed. These 2 shoots were successfully rooted.

- ✓ Out of 288 explants (24 explants, 2 treatments, 4 varieties and 2 lines) 2 shoots and subsequent 2 whole plants were achieved.

### 3.3.3 Experimental Set 3

Experimental Set 3 aimed to test the combined effect of L-ascorbic acid and silver nitrate on different genotypes as well as varieties and lines with respect to callus induction. For callus induction, 12 different treatments were prepared by using MS medium including micro and macro elements and vitamins supplemented with 2 different cytokinin-type growth regulators (BAP (1.0 and 2.0 mg/L) and KIN (1.0 mg/L)), 2 different auxin-type growth regulators (NAA (0.25 mg/L) and 2,4-D (0.25 and 1.0 mg/L)) and 2 different concentrations of sucrose (10 and 30 g/L) as summarized in Table 3.7. All media included L-ascorbic acid at a fixed concentration (20 mg/100 mL) as well as silver nitrate at a fixed concentration (2.5 mg/L) (Gürel, 2000).

**Table 3.7:** Description of Experimental Set 3

| Treatments* | [cytokinin] (mg/L) | [auxin] (mg/L) | [sucrose] (g/L) |
|---|---|---|---|
| 1 | 1.0 BAP | 0.25 NAA | 10 |
| 2 | 1.0 BAP | 0.25 2,4-D | 10 |
| 3 | 1.0 KIN | 0.25 NAA | 10 |
| 4 | 1.0 KIN | 0.25 2,4-D | 10 |
| 5 | 2.0 BAP | 0.25 NAA | 10 |
| 6 | 1.0 KIN | 1.0 2,4-D | 10 |
| 7 | 1.0 BAP | 0.25 NAA | 30 |
| 8 | 1.0 BAP | 0.25 2,4-D | 30 |
| 9 | 1.0 KIN | 0.25 NAA | 30 |
| 10 | 1.0 KIN | 0.25 2,4-D | 30 |
| 11 | 2.0 BAP | 0.25 NAA | 30 |
| 12 | 1.0 KIN | 1.0 2,4-D | 30 |

* Numbers are given to treatments for easing the evaluation of Duncan's Multiple Range Tests.

The ANOVA results for callus frequency and callus weight values obtained from Experimental Set 3 treatments are given in Tables 3.7.1 and 3.7.2, respectively.

**Table 3.7.1:** Results of ANOVA for Experimental Set 3 (callus frequency)

Dependent Variable: **calf (callus frequency)**

| **Source** | **DF** | **Mean Square** |
|---|---|---|
| rep | 2 | 312.50019 |
| trt | 11 | 1324.24103 |
| gen(trt) | 24 | 1435.61650** |
| vari(trt) | 36 | 302.90817** |
| Error | 142 | 20.90688 |

** Significant at P<0.01

According to the table above, ANOVA results revealed significant effects of responses of genotypes as well as varieties and lines on callus induction frequency with respect to treatments (at P<0.01).

**Table 3.7.2:** Results of ANOVA for Experimental Set 3 (callus weight)

Dependent Variable: **calw (callus weight)**

| **Source** | **DF** | **Mean Square** |
|---|---|---|
| rep | 2 | 0.00004859 |
| trt | 11 | 0.00379446 |
| gen(trt) | 24 | 0.00582636** |
| vari(trt) | 36 | 0.00236081** |
| Error | 142 | 0.00041882 |

** Significant at P<0.01

According to the table above, ANOVA results revealed similar significant effects of responses of genotypes as well as varieties and lines on callus weight with respect to treatments (at P<0.01).

In addition to ANOVA, Duncan's Multiple Range Tests (at 0.05 significance level) were also performed to investigate which treatment revealed a significantly different effect on callus frequency and callus weight at genotype as well as variety and line levels. The results of these tests for Experimental Set 3 are given in Table 3.7.3.

**Table 3.7.3:** Results of Duncan's Multiple Range Tests for the means of genotype callus frequency and callus weight values (Experimental Set 3)

| Genotype* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 1 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 2 | 1 | 6 | 28.13 ± 12.68 c | 0.0448 ± 0.0213 cde |
| 3 | 1 | 6 | 3.13 ± 2.13 hi | 0.0019 ± 0.0013 gh |
| 1 | 2 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 2 | 2 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 3 | 2 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 1 | 3 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 2 | 3 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 3 | 3 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 1 | 4 | 6 | 3.13 ± 2.13 hi | 0.0059 ± 0.0040 gh |
| **2** | **4** | **6** | 28.13 ± 2.13 c | **0.1112 ± 0.0354 a** |
| 3 | 4 | 6 | 21.91 ± 7.18 d | 0.0036 ± 0.0010 gh |
| 1 | 5 | 6 | 3.13 ± 2.13 hi | 0.0050 ± 0.0034 gh |
| **2** | **5** | **6** | **65.63 ± 3.87 a** | 0.0571 ± 0.0047 cd |
| 3 | 5 | 6 | 3.13 ± 2.13 hi | 0.0025 ± 0.0017 gh |
| 1 | 6 | 6 | 25.00 ± 0.00 cd | 0.0238 ± 0.0059 efg |
| 2 | 6 | 6 | 21.88 ± 2.13 d | 0.0356 ± 0.0036 def |
| 3 | 6 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 1 | 7 | 6 | 15.63 ± 7.17 e | 0.0127 ± 0.0071 fgh |
| 2 | 7 | 6 | 25.00 ± 5.82 cd | 0.0626 ± 0.0212 bc |
| 3 | 7 | 6 | 12.50 ± 4.56 ef | 0.0024 ± 0.0009 gh |
| 1 | 8 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 2 | 8 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 3 | 8 | 6 | 9.38 ± 4.49 fg | 0.0026 ± 0.0012 h |
| 1 | 9 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 2 | 9 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 3 | 9 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 1 | 10 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 2 | 10 | 6 | 25.00 ± 3.61 cd | 0.0523 ± 0.0074 cd |
| 3 | 10 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 1 | 11 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |
| 2 | 11 | 6 | 46.88 ± 4.49 b | 0.0812 ± 0.0137 b |
| 3 | 11 | 6 | 15.63 ± 4.49 e | 0.0072 ± 0.0019 gh |

**Table 3.7.3 (cont'd)**

| Genotype* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 12 | 6 | 6.25 ± 4.27 gh | 0.0239 ± 0.0163 efg |
| 2 | 12 | 6 | 21.88 ± 5.53 d | 0.0862 ± 0.0446 b |
| 3 | 12 | 6 | 0.00 ± 0.00 i | 0.0000 ± 0.0000 h |

* For numbers refer to Table 3.2 and 3.7.
[1] Same letters show statistical insignificance at 0.05 level.
[1] Bold scripts display the statistically significant PGR combinations.

According to Table 3.7.3, genotype 2 (triploid) produced significantly the highest callus frequency values when treatment 5 (2.0 mg/L BAP + 0.25 mg/L NAA and 10 g/L sucrose) was employed. Catlin (1990) obtained callus and plantlet from cotyledons on medium containing 0.2 mg/L BAP only. Organogenic callus from hypocotyl explants of sugar beet was also initiated at combined concentrations of 0.3 mg/L BAP and 0.1 mg/L NAA (Jacq *et al*., 1992). Treatment 5 did not create the same effect on callus weight, whereas the same genotype yielded significantly the highest callus weight when treatment 4 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and 10 g/L sucrose) was employed. As for callus weight, similar to Experimental Set 1, KIN in combination with 2,4-D was found to be more effective.

Callus frequency and callus weight data for Experimental Set 3 were also analyzed by Duncan's Multiple Range Tests at variety and line levels. The results are given in Table 3.7.4.

**Table 3.7.4:** Results of Duncan's Multiple Range Tests for the means of variety and line callus frequency and callus weight values (Experimental Set 3)

| Variety and Line* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 1 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 2 | 1 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 3 | 1 | 3 | 56.25 ± 3.61 b | 0.0896 ± 0.0161 bc |
| 4 | 1 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 5 | 1 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 6 | 1 | 3 | 6.25 ± 3.61 gh | 0.0038 ± 0.0022 gh |
| 1 | 2 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 2 | 2 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 3 | 2 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 4 | 2 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 5 | 2 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 6 | 2 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 1 | 3 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 2 | 3 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 3 | 3 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 4 | 3 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 5 | 3 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 6 | 3 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 1 | 4 | 3 | 6.25 ± 3.61 gh | 0.0117 ± 0.0068 fgh |
| 2 | 4 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| **3** | **4** | **3** | 31.25 ± 3.61 cd | **0.1752 ± 0.0457 a** |
| 4 | 4 | 3 | 25.00 ± 0.00 de | 0.0472 ± 0.0101def |
| 5 | 4 | 3 | 6.25 ± 3.61 gh | 0.0019 ± 0.0011 gh |
| 6 | 4 | 3 | 37.57 ± 0.07 c | 0.0054 ± 0.0008 gh |
| 1 | 5 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 2 | 5 | 3 | 6.25 ± 3.61 gh | 0.0101 ± 0.0058 gh |
| **3** | **5** | **3** | **68.75 ± 3.61 a** | 0.0620 ± 0.0046 cd |
| 4 | 5 | 3 | 62.50 ± 7.23 ab | 0.0522 ± 0.0082 cde |
| 5 | 5 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 6 | 5 | 3 | 6.25 ± 3.61 gh | 0.0050 ± 0.0029 gh |
| 1 | 6 | 3 | 25.00 ± 0.00 de | 0.0327 ± 0.0063 defg |
| 2 | 6 | 3 | 25.00 ± 0.00 de | 0.0149 ± 0.0072 fgh |
| 3 | 6 | 3 | 18.75 ± 3.61 ef | 0.0326 ± 0.0048 defg |

**Table 3.7.4 (cont'd)**

| Variety and Line* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 4 | 6 | 3 | 25.00 ± 0.00 de | 0.0387 ± 0.0057 defg |
| 5 | 6 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 6 | 6 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 1 | 7 | 3 | 31.25 ± 3.61 cd | 0.0253 ± 0.0097 efgh |
| 2 | 7 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 3 | 7 | 3 | 31.25 ± 3.61 cd | 0.1083 ± 0.0077 b |
| 4 | 7 | 3 | 18.75 ± 10.84 ef | 0.0168 ± 0.0097 fgh |
| 5 | 7 | 3 | 12.50 ± 7.23 fg | 0.0030 ± 0.0017 gh |
| 6 | 7 | 3 | 12.50 ± 7.23 fg | 0.0018 ± 0.0010 gh |
| 1 | 8 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 2 | 8 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 3 | 8 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 4 | 8 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 5 | 8 | 3 | 18.75 ± 3.61 ef | 0.0052 ± 0.0005 gh |
| 6 | 8 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 1 | 9 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 2 | 9 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 3 | 9 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 4 | 9 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 5 | 9 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 6 | 9 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 1 | 10 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 2 | 10 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 3 | 10 | 3 | 31.25 ± 3.61 cd | 0.0580 ± 0.0027 cde |
| 4 | 10 | 3 | 18.75 ± 3.61 ef | 0.0466 ± 0.0154 def |
| 5 | 10 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 6 | 10 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 1 | 11 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 2 | 11 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 3 | 11 | 3 | 56.25 ± 3.61 b | 0.1021 ± 0.0224 b |
| 4 | 11 | 3 | 37.50 ± 0.00 c | 0.0603 ± 0.0002 cd |
| 5 | 11 | 3 | 6.25 ± 3.61 gh | 0.0043 ± 0.0025 gh |
| 6 | 11 | 3 | 25.00 ± 0.00 de | 0.0101 ± 0.0018 fgh |

**Table 3.7.4 (cont'd)**

| Variety and Line* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 12 | 3 | 12.50 ± 7.23 fg | 0.0478 ± 0.0276 def |
| 2 | 12 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| **3** | **12** | **3** | 31.25 ± 3.61 cd | **0.1538 ± 0.0727 a** |
| 4 | 12 | 3 | 12.50 ± 7.23 fg | 0.0186 ± 0.0108 fgh |
| 5 | 12 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |
| 6 | 12 | 3 | 0.00 ± 0.00 h | 0.0000 ± 0.0000 h |

* For numbers refer to Table 3.2 and 3.7.
[1] Same letters show statistical insignificance at 0.05 level.
[1] Bold scripts display the statistically significant PGR combinations.

According to Table 3.7.4, variety 3 (triploid, Visa (H68121)) produced significantly the highest callus frequency value when treatment 5 (2.0 mg/L BAP + 0.25 mg/L NAA and 10 g/L sucrose) was employed. However, this treatment did not create the same effect on callus weight, whereas the same variety yielded significantly high callus weight values when treatments 4 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and 10 g/L sucrose) and 12 (1.0 mg/L KIN + 1.0 mg/L 2,4-D and 30 g/L sucrose) were employed. However, there was no significant difference between these two treatments.

Unfortunately, shoot regeneration studies for Experimental Set 3 as given in Table 3.8 resulted with no shoots.

**Table 3.8:** Experimental Set 3 shoot regeneration treatments and supplementary material to MS media

| Treatments | [plant growth regulator] (mg/L) | [sucrose] g/L |
|---|---|---|
| 1 | - (Hormone Free) | 10 |
| 2 | 1.0 BAP | 10 |
| 3 | 1.0 Zeatin + 1.0 TIBA | 10 |

Ozden-Tokatli *et al*. (2005) reported that ethylene can play a negative regulatory role in micropropagation of pistachio and silver nitrate seems to be effective in counteracting this regulation. $AgNO_3$-containing media completely inhibited browning in pistachio tissues. Addition of $AgNO_3$ to the culture medium improved the regeneration frequency and shoot growth, and reduced basal callus formation in all regenerated explants indicating the possible role of ethylene on basal callus formation of pistachio shoots.

Inhibition of tissue browning was also reported in mangosteen leaf cultures with the use of silver nitrate in the media (Goh *et al*., 1997). However, it was reported that ethylene could cause considerable callus growth at high concentrations in mangosteen leaf explants (Goh *et al*., 1997). The same concomitant inhibition of callus growth also occurred in cassava (Zhang *et al*., 2001) with the addition of silver nitrate to the medium.

According to Gürel (2000), for haploid embryo induction, sugar beet lines were cultured on MS medium containing 2.0 mg/L BAP in combination with 2.5 or 5.0 mg/L $AgNO_3$, or with 0.5 % charcoal. The inclusion of $AgNO_3$ in the culture medium either decreased or completely inhibited embryo formation from ovules. The addition of 0.5 % activated charcoal increased the rate of haploid embryo formation.

In Experimental Set 3 callus formation was greatly reduced compared to the other sets. This is in agreement with some previous studies (Goh *et al*., 1997; Zhang *et al*., 2001; Ozden-Tokatli *et al*., 2005). Since shoot regeneration could not be achieved, we are recommending not to use $AgNO_3$ in sugar beet tissue culture, at least for the employed concentration in this study.

The overall results of Experimental Set 3;

- ✓ Responses of genotypes as well as varieties and lines had significant effects on both callus frequency and callus weight values with respect to treatments.

- ✓ Within genotypes, genotype 2 (triploid) produced significantly the highest callus frequency values when treatment 5 (2.0 mg/L BAP + 0.25 mg/L NAA and 10 g/L sucrose) was employed. However, this treatment did not create the same effect on callus weight, whereas the same genotype yielded significantly the highest callus weight when treatment 4 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and 10 g/L sucrose) was employed.

- ✓ Within varieties and lines, variety 3 (triploid, Visa (H68121)) produced significantly the highest callus frequency value when treatment 5 (2.0 mg/L BAP + 0.25 mg/L NAA and 10 g/L sucrose) was employed. However, this treatment did not create the same effect on callus weight, whereas the same variety yielded significantly high callus weight values when treatments 4 (1.0 mg/L KIN + 0.25 mg/L 2,4-D and 10 g/L sucrose) and 12 (1.0 mg/L KIN + 1.0 mg/L 2,4-D and 30 g/L sucrose) were employed. However, there was no significant difference between these two treatments.

- ✓ Unfortunately, the employed types and combinations of growth regulators were unsuccessful for shoot regeneration. Out of 1728 explants (24 explants, 12 treatments, 4 varieties and 2 lines) no shoot production was achieved.

### 3.3.4 Experimental Set 4

Experimental Set 4 was mainly designed for 1) testing the probable effects of Zeatin, as the cytokinin source, on callus frequency and callus weight of different genotypes as well as varieties and lines, 2) by combining two studies (Yıldız *et al*., 1997 and Gürel, 2000) testing the probable effects of "cold treatment" of callus on subsequent shoot regeneration of different genotypes as well as varieties and lines.

For callus induction, 2 different treatments were prepared by using MS medium including micro and macro elements and vitamins supplemented with plant growth regulators and sucrose as given in Table 3.9.

**Table 3.9:** Description of Experimental Set 4

| Treatments* | [plant growth regulator] (mg/L) | [sucrose] g/L |
|---|---|---|
| 1 | 1.0 Zeatin + 0.25 2,4-D + 1.0 TIBA | 10 |
| 2 | 1.0 BAP + 0.25 NAA | 10 |

* Numbers are given to treatments for easing the evaluation of Duncan's Multiple Range Tests.

The ANOVA results for callus frequency and callus weight values obtained from Experimental Set 4 treatments are given in Tables 3.9.1 and 3.9.2, respectively.

**Table 3.9.1**: Results of ANOVA for Experimental Set 4 (callus frequency)

Dependent Variable: **calf (callus frequency)**

| **Source** | **DF** | **Mean Square** |
|---|---|---|
| rep | 2 | 117.187500 |
| trt | 1 | 0.000000 |
| gen(trt) | 4 | 1054.687500** |
| vari(trt) | 6 | 97.656250ns |
| Error | 22 | 60.369318 |

** Significant at P<0.01
ns Not significant

According to the table above, ANOVA results revealed significant effects of responses of genotypes (at P<0.01) on callus induction frequency with respect to treatments. However, effects of varieties and lines were insignificant on callus induction frequency.

**Table 3.9.2:** Results of ANOVA for Experimental Set 4 (callus weight)

Dependent Variable: **calw (callus weight)**

| **Source** | **DF** | **Mean Square** |
|---|---|---|
| rep | 2 | 0.00010890 |
| trt | 1 | 0.00003885 |
| gen(trt) | 4 | 0.00081591** |
| vari(trt) | 6 | 0.00009974ns |
| Error | 22 | 0.00004669 |

** Significant at P<0.01
ns Not significant

According to the table above, ANOVA results revealed similar significant effects of responses of genotypes (at P<0.01) on callus weight with respect to treatments, but the effect of responses of varieties and lines were insignificant on callus weight with respect to treatments.

In addition to ANOVA, Duncan's Multiple Range Tests (at 0.05 significance level) were also performed to investigate which treatment revealed a significantly different effect on callus frequency and callus weight at genotype level (Table 3.9.3). Since the ANOVA results at variety and line levels were insignificant, Duncan's Multiple Range Tests were not performed for those data.

**Table 3.9.3:** Results of Duncan's Multiple Range Tests for the means of genotype callus frequency and callus weight values (Experimental Set 4)

| Genotype* | Treatment* | N | Mean ± SE[1] (calf) | Mean ± SE[1] (calw) |
|---|---|---|---|---|
| 1 | 1 | 6 | 0.00 ± 0.00 d | 0.0000 ± 0.0000 d |
| **2** | **1** | **6** | **28.13 ± 2.13 a** | **0.0228 ± 0.0012 a** |
| 3 | 1 | 6 | 0.00 ± 0.00 d | 0.0000 ± 0.0000 d |
| 1 | 2 | 6 | 9.38 ± 6.40 c | 0.0092 ± 0.0063 c |
| 2 | 2 | 6 | 18.75 ± 5.10 b | 0.0199 ± 0.0046 b |
| 3 | 2 | 6 | 0.00 ± 0.00 d | 0.0000 ± 0.0000 d |

* For numbers refer to Table 3.2 and 3.9.
[1] Same letters show statistical insignificance at 0.05 level.
[1] Bold scripts display the statistically significant PGR combinations.

According to the table above, genotype 2 (triploid) produced significantly high callus frequency and callus weight when treatment 1 (1.0 mg/L Zeatin + 0.25 mg/L 2,4-D + 1.0 mg/L TIBA and 10 g/L sucrose) was employed. In a similar manner, plants were regenerated from petiole explants of six elite sugar beet (*Beta vulgaris* L.) cultivars. The significant variation observed in regeneration percentages from the six cultivars tested, emphasized the importance of genotype for organogenesis in sugar beet (Grieve *et al*., 1997).

Saunders and Shin (1986) reported that Zeatin alone results in induction of buds, but at a higher concentration of 10 mg/L. However, all the calli obtained from treatment 1 failed to enlarge and eventually died, possibly due to low Zeatin concentration employed.

The calli obtained from treatment 2 (1.0 mg/L BAP + 0.25 mg/L NAA and 10 g/L sucrose) were transferred to MS medium supplemented with BAP (1.0 mg/L) and 10 g/L sucrose and incubated at 4 $^{0}$C in dark for 2 weeks (Gürel, 2000). Next, cold-treated calli were transferred to MS medium supplemented with BAP (2.0 mg/L) and 10 g/L sucrose and incubated in growth room for 4 weeks.

In this study, we could not regenerate any shoots by using "cold treatment" approach. Some workers reported that cold pre-treatment increased shoot formation from callus (Coumans *et al*., 1982 and Gürel, 2000). In contrast, De Greef and Jacobs (1979) reported that treatment of callus with cold (4 $^{0}$C) for 3-9 weeks did not increase shoot formation from the callus.

The overall results of Experimental Set 4;

- ✓ Responses of only genotypes had significant effects on both callus frequency and callus weight values with respect to treatments.

- ✓ Within genotypes, genotype 2 (triploid) produced significantly the highest callus frequency and callus weight values when treatment 1 (1.0 mg/L Zeatin + 0.25 mg/L 2,4-D, + 1.0 mg/L TIBA and 10 g/L sucrose) was employed. However, these calli obtained from treatment 1 failed to enlarge and eventually died.

- ✓ Calli obtained from treatment 2 (1.0 mg/L BAP + 0.25 mg/L NAA and 10 g/L sucrose) although subjected to cold treatment and subsequent shoot regeneration according to Gürel (2000), no shoots were recovered.

- ✓ Out of 288 explants (24 explants, 2 treatments, 4 varieties and 2 lines) no shoot production was achieved.

### 3.3.5 Plant Recovery Efficiency of the Four Experimental Sets

Throughout the four experimental sets a total of 3456 (1152 (set 1), 288 (set 2), 1728 (set 3), 288 (set 4)) explants were used and only 2 shoots were regenerated. These 2 shoots successfully produced 2 whole plants. As a result, plant recovery efficiency was 2 plants out of 3456 explants, which makes a rather low value of 0.058 %.

In this study, experimental sets with various media compositions were conducted to achieve indirect organogenesis of sugar beet (*Beta vulgaris* L.). However, a very low value of plant recovery efficiency was obtained. According to Baloğlu (2005), different media were used to achieve indirect organogenesis of sugar beet cultivar ELK345. Cotyledon, hypocotyl, petiole and leaf explants were used. Except cotyledon, other explants produced calli. However, no shoot development was observed from calli of these explants. Baloğlu (2005) reported that shoot base tissue of sugar beet cultivar 1195 was employed for direct organogenesis. Average percentage of shoot regeneration per leaf blade was found to be 22.4 %.

In our study, both of the 2 shoots developed roots with the use of 3.0 mg/L IBA. Previously, Gürel *et al*. (2003) reported shoot regeneration from petiole explants and subsequent root regeneration from regenerated shoots. They obtained 42.6 % of root formation from regenerated shoots using 3 mg/L NAA. This difference may be due to the utilization of different sugar beet lines and the use of different auxin-type plant growth regulators.

### 3.3.6 Supplementary Experiments

From five different literature sources (De Greef and Jacobs, 1979; Saunders and Daub, (1984), Lindsey and Gallois, 1987; Tétu *et al*., 1987; Zhong *et al*., 1993) four treatments were selected and applied to our genotypes as well as varieties and lines. For callus induction, 4 different treatments were prepared by using MS medium including micro and macro elements and vitamins supplemented with plant growth regulators and sucrose as given in Table 3.10.

**Table 3.10:** Description of Supplementary Experiments

| [plant growth regulator] (mg/L) | [sucrose] g/L | Literature Abbreviations |
|---|---|---|
| - (Hormone Free) | 10 | De Greef and Jacobs (1979) + Saunders and Daub (1984) |
| 1.0 BAP | 10 | Lindsey and Gallois (1990) + Zhong *et al*. (1993) |
| 1.0 KIN | 10 | Zhong *et al*. (1993) |
| 1.0 NAA + 5.0 BAP | 10 | Tétu *et al*. (1987) |

De Greef and Jacobs (1979) described a single hormone-autonomous, continuously regenerating cell line that was isolated fortuitously and could not be obtained again. Saunders and Daub (1984) reported that hormone-autonomous callus could repeatedly be induced directly from shoot cultures of several genotypes.

According to Lindsey and Gallois (1990), different explants were cultured on a range of media to determine the best combination of tissue and medium for shoot regeneration. Over a 6-week culture period, the highest numbers of shoots were obtained on media containing 1.0 mg/L BAP.

According to Zhong *et al*. (1993), petiole explants were transferred to MS media supplemented with either 1.0 mg/L BAP or 1.0 mg/L KIN. Adventitious shoot production was assessed. BAP was found to be much more active than KIN.

According to Tétu *et al*. (1987), various explants were cultured on MS medium supplemented with 5.0 mg/L BAP and 1.0 mg/L NAA. After 4 weeks of culture, friable calli were obtained.

Unfortunately, we could not be able to obtain calli from any of these treatments.

# CHAPTER IV

# CONCLUSION

- Within sugar beet genotypes, with respect to the treatments, triploids or tetraploids responded to treatments significantly superior over diploids.

- The effect of the concentration of sucrose on callus induction might seem to be important, high concentration (30 g/L) caused discoloration and irresponsiveness of formed calli at callus enlargement and subsequent shoot regeneration stages. Therefore, low concentration (10 g/L) is advised to be used at these stages.

- L-ascorbic acid inclusion into the medium, especially at 20 mg/100 mL concentration, yielded the only two spontaneous shoots obtained from the tetraploid EA2075 variety. These shoots were successfully rooted and whole plants were obtained.

- The effect of silver nitrate in combination with L-ascorbic acid, on the prevention of tissue discoloration was investigated. However, not only the symptoms of discoloration did not diminish, but also shoot recovery could not be achieved.

- Since 3456 explants were used during this study, and 2 whole plants were recovered, the efficiency of plant recovery has been calculated as a rather low value of 0.058 %.

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# APPENDIX A

## A.1 Murashige and Skoog (MS) Medium Micro and Macro Elements

| Component | 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 |
| $CaCl_2$ | 332.02 |
| $KH_2PO_4$ | 170.00 |
| $KNO_3$ | 1900.00 |
| $MgSO_4$ | 180.54 |
| $NH_4NO_3$ | 1650.00 |

## A.2 Murashige and Skoog (MS) Medium Vitamins

| Component | mg/L |
|---|---|
| Glycine | 2.00 |
| Myo-inositol | 100.00 |
| Nicotinic Acid | 0.50 |
| Pyridoxine-HCl | 0.50 |
| Thiamine-HCl | 0.10 |

# APPENDIX B

## B.1 Germination Medium

(per liter)

| | |
|---|---|
| Murashige and Skoog (MS) medium including vitamins | 4.43 g |
| Sucrose | 30 g |
| Plant agar | 8.0 g |

pH adjusted to 5.7-5.8 before autoclaving

## B.2 Rooting Medium

(per liter)

| | |
|---|---|
| Murashige and Skoog (MS) medium including vitamins | 4.43 g |
| Sucrose | 30 g |
| IBA | 3.0 mg |
| Plant agar | 8.0 g |

pH adjusted to 5.7-5.8 before autoclaving