In vitro Explant Growth and Shoot Regeneration from Petioles of Sugar Beet (Beta vulgaris L.) Lines at Different Ploidy Levels

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ABSTRACT

This study was carried out to compare 'ELK 345' (diploid) and 'CBM 315' (tetraploid) sugar beet lines with respect to in vitro explant growth, shoot regeneration capacity of petiole explants, in vitro rooting and plantlet establishment. Petioles were cultured on MS (Murashige and Skoog) medium supplemented with 1 mg l⁻¹ 6-benzylaminopurine (BAP) and 0.2 mg l⁻¹ naphthaleneacetic acid (NAA). Regenerated shoots were successfully rooted on MS medium containing 3 mg l⁻¹ indole-3-butyric acid (IBA). It was observed that petiole explants of the tetraploid line 'CBM 315' gave rise to the highest scores with respect to explant fresh and dry weights, shoot regeneration percentage, shoot number per petiole, shoot length, total shoot number per Petri dish, successful rooting and plantlet establishment. Petiole explants of diploid line ('ELK 345') produced 12.61 shoots per explant while petioles of tetraploid line ('CBM') produced 20.23 shoots per explant.

Additional key words: In vitro explant growth, shoot regeneration, Beta vulgaris, ploidy level
Sugar beet (*Beta vulgaris* L.), important sucrose-producing crop worldwide in temperate regions, supplies about 20% of the sugar consumption (Turkish Sugar Co., 2010). Developing new sugar beet varieties with conventional plant breeding methods is slow and labor intensive. Tissue culture methods integrated with conventional breeding programs are playing an increasingly significant role in the improvement of sugar beet (D’Halluin et al., 1992; Gurel, 2000; Hisano et al., 2004). Advanced *in vitro* culture and genetic transformation technologies have been incorporated with classical breeding programs of sugar beet, aiming at the production of herbicide- and salt-tolerant, disease- and pest-resistant cultivars (Tenning, 1998; Shimamoto and Domae, 1999; Gurel et al., 2001; Yang et al., 2005; Gurel et al., 2008). However, sugarbeet is recalcitrant with respect to *in vitro* culture and genetic transformation (Tetu et al., 1987; Krens et al., 1989; Gurel et al., 2008).

Genetic variation is a prerequisite for successful plant breeding. *In vitro* culture techniques seem to offer certain advantages in this respect through somatic hybridization, induction of mutants and selection of disease free and disease resistant plants (Thirugnanakumar et al., 2009). Any progress in increasing the adventitious shoot regeneration frequency in sugar beet *in vitro* culture is extremely valuable for the development of new cultivars. To our knowledge, there is no report on the effect of ploidy level on *in vitro* tissue culture response in sugar beet. Thus, this study was conducted to examine the effect of the ploidy level on *in vitro* explant’s growth, adventitious shoot regeneration, rooting and plantlet establishment from petiole explants of two sugar beet (*Beta vulgaris* L.) lines.

### MATERIALS AND METHODS

**Plant material**

Two sugar beet (*Beta vulgaris* L.) breeding lines, 'ELK 345' (diploid multigerm, good root yield, good sugar yield) and 'CBM 315' (tetraploid multigerm, good root yield, good sugar yield) were used as sources of explants. All breeding lines which were obtained from the Sugar Research Institute, Etimesgut, Ankara, were originated from inbred lines.

**Surface sterilization and germination of seeds**

Seeds were placed in sterile bottles having 100% commercial bleach (5% sodium hypochlorite) and were shaken for 5 h at room temperature. This was followed by 3-4 washes with sterile water and a 24 h rinse in sterile water to increase permeability of the true seed coat as reported by Yildiz et al. (2007). After sterilization, the seed coat was removed from the sterile true seeds. Removal of the seed coat enabled the true seeds to germinate more rapidly. Sterilized seeds were germinated in babyfood-jars containing 30 ml of MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose (w/v), 0.7% (w/v) agar,
0.5 mg l⁻¹ 2,3,5-triiodobenzoic acid (TIBA), and 1 mg l⁻¹ BAP for promoting organogenic cell lines in cultured explants (Jack et al., 1992).

**Explant source and culture conditions**

Petiole explants (0.7 mm in length) were excised from 8-week-old sterile seedlings. For shoot regeneration, petioles were cultured for 5 weeks on MS medium supplemented with 1 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA at 1% sucrose as reported by Yildiz et al. (2007). Petioles were submerged in sterile distilled water with a gentle shaking for 20 min before placing on regeneration medium to increase the adventitious shoot regeneration frequency as reported by Yildiz and Özgen (2004). The pH of the medium was adjusted to 5.8 and autoclaved at 120°C for 20 min. Growth regulators were added to the media before autoclaving.

All cultures were incubated under a cool white fluorescent light (27 µmol m⁻² s⁻¹) with a 16 h light/8 h dark photoperiod in a growth chamber at 25±1°C.

**Observations**

Cell size, measured by width and cell length, was recorded in µm using a microscope at 60X magnification in the petiole’s epidermis layer of sterile seedlings of diploid and tetraploid lines before culture initiation. Fresh and dry weights of petioles with shoots were calculated at the end of the culture. Petiole segments were weighed to determine the fresh weight. The dry weight was obtained after drying explants at 105°C for 2 h. All measurements were made using an analytical scale, with precision of 0.001 g. Data collected included shoot regeneration percentage, shoot number per petiole, shoot length, total shoot number per Petri dish, number of shoots rooted and percentage of shoots rooted.

**Rooting and transplanting**

Seventy regenerated shoots from diploid and tetraploid lines were transferred to sterile baby food-jars containing 30 ml MS medium with 3 mg l⁻¹ IBA; they were incubated for two weeks at 25±1°C to induce root formation. Rooted shoots were then transferred to pots in a growth room for two weeks where light, temperature and humidity were controlled. Humidity was decreased gradually from 100% to 40% during two weeks for acclimatization of seedlings. After two weeks, plantlets were moved to a greenhouse for 10 days and finally they were transplanted to the field.

**Statistical analysis**

In the study, three replicates were used. Petri dishes (100 x 10 mm) containing 10 explants were considered the experimental units. The study was set in three parallels to confirm the accuracy of the study. Data were statistically analyzed by Independent-Samples t test in the ‘SPSS for Windows’ program. Values presented in percentages were
RESULTS AND DISCUSSION

Tissue necrosis, which is caused by the oxidation of phenolic compounds by several enzymes such as polyphenol oxidase (PPO) and peroxidase (POD), is one of the main problems in sugar beet tissue culture (Whitaker and Lee, 1995; Yildiz et al., 1997; Gurel et al., 2001). Because phenolic compounds have been found in different beet tissues (Wende et al., 1999; Kaur and Kapoor, 2002), sugar beet has been placed in a high phenolic concentration group. However, the protocol described by Yildiz et al. (2007) has eliminated the problem of tissue necrosis in sugar beet in vitro culture. Consequently, high frequency shoot regeneration, which is a prerequisite for an efficient transformation system, was achieved. In the current study this protocol was used successfully to obtain adventitious shoots.

We used petioles as an explant and 1 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA as the combination of growth regulators for shoot regeneration because studies reported the petiole was the most responsive explant for in vitro culture of sugar beet and the most effective combination of the plant growth regulators was 1 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA (Tetu et al., 1987; Detrez et al., 1988, 1989; Freytag et al., 1988; Ritchie et al., 1989; Toldi et al., 1996; Grieve et al., 1997; Zhang et al., 2001; Yildiz et al., 2007).

There are differences between diploid and polyploid plants from morphological, physiological, cellular and biochemical aspects (Berkov and Philipov, 2002). Berkov and Philipov (2002) have reported that the roots and leaves of tetraploid Datura stramonium plants had a higher alkaloid content than diploid plants. It was reported that the number of chlorophyll-containing chloroplasts increased from diploids to tetraploids in black wattle (Beck et al., 2003). The overall chlorophyll content in the diploids of the black wattle was 40% less than that of the tetraploids (Mathura et al., 2006). In alfalfa, chlorophyll content and other proteins were shown to almost double from diploid to tetraploid plants (Molin et al., 1982). Similarly, it was reported that in C₄ grass Panicum virgatum, chlorophyll content and other soluble proteins were found to be 40-50% higher in octaploids than in tetraploids (Warner et al., 1987). Yildiz et al. (2005) conducted a study of two sugar beet lines (CBM 315-tetraploid and ELK 345-diploid) at different ploidy levels to determine in vitro susceptibility to Agrobacterium tumefaciens infection. This study showed statistically significant differences in all parameters examined between lines of different ploidy levels (Tables 1 through 4).

In our study, data related to the cell size were obtained in µm using a microscope at 60X magnification on the petiole’s epidermis layer of the sterile seedlings of diploid and tetraploid lines before culture ini-
The largest cells measured were from the tetraploid line in all cases. The differences between cell sizes of the diploid and tetraploid lines were statistically significant at the 0.01 level. The largest mean cell width recorded was 48.64 µm in the tetraploid line, and was 33.88 µm in the diploid line (Table 1). Similarly, the largest mean cell length obtained was 138.66 µm in the tetraploid line, and 94.15 µm in the diploid line. From the results, it was evident that both parameters relating cell size were almost 50% larger in the tetraploid line in all cases (Table 1). Our finding were verified by Smith et al. (2003) reporting that polyploidy increases the cell size and volume of perennial ryegrass (Lolium perenne L.).

The tetraploid line 'CBM 315' had a significantly higher fresh weight ($p < 0.05$) than the diploid line 'ELK 345' in all three experiments (Table 2). Dry weights also were higher in the tetraploid line, and the differences between these lines were statistically significant at 0.01 level in all experiments (Table 2). During culture, petiole explants of the tetraploid line were observed to grow faster than the ones

**Table 1.** Cell sizes in the epidermis layer of petioles of sterile seedlings of diploid and tetraploid lines.

<table>
<thead>
<tr>
<th>Cell size</th>
<th>Cell width (µm)</th>
<th>Cell length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'ELK 345'</td>
<td>'CBM 315'</td>
</tr>
<tr>
<td></td>
<td>Diploid</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>1st experiment</td>
<td>33.84±1.657</td>
<td>49.04±2.162</td>
</tr>
<tr>
<td>t value</td>
<td>5.580**</td>
<td></td>
</tr>
<tr>
<td>2nd experiment</td>
<td>36.72±1.143</td>
<td>50.46±1.739</td>
</tr>
<tr>
<td>t value</td>
<td>6.603**</td>
<td></td>
</tr>
<tr>
<td>3rd experiment</td>
<td>31.08±2.425</td>
<td>46.42±1.550</td>
</tr>
<tr>
<td>t value</td>
<td>5.329**</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>33.88</td>
<td>48.64</td>
</tr>
</tbody>
</table>

Significantly different from zero at ** $p < 0.01$

1 Mean of three experiments
Table 2. Fresh and dry weights, water and dry matter contents of petiole explants of 'ELK 345' (diploid) and 'CBM 315' (tetraploid) lines 5 weeks after culture initiation on MS medium containing 1 mg l\(^{-1}\) BAP and 0.2 mg l\(^{-1}\) NAA

<table>
<thead>
<tr>
<th></th>
<th>Fresh weight (g)</th>
<th>Dry weight (g)</th>
<th>Water content (%)</th>
<th>Dry matter content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'ELK 345' Diploid</td>
<td>'CBM 315' Tetraploid</td>
<td>'ELK 345' Diploid</td>
<td>'CBM 315' Tetraploid</td>
</tr>
<tr>
<td>1st experiment</td>
<td>0.168±0.009</td>
<td>0.226±0.011</td>
<td>0.012±0.001</td>
<td>0.023±0.002</td>
</tr>
<tr>
<td>t value</td>
<td>4.226*</td>
<td>4.621**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd experiment</td>
<td>0.161±0.091</td>
<td>0.251±0.028</td>
<td>0.012±0.001</td>
<td>0.022±0.002</td>
</tr>
<tr>
<td>t value</td>
<td>3.120*</td>
<td>4.542**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd experiment</td>
<td>0.187±0.017</td>
<td>0.284±0.019</td>
<td>0.013±0.0003</td>
<td>0.023±0.001</td>
</tr>
<tr>
<td>t value</td>
<td>3.794*</td>
<td>6.708**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean(^1)</td>
<td>0.172</td>
<td>0.254</td>
<td>0.012</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Significantly different from zero at * \(p < 0.05\) and ** \(p < 0.01\)

\(^1\) Mean of three experiments
of the diploid line. By the end of the culture, petiole explants of the tetraploid line were larger, well developed, and the number of shoots regenerated also was higher (Figure 1a-b) than the diploid line (Figure 1c). The largest mean fresh and dry weights of petiole explants were from the tetraploid line – 0.254 g and 0.023 g, respectively (Table 2). In the diploid line, the mean fresh and dry weights of petioles were 0.172 g and 0.012 g, respectively (Table 2). The difference between fresh and dry weights signifies the tissue water content. From these results, the tissue water content was calculated as 0.231 g (0.254-0.023) in the tetraploid line 'CBM 315', and 0.160 g (0.172-0.012) in the diploid line 'ELK 345'.

The cells with higher ploidy levels in a yeast, Candida sp., have larger vacuoles (Jibiki et. al., 1993); vacuoles play an important role in regulating the osmotic pressure of the cell (Guertin and Sabatini, 2005). Higher osmotic pressure in polyploid tomato plant cells, as re-
ported by Tal and Gardi (1976), could cause higher tissue metabolic activity by increasing water and hormone uptake from the medium. Cell enlargement by water absorption, cell vacuolation, and turgor-driven wall expansion is the main reason of fresh weight increase, as reported by Dale (1988). Increase in dry weight has been related closely to cell division and new material synthesis (Sunderland, 1960). Thus, increase in the fresh and dry weights of petiole explants of the tetraploid line at the end of culture in our study most likely were due chiefly to an increase in the absorption of water and other components from the basal medium via the higher cellular osmotic pressure. Additionally, lower osmotic pressure of the cells of diploid line probably correlated to lower fresh and dry weights of petioles by decreasing the absorption of water and other components from the medium. Tissue water content results indicated that the tetraploid line had higher osmotic pressure, which most likely caused higher absorption of water and other components from the growth. Higher results of all parameters in our study could be attributed to higher cell osmotic pressure of the tetraploid line 'CBM 315'. Yildiz and Özgen (2004) have reported that increasing tissue water content, which caused higher tissue metabolic activity, resulted in higher results of all parameters examined in flax (*Linum usitatissimum*).

It has been shown that increase in ploidy level leads to a larger cell that has a higher growth rate (Jibiki et al., 1993). Tetraploid genotypes had a higher water content (Tal and Gardi, 1976) and more organic solutes than diploid genotypes (Reinink and Biom-Zandstra, 1989). Warner and Edwards (1989) have reported that the chromosome number determines the size of leaves, the size of cells, the number of chloroplasts per cell, and amounts of photosynthetic enzymes and pigments in cell. As the chromosome number increased, DNA content per cell, enzyme activity per cell, cell volume, and photosynthesis per cell all increased. It also was reported that the photosynthetic capacity of larger cells in polyploid plants are higher than smaller cells with lower chromosome numbers (Molin et al., 1982; Warner et al., 1987; Wintermans and De Mots, 1965).

In all of our experiments, the highest results were obtained in the parameters of shoot regeneration percentage, shoot number per petiole, shoot length, total shoot number per Petri dish, number of shoots rooted, and the percentage of shoots rooted from petiole explants of the tetraploid line. The differences between petiole explants of the diploid and tetraploid lines for all parameters examined were statistically significant at $p < 0.01$, with the exception of shoot regeneration percentage, which were significantly different in all experiments at $p < 0.05$ (Table 3, Table 4, Figs. 1, 2).

Shoot primordia on petiole explants appeared in the first week of the culture in the tetraploid line but they developed 16 days after culture initiation in the diploid line. The highest mean shoot regeneration percentage and mean shoot number per petiole was recorded as 69.99% and 20.23 in the tetraploid line and they were 45.57% and
Table 3. Adventitious shoot regeneration from petiole explants of 'ELK 345' (diploid) and 'CBM 315' (tetraploid) lines 5 weeks after culture initiation on MS medium containing 1 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA

<table>
<thead>
<tr>
<th>Shoot regeneration (%)</th>
<th>Shoot number per petiole</th>
<th>Shoot length (cm)</th>
<th>Total shoot number per Petri dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>'ELK 345' Diploid</td>
<td>'CBM 315' Tetraploid</td>
<td>'ELK 345' Diploid</td>
<td>'CBM 315' Tetraploid</td>
</tr>
<tr>
<td>40.0±5.773</td>
<td>66.7±3.333</td>
<td>12.53±0.617</td>
<td>22.80±1.106</td>
</tr>
<tr>
<td>46.7±3.333</td>
<td>70.0±5.774</td>
<td>13.70±0.656</td>
<td>19.70±0.929</td>
</tr>
<tr>
<td>50.0±5.774</td>
<td>73.3±3.333</td>
<td>11.60±0.625</td>
<td>18.20±0.557</td>
</tr>
<tr>
<td>45.57 ±69.99</td>
<td>12.61 ±20.23</td>
<td>2.0 ±2.8</td>
<td>57.50 ±141.77</td>
</tr>
</tbody>
</table>

Significantly different from zero at *p < 0.05 and **p < 0.01

1 Mean of three experiments
12.61 in the diploid line (Table 3). Regenerated shoot length was found to be higher in the tetraploid line 5 weeks after culture initiation. The mean shoot length was 2.8 cm in the tetraploid line, and it was 2.0 cm in the diploid line (Table 3).

The mean total shoot number per Petri dish, which can be determined by shoot regeneration percentage and shoot number per petiole, was recorded as 141.8 in the tetraploid line and 57.5 in the diploid line (Table 3). Shoots regenerated from petiole explants of the diploid and tetraploid lines were rooted on MS medium containing 3 mg l⁻¹ IBA for 2 weeks. The best results were obtained in shoots regenerated from petiole explants of the tetraploid line in all three experiments (Table 4). From the results, it was evident that plantlets grown from petioles of tetraploid line were more vigorous and larger than the plantlets grown from petioles of the diploid line (Fig. 2a). Of the 70 shoots transferred to rooting medium, 61.3 shoots (87.6%) from tetraploid line 'CBM 315' and 51.7 shoots (73.8%) from the diploid line 'ELK 345' were rooted successfully (Table 4). Transferred plants reached harvest ma-

**Fig. 2.** Two-week-old rooted seedlings derived from (a) 'CBM 315' (tetraploid) and (b) 'ELK 345' (diploid) line. (Bar = 1.5 cm)
turity in the field and no morphological abnormalities were observed. To our knowledge, this is the first report of the effect of ploidy levels on tissue culture response, rooting, and plantlet establishment in vitro. Superiority of the performance of the tetraploid sugar beet line ('CBM 315') in our study with respect to tissue culture response agreed with the results of Yildiz et al. (2007). In the study conducted by Yildiz et al. (2007) in sugar beet, two diploid lines ('ELK 345' and 'M 114') and one tetraploid line ('CBM 315') were used to evaluate the effect of different sucrose concentrations in the medium on tissue necrosis and shoot regeneration capacity of the explant. They reported that tetraploid line gave rise to the more shoots per explant than did the two diploid lines. Our findings were in agreement with Stebbins (1947) who reported that tetraploids have higher vegetative growth.

Table 4. In vitro root development of shoots regenerated from petiole explants of 'ELK 345' (diploid) and 'CBM 315' (tetraploid) lines on rooting medium enriched with 3 mg l⁻¹ IBA 2 weeks after culture initiation.

<table>
<thead>
<tr>
<th></th>
<th>Number of shoots rooted</th>
<th>% of shoots rooted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'ELK 345'</td>
<td>'CBM 315'</td>
</tr>
<tr>
<td>Diploid</td>
<td>53±1.155</td>
<td>64±1.000</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>64±1.000</td>
<td></td>
</tr>
<tr>
<td>t value</td>
<td>7.201**</td>
<td></td>
</tr>
<tr>
<td>1st experiment</td>
<td>52±1.528</td>
<td>59±1.155</td>
</tr>
<tr>
<td>t value</td>
<td>3.656*</td>
<td></td>
</tr>
<tr>
<td>2nd experiment</td>
<td>50±1.528</td>
<td>61±1.399</td>
</tr>
<tr>
<td>t value</td>
<td>5.745**</td>
<td></td>
</tr>
<tr>
<td>3rd experiment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean¹</td>
<td>51.7</td>
<td>61.3</td>
</tr>
</tbody>
</table>

Significantly different from zero at *p < 0.05 and **p < 0.01

¹ Mean of three experiments
LITERATURE CITED


