Sucrose Effects On Phenolic Concentration and Plant Regeneration From Sugarbeet Leaf and Petiole Explants

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ABSTRACT

Developing new sugarbeet varieties with conventional plant breeding methods is slow and labor intensive. Integrating tissue culture methods with conventional methods could facilitate this process for some traits. This study investigated the effect of culture media sucrose concentrations on phenolic concentration and tissue necrosis of explant, and shoot regeneration frequency. Petiole and leaf disc explants of three sugarbeet (*Beta vulgaris* L.) breeding lines were excised from sterile seedlings and cultured on media containing different sucrose concentrations. The lowest phenolic concentration and tissue necrosis were recorded from both explants of all lines at 0% sucrose concentration. However, at this concentration, explant growth slowed and no shoots were regenerated. The best shoot regeneration success was obtained from media supplemented with 1% sucrose, 1 mg l-1 6-benzylaminopurine (BAP) and 0.2 mg l^{-1} naphthaleneacetic acid (NAA). At higher sucrose concentrations in the culture media, phenolic concentration and tissue necrosis increased and sharp decreases were observed in shoot regeneration of the three sugarbeet lines.

Additional Key Words: *Beta vulgaris*, shoot regeneration, tissue necrosis, phenolics

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Abbreviations: BAP – 6-benzylaminopurine; MS – Murashige and Skoog; NAA – naphthaleneacetic acid; PPO – polyphenoloxidase; PO – peroxidase; TIBA – 2,3,5-triiodobenzoic acid

Sugarbeet (*Beta vulgaris* L.), an important sucrose-producing crop in temperate regions of the world, supplies about 37% of the sugar consumed worlwide. It is a naturally cross-pollinating, heterogeneous, biennial and varietal improvement by conventional breeding methods is slow and laborous. Tissue culture methods integrated with conventional breeding programs are playing an increasingly significant role in the improvement of sugarbeet (D'Halluin et al., 1992). However, sugarbeet is recalcitrant with respect to *in vitro* culture and genetic transformation (Tetu et al., 1987; Krens et al., 1989).

The tissue blackening or browning caused by the oxidation of phenolic compounds which are exuded from cut surfaces of explants of woody plants, fruits, and vegetables has caused serious problems in establishing *in vitro* cultures. The exudates inhibit growth and eventually cause tissue necrosis. Enzymes such as polyphenoloxidase (PPO) and peroxidase (POD) participate in blackening *in vitro* cultures (Pizzocaro et al., 1993; Down and Norton, 1995; Whitaker and Lee, 1995) and catalyze oxidation of phenolic compounds. It has been suggested that the degree of blackening is related to phenolic concentration and enzyme activity of the tissue, particularly PPO (Kahn, 1975). A reduced peroxidase and polyphenoloxidase activity increases the ability of tissues to initiate growth *in vitro* (Andersone and Ievinsh, 2002).

PPO-catalyzed tissue blackening can be prevented by chemical, enzymatic and physical treatments (Laurila et al., 1998), however these treatments often cannot be used in *in vitro* culture. It has been recommended that activated charcoal, ascorbic acid, citric acid, and sodium chloride be added to culture medium to limit *in vitro* tissue blackening (Pizzocaro et al., 1993).

Phenolic compounds have been found in different beet tissues (Bokern et al., 1991; Harborne et al., 1999; Wende et al., 1999). Kaur and Kapoor (2002) placed sugarbeet in a high phenolic concentration group based upon the 323±11.7 mg/100 g phenolics in its root. Dias and Costa (1983) reported that peroxidase activity of sugarbeet leaves increased at low NaCl concentrations. Kevers et al. (1981, 1983) showed that organogenic sugarbeet calli had high peroxidase levels and they reported that provitamin D_2 (ergosterol), vitamins D_2, and D_3 reduced the calcium-mediated peroxidase secretion in sugarbeet cells in suspension cultures. Gaspar et al. (1982) stated that the process of peroxidase secretion occured in whole tissues as well as suspension cells. Yıldız et al. (1997) reported that activated charcoal reduced tissue blackening and encouraged shoot regeneration from petiole explants of sugarbeet, but the frequency of regenerated shoots was not sufficient for routine transformation and *in vitro* propagation of genotypically distinct cultivars.

The present study evaluated the effect of sucrose concentrations in culture media on the phenolic concentration of explants, tissue blackening and shoot regeneration frequencies on leaf disc and petiole explants of three sugarbeet breeding lines.

MATERIALS AND METHODS

Plant Material

Three sugarbeet (*Beta vulgaris* L.) breeding lines, 'ELK 345' (diploid multigerm, good root yield, good sugar yield), 'CBM 315' (tetraploid multigerm, good root yield, good sugar yield) and 'M 114' (diploid monogerm, 0-type, good root yield, medium sugar yield) were used as sources of explants. All lines were obtained from the Turkish Sugar Factories, Sugar Research Institute, Etimesgut, Ankara.

Surface sterilization and germination of seeds

Seeds were placed in sterile bottles having 100% commercial bleach (5% sodium hypochlorite) and shook for 5 h at room temperature, followed by 3-4 washes and a 24 h rinse in sterile water to increase permeability of true seed coat. 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. To obtain uninfected seedlings, 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} TIBA and 1 mg l^{-1} BAP for promoting organogenic cell lines in cultured explants (Jack et al., 1992).

Adventitious shoot regeneration and tissue necrosis

Leaf disc (1 cm^2 containing mid vein) and petiole (0.7 mm in length) explants were excised from 8-week-old sterile seedlings and cultured for 5 weeks on MS medium supplemented with 0%, 1%, 2%, and 3% (w/v) sucrose, 0.7% (w/v) agar, BAP (0.5, 1, and 2 mg l^{-1}) and 0.2 mg l^{-1} NAA for shoot regeneration.

Tissue necrosis was determined after regenerated shoots were excised at the end of culture by means of a computer program and a desktop scanner. First, total surface area of explants in mm^2 per Petri dish was calculated. Then green parts were removed from the explant with a scalpel and the necrotic areas were measured by the same method. Finally, percent tissue necrosis was reported as the ratio of necrotic area to total explant area in a Petri dish.

Determination of phenolic concentration

Phenolics in the extracts were determined according to the Folin-Ciocalteau procedure (Singleton, and Rossi, 1965), with modifications. Five weeks after culture initiation, explants from each Petri dish were homogenized after determining necrotic areas, with a mortar and pestle, in liquid nitrogen. Three ml of 99% methanol-1% HCl solution was added to 1 g of ground explants and shook for 4 h. This homogenate was centrifuged at 6000 g for 15 min at room temperature and the supernatant was saved. Samples (10 μl, three replications) were diluted with 765 μl of distilled water and 75 μl of Folin-Ciocalteau reagent, after 3 min, 750 μl of sodium carbonate (6%) (w/v) were added and the contents were mixed thoroughly then allowed to stand for 90 min. At the end of this period, the color developed and absorbance was measured at 725 nm on a Pharmacia Biotech Ultrospec 2000 spectrophotometer against an identical mixture (control) in which there was no sample. The phenolic concentration was expressed as gallic acid equivalents in mg/100 g of fresh weight material.

Culture conditions

All cultures were incubated under 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. 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.

Rooting and transplanting

Shoots (approximately 3 cm high and 4-5 leaves) produced from petiole explants were transferred to sterile baby food-jars containing 30 ml MS medium with 3 mg l^{-1} IBA and incubated for 2 weeks at 25°C to induce root formation. Rooted shoots were transferred to pots in a growth room for 2 weeks where light (27 μmol m^{-2} s^{-1}), temperature (25°C) and humidity were controlled. Humidity was decreased gradually from 100% to 40% during two weeks. After 2 weeks, plantlets were moved to a greenhouse for 10 days and finally they were transplanted to the field.

Statistical Analysis

Three replicates were used for both shoot regeneration and the determination of phenolic concentration. Petri dishes containing 10 explants were considered the experimental units. All experiments were repeated two times. Means were separated using Duncan's multiple range tests with α=0.01. Data in percentages were subjected to arcsine ($\sqrt{x}$) transformation before statistical analysis (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Surface sterilization and germination of seeds

The difficulty in establishing aseptic cultures from sugarbeet seeds and seedlings have been reported previously (Hussey and Hepter, 1978). Although it was reported that surface sterilization after seed coat removal was effective and practical (Freytag et al., 1988), the sterilization method used here gave a high proportion of seeds free from contamination as well as high germination frequency. The number of seeds sterilized were 563 in 'ELK 345', 498 in 'CBM 315' and 817 in 'M 114'. The contamination frequency ranged from 0.9 to 1.6%. Of the uninfected seeds, the germination frequency was very similar for lines 'ELK 345' (99.5%) and 'CBM 315' (99.0%), and slightly reduced (93.2%) for 'M 114' All seeds that germinated produced normal seedlings.

Phenolic concentration of explants

All measurements of phenolic concentrations were performed 5 weeks after culture initiation. Harms et al. (1983) reported that secretion of phenolic compounds as a wounding response inhibited *in vitro* explant growth in sugarbeet and garden beet. The highest phenolic concentration was in explants grown on media with 3% sucrose while the lowest were from 0% sucrose, in both leaf discs and petioles (Table 1). The phenolic concentration of tissues decreased significantly ($P<0.01$) as sucrose concentration decresaed. For example, the phenolic concentration of leaf disc explants decreased from 329.5 mg/100 g fresh weight at 3% sucrose concentration to 32.2 mg/100 g fresh weight at 0% sucrose concentration in line 'ELK 345' (Table 1). Similar influence of sucrose on phenolic concentration in avacado was reported by Kahn (1975). A significant correlation between sucrose concentrations and phenolic concentration in both explants of all lines affirmed the relationship between increased phenolic concentration and increased sucrose concentrations.

Tissue necrosis

Tissue necrosis decreased with decreasing sucrose concentrations (Table 1). One week after leaf disc and petiole explants were placed on regeneration media containing 3% sucrose, tissue blackening began to spread from cut ends of explants, reaching a maximum level 5 weeks after culture initiation (Figure 1a and b). Similar results were reported for potato (Partington et al., 1999). The lowest necrotic scores for all three lines were recorded on media containing 0% sucrose, irrelevant of explant source (Table 1). The necrotic area increased progressively

Table 1. Effect of sucrose on tissue necrosis and phenolic concentration of sugarbeet leaf disc and petiole explants cultured on medium containing 1 mg l^{-1} BAP and 0.2 mg l^{-1} NAA

		Sucrose concentration (%) (w/v)								
		0		1		2		3		
Lines	**Explant**	**Necrotic / total area mm²**	**Phenolics (mg/ 100 g)**	**Necrotic / total area - mm² %**	**Phenolics (mg/ 100 g)**	**Necrotic / total area - mm² %**	**Phenolics (mg/ 100 g)**	**Necrotic / total area - mm² %**	**Phenolics (mg/100 g)**	**r‡**
ELK 345	Leaf Disc	0.53/21 (2.5)*	32.2 d†	31.49/192 (16.4)	94.0 c	37.76/123 (30.7)	184.4 b	35.44/94 (37.7)	329.5 a	0.982**
	Petiole	0.43/24 (1.8)	13.9 d	12.73/119 (10.7)	51.6 c	25.00/72 (34.7)	152.2 b	30.46/68 (44.8)	204.2 a	0.985**
CBM 315	Leaf Disc	1.17/45 (2.6)	13.4 d	32.80/328 (10.0)	47.2 c	58.91/187 (31.5)	132.5 b	62.83/139 (45.2)	196.0 a	0.988**
	Petiole	1.65/33 (5.0)	6.8 d	55.77/146 (38.2)	35.0 c	48.91/117 (41.8)	98.5 b	43.67/87 (50.2)	149.2 a	0.990**
M 114	Leaf Disc	0.99/21 (4.7)	10.6 d	22.62/87 (26.0)	98.2 c	32.59/56 (58.2)	204.8 b	27.76/41 (67.7)	316.7 a	0.999**
	Petiole	0.91/12 (7.6)	2.6 d	41.62/72 (57.8)	44.0 c	27.64/42 (65.8)	78.5 b	19.12/27 (70.8)	128.7 a	0.997**

Each value is the mean of two experiments (both experiments were 3 replications each with 10 explants)

† Phenolic concentrations within a row followed by different letters are significantly different at the 0.01 level.

‡ Correlations between sucrose concentrations and phenolic concentration

* Necrosis (%) = (Necrotic area / Total area) • 100

** Significantly different than 0 at the 0.01 level

when sucrose concentration increased. Although the least amount of tissue necrosis was observed on media containing 0% sucrose, these explants did not grow and no shoots were regenerated. On 1% sucrose, necrosis of leaf disc explants was reduced by almost 57% (from 37.7% to 16.4%) in 'ELK 345', 78% (from 45.2% to 10.0%) in 'CBM 315' and 62% (from 67.7% to 26.0%) in 'M 114' as compared with 3% sucrose (Table 1, Figure 1a and c). A similar decrease in necrosis was observed when petiole explants were cultured on 1 and 2% sucrose as compared to 3% (Table 1, Figure 1b and d, data not shown).

The highest explant growth (total area, mm2) was noted on 1% sucrose (Table 1). Gürel et al. (2001) reported that most of the hypocotyl, cotyledon, leaf and petiole explants of line 'CBM 315' turned dark in color and showed no further growth on MS medium containing 3% sucrose, BAP, or KIN in combination with NAA or 2,4-D at 0.0, 0.5 or 1.0 mg l-1; they attributed this phenomenon to high concentrations of phenolics. In our study, at higher sucrose concentrations, because of increasing phenolic concentration and the subsequent necrosis, tissue growth was inhibited (Table 1). Explant expansion and total growth decreased significantly with increasing phenolic concentration which was correlated with increasing sucrose concentration. In leaf discs, explant area at 1% sucrose was recorded as 192 mm2 in 'ELK 345', 328

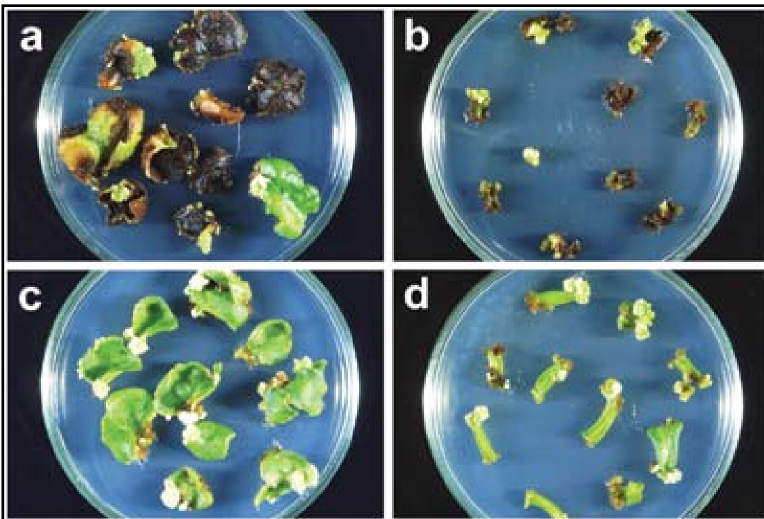

Fig. 1. Tissue necrosis in leaf disc (a, c) and petiole (b, d) explants of line 'CBM 315' five weeks after culture initiation on regeneration medium containing 1 mg l^{-1} BAP + 0.2 mg l-1 NAA, 3% (a, b) and 1% (c, d) sucrose

in 'CBM 315,' and 87 in 'M 114' as compared to 94, 139 and 41 mm2, respectively, at 3% sucrose (Table 1). Similar results were obtained from petiole explants (Table 1).

Sucrose as a carbon source supports growth of plant cells in culture (Gamborg and Phillips, 1995). A sucrose concentration of 1-5% is generally used for *in vitro* tissue culture, since it is also synthesized naturally by the tissue (Pierik, 1987). The photosynthetic activity of the plantlets of rose (Langford and Wainwright, 1987), carnation (Kozai, and Kubota, 1988) and strawberry (Hdider and Desjardins, 1994) grown *in vitro* depends on the amount of carbon in the medium. Langford and Wainwright (1987) reported that the highest net photosynthetic rate was obtained when rose plantlets were grown in media containing less than 3% sucrose. Julkunen-Tiitto (1996) reported that willow plantlets cultured *in vitro* with 8% sucrose died within a few days without any growth. At 6% sucrose, the plantlets grew well, but at 1.5% or less sucrose, they were slow-growing and root development was poor. The availability of carbohydrates is a major factor affecting secondary metabolic production (Bryant et al., 1983). It was reported that sugar decreased the inhibitory effect of malto-dextrin and consequently increased blackening in apple (Xu et al., 1993). Increasing the growth media sucrose concentration resulted in increased phenolics in willow (Julkunen-Tiitto, 1996). Curtis and Shetty (1996) noted that elevated phenolic levels were associated with increased sucrose in oregano. These reports are consistent with our findings that sucrose accelerated the accumulation of phenolics and consequently caused tissue death. Sucrose is the principal storage carbohydrate in sugarbeet that is produced through photosynthesis and transported to root cells (Kursanov, 1974). As there were no roots in our *in vitro* cultures, it is possible that synthesized sucrose accumulated in the leaf disc and petiole explants as well as in the culture media. Thus increasing the overall sucrose concentration leading to cell death.

Adventitious shoot regeneration

Shoot regeneration from leaf discs and petioles was observed only when these explants were cultured on 1% sucrose. Therefore, we examined adventitious shoot regeneration of leaf disc and petiole explants only on media containing 1% sucrose.

The highest shoot regeneration from leaf disc explants of lines 'ELK 345' and 'CBM 315' were observed on media supplemented with 1 mg l^{-1} BAP+0.2 mg l^{-1} NAA (Table 2). Shoot number per leaf disc increased from 1.7 to 6.1 in 'ELK 345' and from 2.7 to 7.7 in 'CBM 315' when the BAP concentration was increased from 0.5 to 1 mg l^{-1} (Table 2).

Table 2. Adventitious shoot regeneration from sugarbeet leaf disc and petiole explants on different MS mediums containing1% sucrose

Explant	Growth regulators (mg l^{-1})	ELK 345 Regeneration (%)	ELK 345 Shoot number per explant	CBM 315 Regeneration (%)	CBM 315 Shoot number per explant	M 114 Regeneration (%)	M 114 Shoot number per explant
Leaf disc	0.5 BAP + 0.2 NAA	10	1.7 c[†]	20	2.7 c	0	0
	1 BAP + 0.2 NAA	15	6.1 a	25	7.7 a	0	0
	2 BAP + 0.2 NAA	10	3.6 b	20	5.7 b	0	0
Petiole	0.5 BAP + 0.2 NAA	20	4.3 c	50	8.3 c	20	2.3 c
	1 BAP + 0.2 NAA	50	11.7 a	70	23.7 a	20	8.7 a
	2 BAP + 0.2 NAA	30	7.7 b	50	14.3 b	10	5.7 b

Each value is the mean of two experiments (both experiments were 3 replications each with 10 explants)
[†]Values in a column followed by different letters are significantly different at the 0.01 level.

No shoots regenerated from line ‘M 114’ on any of the tested media.

Similar results were obtained with petiole explants, except for shoots that regenerated from line ‘M 114’. The highest frequency of shoots per petiole explant was obtained from line ‘CBM 315’, 23.7 (Table 2). The differences among 3 different growth media in shoot number per explant were statistically significant ($P<0.01$, Table 2).

Shoot regeneration and shoot number per explant obtained from petiole explants of all lines were higher than the frequency from leaf discs, consistent with findings of Krens and Jamar (1989). We observed little callus formation on these explants, similar to observations report by Saunders and Shin (1986). Our results (Table 2) clearly showed that 1 mg l^{-1} BAP was optimal as reported previously (Hussey and Hepter, 1978; Freytag et al., 1988; Grieve et al., 1997).

In vitro cultures have been initiated from sugarbeet cotyledons (Catlin, 1990), petioles (Detrez et al., 1988) and leaves (Ritchie et al., 1989). The highest reported shoot regeneration frequencies from leaf, petiole and intact leaves of sugarbeet are 45.7% (Zhong et al., 1993) and 29.5% (Doley and Saunders, 1989). In the present study, the highest shoot regeneration frequencies and shoot number per petiole were 70% and 23.7, respectively, from line ‘CBM 315’ (Table 2). Although the same lines were used in previous studies, the highest shoot number per explant was obtained in our study (8.7 in line ‘M 114’, 11.7 in line ‘ELK 345’ and 23.7 in ‘CBM 315’) and was more than 10 times that reported by others (Gürel et al., 2001; 2003). We attribute the high shoot number per explant results mainly to the use of 1% sucrose in the growth media.

Rooting and transplanting

Shoots from petiole explants were transferred to rooting medium. Of the 80 shoots transferred to rooting medium, 80% of the ‘ELK 345’, 84% of the ‘CBM 315’ and 74% of the ‘M 114’ shoots formed rooted. More than 85% of the transferred plants reached harvest maturity in the field and no morphological abnormalities were observed.

To our knowledge, this is the first report in sugarbeet indicating that media sucrose concentration affected tissue blackening and consequently tissue necrosis caused by phenolic compounds. This study revealed that tissue blackening hindered shoot regeneration in sugarbeet and that the procedure we describe inhibits tissue blackening, thus the highest frequencies of shoot regeneration were obtained when the culture media was supplemented with 1% instead of 2 or 3% sucrose.

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