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Research Article

Callus Development and Indirect Shoot Regeneration from Seedling Explants of Sugar Beet (*Beta vulgaris* L.) Cultured *In Vitro*

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Abstract: For callus production, hypocotyl, cotyledon, petiole and leaf explants from seedlings of different sugar beet (*Beta vulgaris* L.) breeding lines were cultured on MS medium containing BAP or KIN in combination with NAA or 2,4-D at 0.0, 0.5 or 1.0 mg/l. The use of both auxins in combination with 0.5 mg/l BAP or 0.5 mg/l KIN resulted in greater amounts of callus from all types of explants. However, hypocotyl and cotyledon explants produced significantly more callus than petiole and leaf explants when the means of all lines were taken into account. Two types of callus were usually obtained, white and friable callus with large cells (Type I) and green and compact callus with smaller cells (Type II). For shoot induction, Type I callus was transferred to MS medium containing 2.5 mg/l TDZ or 1.0 mg/l BAP in combination with 0.3 mg/l IAA, together with or without a cold pre-treatment at 4°C for two weeks. More shoots developed from the pre-treated callus and elongated shoots could be readily rooted on MS medium containing 3.0 mg/l IBA.

Key Words: Sugar beet, seedling explants, callus formation, indirect shoot regeneration.

Kültüre Alınmış Şeker Pancarı (Beta vulgaris L.) Fide Eksplantlarından Kallus Gelişimi ve İndirek Sürgün Rejenerasyonu

Özet : Kallus üretimi için, farklı şeker pancarı (*Beta vulgaris* L.) ıslah hatlarının fidelerinden alınan hipokotil, kotiledon, yaprak sapı ve yaprak eksplantları, BAP veya KIN ile NAA veya 2,4-D'nin 0.0, 0.5 veya 1.0 mg/l düzeyindeki kombinasyonlarını içeren MS ortamında kültüre alınmışlardır. Her iki oksinin de 0.5 mg/l BAP veya 0.5 mg/l KIN ile kombinasyonları, bütün eksplant tiplerinde daha fazla miktarda kallus oluşturmuş fakat, bütün hatların ortalamaları dikkate alındığında, hipokotil ve kotiledon eksplantları yaprak sapı ve yaprak eksplantlarından daha fazla kallus oluşturmuştur. Genellikle, iki tip kallus elde edilmiştir; büyük hücrelerden oluşan beyaz ve gevşek kallus (Tip I) ve küçük hücrelerden oluşan yeşil ve sıkı yapılı kallus (Tip II). Sürgün üretimi için, Tip I kallusun bir kısmı karanlık ortamda 4 °C'de iki haftalık bir soğuk ön-işlem uygulandıktan sonra, diğer bir kısmı ise ön-işlem uygulanmadan 2.5 mg/l TDZ veya 1.0 mg/l BAP ile 0.3 mg/l IAA'nın kombinasyonunu içeren MS ortamına aktarılmışlardır. Soğuk ön-işlemi uygulanan kallusta daha fazla sayıda sürgün elde edilmiş ve büyüyen sürgünler 3.0 mg/l IBA içeren MS ortamında kolayca köklenmişlerdir.

Anahtar Sözcükler: Şeker pancarı, fide eksplantları, kallus oluşumu, indirek sürgün rejenerasyonu

Introduction

A basic requirement for the application of tissue cultures in crop improvement is either to make use of or devise an *in vitro* plant regeneration system. Callus can easily be obtained from various parts of the sugar beet (*Beta vulgaris* L.) plant including hypocotyls, cotyledons, leaves, petioles, roots, flower stalks, anthers, embryos and seeds. Two groups of researchers reported a hormone-autonomous (habituated) callus which did not require plant growth regulators for growth and subsequent regeneration (De Greef & Jacobs, 1979; Saunders & Shin, 1986). Formation of adventitious shoots or roots from callus was achieved by several researchers although at low frequencies. Hooker and Nabors (1977) and De Greef and Jacobs (1986), for example, obtained one plantlet only from callus derived

from young seedling explants. The composition of the culture medium is important in determining the morphogenetic pathway. A cytokinin (usually BAP) and an auxin (mostly IAA, NAA or 2,4-D) are normally included in the primary culture medium for callus formation followed by incorporation of a lower auxin to cytokinin ratio for shoot induction and a higher ratio for rooting in the subsequent media. Genotypic variation is also important in relation to callus production and subsequent organ formation, some genotypes being more amenable to organogenesis than others and young tissues being more responsive than older tissues (Bhat, Ford-Llyod & Callow, 1985; Keimer, 1985; Mikami, Sudoh & Kinoshita, 1989; Gürel, 1997).

The present report describes an optimised regeneration system in four sugar beet breeding lines, previously developed at the Sugar Institute (Ankara), from callus derived from different seedling explants cultured on a variety of medium compositions. The regenerant plants will then be used in the further cycles of our on-going breeding programmes.

Materials and Methods

Seeds of four sugar beet breeding lines (Table 1) were treated with 70% (v/v) alcohol for 5 min, and sterilised with 7.5% (v/v) sodium hypochloride for one hour in the presence of 0.5 ml Tween 20 per 100 ml solution. Seeds were then rinsed several times with sterile distilled water and left in sterile distilled water for 16-20 hours. After sterilisation, seeds were cultured on MS medium (Murashige & Skoog, 1962) containing 3% (w/v) sucrose, 0.8% (w/v) agar (Oxoid No. 3), 0.5 mg/l TIBA and 1.0 mg/l BAP. The pH was adjusted to 5.8 with 0.1 M NaOH before adding agar and then autoclaving was carried out for 15 min at 15 lb/sq in.

Table 1. Main characteristics of the sugar beet breeding lines used in the callus culture experiments.

Lines	Characteristics
M114	Diploid monogerm, O-type, good root yield, medium sugar yield
M1017	Diploid monogerm family, N-type beet
ELK345	Diploid multigerm, good root yield, better sugar yield
ÇBM315	Tetraploid multigerm, good root yield, good sugar yield

For callus induction, hypocotyl and cotyledon explants were taken from 10-12 day-old seedlings, and petiole and leaf explants were excised from 22-25 day-old seedlings when they had 3-4 true leaves. Isolated explants (3-4 mm) were cultured on MS medium containing BAP or KIN in combination with NAA or 2,4-D at 0.0, 0.5 or 1.0 mg/l each. All of the media combinations were supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. Explants were cultured at 25±1°C under a 16 h photoperiod.

For shoot regeneration, white and friable callus obtained from the above experiments was transferred to MS medium containing 2.5 mg/l TDZ alone or 0.3 mg/l BAP in combination with 0.1 mg/l IAA under dark conditions together with or without a cold pre-treatment at 4 °C for two weeks. Callus was then transferred onto a shoot regeneration medium containing either 2.5 mg/l TDZ alone or 1.0 mg/l BAP in combination with 0.3 mg/l IAA and cultures were incubated at 25±1°C under a 16 h photoperiod. The developed shoots were then rooted on MS medium containing 3.0 mg/l IBA only.

Experiments were repeated three times, each with 10 replicates, and callus and shoot formation were recorded after 60 days' incubation. The amount of callus was estimated by a '0-4 Scale' scoring system (Table 2) and the number of shoots developed from the callus was counted. Analysis of variance was carried out and the differences between the treatments were determined by Duncan's Multiple Range Test.

Table 2. A "0-4 Scale" scoring system developed for measuring the amount of callus obtained from hypocotyl, cotyledon, petiole and leaf explants of sugar beet seedlings.

Score	Description
0	No visible callus
1	Small proliferation at cut ends only
2	5 mm callus at cut ends
3	5-10 mm callus from all over the explant
4	> 10 mm callus from all over the explant

Abbreviations

BAP: 6-benzylaminopurine, 2,4-D: 2,4-dichlorophenoxyacetic acid, GA3: gibberellic acid, IAA: indole-3-acetic acid, IBA: indole butyric acid, KIN: kinetin, NAA: naphtalene acetic acid, TDZ: thidiazuron, TIBA: 2,3,5-triiodobenzoic acid.

Results

Callus Formation from Hypocotyl, Cotyledon, Petiole and Leaf Explants

The amount of callus developed from hypocotyl explants increased with increasing BAP concentrations in the culture medium. The highest BAP concentration (1.0 mg/l) resulted in significantly more callus production than other BAP (0.1 and 0.5 mg/l) and KIN (0.1, 0.5 and 1.0 mg/l) levels (Table 3). However, higher amounts of callus were obtained when 0.5 or 1.0 mg/l 2,4-D was combined with a cytokinin, especially with 0.5 or 1.0 mg/l BAP. Combinations with 2,4-D in general produced significantly more callus than with NAA, with a mean of 1.9-2.2 compared to 1.0-1.7, respectively.

Cotyledon explants responded slightly differently from hypocotyl explants. 0.5 or 1.0 mg/l BAP produced significantly more callus than the control treatment and 0.5 or 1.0 mg/l KIN (Table 4). The auxins also played an important role in callus induction from cotyledon explants and lower concentrations were found more effective.

In the absence of cytokinins, petiole explants from all lines gave the lowest amount of callus (0.2) (Table 5). 1.0 mg/l BAP resulted in the highest amount of callus (1.4), which was significantly different from the means of the control and other treatments. KIN also produced more callus than the control treatment although it was less effective than BAP. On the other hand, petiole explants also produced significantly more callus in the

Table 3. Effects of different concentrations and combinations of auxins (NAA and 2,4-D) and cytokinins (BAP and KIN) on mean callus formation from hypocotyl explants.

Growth Regulators (mg/l)		BAP			KIN		Means[1]
		0.0	0.5	1.0	0.5	1.0	
NAA	0.0	0.1 ± 0.1	0.8 ± 0.2	1.7 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	1.0 d
	0.5	1.9 ± 0.2	1.7 ± 0.1	2.0 ± 0.2	1.5 ± 0.2	1.3 ± 0.2	1.6 c
	1.0	1.4 ± 0.2	2.0 ± 1.2	1.8 ± 0.2	1.9 ± 0.1	0.8 ± 0.2	1.7 c
2,4-D	0.5	0.9 ± 0.2	2.3 ± 0.8	2.9 ± 0.1	1.4 ± 0.2	1.8 ± 0.2	1.9 b
	1.0	2.1 ± 0.2	1.9 ± 0.1	2.5 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2.2 a
Means[1]		1.5 c	1.8 b	2.2 a	1.6 bc	1.3 d	

[1]Means with the same letter within each row or column are not significantly different at p=0.05 according to Duncan's Multiple Range Test.

Table 4. Effects of different concentrations and combinations of auxins (NAA and 2,4-D) and cytokinins (BAP and KIN) on mean callus formation from cotyledon explants.

Growth Regulators (mg/l)		BAP			KIN		Means[1]
		0.0	0.5	1.0	0.5	1.0	
NAA	0.0	1.8 ± 0.2	2.0 ± 0.2	1.8 ± 0.2	1.1 ± 0.2	1.5 ± 0.2	1.6 bc
	0.5	2.0 ±0.2	2.0 ± 0.2	2.2 ± 0.2	1.6 ±0.2	1.5 ± 0.2	1.8 ab
	1.0	1.5 ± 0.2	1.8 ± 0.1	1.4 ± 0.1	1.8 ± 0.2	1.0 ± 0.2	1.5 c
2,4-D	0.5	2.1 ± 0.3	1.8 ± 0.2	2.1 ± 0.1	1.5 ± 0.1	2.0 ± 0.2	1.9 a
	1.0	1.9 ± 0.7	1.8 ± 0.2	2.3 ± 0.1	1.8 ± 0.1	1.5 ± 0.2	1.7 abc
Means[1]		1.7 bc	1.9 a	1.9 a	1.6 c	1.5 c	

[1]Means with the same letter within each row or column are not significantly different at p=0.05 according to Duncan's Multiple Range Test.

Table 5. Effects of different concentrations and combinations of auxins (NAA and 2.4-D) and cytokinins (BAP and KIN) on mean callus formation from petiole explants.

Growth Regulators (mg/l)		BAP			KIN		Means[1]
		0.0	0.5	1.0	0.5	1.0	
NAA	0.0	0.2 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.4 c
	0.5	0.3 ± 0.1	0.2 ± 0.1	1.5 ± 0.2	1.2 ± 0.1	1.1 ± 0.2	0.9 b
	1.0	0.0 ± 0.0	1.3 ± 0.1	1.2 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.8 b
2,4-D	0.5	0.4 ± 0.1	2.3 ± 0.2	0.8 ± 0.2	1.1 ± 0.2	1.5 ± 0.2	1.2 a
	1.0	0.0 ± 0.0	1.4 ± 0.2	2.4 ± 0.2	1.5 ± 0.2	0.2 ± 0.1	1.2 a
Means[1]		0.2 e	1.1 b	1.4 a	0.9 c	0.8 d	

[1]Means with the same letter within each row or column are not significantly different at p=0.05 according to Duncan's Multiple Range Test.

presence of 0.5 or 1.0 mg/l 2,4-D than NAA or no growth regulators.

Media containing 0.5 or 1.0 mg/l BAP were generally more effective in inducing callus from leaf explants than those containing KIN or no growth regulators (Table 6). As to the auxins, 2,4-D yielded more callus than NAA from all explants tested.

Comparison of different explant types and lines in terms of callus production revealed a genotypic variation between the types of explant and lines, with ÇBM315 being the least responsive while M1017 was the most productive, with means of 0.8 and 1.7, respectively (Table 7). As to the comparison of explant types, hypocotyl and cotyledon explants were more responsive for callus production than petiole and leaf explants, with means of 1.5 and 1.6 compared to 1.0, respectively.

Two types of callus were usually obtained; white and friable callus or green and compact callus, referred to as Type I and Type II, respectively. Type I callus mostly consisted of large and translucent cells (Figure 1), while Type II callus contained small and green cells (Figure 2). Type I callus was usually formed on medium containing either 0.5 mg/l BAP or KIN, or on medium containing no plant growth regulators (i.e., hormone-free medium). However, Type II callus often developed on media containing 1.0 mg/l BAP or KIN in combination with 1.0 mg/l NAA or 2,4-D.

Table 6. Effects of different concentrations and combinations of auxins (NAA and 2.4-D) and cytokinins (BAP and KIN) on mean callus formation from leaf explants.

Growth Regulators (mg/l)		BAP			KIN		Means[1]
		0.0	0.5	1.0	0.5	1.0	
NAA	0.0	0.4 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	0.8 ± 0.2	0.8 cd
	0.5	0.2 ± 0.1	0.3 ± 0.1	1.3 ± 0.2	0.6 ± 0.1-	1.0 ± 0.1	0.7 d
	1.0	0.0 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.8 c
2,4-D	0.5	0.2 ± 0.1	2.0 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.1 b
	1.0	0.0 ± 0.0	1.7 ± 0.1	2.1 ± 0.1	1.6 ± 0.1	1.6 ± 0.2	1.4 a
Means[1]		0.2 c	1.2 a	1.2 a	1.1 b	1.1 b	

[1]Means with the same letter within each row or column are not significantly different at p=0.05 according to Duncan's Multiple Range Test.

Table 7. Effects of different explant types on mean callus formation from different sugar beet breeding lines.

Lines	EXPLANT TYPE				
	Hypocotyl	Cotyledon	Petiole	Leaf	Means[1]
M114	2.0 a	1.9 a	0.5 d	0.7 c	1.3 ab
ELK345	1.3 b	1.8 ab	0.9 b	1.2 b	1.3 ab
ÇBM315	0.6 c	1.1 c	0.7 c	0.8 c	0.8 b
M1017	1.9 a	1.6 b	1.9 a	1.5 a	1.7 a
Means[1]	1.5 a	1.6 a	1.0 b	1.0 b	

[1]Means with the same letter within each row or column are not significantly different at p=0.05 according to Duncan's Multiple Range Test.

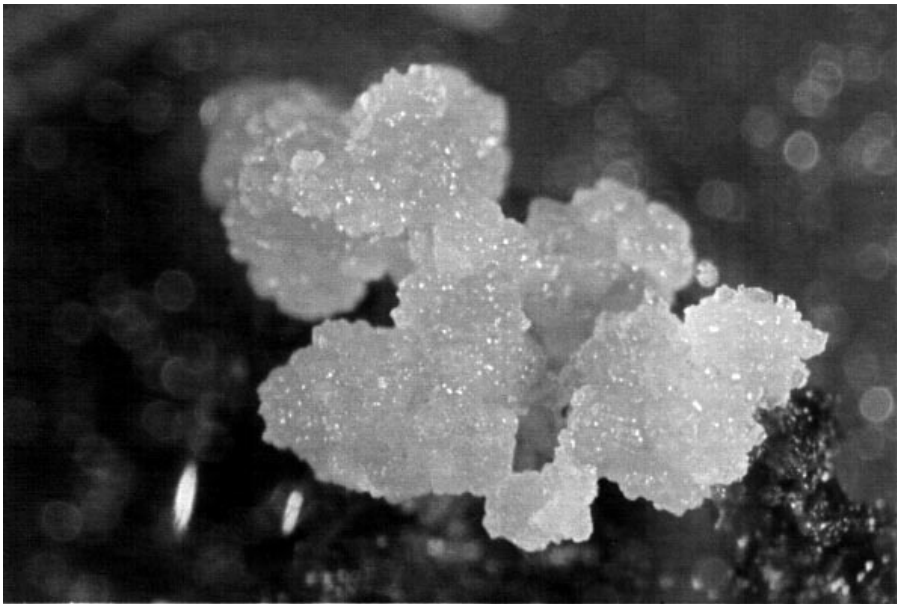

Figure 1. White and friable callus (Type I) derived from leaf explants of line M114 cultured on medium containing 0.5 mg/l BAP only.

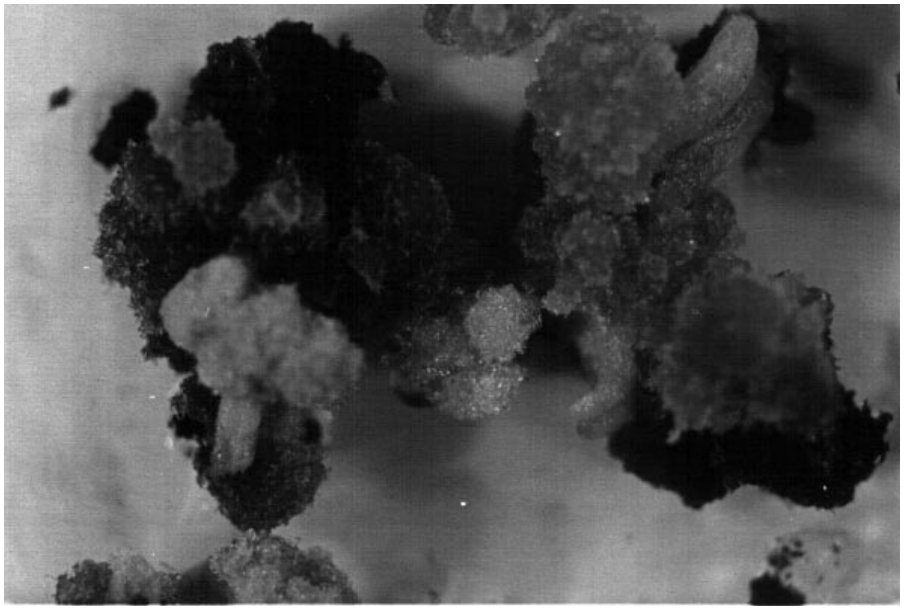

Figure 2. Green and compact callus (Type II) derived from petiole explants of line M114 cultured on medium containing 0.5 mg/l BAP and 1.0 mg/l NAA.

Shoot Regeneration from Callus

The development of shoots (Figure 3) from white and friable callus (regardless of the explant type) was observed after 3-4 weeks of culture on medium containing i) 1.0 mg/l BAP in combination with 0.3 mg/l IAA or ii) 2.5 mg/l TDZ alone. Slightly more shoots were obtained from cold-treated explants of each medium but the differences between the treatments as well as lines were not significant at p= 0.5 (Table 8). Over 90% of the regenerated shoots could be readily rooted when cultured on medium containing 3.0 mg/l IBA only (Figure 4).

Discussion

Callus Development

Callus induction has been reported from almost all types of tissues of sugar beet on various media formulations, which usually contained a combination of a cytokinin and an auxin (Saunders & Shin, 1986; Gürel, 1997; Van Geyt & Jacobs, 1985; Freytag et al., 1988; Saunders & Tsai, 1999). Catlin (1990) obtained callus and plantlets from cotyledons on medium containing 0.2 mg/l BAP only. An auxin-dependent callus was initiated from leaf pieces of sugar beet on medium supplemented with 1.0 mg/l IAA and 0.1 mg/l KIN (Coumans et al., 1982). The most favourable media for callus induction contained a combination of 0.1 mg/l 2,4-D and 1.0 mg/l BAP or 2.2 mg/l TDZ alone (Roussy et al., 1996). Organogenic callus from hypocotyl explants of sugar beet was initiated at concentrations of 0.3 mg/l BAP and 0.1 mg/l NAA (Jacq et al., 1992). Hooker and Nabors (1977) recorded an interaction between the type and concentrations of plant growth regulators, and explant source, a combination of 2,4-D and BAP resulting in a

Figure 3. Shoots developed from white and friable callus (Type I) on medium containing 2.5 mg/l TDZ only after a two weeks' pre-culture under cold and dark conditions.

Table 8. Effects of cold pre-treatment and different concentrations and combinations of cytokinins (BAP or TDZ; mg/l) and auxin (IAA; mg/l) on mean shoot development (expressed as the mean number of shoots per explant) from white and friable callus of different sugar beet breeding lines.

	With cold treatment		Without cold treatment		
Lines	BAP (1.0) IAA (0.3)	TDZ (2.5)	BAP (1.0) IAA (0.3)	TDZ (2.5)	Means[1]
M114	0.6 ± 0.2	0.5 ± 0.2	0.3 ± 0.2	0.2 ± 0.2	0.4
ELK345	0.8 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.6
ÇBM315	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	0.5
M1017	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5
Means[1]	0.6	0.5	0.4	0.4	

[1]The differences between the means were not significant.

Figure 4. A rooted shoot on medium containing 3.0 mg/l IBA only.

higher callus production than the combinations of IAA or NAA with KIN. Similarly, we found that 2,4-D was more effective for callus induction than NAA as a source of auxin in all types of explants tested. Krens and Jamar (1989) reported that auxins were more effective when combined with low BAP levels (0.05 mg/l). We observed otherwise but it should be noted that we did not use a wide range of BAP levels. Among the cytokinins used in our experiments, BAP was clearly more effective than KIN, especially when used at 1.0 mg/l in combination with 0.5 or 1.0 mg/l 2,4-D.

Normally, two types of callus were observed in our experiments. Basal medium or media containing 0.5 mg/l BAP and 0.5 or 1.0 mg/l KIN usually produced white and friable callus (Type I) regardless of the presence of auxins, while medium containing high concentrations of BAP (1.0 mg/l) alone or in combinations with auxins produced green and compact callus (Type II). The production of white and friable callus from various sugar beet explants has also been reported by others (Saunders & Daub, 1984; Nakashima et al., 1988; Ritchie, Short & Davey, 1989; Konwar & Coutts, 1990; Shimamoto et al., 1993; Zhong, Smith & Thomas, 1993). Recent studies have suggested that the organogenic potential is related to callus structure. White and friable callus consisting of large cells was able to produce roots and shoots (Saunders & Daub, 1984; Konwar & Coutts, 1990; Shimamoto et al., 1993), while green and compact callus with small cells showed no organogenic capacity (Ritchie, Short & Davey, 1989; Tetu, Sangwan & Sangwan-Norreel, 1987).

Callus initiation was also influenced by genotypes. Line M1017 was the most productive, whereas line ÇBM315 was the least. De Greef (1978) recorded a genotypic variation in the callusing ability of leaf explants of sugar beet, with some genotypes being more responsive than others. This may suggest that there is a considerable variation in the distribution of the genes responsible for this response among different populations of beet (Bhat, Ford-Llyod & Callow, 1985). Most of the explants of line ÇBM315 turned a dark colour and showed no further growth. This may be attributed to high levels of phenolic substances produced by this breeding line. Such a wounding response, which inhibited callus growth, was also noticed by Harms, Baktır & Oertli (1983) in sugar beet and garden beet.

Variations also existed between different types of explants in their ability to form callus. In our experiments, cotyledon and hypocotyl explants produced considerably more callus than petiole and leaf explants, indicating that the source of explant is an important factor in determining the rate of success in such tissue culture experiments. This may also suggest that levels of endogenous hormones or their sensitivity might vary between organs.

Shoot Regeneration from Callus

The process of shoot regeneration is generally signalled by the appearance of green globular areas on callus followed by the emergence of either leaf-like structures or shoot bud initials. It is well known that shoot formation from sugar beet is often induced when callus is cultured on medium containing BAP (Shimamoto et al., 1993; Saunders, 1982), TDZ (Roussy et al., 1996), KIN and GA_3 (Coumans et al., 1982) or IBA and BAP (Freytag et al., 1988). On the other hand, Saunders (1982) obtained bud formation at low frequencies from habituated callus in the absence of growth regulators. We observed no significant difference between medium containing a combination of 1.0 mg/l BAP and 0.3 mg/l IAA, and medium containing 2.5 mg/l TDZ alone, regardless of a cold pre-treatment at 4°C for two weeks. However, cold pre-treatment slightly increased shoot formation from callus in all breeding lines used, which is in agreement with the results of other researchers (Coumans et al., 1982). In contrast to our findings, De Greef & Jacobs (1979) reported that pre-treatment of callus with cold for 3-9 weeks had no effect on shoot formation from callus. Histological studies showed that organogenic callus was characterised by regions of meristematic cells located near the callus surface (Shimamoto et al., 1993; Abe et al., 1991) and most of these shoots were considered to be of organogenic origin, based on their rosette leaf patterns (Yu, 1989).

Development of a reliable *in vitro* regeneration protocol for sugar beet is difficult due to its highly heterogenous nature. The system applied in this study produced some plant regeneration but it appears that to achieve an efficient rate of plant regeneration in sugar beet might require much further refinements in the design of the composition of culture media. In addition, the use of a wider range of genotypes would be necessary.

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