Tr. J. of Botany
22 (1998) 233-238

Research Article

Plant Regeneration from Unfertilized Ovaries of Sugar Beet (*Beta vulgaris* L.) Cultured *In Vitro*

Ekrem GÜREL
Abant Izzet Baysal University, Faculty of Science & Arts. Department of Biology 14280, Bolu-TURKEY
Songül GÜREL
Sugar Institute, Plant Breeding Department, Etimesgut 06790, Ankara-TURKEY

Received: 22.08.1997
Accepted: 27.03.1998

Abstract: A. method is described for plant regeneration from unfertilized ovaries isolated from a diploid male sterile sugar beet (*Beta vulgaris* L.) breeding line that was developed at the Sugar Institute, Ankara, Turkey. Ovary explants were cultured on Murashige & Skoog (MS) medium containing 2.0 mg/l benzylaminopurine (BAP). Two treatments were tested by incubating all of the explants in darkness for 15 days, and then transferring one half to light and keeping the other half in darkness throughout the culture. Callus formation occurred in both treatments more or less at similar rates: however, there was a significant difference between the treatments with regard to the shoot-forming capacity of the explants, those transferred to light after an initial incbation in darkness producing more shoots (14.6%) than those kept in darkness continuously (4.2%). Root induction was readilyachieved within two weeks when shoots were transferred to MS medium supplemented with 2.0 mg/l naphthaleneacetic acid (NAA) and 2.0 mg/l silver nitrate ($AgNO_3$). An inverse relationship between the callus and shoot-forming capacity of the individual explants was apparent. The determination of ploidy levels of the regenerants was performed by chromosome counting in leaf samples of regenerated plants and the results revealed that all of the regenerants were diploid.

Key Words: *Beta vulgaris* L., tissue culture, ovary culture, plant regeneration

In Vitro Şartlarda Döllenmemiş Şeker Pancarı (*Beta vulgaris* L.) Yumurtalıklarından Bitki Rejenerasyonu

Özet: Bu çalışmada, Ankara Şeker Enstitüsünde geliştirilen diploid erkek kısır bir şeker pancarı (*Beta vulgaris* L.) ıslah hattından alınan döllenmemiş yumurtalıklardan elde edilen bitki rejenerasyonuna ait yöntem tanımlanmıştır. Yumurtalık eksplantları, 2.0 mg/l benzilaminpürin (BAP) içeren Murashige & Skoog (MS) ortamında kültüre alınmıştır. İki farklı muamele denenmiş olup; eksplantaların tamamı 15 gün karanlıkta tutulduktan sonra, yarısı normal (i) *ışık* ortamına aktarılırkan, diğer yarısı da bütün kültür boyunca (ii) *karanlık* ortamda bırakılmıştır. Her iki ortamda kültüre alınan eksplantlarda aşağı yukarı aynı oranlarda kallus oluşmuştur. Ancak, muameleler arasında eksplantaların sürgün-oluşturma kapasiteleri bakımından önemli farklar meydana gelmiş olup; karanlıktaki ön inkübasyon döneminden sonra ışığa aktarılanlar, sürekli karanlıkta tutulanlara göre daha fazla sayıda sürgün oluşturmuştur (% 14.6'ya karşın % 4.2). Ayrıca, eksplantların kallus ve sürgün-oluşturma kapasiteleri arasında ters orantılı bir ikili gözlenmiştir. Sürgünler 2.0 mg/l naftelenasetik asit (NAA) ve 2.0 mg/l gümüş nitrat (AgNO3) içeren MS ortamına aktarıldıklarında, iki hafta içerisinde köklenme başlanmıştır. Elde edilen bitkilerin ploidi seviyeleri rejenerantların yaprak örneklerinde yapılan kromozom sayımları ile belirlenerek, elde edilen bütün bitkilerin diploid olduğu gözlenmiştir.

Anahtar Sözcükler: *Beta vulgaris* L., doku kültürü, yumurtalık kültürü, bitki rejenerasyonu

Introduction

Plant tissue cultures have been extensively used in plant science with the aim of developing new crop varieties. Establishment of a reliable protocol for the clonal propagation of elite plants, which could be a genetically-engineered material or a single plant or line improved through calssical breeding studies, has been one of the main objectives of such cultures. However, there is a serious drawback in this direction, which is the failure to develop widely-applicable method for the regeneration

which greatly favoured the further growth of the developing roots. Regenerants (plantlets) were then transferred to soil pots containing equal parts sterile soil, sand and compost when they reached 12-15 leaf stage in culture (Figure 1e). The plantlets, however, needed to be sprayed with water regularly, as they tended to wilt otherwise. The timing order of the process appearing during *in vitro* plant regeneration from ovary explants of our sugar beet material is summarized in Table 1, which suggests that regenerant plants can be obtained within nearly a three-month period. Chromosome counting, which was carried out on leaves taken from the regenerant plants immediately before they were transferred to the soil pots, revealed that all progenies were diploid (2n=18).

When a comparison was made between the explants of the treatments at 25 days' incubation, it was clear that ovaries pre-cultured in darkness for 15 days (i.e., light treatment) grew larger and faster than those cultured in darkness continuously (i.e., dark treatment). In addition, the shoots that developed in complete darkness were pale white in colour while those that developed under light conditions, after an initial incubation in darkness for 15 days, were light-to-dark green in colour. Also, in both treatments, observations revealed that there was an inverse relationship between the callus- and shoot-froming capacity of the individual eqplants; either those which primarily formed callus were reluctant to develop shoots or those explants producing shoots were inclined to form either no callus at all or only very little.

Tablo 1. The timing order of plant regeneration from unfertilized ovaries of a diploid male sterile sugar beet breeding line cultured on MS medium supplemented with 2.0 mg/l BAP for callus and shoot formation, and 2.0 mg/l NAA and 2.0 mg/l $AgNO_3$ for root formation

Order	Culture Stage	Time (Days)
1	Culture Initiation	0
2	Callus Induction	13-15
3	Shoot Initiation	20-22
4	Shoot Development	42-45
5	Root Initiation	60-65
6	Production of a Complete Regenerant	80-90

Prolonged culture on medium containing 1.0 mg/l BAP and 0.2 mg/l NAA caused a considerable increase in callus formation at the base of the shoots and also in the number of vitrified shoots. When these cultures were transferred to a fresh basal medium (BM) containing no plant growth regulators (i.e., hormone-free medium), the degree of vitrification of the individual shoots decreased markedly and the shoots grew much more vigorously. In addition, the problem of vitrification was, to a considerable extent, overcome by storing the vitrified shoots at 4°C in a refrigerator (i.e., cold treatment) for 5-7 days and then transferring back to the incubation room. Similarly, the transfet to BM was also effeciteve in reducing callus formation.

Discussion

Plant regeneration from unfertilized ovaries of a sugar beet (*Beta vulgaris* L.) breeding line (21124) was achieved in this study. Our main objective was to produce haploid plants through *in vitro* gynogenesis, which was previously reported by some researchers (6, 10-12), and to use them in an on-going breeding program aimed at the production of homozygous lines. We therefore hoped to obtain haploid plants. However, chromosome counting revealed that all the regenerants were diploid, and this method, therefore, turned out to be an efficient means of plant regeneration, rather than of haploid plant production.

Sugar beet is known as a recalcitrant species in terms of tissue culture responses (5, 13). Callus can be readily produced when cultured on media containing plant growth regulators, but regeneration is unpredictable. Although indirect organogenesis from callus and direct organogenesis from sugar beet tissue without prior callus formation have been observed (1, 4, 14-16), the necessary conditions have not been well defined yet. The rate of success obtained in this study for the regeneration of our sugar beet breeding material, therefore, is more significant in consideration of the recalcitrancy of this economically important crop species.

In our study, we used ovary explants for plant regeneration since anther culture (i.e., androgenesis) has proven unsuccessful in several sugar beet materials, resulting in either callus formation (11, 17) or roots only (18), while ovules/ovaries (i.e., gynogenesis) have been reported to produce haploid plants, althougn at low frequencies (6, 7, 10). We used ovary explants isolated from secondary or tertiary inflorescences since previous studies reported that the primary branches produced considerably less regeneration (11). The colour of the anthers at the time of isolation varied from pale-yellow to brown depending on the physiological ages of the buds, as also reported by other researchers (7).

MS medium containing 2.0 mg/l BAP seemed efficient for the induction of shoots in both light and dark treatments, although the light treatment produced more

of different varieties of the same species or even sometimes different plants of the same vaiety, let alone closely related plant species (1, 2). There seems to be several factors which determine the ability of regeneration from many plant tissues, such as the type (source) of explant, genotype and the physiological age of the explant tissue, as well as other cultural and environmental factors (3), and the development of a reproducible method can oftne be achieved by an extensive and, perhaps more significantly, sensitive optimization of these parameters (4), although some species, for instance sugar beet (*Beta vulgaris* L.), are recalcitrant to *in vitro* regeneration (5).

Sugar beet is one of only two crop species from which sugar can be produced commercially; sugar cane and sugar beet provide 63% and 37% of the world's total sugar production, respectively. It is a heterosygous species and, thus, the production of homozygous lines is normally difficult. Anther or ovule cultures are frequently used to minimize this problem by producing haploid lines following chromosome doubling (6). Such cultures can also be used for plant regeneration, but the frequency is rather low when compared with other tissue culture techniques (7). The results of our experiments are presented, in which attempts were initially made to obtain haploid plants from ovary explants of a sugar beet breeding line but only diploid progeneis were obtained.

Materials and Methods

Plant Material and Sterilization

A diploid (2n=2x=18) male sterile sugar beet (*Beta vulgaris* L.) breeding line (21124) developed at the Sugar Institute, Plant Breeding Department, Ankara, Turkey, was used in this study. Secondary or tertiary inflorescence branches 5-8 cm in length with unopened (unfertilized) flower buds were removed from field-grown plants in the early hours of morning (06:00-07:00 am) in July. The inflorescene pieces were first washed with tap water. The branches were then dried between sterile distilled water. The branches were then dried between sterile filtre papers before 3-4 mm long buds were dissected under a dissecting microscope in the laminar flow to remove the whole ovary. The ovary explants were longitudinally cut into two half pieces and each half was used as an explant.

Shoot and Root Development

For shoot induction, ovaries were a) either incubated in darkness for 15 days and then transferred to a 16 hour light/8 hour dark regime (light treatment), or b) kept in complete darkness throughout the culture by covering the containers with aluminium foil (dark treatment). A total of 144 explants per treatment (experiments were repated three times, using 48 explants per treatment at each repeat) were cultured in 9 cm plastic Petri dishes (12 explants per dish) containing 20 ml of MS basal medium (8) supplemented with 2.0 mg/l BAP (6-benzly amino purine), 3% sucrose and 0.8% agar (Oxoid No.3), pH 5.8, which had been sterilised by autoclaving for 15 mins at 103.5 kPa. This medium was designated the shoot-inducing medium (SIM). The developing shoots were transferred to fresh MS medium containing 1.0 mg/l BAP and 0.2 mg/l NAA (naphthalene acetic acid) for further growth and development. The shoots were then transferred to glass jars containing the root-inducing medium (RIM), which was the same as the SIM medium with the exception of using 2.0 mg/l NAA instead of 2.0 mg/l BAP and 2.0 mg/l $AgNO_3$ (silver nitrate). When root fromation was induced, the shoots with very small roots were transferred to MS basal medium containing no plant growth regulators (hormone-free medium) for further growth of the roots. Petri dishes or glass jars were sealed with Parafilm, and both shoot and root cultures were incubated at 25±1°C and 55-60% relative humidity. The rooted shoots were finally transferred to 10 cm soil pots containing equal parts of sterile soil, sand and compost. The soft and fragile leaves of the plantlets were half-covered with plastic seheets and sprayed with distilled water containing half-strength MS salts every three hours during the day for the first two weeks to prevent wilting.

Ploidy Determination

Ploidy levels of the regenerants were determined by chromosome counting in leaves before the plantlets were transferred to the soil pots. The leaf samples taken from the lamina tip of the latest (youngest) leaf of the regenerated plants were first soaked in 8-hydroxychinoline (0.002 mol/l) for 4 hours and then washed with tap water four times. The materials were then fixed in a solution containing 2 volume 96% ethanol: 1 volume HCl for 20 minutes. In order to remove the fixative from the leaves, they were washed with tap water several times and kept in distilled water. Small pieces of the leaf tissue were excised and put on a slide glass and then a drop of 3% aceto orcein was applied to the samples and chromosomes were counted under a light microscope (9)

Results

The first observations were made at 15 days when half of the ovary explants were transferred from dark to

light. Changes in size and colour of the ovary explants were apparent in both light and dark treatments. Most of the explants had already produced varying amounts of callus by 15 days, until which time all the explants of both treatment groups had been kept in darkness (Figure 1a). Apart from callus fromation, some small projecting ridges appeared around the explants cultured on medium contianing 2.0 mg/l BAP, and by 21 days, some of these ridges began to resemble small leafy structures (Figure 1b), most of which then developed into shoots during the next 3 weeks, i.e., by 42-45 days on the same medium, while others formed callus. There was a significant difference between the treatments in terms of the number of explants producing shoots: 14.6% of the explants in the light treatment and 4.2% in the dark treatment. The newly developed shoots were then subcultured for three weeks on MS medium containing a lower BAP (1.0 mg/l) concentration and NAA (0.2 mg/l) (Figure 1c), which was found favourable for shoot multiplication and development in both treatments.

After shoot growth and multiplication were completed, the multiple shoots were first singled out and then transferred to the RIM medium, which contained 2.0 mg/l NAA and 2.0 mg/l silver nitrate. Rooting of the shoots was readily achieved, nearly three fourth (72.3%) of the shoots in the light treatment and half (52.4%) of the shoots in the dark treatment producing roots within two weeks on the RIM medium (Figure 1d). After root induction was completed and very small roots began to appear at the base of the shoots on the RIM medium, they were transferred to BM (hormone-free) medium, shoots, 14.6% of the explants produced shoots in the light treatment compared to 4.2% in the dark treatment.

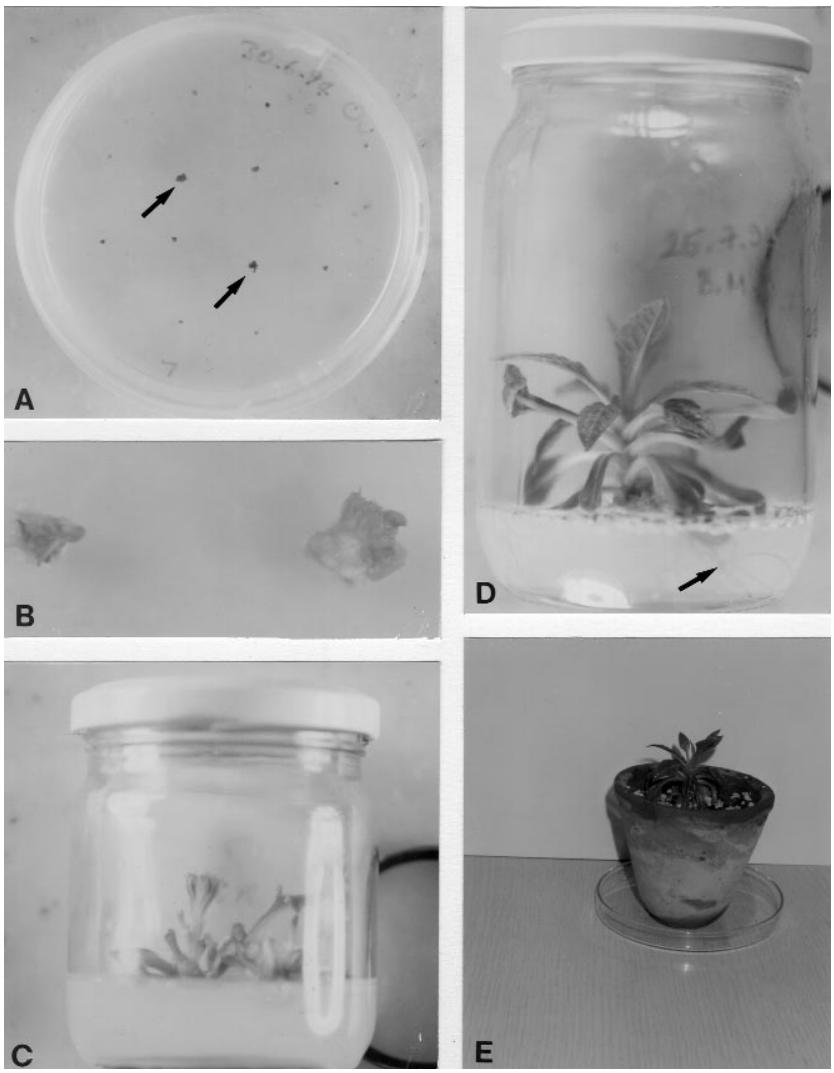

Figure 1. Plant regeneration from unfertilized ovaries of a sugar beet (*Beta vulgaris* L.) breeding line. a) Ovary explants (arrow) at 15 days' incubation on the SIM medium contianing 2.0 mg/l BAP, b) Small leafy structures developed from the ovary explants on the SIM medium, c) Multiple shoots obtained by subculturing on MS medium supplemented with 1.0 mg/l BAP and 0.2 mg/l NAA, d) Rooting of the shoots (arrow) on the RIM medium containing 2.0 mg/l NAA and 2.0 mg/l $AgNO_3$, e) Regenerant plants transferred to soil pots containing one third of sterile

There was also a clear difference between the treatments with respect to the appearance of the explants. Those explants incubated in light were a light-to-dark green colour whereas those kept in dark continuously were pale white in colour, most likely due to the synthesis of chlorophyll as the shoot development proceeded under a normal light/dark regime.

Rooting of the shoots was readily achieved when cultured on a medium supplemented with 2.0 mg/l NAA and 2.0 mg/l silver nitrate. Auxins are well known to induce adventitious root formation in many plant species, and 2.0 mg/l silver nitrate was previously found to promote root development in leaf explants of sugar beet, causing a three-fold increase in the number of emerged roots and a five-fold increase in the number of root primordia present in the midrib-petiole junction segments when compared to the control explants (15). There are other studies reporting the promotive effect of silver nitrate for shoot (19), callus (20) or root formation (21) in other species. In our study, the further growth of the developing shoots was greatly enhanced when the rooted shoots were transferred from the RIM medium, which contained a relatively high concentration of NAA (2.0 mg/l), to MS basal medium supplemented with no plant growth regulators. This was consistent with our previous research, in which a high NAA concentration (1-30 mg/l) was found to inhibit root growth after induction, whereas transferring leaf explants from high NAA to hormone-free medium, after an initial incubation for 1-4 days at high NAA, greatly enhanced subsequent root growth (5).

Prolonged culture on MS containing 1.0 mg/l BAP and 0.2 mg/l NAA, which was used as a medium for the further growth of the shoots, caused both increased callus formation and vitrification of the shoots. Vitrification has also been reported in other research on ovule culture in the sugar beet (7), and we were able to overcome this problem by cold treatment at 4°C for 5-7 days, which is a common practice in the culture of many plant species (22).

The shoots appeared to develop directly from the explant tissue (i.e. direct organogenesis) since not much callus formation was observed in the vicinity of the shoots. However, histological study is needed to confirm this. In addition, since all of the plantlets obtained were diploid, we suggest that they developed from the somatic cells surrounding the ovary, a commonly observed phenomenon in anther or ovary cultures of sugar beet (18). On the other hand, spontaneous dihaploidization could also be accounted for the diploid regenerants but this appears to be rather remote since it was expected to obtain at least few, if not many, haploid plants from the developing ovaries. In conclusion, a plant regeneration system from unfertilized ovaries of sugar beet was developed, and this procedure may help to improve the *in vitro* manipulation of sugar beet, which is known as recalcitrant.

References

1. Saunders JW, Shin K, Germplasm and physiological effects on induction of high-frequency hormone autonomous callus and subsequent shoot regeneration in sugar beet. Crop Sci., 26:1240-1245, (1986).
2. Gürel E, In vitro development from leaf explants of sugar beet (Beta vulgaris L.): Variability between cultivars, plants and organs. Turkish J. Both., 21:131-136, (1997).
3. Krens FA, Jamar D, The role of explant source and culture conditions on callus induction and shoot regeneration in sugar beet (Beta vulgaris L.). J. Plant Physiol., 134:651-655, (1989).
4. Tetu T, Sangwan RS, Sangwan-Norreel BS, Hormonal control of organogenesis and somatic embryogenesis in Beta vulgaris L. callus. J. Exp. Bot., 38:506-517, (1987).
5. Gürel E, Wren MJ, In vitro development from leaf explants of sugar beet (Beta vulgaris L.): Rhizogenesis and the effect of sequential exposure to auxin and cytokinin. Ann. Bot., 75:31-38, (1995).
6. Bossoutrot D, Hosemans D, Gynogenesis in Beta vulgaris L. From in vitro culture of unpollinated ovules to production of doubled haploid plants in soil. Plant Cell Rep., 4:300-303, (1985).
7. Galatowitsch MW, Smith GA, Regeneration from unfertilized ovule callus of sugar beet (Beta vulgaris L.). Can. J. Plant Sci., 70:83-89, (1990).
8. Murashige T, Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15:473-497, (1962).
9. De Jong JH, De Bock TSM, Use of haploids of Beta vulgaris L. for the study of orcein and giemsa stained chromosomes. Euphytica, 27:41-41, (1978).
10. Van Geyt JPC, Speckmann GJ, D'Halluin K, Jacobs M, In vitro induction of haploid plants from unpollinated ovules and ovaries of the sugar beet (Beta vulgaris L.). Theor. Appl. Genet., 73:920-925, (1987).

11. Doctrinal M, Sangwan RS, Sangwan-Norreel BS, In vitro gynogenesis in Beta vulgaris L.: Effect of plant growth regulators, temperature, genotypes and season. Plant Cell Tiss. Org. Cult., 17:1-12, (1989).

12. Manowlovic V, Nikoic Z, Radovic S, Sugar beet haploids obtained from unpollinated ovules. IIRB Annual Meeting, pp. 147-150, 19-22 June, France, (1995).

13. Bornman CH, Regeneration in vitro of economically important crop plants in the Nordic countries. Hereditas Suppl., 3:7-13, (1985).

14. Detrez C, Tetu T, Sangwan RS, Sangwan-Norreel BS, Direct organogenesis from petiole and thin cell layer explants in sugar beet cultured in vitro. J. Exp. Bot., 39:917-926, (1988).

15. Gürel E, Callus development and organogenesis in cultured leaf explants of sugar beet (Beta vulgaris L.). The Proceedings of the Second Arab Conference on Perspectives of Modern Biotehnology, pp. 314-330, April 24-28, 1993, Amman, Jordan, (1993).

16. Gürel E, Gürel S, Plant regeneration from leaf explants of sugar beet (Beta vulgaris L.) cultured in vitro. J. Kükem, 19:29-37, (1996)

17. Rogozinska, JH, Goska M, Kuzdowicz A, Induction of plants from anthers of Beta vulgaris cultured in vitro. Acta Soc. Bot. Polon., 3:471-479, (1977).

18. Rogozinska JH, Goska M, Attempts to induce haploids in anther culture of sugar, fodder and wild species of beet. Acta Soc. Bot. Polon., 51:91-105, (1982).

19. Khalid M, Chraibi B, Latche A, Roustan JP, Fallot J, Stimulation of shoot regeneration from cotyledons of Helianthus annuus by ethylene inhibitors, silver nitrate and cobalt. Plant Cell Rep., 10:204-207, (1991).

20. Williams J, Pink DAC, Biddington NL, Effect of silver nitrate on long term culture and regeneration of callus from Brassica oleracea. Plant Cell Tiss. Org. Cult., 21:61-66, (1990).

21. Liu J, Mukherjee I, Reid DM, Adventitious rooting in hypocotyls of sunflower (Helianthus annuus) seedlings. III. The role of ethylene. Physiol. Plant., 78:268-276, (1990).

22. Deberg P, Harbaoi Y, Lemeur R, Mass propagation of globe artichoke (Cynara scolymus): Evaluation of different hypotheses to oevrcome vitrification with special reference to water potential. Physiol. Plant., 53:181-187, (1981).