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Research Article

Development of an Efficient Plant Regeneration System in Sunflower (*Helianthus annuus* L.)

Ekrem GÜREL
Abant İzzet Baysal University, Faculty of Arts & Sciences, Department of Bilology, 14280 Bolu-TURKEY

Kemal KAZAN
CRC for Tropical Plant Pathology, University of Queensland, Queensland 4072, AUSTRALİA

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Abstract: With the aim of developing an efficient plant regeneration system from cells or tissues of sunflower (*Helianthus annuus* L.), we compared several regeneration protocols using different explant types and hormonal combinations. Somatic embryogenesis could be induced on cotyledon explants, especially from the basal (proximal) portion of the cotyledons, but genotypic vairation appeared to be the most critical factor for both somatic embryo and root production. Such a variation was more prominent when 10 different sunflower cultivars were compared for shoot production from shoot-tip explants. Further refinement of the culture conditions may be neceassary to improve the efficiency of somatic embryo production. Thin cell layers from hypocotyl segments were not found to be successful for plant regeneration.

Key Words: *Helianthus annuus* L., sunflower, tissue culture, plant regeneration.

Ayçiçeğinde (*Helianthus annuus* L.) Etkin bir Bitki Rejenerasyon Sisteminin Geliştirilmesi

Özet: Ayçiçeği (*Helianthus annuus* L.) hücre veya dokularından etkin bir bitki rejenerasyon sistemi geliştirmek amacıyla, farklı eksplant tipleri ve hormon kombinasyonlarının kullanıldığı değişik rejenerasyon protokolleri karşılaştırılmıştır. Özellikle ait (bazal) kısımlardan olmak üzere, kotiledon eksplantlarından somatik embriyogenezis elde edilmiş, fakat genotipik varyasyonunun hem somatik embriyo hem de kök üretimi için en kritik faktör olduğu gözlenmiştir. Sürgün-ucu ekpslantlarından sürgün üretimi bakımından 10 farklı ayçiçeği çeşidi karşılaştırıldığında, söz konusu varyasyon çok daha belirgin olarak ortaya çıkmıştır. Somatik embriyo üretimini artırmak amacıyla kültür şartlarının geliştirilmesi gerekli görülebilir. Hipotokil segmentlerinden elde edilen ince hücre katmanları ise bitki rejenerasyonu açısından başarılı bulunmamıştır.

Anahtar Sözcükler: *Helianthus annuus* L., ayçiçeği, doku kültürü, bitki rejenerasyonu.

Introduction

Sunflower (*Helianthus annuuss* L.) is one of the most important oil crops in the world and high oil content together with increased disease resistance has been the main aim for the improvement of this crop. Due to the limited natural variation, the genetic improvement of the cultivated sunflower for such agronomic traits has been broadly based on the ability to transfer the desired genes from wild relatives through conventional breeding methods, but unfortunately the utilization of many wild spicies has been limited by natural barriers to the reproductive process. Successful application of gene transfer techniques for the improvement of technological and agricultural qualities of sunflower varieties, therefore becomes the most efficient and promising alternative. However, the development of an applicable gene transfer system requires first an efficient plant regeneration system from the target cells following the transformation process.

There are several reports describing plant regeneration from different explants of sunflower including immature embryos (1, 2), cotyledons (3, 4) and thin cell layers from hypocotyls (5). However, inefficient plant regeneration, difficulties in rooting, abnormalities in the morphogenesis and premature flowering are the major problems. The only efficient type of explant currently used for genetic transformation of sunflower genotypes appears to be the shoot-tip meristems (6-8). This is simply because of the ease of regeneration of plants from the meristematic tissue in which shoot and

leaf primordia already exist, but the chimeric nature of the regenerants developing from such meristematic tissue is a serious problem (9). It is, therefore, important to develop a reliable transformation protocol which would use a plant regeneration system from somatic tissues through organogenesis or somatic embryogenesis.

In an effort to facilitate the trnasfer of genes encoding novel antimicrobial peptides into sunflower through *Agrobacterium tumefaciens*, we have previously identified the most responsive material to bacterial infection using split or non-split shoot-tip meristem explants after wounding by particle bombardment (unpublished data). In the present study, with the aim of developing a more efficient plant regeneration system from genetically modified cells or tissues of sunflower in a way to prevent the production of chimeric transgenics, we examined different regeneration protocols and types of explants including shoot-tips, cotyledons, cotyledonary petioles, hypocotyys and thin cell layers from hyocotyls in a subset of diverse genotypes (10).

Materials and Methods

Comparison of BAP and NAA Combinations

Sunflower seeds from a commercial hybrid cultivar (Hysun 45) were rinsed twice with sterile distilled water and then soaked in it for 2 h at room temperature. They were then washed in 70% ethanol for 2 min, dehulled and rinsed in 70% ethanol for another 2 min followed by surface sterilization in 2.5% sodium hypochlorite for 10 min under vacuum infiltration. The seeds were then rinsed again in 70% ethanol for 2 min and finally rinsed in sterile distilled water 3-4 times followed by 5-6 hours imbibition in sterile distilled water at room temperature. Seed coats were then removed before culturing in deep plastic Petri plates 9 cm in diameter, each containing 25 ml of MSO medium which contained full-strength MS (11) salts and vitamins, 30 g/l sucrose and 8 g/l bacto agar (Difco) at pH 5.7. After three days' germination in darkness at 28°C, four types of explants were prepared from these seedlings; a) half-shoot apices (shoot-tips were cut into two longitudinal halves), b) 0.5 cm length hypocotyl segments, c) intact cotyledons and d) cotyledonary petiole (the base of cotyledons to the point where attached to the hypocotyl). The explants were cultured at 16 different hormonal comibinations consisting of four differerent levels of BAP, benzylamino purine, (0.0, 0.5, 1.0 and 2.0 mg/l) and NAA, naphtleneacetic acid, (0.0, 0.1, 0.25 and 0.50 mg/l). 30 explants per treatment were used, with 5 explants in each plate, and the experiment was repeated twice. Cultures were incubated at 25/20°C day/light temperature in a 16-hr photoperiod under white fluorescent light (160 μE m^{-2} s^{-1}) and the results were evaluated after 15 days in culture by scoring callus, root and shoot formation. For callus assessments, a 0-6 scoring system (12) was used whereas root and shoot assessments were made by counting the number of roots and shoots. No further steps were taken to obtain rooted shoots or complete regenerants.

Comparison of Different Genotypes

Seeds from 10 different genotypes, four commercial hybrids (Hysun 25, Hysun 36, Hysun 45 and Hyoleic 31), five public inbred lines (HA 89, HA 341, DL 9542, DL 9546 and DL 9548) and one restorer line (RHA 271), were sterilised as described above and cultured on MSA medium which contained full-strength MS medium supplemented with 0.1 mg/l BAP, 500 mg/l casaminoacid, 30 g/l sucrose, 8 g/l bacto agar (Difco) at pH 5.7. After two days' incubation in darkness at 28°C, the cotyledons and radicles were removed and the explants were transferred back to the same medium at 25/20°C day/light temperature in a 16-hr photoperiod under white fluorescent light (160 μE m^{-2} S^{-1}). 45-60 shoot-tip explants per cultivar were used, with 5 explants in each plate, and the number of shoots was counted at the end of 10 days' incubation. This experiment was repeated three times.

Somatic Embryogenesis from Cotyledons

The method used here was modified from a recent study (4). Seeds from three commercial hybrids (Hysun 25, Hysun 45 and Hyoleic 31) and one restorer line (RHA 271) were sterilised as described above and cultured on MSO medium in darkness for 6 days. Intact cotyledons were removed from the seedlings and cut transversely into two equal parts, i.e. upper/distal and lower/proximal part. Each half was cultured separately on SEM (somatic embryo medium) for 15 days in darkness at 28°C. The SEM medium contained full-strength MS medium supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 0.1 mg/l GA3 (giberellic acid), 100 mg/l myo-inositol, 0.5 mg/l KNO3, 0.4 mg/l thiamine, 30 g/l sucrose and 8 g/l agar at pH 5.7. Then, explants were transferred to fresh SEM medium at 25/20°C day/light temperature in a 16-hr photoperiod under white fluorescent light (160 μE m^{-2} s^{-1}) for 5 days and then finally transferred to REG (regeneration medium) for 7 days for somatic embryogenesis. The REG medium was the same as SEM except for that it contained 0.5 mg/l NAA instead of 1.0 mg/l and did nod contain thiamine. 40 explants per treatment were used, with 5 explants in each plate, and

the number of somatic embryos and roots were counted but the callus formation was measured by determining the amount of callus and was expressed as callus increase (g) after 28 days' incubation. This experiment was repeated twice.

Thin Cell Layer Experiment

A modified protocol from a recent work (5) was followed for this experiment. Seeds from two hybrid cultivars, Hysun 45 and Hyoleic 31, were sterilised and germinated as described above for somatic embryogenesis experiments. The thin cell layer explants (TCLs) were prepared by removing a very thin epidermal layer with a sharp scalpel from 1 cm long hypocotyl segments of 8-day-old seedlings. The explants were cultured for 5 days in darkness in liquid MSL medium which contained full-strength MS medium supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 2 g/l casein hydrolysate and 30 g/l sucrose at pH 5.7. 50 explants per cultivar were used, culturing 10 explants in each of five 125 ml flaks. The flasks were shaken continuously at 160 rpm at 28°C for 5 days and then transferred to liquid B5L medium containing Gamborg's B5 salts and vitamins (13) supplemented with 90 g/l sucrose only at pH 5.7 for 8 days under the same conditions. This experiment was not repeated.

Results & Discussion

Effects of BAP and NAA Combinations on Callus, Root and Shoot Formation

Our initial experiments compared the effects of 16 combinations of BAP and NAA concentrations on callus, root and shoot formation from four different types of explants; shoot-tips, hypocotyl segments, cotyledons and cotyledonary petioles (Tables 1-3). In terms of callus development, the general pattern was that the amount of callus steadily increased with increasing BAP concentrations at all NAA levels, regardless of the explant type, although callus formation was usually highest when NAA was absent (Table 1). However, when different types of explants were compared, it appeared that shoot-tip and hypocotyl explants produced slightly more callus than cotyledon and cotyledonary petiole explants. The relationship between callus formation and BAP/NAA combinations is consistent with previous studies (3,14). Since somaclonal variation is not desirable, the higher ability of callus production of shoot-tip and hypocotyl explants would not be of much use, as also reported by others (15).

With regard to the rooting abilities of the explants, it was clear tahat BAP at any concentration completely blocked root induction since root development occurred in those treatments which contained NAA only in all types of explant (Table 2). It was also observed that none of the explants produced roots when NAA was also absent, i.e. hormone-free medium. However, while 0.10 and 0.25 mg/l NAA appeared to be inductive, 0.50 mg/l was found to be inhibitory. When comparing explants types, the cotyledons were the least responsive to the root-inducing effect of the auxin NAA. These findings were, however, promising since previous studies demonstrated that the rooting of the regenerated shoots was difficult (16).

In assessing the shoot-forming abilities of four different types of explants, the results celarly indicated that shoot-tips were the most responsive (Table 3). This is rather predictable since a meristematic tissue already

Table 1. Effects of different levels of BAP and NAA on callus formation (expressed as mean callus per explant according to the 0-6 scoring system) from shoot-tips, hypocotyls, cotyledons and cotyledonary petioles of cv. Hysun 45.

Explant	NAA (mg/l)	BAP (mg/l)			
		0.0	0.5	1.0	2.0
Shoot-tip	0.0	0.8	1.4	2.6	3.4
	0.1	2.4	3.8	4.0	4.0
	0.25	2.2	3.2	3.0	1.4
	0.5	0.8	2.0	3.0	1.4
Hypocotyl	0.0	1.2	2.6	4.0	2.8
	0.1	2.0	4.0	3.8	4.0
	0.25	2.7	3.2	3.2	2.4
	0.5	1.0	2.3	1.4	1.2
Cotyledon	0.0	0.8	1.4	0.8	1.2
	0.1	0.0	2.0	2.2	2.0
	0.25	1.0	2.2	1.2	2.2
	0.5	0.0	1.6	1.7	1.2
Cot. Petiole	0.0	0.8	1.6	1.0	2.4
	0.1	1.2	2.6	2.8	2.2
	0.25	1.6	2.4	2.4	2.0
	0.5	0.6	0.0	2.2	2.3

Explant	NAA (mg/l)	BAP (mg/l) 0.0	0.5	1.0	2.0
Shoot-tip	0.0	0.0	0.0	0.0	0.0
	0.1	0.1	0.0	0.0	0.0
	0.25	0.4	0.0	0.0	0.0
	0.5	0.0	0.0	0.0	0.0
Hypocotyl	0.0	0.0	0.0	0.0	0.0
	0.1	1.4	0.0	0.0	0.0
	0.25	1.5	0.0	0.0	0.0
	0.5	0.0	0.0	0.0	0.0
Cotyledon	0.0	0.0	0.0	0.0	0.0
	0.1	0.0	0.0	0.0	0.0
	0.25	0.6	0.0	0.0	0.0
	0.5	0.0	0.0	0.0	0.0
Cot. Petiole	0.0	0.0	0.0	0.0	0.0
	0.1	0.2	0.0	0.0	0.0
	0.25	0.6	0.0	0.0	0.0
	0.5	1.2	0.0	0.0	0.0

Table 2. Effects of different levels of BAP and NAA on root formation (expressed as mean root number per explant) from shoot-tips, hypocotyls, cotyledons and cotyledonary petioles of cv. Hysun 45.

exists in such explants, which then undergoes organogenesis readily (17). This is further supported by the fact that there was no considerable difference among the different combinations of BAP and NAA in terms of the mean number of shoots produced. Apart from the shoot-tip explants, only hypocotyl explants produced few adventitious shoots at combinations of 0.0, 0.5 and 1.0 mg/l BAP only, producing no shoots with either higher cytokinin alone (2.0 mg/l BAP) or any other treatments. This finding is consistent with the generally known inhibitory effect of auxins on shoot induction (3). None of the cotyledon or cotyledonary petiole explants were able to produce shoots in this experiment, perhaps requiring further precision of the culture parameters.

Comparison of Different Genotypes for Regeneration from Meristematic Tissue

We compared 10 different sunflower gneotypes in terms of shoot formation (Table 4). As the data indicates, there were significant differences between genotypes, with the mean number of shoots ranging from 7.1 in cv. Hysun 45 to none at all in cv. DL 9542. Also, these results show that the hybrid cultivars (Hysun 25, Hysun 36,

Explant	NAA (mg/l)	BAP (mg/l) 0.0	0.5	1.0	2.0
Shoot-tip	0.0	1.6	1.2	1.8	2.4
	0.1	1.7	2.0	1.4	0.4
	0.25	2.2	1.4	1.8	1.0
	0.5	2.0	0.7	1.4	1.2
Hypocotyl	0.0	0.4	1.2	0.9	0.0
	0.1	0.0	0.0	0.0	0.0
	0.25	0.0	0.0	0.0	0.0
	0.5	0.0	0.0	0.0	0.0
Cotyledon	0.0	0.0	0.0	0.0	0.0
	0.1	0.0	0.0	0.0	0.0
	0.25	0.0	0.0	0.0	0.0
	0.5	0.0	0.0	0.0	0.0
Cot. Petiole	0.0	0.0	0.0	0.0	0.0
	0.1	0.0	0.0	0.0	0.0
	0.25	0.0	0.0	0.0	0.0
	0.5	0.0	0.0	0.0	0.0

Table 3. Effects of different levels of BAP and NAA on shoot formation (expressed as mean shoot number per explant) from shoot-tips, hypocotyls, cotyledons and cotyledonary petioles of cv. Hysun 45.

Table 4. Comparison of different sunflower genotypes in terms of shoot formation from shoot-tip explants after 10 days incubation.

Genotypes	Mean Number of Shoots per Explant
Hysun 45	7.1
Hysun 36	6.5
Hyoleic 31	5.8
Hysun 25	3.9
DL 9548	2.4
HA 341	2.0
DL 9546	1.8
RHA 271	0.4
HA 89	0.3
DL 9542	0.0

Hysun 45 and Hyoleic 31) were distinctly better than others. This might be attributed to the high vigour of the hybrid material. It may be suggested that determining the right source of plant material might be a more significant factor than determining the right treatment when optimizing a regeneration protocol in sunflower. The genotypic variation can be a serious problem in all tissue culture work, especially with heterozygous species like sunflower (18-20), Previous studies demonstrated that genetic variability was enormous when 30 cultivars were compared, with nearly half of the cultivars producing no shoots at all and the other half ranging up to 70% regeneration capacity (18). To minimize the effect of genetic variation on the success of a regeneration system in a given species, one possible way would be to use a large number of explants. Another alternative would be to develop a method for secreening plant material to identify those with a low potential for organogenesis, as proviously attempted in other species (21). It should be also noted that the mean number of shoots obtained in this experiment were considerably higher than in the previous experiment. This is because of the use of intact shoot-tip explants in this set of experiments whereas the previous one used split shoot-tips. Our preliminary experiments (unpublished data) compared split and intact shoot-tip explants and the results showed that the former type of explants produced fewer shoots, most likely due to the damage caused to the meristematic region by cutting with a scalpel, thus reducing the number of new shoots developing from the wounded meristems.

Regeneration of Somatic Embryos from Cotyledons

Embryo development could be observed after three weeks ‘ incubation. No dissecting or light microscopy was done to determine the origin and developmental sequence of embryos (i.e., globular, heart and torpedo stages). However, samples were routinely observed under a stereo microscope, and both the bipolar structure of the newly formed embryos, most likely being at the late torpedo to cotyledonary stage, and the subsequent development of roots and shoots spontaneously were regarded as proof of somatic embryo formation. It should also be noted that these embryos appeared to be of direct origin since no callus formation could be observed in the vicinity of the developing embryos.

Three commercial hybrid cultivars (Hysun 25, Hysun 45 and Hyoleic 31) and one restorer line (RHA 271) were compared for somatic embryo, root and callus formation using upper and lower parts of cotyledons as the source of explants (Table 5). The most prominent observation was that there were clear differences between genotypes and between upper and lower part explants for somatic embryo production. Out of the four cultivars, only Hyoleic 31 produced somatic embryos but there was about a four-fold difference between lower and upper part explants of the same cultivar, 1.47 embryos per explant compared with 0.40, respectively. This difference may be attributed to the polar transport of endogenous auxins if they have any role in embryo induction (22). It might be also related to the age of the tissue used since it is well known that the lower (proximal) part of leaf tissue is physiologically older than the upper (distal) part as the leaf expands (23) although it is difficult to assume that such a great difference could be the result of such an apparently minor effect. Somatic embryos obtained in our experiment readily developed into complete plants within 15-20 days when transferred to MS basal medium although no particular attempt was made to produce whole regenerants.

It is also interesting to observe that the *in vitro* embryogenic and organogenic capacities were restricted to the hybrid cultuvars only as cv. RHA 271 produced neither somatic embryos nor adventitious roots but callus only. Nevertheless, the hybrid genotypes exhibited a differential pattern, i.e. cv. Hyoleic 31 was productive for all parameters while cvs. Hysun 25 and Hysun 45 failed to produce somatic embryos and formed roots and callus only. It should also be noted that despite a several-fold difference between upper and lower part explants in terms of somatic embryo production, both types of explants of all cultivars produced similar amounts of root

Genotypes	Explant	Mean No. Embryos	Mean No. Roots	Mean Callus Increase (g)
Hyoleic 31	Upper	0.40	2.28	1.13
	Lower	1.47	2.28	1.45
Hysun 25	Upper	0.00	0.27	0.94
	Lower	0.00	0.65	1.04
Hysun 45	Upper	0.00	0.95	0.59
	Lower	0.00	1.04	0.76
RHA 271	Upper	0.00	0.00	0.53
	Lower	0.00	0.00	0.85

Table 5. Somatic embryo (SE), root and callus formation from upper part and lower part of cotyledons taken from 6-day-old seedlings of four different sun-flower genotypes.

or callus. This may suggest that the two different types of internal organization leading to somatic embryogenesis or organogenesis involve different developmental processes.

Attempts to Regenerate Plants from Thin Cell Layers

Thin cell layer explants were prepared by isolating epidermal plus a few sub-epdermal cells from the hypocotyls of 8 days old seedlings of two hybrid cultivars, Hysun 45 and Hyoleic 31, and cultured under conditions defined to be successful in a very recent report (5). Our results were not consistent with their finding since we obtained only two highly vitrified somatic embryos from cv. Hyoleic 31. In addition, we observed that high sucrose concentration used at the second stage of this protocol caused the suspension culture to become viscous and glutinous which apparently reduced the aeratin of the culture medium and increased the osmotic potential excessively. Therefore, high viscosity, less aeration and high osmiticum all caused by high sugar concentraiton might be regarded as possible reasons for failure. On the other hand, the gneotypic variation could also be responsible since it is a critical factor in the success of a regeneration system (19, 20).

Conclusions

In tissue culture studies, direct organogenesis or somatic embryogenesis is preferred if somaclonal variation is a concern. Furthermore, since somatic embryos can readily develop into a functional plant, they are much preferred to adventitious shoots in those species in which rooting is a problem. With regard to transformation studies, adventitious shoot or somatic embryo production is even more signigicant since chi meric tarnsgenics derived from meristems are of little value. The success of inducing somatic embryos from cotyledon explants in our attempts, therefore, offers the possibility of using this protocol for the genetic transformation of our sunflower material through *Agrobacterium tumefaciens.* However, it may be necessary to refine the culture conditions in order to improve the efficiency of the system. Genotypic variation seems to be an important factor for a tissue culture work, thus determining the most responsive material appears to be the first priority. Thin cell layer explants did not seem to work, at least with our material, although we tried to reproduce the results of a previous protocol, and also seemed to offer little success as they require liquid culture conditions incubated in darkness. In cases where shoot-tip explants are to be used, the hormonal combination of the culture medium may not be very critical as new shoots can readily develop from a large number of explants, most likely due to the existense of meristems.

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