Plant Cell Physiol. 33(2): 157–164 (1992)

Cotyledons: an Explant for Routine Regeneration of Sunflower Plants

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The potential of plant regeneration from sunflower cotyledon explants was studied under conditions either to allow direct regeneration (organogenesis) or callus proliferation with high regeneration capacity.

Analysis of histological modifications caused by tissue culture conditions showed that the regeneration protocol described here permitted the development of abundant meristematic centers in discrete regions of the basal part of the cotyledon.

The protocol presented here proved to be suitable for the regeneration of 10 genotypes, among 20 assayed, including commercial inbred lines and hybrids of common use for production in Argentina.

Furthermore, *Agrobacterium* infection experiments showed that basal halves of cotyledons were transformed, evidencing tumour proliferation.

Key words: *Helianthus annuus L* — *Helianthus argophyllus* — Plant regeneration.

The application of biotechnology for the improvement of sunflower has been limited due to difficulties associated with the lack of an efficient and reproducible method for plant regeneration. Although fertile sunflower plants have been regenerated from a variety of cultured tissues, these methods are often limited to certain genotypes and have not been successfully applied to commercial lines or hybrids.

Successful regeneration from de-differentiated tissues, arising from explants, has been reported (Sadhu 1974, Binding et al. 1981, Greco et al. 1984, Paterson and Everett 1985, Finer 1987, Freyssinet and Freyssinet 1988, Wilcox-McCann et al. 1988, Jeannin et al. 1989). In addition, regeneration from cultured cotyledons has also been successful. In these experiments, immature and mature stages of cotyledons have shown the most promising results. Diverse genotypes of sunflower have been regenerated from cotyledons of immature embryos (Finer 1987, Freyssinet and Freyssinet 1988, Wilcox-McCaan et al. 1988, Jeannin et al. 1989). However, this method is cumbersome due to the fact that the donor plants must be grown under controlled conditions to maturity.

Mature cotyledons as a source of explants for shoot regeneration were reported by Greco et al. (1984), Power (1987), and Nataraja and Ganapathi (1989). In a previous paper, Knittel et al. (1991) reported a high frequency of regeneration from sunflower cotyledons obtained shortly after germination. This method has been used successfully to regenerate other plant species (Hinchee et al. 1988, Mante et al. 1989).

In this paper we present results on the regeneration potential of cotyledon explants from cultured cotyledon pieces under different conditions. The optimal culture conditions were then used to test this method for regenerating several sunflower genotypes (including commercial inbred lines and hybrids).

The regeneration process is related to the formation of adventitious buds which starts with "de novo" formation of meristematic centers. In this study, we found that the histological analysis of explants after varying amounts of time in culture proved to be essential to identify cultures with high capacity of regeneration. Furthermore, we

Abbreviations: BAP, 6-benzylaminopurine; MS-Ha, Murashige-Skoog medium (1962) modified by Paterson and Everett (1985); MSS, hormone free Murashige-Skoog medium (1962) supplemented with 10 g liter^{-1} sucrose; NAA, alpha-naphtaleneacetic acid.

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tested the susceptibility of this cultured tissue to *Agrobacterium* infection in order to evaluate its potential use for genetic engineering.

Materials and Methods

Plant material—Inbred lines: Ha 300b was kindly provided by Sanofy Elf Biorecherches (SEBR) Labège, France and propagated in the Agriculture Experimental Stations of Balcarce and Pergamino (INTA). Pha 67a and Pha 67b; Impira INTA Selección 11 Castelar (lines with specific resistance to rust infection); HaR 2; Rha 271; Rha 801; Rha 274; Ha 89a, Ha 124 and Ha 822a are inbred lines cultured at the Agriculture Experimental Station of Pergamino (INTA). Impira INTA Selección 11 Castelar is an inbred line selected by alternate mass selection and selfing of a population generated by an interspecific cross between *Helianthus argophyllus* and *H. annuus* cultivar Saratov Selección Pergamino (Cialzeta and Antonelli 1971). The later is a selection of a Russian cultivar obtained at INTA-Pergamino. Impira INTA Selección 11 Castelar carries at least one major resistance gene, conferring resistance to *Puccinia helianthi* Schw isolates 767, 793, 823 and 890 (Heinz 1984) and to the North American isolate 4 (Yang et al. 1986).

Pha 67a and Pha 67b are inbred lines selected by Ing. Agr. A. Luciano by selfing cultivar Pehuen INTA. Pehuen INTA was selected from an intercross between Russian cultivars VNIIMR 8932, VNIIMR 6540 line RA 3-R from Pergamino and line 119-2 from Mordem Experimental Station (U.S.A.). Both lines carry race-specific resistance genes for sunflower rust.

NX 142, NX 136 and XL 643 are commercial lines which were generously supplied by Duperial (ICI Seeds, Venado Tuerto, Argentina). The hybrid seeds used were supplied by SEBR (Mirasol) and ICI (Contiflor 3, 7, 8, 9 and 15 and Contioil 1000).

Surface sterilization and germination of seeds—Conditions for sterilization and germination of seeds were as described previously (Knittel et al. 1991) with minor modifications. Seeds were sterilized in 70% ethanol (30 s), followed by 3.5% sodium hypochlorite (5 min under vacuum and then 30 min at atmospheric pressure), and rinsed 5 times with sterile distilled water. The sterilized seeds were imbibed in sterile distilled water overnight in the dark (25°C) and then allowed to germinate on solidified (7 g liter^{-1} agar) MSS medium (Murashige and Skoog 1962) supplemented with 500 mg liter^{-1} nystatine. The germinating seeds were kept in the dark for 3 days and then transferred to the light (16 h day/8 h night photoperiod at 92.0 μE m^{-2} s^{-1} provided by Philips fluorescent tubes TLD, 36 W 54 DL8) for varying periods (1–6 days) depending on the genotype. The temperature was maintained at 25°C.

Explant manipulations and hormone treatments—Cotyledons were excised from the plantlet taking great care to remove apical and axillary meristems. Cotyledons were routinely divided into two halves (basal and distal). In some experiments the basal proximal half was further subdivided longitudinally in two or four segments. The basal proximal region of the explant was then placed in contact with the medium by partial submersion. The medium was a modified version of the Murashige-Skoog (1962) as described by Paterson and Everett (1985) (MS-Ha) and was supplemented with 1.00 mg liter^{-1} BAP. Media were sterilized by autoclaving (20 min, 1 atm, 120°C) while hormones and vitamins were sterilized by filtration (0.22 microns, Millipore membranes, Bedford, U.S.A.) and added after autoclaving.

Inbred line Ha 300b half cotyledons were cultured in MS-Ha containing different concentrations of ANA and BAP, ranging from 0.0 to 1.0 mg liter^{-1}. Calli produced by culturing half cotyledons on 0.75 mg liter^{-1} NAA and 0.10 mg liter^{-1} BAP were transferred to MS-Ha medium containing 1.00 mg liter^{-1} BAP to induce plant regeneration.

Each value is the mean of at least 3 independent experiments using 25–30 explants. Responses were evaluated 10 and 30 days after initiation of culture.

Regenerated shoots (5–10 mm in size) were excised and rooted on solidified rooting medium (5 g liter^{-1} agar) containing one fourth the normal concentration of macro and microelements (Murashige-Skoog medium 1962) and sucrose (10 g liter^{-1}). Plantlets with well developed roots were transferred to soil and grown in a greenhouse.

Histological analysis—Five samples of cultured explants were taken every 2 days and the material was fixed in FAA (10% formaldehyde, 50% ethanol, 5% acetic acid and 35% deionized water) for 48 hours and treated according to Johansen (1940). Wax blocks were sectioned by using a rotary microtome, and the slices mounted on slides and stained with safranin-alcian blue.

Agroinfection—Cotyledons slices were co-cultured with *Agrobacterium tumefaciens* strain B6S3 (Otten et al. 1984) according to Horsch et al. (1987). Control explants were co-cultured with *A. tumefaciens* strain GV 3850 (pGV 3850) which carries a nonfunctional Ti plasmid in which T-DNA region was replaced by a portion of pBR 322 (Zambryski et al. 1983). After agroinfection the explants were subcultured into MSS medium supplemented with 500 μg ml^{-1} of Cefotaxime (generously donated by Lab. Argentia, Buenos Aires, Argentina). The explants were evaluated 20 days later.

Results

Influence of culture conditions and cotyledon dissection—The response of 30 day-cultures of half cotyledon explants with varying combinations of auxins and cytokinins

Table 1 Differential response of half cotyledon explants (Ha 300b) to auxin and cytokinin combinations

BAP[a]	NAA[a]				
	0.00	0.10	0.50	0.75	1.00
0.00	r (100)	r (55)	fc (100)	fc (100)	fc (100)
0.10	s (70) [3- 4]	nc (80)	fc (75)	fc (100)	fc (100)
0.50	s (80) [5- 7]	nc (89)	nc (100)	fc (90)	fc (90)
0.75	s (87) [8–10]	nc and s (87)	nc (65)	fc (100)	fc (100)
1.00	s (90) [10–12]	s (60) [8–10]	s (36) [4–5]	nc (87)	fc (70)

Numbers in parentheses indicate the percentage of responding explants. Numbers in square brackets indicate number of shoots per explant. s, shoot; nc, nodular callus; fc, friable callus; r, roots.
[a] Concentration of BAP and NAA are given in mg liter^{-1}.

is shown in Table 1. Direct shoot regeneration was reproducibly obtained in media containing only BAP (0.50, 0.75 and 1.00 mg liter^{-1}) or BAP (1.00 mg liter^{-1}) with NAA concentrations of 0.50 mg liter^{-1} and bellow. Increasing

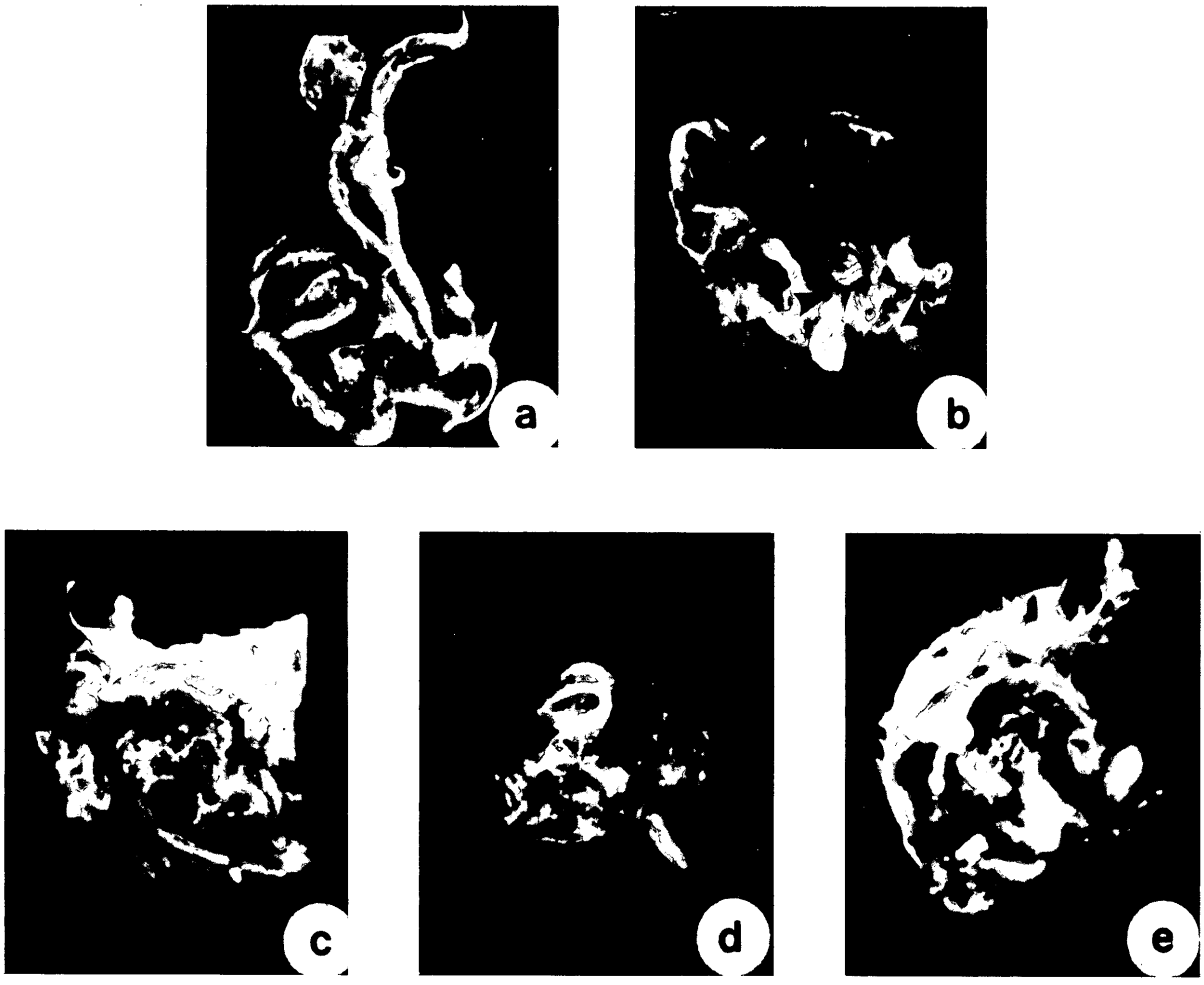

Fig. 1 Differential response of explants of Ha 300b after 30 days of culture in MS-Ha medium containing 0.75 mg liter^{-1} BAP with increasing concentrations of NAA. a) NAA=0.0 mg liter^{-1} which resulted in an average regeneration frequency of 5 large shoots per explant. b) NAA=0.10 mg liter^{-1} which resulted in an average regeneration frequencies of 3 small shoots (arrowed) per explant. c), d) and e) NAA=0.50, 0.75 and 1.00 mg liter^{-1}, respectively, resulted in callus induction, but absence of adventitious shoots.

concentrations of BAP resulted in higher proportion of explants with shoots and a higher number of regenerating shoots per explant. In contrast, the addition of NAA decreased the percentage of explants with shoots and the number of regenerating shoots per explant in an inverse relation to the concentration of auxin (Fig. 1). In media supplemented with auxin, shoot differentiation was replaced by callus proliferation and depending on the auxin concentration, two types of calli were produced (nodular or friable).

About seventy percent of the calli that developed in the presence of 0.10 mg liter^{-1} BAP and 0.75 mg liter^{-1} NAA, differentiated to produce 2-5 shoots per callus in 15 days after transfer to a medium containing 1.00 mg liter^{-1} BAP.

A marked reduction (90 to 40%) in the number of ex-

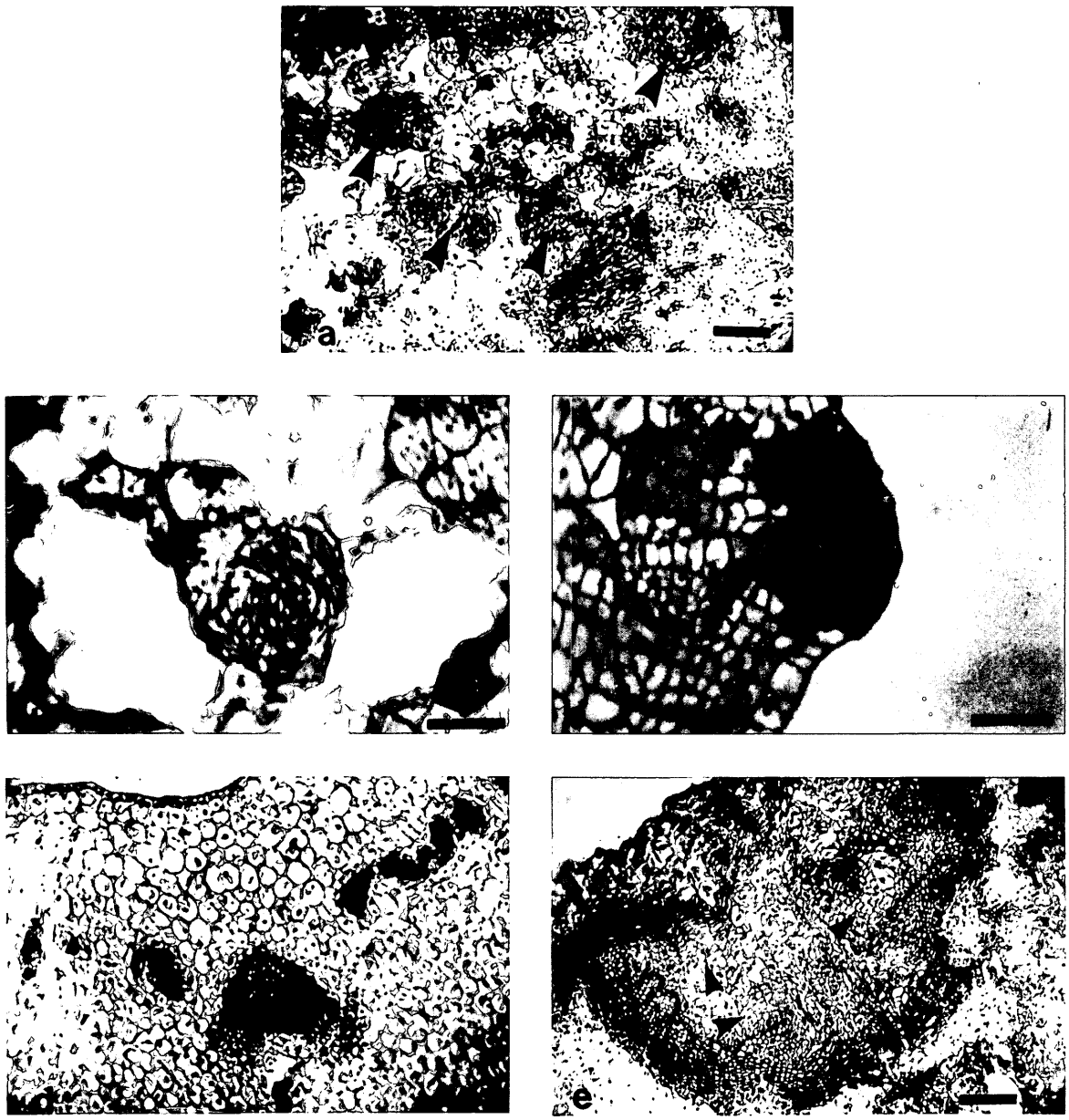

Fig. 2 Transversal section of cotyledon explants on MS-Ha medium supplemented with 1.00 mg liter^{-1} BAP. a) Arrows indicate meristematic centers of the basal region of typical explants after six days of culture. Bar = 100 microns. b) Detailed view of a meristematic center belonging to an explant after six days of culture. Differences in size and tissue organization between meristematic and parenchimatous cells can be observed at the right corner. Bar = 10 microns. c) Meristematic cell clusters breaking through the epidermis of an explant after ten days of culture. No connections between meristematic centers and the vascular axis of cotyledons can be observed. Bar = 10 microns. d) Cotyledon tissue prior culturing. Bar = 100 microns. e) View of the distal region of the explant after ten days of culture. Periclinal cell divisions are indicated by the arrow. Bar = 100 microns.

plants that differentiated into shoots was observed when the basal half of cotyledons (Ha 300b) were longitudinally divided into quarters and these quarters furtherly sliced and used as explants. Furthermore, fewer shoots per explant were formed: i.e., 2–5 in sliced explant as opposed to 10–12 in intact cotyledons halves.

Histological analysis—The kinetics of shoot formation followed at the histological level is shown in Fig. 2a and 2b. Anatomical examination of cultured explants shows "de novo" formation of meristematic centers in the basal region of the cotyledons with small cells growing and surrounding a hypothetical axis (Fig. 2b). This structure suggests that cells are organized with a higher degree of complexity than in parenchymatous-type cells (Escandón 1990). Meristematic centers may develop under and emerge through the epidermal layer surrounded by parenchymatous-type cells produced through proliferating tissue (Fig. 2c). This is clearly observed 10 days after culture contrasting with the morphological characteristics at the onset of the culture (Fig. 2d). Explants derived from distal and proximal regions of cotyledons developed differently (Fig. 2a and 2e). Mitotic activity in the distal region followed periclinal divisions, meanwhile the proximal region followed divisions around an hypotethical axis giving rise to proliferation of meristematic centers.

Influence of the genotype—In order to study the genotypic influence on regeneration efficiency, 13 sunflower inbred lines and 7 hybrids under our optimal condition for direct regeneration (MS-Ha supplemented with BAP) were tested. This experiment clearly demonstrated that regeneration efficiency depends on the genotype (Table 2). About 50% of tested genotypes regenerated, some of them with very diverse genetic backgrounds including those derived from crossings with *H. argophyllus*. Regeneration of shoots varied from 90% of explants giving rise to viable shoots (Ha 300b), to genotypes were no shoot developed (Ha 124b and Contioil 1000). These results also indicate that plant regeneration is under genetic control and it does not depend on the hybrid origin of the explant. Shoot regeneration was restricted to the basal region of the cotyledon, and the number of shoots per explant varied from 1 to 12.

Regeneration of mature plants from the shoots was observed at a high efficiency. In certain genotypes (e.g., Ha

Table 2 The effect of genotype on shoot regeneration efficiency

Type	Genotypes	Shoots/Cotyledon	Explants responding (%)
Inbred lines	Ha 300b	10–12	90
	Ha 822a	0	0
	Ha 124a	0	0
	Ha 89a	0	0
	NX 142	0[a]	0[a]
	NX 136	1– 2	16
	XL 643	0	0
	HaR 2	4– 6	31
	Impira	6– 7	50
	Pha 67a	8– 9	30
	Pha 67b	0	0
Inbred restorer lines	Rha 271	4– 6	20
	Rha 801	5– 6	20
	Rha 297	5– 6	27
Hybrids	Mirasol	0	0
	Contiflor 3	3– 4	83
	Contiflor 7	3– 4	20
	Contiflor 8	0	0
	Contiflor 9	0	0
	Contioil 1000	0	0

1–6 day old half cotyledons from different genotypes were cultured in standard medium plus BAP (1.00 mg liter^{-1}). 0 represents absence of shoot regeneration. However, this absence of shoot regeneration does not mean absence of response since callus or root development was usually observed.

[a] This genotype showed development of 3–4 shoots/cotyledon in 15% of the explants when medium was supplemented with 1.00 mg liter^{-1} BAP and 0.75 mg liter^{-1} NAA.

Fig. 3 Representative R_0 plantlet showing an off-type phenotype.

300b, Pha 67a, Contiflor 3 and Impira INTA Selección 11 Castelar) 90–95% of shoots produced roots and the plantlets could be transferred to soil. In other genotypes (e.g., NX 142 and NX 136) this value was 50% or better. For the majority of the genotypes the rooting efficiency was between 25–40%.

Shoot vitrification was not observed when regenerated shoots were subcultured into rooting medium. However, a brief passage to medium containing auxins (for example in a ratio NAA/BAP=1 : 1) induced abundant shoot vitrification in most of the material tested (data not shown).

Regenerated plants were generally branched with small inflorescences in the buds of almost every branch (Fig. 3) and coincided with those described by Knittel et al. (1991). The inflorescences were composed of a low number of flowers (2–10), most of them sterile. In some cases seeds could be recovered from the apical inflorescence. This aberrant phenotype was genotype independent. About 20 plantlets of each genotype showing regeneration response were transferred to the greenhouse.

To study susceptibility of cotyledons to *Agrobacterium* infection, explants were co-cultured with *A. tumefaciens*. Development of tumourous tissue originating from the explants treated with wild-type *A. tumefaciens* B6S3 is shown in Fig. 4. Development of this tumourous tissue was restricted to the basal part of the explant. Explants treated with control *A. tumefaciens* GV 3850 (pGV 3850) did not show tissue proliferation under the same culture conditions.

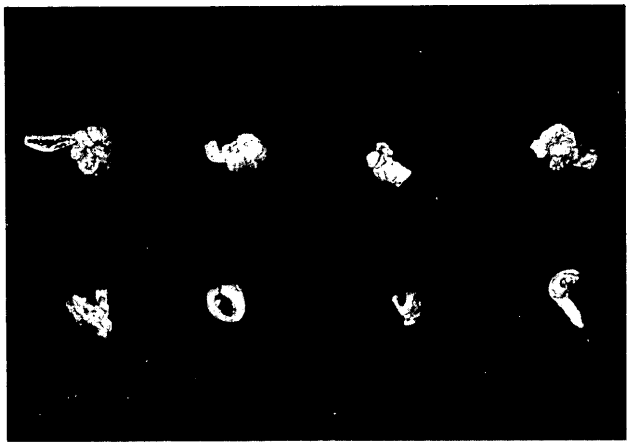

Fig. 4 Explants infected with wild-type *Agrobacterium* and cultured for 20 days in MSS. The resulting tumours are shown in the upper lane. Control explants infected with the disarmed vector *A. tumefaciens* strain GV 3850 were cultured in the same conditions and are shown in the lower lane. No tumour tissues were observed.

Discussion

Are cotyledons a suitable source of explants for a routine sunflower regeneration protocol? Up to now the methods reported by Sadhu (1974), Binding et al. (1981), Greco et al. (1984) and Paterson and Everett (1985) were limited to certain genotypes. In this report, together with those recently reported by Knittel et al. (1991), it is demonstrated that cotyledons are an excellent choice of explant for sunflower regeneration. This method has been successfully used with a large number of genotypes. Fifty percent of tested genotypes, including commercial hybrids and inbred lines, regenerated shoots.

Both, hormone and media composition as well as manipulation of the explants have been found to be important factors for the efficient regeneration of shoots from sunflower cotyledons (Knittel et al. 1991).

Knittel et al. (1991) divided basal halves of cotyledons in abaxial and adaxial quarters. In these conditions best results were achieved using 1.00 mg $liter^{-1}$ BAP and 0.50 mg $liter^{-1}$ NAA. In the present study best hormonal balance for regeneration was found to be different, even though the same genotype and the same age of cotyledons were used. A possible explanation for these differing results may be explained by a position effect. If the cell or group of cells responsible for regeneration are sensitive to nutrient and hormonal gradients, then the position of the explant in the medium would affect the development of potential meristematic centers (Aitchinson et al. 1977). This hypothesis is reinforced by the fact that an increase in the cytokinin/auxin ratio favours the formation of meristematic centers and the production of buds that result in highly viable shoots in tobacco (Meins et al. 1982). Further support of this hypothesis comes from the observation that position has an effect on development between meristematic centers within the same explant. Knittel et al.

(1991) observed that regeneration of shoots from cotyledon cultures was primarily restricted to the basal region of the explant. Histological analysis of this region showed the "de novo" induction of multiple meristematic centers (Figs. 2a and 2b). In contrast, in the distal region the cells were arranged in compact radial rows devoid of intercellular spaces and contained periclinar divisions. This unusual mitotic activity was probably induced by culture conditions and likely to originate in secondary meristems (i.e., cambium). However, no tissue or group of cells resembling a meristematic center were observed in these regions (Fig. 2e). Differences in development between meristematic centers on the same explant may be explained through an "apical dominance" effect where some of the cells with a leading relative position with respect to development, cause an inhibitory effect on other cells with regeneration capacity.

The absence of a connection between regenerated shoots and the vascular axis of cotyledons and the number of shoots per explant are evidence for the adventitious origin of these regenerated shoots (Fig. 2c).

In order to facilitate the exposure of meristematic centers to DNA delivery systems such as cocultivation with agrobacteria in transformation experiments, half cotyledons were assayed under different conditions to optimize their regeneration ability, and then were sliced longitudinally to test the effect of explant dissection. Line Ha 300b was chosen for these experiments since this genotype showed the most reproducible results and allowed direct comparison with previous experiments (Knittel et al. 1991). When the cotyledons were further dissected a marked reduction in the number of explants that differentiated into shoots was observed. In addition, fewer shoots were formed per explant: 2–5 shoots per explant in sliced cotyledons as compared to 10–12 in intact cotyledon halves. This loss of regeneration capacity may be due to the ethylene production caused by the wound made to the explant (Paterson-Robinson and Adams 1987). To test this hypothesis sliced cotyledons are presently being cultured in the presence of silver nitrate and ethylene antagonists.

Other factors such as explant manipulation (dissection and orientation on the medium) as well as explant age and physical culture conditions appear to be equally important. This may help explain the differences in the regeneration ability and number of the shoots between protocols reported here and those reported by Knittel et al. (1991).

One of the objectives of this project on plant regeneration was to facilitate genetic transformation of sunflower. In fact, the use of sliced cotyledons would favor the interaction of cells with regeneration potential, with agrobacteria. The development of tumours on explants exposed to agrobacteria indicates that the use of cotyledons is a suitable approach for transformation experiments. The suitability of cotyledons is further supported by the fact that *Agrobacterium* infection was localized in the basal region where meristematic centers were found. As an alternative, the meristematic centers are suitable targets for transformation experiments using a particle gun to deliver DNA. However, attention must be drawn to the inhibiting effect of wounding observed in this work.

In this report, we describe a regeneration protocol that was reproducible and efficiently regenerated plants from different genotypes, although it may need some fine-tuning of the conditions for each case.

We want greatly acknowledge Dr. Gustavo Gibertti for his skillful histological expertise, to Dr. Arturo Martinez for scientific revision of the manuscript, to Dr. Les Szabo (University of Minnesota) for the linguistic revision and to Nilda López for skillful technical assistance. Maria Fernanda Ceriani holds a fellowship from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas). H. Esteban Hopp is a research scientist of CIC (Comisión de Investigaciones Científicas de la Pcia. de Buenos Aires).

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(Received May 7, 1991; Accepted December 20, 1991)