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Protoplasts from cotyledon and hypocotyl of sunflower (*Helianthus annuus* L.): shoot regeneration and seed production

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Summary. Cotyledon and hypocotyl protoplasts of *Helianthus annuus* inbred line 47 302 bcd were embedded in alginate and plated on L4 medium (Lenée and Chupeau 1986). After one month, the calli were transferred on MSSH regeneration medium (Murashige and Skoog 1962; Schenk and Hildebrandt 1972) where they regenerated shoots (overall efficiency 10^{-2}%). The shoots were elongated on B5 (Gamborg *et al.* 1968) medium first without hormones, then supplemented with GA3 and BAP (both 0.05 mg/l). In order to overcome the difficulty to induce rooting by classical methods, the elongated shoots were grafted on a sunflower rootstock. The grafted shoots produced flowers and seeds. Different factors have been shown to have an important influence on the capacity to regenerate shoots: the genotype, the physical culture conditions at the callus regeneration step (e.g. protoplasts embedded in alginate), and the media composition.

Key words: *Helianthus annuus* - Protoplasts - Plant regeneration

Abbreviations: BAP: 6-benzylaminopurine - GA3: gibberellic acid - IBA: indole-3-butanoic acid - IAA: indole acetic acid - MES: 2-N-morpholinoethane sulfonic acid - NAA: 1-naphthalene acetic acid - 2,4D: 2,4 dichlorophenoxyacetic acid.

Introduction

Sunflower (*Helianthus annuus* L.) plants have been regenerated from various explants, including immature zygotic embryos (Finer 1987; Jeannin and Hahne 1991), cotyledons from young seedlings (Knittel *et al.* 1991), hypocotyl (Greco *et al* . 1984), shoot tips (Paterson 1984) and stem parenchyma (Bohorova *et al.* 1985). However this regeneration potential has not generally been found in protoplasts obtained from these explants. Although an isolated report without experimental details of Binding *et al.* (1981) mentioned shoot regeneration from shoot tip derived protoplasts, only recently, Burrus *et al.* (1991) reported the regeneration of a small number of fertile plants from hypocotyl protoplasts of a private genotype. This genotype has been specially selected for this purpose and is derived from a backcross of an interspecific hybrid between *H. annuus* and *H. petiolaris*. Plant regeneration from protoplasts has been reported in *H. giganteus* and *H. petiolaris*, two wild species closely related to the cultivated sunflower (Krasnyanski *et al.* 1992; Chanabé *et al.* 1991). No data were given concerning the fertility of the regenerated plants. In the case of *H. petiolaris*, only one callus has given rise to successful regeneration.

We describe here the regeneration of fertile plants derived from hypocotyl- as well as cotyledon- derived protoplasts of an inbred line of *H. annuus* without a significant content of a wild genome.

Materials and methods

Donor plants. Seeds of *Helianthus annuus* inbred line code number 47 302 bcd (obtained from Société des Maïs Européens, Mainvilliers, France) and of the other genotypes were sterilized and germinated as described in Fischer and Hahne (1992) .

Protoplast isolation and culture. The cotyledons were cut into thin strips which were incubated in an enzyme solution containing the saline solution "S" according to Lenée and Chupeau (1986) (without bromocresol purple), cellulase 345L (0.4%), pectinase M2L (0.1%) (both from Cayla, Toulouse, France), and pectinase Rohament P5 (0.35%) from *Aspergillus niger* (Serva). Hypocotyl protoplasts were obtained as described by Lenée and Chupeau (1986). The following steps (purification and culture) were the same for both protoplast types. After overnight incubation at 25°C in darkness, the mixture was filtered through a layer of hydrophilic cotton. The isolated protoplasts were collected by centrifugation (90 x g, 5-10 min) and then washed twice in solution S. The cotyledon and hypocotyl protoplasts were plated at a density of 4.10^4 protoplasts/ml in alginate-solidified L4 medium (Lenée and Chupeau 1986) containing 3 mg/l NAA and 1 mg/l BAP (L4 -1). The protocol used for the embedding of

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freshly isolated protoplasts in alginate (Damm and Willmitzer 1988; Damm *et al.* 1989) was slightly modified. Aliquots (0.5 ml) of a protoplast suspension (8.10^4 protoplasts/ml in 0.6 M mannitol) were carefully mixed with an equal volume of a solution containing 2% sodium alginate (Roth) in 0.5 M mannitol. The mixture was plated on 20 mM $CaCl_2$ agar (Damm *et al.* 1989). After 30 min. incubation at room temperature, a solution containing 50 mM $CaCl_2$ in 0.4 M mannitol was added in order to complete the polymerization of alginate. After 30 min the layers were removed from the $CaCl_2$ agar and transferred into Petri dishes containing 4 ml of L4 medium. The protoplasts were incubated in darkness at 25°C for 10 days and then transferred to light. After 5-7 days, the L4 medium was replaced by L'4 (NAA concentration reduced to 0.1 mg/l; Lenée and Chupeau 1986). Subsequently, one third of the medium was replaced by L"4 (L'4 with 1% sucrose and 550-600 mosm) at weekly intervals. The freshly embedded protoplasts have also been plated in L4 with another hormonal combination (L4-2: 2,4-D 1mg/l; BAP 1 mg/l).

Plant regeneration from protoplast derived calli. Once the protoplast derived calli reached 2 mm in size, they could be transferred to regeneration medium (MSSH, see table 1). The osmotic pressure of the medium was adjusted to 500 mosm kg^{-1} with mannitol. After 10 days, the osmotic pressure was reduced to 300 mosm kg^{-1} by reducing the mannitol. As soon as the shoots reached a size of 3-5 mm, they were isolated from the callus and elongated by transferring them onto B5 medium (Gamborg *et al.* 1968) without hormones for approx. 3 weeks. The elongating shoots were then subcultured to the same medium, but supplemented with 0.05 mg/l GA_3 and 0.05 mg/l BAP, for two additional weeks. If necessary, the shoots were transferred to B5 medium with 0.05 mg/l BAP for several additional weeks until they reached a size sufficient for root induction or grafting.

Two other sequences of shoot regeneration media have also been tested (see table 1).

Root induction and grafting of the shoots. Shoots that had reached a size of 10-20 mm could be grafted onto a sunflower rootstock. All grafting operations were performed in the greenhouse. We used 4-6 week old plants of inbred line HA 300 B (obtained from Sanofi Elf Biorecherches, Labège, France). These plants were decapitated and incised lengthwise (1-1.5 cm). The *in vitro* shoot was bevelled on two sides and introduced in the slit of the rootstock in the vicinity of the vascular bundles. Scion and rootstock were wrapped with moistened raffia and then the upper part of the plant and the graft region were covered with a plastic bag during 2 weeks in order to ensure a high degree of humidity. Flowers were hand-pollinated either with their own pollen (selfed) or with pollen of HA 300.

Table 1. Medium composition

Media type	Medium*	Supplements (mg/l)							Osmotic pressure (mosm/kg)
		NAA	2,4-D	BAP	GA3	Peptone	Sucrose	Vitam.	
Protopl. culture	L4-1	3		1			20 000	L4	660
	L4-2		1	1			20 000	L4	660
Reg-1	MS		1	0.02			30 000	MS	500
	MS	0.05		0.2			30 000	MS	300
Reg-2	MS mod	1		1	0.1	500	30 000	MS	500
	MS mod	1		1	0.1	500	30 000	MS	300
Reg-3	MSSH	0.05		0.2			20 000	SH	500
	MSSH	0.05		0.2			20 000	SH	300

*L4 -see Lenée and Chupeau (1986); MS -see Murashige and Skoog (1962); SH -see Schenk and Hildebrandt (1972)

Results

Protoplast culture and shoot regeneration

When embedded in an alginate matrix floating in liquid L4 medium (Lenée and Chupeau 1986), both hypocotyl and cotyledon protoplasts started to divide two days after isolation. The initial development of these protoplasts gave rise to very compact structures (fig 2), the first divisions generally occurring within the confinement of the original protoplasts (fig 2). After approx. 3 weeks of culture, these compact structures had developed into less compact entities with a more typical callus aspect (fig 2).

The calli could be transferred to regeneration medium after 3 to 4 weeks. At this time , most calli had reached a diameter of 1-2 mm. The majority of them were white with a more or less brownish tint. However, a small number of green calli developed in every dish. These were preferentially transferred to regeneration medium.

The organogenic calli, entirely green at the time of transfer to MSSH, developed into white calli containing green meristematic nodules on this medium. Shoots appeared rapidly after transfer to MSSH. Sometimes they developed already on MSSH 500 mosmkg^{-1}(6 to 10 days after transfer from the alginate layer), but the majority appeared approx. 6 days after transfer to MSSH 300 mosm kg^{-1}(16 days after transfer from the alginate layer; fig 2). The regeneration protocol is summarized in fig 1.

As soon as the shoots reached a size allowing their excision (4-5 mm), they were transferred from the callus onto a cellulose plug imbibed with B5 medium without hormones. Removal of hormones (especially BAP) was found to reduce vitrification which otherwise could become a problem. After approx. 3 weeks, the B5 medium was supplemented with GA_3 and BAP (both at 0.05 mg/l) in order to promote shoot elongation. Higher concentrations of GA_3 resulted in weak shoots.

From 4.10^4 protoplasts plated initially in a Petri dish, 1 to 5 calli regenerated shoots corresponding to an overall efficiency of up to 10^{-2}%. The fact that we are able to select the calli with organogenic potential (green calli) allowed us to reduce the number of calli transferred to regeneration medium. 26% of these calli derived from cotyledon protoplasts and 35% derived from hypocotyl protoplasts, responded with shoot formation after transfer to MSSH.

Variation of important factors

The sunflower inbred line used in this study has been identified in the context of a genotype screen. Sixteen genotypes, having different geographic origins (table 2),

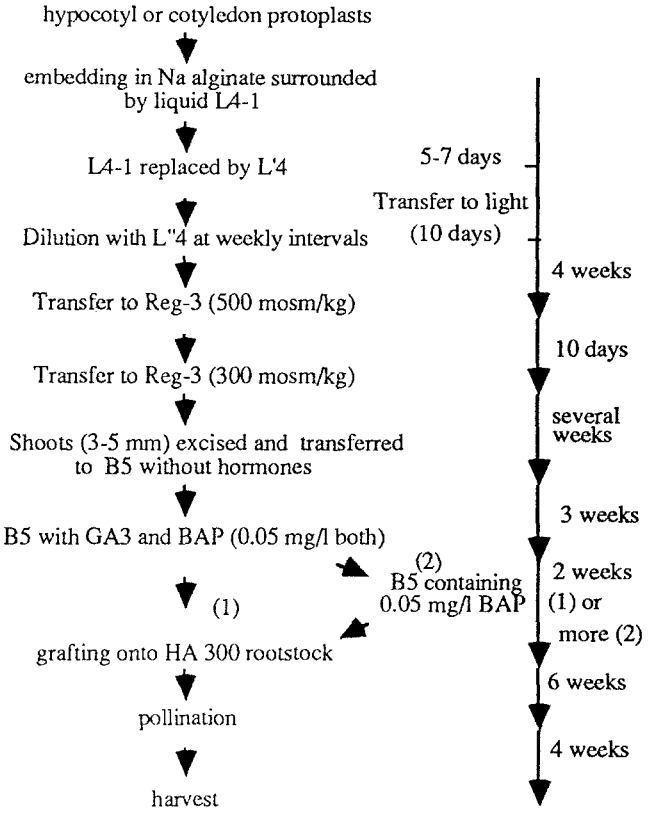

Fig. 1. Regeneration protocol of fertile plants from cotyledon and hypocotyl protoplasts of Helianthus annuus inbred line 47 302 bcd. For media, see materials and methods. (1) and (2): alternative routes.

have been evaluated on the media listed in table 1, according to the following protocol. Cotyledon protoplasts of these genotypes were embedded in alginate and plated in L4-1 or L4-2 media (table 1). Media were replaced as described above. After one month, the calli of each line were subjected to three different regeneration schemes, consisting of a sequence of two media each (table 1). Shoot regeneration was observed only with the combination of line 47 302 bcd and the sequence of media shown in fig 1.

While the frequency of callus production from protoplasts is relatively high, the yield of regenerated shoots is still low, albeit very reproducible. A number of variables have been tested in order to improve the frequency of potentially morphogenic (green) calli, and the quality of the obtained shoots.

Promoting good gas exchange (sealing the dishes with a polyethylene film instead of parafilm) enhanced the number of green calli. When the dishes were sealed too tightly, the developing calli assumed a brownish colour. This observation was interpreted to indicate a possible inhibitory effect of ethylene production. Silver ions have been shown to stimulate shoot regeneration from potato protoplasts (Perl *et al.* 1988) and sunflower cotyledons by inhibiting ethylene action (Chraibi *et al.* 1991). However, the addition of silver nitrate (2 mg/l) to the medium directly after isolation or after 2 weeks of culture did not improve the quality of the colonies. In the latter condition the colonies turned brown while in the former, division activity was abolished altogether. Variations of the osmotic pressure or the sucrose content at different steps of the protocol, or variations of the embedding technique (beads instead of a continuous alginate layer), were without effect.

Vitrification is a developmental abnormality that affects numerous plants cultured *in vitro*, and the shoots regenerated from our protoplast cultures suffered from this effect. Different factors have been shown to promote vitrification: excess of cytokinins (Bornman and Vogelman 1984), high relative humidity (Bottcher *et al.* 1988), high water potential of the medium (Debergh *et al.* 1981), high ammonium levels (Daguin and Letouze 1986), low concentration of the gelling agent (Leshem 1983). Vitrification may be limited by controlling the gas exchange (Dillen and Buysens 1989) and the carbohydrate concentration and sources (Rugini 1986; Orlikowska 1987). In our case, the addition of silver nitrate (2 mg/l) or a commercial antivitrifying agent (EM2, 5 g/l, Pronatec, France) to the regeneration medium did not reduce vitrification, nor did a reduction of BAP (0 or 0.1 mg/l) or sucrose (1% instead of 2%) in the regeneration medium. Likewise, replacing nitrate and ammonium by glutamine as the sole nitrogen sources in the regeneration medium, or agar by gelrite (0.3%) did not affect the degree of vitrification.

The regenerated shoots failed to produce roots, in spite of intense efforts to promote root induction. We therefore developed a grafting technique which allows transfer to the greenhouse and seed production.

Grafting and seed production

Shoots with a length of 0.5-2 cm were grafted onto decapitated sunflower plants directly in the greenhouse. The age of the rootstocks did not appear to be critical, but younger plants are easier to manipulate than those where the stem is too lignified. However, 1-2 pairs of leaves should be left on the rootstock after grafting. Four to six week old plants seem to be a good compromise. Grafting was successful with an amazing range of quality of scions. The regenerated shoots were often vitrified to various degrees or carried well developed flower buds. Successful graft unions have been obtained with all these different shoot types. The influence of the rootstock resulted in an improvement of the development, and the more or less normal shoots obtained by this approach set flowers in the majority of cases. The height of the grafted shoots varied from 2 cm to more than 50 cm at the time seeds were harvested.

The grafted shoots developed rapidly and could be pollinated within 6 weeks after grafting. In comparison, 8 weeks were needed for normal plants in our greenhouse conditions from sowing to pollination. To date, 53 shoots have been grafted, 33 formed flowers and 20 gave seeds. The number of seeds per head varied from 2 to 235.

From protoplast isolation to the harvest of seeds, the regeneration procedure took 6 months.

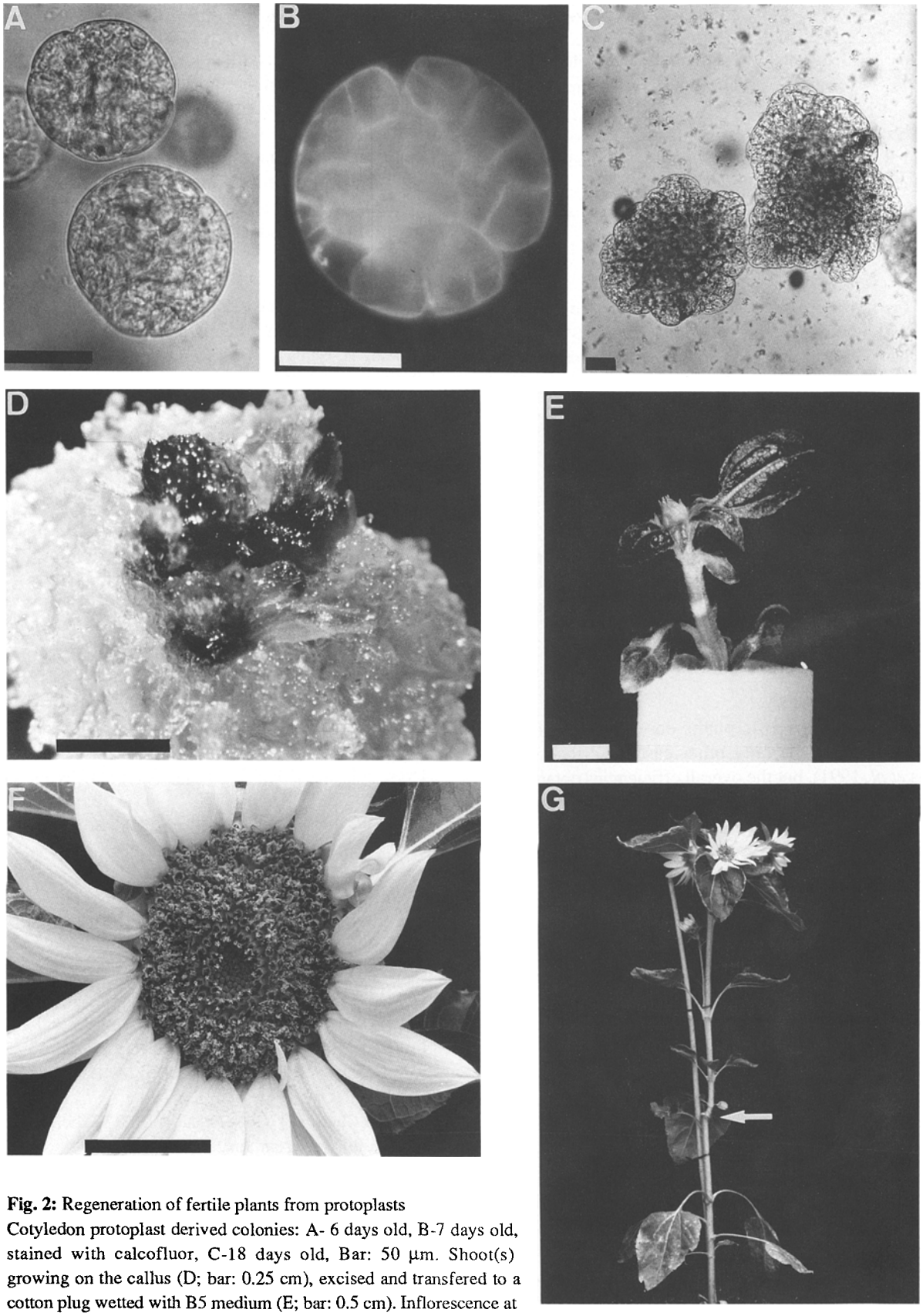

Fig. 2: Regeneration of fertile plants from protoplasts
Cotyledon protoplast derived colonies: A- 6 days old, B-7 days old, stained with calcofluor, C-18 days old, Bar: 50 µm. Shoot(s) growing on the callus (D; bar: 0.25 cm), excised and transfered to a cotton plug wetted with B5 medium (E; bar: 0.5 cm). Inflorescence at the pollination stage (F; bar: 3.5 cm). G-Overwiev of the grafted plant (pollination stage). Note the graft area (arrow). Total height 70 cm.

Table 2. Genotype screen.

Genotype	Geograph. origin	Callus formation L4-1	L4-2	Callus behaviour on reg. media*
47 302 bcd	CIS+USA	+	+	shoots (Reg.3)
47 114 i	France	+	ND	nodules (Reg.3)
47 134 n	France	+	ND	c
22 1195	China	+	ND	c
22 1196	China	+	+	c
47 252 s	Canada	+	+	c
67 732 h	USA	+	+	c
47 264 (3)	USA	+	+	nodules (Reg.3)
67 282 y	USA	+	+	ND**
67 182 h	USA	+	+	nodules (Reg.3)
67 304 a	USA	+	+	nodules (Reg.3)
47 38 x	South Africa	+	+	c
47 44 d	South Africa	+	ND	c
28 66 626	USA	+	+	nodules (Reg.3)
51 144 (3)	South America	+	ND	nodules (Reg.3)
41 1412 (1)	South America	+	+	nodules (Reg.3)

ND: not determined; * medium on which the calli developed shoots or nodules is indicated in ( ); c callus without visible signs of differentiation; ** no transfer performed (brown calli). The genotypes have all been obtained from the SDME.

Discussion

The regeneration of fertile plants described here differs considerably from the only other case described so far (Burrus *et al.* 1991), but the overall efficiencies obtained are similar. The genotype used in our study was a sunflower inbred line, while the regeneration potential in the genotype used by Burrus *et al.* (1991) appears to be due to the introgression of *H. petiolaris*. The media used in the two protocols are quite different. A characteristic element of our regeneration medium is its low concentration of growth regulators.

The two protocols appear to be specific for their respective genotypes. The protocol developed for line 47 302 bcd did not yield shoots in any other tested genotype, although calli with green nodules could be obtained in several cases. In contrast, we could subject both hypocotyl and cotyledon protoplasts to the identical treatment and obtain comparable regeneration frequencies.

The regeneration of calli in an alginate matrix is essential to obtain shoot regeneration later on. Protoplasts cultured in liquid medium never yielded plants. Previous histological studies (Fischer and Hahne 1992) showed that the quality of the colonies is best in alginate solidified medium rather than in liquid or agarose solidified medium.

In vitro grafting has been employed previously to recover regenerants which did not develop sufficiently to be transferred to the greenhouse, e.g., in pea (Natali and Cavallini 1989) or cocoa (Aguilar *et al.* 1992). We have grafted the *in vitro* shoots directly on greenhouse-grown plants with a high success rate (62% of the grafted shoots developed flowers). Although this technique may appear complicated for routine application, it avoids several transfers *in vitro* of the shoots, and it may shorten the total time spent *in vitro* considerably. In general the grafted shoots developed more vigorously than rooted sunflower regenerants obtained in other experiments, and the seed yield was comparatively high on grafted plants. Thus, even in the case where rooting *in vitro* is possible, the grafting approach may be advantageous.

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