MORPHOGENETIC COMPETENCE IN THE GENUS *HELIANTHUS*: INCREASED TOTIPOTENCY IN PLANTS PREVIOUSLY REGENERATED IN TISSUE CULTURE AND CHARACTERIZATION OF AN EPIPHYLLIC VARIANT

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Summary: Leaves of *Helianthus tuberosus* (2n = 6x = 102) and *H. annuus* x *H. tuberosus* (2n = 4x = 68) chiefly respond to growth regulators with callus and roots production, while adventitious organogenesis or somatic embryogenesis are induced occasionally. A remarkable regeneration frequency (about 30 %) is achieved from leaves of one only genotype (HTPI-15) of *H. tuberosus*. In contrast, a high morphogenetic competence is showed, even without growth regulators, by cells of regenerated plants of *H. tuberosus* and of the interspecific hybrid subjected to a following *in vitro* culture cycle. Moreover, the high morphogenetic potential displayed by 2 of the 24 triploids (2n = 3x = 51) obtained by the backcross from regenerated plants of *H. annuus* x *H. tuberosus* with *H. annuus*, proves that this characteristic is transmissible through pollination. A variant clone (EMB-2) derived from a regenerated plant of the interspecific hybrid *H. annuus* x *H. tuberosus* shows a particular deviation from the usual pattern of development producing, both *in vitro* and *in vivo*, epiphyllous embryos and/or shoot-like structures usually arranged in clusters or rows along pre-existing veins. Alterations of the endogenous hormonal levels or mutations in genes involved in the switch from indeterminate to determinate cell fate may be responsible for the ectopic development of shoots and embryos on leaves of the EMB-2 variant.

Introduction

In *Helianthus* spp. screening for *in vitro* morphogenesis (Paterson and Everett 1985; Knittel *et al*. 1991), and genetic analyses (Sarrafi *et al*. 1996; Berrios *et al.* 1999), indicate that at least some specific events of the somatic embryogenesis and/or organogenesis may be under genetic control. In spite of that, also epigenetic mechanisms seem to be involved in the expression of cell totipotency. In the genus *Helianthus* a high embryogenic potential can be acquired by cells of regenerated plants (Pugliesi *et al*. 1993a; Fambrini *et al*. 1996; 1997). Indeed, *in vitro* regeneration process seems to be the essential requirement to make differentiated cells able to produce somatic embryos and/or buds at high frequencies, also without the presence of exogenous growth regulators. Therefore, regenerated plants appear to have undergone in culture a genetic or epigenetic selection that has improved the embryogenic and/or organogenic capacity of explant tissues. In this work, we investigated the high morphogenetic competence induced by the regenerative events in *H. tuberosus* and *H. tuberosus* x *H. annuus*, and the transmissibility of this trait in progenies obtained by interspecific hybridization. The data have been discussed, prospecting that genotype and foregoing regenerative occurrence are two key factors for *in vitro* morphogenesis in the genus *Helianthus*. Moreover, we conducted the histological characterization of the EMB-2 variant derived from a regenerated plant of *H.annuus* x *H. tuberosus*, that showed an unusual pattern of organization of the plant body, diffrentiating, both *in vitro* and *in vivo*, epiphyllous embryos and/or buds.

Materials and Methods:

Plant Material

The accession S. Pietro (HTPI) of *Helianthus tuberosus* (2n = 6x = 102) and tetraploid hybrids (2n = 4x = 68) *H. annuus* x *H. tuberosus* (HA x HT), obtained by crossing the inbred line HA89 cms of *H. annuus* (2n = 2x = 34) with *H. tuberosus*, were used as started materials in the first regeneration cycle (R_0). After sterilization (Fambrini *et al*. 1996), the naked seeds were placed on solidified (8 g l^{-1} Bactoagar) MS basal medium (Murashige and Skoog 1962) without growth regulators, in growth chamber under a temperature of 25 ± 1 °C and a 16-h photoperiod. Irradiation was 35 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Every two weeks, each plantlet was multiplied by single-node cuttings (Fambrini *et al*. 1997).

First regeneration cycle (R_0) from leaf explants of H. tuberosus *and* H. annuus *x* H. tuberosus

Leaf explants from *H. tuberosus* (HTPI) and from 30 multiplied interspecific hybrid plantlets (HA x HT) were placed in Petri dishes on regeneration medium (BN) composed of MS basal medium supplemented with 0.1 mg l^{-1} NAA and 0.2 mg l^{-1} BA. The medium contained 30 g l^{-1} of sucrose and 8 g l^{-1} Bactoagar. Cultures were incubated in growth chamber in the same conditions as described above. Eight to twelve explants were placed in each plate. After 3 - 4 weeks of culture, shoots (length 10 - 15 mm), formed from buds or somatic embryos, were individually separated and subcultured for rooting on MS medium without growth regulators. After rooting, the plants were transferred into pots and placed in a growth chamber at 22 ± 1 °C under 16-h photoperiod and irradiance of 165 μmol m^{-2} s^{-1} provided by fluorescent tubes. After 15-20 d, the plantlets were transferred to greenhouse and grown to maturity.

Regeneration from leaf explants of regenerated plants (R_1) and sexual transmission of the high regeneration potential

Forty-five random selected regenerated plantlets (R_1) from the genotype HTPI-15 of *H. tuberosus* and 9 regenerated plantlets (R_1) of *H. annuus* x *H. tuberosus* (HA x HT-R), were micropropagated by single-node cuttings as described above. The clones obtained constituted the material for new regeneration cycles. Moreover, the triploids (2n = 3x = 51: BC-R) obtained by backcrossing regenerated plants of the tetraploid hybrids (HA x HT-R) with the parental line HA89 of *H. annuus* were used as material to evaluate the sexual transmission of the high regeneration potential. The procedure of tissue culture and the grown conditions were the same as described above.

Histological analysis of the epiphyllic variant

An histological analysis has been conducted in the clone EMB-2 derived from a regenerated plant (HA x HT-R) of *H. annuus* x *H. tuberosus*. Leaf segments of EMB-2 plants grown in greenhouse were collected and fixed for 24-h in FAA (formalin/glacial acetic acid/ethanol, 5/5/90 v/v), at room temperature before being transferred to 70% ethanol. The materials dehydrated in ethanol, were cleared in xylene and embedded in paraffin. Serial sections, 10 μm thick, were cut using a rotary microtome (Reichert), and transferred onto glass slides. The paraffin was removed and the material was stained in Delafield's hematoxylin and mounted in DPX.

Results and Discussion

In our experiments we demonstrate that in addition to the genotypic influence on the *in vitro* behaviour (Table 1), an acquisition of high morphogenetic potential is induced by a previous regenerative event (Tables 1 and 2) and inherited in seed derived progenies (Table 3).

Table 1. Effect of the regeneration event on morphogenetic potential of *Helianthus tuberosus* leaves (accession HTPI), cultured in BN medium. R0 = first cycle of *in vitro* tissue culture; R1 = second cycle of *in vitro* tissue culture (regenerated plants).

Cycle	Clone	N° of cultured explants	Regeneration (%)Y
R0	HTPI-1 to-14 (14 clones)	1223	0.08 a
R0	HTPI-15 (1 clone)	276	30.8 c
R1	HTPI-15-N (15 clones)	575	8.2 b
R1	HTPI-15-M (30 clones)	840	94.7 d

Y The data were treated using analysis of variance procedures and means were separated using Tukey's test after arcsin transformation of the regeneration percentage. Frequencies followed by different letters are significantly different ($P = 0.05$).

In the genus *Helianthus*, some studies reported a strong correlation between the genetic background of the donor plant and its *in vitro* regeneration response (Paterson and Everett 1985; Knittel *et al*. 1991; Sarrafi *et al*. 1996; Berrios *et al*. 1999). In our case, *H. tuberosus* is a highly heterozygous open-pollinated species, and the seeds show a wide genetic variability that would explain why only one genotype (HTPI-15) was able to regenerate plants, at high frequency (Table 1). The complete absence of morphogenetic competence displayed by the inbred line HA89 of *H. annuus* (data not shown) suggests that the regeneration detected in leaves of *H. annuus* x *H. tuberosus* (Table 2), could reside in physiological and/or genetic factors of the hexaploid species *H. tuberosus* and/or in a more suitable interaction between nucleus and cytoplasm (Nestares *et al*. 1998).

Table 2. Effect of the regeneration event on morphogenetic potential of *H. annuus* x *H. tuberosus* leaves cultured in BN medium. R0 = first cycle of *in vitro* tissue culture; R1 = second cycle of *in vitro* tissue culture (regenerated plants).

Cycle	Medium	N° of cultured explants	Regeneration (%)Y
R0: (HA x HT)	MS	162	0 a
(30 clones)	BN	1523	1.38 b
R1: (HA x HT-R)	MS	970	27.11 c
(9 clones)	BN	1015	60.49 d

A remarkable embryogenic potential is displayed by explants of regenerated plants (R_1) subjected to a second culture cycle *in vitro*, even in absence of growth regulators (Tables 1 and 2). An increase of the *in vitro* regeneration potential, from regenerated plants was reported by several authors (Konar *et al*. 1972; Pugliesi *et al*. 1993a; Pedroso and Pais 1995; Fambrini *et al*. 1997). In many instances and likely in *H. tuberosus* and in the interspecific hybrid, the first *in vitro* culture cycle could induce and/or select cells with genetic (somatic mutations) or epigenetic changes that lead to an increased morphogenetic potential in regenerated plants. Otherwise from the results obtained with the interspecific hybrids *H. annuus* x *H. tuberosus* (Table 2), in *H. tuberosus* a high morphogenetic potential is not always displayed by regenerated plants (Table 1). In fact on BN medium, a high regeneration percentage (94.7 %) characterized 30 clones, designed HTPI-15-M (M = highly morphogenetic). In these clones a high regeneration frequency (47.3 %) was also detected on MS medium deprived of growth regulators (data not shown). In contrast, 15 clones, designed HTPI-15-N (N = no-highly morphogenetic), showed a significant lower regeneration ability (Table 1). In the first regeneration cycle of HTPI-15 we observed contemporary adventitious organogenesis and somatic embryogenesis. Likely, some leaf cells of the genotype HTPI-15 of *H. tuberosus* had attained different states of competence and therefore expressed this potential by developing embryos or adventitious meristems. Since, in *H. annuus* x *H. tuberosus* regeneration occurred only through embryogenesis (Fambrini *et al*. 1996), we could suppose that a different stability characterizes the two distinct levels of competence and thus, only plants of *H. tuberosus* regenerated through somatic embryogenesis acquired embryogenic competence, while plants regenerated *via* organogenesis didn't retain this morphogenetic potential. In support to our hypothesis it is necessary to remind that in many species this phenomenon has been chiefly exhibited by plants obtained throughout somatic embryogenesis (Konar *et al*. 1972; Maheswaran and Williams 1984; Nadel *et al*. 1990; Pugliesi *et al*. 1993a).

Table 3. Regeneration from leaf explants of triploid (*H. annuus* x *H. tuberosus*: HA x HT-R) x *H. annuus* (2n = 3x = 51: BC-R) cultured in BN medium.

Clone	N° of cultured explant	Regeneration (%)Y
BC-R (22 clones)	1212	0.08 a
BC-R-1-2 (1 clone)	257	56.4 b
BC-R-1-9 (1 clone)	33	60.6 b

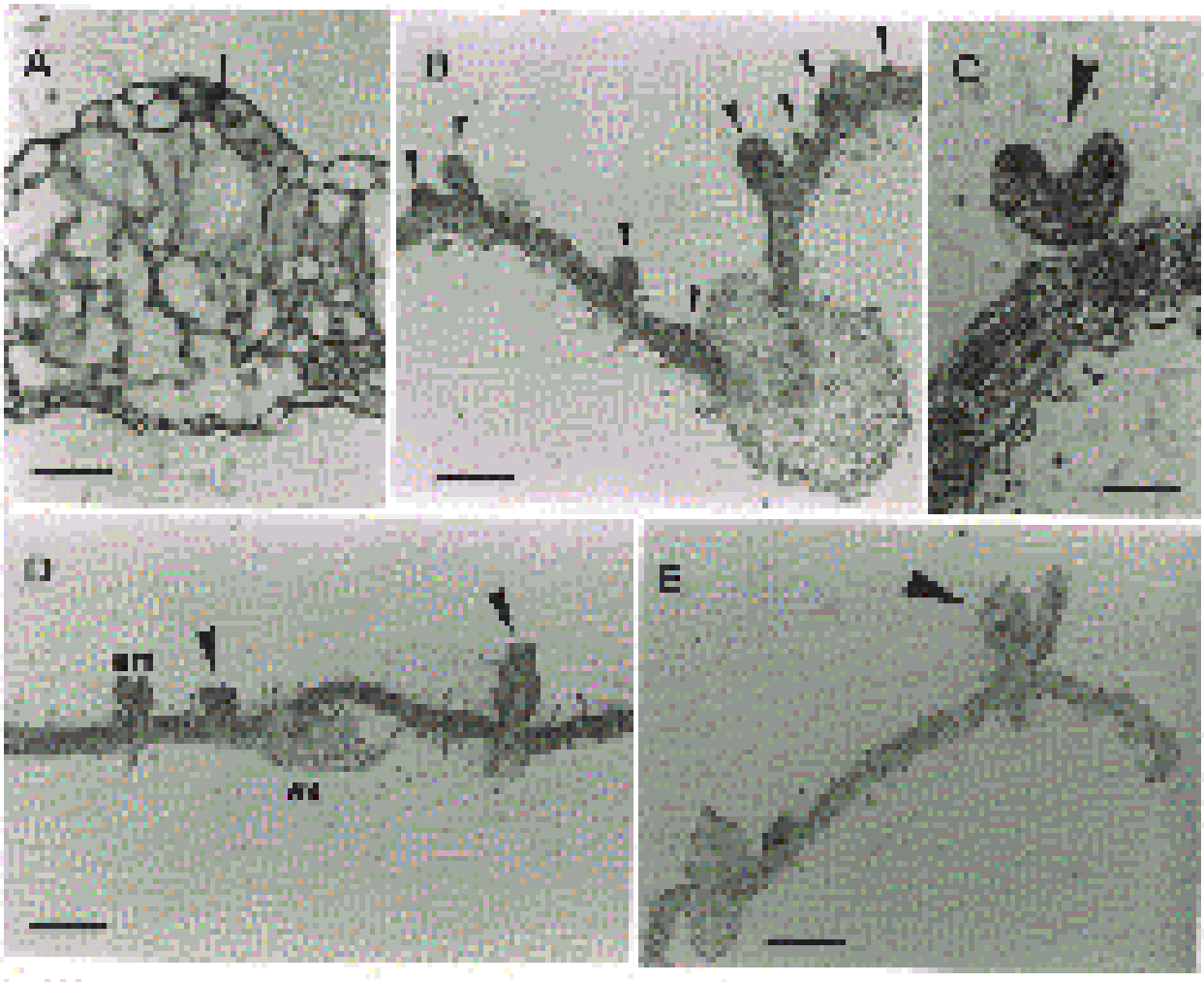
To evaluate whether the acquisition of the high embryogenic competence of our material was genetically controlled and therefore transmissible to the progenies, we backcrossed a regenerated tetraploid hybrid (HA x HT-R) with *H. annuus* but only few triploid seeds (2n = 3x = 51) were obtained. The high morphogenetic potential shown by 2 of the 24 individuals of the progeny obtained (BC-R) proves that this characteristic is transmissible through pollination (Table 3); however the limited amount of individuals which it was possible to analyse does not allows to come to a conclusion of the genetic control of the trait.

Although all clones derived from regenerated plants of *H. annuus* x *H. tuberosus* (HA x HT-R) were characterized by a high morphogenetic competence (Table 2), the clone EMB-2 distinguished itself from the other. In fact in EMB-2 plants the normal differentiation process is perturbed such that groups of leaf epidermal cells acquire characteristics of meristematic or embryonic cells, producing shoots and embryo-like structures, respectively. Most of the morphogenetic structures, usually arranged in clusters or rows along pre-existing veins, could be isolated from leaves and, after rooting in MS basal medium, they produced plantlets that exhibited epiphyllous embryos and shoots as the original clone.

To study the *in vivo* EMB-2 behaviour, some of the *in vitro* rooted plantlets were transplanted and grown in greenhouse. Although most of the deformed plants survived only a

few days, some plants were able to reach the floral stage. There was a great variability in the degree and pattern of development of the ectopic morphogenetic structures upon leaves, both within a single plant and among different plants. The histological analysis showed that epidermal cells of adaxial surface initiate ectopic structures by periclinal division (Fig. 1A), especially in proximity to the veins and at the leaf margin. Repeated divisions of these cells caused folding of the superficial tissue giving rise to an elevated dome or cushion (Fig. 1B). Subsequently, these cell clusters increased their volume by division and developed embryos and/or shoots (Figs. 1C-E). Usually, these epiphyllous structures were formed directly opposite to the xylem pole of a well-differentiated vein (Figs. 1D-E), that frequently showed an abnormal enlargement (Fig. 1D). EMB-2 was propagated by tubers and although many shoots of the new plants showed abnormal leaves with ectopic vitrescent morphogenic structures and died without further growth; however, several normal shoots produced plants characterized by epiphyllous structures similar to the parent plant.

Fig. 1 Origin and development of epiphyllous structures in the clone EMB-2 of the interspecific hybrid *H. annuus* x *H. tuberosus* as indicated by leaf cross-sections stained with hematoxylin. (A) Periclinal division



(arrow) in the epidermal cell of adaxial leaf surface. Scale bar = 40 μm. (B) Initial stages of the epiphyllous structures (arrowheads) on the leaf adaxial surface. Scale bar = 400 μm. (C) Epiphyllous embryo (arrowhead). Scale bar = 140 μm. (D) Ectopic structures (arrowheads) on the leaf adaxial surface. (em) = embryo; (ev) = enlarged vein. Scale bar = 600 μm. (E) Epiphyllous shoot (arrowhead). Scale bar = 450 μm.

Konov *et al*. (1998) have demonstrated the close relationship between epiphyllous structures in sunflower and exogenous treatments with cytokinins. In the ectopic manifestations of EMB-2 leaves some differences are evident in respect to the epiphylly observed in micropropagated sunflower. The EMB-2 clone, derived from a single somatic embryo induced in a low cytokinins concentration medium (Fambrini *et al*. 1997), was propagated by single- node cutting for more than three years in basal medium (MS) without growth regulators and no further growth hormone addition was required to exhibit regularly epiphyllous structures. Moreover, EMB-2 leaves developed epiphyllous embryo-like structures (Figs. 1C-D), never described in micropropagated sunflower (Konov *et al*. 1998). In EMB-2 not all leaf cells exhibited the same potential to form adventitious morphogenetic structures; it is noteworthy that epiphyllous embryos and shoots are often associated with the veins (Figs. 1D-E). We could suppose a mutation, induced throughout the *in vitro* regeneration process, that alters the hormonal endogenous levels (i.e. cytokinins) of the EMB-2 plants. Auxin is thought to be a major regulatory factor in the vascular differentiation, and various cytokinins promote xylem differentiation (Aloni 1987). In addition, subepidermal cells in proximity of the vascular strand are responsible for the initiation of shoots from leaf sectors of transgenic tobacco expressing *ipt* gene, which leads to the synthesis of cytokinin (Estruch *et al*. 1991). Alternatively, the EMB-2 phenotype could be explained by mutations in regulatory gene(s), homologous to *Knotted1* (*Kn1*) or *LEAFY COTYLEDON1* (*LEC1*) genes, whose postembryonic expression may activate genes that suppress vegetative development (Williams-Carrier *et al*. 1997; Lotan *et al*. 1998). On the other hand, a close relationship between homeobox *Knotted*-like genes and cytokinins on differentiation processes, has been demonstrated (Frugis *et al*. 1999; Rupp *et al*. 1999). It is impossible, at present, to discriminate the nature (genetic or epigenetic) of the epiphylly expressed by EMB-2 plants. Further studies will be necessary to identify the biochemical basis of this phenomenon by more detailed physiological characterization (i.e. endogenous hormonal levels). Moreover, the availability of a transformation technique for the interspecific hybrids *H. annuus* x *H. tuberosus* (Pugliesi *et al*. 1993b) will enable the phenotypic comparison among transgenic plants overexpressing *LEC1* or *Kn1*-related genes and the EMB-2 variant. This is the first case of *in vivo* epiphilly found in the biggest family of the Dicotyledoneae, and it could in the future prove to be an important kind of material to study the molecular basis of cell totipotency.

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