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Morphogenesis in *Helianthus tuberosus*: genotypic influence and increased totipotency in previously regenerated plants

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Abstract

Leaf tissues of 38 genotypes, derived from four accessions, of the hexaploid species *Helianthus tuberosus* (2n=6x=102) responded to growth regulators (BA, NAA) chiefly by forming callus, while adventitious organogenesis or somatic embryogenesis were induced occasionally. A remarkable regeneration frequency (about 30 %) was achieved only from leaves of genotype HTPI-15. Explants of many regenerated plants of HTPI-15 subjected to a second culture cycle *in vitro* displayed a high morphogenetic potential (regeneration frequency > 90 %). White globular structures were initiated on the adaxial surface of these leaves without a callus phase. Somatic embryogenesis was asynchronous and embryoids, of different developmental stage, were simultaneously detected on each explant. Although many embryos developed single or malformed cotyledons or germinated precociously, without the differentiation of a complete root system, phenotypically normal plants were regenerated after rooting on regulator-free half-strength MS medium.

Additional key words: cellular determination, competence, somatic embryogenesis.

Introduction

In the last years plant regeneration in the genus *Helianthus* has been achieved through organogenesis and somatic embryogenesis (Pélissier *et al.* 1990, Knittel *et al.* 1991, Pugliesi *et al.* 1991, 1993). Screening for *in vitro* morphogenesis (Paterson and Everett 1985, Witrzens *et al.* 1988, Pélissier *et al.* 1990, Knittel *et al.*

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Abbreviations: BA - N^6-benzyladenine; IAA - indole-3-acetic acid; MS medium - Murashige and Skoog medium; NAA - α-naphthalenacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid.

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1991), and genetic analysis (Sarrafi *et al.* 1996), 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 cellular totipotency (Henshaw *et al.* 1982, Wareing and Al-Chalabi 1985, Halperin 1986). Cotyledons, hypocotyls and immature zygotic embryos are the explants more commonly used in tissue culture of sunflower (Alibert *et al.* 1994); in contrast, leaves are not able to prominent adventitious differentiation (Pugliesi *et al.* 1993).

Plants regenerated from leaves of the interspecific hybrid *H. annuus* × *H. tuberosus* by means of somatic embryogenesis acquired a high morphogenetic competence (Pugliesi *et al.* 1993, Fambrini *et al.* 1997). In regenerated plants the embryogenic potential was not restricted to a particular organ, it was maintained throughout plant ontogeny and it was not lost after repeated sub-culture (Fambrini *et al.* 1997). There are many reports on the increased regeneration potential by one or several regeneration cycles (Konar and Nataraja 1965a, 1965b, Lupotto 1983, Maheswaran and Williams 1984, Bohorova *et al.* 1985, Nolan *et al.* 1989, McLean and Novak 1989, 1998).

In this work, to extend previous studies on the hybrid *H. annuus* × *H. tuberosus* (Fambrini *et al.* 1996, 1997), we exploited the morphogenetic competence in leaves of the genotypes of *H. tuberosus* and *H. annuus* already used in the interspecific hybridization (Fambrini *et al.* 1997). Since *H. tuberosus* is a highly heterozygous open-pollinated species, the behaviour *in vitro* of several accessions of different geographical origin was also investigated. Moreover, we evaluated, in a second *in vitro* cycle, the morphogenetic potential of explants collected from regenerated plants.

Materials and methods

Plants: Four accessions of *Helianthus tuberosus* (2n=6x=102) and the inbred line HA89 cms of *Helianthus annuus* (2n=2x=34) were used as starting materials. The accessions of *H. tuberosus* were: HT S. Pietro/100 (HTPI), provided by the Agricultural Department of Plant Biology, University of Pisa; HT/272 (HTSI), provided by the Botanical Garden of University of Siena; Ames/2729 (HTAM), provided by North Central Regional Plant Introduction Station of Ames, USA; I/22 (HTGR), provided by Botanical Garden of Greifswald, Germany. The inbred line HA89 of *H. annuus* and the accession HTPI of *H. tuberosus* were the materials previously used in the interspecific hybridization (Fambrini *et al.* 1997). Achenes were kept in sterile distilled water for 120 min. The naked seeds were surface-sterilized for 1 min in 70 % (v/v) ethanol and for 20 min in 2.8 % (v/v) sodium hypochlorite solution containing 0.01 % Triton X-100 and then rinsed in sterile distilled water. For germination, they were placed on solidified (8 g dm^{-3} *Bactoagar*, Basingstoke, England) MS basal medium (Murashige and Skoog 1962) without growth regulators. After one week, 39 seedlings (15 of HTPI, 4 of HTGR, 15 of HTAM and 5 from HTSI) were transplanted to 20 cm^3 solidified MS medium in 150 cm^3 Erlenmeyer flasks and incubated under a temperature of 25 ± 1 °C and a

16-h photoperiod in growth chamber. Irradiance was 35 μmol(photon) m^{-2} s^{-1} provided by cool-white fluorescent lamps (*Philips*, Eindhoven, The Netherlands). Every of two weeks, each plantlet was multiplied by single-node cuttings to obtain 39 clones (Fambrini *et al.* 1996, 1997). Plantlets of the inbred line HA89 were grown *in vitro* in the same conditions as previously described (Pugliesi *et al.* 1993).

Regeneration from leaf explants of *H. tuberosus* and *H. annuus*: Leaf explants (0.5 - 1.0 cm^2) from an inbred line of *H. annuus* and several genotypes of *H. tuberosus* were placed in Petri dishes on MS basal medium supplemented with different concentrations of α-naphthaleneacetic acid (NAA) and N^6-benzyladenine (BA) (Tables 1, 2). The media contained 30 g dm^{-3} sucrose and were solidified with 8 g dm^{-3} *Bactoagar*. Eight to twelve explants were placed in each plate.

Plant development: 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 half strength MS medium without growth regulators and with a reduced sucrose concentration (15 g dm^{-3}). The cultures were maintained in growth chamber in the above mentioned conditions. After two weeks, the percentage of rooted shoots was recorded. After rooting, the plants were transferred into pots containing a mixture of vermiculite, peat and soil, and were then covered with plastic bags to maintain high humidity and placed in a growth chamber at 22 ± 1 °C under 16-h photoperiod and irradiance of 250 μmol m^{-2} s^{-1} provided by fluorescent tubes (*Sylvania*, Wembley, England). After 15 - 20 d, the plastic bags were removed and the plantlets transferred to greenhouse and grown to maturity.

Regeneration from leaf explants of regenerated plants: 45 random selected regenerated R_1 plantlets from the genotype HTPI-15 of *H. tuberosus* were micropropagated by single-node cuttings as above described. The 45 clones obtained, constituted the material for new regeneration cycles. Leaf explants were cultured on basal medium without growth regulators (MS) or on basal medium supplemented with 0.2 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA (BN). The procedure of tissue culture and the growing conditions were the same as described above.

Statistical analysis: Statistical analyses were made after arcsin transformation of the percentage of explants producing buds or somatic embryos of three independent experiments with several replications (3 - 6 Petri dishes). Significance of differences between means was assessed by Tukey's test ($P = 0.05$). Homogeneity of variances was evaluated by Bartlett's test ($P = 0.05$). In Table 3, statistical significance was assessed by Student's *t*-test ($P = 0.05$).

Results

Regeneration from leaf explants of *H. tuberosus* and *H. annuus*: Leaf explants of 15 different genotypes of the accession HTPI were cultured on media with different

concentration of BA and NAA. Only one leaf explant from 2 200 leaf explants of genotypes HTPI-1 to HTPI-14 produced one adventitious embryo (Table 1). In contrast, the genotype HTPI-15 showed a high percentage of regeneration (Table 1). Although in this clone we observed somatic embryogenesis on a medium without growth regulator, the presence of NAA and BA in the substrates remarkably increased regeneration percentage (to 25 - 30 %). Always the morphogenetic structures originated directly from the adaxial leaf surface or at the cut margin of the leaf, especially in the proximity of the veins. Nevertheless, only 1 - 3 embryos were initiated in each regenerating explant. Occasionally in some explants, together with somatic embryos, we observed adventitious buds. Often precocious germination of the shoot apical meristem occurred in the pro-embryos, while uncommon was the development of an adequate root system. In addition, some abnormal embryos developed an altered number of cotyledons. Differentiation of roots was obtained after 15 - 20 d (about 80 %) by transferring the embryos and/or shoots to growth regulator-free half-strength MS medium.

Table 1. Effect of genotype and growth regulators on somatic embryogenesis from leaf explants of *Helianthus tuberosus*. Means followed by the same letter are not significantly different at $P = 0.05$.

Clone	BA [mg dm^{-3}]	NAA [mg dm^{-3}]	Number of explants	Embryogenesis [%]
HTPI-1 to	0	0	335	0
HTPI-14	0	0.1	363	0
(14 clones)	0.2	0.1	1223	0.08
	0.5	0.1	319	0
HTPI-15	0	0	87	6.9a
	0	0.1	76	5.3a
	0.2	0.1	276	30.8b
	0.5	0.1	74	24.3b

Then, we have exploited the morphogenetic potential of three different accessions of *H. tuberosus*. Leaf explants were cultured on the same medium as in the former experiment. In 23 genotypes we observed an insignificant morphogenetic potential (Table 2). In contrast, one genotype (HTSI-5) showed a higher regeneration frequency (4.23 %).

Table 2. Somatic embryogenesis from leaf explants of three accessions of *Helianthus tuberosus* cultured in MS medium supplemented with 0.2 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA.

Accession		Number of explants	Embryogenesis [%]
HTSI-1 to HTSI-4	4 clones	385	0.52
HTSI-5	1 clone	142	4.23
HTGR-1 to HTGR-4	4 clones	367	0.27
HTAM-1 to HTAM-15	15 clones	896	0.11

In our experimental conditions, a complete inability to regenerate adventitious shoots or somatic embryos was displayed by leaves of *H. annuus* on all hormone combinations (data not shown).

Morphogenesis from regenerated plants: From 85 regenerated R_1 plantlets of the genotype HTPI-15, 45 of them were random selected and micropropagated through single node cuttings on MS basal medium. The 45 clones obtained were used as a source of explants in the second *in vitro* culture cycle. The leaves were cultured on MS basal medium without growth regulators or on BN medium (Table 3). On BN medium, a high regeneration percentage (94.7 %) characterized 30 clones, designed HTPI-15-M (M = highly morphogenetic). In these clones a remarkable regeneration

Table 3. Somatic embryogenesis from leaf explants of regenerated plants of *Helianthus tuberosus*, cultured on medium without growth regulators (MS) or MS supplemented with 0.2 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA (BN). Means followed by the same letter are not significantly different at $P = 0.05$. Statistical analysis was done separately for MS and BN media.

Medium	Accession		Number of explants	Embryogenesis [%]
MS	HTPI-15-N	15 clones	279	0 a
	HTPI-15-M	30 clones	522	47.3 b
BN	HTPI-15-N	15 clones	575	8.2 a
	HTPI-15-M	30 clones	840	94.7 b

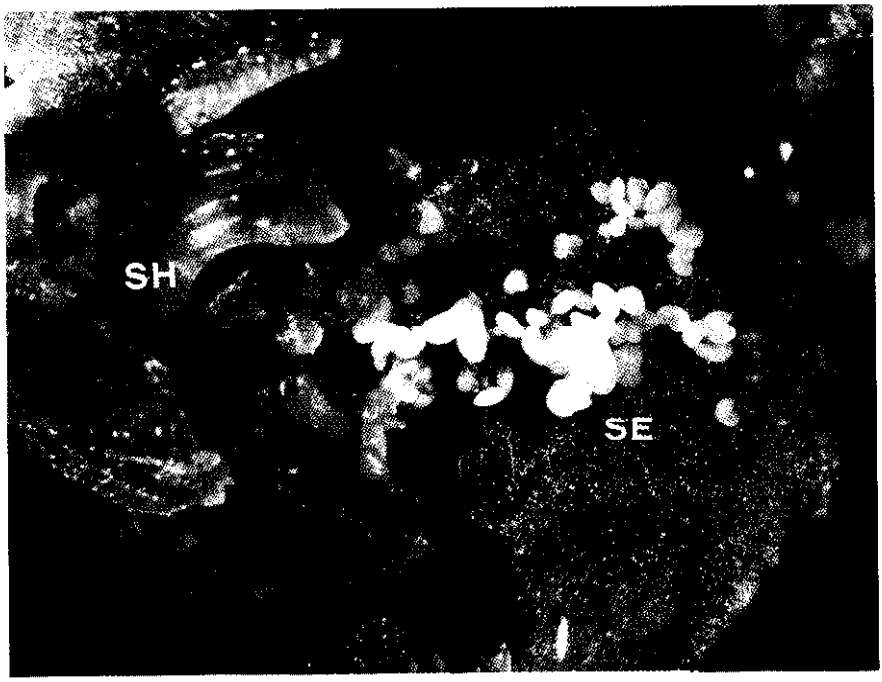

Fig. 1. Cluster of somatic embryos (SE) and single shoot (SH) developed from a leaf explant of HTPI-15-M.

frequency (47.3 %) was also detected on MS medium deprived of growth regulators. In contrast, 15 clones, designed HTPI-15-N (N = no-highly morphogenetic), showed a significantly lower regeneration ability (Table 3). Moreover, as observed in the first regeneration cycle, in each regenerating leaf explant of HTPI-15-N a limited number (1 - 3) of morphogenetic structures were initiated. In contrast, after 5 - 7 d of culture on BN medium, leaves of HTPI-15-M developed, without a prominent callus phase, many (5 - 20 per explant) white globular pro-embryos. These structures occurred mainly on the adaxial surface, and in 8 - 12 d appeared markedly elongated. After 2 - 3 weeks of incubation, they reached the cotyledon-like stage. Somatic embryogenesis was asynchronous and embryoids, corresponding to every recognizable developmental stage were simultaneously observed (Fig. 1). Many embryos developed single or malformed cotyledons, and often, multiple embryos with many root-shoot axes originated from one morphogenetic unit. In spite of these abnormalities, the high regeneration rate allowed to recover many phenotypically normal plants. The regenerating explants subcultured on MS medium, without growth regulators, differentiated shoots from precociously germinated embryos. These shoots were rooted as previously described, transplanted in greenhouse and grown until tubers production.

Discussion

We demonstrate that both genotypes (Tables 1, 2) and previous regeneration (Table 3) are factors that greatly influenced the morphogenetic competence expressed by leaf explants of *H. tuberosus*. 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, Pélissier *et al.* 1990, Knittel *et al.* 1991, Sarrafi *et al.* 1996).

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 (Tables 1, 2). The complete absence of morphogenetic competence displayed by the inbred line HA89 of *H. annuus* suggest that the regeneration previously detected in leaves of *H. annuus* × *H. tuberosus* (Fambrini *et al.* 1997), 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 (Ohki *et al.* 1978, Nestares *et al.* 1998).

An increase of the *in vitro* regeneration potential, from regenerated plants was reported by several authors (Konar *et al.* 1972, Lupotto 1983, Saunders and Doley 1986, Robertson *et al.* 1988, Nolan *et al.* 1989, Nadel *et al.* 1990, Pugliesi *et al.* 1993, Pedroso and Pais 1995, Fambrini *et al.* 1997, Ovečka *et al.* 1997). In many instances and likely in *H. tuberosus*, the first *in vitro* culture cycle could induce and/or select cells with genetic (somatic mutations) or epigenetic changes that lead to an increased embryogenic potential in regenerated plants.

In the first regeneration cycle of HTPI-15 we observed contemporary adventitious organogenesis and somatic embryogenesis. Distinctive cellular competences are

established very precociously just at the first stage of the embryogenesis as resulted in a differential gene expression (Steeves and Sussex 1989). Some indirect evidences that embryogenic and organogenic competences are distinct states, which are not necessarily shared by the same cells, come from the observation that carrot organogenic cultures are sometimes more readily produced in the presence of the weaker auxins, IAA and NAA, and embryogenic cultures in the presence of the strong 2,4-D. Steward (1967) argued that the more powerful growth regulators were required to eliminate the developmental constraints which accumulate in more mature carrot cells. It might be evidenced that the regression brought about by the weaker growth regulator is incomplete, only restoring an organogenic competence. Moreover, where embryogenic and organogenic competence have been monitored in long-term cultures, it has generally been found that any decline in the two properties follows independent time courses suggesting that the two types of competence were not inseparable (Gould 1978). In our case, we could presume that some leaf cells of the genotype HTPI-15 of *H. tuberosus* had attained different states of competence and therefore express this potential developing embryos or adventitious meristems. In contrast to the results obtained with the interspecific hybrids *H. annuus* × *H. tuberosus* (Fambrini *et al.* 1997), in *H. tuberosus* a high morphogenetic potential is not always displayed by regenerated plants (Table 3). Since, in *H. annuus* × *H. tuberosus* regeneration occurred only through embryogenesis (Fambrini *et al.*, 1997), 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 did not retain this morphogenetic potential. In support to our hypothesis it is necessary to remind that in many species this phenomenon has chiefly exhibited by plants obtained throughout somatic embryogenesis (Konar *et al.* 1972, Lupotto 1983, Maheswaran and Williams 1984, McLean and Novak 1989, Nolan *et al.* 1989, Nadel *et al.* 1990, Pugliesi *et al.* 1993, Fambrini *et al.* 1997).

In conclusion, we have demonstrated that in addition to the genotypic influence on the *in vitro* response of *H. tuberosus*, an acquisition of high embryogenic potential is effectively consequent to a previous regeneration. Seed-progeny tests could elucidate if the dissimilar morphogenetic potential observed among several genotypes of *H. tuberosus* and the stable embryogenic competence showed by 30 regenerated plants of HTPI-15 are both inherited traits.

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