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Organogenesis of anther-derived calluses in long-term cultures of *Oenothera hookeri* de Vries

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Abstract

Anthers of *O. hookeri* containing uninucleate microspores were cultured, *in vitro*, at 25 °C (16 hours photoperiod) on solid MS medium. After 10–15 days, on media with 2,4-dichlorophenoxyacetic acid, 1-naphthaleneacetic acid and 6-benzylaminopurine, anthers developed friable calluses. After unsuccessful treatments on embryogenic – and/or organogenic – induction media, calluses were placed on a hormone-free MS medium for 24 months with routine transfers every 3 weeks. After this period, the calluses developed buds and subsequently plants. Ro generation plants, were morphologically distinct.

Abbreviations: BA – 6-benzylaminopurine, 2,4-D – 2,4-dichlorophenoxiacetic acid, IBA – indole-3-butyric acid, MS – Murashige & Skoog (1962) medium, NAA – 1-naphthaleneacetic acid

Introduction

Anthers of several plant species grown *in vitro*, generate plants directly by embryogenesis or indirectly via organogenesis (Williams & Maheswaran 1986; Roca et al. 1991; Szarejko et al. 1991). In this respect embryoids, and/or calluses can have their origin in reproductive cells (microspores/pollen) or diploid anther tissues (Vasil 1980).

In general, androgenesis, *in vitro*, is genotype-dependent and usually rapid (Vasil 1980; Roca et al. 1991). Evidence of morphogenesis in anther-derived calluses sustainable beyond 60 days, is rare (Tsay et al. 1986).

The ontogeny stages of pollen development in the genus *Oenothera* are well studied (Noher de Halac et al. 1992), but until now, the formation of organized multicellular structures, was never sustainable as for example with *O. coronifera* (Jean et al. 1976). In this study, we describe the establishment and prolonged maintenance of calluses with subsequent organogenesis and plant regeneration (non-haploid) from *O. hookeri* De Vries anther cultures.

This study aims at contributing to the understanding and control of morphogenesis in anther-derived calluses that are recalcitrant in terms of plant regeneration.

Materials and methods

Anther donor diploid plants (2n = 2x = 14) were raised from seeds an the early flowering mutant of *Oenothera hookeri* De Vries (provided by Dr. C. Harte, Developmental Biology Institute of the University of Cologne, Germany) (Halac et al. 1992).

Seeds were germinated in vermiculite and plantlets were grown under constant illumination of 12,5 μmol m^{-2} s^{-1} (26 ± 2 °C) for 3–4 weeks. Seedlings (3 cm in length) and with 5–6 fully expanded leaves, were individually transplanted into pots with ordinary soil and maintained under the same light conditions. Flowering was obtained after 45–60 days. Isolated flower buds were placed in distilled water and exposed to a cold pre-treatment at 5 °C (2 days). Buds were disinfected in 70% (v/v) ethanol (1 min), 0.8% (v/v) sodium

Table 1. Inductive media composition and percentage of callus developing anthers.

Hormone type and concentration (mg/l)				No. of anthers tested	No. of anthers giving callus	Anther efficiency[2] (%)
Media[1]	2,4-D	NAA	BA			
1	0.0	0.0	0.0	420	0	0
2	1.0	1.0	0.0	400	120	30
3	2.0	2.0	0.0	560	244	43.5
4	1.0	0.0	1.0	220	56	25.4
5	2.0	0.0	2.0	226	116	49.5

[1]Basic MS medium, pH 5.8.
[2]The percentage of calluses produced per 100 plated anthers.

hypochlorite (15 min), followed by 3 rinses in sterile distilled water. To determine pollen developmental stage, one anther per flower was squashed and stained with aceto-carmine.

Basic MS medium containing 30% (w/v) sucrose and 0.8% (w/v) agar (Sigma Chemical Co.) (pH 5.8) was used for all treatments. Filter-sterilized hormones were added to the medium as required. To initiate callus growth, 7 anthers per flower from 261 flowers were cultured for 4 weeks in (60 × 60 mm) Petri dishes sealed with extensible P.V.C. (Vitoplas, Vitopel SA., Córdoba, Argentina) and incubated in a growth chamber, under fluorescent illumination (16 h photoperiod, 3.75–12.5 μmol m^{-2} s^{-1} at 25 °C ± 1 °C) and then 8 hours in the dark (20 °C ± 1 °C). Three replicate treatments per growth regulator regime were performed.

Anthers at the tetrad stage, with uninucleate microspores or with young pollen grains were cultured on 50 ml MS medium supplemented with 2,4-D, NAA and BA (Table 1). After 2 weeks, anthers that developed calluses were transferred to MS medium with different growth regulators to induce long-term embryogenesis and/or organogenesis. Twenty five combinations of NAA (0.0, 0.02, 0.2, 2.0, 4.0 mg/l) with BA (0.0, 0.02, 0.2, 2.0, 4.0 mg/l) and 16 combinations of NAA (0.0, 0.1, 1.0, 2.0, 3.0 mg/l) with 2,4-D (0.0, 0.1, 1.0, 2.0, 3.0 mg/l) were assessed. After 30–45 days proliferating calluses were transferred to fresh hormone free MS medium (25 °C ± 1 °C, 16 h photoperiod 12,5 μmol m^{-2} s^{-1}) with transfers every 30–45 days for approximately 700 days (20 subcultures). They were finally placed at a 16 h/day photoperiod with a light intensity of 3.75 μmol m^{-2} s^{-1}. Calluses which had produced vegetative buds were cut into 5 mm × 5 mm portions and placed on to the same media for another 60 days. For rooting, 1–2 cm long shoots were separated from the calluses and placed in 10 × 100 mm tubes with 15 ml MS medium, with 4 different levels of IBA (0.0, 0.5, 1.0, 2.0, 3.0 mg/l). After 30 to 40 days, rooted plantlets were placed in pots containing a 1:1 (v/v) mixture of sterilized soil and vermiculite and covered with transparent nylon bags. After 3 weeks they were transferred and flowered in the glass house at room temperature (28–34 °C).

Aceto carmine-stained (Bradley 1948) and thin sectioned (Noher de Halac et al. 1992) organogenic and non-organogenic portions of callusus were observed under a Standard 14 Zeiss microscope using bright field optics. Regenerating calluses were sampled as previously described (Noher de Halac & Harte 1985) and observed under a Philips 500 S.E.M.

Results

The most appropriate developmental stage of anthers for callus formation, is the uninucleate microspores stage obtained from 7–8 mm long buds. Anthers containing tetrades and young pollen grains, were totally unresponsive.

The percentage of calluses obtained from 1826 plated anthers with microspores at the uninucleate stages (261 flowers) in inductive media of 5 different hormone compositions is given in Table 1. Anthers placed on the control medium 1 (without hormones) did not give calluses, while the highest yield calluses (43.5% and 49.5%, Table 1) was obtained with media 3 and 5 (Table 1).

Calluses were visible after 10–15 days culture (Fig. 1). Anthers that did not form callus turned black and

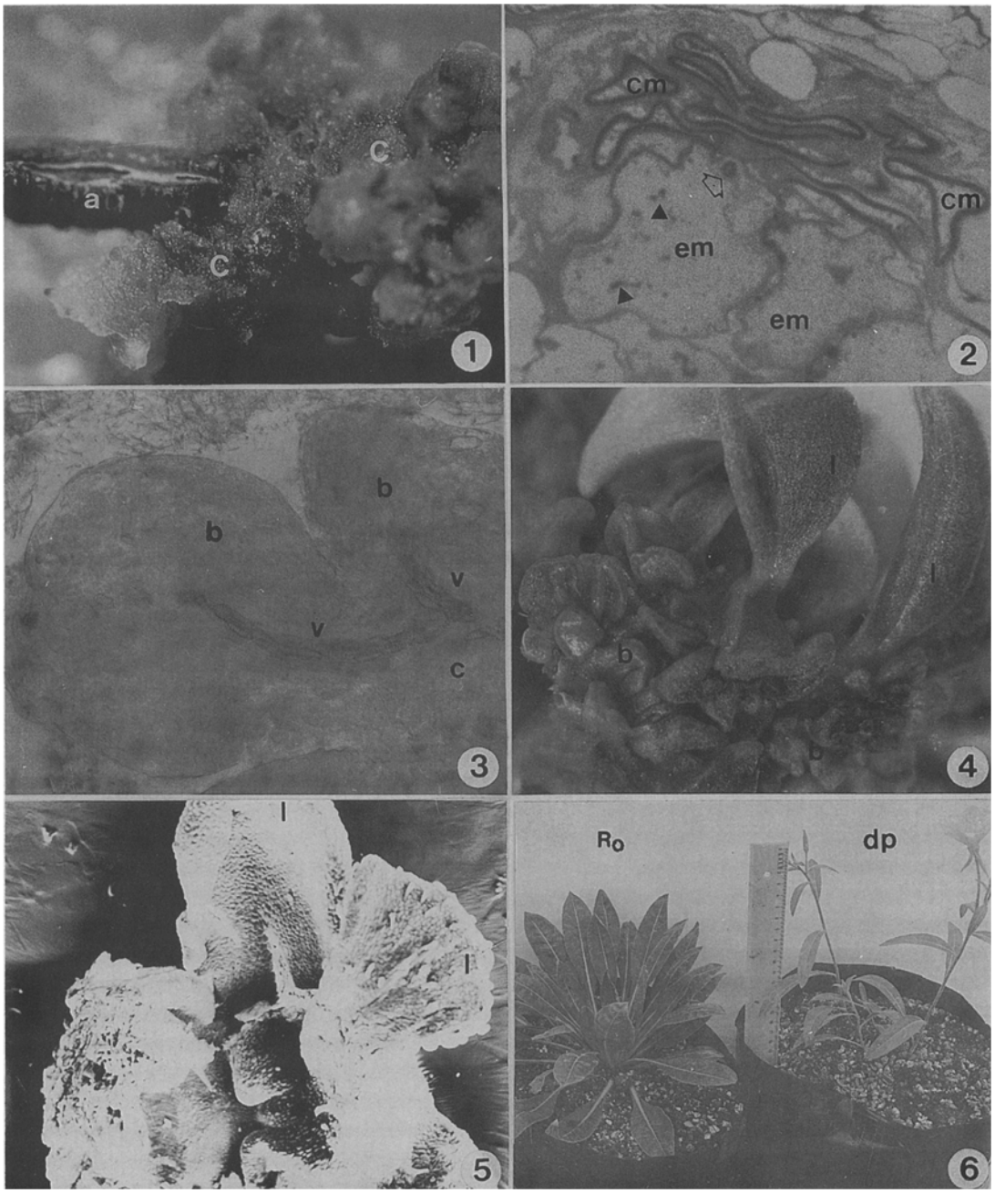

Fig. 1. One month old callus (c) attached to the anther wall (a). Medium: MS + 2 mg/l 2,4-D + 2 mg/l NAA. × 21

Fig 2. Section of an anther after 16 days in culture. Stained with alkaline Toluidine Blue. Two microspores are enlarged (em) and show a nucleus (arrow) and several dark bodies (arrow heads). Other microspores are collapsed (cm). × 520

Fig 3. One year old callus (c) with globular meristematic nodules (b) and vascular connections (v), squashed and stained with aceto carmine. × 413

Fig 4. Two year old shooting callus, with meristematic nodules (b) and leaves (l). Medium: MS lacking hormones. × 21.

Fig 5. Scanning electron microphotograph of a shoot with hairy leaves (l). × 75.

Fig 6. Comparison between an anther donor plant (dp) and a (Ro) regenerated plant from calli of *O. hookeri*.

Table 2. Percentage of rooted shoots on MS medium with different IBA concentrations.

IBA concentration (mg/l)[1]	No. of shoots transferred	No. of rooting shoots	(%)
0.0	42	2	5.02
0.5	42	23	54.07
1.0	42	27	64.02
2.0	50	42	84
3.0	50	49	98.4

[1]Basic MS medium, pH 5.8.

stopped growing. Sections of 16 day old cultured anthers showed a few enlarged microspores with 1 healthy nucleus and several stained bodies (Fig. 2).

Rooting was induced in 30–40 day old calluses after 7 days transfer to hormone-free MS media but these did not proliferate.

After more than 20 transfers on hormone-free media, with a light intensity of 3.75 μmol m^{-2} s^{-1}. 10 brown calluses resumed growth, turning green (1.86%). Organogenic calluses were green with a rough, moist surface and grew rapidly. The first visible green nodules were squashed on a slide and when stained with aceto carmine, showed a vascular connection strand (Fig. 3). These meristematic nodules developed foliar organs covered by unicellular hairs (Figs. 4, 5). Shoots were generally green but four (0.74%) plants were albino.

Shoots were induced to root, after 60 days in hormone-free media, and then cultured for 30 days on media with IBA alone. The number of shoots giving roots was higher as the IBA concentration increased (Table 2). After rooting, plantlets exhibited a geotropically positive and fibrous root system. Once transplanted, the growth of plantlets was initially slow, (4 cm high by 2 months) as compared to donor plants (30–40 cm high).

Of 250 juvenile plantlets, 150 survived the transplantation phase. Of the 150, 83 plantlets reaching maturity and 43 of these individuals flowered one year later. The plants regenerated (Ro) from the calluses had a typical morphological variation. Ro generation showed a rosette at the base of the shoot while anther donor plants exhibited elongated internodes (Fig. 6). This variation was observed in all regenerated plants.

Chromosome counting of root meristems in Ro plants showed that all plants examined were diploid (2 n = 2x = 14) and completely fertile.

Discussion

Our report is the first one on plants developed from anthers in the genus *Oenothera*. Experiments of *in vitro* culture of explants of other young organs of the plants have been performed by several authors, but a full cycle leading to plant production has not been reported (Stubbe & Hermann 1982; Suzuki et al. 1990; Harte personal communication). Jean et al. (1976) observed the proliferation of isolated microspores in culture in *O. coronifera* Renner, but the multicellular structures obtained, did not develop.

In our experiments, diploid (2 n = 14) plants were obtained through indirect androgenesis (Narayanaswamy & George 1982; Williams & Maheswaran 1986; Roca et al. 1991; Szarejko et al. 1991). The diploid chromosome number is expected if proliferation of the diploid tissues of the anther is assumed. Masses of callus tissue emerged from the anthers 10 days after initiation of cultures, while few microspores increased in volume showing several small stained bodies which could be micronuclei. After 16 days in culture the enlarged microspores did not develop.

In our study we show that anther callus production is dependent on the developmental stage of the anther, as well as on the hormone composition of the media (Table 1). Calluses were considered recalcitrant for nearly 20 subcultures on fresh hormone containing and hormone free media. Then, a few of these recalcitrant callus (1.86% of the population) shooted.

More studies are necessary to understand the pathway by which environmental and genetical factors induce shooting after callus tissues have been subjected to such long culture times.

Factors influencing the organogenic response can only be speculated on: The shooting event is either hormone independent or it may rely on a still unknown endogenous hormone balance. On the other hand, light intensity decreased from 12.5 to 3.75 μmol m^{-2} s^{-1}, 21–30 days before shooting, but this can be excluded as the inductive factor, since a repetition of the experiments with fresh anthers, established and kept under the lowest light condition, resulted in the repetition of the long recalcitrant phase and the late shooting event.

Once the shooting response was obtained, calluses remained productive for more than 36 transfers to hormone free media. Few authors mention time spans for embryogenic and/or organogenic capacity of calluses. As an example, we found that Tsay et al. (1986) kept productive calluses of *Zea mays* for one year. *Nicotiana* somatic calluses were maintained for more than 20 years (Melchers 1977), but as for as we know organogenesis was not obtained.

Morphology of the generation Ro plants differed from the anther donor plants (Fig. 6). The most important differential trait is the presence or lack of the rosette at the base of the shoot, a leading feature of taxonomic significance in the genus *Oenothera* (Dietrich 1977).

If the changes in *O. hookeri* calluses, that could have occurred in a single cell or a group of cells, are genetic or epigenetic is still an open question as for other species (Vasil 1980; Ting & Gu 1990; Gozukirmizi et al. 1990).

Our results show that: a) Plants of the Ro generation are not haploid, since chromosome number is coincident with the diploid number of the species (2n = 14); thus morphology differences cannot be explained on this basis, b) Neither chromosome number alteration, nor alteration in ploidy level could account for the morphological differences observed, c) The Ro population has a homogeneous morphology (Halperin 1986), d) Morphological changes are stable at least for two more generations. On the other side, the mechanisms for chromosome number variation *in vitro* (polyploidy, aneuploidy) are sufficiently understood and by contrast, little is known on the causes of chromosome structural changes, gene mutations and molecular DNA changes in these conditions (Lee & Phillips 1988; D'amato 1991).

One evidence of somaclonal variability is, however, the appearance of albinos among our plantlet population in a ratio of 0.74%. These individuals showed that at least certain possible genotypes are selected out of the population by a lethal factor. Interestingly enough, the time lapse of callus culture, has been postulated to influence the formation of albinos in *Triticum aestivum* (Chen 1986; Liang et al. 1990).

One of the main conclusions of this work is that in *O. hookeri*, certain endogenous and/or exogenous conditions, activate recalcitrant anther-derived calluses to shooting, followed by the development of complete plants. The nature of the inductive stimulus remains, however, still unknown.

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