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Correlation of sequential floral and male gametophyte development and preliminary results on anther culture in *Opuntia ficus-indica*

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Abstract

Before approaching anther culture as a tool to trigger an androgenic response in a new species, it is advisable to characterize and correlate flower and male gametophyte development to enable reproducible identification of the appropriate starting material. Buds and flowers of *Opuntia ficus-indica* cv. Gialla were classified in eight stages according to their total length at the earlier stages and the length of the corolla in flowers with emerging sepals. Due to the low condensation of chromatin in the microspore nucleus as well as in the vegetative nucleus of the bi- and tricellular pollen along with the high autofluorescence of the intricate exine, DAPI staining turned out not to be feasible in this species. Therefore an approach based on light-microscopy observation of semithin sections was used. These sections were stained with toluidine blue for general structure recognition and I_2KI to study starch deposition. Correlations were made between the sequential floral and male gametophyte development. Using this approach we determined the timing of pollen formation and observed that pollen development is impaired in plants producing seedless fruits. Furthermore, anther culture was carried out with anthers collected from flower buds at stages 2 and 3. Most of the anthers produced callus, however no regeneration was obtained.

Introduction

Opuntia ficus-indica L. Mill., native of Mexico, is cultivated for its fruits as well as a forage crop for animals. In Italy, it is mainly spread in Sicily. This species perfectly adapted to arid zones characterized by droughty conditions, erratic rainfall and poor soils subjected to erosion. Its taxonomy is difficult to establish for a number of reasons: the phenotypes, which vary greatly according to ecological conditions; the high number of populations with different ploidy and vegetative and/or sexual reproductive mode, as well as the existence of numerous hybrids (Reynolds and Arias 2001). In Italy, the population of prickly pear is principally composed of three ecotypes or varieties: “Gialla”, “Rossa” and “Bianca”, which are characterized by different fruit skin colours, and all of them have a high degree of heterozygosity and variability, *e.g.* regarding the quantity of seeds per fruit (seedless plants and parthenocarpy) (Weiss *et al.* 1993).

Opuntia ficus-indica L. Mill. breeding to develop new cultivars was hampered by some reproductive

aspects such as cleistogamy, nucellar embryony and a low seed germination rate (Chessa and Nieddu 2002, Chessa *et al.* 2000). Biotechnology represents a valid tool to improve the speed and the efficiency of the traditional breeding methods. Little research has been carried out on tissue culture in *Opuntia* such as the establishment of callus and cell suspension cultures (Llamoca-Zarate *et al.* 1999) as well as studies on the induction of embryonic globular structures (Pinheiro da Costa *et al.* 2001).

Haploid plants, with a gametophytic set of chromosomes in the sporophyte, and homozygous doubled haploid plants are of interest for genetic and developmental studies as well as for plant breeding. In fact they can be potentially employed in selection, mutation research, genetic analysis and genetic transformation.

Anther or isolated microspore culture are the most effective and widely used method of producing haploids and doubled haploids (Heberle-Bors 1985).

I*n vitro* pollen embryogenesis is affected by numerous factors: genotype, pretreatment applied to anthers or to floral buds, pollen developmental stage, donor plant growth conditions, culture media, and conditions of incubation (Touraev *et al.* 1997). Regarding the stage of pollen development, it was observed that only certain, immature stages are amenable to cell reprogramming towards proliferation and embryogenesis. When aiming to apply this technology to a new species, a staging of pollen development is needed to limit the number of explants to be tested *in vitro*.

The present research was carried out to study the correlation of sequential floral and male gametophyte development and to investigate the response to *in vitro* culture of anthers collected from flower buds of two different candidate stages of development.

Material and methods

Plant material

Flower buds and flowers of different sizes of *Opuntia ficus indica*, cv. "Gialla" were collected in spring 2004 from plants growing in open fields in inland Sicily. Fruit-seeded and fruit-seedless plants were selected as sample source. The specimens were kept refrigerated and transported to the laboratory for classification according to the total length of the buds and also to the length of the corolla in flowers with emerging sepals.

Sample fixation and processing

The anthers were carefully excised from the flowers and fixed in 4 % formaldehyde in phosphate-buffered saline (PBS), pH 7.4 overnight at 4 °C. After washing in PBS, the samples were dehydrated in a series of methanol of increasing concentration with a progressive lowering of temperature (PLT) in a LEICA AFS device (Coronado *et al.* 2002) and infiltrated in LRwhite resin (Agar Scientific) at –20 °C, as previously described (González-Melendi and Shaw 2002). Polymerisation of the resin was performed at the same temperature under UV light.

Light microscopy

Formaldehyde-fixed anthers were stained with a 10 $\mu g \cdot ml^{-1}$ DAPI solution containing 1 % Tween 20, for different times and incubated either at room temperature or at 4 °C. Then, the anthers were washed in water and squashed onto slides. The preparations were observed on a Zeiss fluorescence microscope under UV light. Sections of 1 µm from resin-embedded specimens were collected on slides. These were either unstained and observed under phase contrast or stained and observed under bright field. To stain the sections we used a 0.1 % toluidine blue solution in water, for general structure observation, and I_2KI , to detect starch accumulation (O'Brien and McCully 1981). Photographs were taken in a Leitz photomicroscope equipped with a digital camera (Olympus DP 10).

Anther culture

After cold pre-treatment for 15 days at 4 °C, flower buds of stages 2 and 3 (Table) were sterilized by immersion for 15 min in 70 % (v/v) ethanol followed by immersion in a sodium hypochlorite solution (about 0.5 % active chlorine in water) and a few drops of Tween 20 for 20 min and finally 3 rinses each of 5 min with sterile, distilled water. The pet-

Table. Classification of buds and flowers according to morphological features as illustrated in figure 1. Buds and flowers were classified in three main categories: a) Buds without emerging sepals and white anthers, b) Flowers with emerging sepals and yellow anthers and c) Open flowers and yellow anthers for a rapid in-field selection. Then a more careful dissection of floral development showed eight classes of buds and flowers according to measurable features (total length and length of the corolla) to study its correlation with pollen development.

Stages	Total length (cm)	Corolla length (cm)
Buds without emerging sepals. White anthers		
1	1.5 – 1.8	
2	1.9 – 2.7	
3	2.8 – 3.1	
4	3.2 – 3.8	
Flowers with emerging sepals. Yellow anthers		
5	3.9 – 4.8	0.65 – 0.75
6	4.9 - 6	0.8 -1
7	6.1 – 6.8	1.1 – 1.3
Open flowers. Yellow anthers		

als were aseptically removed with small forceps and the anthers carefully dissected and placed in 6-cm diameter Petri dishes containing 10 ml of solid induction medium (Germanà and Chiancone 2003). About 50 anthers were placed in each Petri dish and 15 dishes were prepared for each developmental stage (2 and 3). The Petri dishes were sealed with parafilm, incubated at 27±1 °C, for 30 days in the dark and then placed under cool white fluorescent lamps (Philips TLM 30W/84) with a photosynthetic photon flux density of 35 μmol·m^{-2}·s^{-1} and a photoperiod of 16 hours.

After 7 months in culture, anthers with callus, opened, not-developed and swollen were counted. Analysis of variance using Fischer's LSD test at the 95 % confidence level was applied.

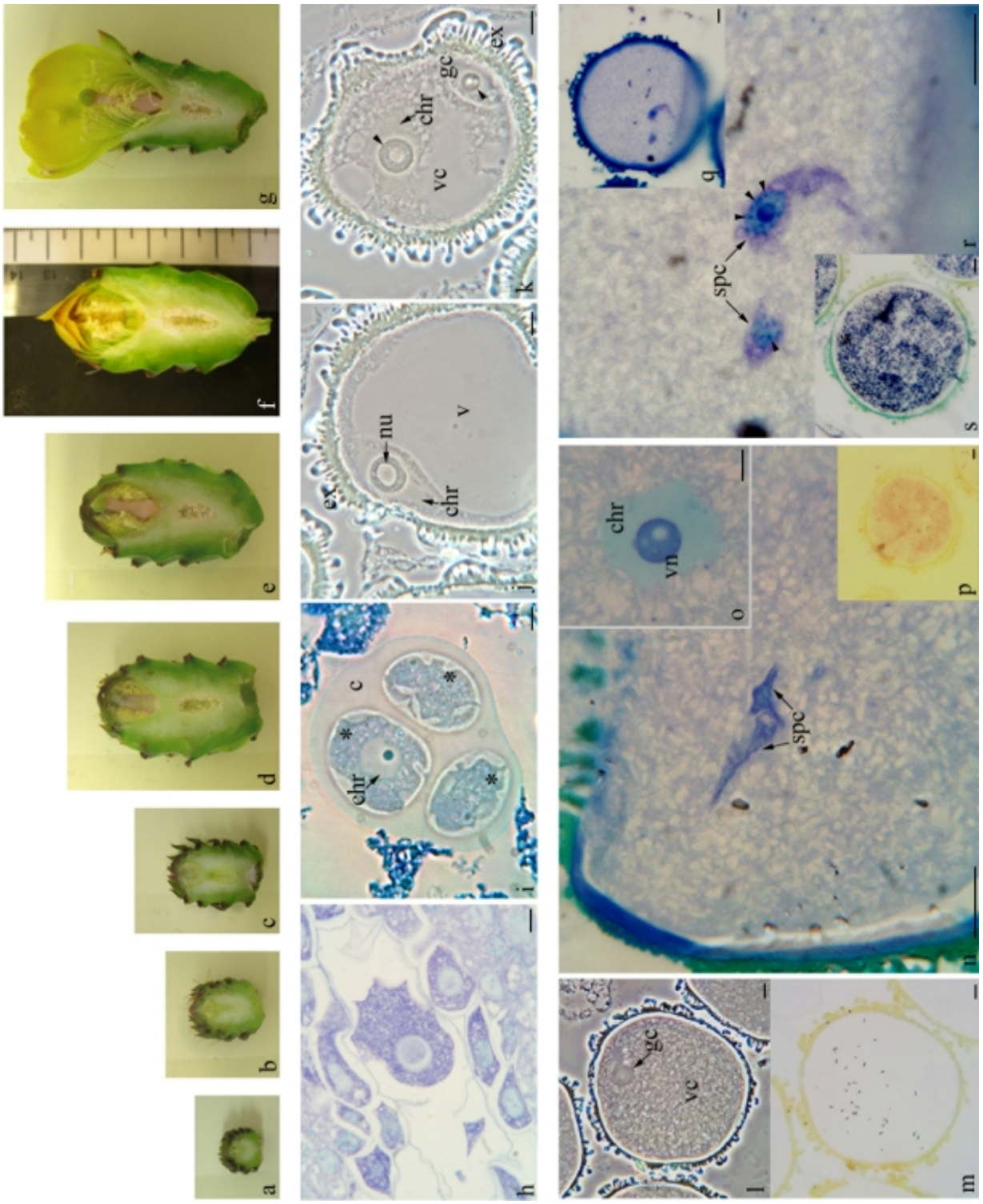
Results and discussion

Male gametophyte development

The specimens collected from the fields were classified in three groups based on qualitative, easily-recognised features for a rapid, in-field selection. Then, for a further and more precise classification, eight classes of buds and flowers were determined according to their total length and also to the length of the corolla of flowers with emerging sepals (Table). The first group included stages 1 to 4 (Fig. 1a-c), corresponding to buds of different sizes with small, white anthers inside. The second group corresponded to stages 5 to 7 (Fig. 1d-f), represented by flowers of different sizes with emerging sepals and yellow anthers. The third group coincided with stage 8 (Fig. 1g), corresponding to open flowers with yellow anthers.

To find out the sequential pollen developmental phases within these eight stages, DAPI staining was attempted which is an easy, rapid and low cost method of staging based on the specific labelling of DNA (Vergne *et al.* 1987). The intensity of fluorescence is a linear function of the degree of chromatin condensation. Then, the number of nuclei, their relative position and, principally, the distinction between the vegetative and generative nuclei of the pollen grain based on their different pattern of chromatin condensation (Testillano *et al.* 2005) can be determined. Although DAPI staining has been successfully applied to characterise pollen development in different species (Ramírez *et al.* 2003) and to study diploidization in microspore-derived embryogenesis (González-Melendi *et al*. 2005), its use is not always possible. This appears to be the case in the species studied in the present report. After long treatments with DAPI, even overnight at 4 °C, no staining was observed. Occasionally, at late stages of floral development one or two small, fluorescent foci could be observed. Earlier stages did not show any staining. Only the autofluorescence of the intricate exine of the pollen grain of *Opuntia* was clearly recognised (Fig. 2).

As an alternative to correlate the floral and male gametophyte development, the specimens can be embedded in a resin and sliced in semithin sections for light microscopy observations. There is a wide range of embedding protocols and media depending on the final use of the preparations and the resolution of the study, either light or electron microscopy. We chose a low-temperature embedding protocol that combines good structural and biochemical preservation of the specimens and can be used for light and electron microscopy observations.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← Fig. 1a-g: Sequential stages of floral development in *Opuntia ficus indica* according to Table 1. Flower buds. a: stage 1; b: stage 2; c: stage 3. Flowers with emerging sepals. d: stage 5; e: stage 6; f: stage 7. Open flowers. g: stage 8. h-s: Semithin sections across anthers from different floral stages. h: meiocytes at stages 1/2. Toluidine blue staining. i: tetrad at stages 3-4. Toluidine blue staining. Three microspores (asterisks) are clustered together by a wall of callose (c). The pattern of chromatin (chr) is deconensed. j: late vacuolate microspore at stage 5. Phase contrast. A large vacuole (v) pushes the cell nucleus towards the cell wall. The nucleolus (nu) shows a large vacuole of activity (clear area inside) that is a typical feature of cells in the G2 phase of the cell cycle. The exine (ex) shows an intricate pattern. k: young bicellular pollen grain right after pollen mitosis I (stage 5), showing the large vegetative (vc) and the small generative (gc) cells. Phase contrast. l: Bicellular pollen grain at stage 6 with the small generative cell (gc) engulfed within the vegetative cell (vc). Phase contrast. m: I_2KI staining on a consecutive section of the same pollen grain shown in l shows some starch deposition on small foci in the vegetative cytoplasm. n: Tricellular pollen grain at stage 7 showing the two sperm cells (spc) close together. Toluidine blue staining. o: Detail of a vegetative nucleus (vn) at stage 7, showing a decondensed pattern of chromatin (chr) and a moderately active nucleolus. Toluidine blue staining. p: I_2KI staining at stage 7 shows that starch accumulation spreads to the whole cytoplasm of the vegetative cell. Its reddish colour indicates a recent formation. q: Tricellular pollen grain at stage 8 Toluidine blue staining. r: Close up of the sperm cells (spc) located apart from each other in the vegetative cytoplasm. The nuclei of the sperm cells show some patches of condensed chromatin (arrowheads) and a compact nucleolus. s: I_2KI staining shows starch accumulation through the cytoplasm of the vegetative cell. Its dark colour indicates that starch deposition has been completed. Bars (h – s) = 1 μm.

This approach has been recently reported in *Capsicum* to compare the gametophytic development with the earliest events of microspore-derived embryogenesis (Bárány *et al.* 2005) and for correlative light and electron microscopy observation of the same specimens in *Hordeum vulgare* pro-embryos of different ploidy (González-Melendi *et al.* 2005). Although more time consuming, this method provides a more detailed information about the physiological state of the specimens than DAPI staining since not only the nuclei but also many cytological aspects of the cytoplasm can be observed.

Different phases of meiosis, up to the tetrad, were found in the first group of buds (stages 1-4, Fig. 1h-i). The nuclei of the microspores at the tetrad stage showed a highly decondensed pattern of chromatin after toluidine blue staining. However, in other species such as *Capsicum annuum*, large

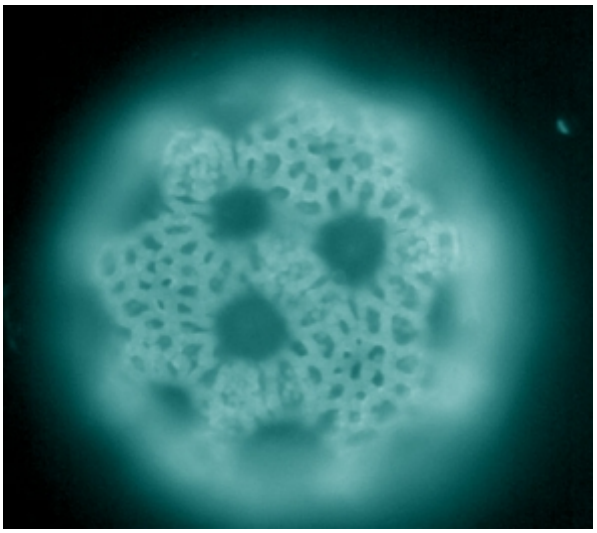

Fig. 2. Intricate pattern of the autofluorescent exine of a microspore (DAPI staining). Bar = 1 μm

patches of condensed chromatin can be found at this stage (González-Melendi *et al.* 2000). The free young microspore stage (after dissolution of the callose, which keeps the microspores clustered together in the tetrad) seems to be very short since it was not detected in our samples and might be found in the transition phase from stage 4 to 5, the latter being characterised by the vacuolate microspore (Fig. 1j) with a large vacuole in the cytoplasm that polarises the position of the nucleus. The chromatin is decondensed and the nucleolus shows an architecture (a big vacuole of activity in the centre of the nucleolar body) which announces the G2 phase of the cell cycle of the microspore (Risueño and Medina 1986). Then an asymmetric division (pollen mitosis I) occurs, which produces the unequal vegetative and generative cells of the bicellular pollen

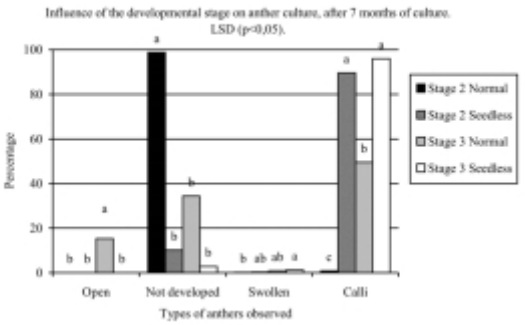

Fig. 3. Influence of developmental stage on *in vitro* anther culture. Within each anther type, values with different letters are significantly different at p≤0,05 (Fischer's LSD test

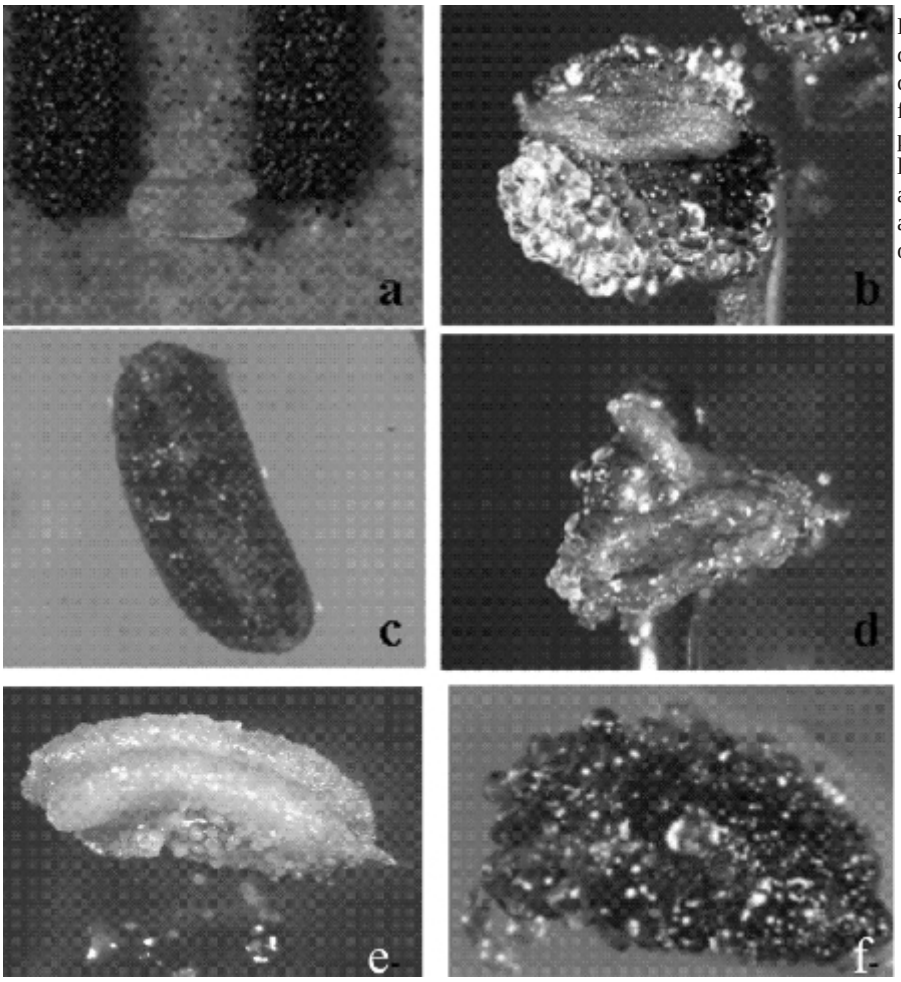

Fig. 4. Anther at the stage 2 before culture (a) and after 5 months of culture (b-f); (b) white callus formed by an anther of a fertile plant, (c) swollen anther, (d) yellowish-white callus formed by an anther of a fertile plant, (e) open anther, (f) red callus from an anther of a seedless plant.

grain (Fig. 1k). At stage 6, the generative cell detaches from the cell wall and moves into the cytoplasm of the vegetative cell (Fig. 1l). Starch accumulation starts in the vegetative cytoplasm at this developmental stage, as observed after I_2KI staining (Fig. 1m). Pollen mitosis II occurs at stage 7 (Fig. 1n-p). Two sperm cells, next to each other, can be observed surrounded by the vegetative cytoplasm (Fig. 1n). This is the tricellular pollen stage. When the sections are stained with toluidine blue, the vegetative nucleus shows highly decondensed chromatin (Fig. 1o). Starch deposition becomes more evident and abundant at this stage. The reddish colour indicates newly formed starch (Fig. 1p). In open flowers (stage 8) we found mature tricellular pollen grains (Fig. 1q) with highly abundant starch staining of dark colour (Fig. 1s), which indicates that starch accumulation had already ceased. The sperm cells are separated to each other. Their nuclei contained compact nucleoli and patches of condensed chromatin (Fig. 1r). This might explain the faint DAPI staining observed at this stage in our former trials. At earlier stages, the nuclei of the microspores and vegetative cells showed highly dispersed chromatin, which likely made the visualisation of DAPI staining difficult under the highly autofluorescent exine (Fig. 2).

In plants producing seedless fruits, pollen development was impaired. At floral stages corresponding to tricellular pollen according to the timing in seed-forming plants, further developmental phases than vacuolate microspores were not observed. Thus, no functional pollen would be available for fertilisation at anthesis. In such plants, fruit set could only be explained by parthenocarpy as reported to occur in this species (Weiss *et al.* 1993, Chessa and Nieddu 2002).

In vitro anther culture

A high percentage of anthers collected from flower buds of stage 2 in fertile plants did not undergo any modification (non-developed anthers). A very low percentage of swollen anthers (Figs. 3, 4c) was registered in the cultures of stages 2 and 3 of fruit-seeded and seedless plants. Anthers of stage 3 compared to those of stage 2 developed a significantly higher percentage of callus formation in both types of plants (49.5 % in fertile and 96.0 % in seedless ones). Anthers collected from seedless plants showed higher callus production (probably of somatic origin), compared with seed-forming

plants (89.6 % in stage 2 and 96 % in stage 3, Fig. 3).

The appearance of the calli obtained from anthers of the two plant types was quite different (Fig. 4). In fertile plants, calli were mainly yellowish-white (Figs. 4b, 4d) as previously reported by Pinheiro da Costa *et al.* (2001), who produced calli from *in vitro* germinated zygotic embryos of *Opuntia ficus-indica*. On the contrary, anthers collected from plants producing seedless fruits gave mainly red calli (Fig. 4f). About 15 % of stage 3 anthers from fertile plants showed opened lobes releasing the microspores onto the culture medium (referred to as 'open' in Fig. 3) (Fig. 4e).

Conclusions

The methodical approach applied in this study permitted a descriptive analysis of pollen development in a species which turned out not to be amenable to DAPI staining. By correlating floral with pollen development we were thus able to select appropriate starting material for anther cultures aiming to induce androgenesis. The detailed information provided in the present study was not available up to date. Resin-embedding of specimens and sectioning was compatible with cytochemical stains to visualise the subcellular organisation and the detection of components throughout pollen development. An additional advantage of this approach is that these samples can also be used for studies at higher resolution using the electron microscope.

The preliminary study on anther culture allowed the production of calli, however no regeneration was obtained thus far. Further experiments are in progress to characterize the calli obtained and to regenerate plants.

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