REVIEW

Pseudo-embryogenic Structures in Anther and Isolated Microspore Cultures *in vitro*: a Cautionary Guide

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Abstract: This review describes sources of structures of non-microspore origin observed in anther and microspore cultures. Various characteristics of these structures may cause a wrong diagnosis of these structures as embryos or cell/tissue clusters of microspore origin. Here we suggest such structures to be named as pseudo-embryogenic structures. The introduction of pseudo-embryogenic structures and their origins could be helpful to distinguish them from true microspore-derived structures. Prompted by certain environmental cues, somatic cells existing as a contamination in immature pollen (microspores) cultures can lead to the formation of 'pseudo-embryos' commonly known as embryoids. The pseudo-embryogenic structures may be classified in the following groups: (*i*) pseudo-star-like structures; pseudo-multicellular structures; (*ii*) pseudo-embryos with pseudo-suspensors; (*iii*) contaminating bacteria appearing as callus colonies; (*iv*) calli and embryos of somatic origin; (*v*) giant tetrad-like structures; (*vi*) anther wall cells. The exact origin of these structures is discussed in this paper, and some recommendations are proposed in order to avoid misinterpretation.

Keywords: anther culture; microspore embryogenesis; pseudo-embryogenic structures

Modern plant breeding strategies mainly depend on the commercial availability of homogeneous genetic material with guaranteed levels of identity. Doubled haploid (DH) plants provide an efficient short-cut for the production of homozygous plants (Forster *et al.* 2007). Haploidy refers to a plant having the gametic number of chromosomes in the sporophyte or plant phase. The chromosome number of haploid plants may be doubled spontaneously or artificially using anti-microtubular drugs, leading to doubled haploids (Touraev *et al.* 2001). The major advantage of doubled haploids in plant breeding is the immediate achievement of complete homozygosity upon chromosome

doubling which may be used in the fixation of desired genotypes in one generation. The discovery of doubled haploidy has changed plant breeding dramatically, and breeding programs of many globally important crops are nowadays mainly based on the use of this innovative and efficient technology (Forster *et al.* 2007).

DH plants can be obtained by several methods including wide hybridization followed by embryo rescue, parthenogenesis, gynogenesis or microspore embryogenesis (ME) in anther or isolated microspore cultures (Touraev *et al.* 2001; Sari *et al.* 2006; Bal & Abak 2007; Shariatpanahi & Touraev 2010). Microspore embryogenesis is the most developed and best documented approach to produce doubled haploids and can be induced either in excised and *in vitro* cultured anthers or in isolated microspore cultures. At first, the induction of ME requires a developmental reprogramming from the gametophytic (pollen) pathway into the sporophytic (embryo) pathway by applying various stress pretreatments (Shariatpanahi *et al.* 2006).

Embryogenic microspores formed after stress pretreatment are characterized by various physiological and morphological changes (Touraev *et al.* 2001; Shariatpanahi *et al.* 2006). In tobacco and wheat, following the induction, the nucleus moves to a central position surrounded by vacuole-like structures (Garrido *et al.* 1995; Touraev *et al.* 1996b). Borders of these vacuoles appear possibly as strings attached to the centrally located nucleus developing towards the edges of the microspore wall, termed as star-like by Touraev *et al.* (1996a, b). However, the formation of star-like microspores following the induction is not a common phenomenon but only observed in microspore cultures of some species, confirmed by cell tracking experiments in wheat, barley and tobacco (Indrianto *et al.* 2001; Maraschin *et al.* 2005). In other species, such as *Brassica*, swelling was found to be a sign of the embryogenic status of cultured microspores (Hause *et al.* 1993). To date, there are no clear cytological markers that could be used to cautiously identify embryogenic microspores, posing a problem particularly in poorly investigated species. Usually, when a new species is tested for its prospects in ME, the presence or absence of star-like structures as well as the swelling are examined at first. However, this strategy can result in misleading observations, especially when it is difficult to obtain clean microspore cultures without somatic cell debris. In anther cultures, on the other hand, somatic cells of the anther wall can enter dedifferentiation and divide, forming prominent multicellular structures that can easily be wrongly interpreted as microspore-derived structures.

In this review, we describe and give insight into the origin of commonly misinterpreted multicellular structures observed in microspore/anther cultures. These structures are of non-microspore origin and hereafter will be called "pseudo-embryogenic structures". This report will provide researchers with a practical and visual guide for better identification of true embryogenic microspores and true structures of microspore origin. Hitherto, we have determined a total of six different pseudo-embryogenic structures in seven subchapters with accounts provided below.

Pseudo-star-like structures

In isolated microspore cultures of *Mimulus aurantiacus* circular structures were observed at a high frequency (unpublished). These structures contained several compartments surrounding a circle in the centre (Figure 1b, d). They appeared somewhat similar to true star-like microspores of wheat (Figure 1a). When these structures were examined using FDA and DAPI stains for viability and status of the nucleus, respectively, they appeared multicellular. True microspore-derived multicellular structures (Figures 1c, e) are compared with pseudo-structures (Figures 1d, f). In the isolated microspore cultures of eggplant, sporophytically developing microspores are swelled and compartmentalized, containing several nuclei (Bal *et al.* 2009) similar to trichome head as shown in Figure1f.

In some cases, large starch granules may accumulate in microspores depending on the carbon source provided in the medium, leading to misinterpretation of these granules for multicellular structures. Indeed, in microspores or immature pollen grains of *Dipladenia*, starch grains resemble individual nuclei and the whole microspore appears multicellular (Figure 2). Detailed cytology of *Mimulus* anther and microspore cultures revealed clearly that the structures observed in *Mimulus* cultures did not originate from microspores but from trichome heads that are readily available on the styles of *Mimulus* flower buds.

Also in other species, most of the pseudo-embryogenic structures mainly originate from trichomes
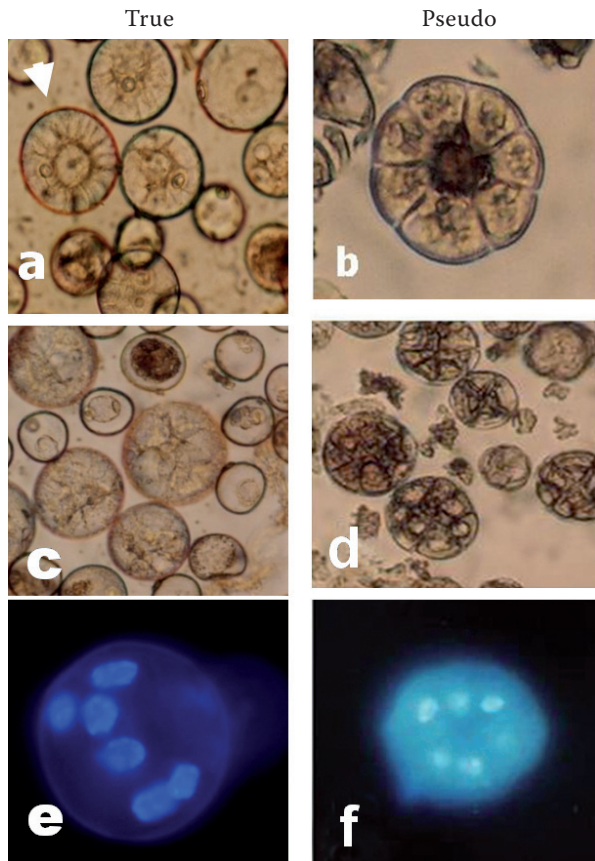
True   Pseudo

Figure 1. True star-like and multicellular structures in bread wheat (a, c), trichome heads appearing as star-like structures in *Mimulus* (b, d), DAPI staining of the trichome head with several nuclei in eggplant (f), true multinucleated structure in *Mimulus* (e)
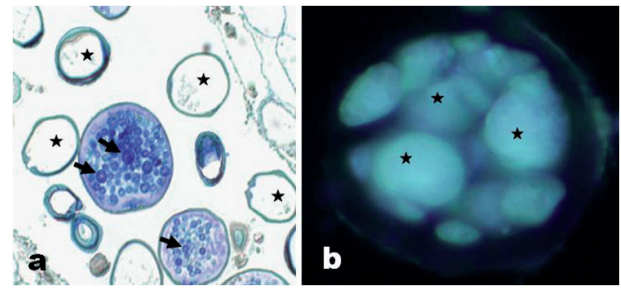

Figure 2. Microspores of *Dipladenia* (a) and *Mimulus* (b) appearing as multicellular due to starch grains stained in microspores in anther cultures. Starch granules (a, arrows), microspores without development (a, asterisks), starch grains resemble individual nuclei (b, asterisks)

(Figure 3). Trichomes, also called plant hairs, are a group of organized cells protruding from the epidermis with various shapes and functions, classified as either glandular hairs with a secretory function or non-glandular hairs. The latter are also named bulbous trichomes due to their morphology (Uphof 1962; Cutter 1978). Trichomes cover different parts of the flower bud in many plant species and may detach during microspore isolation, thus contaminating the microspore culture. This often occurs when the isolation of microspores is carried out by blending the whole buds instead of stirring the anthers. The broken trichome heads may not even be completely removed after Percoll gradient centrifugation (unpublished). The com-
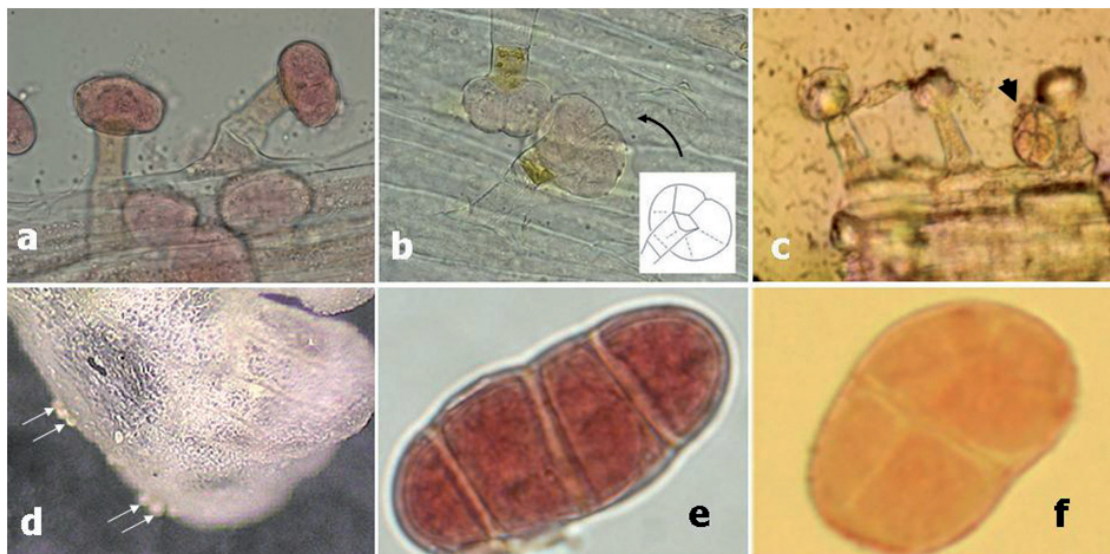

Figure 3. Bulbous trichomes in *Valeriana* (a, b) and *Solidago* (c) detected in microspore and anther cultures, respectively. Several trichomes are visible on the tissues of flower buds and anthers in *Dipladenia* (d, arrows; e) and *Valeriana* (f)

partmentalization within the circular structure is distinguishable in the trichome head (Figure 3b).

Contaminations with trichome heads may also occur in anther cultures. The microscopic analysis is usually carried out after a period of time that is adequate for the development of true microspore-derived embryos. At the time of microscopy the trichome heads may be mistakenly identified as microspore-derived. Pseudo-embryogenic structures are often morphologically similar to true microspore-derived embryos with their characteristic swelling, larger size and multicellular nature, illustrated in anther cultures of *Dipladenia* (Figures 3d, e) and *Valeriana* (Figure 3f). Sharma *et al.* (2003) reported an eight-celled head of a menthol mint trichome, which is one of the most common pseudo-embryogenic structures.

In cases where star-like structures occur, the star-like morphology becomes visible only following the incubation of microspores in culture medium for a certain period of time. However, pseudo-multicellular structures are visible immediately at the beginning of cultures. There may be occasions where cultures may not be observed thoroughly at the beginning of the culture. In such a case, caution is necessary at the time of data recording, and the researcher should be on alert against the presence of star-like structures in cultures. The structures observed may in fact be trichomes present since the beginning of the cultures. Hence the existence of structures with star-like appearance in fresh cultures can be considered as a sign of non-microspore origin. The comparative approach described above is also applicable in the identification of true, i.e. microspore-derived multicellular structures, and pseudo-multicellular structures.

Bulbous trichomes appearing as microspore-derived embryos bearing suspensors

In some other cases, bulbous trichomes contaminating anther and microspore cultures may be considered mistakenly as multicellular structures bearing suspensors (Figure 4). Usually, these trichomes are attached to somatic tissues with a tube-like extension at the multicellular trichome head (Figure 4b). During the isolation of anthers, the basis of the tube may be broken and disconnected from the somatic tissue, possibly leading to a misinterpretation as pro-embryos with suspensors. This mistake may be reinforced when several trichomes of different sizes are wrongly perceived as various stages of embryo development (Figure 4). True microspore-derived embryos bearing suspensors are shown in Figures 4a, c, e. It is interesting that also in a liquid culture, some of the bulbous trichomes, when observed through the inverted microscope, are positioned horizontally in the medium (Figure 4d). When stained with DAPI, the multicellular nature of the bulbous trichome head becomes apparent (Figure 4f).

In comparison with microspore cultures employing isolation with a blender, in anther cultures the frequencies of pseudo-star-like and pseudo-multicellular structures are expected to be low. In
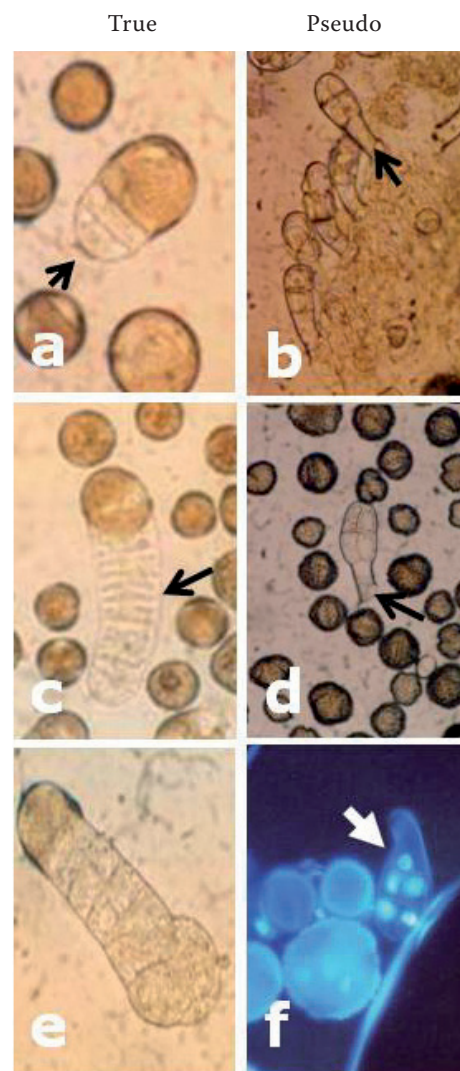

True   Pseudo

Figure 4. Trichomes attached to somatic tissues with a tube-like extension (b, arrow), broken trichome in the culture (d, arrow), broken trichome stained with DAPI (f, arrow), true microspore-derived embryos bearing suspensors in *Brassica napus* (a, c, e)
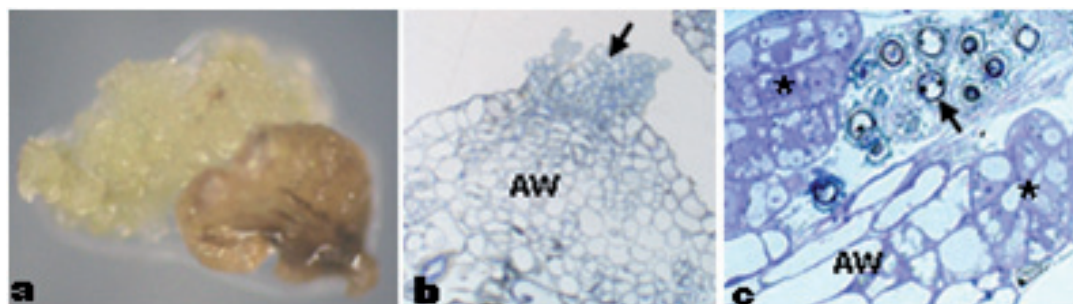
Figure 5. Somatic tissue-derived callus on the surface of cultured anther in *Mimulus* (a), callus development (arrow) from the diploid tissue of the anther wall (AW) in *Bougainvillea* (b), embryo-like structures (asterisks) in *Coreopsis* developed from the sporophytic tissues of the anther wall (c)

microspore cultures requiring blending of whole buds for microspore isolation, all trichomes in a bud are inevitably mixed with the microspore suspension, and removing them all will prove difficult. However, in anther cultures trichomes may be present at a low frequency because they originate only from sporadic contamination of trichomes during manipulation for anther excision. In this case, a small number of trichomes from various parts of a flower may attach to the anthers and remain there until well after inoculation of the anthers. Subsequently, when checking for the fate of microspores under the microscope, these few contaminating trichomes may be misleading and cause a wrong diagnosis. In a working ME protocol a considerable number of true microspore-derived structures develop. Therefore, embryo-like structures of low frequency in anther cultures can be considered as a sign of non-microspore origin.

Calli and embryos of somatic origin in cultured anthers and microspores

The formation of callus from somatic tissues of cultured anthers is a rather common phenomenon reported in many species (Lee *et al.* 1988; Gribaudo *et al.* 2004; Chen *et al.* 2005), and it remains a source of misleading conclusions on the origin of anther-derived plants (Figure 5a). Some of the anther wall cells, e.g. endothecium of connective tissue cells, dedifferentiate and enter multiple divisions, giving rise to calli (Figure 5b) or to embryo-like structures (Figure 5c) and even to diploid plants. Cytological sections of such anthers allow understanding precisely the origin of these calli and show their somatic origin. It should be noted that, generally, the favourable developmental conditions of such calli or embryo-like structures in anther cultures are not conducive to embryogenesis in microspores. Subsequently, with some protocols only somatic tissues were reported to have developed (Mauro *et al.* 1986; Chen *et al.* 2005). Occasionally, also microspores develop along with somatic cells into embryos, however at a low frequency and resulting in mixoploid regenerants (unpublished results).

The formation of calli/embryos from somatic tissues is not restricted to anther cultures but can be observed also in isolated microspore cultures. This may happen when a microspore suspension is not clean enough, i.e. it is mixed with somatic tissues. Usually, a pure population of microspores is prepared by filtering the isolate and using stepwise washing procedures, followed by Percoll gradient centrifugation to obtain even more homogeneous and clean microspore populations. However, this may be difficult to achieve due to inexperience in the handling of a particular species or very small buds, not allowing microspore isolation by any method except for the isolation with a blender. In the case of *Sanvitalia procumbens*, for example, the size of buds is small (3–4 mm in diameter) containing several florets of 0.5–1.0 mm in diameter. Hence, the isolation of microspores by stirring anthers is not feasible, and the blender isolation using whole buds remains the only alternative. However, this method does not allow the complete removal of somatic tissues. *Sanvitalia* somatic tissues did not show any propensity to develop and hitherto no development or regeneration was observed (Bal *et al.* 2006; Bal & Touraev 2008). However, in isolated microspore cultures of *Arabidopsis thaliana* somatic cell debris present in microspore cultures readily develops into callus colonies and embryos at various stages

(Figure 6b, d). It is therefore important that before concluding on the status of structures observed as microspore-derived embryos, the results should be repeated in highly homogeneous cultures making sure that no somatic tissues are mixed with the microspore suspension.

Establishment of pure cultures, i.e. without somatic debris or tissues, is essential in ME. In anther cultures, depending on both the species and hormonal balance, the frequency of structures regenerated from somatic cells of the anther wall may be higher. Origins of regenerants in anther culture should be carefully identified since embryos originating from somatic tissues may detach from the anther wall before their identification in culture. In some species such as grapevine, calli derived from somatic anther cells can be used to initiate embryogenic cultures (Mauro *et al.* 1986) for further genetic transformation. In such a case the identification of callus origin may be carried out using cytological or biochemical methods, such as isozyme analysis (Nurhidayah *et al.* 1996; Naess *et al.* 1998).

Bacteria appearing as callus colonies

Contamination agents such as bacteria in cultures may also be misinterpreted and considered to be of microspore origin (Figure 7). In microspore cultures of tomato (*Lycopersicon esculentum* Mill.)

structures similar to callus colonies were observed (Figures 7b, d). They appeared on the surface of a culture well as yellowish compact colonies of varying sizes (unpublished). However, detailed analysis identified them as bacterial colonies (Figure 7b). True microspore-derived embryos in wheat are shown in Figures 7a, c.

This case is of particular importance in species that are naturally colonized by endophytic microorganisms such as bacteria or even fungi. The first steps of development of these contaminants in cultures may thus be wrongly interpreted as microspore-derived callus colonies. It is then indispensable to use antibiotics in different steps of the process against the endophytes in the initial explants.

Giant tetrad-like structures

In isolated microspore cultures of tomato, in addition to microspores and multicellular structures, relatively large tetrad-like structures were observed (arrows in Figure 8a; Bal & Abak 2005). Initially it was believed that such cells may have been tetrads swelled under the conditions of that particular culture. Later, however, these structures were claimed to originate from trichomes that develop on the

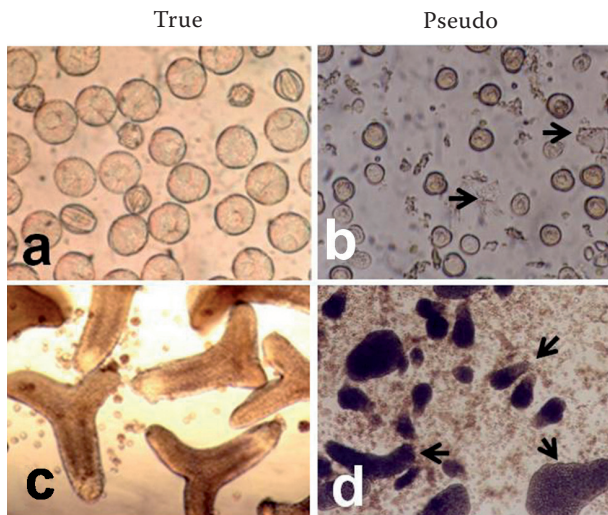True   Pseudo

Figure 6. Pseudo-embryos in *Arabidopsis thaliana* (b, d), somatic tissues mixed in the medium in a heterogeneous culture (b, arrows), true microspore-derived embryos in *Brassica napus* (a, c)

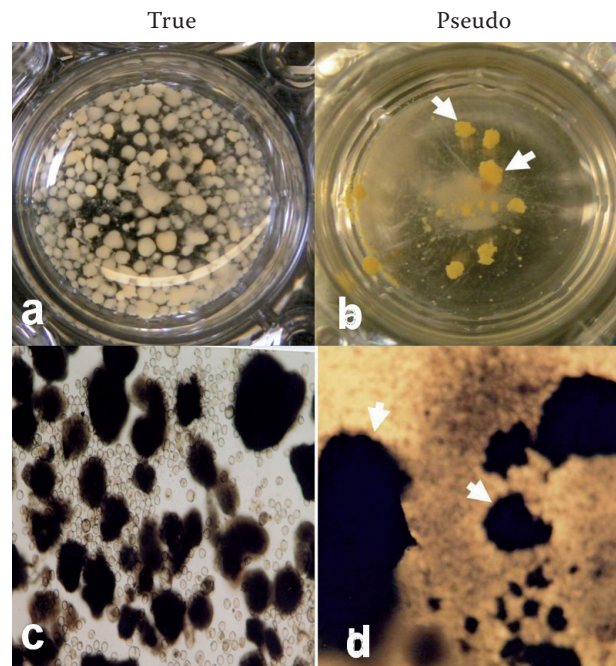True   Pseudo

Figure 7. Bacterial colonies in tomato microspore cultures (b, d), true embryos derived from microspores in bread wheat (a, c)
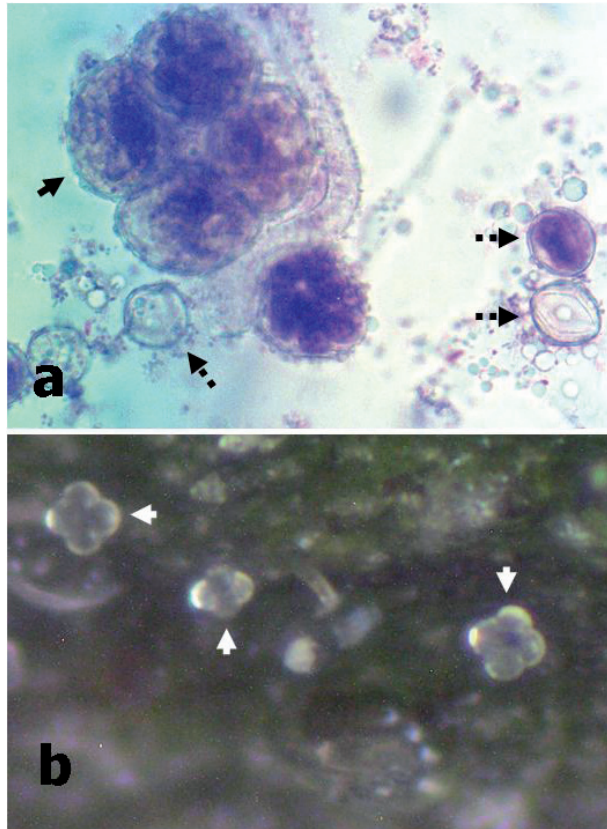
Figure 8. A giant tetrad-like structure originated from trichomes in isolated microspore culture of tomato (a, b, arrows)

surface of flower buds, leaves and green tissues of tomato (arrows in Figure 8b; Aragao *et al.* 2000). The microspore isolation in this particular occasion was carried out by macerating intact buds with a pestle, resulting in a mixture of trichome heads and isolated microspores in the culture. These structures, however, were not observed when microspores were isolated directly from anthers by stirring.

Pseudo-embryos derived from anther wall cells

The significance of using microspore culture was realized since sometimes in anther culture even the anther wall would also produce the callus resulting in pseudo-embryos and thus, tracing the origin of plants was so difficult (Jain *et al.* 1997). Certain inhibitors and toxic compounds that are released by degenerating the anther wall diffuse directly into the liquid solution rather than remaining localized around the anther in the agar medium. Float anther culture has been used in many spe-

cies (Tyagi *et al.* 1979; Xu *et al.* 1981; Chen & Chen 1983) mixing up true and pseudo embryos.

The response of anthers to elevated temperature suggests that a heat shock response may be involved (Lindquist 1986). It may disrupt the normal integrated development of the somatic anther tissue and subsequently it may synchronize the physiological states of the two tissues and thereby stimulate the induction process of true embryos (Dunwell *et al.* 1983).

Effects of other factors on anther culture

Results of some of the recent studies also reveal that the replacement of sucrose by maltose enhanced the success of anther culture (Trottier *et al.* 1993). Maltose as a sole carbon source in the culture medium also proved critical for the development of microspore-derived embryos in *Triticum aestivum* (Mejza *et al.* 1993). It was observed from the results of cultures of some species (e.g. rice) that sucrose is an essential component for the induction of callus in anther culture to pseudo-embryos (Sharmin & Bari 2004).

In anther or microspore cultures on hormone-free media, a strong selection pressure exists which allows further growth of true embryos only (Aionesei *et al.* 2005). However, according to some researches, in an optimal plant growth regulator combination, many dividing microspores evolve into true embryoids that have very high germination rates. Additionally, the inclusion of plant growth regulator (PGR) such as abscisic acid (ABA) (Hu *et al.* 1995), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 6-benzylaminopurine (BAP) (Liu *et al.* 2002) in the pretreatment phase is also beneficial for embryogenic induction.

Furthermore, when ovaries were added to wheat microspore cultures, the number of pre-embryoids, embryoids and regenerated plants increased significantly (Hu & Kasha 1997; Zheng *et al.* 2001, 2002). Without ovaries, the frequency of multicellular structure was reduced (Liu *et al.* 2002). So it can be concluded that the replacement of PGRs by ovaries increased the number of true embryos.

CONCLUSIONS

The data presented in this review were acquired by research on anther and microspore cultures of

many different species over the years mainly in the authors' laboratories. Numerous papers dealing with anther and microspore cultures unintentionally but mistakenly reported earlier on structures of non-microspore origin similar to those described here. To avoid that, and correctly interpret the origin of structures observed through the microscope, the above-mentioned points should be taken into account when microspore and anther cultures are evaluated.

A useful method commonly used for the final control in a haploid/doubled haploid production technology is a detection of the haploid status of produced embryos/plantlets (before induced or spontaneous diploidization) by flow cytometry or molecular markers. More recently, isozyme native polyacrylamide gel electrophoresis has been used as an efficient technique for the identification of haploids/doubled haploids, and also for determination of the frequency of spontaneous diploidization of regenerated plants of microspore origin (Smykalova *et al.* 2012).

It is necessary to have comprehensive knowledge of the morphology and cytology of *in vivo* developing pollen under the microscope. This helps to avoid the confusion of microspores/pollen grains with other structures. Reviewed papers sometimes report on multicellular structures with two or three cells which are in fact bi-cellular or three-cellular pollen grains. Such mistakes can easily be avoided by comparing the *in vitro* microspore-derived structures with pollen developed *in vivo/in vitro*.

In addition to the accounts of pseudo-embryogenic structures presented here, it is possible that there are other cases encountered by others. Therefore, researchers must be prepared for additional misleading situations. In order to avoid a wrong diagnosis, a good practice in ME research is of utmost importance.

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