ANDROGENESIS
IN RECALCITRANT SOLANACEOUS CROPS

José M. Seguí-Simarro*, Patricia Corral-Martínez, Verónica Parra-Vega and Beatriz González-García.

COMAV - Universidad Politécnica de Valencia. CPI, Edificio 8E - Escalera I, Camino de Vera, s/n, 46022. Valencia, Spain.

* Corresponding author
e-mail: seguisim@btc.upv.es. Tel. +34 96 387 7000 ext 88472. Fax +34 96 387 9422

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Abstract

Tomato, eggplant and pepper are three solanaceous crops of outstanding importance worldwide. Thus, a fast and cheap method to produce pure (homozygous) lines is a priority for breeders. Traditionally, pure lines are produced by classical inbreeding and selection techniques, which are time consuming (several years) and costly. Alternatively, it has become possible to accelerate the production of homozygous lines through a biotechnological approach: the induction of androgenesis to generate doubled haploid (homozygous) plants. This biotechnological *in vitro* tool reduces the process to only one generation, which implies important time and costs savings. These facts make androgenic doubled haploids the choice in a number of important crops where the methodology is well set up. Unfortunately, recalcitrant solanaceous crops such as tomato, eggplant and pepper are still far from an efficient and reliable technology to be applied to different genotypes in breeding programs on a routine basis. In eggplant and pepper, only anther cultures are known to work relatively well. Unfortunately, a more efficient and promising technique, the culture of isolated microspores, is not sufficiently developed yet. In tomato, none of these methods is available. However, recent advances in the knowledge of zygotic and androgenic embryo development are filling the gaps and opening new ways to achieve the final goal of an efficient protocol in these three recalcitrant species. In this review, we outline the state of the art on androgenic induction in tomato, eggplant and pepper, and postulate new experimental ways in order to overcome current limitations.

Introduction

It is well known that crop productivity can be increased through the use of hybrids, made by crossing homozygous (pure) lines with defined traits. Pure lines are traditionally generated by techniques based on classical breeding, through successive rounds of selfing and selection. This requires a considerable amount of time and resources. However, in recent years alternative biotechnological approaches, by far more efficient and sustainable than traditional methods, are being used in some species. These techniques, based on androgenesis (Seguí-Simarro 2010), produce pure, doubled haploid (DH) lines through the *in vitro* regeneration of plants from microspore/pollen-derived embryos or calli. In this biotechnological pathway, alternative to normal pollen development, microspores or pollen grains are *in vitro* deviated from the gametophytic pathway and induced to form haploid embryos or calli, either directly in the culture medium, or within the anther. *In vitro* androgenesis can be induced in several angiosperms, both monocots and dicots (see Dunwell 2010 for a recent review). Through *in vitro* androgenesis, plants can be directly regenerated by microspore-derived, haploid embryogenesis, or indirectly from an intermediate haploid callus phase. These plants will be either DH -if they duplicate their original haploid genome- or just haploid as the original microspore. In this latter case, additional treatments to promote genome doubling would be needed (Seguí-Simarro and Nuez 2008a). In both cases, the resulting plants will be 100% homozygous, having a genetic background exclusively coming from the male spores of the donor plant. In other words, they will be pure lines.

This biotechnological approach to doubled haploidy has a great number of important advantages, for both basic and applied research (reviewed in Dunwell 2010). Among them, the most important is the dramatic reduction in time and costs needed for the production of pure lines for breeding purposes. This has been the reason that boosted the search for reliable protocols to induce microspore embryogenesis in species of agronomic interest. In 2003, protocols for DH production were already available for more than 250 crops, from herbaceous such as wheat, barley, rice, rapeseed (canola), tobacco or maize (reviewed in Maluszynski et al. 2003) to trees such as mandarin, bitter orange or cork, among others (reviewed in Srivastava and Chaturvedi 2008). Nowadays, this number will surely be higher. However, except for model species such as rapeseed,

barley or tobacco, the efficiency in obtaining DHs is still very low (Palmer and Keller 2005). This is even more critical in horticultural crops of high agronomic interest like those belonging to the Solanaceae family.

The Solanaceae family of flowering plants comprises between 3,000 and 4,000 species in about 90 genera (Knapp et al. 2004). This family is one of the most important in terms of agricultural interest, and includes five major cultivated crop plants, namely potato (*Solanum tuberosum*), pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*). The Solanaceae are a typical example of an ethnobotanical family, meaning that it is extensively exploited and utilized by humans since the beginning of the agricultural age. It is one of the most important sources of food and spices, mostly from these agricultural crops. Despite the tremendous importance of this family for world's agriculture, DH technology is not yet efficiently implemented in some of these interesting crops. Curiously, the first observation of *in vitro*, microspore-derived androgenesis was reported in a solanaceous plant by Guha and Maheshwari (1964), who described the formation of pollen-derived plants within anthers of *Datura innoxia*. Among the five major solanaceous crops (pepper, tobacco, potato, eggplant and tomato), only in tobacco enough progress has been made to consider this species as a model system for the study of microspore embryogenesis. At present, refined protocols have been developed to produce DHs from tobacco anther and isolated microspore cultures on a routine basis and with an acceptable efficiency (reviewed in Belogradova et al. 2009). Potato cannot be considered as a model system, but methods to induce haploids, from both anther and microspore culture, are already available (reviewed in Rokka 2009).

The rest of interesting solanaceous crops (tomato, eggplant and pepper) are still considered recalcitrant. Although in some cases DHs have been obtained, we are still far from the efficiency achieved in tobacco. Despite of the genetic proximity of these solanaceae, they seem fairly distant in terms of androgenic response. Up to now, only anther culture seems to work in eggplant or pepper. In tomato no efficient protocols are available. In this work we revise the current knowledge on the experimental induction of androgenesis through anther and isolated microspore cultures in tomato, eggplant and

pepper, highlighting the different experimental approaches and their most significant improvements. Finally, we outline new hypotheses about potential solutions to overcome the principal bottlenecks currently detected in these three agronomically interesting crops.

Androgenic DHs in tomato

Tomato is the first vegetable crop worldwide, both in terms of production (129,942,416 Tm in 2007) and cultivated area (4,643,957 Ha in 2007). Among the 155 different crops analyzed in the 2007 FAOSTAT database (FAOSTAT 2009), tomato ranks 12th in production and 38th in area harvested. These data give an idea of the tremendous importance of tomato for global agriculture. Despite of it, little is known in the tomato DH field, where no reliable and standardized methods are available so far. Over the past 40 years, many inductive and culture conditions have been assessed (reviewed in Bal and Abak 2007). However, throughout these years only two laboratories have published the complete regeneration of anther-derived tomato plants (Figure 1) with a demonstrated haploid or DH origin (Shtereva et al. 1998; Zagorska et al. 1998; Seguí-Simarro and Nuez 2005, 2007). These regenerants showed a degree of morphological variability ranging from high (Zagorska et al. 2004) to low (Seguí-Simarro and Nuez 2007). In all cases, mixoploidy and a low general efficiency was observed. It is obvious that tomato is extremely recalcitrant, and a much more in-depth study of the factors involved in tomato androgenesis is still essential to obtain successful results.

The most critical of these factors seems to be the genotype and the developmental stage. As usual in most of the androgenic systems known to date, the genotype plays an important role. In particular, male-sterile mutant lines have been shown to be especially sensitive to induction (Zamir et al. 1980; Zagorska et al. 1998; Seguí-Simarro and Nuez 2007). It is noteworthy that male-sterile phenotypes are usually manifested at the late meiocyte stage, which in most cases overlap with the inductive window of tomato anther cultures. Since the first published works, the identification of the right stage to induce the tomato gametophyte has also been a matter

of debate. Indeed, until a few years ago, the most suitable developmental stage was not clear. Already in 1978, (Dao and Shamina 1978) published the generation of callus through the culture of anthers with microspores at the tetrad stage, whereas anthers containing late, vacuolate microspores or young bicellular pollen directly produced embryoids. This work suggested that in tomato different stages may be responsive under different treatments. In recent years, three studies were published reinforcing this hypothesis. First, (Seguí-Simarro and Nuez 2005) narrowed the best stage for anther culture to the meiocyte, just before compartmentalization of the tetrad. This developmental window implies that recombination must be successfully finished without disruption, but microspore formation (tetrad walling) has to be prevented. Second, the formation of multicellular structures from isolated unicellular microspores was described upon exposure to starvation, cold and colchicine (Bal and Abak 2005). Third, in 2007 it was finally demonstrated that in tomato, androgenesis can be induced at two different stages (Seguí-Simarro and Nuez 2007), although with greatly different results and implications, as follows.

On the one hand, this study confirmed that it is possible to induce androgenesis from meiocytes as long as they are *in vitro* cultured within the anthers, as previously shown (Shtereva et al. 1998; Seguí-Simarro and Nuez 2005). This way, haploids, DHs, but also mixoploids can be obtained from meiocytes. After induction, meiocyte-derived calli may arise (1) from haploid cells still enclosed within the tetrad, which stop their gametophytic program and start proliferation, or (2) from meiocyte-derived diploid cells, coming from the fusion of two haploid nuclei separated by defective, incomplete, or absent post-meiotic cell walls. In the first alternative, callus is originated from a haploid meiotic cell (a potential microspore), which undergoes proliferation. Thus, one could argue that microspores are the true callus precursors. Nevertheless, it has to be remarked that induction must take place prior to microspore formation, i.e. tetrad compartmentalization. Then, haploid callus cells duplicate their genome by nuclear fusion, giving rise to true DH cells which may regenerate a DH plant (Seguí-Simarro and Nuez 2007). The second process implies the disturbance of normal mechanisms of post-meiotic cytokinesis during tetrad walling, caused by the stressing conditions applied to the anther culture. Defective compartmentalization of haploid nuclei favors their coalescence and their eventual fusion. As opposed to the first alternative, this

fusion would not give rise to a DH, since fusion of two reduced meiotic cells generates new allele combinations not necessarily homozygous. We have recently confirmed this notion by showing additional genetic evidences of nuclear fusion (Corral-Martínez et al, manuscript under review). We obtained diploid meiocyte-derived calli from anthers of tomato hybrids heterozygous for 4 independent microsatellite (SSR) molecular markers and three linked morphological markers. Among these calli, nearly 10% showed heterozygosity for some of the markers, but not for all. Partial heterozygosity excluded both a haploid and a somatic origin. In turn, this reinforced the notion of haploid nuclear fusion as the source of a part of the observed non-DH regenerants.

The fact that meiocytes are cultured within their anthers makes also possible the occasional appearance of somatic calli and regenerants, coming from connective or filament tissues of the anther walls. Indeed, filament tissues typically exhibit a high proliferative response when cultured *in vitro* (Seguí-Simarro and Nuez 2006), and it is believed that tomato anther tissues at meiotic stages are more sensitive to tissue culture than those of older stages (Bal and Abak 2007). We have recently demonstrated that up to 83% of the total of calli produced are originated from the stubs that emerge from the interlocular septa separating the two pollen sacs of a theca (Corral-Martínez et al, manuscript under review). These undesirable phenomena, inherent to this technology, represent a major problem when it comes to translating this experimental methodology to production technology, because it requires the genetic evaluation of every single regenerant to determine their origin and ploidy. This makes the process costly and slows down the final result.

On the other hand, it has also been shown that when microspores at the vacuolate stage are isolated and grown in liquid medium, it is possible to induce proliferative divisions in these microspores, generating embryo-like structures (Seguí-Simarro and Nuez 2007). This alternative eliminates the influence of somatic tissues, thus avoiding the problems mentioned above. Unfortunately, up to now very few genotypes have been evaluated for this method, and in the very scarce positive results obtained, it has not been possible to promote growth beyond the first divisions of the embryogenic microspore (Seguí-Simarro and Nuez 2007). Our current belief is that culture conditions are not well suited to promote embryo development in an *in vitro*

environment, devoid of the protective and nutritive role of the zygotic endosperm. This is not surprising since embryos rescued from interspecific or intergeneric crosses frequently abort at different developmental stages due to disturbances in endosperm development (reviewed in Bhatia et al. 2004). An alternative possibility would be the unmasking of lethal genes in the dividing haploid cells, which would preclude further development.

In summary, at present it is difficult to envisage an efficient method to obtain DHs in tomato on a routine basis. DHs have been proven possible in tomato through anther culture, although the low efficiency inherent to the method, the difficulty to improve it, and the presence of a number of collateral, undesirable products make this option not recommendable for practical purposes. On the other hand, the culture of isolated microspores seems feasible, but there is still a long way to explore. Recent studies have opened a hopeful way to the long-awaited androgenic DH through the induction of isolated microspores. However, this topic is still largely at its infancy, and much more efforts must be devoted at both the induction stage and the embryo growth and differentiation stages.

Androgenic DHs in eggplant

Eggplant (*Solanum melongena* L.) is also among the most important vegetables worldwide. In terms of production and area harvested, eggplant was in 2007 in the 6th and 7th place in the rank of vegetable crops with 32,154,900 Tm and 1,842,331 Ha, respectively (FAOSTAT 2009). Eggplant appears to respond to the induction of androgenesis better than tomato. In fact, through anther culture it is possible to induce the deviation of the eggplant microspore to transform into a haploid or DH embryo and eventually a plant (Figure 2). The first report of plant regeneration from anther cultures dates from 1973 (Raina and Iyer 1973). Upon treatment with colchicine, they were able to induce callus proliferation and plant regeneration from anther-derived calli. However, according to the author's own interpretation, a somatic origin for the calli was likely, being produced from connective tissue. By the end of this decade, other reports published the production of haploid eggplant individuals (Isouard et al. 1979). Misra et

al. (1983) reported in 1983 the induction of callus derived from microsporogenous tissue, being able to regenerate shoots and roots. However, it was in 1982 when the work of Robert Dumas de Vaulx and Daniel Chambonnet established the basis for a general, reliable and reproducible protocol for haploid embryo production and DH plant regeneration from eggplant anther cultures (Dumas de Vaulx and Chambonnet 1982). In their original method, incubation of anthers at 35ºC during eight days in darkness and then at 25ºC in a medium containing 2,4-D and kinetin was proposed to promote the development of MDEs within the cultured anthers. Later on, it was shown that androgenesis in eggplant is dependent on the genotype of the donor plant and the developmental stage of the microspore, as well as on culture conditions, including temperature, type and concentration of growth regulators (Rotino 1996; Rotino et al. 2005). Genetic variability was also observed in plants regenerated from anther cultures (Rotino et al. 1991). These results confirmed that in eggplant anther cultures, microspore embryogenesis typically proceeds as in other better studied species. Consequently, it was applied to plant breeding. At present, the Dumas de Vaulx method is the basis of many protocols, now routine methods used in breeding programs for the production of pure DH lines, adapted to particular cultivars (Figure 2; Sanguineti et al. 1990; Rotino 1996; Rizza et al. 2002; Rotino et al. 2005; http://www.scri.sari.ac.uk/assoc/COST851/Default.htm).

As seen, eggplant embryos can be successfully induced from microspores cultured within the anther. However, this method does not exclude the occasional appearance of somatic embryos from anther tissues, as demonstrated for tomato, and also suggested for some of the pioneering works in eggplant. In addition, we must take into account the uncontrollable secretory effect of the tapetal layer that surrounds the pollen sac, which prevent from having a strict control of culture conditions. In addition, anther cultures have a limited efficiency, producing only a few embryos per cultured anther. These limitations can be overcome by the direct isolation and culture of microspores. In those species where isolated microspore cultures are well set up, it is possible to obtain hundreds or even potentially thousands of embryos from the microspores contained in a single anther. As in tomato, the development of a method for androgenesis induction from isolated eggplant microspores would be highly desirable, and would avoid the problems mentioned above of tapetum effect, appearance of

somatic regenerants, and low efficiency. In this regard, it is striking that very few studies have been published on the successful production of DH plants from isolated microspores (Miyoshi 1996; Corral-Martínez et al. 2008; Bal et al. 2009). In the studies of Miyoshi, plantlets were obtained from microspore-derived calli, with an efficiency of 20-65 calli/anther and 0.001-0.02 plants/calli, better than anther cultures according to the author. More recently, by combining a 33ºC heat shock with mannitol treatments Bal et al. (2009) were able to induce divisions in isolated eggplant microspores, although no plant regeneration was reported, neither from callus nor from embryos.

At present, our group is developing a method (Figure 3) to produce eggplant DHs from isolated microspores more efficiently (Corral-Martínez et al. 2008; Corral-Martínez et al., in preparation). In this case, plants apparently come from calli, as in the Miyoshi method. However, after careful examination of the early stages of microspore proliferation, it seems that embryogenic microspores (Figure 3A) develop as true MDEs up to the globular embryo stage (Figure 3B). Instead of experiencing the radial-to-bilateral symmetry transition typical of zygotic embryos, globular MDEs revert to an undifferentiated state as callus-like structures (Figure 3C). Nevertheless, callus-derived haploid and DH plants can be regenerated through organogenesis (Figures 3D, E). These results are extending the still scarce knowledge on eggplant microspore cultures. In addition, they clearly point to deficiencies in the composition of the post-inductive culture medium as responsible for this abnormal androgenic pattern. The future study and refinement of the experimental protocol to promote a normal MDE development and maturation will surely contribute to the definition of a true embryogenic pathway as demonstrated for androgenic model systems.

Androgenic DHs in pepper

Peppers (the genus *Capsicum*) consists of approximately 20–27 species, five of which are domesticated: *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens* (Pickersgill 1997). Some of them are used in fresh as vegetables, and others are dried and used as spices. As to the response to androgenesis induction, pepper is the third solanaceous crop that could be defined as recalcitrant. The first reports on the

production of haploid *Capsicum* plants by anther culture involved the use of Asian varieties (George and Narayanaswamy 1973; Kuo et al. 1973). A year after, androgenesis induction in European varieties was reported (Novak 1974). Thereafter a number of cultivars, mostly of *C. annuum*, and interspecific crosses have been tested for responsiveness to anther culture (Sibi et al. 1979; Morrison et al. 1986; Kristiansen and Andersen 1993; Mityko et al. 1995; Nowaczyk et al. 2006; Gémes Juhász et al. 2009). Pepper anther cultures have been used as a tool for basic studies, but their most important application has been their use in breeding programs, finally aimed to produce commercial hybrids with maximum heterosis and new or improved traits.

The first pepper anther cultures produced calli, from which plants were regenerated through organogenesis. Further refinement of the experimental procedures allowed the group of Dumas de Vaulx for the direct induction of embryogenesis (Figure 4) without an intervening callus phase, and for a significant improvement in the efficiency of the process of DH production (Sibi et al. 1979; Dumas de Vaulx et al. 1981). Basically, the refinement consisted on the use of a high temperature treatment to induce microspore divisions, and the use of two different medium compositions and growth conditions for induction and embryo development. As in the case of eggplant, the work of the group of Dumas de Vaulx established the basis for a general, reliable method for anther culture in pepper, which can be applied with slight modifications to many different pepper varieties. A further improvement of the culture conditions consisted on the addition of activated charcoal to the culture medium to adsorb toxic compounds. In 1997, Dolcet-Sanjuan et al. published a method for embryo induction in a single step, with a biphasic medium consisting of a liquid medium phase poured over a semi-solid, agar-based phase with activated charcoal. This method was based on other previously published (Morrison et al. 1986), but implemented with a CO_2 atmosphere. This method allowed for the production of embryos of genotypes that did not respond to the method of Dumas de Vaulx, and increased the efficiency of other, poorly responding genotypes. However, the technical difficulty of having a growth chamber with a CO_2 supply has precluded many laboratories from adopting the Dolcet-Sanjuan method on a routine basis. At present, the Dumas de Vaulx is the most used routine method. Nowadays, particular adaptations of this general protocol are still being used by hybrid seed companies worldwide to generate pepper DHs for breeding purposes.

Despite of its popularity and simplicity, pepper anther cultures present the same problems described above for eggplant (uncontrolled tapetum secretion, the possibility of somatic regenerants and low efficiency), so it is also desirable to develop a protocol for obtaining pepper DH through isolated microspore culture. This is why several groups have explored this pathway in the last decades, describing the formation of early embryos. However, in most cases embryo development has not been possible beyond the globular stage (reviewed in Regner 1996). It was in 2006 when Supena et al. (2006a; 2006b) were able to regenerate haploid plants from microspores isolated from the anther by a non-mechanical method. This method (named the shed-microspore method) involved a biphasic (liquid-solid) medium where the inoculated anthers float on the liquid phase and, upon dehiscence, open and pour their microspore contents. Embryogenic microspores sink and accumulate at the liquid-solid interphase. Thus, microspores are isolated from the anther and allowed to form viable embryos out of the direct influence of the anther. This elegant method was demonstrated to work in 10 different varieties of Indonesian hot pepper. Although efficiencies were variable, in general this method outperformed the previously described culture techniques. Although promising, this method is still recent, and has been only proven as efficient for Asian pepper types. It would be interesting to study this method in other, European sweet types.

A different approach to microspore culture is the direct, mechanical isolation and culture in liquid medium. Using this method, Supena et al. (2006a) reported a high rate of microspore induction in the same Indonesian hot types evaluated for the shed-microspore method, but a very low percentage of them transformed into embryos, with only a maximum of 0.1 plants regenerated per bud. More recently, Kim et al. (2008) assessed a different protocol for direct culture of isolated microspores of hot pepper types with an elevated efficiency in terms of embryo production, but not of embryo quality. According to this study, a pretreatment of microspores at 32ºC in sucrose-free medium, the election of sucrose as both an osmoticum and a carbon source for embryo growth, and the use of an optimal microspore plating density are crucial parameters in order to obtain an acceptable response in terms of quality of embryos, capable to undergo germination. In 2009, Gémes Juhász et al. tested over 2000 genotypes,

including hot, spice and sweet types. They proposed that the addition of maltose during the induction phase and the co-culture of pepper embryos with wheat ovaries may have a positive effect in the quantity and quality of embryos produced through microspore culture. The experience of our group (Figure 5; Parra-Vega et al. 2010), working with sweet pepper types (Lamuyo, California and sweet Italian) confirms most of the conclusions deducted from these studies. In sweet genotypes, it is possible to induce the development of pepper microspores (Figure 5A) through the different embryogenic stages (Figures 5B-E) and finally regenerate plantlets (Figure 5F). However, many of the embryos produced show morphological abnormalities, ranging from mild to severe. Besides, some of them assume an undifferentiated, callus-like growth, similar to that described for eggplant (data not shown). It would therefore be necessary a deeper knowledge of the requirements of these embryos, especially in stages beyond globular, when tissue differentiation starts in parallel to embryo elongation and maturation.

In summary, at present there are four main types of protocols shown to experimentally induce microspore embryogenesis in pepper: (1) the Dumas de Vaulx method, (2) the biphasic method of Dolcet-Sanjuan, (3) the shed-microspore method, and (4) the direct microspore isolation method. The four have proven useful in obtaining DH plants. However, not all of them are in principle applicable to all genotypes. In fact, the genotype of the donor plant is, as in many other species, one of the most decisive factors. In some cases, the optimization of growth conditions for a given genotype is not sufficient to overcome the barriers imposed by the genotype itself (Kristiansen and Andersen 1993). However, the possibility of applying different types of inductive protocols allows for the choice of the most convenient for each variety. As an example, the variety ‘Piquillo’ shows a null response to the method of Dumas de Vaulx (Mityko et al. 1995), and a positive response to the biphasic method of Dolcet-Sanjuan et al. (1997). Therefore, before starting a breeding program based on DH production it is advisable to assess the response of each variety to the different protocols available.

Towards the improvement of microspore embryogenesis in tomato, eggplant and pepper

In view of the progress in the last four decades, we can conclude that androgenic DHs in recalcitrant solanaceae as tomato, eggplant and pepper are possible, but not easy. The DH technology is sufficiently developed in eggplant and pepper to produce DHs through anther culture in a number of genotypes. However, this approach implies several limitations, including a generally moderate efficiency. In tomato, anther culture has also been demonstrated possible, but for very few genotypes. In addition to the general problems of anther culture, the unavoidable limitations inherent to the nature of the inducible stage make this approach barely applicable in practice.

It seems also possible to obtain DHs through isolated microspore culture. Although more promising than anther culture, this way still appears difficult and needs major improvements to become a routine procedure. In our opinion, two key bottlenecks impose strong limitations to the efficiency of this technique in recalcitrant solanaceous crops: induction efficiency and embryo progression. As to the first bottleneck, it is widely known that the genotype is the most influential factor for the androgenic response and further embryo development (Seguí-Simarro and Nuez 2008b; Dunwell 2010; Seguí-Simarro 2010). In eggplant and pepper, this is also true, but to a lesser extent than in tomato. In tomato, the recalcitrancy inherent to the genotype remains a major problem. The androgenic potential of a significant number of genotypes has been evaluated by anther culture (see Zagorska et al. 1998; Seguí-Simarro and Nuez 2007 for some examples). Just 14 have been evaluated for microspore culture (Bal and Abak 2005; Seguí-Simarro and Nuez 2007). From them, only 5 responded, with very few microspores dividing symmetrically. Thus, the absence of a minimally responding genotype is the main limitation to develop a protocol for microspore culture in tomato. This species has experienced three major bottlenecks in the process of domestication (Rick 1976) that led to the current situation where all of the varieties of cultivated tomatoes share a very narrow genetic background (Foolad 2007). In fact, it is estimated that cultivated tomato represents only 5% of the total genetic variability that can be found in all species of tomato, including wild relatives (Miller and Tanksley 1990). Thus, it would not be surprising to observe a similar androgenic response (very low, if any) in the rest of tomato cultivars not assessed so far. On the other hand, it would be very interesting to assess other genotypes related to

cultivated tomato (tomato wild relatives), but with a much broader genetic background, in which the probability of finding favorable gene combinations may be higher. This could be a promising research line for future investigations in tomato.

Embryo progression is the second major drawback. It seems that for most of the published methods, efficiency is significantly lost during the transition of a proliferating, yet undifferentiated globular embryo into a heart-shaped, bilateral embryo. The presence of anatomical and functional abnormalities, ranging from mild to severe, seems to be a generalized problem evidenced in most of the known androgenic systems. It is remarkably frequent in eggplant and pepper MDEs derived from anther and isolated microspore cultures. Compared to zygotic embryos (Figures 6A, B), these MDEs exhibit stunted or even absent cotyledons (Figures 6C, D), evidencing serious deficiencies in lateral expansion (Supena et al. 2006b; Kim et al. 2008; Parra-Vega et al. 2010). But the most dramatic abnormality is the lack of a functional, zygotic-like shoot apical meristem (SAM), which precludes further germination of these embryos. In eggplant microspore cultures, disruption of the normal embryogenic process occurs earlier, with the formation of calli instead of embryos (according to Miyoshi 1996), or the deviation of globular embryos towards callus-like proliferation (Figure 3; Corral-Martínez et al. 2008). In tomato microspore cultures, problems appear even before, since the proembryo is arrested after the first proliferative divisions (Seguí-Simarro and Nuez 2007). In view of these results, it would be advisable to devote more efforts to the knowledge of the particular requirements of embryo development, in order to facilitate their transition towards a mature embryo.

Experiments with model systems for microspore culture have revealed a number of factors directly or indirectly involved in the development of MDEs. Many of them have in common their presence, with an important role, during zygotic embryogenesis, and their synthesis and/or regulation by the endosperm or other ovule/seed tissues. It is reasonable to think that in embryogenic *in vitro* systems devoid of any maternal tissue, these factors, either absent or poorly regulated, may be behind some of the observed deviations. In recent years, the group of Claudio Stasolla has made significant contributions to the knowledge of the factors involved on proper SAM establishment and development. For example, in 2010 they demonstrated the role of the

SHOOTMERISTEMLESS (*STM*) gene in the promotion of high quality, zygotic-like SAMs in two *in vitro* embryogenic systems such as Arabidopsis somatic embryogenesis and rapeseed microspore embryogenesis (Elhiti et al. 2010). Transformed lines overexpressing *STM* showed an increased embryo-forming competence, a reduced requirement for exogenously applied auxins (2,4-D), and an increased expression of genes related to embryo promotion, SAM establishment, DNA hypomethylation, hormone perception and glutathione metabolism. All of these processes are behind the acquisition of a proper SAM identity and its further development. For example, genes related to glutathione metabolism regulate the interconversion between the oxidized and reduced forms of glutathione (GSSG and GSH, respectively) for the maintenance of an optimal intracellular redox potential. This is essential to avoid SAM degeneration in rapeseed. This degeneration is due to the formation of intercellular spaces that cause the loss of physical contact between meristematic cells, which in turn make them lose their identity and acquire parenchymatic features (Yeung and Stasolla 2001; Belmonte et al. 2006; Stasolla et al. 2008). The control over the redox potential may be exerted by the exogenous application of an inhibitor of GSH synthesis (buthyonin sulfoximine; Stasolla et al. 2008) or of brassinosteroids, as demonstrated by Belmonte et al. (2010). Brassinosteroids have also shown positive effects in increasing the androgenic response in highly recalcitrant rapeseed genotypes ('Westar', for example), in a stable and inheritable way (Malik et al. 2008). In other words, the use of brassinosteroids may both increase SAM quality and modify the androgenic response of a plant and its sexual offspring.

Other growth regulators such as ethylene, abscisic acid (ABA) or indole acetic acid (IAA) may also have a key role in the improvement of eggplant and pepper embryo quality. As discussed in previous reviews (Seguí-Simarro and Nuez 2008b), problems in the transition from globular to heart-shaped embryos, defects in the lateral expansion of cotyledons or in embryo elongation, early germination, and other anatomical problems frequently detected in eggplant and pepper embryos (Figures 2C, 2D, 3C, 4C, 5D, 5E, 6C, 6D) might be corrected through exhaustive studies on the particular hormonal requirements of these species at each embryonic stage.

The relationship between embryo quality and arabinogalactan proteins (AGPs) must also be highlighted. AGPs are extracellular matrix glycoproteins, highly glycosylated with arabinose and galactose (arabinogalactan) residues. AGPs are present in various reproductive tissues, including ovules. In addition to their role in multiple processes related to plant growth and development (Cheung and Wu 1999), they have a decisive role in androgenesis induction and MDE development in several species. In wheat, AGPs and arabinogalactans promote androgenesis induction and microspore survival (Letarte et al. 2006). In maize, they are essential for the viability and development not only of MDE, but also of zygotic embryos (Paire et al. 2003). In rapeseed, they have been shown to be involved in key androgenic developmental processes, such as the formation of early embryogenic patterns, or the subsequent bipolar growth along the apical-basal axis of the embryo (Tang et al. 2006). Although the precise role is not clearly known, it seems clear that AGPs and arabinogalactans play an important function during embryogenesis. In particular, Zhong et al. (2010) recently demonstrated in Arabidopsis zygotic embryos a clear link between AGP functional blockage and important alterations of cotyledon morphology and vasculature, which led to inhibition of embryo germination. The fact that androgenic embryos synthesize these substances, which are generated naturally by the seed endosperm, suggests that the cultured embryo tries to "mimic" the zygotic scenario, compensating for some of the deficits of the *in vitro* culture medium. These notions, along with the fact that AGPs have not been studied beyond the androgenic model systems, invite to speculate that androgenic recalcitrancy could be related to the ability of the MDEs to synthesize these molecules on their own. Indeed, the androgenic response can be enhanced in recalcitrant maize genotypes by applying AGPs (Borderies et al. 2004). Thus, recalcitrant species such as pepper, eggplant and mostly tomato, might be highly deficient for these compounds. We have very recently conducted some preliminary studies adding a mixture of AGPs to embryogenic microspores of eggplant and pepper, and results have been extremely positive in terms of embryo quality (manuscript in preparation). Comparative studies of the presence of AGPs during the different stages of MDE development in these species, clearly recalcitrant, would surely help to validate this hypothesis.

Finally, a new, potentially universal method to obtain DHs has been very recently proposed. In Arabidopsis, it has been possible to selectively eliminate the complete haploid genome of one of the two parentals when sexually crossed (Ravi and Chan). This way, uniparental haploid individuals are directly produced through seeds. In this method, one parental is manipulated to alter the Arabidopsis centromere-specific histone CENH3, in order to reduce its ability to interact with the mitotic chromosome segregation machinery. When *cenh3* null mutants are crossed to wild type individuals, the hybrid zygote will carry chromosomes with both wild type and altered CENH3 histones, but those carrying wild type histones will be favored during chromosome segregation. In other words, the chromosomes coming from the *cenh3* parental will be eventually eliminated, thus generating a haploid, wild type individual which can be converted into a DH through conventional chromosome doubling techniques (Seguí-Simarro and Nuez 2008a). This innovative approach to haploidy has the potential to be applied to virtually any plant species, since CENH3 is universally expressed in all eukaryotic species. Thus, it is a clear candidate to be applied in recalcitrant genotypes such as those hereby described.

Concluding remarks

As seen, microspore culture in tomato, eggplant and pepper still needs major refinements to reach the levels of efficiency achieved in model species. The acquired experience and previous results in these solanaceae, combined with recent discoveries coming from model species is allowing for the identification of a number of factors, most of them common to the three species, around which the search for an efficient protocol should be focused. A better understanding of the role of these factors during MDE embryogenesis would allow for the advance in this search, and would potentially apply in parallel to the protocols of the three species.

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Figure legends

Figure 1: Anther culture and plant regeneration in tomato. A: Meiocyte-containing anther with a young callus (arrowhead) emerging from the anther locule. B: Callus with shoot initials at its surface. C: Developing shoots, leaves, and roots over an old callus. D: Tomato plants regenerated from anther cultures and grown in the greenhouse. Figure reproduced from (Seguí-Simarro and Nuez 2007) © José M. Seguí-Simarro. Bars in A: 2 mm; B, C: 1 cm.

Figure 2: Anther culture and DH plant regeneration in eggplant cv. 'Bandera', based on the Dumas de Vaulx method. A: Eggplant bud at the optimal stage for anther isolation. B: Swollen anther after four weeks in culture, with an embryo emerging at the left side (arrow). C: Individualized torpedo MDE. D: Elongated, germinating MDE. E: Young seedling, two weeks after embryo germination, with the development of the first true leaves. F: *In vitro* grown DH plant, ready to be transferred to pots *ex vitro* and acclimated. G: Acclimated DH plant in the greenhouse. Bars in B, C: 1 mm; D, E: 1 cm.

Figure 3: Microspore culture in eggplant cv 'Bandera'. A: Dividing, embryogenic microspore. B: Globular MDE. C: Macroscopic callus formed after proliferation of a globular MDE unable to transform into heart-shaped MDE. D: Callus-derived regenerant. E: Fully regenerated DH individuals. Bars in A: 50 μm; B-C: 500 μm; D: 5 mm.

Figure 4: Anther culture and DH plant regeneration in pepper cv. 'Herminio', based on the Dumas de Vaulx method. A: Pepper bud (arrow) at the optimal stage for anther isolation. B: Cultured anther, open along the dehiscence line, through which a MDE is emerging (arrow). C: Individualized, germinating MDE. D: Young seedling directly emerged from the anther locule. E: Seedling, two weeks after embryo germination. F: *In vitro* grown DH plant, with a sufficient shoot apical and radicular development to be transferred to pots *ex vitro* and acclimated. Bars: A: 1 mm; C-D: 5 mm.

Figure 5: Microspore culture in pepper cv 'Herminio'. A: Dividing, embryogenic microspore. B: Globular MDE. C: Heart-shaped MDE. D: Torpedo MDE. E: Germinating MDE. F: In vitro regenerated plantlet. Bars in A: 50 μm; B-D: 500 μm; E: 5 mm.

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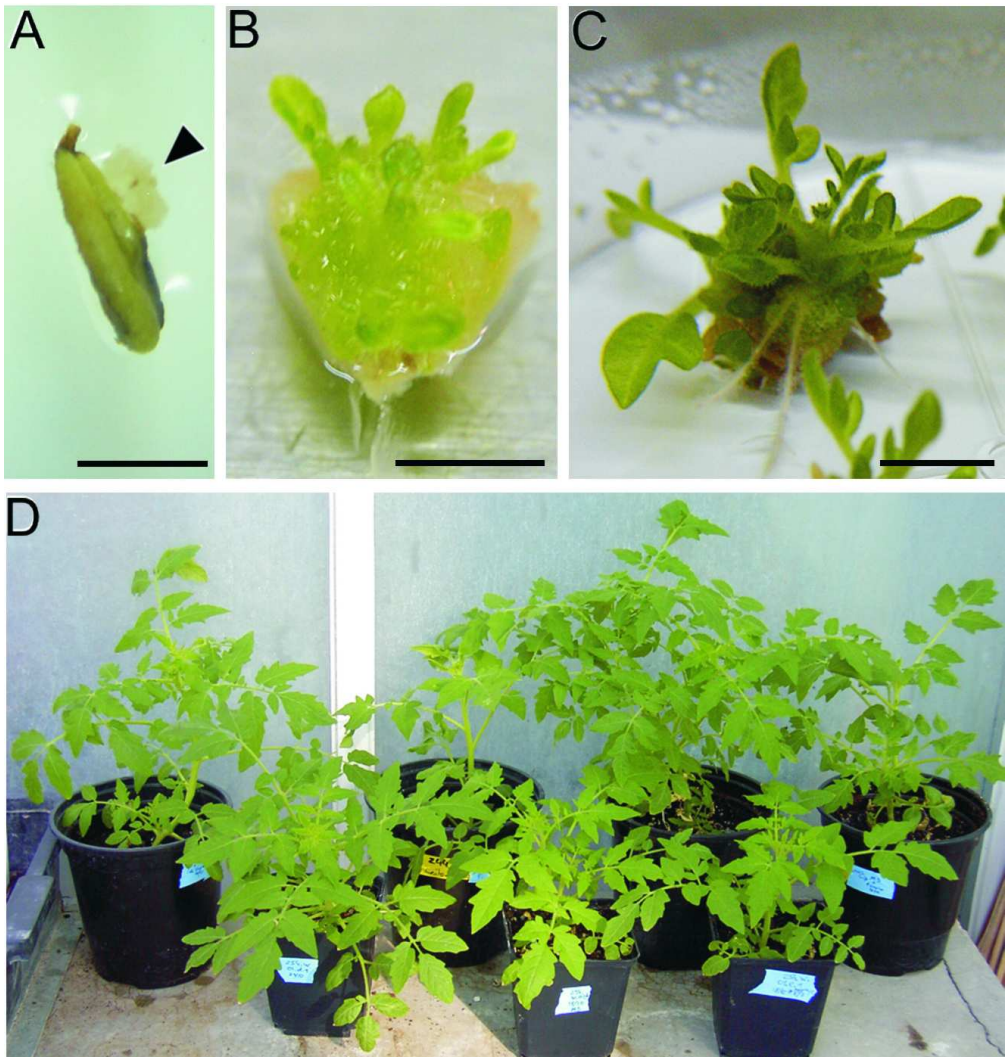

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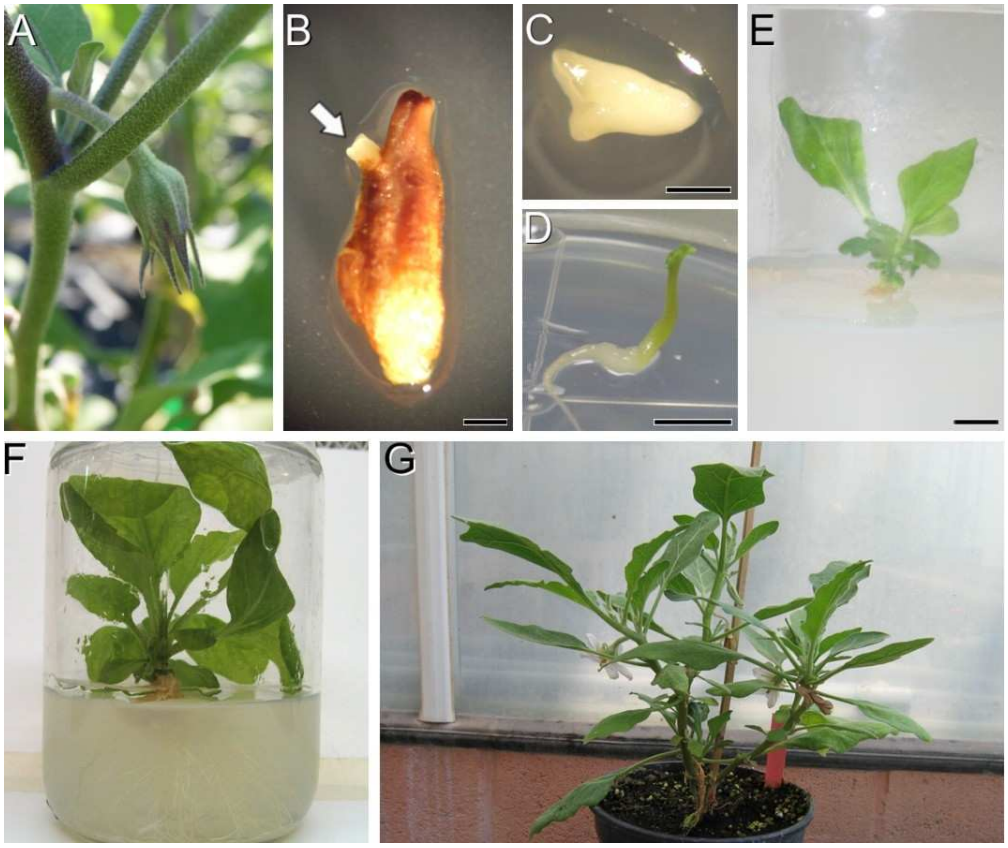

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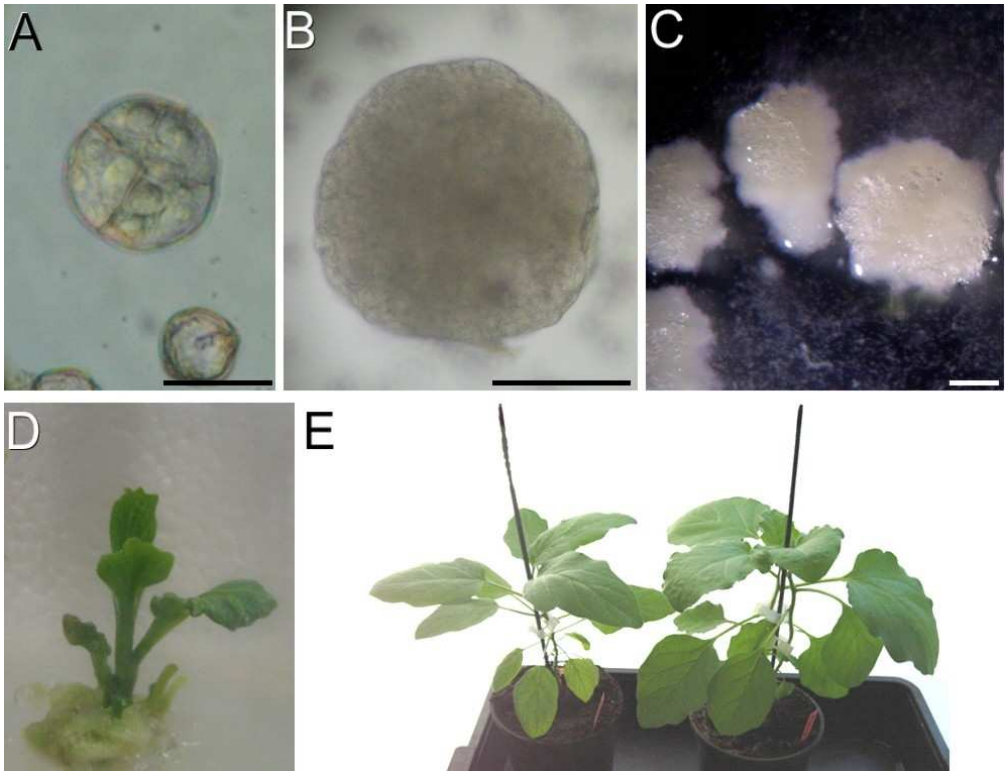

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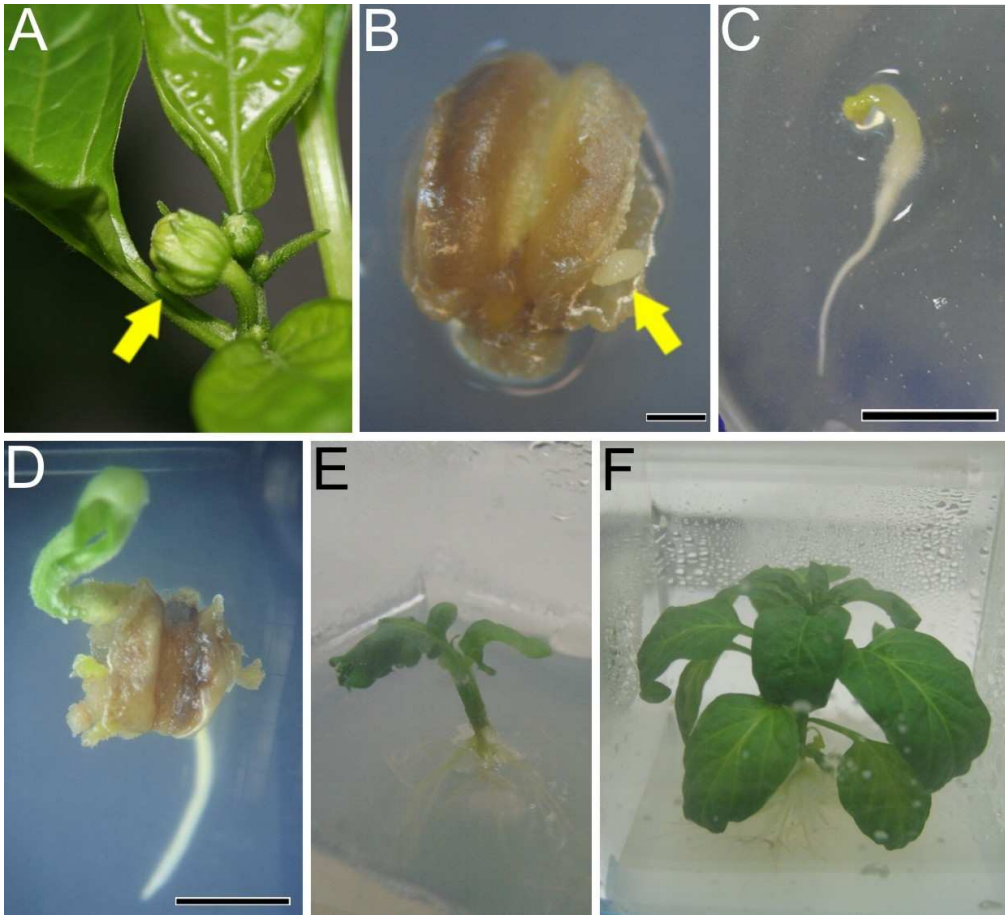

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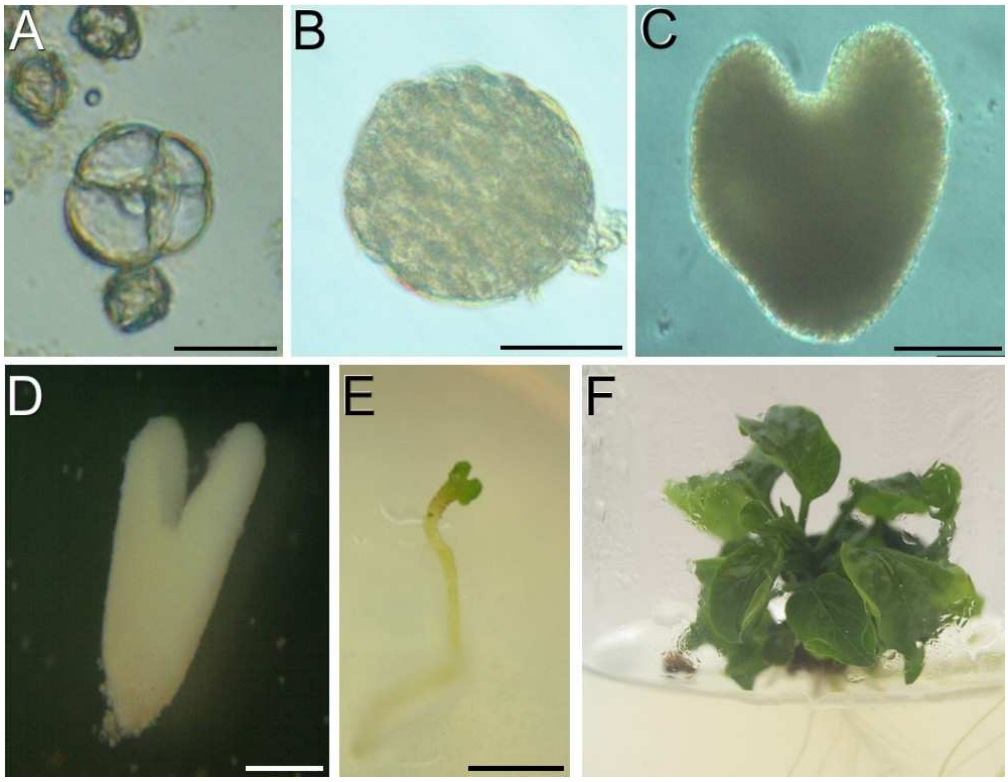

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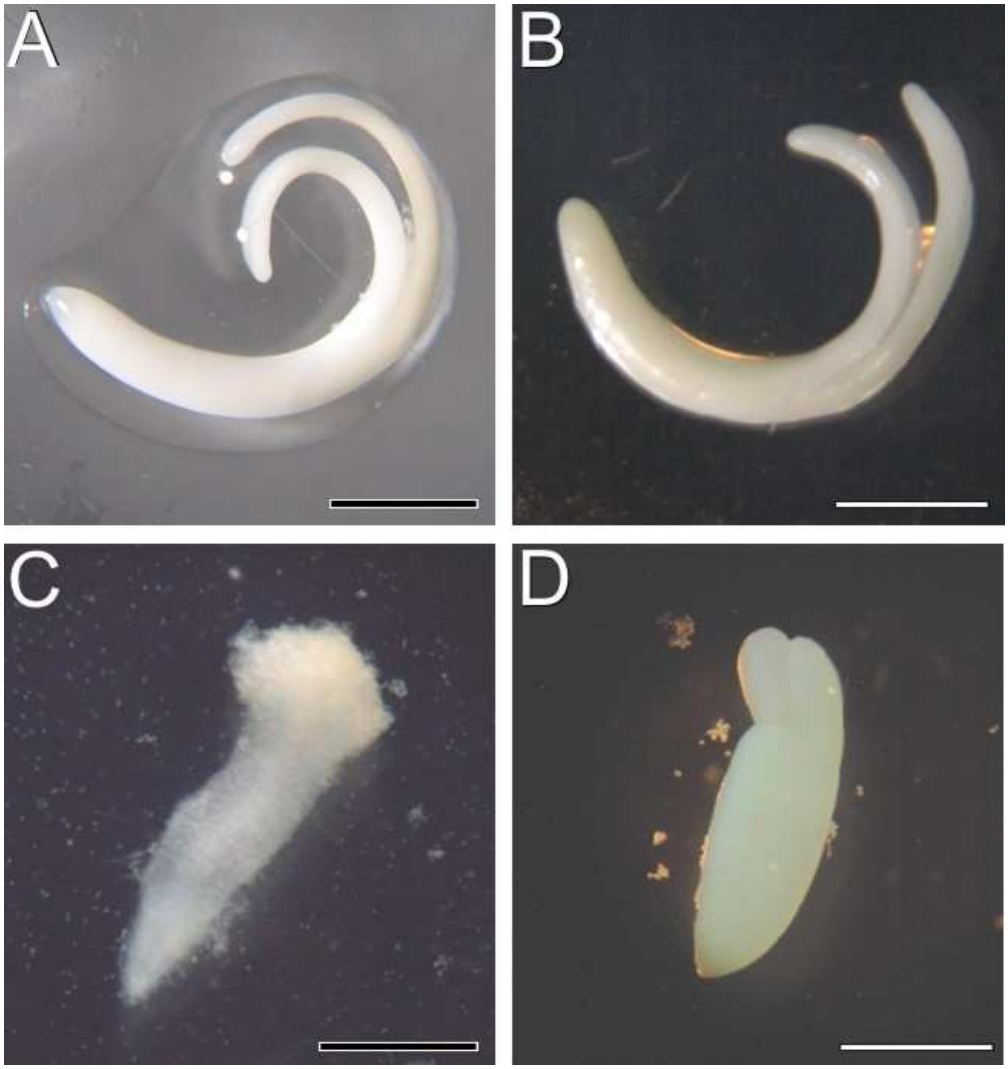

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55x58mm (300 x 300 DPI)