

Pepper (*Capsicum annuum* L.) anther culture: fundamental research and practical applications

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Abstract: To create new varieties resistant to biotic and abiotic stress factors that possess high productivity and improved fruit quality, breeders require modern breeding methods and techniques. Double haploid (DH) lines are an invaluable breeding element due to their guaranteed, completely homozygous nature. One of the fastest methods for obtaining DH lines is anther culture. Microspore embryogenesis provides considerable opportunities in the areas of breeding and biotechnology and represents a model system for fundamental biological research. The effectiveness of pepper anther culture is still low, which affects the application of DH lines in breeding programs. A detailed study of the anther culture process will produce a deeper understanding of the mechanisms that underlie the switch from gametophyte to sporophyte during embryo development. In this respect, the genes expressed during microspore embryogenesis and those that control the cellular response to stress have a key role. This review provides information about the control of embryo formation together with the features of practical application of pepper anther culture.

Key words: Embryogenesis, haploids, DHs, genes, plant breeding, androgenesis

1. Introduction

In general, haploid cells or individuals are those in which the original chromosome number has reduced by half. According to Seguí-Simarro (2010), androgenesis can be defined as a set of biological processes leading to an individual that genetically originated exclusively from a male nucleus. After spontaneous or induced genome doubling, double haploids (DHs) that are completely homozygous are obtained. The production of haploid plants can be induced by methods such as parthenogenesis, interspecific hybridization, pollination with irradiated pollen, hormonal and/or chemical treatment, and application of high-temperature stress (Forster et al., 2007; Murovec and Bohanec, 2013). In anther culture, haploids and DHs occur through direct microspore embryogenesis. This technique has no analogue for fast creation of pure homozygous lines in a single step. In pepper-plant breeding, for example, this method has helped in the development of important new varieties with improved adaptability, productivity, and disease and pest tolerance (Chunling and Baojun, 1995; Venczel and Mitykó, 1995; Arnedo Andrés et al., 2004; Nervo et al., 2007). DH lines are a suitable material for genetic and molecular studies,

and this determines their high value in molecular breeding as well (Madhusudhana, 2015).

Regarding the genus *Capsicum*, the first haploids were derived by in vitro anther culture of *C. annuum* and *C. frutescens* (George and Narayanaswamy, 1973; Kuo et al., 1973; Wang et al., 1973; Novak, 1974). In these early studies, the regeneration of plants achieved was of low efficiency, and research efforts in the next experiments aimed to identify the factors influencing induction of androgenesis. After many experiments, it was found that androgenic response depended on the following: growing conditions, age and genotype of the donor plant (Ercan et al., 2006; Niklas-Nowak et al., 2012; Grozeva et al., 2013; Koleva-Gudeva et al., 2013; Alremi et al., 2014), developmental stage of microspores in the anther (Nowaczyk and Kisiała, 2006; Parra-Vega et al., 2013a; Barroso et al., 2015), composition of the culture medium, concentration and combination of growth regulators, organic and inorganic additives (Büyükalaca et al., 2004; Zhao et al., 2010; Taşkın et al., 2011; Roshany et al., 2013; Olszewska et al., 2014), and pretreatment of flower buds and/or anthers (Koleva-Gudeva et al., 2007; Özkum and Tıpırdamaz, 2007; Irikova et al., 2011b; Nowaczyk et al., 2015).

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Despite the considerable number of studies conducted with pepper anther culture, the reported effectiveness of the process is still low (Irikova et al., 2011a). A high frequency of anther-derived embryos can be obtained, but few of them develop into normal plants (Segui-Simarro et al., 2011a). In recent years, improvements in anther culture concern mainly the optimization of medium composition including the type, concentration, and combination of plant-growth regulators (Olszewska et al., 2014) and the type of basal medium (Alremi et al., 2014). Parra-Vega et al. (2013b) found that a shorter duration (4 days) of anther culture at 35 °C increased embryo production compared to prolonged treatment (8 days), according to the protocol of Dumas de Vaulx et al. (1981). Furthermore, a shorter incubation treatment in darkness decreased callus formation, a common problem in pepper anther culture.

An alternative method to obtain DH plants from a male nucleus *in vitro* is shed-microspore culture, based on anther culture systems with a double-layer medium (Supena et al., 2006). Supena and Custers (2011) achieved the induction of a significantly higher percentage of normal-looking embryos (from 20% to 50%) in Indonesian hot pepper. In order to evaluate the potential of this method, (Ari et al., 2016) studied 64 ornamental pepper genotypes and reported only 1.48 normal-looking embryos per flower bud in the best-responsive genotype.

Haploid and DH plants can be obtained from microspore culture, but at disappointingly low rates. Using this method, Supena et al. (2006) reported that only 0.1 plants regenerated per flower bud. Kim et al. (2008) suggested a different protocol for microspore culture leading to a higher frequency of embryo production and plant regeneration (4 plants/flower bud). The authors pointed out three key factors that positively affect embryogenesis: pretreatment of microspores in sucrose-free starvation medium at 32 °C, culture in modified NLN medium (NLNS) supplemented with sucrose, and optimal microspore density at 8×10^4 – 10×10^4 /mL. Lantos et al. (2009) optimized the method by co-culturing pepper anthers with wheat ovaries; however, the regeneration frequency was lower (0.0 to 1.25 plants/petri dish) than in Kim et al. (2008). Later experiments conducted with sweet pepper aimed to improve the effectiveness of microspore culture through optimization of culture medium, plant growth regulators, and additives (Lantos et al., 2012; Cheng et al., 2013).

The three methods discussed lead to production of haploids and DHs. Unfortunately, none are applicable to a wide spectrum of genotypes. For example, Niklas-Nowak et al. (2012) established different androgenic response rates even in individual plants belonging to the same genotype. Parra-Vega et al. (2013b) obtained a higher percentage of embryos through the shed-microspore approach, compared to conventional anther culture. A

two-step culture system of isolated microspores of hot pepper was most effective for producing cotyledonary embryos, compared to liquid and double-layer culture (Kim et al., 2013). With these results in mind, anther culture remains the most popular and preferred technique for DH production in pepper. This is probably due to its simplicity and the abundance of experimental results and detailed protocols regarding the large number of studied genotypes (sweet, hot, spice-type, etc.). In addition, the potential of microspore and shed-microspore culture for microspore-originating embryogenesis has not been realized yet, due to the small number of tested genotypes and technical difficulties such as microbial contamination and mechanical isolation of microspores (Supena et al., 2006; Gémes Juhász et al., 2009; Liu et al., 2013).

2. Perspectives on anther culture

The main objective of haploid research is to obtain homozygous lines with high practical value, but experiments in this area also have theoretical importance for fundamental biological sciences (genetics, embryology, cytology, and molecular biology). Microspore embryogenesis is important for biotechnological applications and as a model system for studying plant cell totipotency (Maraschin et al., 2005). Physiological, cytological, biochemical, and molecular analyses provide abundant information for cell programming and reprogramming mechanisms (González-Melendi et al., 1995; Bárány et al., 2005; Bárány et al., 2010; Žur et al., 2015). In response to stress *in vitro*, microspores can switch their gametophyte developmental program towards cell division and proliferation, embryogenesis, and plant regeneration. The comparative analysis between the gametophyte and embryogenic pathways of microspores allows for identification of nuclear changes typical for the different states of activity, which are specific to both styles of development (Bárány et al., 2005; Dunwell, 2010). Androgenic development can be divided into 3 main phases: acquisition of embryogenic potential, initiation of cell division, and the formation of structure (Maraschin et al., 2005). One of the key points in the process is the successful release of embryo-like structures from exine wall microspores. As a consequence of genotype-determined differences in the cytological mechanism that controls androgenic induction and haploid chromosome doubling, creation and application of haploids in many plant species, including several pepper varieties, is frequently impeded.

Breeding programs require the availability of a considerable number of genetically stable plants. Androgenesis and the creation of DHs is a routine part of the selection process for some crops (tobacco, barley, rapeseed, and wheat) and is also the shortest route to the production of truly homozygous material (Forster et al.,

2007). In this regard, the most important achievement is the development and optimization of protocols for haploid and DH production under laboratory conditions and utilization of the novel DH lines in practice.

Pepper anther culture also offers an opportunity for the selection of gametoclonal variants with new genomic constitutions (Gyulai et al., 2000) and unique meiotic recombinants (Gémesné Juhász et al., 2001), as well as identification of androgenic lines resistant to viruses, bacteria, or nematodes (Abak et al., 1982; Hwang et al., 1998; Barbary et al., 2014). In pepper breeding projects, haploid plants and DH lines are often included in studies for gene mapping and the identification of useful recessive mutations (Ochoa-Alejo and Ramirez-Malagon, 2001; Yoo et al., 2003; Minamiyama et al., 2006; Djian-Caporalino et al., 2007; Mimura et al., 2012). The application of PCR-based techniques is becoming important, not only for genetic analysis, but also for variety determination of plants of DH origin (Prince et al., 1995; Parra-Vega et al., 2013b). Methods such as RAPD, RFLP, AFLP, and multiplex PCR have been applied in the analysis of DH pepper plants for resistance against Cucumber mosaic virus (CMV), Potato virus Y (PVY), Tomato mosaic virus (ToMV), and *Phytophthora capsici* Leon (Caranta et al., 1997; Mannerlof and Tenning, 1997; Arnedo Andrés et al., 2004; Thabuis et al., 2004; Janzac et al., 2009). The application of SCAR and CAPS markers that are linked to *Pvr4* alleles of genotypes susceptible and resistant to PVY, respectively, indicated that the SCAR marker is more suitable than the CAPS marker (Caranta et al., 1999; Arnedo-Andrés et al., 2002). Nineteen polymorphic bands generated from 7 primers were established through RAPD, SSR, and ISSR-PCR analysis of the R_2 generation of spontaneous diploidized pepper regenerants. Theoretically, all polymorphic PCR bands were derived from locus-specific sequence rearrangements in the genome, and they resulted from meiotic recombination during male gametogenesis. This created new binding sites in the genome of RAPD primers or SSR/ISSR, thus forming new PCR bands (Gyulai et al., 1999, 2000). The direct selection of DH progeny by molecular markers can be facilitated with RAPD analysis (Gémesné et al., 2000). Investigation of molecular polymorphism of donor plants, haploid regenerants, and spontaneous and induced DH plants by RAPD, SSR, and ISSR-PCR analysis revealed that the number of genetic changes occurring during a meiotic recombination is higher than that after colchicine-induced doubling of the genome (Gémesné Juhász et al., 2001).

The experiments carried out with model systems show that direct or indirect microspore embryogenesis is influenced by numerous factors, although the leading contributor is the genotype of the donor plant (Germanà, 2011; Seguí-Simarro et al., 2011b).

3. Key genes influencing embryogenic microspore development

Anther culture is a rewarding experimental system for understanding the changes caused by stress treatment of microspores. It also allows the molecular mechanisms triggering direct embryogenesis to be studied. The key attribute involves genes that are expressed during microsporogenesis and those participating in cellular stress response with the synthesis of heat-shock proteins (for example, HSP70; Bárány et al., 2001) and suppression of the gametophytic program and switching to the embryogenic (Maraschin et al., 2005).

The genes controlling the early stages of embryo formation probably also control the main processes in plant development, such as meristem formation, polarity, and tissue differentiation, and they function during the entire life cycle (Kaplan and Cooke, 1997). The most important issue is the identification of genes that are specific to the androgenic response and that are, in turn, responsible for switching from the gametophytic toward the sporophytic developmental pathway (Wang et al., 2000), as well as associated biochemical and molecular changes during stress treatment (Mordhorst et al., 1997; Touraev et al., 1997; Bárány et al., 2010). Most of the genes expressed during stress treatment are related to stress proteins, cell protection from stress, sucrose and starch metabolism, and proteolysis (Bárány et al., 2001; Mitsushashi et al., 2004; Seguí-Simarro et al., 2011a). The achievement of androgenic potential is based on dedifferentiation, whereas existing transcriptional and translational cellular profiles are probably eliminated or altered in order to block pollen development and trigger embryogenesis. A stress-induced reprogramming of cellular metabolism precedes the activation of key regulators of embryogenesis such as the *BABY BOOM* transcription factor (Maraschin et al., 2005).

Currently, some genes specifically expressed during the early stages of microspore embryogenesis in different plants have been identified, including *BnmNAP*, *ntsm10*, *ZmAEL1*, *ZmAEL3*, *LEC1*, *LEC2*, and *BBM* (Boutillier et al., 1994; Touraev et al., 1997; Vrienten et al., 1999; Magnard et al., 2000; Boutillier et al., 2002; Malik et al., 2007; Tsuwamoto and Takahata, 2008; Malik and Krochko, 2009).

BABY BOOM (*BBM*) and *LEAFY COTYLEDON* (*LEC*) genes were identified in the genome of pepper (*Capsicum annuum* L.), and high levels of expression of these genes during early stages of direct embryogenesis in anther culture were reported (Irikova and Denev, 2008; Irikova et al., 2012). Expression of these genes was not found in nonembryogenic anthers or in mature plants of pepper. The authors suggested that differences observed in the in vitro response of the investigated cultivars were due to the interaction of these genes with other gene products

and factors, such as micro-RNA, that were involved in the regulation of their expression (Irikova et al., 2012).

Lee et al. (2009) identified a new gene, *DEG13*, which is associated with the microspore embryogenesis in pepper and is highly expressed under high-temperature stress. The sequence of nucleotides showed that *DEG13* is homologous with the *POACT88* gene from potato ($\approx 91\%$), as well as with the alcohol dehydrogenase gene from *Arabidopsis* ($\approx 70\%$), both activated by stress. Two gene sequences, *PELTP* and *PEGST*, which were expressed in the early stages of microspore embryogenesis in pepper, were identified. *PELTP* is highly homologous with the *LTP* of pepper and *PEGST* with the *GST* gene of pepper (98% and 99%, respectively). The authors presume that the two genes may play an important role in the early stages of pepper microspore embryogenesis (Zhang et al., 2008).

Knowing the genes responsible for the initiation of androgenesis in microspores cultured in vitro and the factors that control the processes and stages of direct embryogenesis could help create efficient embryogenic anther culture in a number of plant species, including pepper.

4. Application of anther culture in pepper-breeding programs

Although anther culture is one of the fastest methods for obtaining isogenic lines, its application in pepper breeding is still limited because of low plant regeneration efficiency (Grozova et al., 2009; Ercan and Şensoy, 2011; Olszewska et al., 2014). For breeding purposes, a large number of genetically stable, homozygous plants are needed in order to evaluate targeted breeding traits such as morphological characteristics and main parameters of productivity, quality of fruits, and resistance to various diseases. Despite the difficulties in recent years, the number of studies concerning the practical aspect of the process in different *Capsicum* species is steadily increasing (Olszewska et al., 2010, 2011; Shrestha et al., 2010; Luitel et al., 2012; Luitel and Kang, 2013a; Shmykova et al., 2014; Trajkova and Koleva-Gudeva, 2014). It also includes the data reported on the development of varieties and F_1 hybrids employed as parental lines of androgenic origin (Chunling and Baojun, 1995; Pauk et al., 2010). Androgenic pepper lines of R_2 progeny with improved yield characteristics and dry matter content in fruits were also obtained (Kisiała et al., 2011). Shrestha et al. (2011) isolated 5 superior DH lines, and they observed considerable variation in plant and fruit traits among other lines. After detailed characterization of morphological and production parameters of the pepper fruits, androgenic lines with positive traits were found (Koleva-Gudeva and Trajkova, 2012). Higher visual and taste quality, fruit firmness, dry matter content, total

soluble content, titratable acidity, phenolic content, and antioxidant activity were exhibited in pepper DHs (Luitel and Kang, 2013b). Nowaczyk et al. (2014) proved that DH technology can be successfully used for the rapid genetic stabilization of soft-flesh *Capsicum* spp. recombinants.

Different levels of resistance to *Xanthomonas campestris* P.v. *viscatoria* were observed in pepper lines obtained from anther cultivation in vitro (Hwang et al., 1998). Other authors reported on the breeding lines resistant to *Phytophthora capsici*. They confirmed the possibility of transferring a high level of disease tolerance from resistant DH lines into traditional pepper varieties to develop new multiple-disease-resistant genotypes (Nervo et al., 2007). Pepper DH lines homozygous for genes both resistant and susceptible to PVY-1-2 were achieved through anther culture (Arnedo Andrés et al., 2004). DH parental lines with resistance and important qualitative and quantitative traits were used as background germplasm for the creation of new sweet pepper varieties in Hungary (Mitykó and Gémes Juhász, 2006). Pepper lines with high productivity, improved fruit traits, and low susceptibility to *Verticillium* wilt were produced through androgenesis (Todorova et al., 2013). The authors demonstrated the advantage of in vitro pepper anther culture as a means to enrich the genetic diversity and develop pure lines in much less time and with fewer required resources compared to the conventional breeding process.

The Balkan Peninsula is a secondary center of origin of pepper and is characterized as a gene pool of local forms that are typical and well-adapted to this region. The existing specific genetic diversity could offer a starting point for the creation of pepper varieties that correspond to modern breeding requirements, high productivity and disease resistance, but of typical and original taste (Krasteva et al., 2012). Increasing attention has been paid to the content of ascorbic acid and phenolic compounds in the fruits, which contribute to high nutritional value and consumer satisfaction due to their healthy qualities (Pevicharova and Todorova, 2014). Pepper breeding is a long-term and labor-consuming process due to the fast degeneration of germplasm. In vitro techniques could overcome this problem by helping with the restoration of old varieties and contributing to the reintroduction of genes that have been removed by the process of selection. For example, the application of microspore embryogenesis has resulted in the creation of pepper lines adapted in this European region that offer improved productivity, resistance to *Verticillium dahliae* Kleb (Grozova et al., 2009; Koleva-Gudeva and Trajkova, 2012; Todorova et al., 2013; Trajkova and Koleva-Gudeva, 2014) and Tobacco mosaic virus (TMV), and that produce higher fruit antioxidant activity (data not published).

5. Conclusion

The significant advantage of in vitro androgenesis as a breeding tool is that it speeds up both processes: achieving homozygosity and increasing selection efficiency. Pepper belongs to androgenesis-recalcitrant plants, characterized by a low frequency of embryo induction from anthers and a low rate of plant regeneration. Therefore, it is necessary to investigate the changes, cytological and biochemical, that occur in cells during the switch from gametophyte to sporophyte pathways of microspore development in vitro. Clarifying the genetic control of different stages of

embryo transformation is a milestone for improvement of the pepper androgenetic process. Research work with pepper in this vein is still at an early stage, and extensive experimental work is required to identify the genes responsible for microspore embryogenesis and their expression in different embryo transformation stages. Such knowledge could lead to more efficient protocols for embryo induction and regeneration from pepper anthers and microspores. This, in turn, will increase the number of researchers who can apply anther culture as their method of choice in future projects related to pepper breeding.

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