

## TRADITION, QUALITY AND BIOTECHNOLOGY IN HUNGARIAN SPICE PEPPER (*Capsicum annuum* L.) BREEDING

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Spice pepper production has a history of almost 300 years in the southern part of Hungary. In this study the results of two biotechnological improvements are summarized. Anther and isolated microspore culture techniques were improved to release haploid and doubled haploid (DH) lines for spice pepper breeding. Both the anther and isolated microspore culture methods were successfully used in spice pepper haploid production. Microspore culture- derived structures were analysed to identify their different parts. Green plantlets were regenerated from embryos derived from both anther and microspore cultures. Their doubled haploid analogues were integrated into Hungarian spice pepper hybrid seed breeding programmes. One hybrid, Sláger, was released as a new genotype for spice pepper production in 2008 and two hybrid candidates (Délibáb and Bolero) are now being tested in official trials.

**Key words:** *Capsicum annuum* L., embryogenesis, histology, microspore culture, ovary co-culture, pepper

### Introduction

In southern Hungary, spice pepper growing has a history of several hundred years and in the southern region of the Carpathian Basin a great number of families and medium-sized farms are engaged in spice paprika production. Spice paprika powder (red paprika: sweet and hot) is the most important savoury ingredient in the famous Hungarian goulash and other traditional dishes. Hungary is among the world's five biggest spice pepper producers (Somogyi et al., 2003).

The economic conditions in the 21<sup>st</sup> century have raised new challenges for spice pepper production. The demand for hybrid seed is increasing on the part of farmers, particularly for spice pepper production in plastic tunnels. Homozygous lines play an important role in breeding programmes. Heterosis

furnishes new possibilities for the production of spice pepper with higher quality and in larger quantities (Luo et al., 2006). *In vitro* techniques provide a new methodological background for the production of homozygous lines via microspore-derived haploid production (Kasha and Maluszynski, 2003). Methodologically there are two alternatives for *in vitro* haploid production: anther culture or isolated microspore culture. Haploid production is followed by colchicine treatment, which is the last step in DH production.

Anther culture is a well-known method for developing pepper haploids. The use of the procedure to achieve haploid induction was published simultaneously by three different laboratories (George and Narayanaswamy, 1973; Kuo et al., 1973; Wang et al., 1973). Later, Sibi et al. (1979) published a two-step anther culture system, which was further optimized by Dumas de Vaultx et al. (1981). This method was subsequently studied and continuously improved by different laboratories (Mitykó et al., 1995; Dolcet-Sanjuan et al., 1997; Gémes Juhász et al., 1998; 2006; Bárány et al., 2005; Kim et al., 2004) and sporadically applied in different breeding programmes (Thomas et al., 2003; Gémes Juhász et al., 2006; Mitykó and Gémes Juhász, 2006).

Certain aspects of this method, such as genotype dependency, excessive manual work and low efficiency, led to a search for alternative methods for DH plant production. Microspore culture may offer an alternative solution for pepper haploid and DH plant production. Isolated microspores are cultured in liquid medium without somatic tissues, so the embryos and regenerated plants should be derived from haploid cells.

Supena et al. (2006) published the first results of microspore culture-derived haploids in pepper, without giving experimental details, and described androgenesis from shed microspores. Kim et al. (2008) gave a detailed report on the use of the isolated microspore culture method in the hot pepper variety Milyang-jare. Lantos et al. (2009) improved the pepper microspore culture method by using wheat ovaries for alien species ovary co-culture.

The present paper reports the results of anther and isolated microspore cultures of spice pepper genotypes, and gives a short description of the phenomenon of androgenesis in anther and microspore cultures of spice pepper. The *in vitro* haploid induction protocols for spice pepper are based on cereal haploid production methods developed and used in Szeged (Pauk et al., 2003; Lantos et al., 2005) and on internationally published data (Dumas de Vaultx et al., 1981; Supena et al., 2006; Kim et al., 2008).

## Materials and methods

### *Plant material and donor plant growth conditions*

The Hungarian and Spanish (from Junta de Extremadura, Servicio de Investigación Agraria, Finca La Orden, Badajoz, Spain) pepper genotypes used in the experiments are essential breeding materials for various pepper research programmes in Hungary. The donor plants were grown in the greenhouse (natural photoperiod, 25–32°C during the day and 15–19°C at night). The seedlings were grown in PVC bags (100 × 180 mm) containing a 1:1 mixture of peat and sandy soil. The donor plants were fed with Volldünger® fertilizer every two weeks.

*Collection of donor materials and pretreatment for microspore culture*

The donor buds were collected in the optimal developmental stage (late uninucleate and early binucleate stages), sterilized for 20 min in an Erlenmeyer flask containing 50 ml 2% NaOCl solution plus 1 drop of Tween-20, and then rinsed three times with sterile distilled water (Millipore Elix 5). Anthers from sterilized buds were isolated directly into 55 mm diameter glass Petri dishes containing 5 ml 0.3 M mannitol solution and 200 mg l<sup>-1</sup> cefotaxime (antibiotic). The microspores were pretreated at 32°C in the dark for 7 days.

*Anther and microspore culture*

For anther culture, the isolated anthers were placed on CP induction medium (Dumas de Vaulx et al., 1981) and kept in the dark at 32°C for 8 days. After heat stress, the cultures were moved to a growth chamber (25°C, 16-hour photoperiod) and the anthers were transferred to R1 regeneration medium after four days.

For isolated microspore culture, the microspore isolation protocol was carried out on the basis of the cereal microspore isolation procedures successfully used earlier (Pauk et al., 2003; Lantos et al., 2005), but with certain modifications (Lantos et al., 2009). The isolated microspores ( $3 \times 10^4$  microspores ml<sup>-1</sup>) were cultured in 35 mm diameter plastic Petri dishes (Sarstedt Inc., USA, Cat. 83.1800) containing 1.5 ml modified W14 (Ouyang et al., 1989) liquid medium (W14mi) containing 9% maltose, 1000 mg l<sup>-1</sup> glutamine, 0.5 mg l<sup>-1</sup> kinetin and 0.5 mg l<sup>-1</sup> 2,4-D (Ficoll was omitted). 200 mg l<sup>-1</sup> cefotaxime (antibiotic) was added to each culture. Seven sterile isolated wheat ovaries were added directly to the freshly isolated pepper microspore cultures. The Petri dishes were kept at 28°C in a dark thermostat at high humidity (~80%).

*Plantlet regeneration and transfer of plantlets into soil*

Microspore-derived embryoids in the bipolar development stage were transferred into 55 mm diameter Petri dishes containing R1 regeneration medium (Dumas de Vaulx et al., 1981). During the regeneration period, the cultures were kept in a culture room at 24°C with a 16/8 hour day/night photoperiod at a light intensity of 100 µmol m<sup>-2</sup> s<sup>-1</sup>. When the regenerated plantlets reached the 1–2-leaf stage with roots, they were transferred into glass tubes containing growth regulator-free, half-strength MS medium (Murashige and Skoog, 1962) with 2% sucrose.

The well-rooted plantlets were then transferred into a non-sterilized 1:1 mixture of peat and sandy soil in pots. During the following two weeks, the plantlets were acclimatized in a greenhouse growth cabinet at a relative humidity of 80%. Before flowering, the plants were transferred to an individual isolator box made of wood and cloth. The self-pollination of individuals was assisted with a sterilized brush. The fruits were collected from the microspore-derived plants.

*Histological examination*

For histological examination of the *in vitro* structures induced in spice paprika microspore cultures, the samples were fixed in a solution of 4% glutaraldehyde buffered to pH 7.2, dehydrated in a graded ethanol series, and then embedded in Historesin (Leica) for light microscopy. A Microm HM 360 microtome was used to cut 6 µm sections. The light microscopy specimens were examined with an Olympus BH-2 epifluorescence microscope.

## Results

*Androgenesis induction in anther and microspore cultures of spice pepper*

Anthers of selected genotypes were isolated from donor buds containing microspores in the ideal developmental stage (Fig. 1a, b). Five to six weeks after isolation, white embryos developed on the regeneration medium, which

regenerated into green plantlets (Fig. 1c, d). Plantlets with two to four leaves were transferred into glass tubes (Fig. 1e). The well-rooted plantlets were acclimatized in the greenhouse (Fig. 1f).

In the isolated microspore culture, viable microspores were separated from the somatic tissues in a white band using modified gradient centrifugation (Fig. 2a). The isolated viable microspores (Fig. 2b) were cultured in W14mi liquid medium with wheat ovaries. The first cell divisions were observed inside the walls of the microspores at the end of the first week. In the presence of wheat ovaries, multicellular colonies emerged from the walls of the microspores in the second week of cultivation (Fig. 2c). The multicellular structures developed intensively (Fig. 2d), and after 5–6 weeks the well-developed embryoids were checked (Fig. 2e). The microspore culture-derived embryos mainly regenerated into green plantlets with leaf rosettes. However, numerous embryos produced normal plantlets (Fig. 2f). The well-rooted plantlets were acclimatized in the greenhouse.

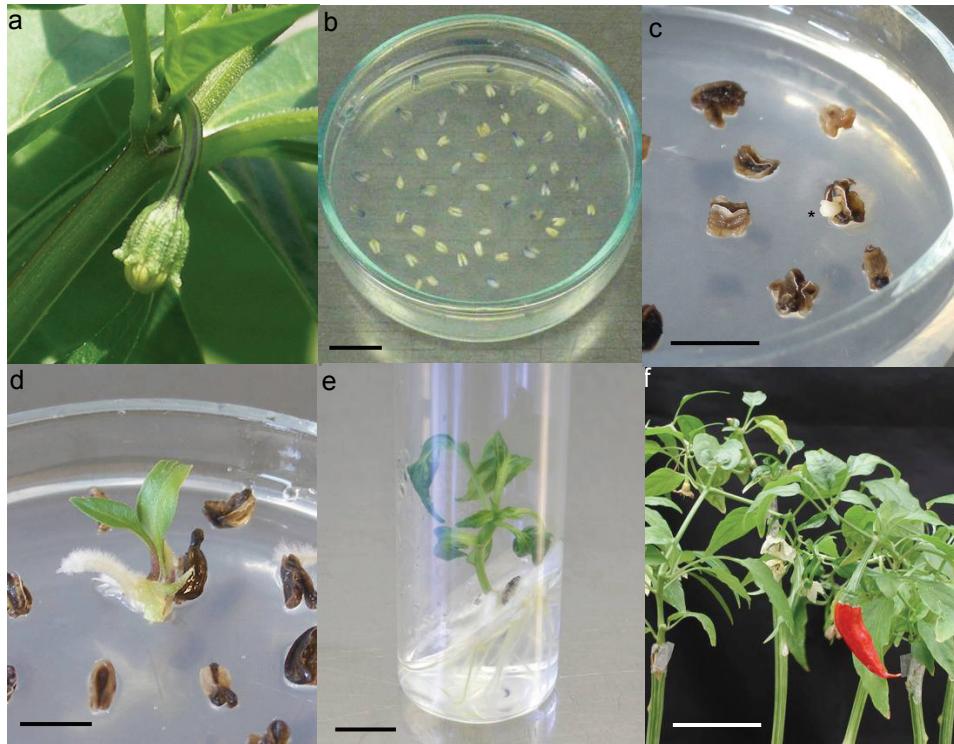


Fig. 1. a A donor bud containing microspores in the optimal stage for the induction of androgenesis, b Isolated anthers on induction medium, c A white embryo derived from anther culture (\*), d Green plantlet regenerated from anther culture, e The same plantlet rooted in a test-tube, f Well-rooted plantlets acclimatized to greenhouse conditions. Bars = 1 cm for a, b, c, d and e; 5 cm for f.

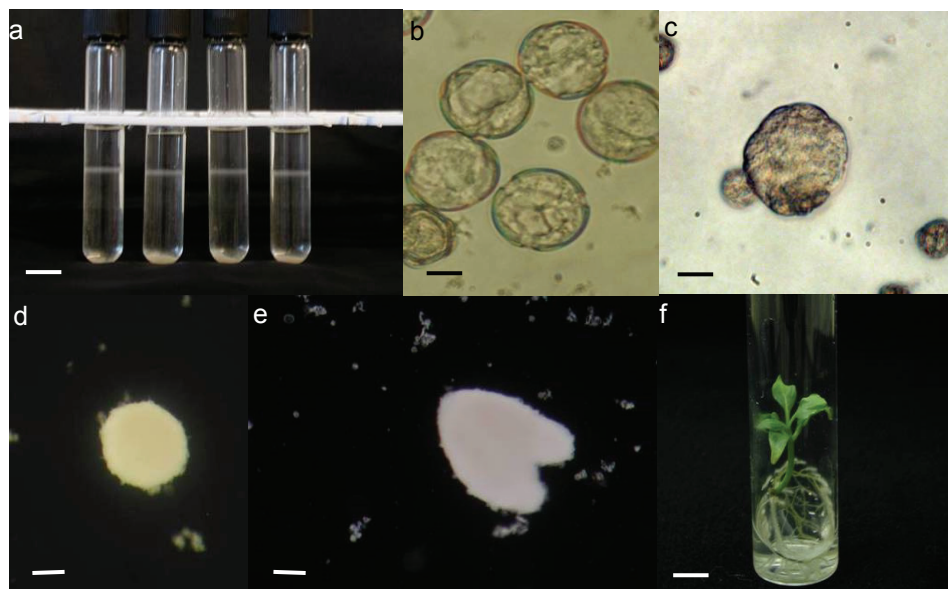


Fig. 2. a Viable microspores form a white band after gradient centrifugation, b Isolated microspores on the first day of culture, c A microspore-derived multicellular structure, d Globular microspore-derived embryos, e A well-developed microspore-derived embryo, f The plantlet was rooted separately in a glass tube. Bars = 1 cm for a and f, 10  $\mu$ m for b and c, 1 mm for d and e

*Histological study of adventitious embryogenesis  
and the regeneration of spice paprika plantlets*

Following the microspore culture experiment, the embryoids obtained were transferred onto R1 regeneration medium. At the end of the first week of the regeneration experiment, well-structured embryoids had emerged and developed. The structure of the microspore-derived embryoids was investigated with an Olympus BH-2 epifluorescence microscope (Fig. 3). The longitudinal section of the slightly elongated embryoids displayed two distinct poles. The root meristem consisted of smaller cells with a lower degree of vacuolization. Vascular differentiation had occurred in the root primordium and the vascular tissues were connected to the central vascular system of the shoot. The opposite pole of the embryoid formed two equally developed primordial cotyledons. A central procambium bundle continued from the hypocotyl into both cotyledons. The central structures were coated with a thick tissue that resembled a cortical layer (Fig. 3). The embryoids were completely covered by hairy epidermis. Epidermal hairs were abundant on the root surface.

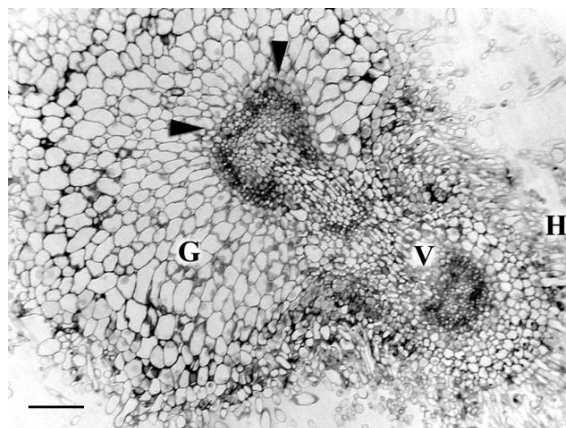


Fig. 3. Longitudinal section of a microspore-derived embryo exhibiting two distinct poles and covered by a hairy epidermis (H). Epidermal hairs are abundant on the root area surface. The vascular tissues (V) of the root primordium are connected to the central vascular system of the shoot. Two primordial cotyledons (arrows) are formed at the opposite pole. The central structures are coated with a thick cortical layer (G). Toluidine blue staining. Bar = 100  $\mu$ m

#### *Application of DH lines in breeding*

The DH lines generated via these methods were integrated into the hybrid programme of the Red Pepper Research and Development Co. Ltd. The homogeneity of the DH lines and the uniformity of hybrids were checked. The homogeneity and agrobotanical traits of the DH lines were checked during seed propagation. No significant variation was found between the parents and their progeny for major phenological and morphological traits.

### **Discussion**

Androgenesis was induced in the Hungarian and Spanish spice pepper genotypes that play a key role in Hungarian spice pepper breeding programmes using anther and microspore culture. The genotype was found to influence the efficiency of the methods. The effect of genotype was investigated in anther culture and shed microspore culture (Mitykó et al., 1995; Gyulai et al., 2000; Ercan et al., 2006; Supena et al., 2006). The genotype influenced the number of embryoids and shoots not only in anther culture but also in isolated microspore culture (Lantos et al., 2009). Plant regeneration from microspore-derived embryos is one of the most critical steps in pepper microspore culture. In the present experiments, the tested genotypes produced microspore culture-derived diploid plants at a rate of 0.2–1.1 plants/Petri dish. Accordingly, further experiments will be required to improve the plant regeneration efficiency and decrease the effect of the genotype in isolated microspore culture.

The longitudinal section of the microspore-derived embryoids displayed two distinct poles. Cell and tissue differentiation were observed on the different parts of the embryoids (root primordium, central vascular system, two primordial cotyledons, procambium and hairy epidermis). These are good indications of the induction of embryogenesis in isolated microspore culture.

Anther culture is already an integral part of pepper breeding (Thomas et al., 2003; Gémes Juhász et al., 2006; Mitykó and Gémes Juhász, 2006), and the new isolated microspore culture will open up new opportunities for breeders. The new anther and microspore culture-derived DH descendants did not exhibit any significant morphological or phenological differences compared to the initial varieties. DH lines play an essential role in Hungarian pepper breeding programmes. The hybrids reached an adequate level of uniformity before they were entered in the national testing system. The hot spice pepper hybrid Sláger was registered in 2008, while the sweet spice pepper hybrids Bolero and Délibáb were still being tested in 2010.

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