CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

ESTACIÓN EXPERIMENTAL DEL ZAIDÍN

DEPARTAMENTO DE BIOQUÍMICA, BIOLOGÍA CELULAR Y MOLECULAR DE PLANTAS

UNIVERSIDAD DE GRANADA

FACULTAD DE CIENCIAS

DOCTORADO EN BIOLOGÌA AGRARIA Y ACUICULTURA

MICROSPORE EMBRYOGENESIS INDUCTION IN HOT PEPPER GENOTYPES

TESIS DOCTORAL

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GRANADA 2013

Editor: Editorial de la Universidad de Granada
Autor: Salvatore Pelliccione
D.L.: GR 527-2014
ISBN: 978-84-9028-814-6

MICROSPORE EMBRYOGENESIS INDUCTION IN HOT PEPPER GENOTYPES

Memoria presentada por el licenciado Salvatore Pelliccione

para optar al grado de Doctor.

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VºBº

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EEZ-CSIC

Universidad de Granada

2013

Adela Olmedilla Arnal, Investigador Científico de OPIS en el Consejo Superior de Investigaciones Científicas (CSIC),

Informa:

Que el trabajo de investigación titulado "MICROSPORE EMBRYOGENESIS INDUCTION IN HOT PEPPER GENOTYPES", realizado por el licenciado Salvatore Pelliccione bajo mi dirección, se considera ya finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en la Universidad de Granada.

Granada, Julio de 2013.

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Este trabajo ha sido realizado en el Departamento de Bioquímica, Biología Celular y Molecular de Plantas de la Estación Experimental del Zaidín (CSIC-Granada) y en las instalaciones de la empresa Monsanto Agricultura España, El Ejido, Almería gracias a una beca predoctoral JAE-Pre-DOC del CSIC cofinanciada por FEDER. La colaboración con la empresa De Ruiter Research España S.L. (en la actualidad Monsanto Agricultura España) se materializó en el contrato empresa/CSIC: 090201080023, nº de OTT: 20090037.

Parte de los resultados de este trabajo han sido presentados en los siguientes congresos y publicaciones:

Publicaciones:

SERRANO I., PELLICCIONE S., OLMEDILLA A. Programmed-cell-death hallmarks in incompatible pollen and papillar stigma cells of *Olea europaea* L. under free pollination Plant Cell Rep 29 (6): 561-572. 2010. (Que facilitó la puesta a punto de las técnicas de Biología celular aplicadas en la Tesis).

Congresos:

PELLICCIONE S., SERRANO I, SERRATO A., SAHRAWY M., OLMEDILLA A. In situ localization techniques for the evaluation of the role of thioredoxins in plant reproduction. Joint Congress of the Spanish and Portuguese Microscopy Societies. Segovia (España) 2009. (Presentación de resultados autorizada por la empresa Monsanto).

PELLICCIONE S., SERRANO I, OLMEDILLA A., RODRÍGUEZ GARCÍA MI. Estudio celular y ultraestructural de la evolución de la superficie del stigma de olivo durante la polinización. XXIII Reunión Bienal de la SME. Bilbao (España) 2007. (Que facilitó la puesta a punto de las técnicas de Biología celular aplicadas en la Tesis).

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A' cos' cchiù important'
è o' rispett'.

Totonn' Pelliccion'

Agradecimientos

En primer lugar agradecer a mi directora de tesis la Dra. Adela Olmedilla por ser mi guía y apoyo durante estos años, desde el punto de vista científico y humano.

A todos los miembros de la EEZ y de Monsanto que colaboraron en la realización de esta Tesis.

A las Dras. Ana Castillo y Moonza Kim por permitirme trabajar en sus laboratorios, dedicarme parte de su tiempo y compartir sus conocimientos sobre embriogénesis conmigo.

A las Dras. Ana Pretova y Beata Barnabas por abrirme las puertas de sus laboratorios y permitirme conocer otras formas de trabajar en el laboratorio.

A las familias Tomás-Jiménez, Fernández-Ortega y Serrano-Valdivia que han sido y siguen siendo mi hogar desde mi llegada a España hasta ahora y han contribuido a que sea más leve cargar con el peso de la ausencia de mi familia.

A mis compañeros Irene, Rosa y Gabriela por tantos momentos compartidos dentro y fuera del laboratorio.

A Mariángeles y Rosabel que han sido un descubrimiento valioso en la mina de la vida.

A Emilia, demonstración viviente de que amigos verdaderos se pueden encontrar a cualquier edad, que ha sido y sigue siendo mi hermana, amiga y en algunas ocasiones como una madre.

Finalmente, mis más sentidos agradecimientos para todas aquellas personas con la que me he cruzado por este camino y que no están nombradas por aquí.

Desidero non solo ringraziare ma soprattutto dedicare questa tesi a tutti quelli che pensano con la propria testa ma sanno anche quando il cuore deve avere la meglio, a quelli che risolvono i loro problemi in silenzio e sono capaci di fare il bene dove gli altri vedono il vuoto, a quelli che scelgono la direzione opposta al traguardo, agli ultimi, agli esclusi, ai perdenti e a tutti quelli che questa società malata considera come tali.

Mamma, Papà, Zio Antonio, Zia Mena, Zio Mario, Zia Concetta, Zio Cenzo, Zia Antonia, Nonna Giovannina, Palmina, Giovanna e Alessandra hanno avuto il merito di insegnarmi a riconoscere in queste persone i veri esempi da seguire. Nei vostri silenzi e nelle vostre azioni sono stato ad osservarvi ed ho carpito i

vostri segreti.....piano piano.....zitto zitto e poi.....poi mi sono reso conto di essere felice, lo sono sempre stato, grazie a voi ed al vostro esempio.

Resumen

El pimiento (*Capsicum annuum*) es una especie ampliamente utilizada y cultivada tanto en campo abierto como en invernadero. En particular, el pimiento picante tiene una gran variedad de aplicaciones tanto alimentarias como farmacéuticas por lo que su interés económico crece día a día.

La inducción de la embriogénesis de las microsporas mediante el cultivo de anteras y de microsporas aisladas es una herramienta biotecnológica muy poderosa en la mejora de plantas a través de la producción de plantas doble-haploides de una forma rápida y eficaz. Sin embargo, en el pimiento picante sólo se ha conseguido inducir la embriogénesis mediante cultivo de anteras o de microsporas en un número muy reducido de genotipos. El objetivo principal de esta tesis ha sido el desarrollo de un protocolo eficiente para la inducción embriogénica mediante cultivo de anteras y microsporas aisladas en pimiento picante. Así mismo, se ha realizado el análisis de los cambios morfológicos e histoquímicos producidos durante este proceso. Para ello, se analizaron y optimizaron distintos protocolos de cultivo de anteras y de microsporas aisladas. Además, se realizó un estudio celular e histoquímico al microscopio óptico de microsporas y anteras en distintas fases de la embriogénesis.

Este trabajo se llevó a cabo en cuatro genotipos (3 picantes: Serrano, Jalapeño y CM334 y 1 dulce: Lamuyo) seleccionados de entre los disponibles en Monsanto, empresa en colaboración con la cual se desarrolló esta tesis. Con los procedimientos ensayados se pudo comprobar la gran influencia que el genotipo tiene en el proceso de inducción de la embriogénesis tanto mediante el cultivo de anteras como de microsporas aisladas. Se comprobó así mismo que las anteras de los genotipos picantes sufrían una considerable oxidación durante el cultivo que podía evitarse mediante el uso de carbón activo que además estimulaba la producción de embriones en el genotipo dulce. Aunque inicialmente se probaron los procedimientos adaptados para genotipos picantes los resultados más eficientes para el cultivo de anteras se obtuvieron con un procedimiento que

optimizamos a partir del de McComb y McComb (1977), que había sido empleado con éxito en otra Solanácea. Este procedimiento (MC1) en el que hemos reducido la concentración del carbón activo y sustituido la sacarosa por maltosa como fuente de carbono no incluye el uso de hormonas. Este método sencillo, repetitivo y económico no requiere de subcultivos que compliquen el procedimiento y aumenten el riesgo de contaminación de las anteras. En el genotipo Serrano, el que mejor responde con este método, produce más de 10 embriones perfectos por cada 100 anteras cultivadas.

En cuanto a la embriogénesis inducida mediante cultivo de microsporas aisladas, los mejores procedimientos son los que utilizan el método de aislamiento de las microsporas de Soriano *et al*. (2008) optimizados utilizando los medios de cultivos y pretratamientos del MC1. Con este método de aislamiento tanto el procedimiento que adicionan n-butanol en el pretratamiento como el que no incluye este alcohol los mejores resultados se obtuvieron en el genotipo Serrano con alrededor de 5 embriones por cada 100 anteras.

El análisis celular de la embriogénesis inducida mediante cultivo de anteras y microsporas aisladas mostró que uno de los primeros signos de inducción embriogénica era el aumento del tamaño de las microsporas, sin embargo dicho aumento no garantizaba la producción de embriones. Además, se comprobó que dicha inducción produce divisiones simétricas y asimétricas en la microsporas siendo las primeras más abundantes.

Por primera vez se ha descrito la formación de estructuras semejantes al suspensor en los embriones inducidos tanto mediante el cultivo de anteras como el de microsporas aisladas en genotipos de pimiento dulce y picante.

Summary

Pepper (*Capsicum annuum*) is a widely used and cultivated crop both in open field and greenhouses. Hot pepper, in particular, has a great variety of food and pharmaceutical applications and that is why its economic interest is constantly increasing.

The induction of microspore embryogenesis by anther and isolated microspore culture is a very powerful biotechnological tool for plant breeding allowing the production of double-haploid plants in a fast and efficient way. However, microspore embryogenesis induction by anther or isolated microspore culture has only been achieved in a reduced number of genotypes.

The main objective of this thesis was the development of an efficient protocol to induce in hot pepper microspore embryogenesis by anther and isolated microspore culture. Furthermore, morphological and histochemical changes produced during this process were analyzed. To carry out these objectives, different procedures of anther and isolated microspore culture were tested and optimized. Moreover, a cellular and histochemical study of anthers and microspores at different stages during microspore embryogenesis was achieved by light microscopy.

This work was carried out in four pepper genotypes (3 hot genotypes: Serrano, Jalapeno and CM334 and 1 sweet genotypes: Lamuyo) selected from those available in Monsanto, a company with whom this thesis was developed.

The procedures tested showed the great influence of genotype in microspore embryogenesis induced by both anther and isolated microspores culture. Moreover, it was found that the anthers of hot genotypes suffered a considerable oxidation during culture that could be avoided by the use of activated charcoal which can stimulated embryo production in the sweet genotype.

Summary

Although initially we tested procedures adapted for hot pepper genotypes, the most efficient results in anther culture were obtained with a procedure optimized from McComb and McComb (1977), which was successfully used in another *Solanaceae*. This procedure (MC1) where the concentration of active charcoal has been reduced and sucrose was replaced by maltose, as carbon source, avoid the use hormones. Therefore, this procedure which is simple, repetitive and not expensive does not require subcultures that complicate the process and increase the risk of contamination of the anthers.

In Serrano, which is the genotype that better responds to this procedure more than 10 perfect embryos per 100 cultured anthers were obtained.

Regarding microspore embryogenesis induction by isolated microspore culture, the best procedures were obtained by using the microspore-isolation method of Soriano *et al*. (2008) optimized by using culture media and pretreatments of MC1 anther culture procedure. With this method of microspore isolation both in the procedure which added n-butanol and in the procedure that do not add this alcohol in the pretreatment, around 5 embryos per 100 anthers were obtained with the hot Serrano genotype.

The cellular analysis during microspore embryogenesis induction by both anther and isolated microspore culture showed that one of the first signs of the embryogenic induction was the increase in microspore size; however this increase did not guarantee the production of embryos. Furthermore, it was found that during the embryogenic induction, asymmetric and symmetric divisions occurs being the first more abundant. The formation of suspensor-like structures was described in hot and sweet pepper genotypes for the first time after microspore embryogenesis induction both in anther and isolated microspores culture.

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Introduction

1. *Capsicum spp.* general aspects.

The genus *Capsicum* is a genus of angiosperms belonging to the *Solanaceae* family, it consists in about 22 wild species and five domesticated species: *C. annuum*, *C. chinense*, *C. frutescens*, C. *pubescens*, and *C. baccatum* (Bosland 1996). The genetic variability among the domesticated species is an interesting characteristic of the genus *Capsicum*. *Capsicum spp*. are diploids ($2n=2x=24$) and have a high range of morphological variations including color, size, taste and shape of the fruits. One of the principal differences between wild and domesticated *Capsicum* is that the fruits of the wild forms are smaller, red-colored and deciduous while domesticated forms are extremely variable in fruit size, fruit and flower color and usually retain the fruit on the peduncle at maturity (Pickersgill 1969, Eshbaugh 1976). Furthermore, *Capsicum* fruits can be classified as sweet or hot depending on their taste and pungency. *Capsicum* plants are semi-perennial but are grown as annual in cultivation. Its size can vary from 20 cm to about 1 m in open field while in greenhouses may reach more than 2 m. Flowers are hermaphrodite, actinomorphic and developed at the node, having white corolla with 5-6 white petals and a short green calyx with 5-6 sepals. The androecia are composed by 6 stamens with white-yellow anthers turning purple when pollen grains matured, while gynoecia have a superior ovary with 2 locules and a pistil with a simple or bi-lobed stigma. The length of the style and the position of the stigma and the anthers vary between genotypes affecting cross pollinations rate. Morphological characteristics of the flowers and mainly of the fruits are used to distinguish different species of *Capsicum* (Fig.1).

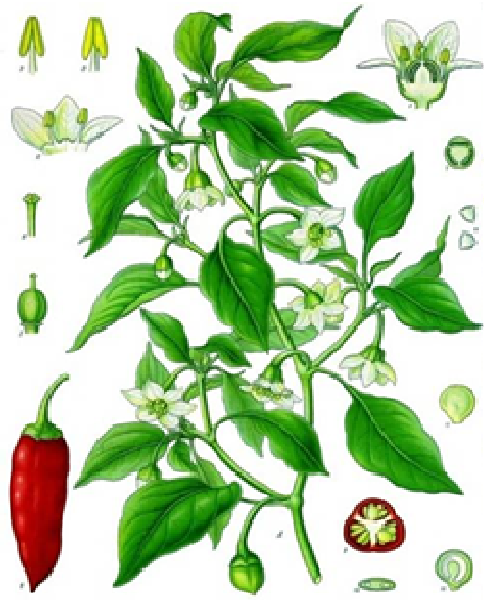


Fig. 1. Capsicum annuum in Franz Eugen Köhler, 1887 showing flowers and fruits details.

The terms used to name species of *Capsicum* are confusing because of the high number of expression used. Words as: pepper, chili, chile, aji, paprika, and *Capsicum* are used interchangeably in the literature, in next pages we will use *Capsicum* when referring to the genus while we will use hot and sweet pepper referring to pungent or non pungent varieties.

Plant flowering begin 1-2 months after seedling and fruiting stand usually one month after flowering. Plants can be self- or cross- pollinated being the self-pollination more frequent. Cross-pollination can be carried out by wind or by different species of insect such as *Coleoptera*, *Aphididae*, or *Apis*. Seeds are plane and circle-shaped and present usually a creamy/yellow color while some wild species have black seed color (Zewdie and Bosland 2003). The seeds of *Capsicum* plants are predominantly dispersed by birds. Hot pepper specifically present a kind of parallel evolution with birds that are insensitive to capsaicin

because of their lack of capsaicin receptors or because their receptors are insensitive to capsaicin.

Capsicum spp. represents one of the ancient crops utilized nowadays and probably the earliest domesticated plant in the ‘’New World’’. It is documented by archeological studies like those carried out at Tehuacán Valley in Mexico where remains of hot pepper where found in human coprolites and at Chavín de Huantar in Peru where representations of *Capsicum* fruits were found in Tello's obelisk, that Native Americans introduced *Capsicum* in their diet and used it for medical and ritual issues since about 8000 years ago (MacNeish 1964, Davenport 1970).

The origin and domestication of the genus *Capsicum* are controversial, in spite of the fact that all of the documented species seems to have originated in Central and South America; nowadays many authors indicate two areas of the domestication of *Capsicum annuum* in Yucatán region and Bolivian part of the Amazonia as the results of two different domesticating events (Eshbaugh 1976, Perry *et al.* 2007, Aguilar-Meléndez 2009). Even considering the domestication of the genus *Capsicum* by Native Americans one of the pillars of *Capsicum* agricultural history, the incontrovertible most important event was the introduction in Europe, Asia and Africa by Spanish and Portuguese expeditions during the Age of Discovery (Fig. 2).

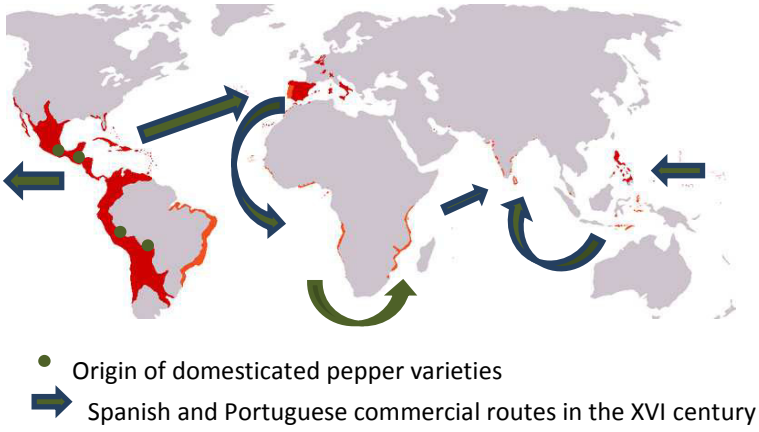
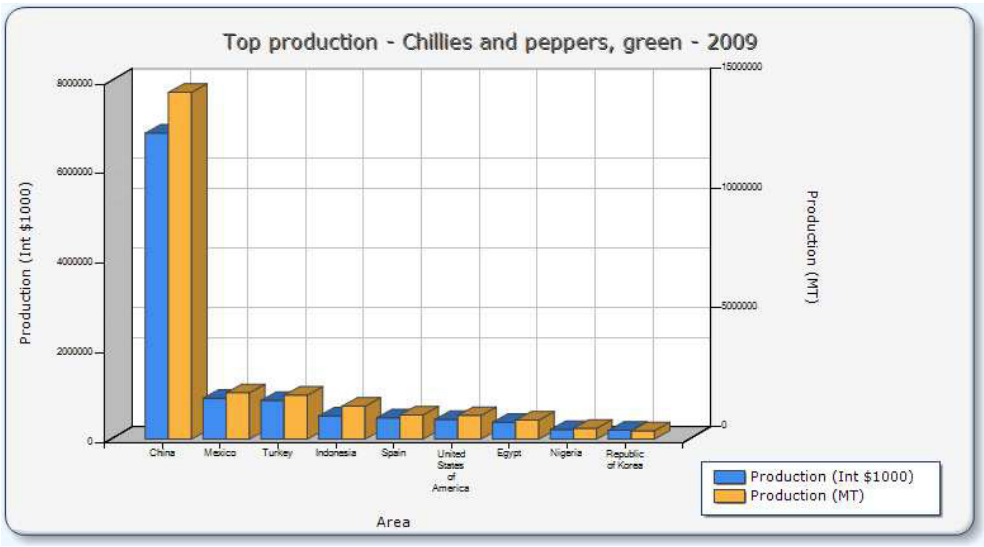


Fig. 2. Origin and possible diffusion of hot pepper in the XVI century (Modified from Cambridge Modern History Atlas, 1912 and Wikipedia.org).

Columbus mentioned the "ají" (name used by native people) in his first expedition when arrived to ''Hispaniola'' and Gonzalo Fernández de Oviedo in its 'Historia General y Natural de las Indias' clearly mentioned this hot pepper as a common component of native's diet. By the time the first European expedition reached America, native people had already developed a lot of varieties of hot peppers (Bosland 1996). Since the introduction in Europe in 1493 by Columbus the diffusion of the *Capsicum* all over the world was very fast, competing with black pepper (*Piper nigrum*), one of the most expensive spices since the Middle Age. The reason of the rapid diffusion was the ability of the *Capsicum* to acclimatize to the 'old world' conditions facilitating its access to everybody. Nowadays many of the domesticated *Capsicum* varieties by Native Americans remain cultivated and consumed only at regional scale and lack an intensive and large-scale cultivation.

2. *Capsicum spp.* economic aspects.

Capsicum spp. are adapted to a wide range of temperatures and precipitation amounts and are a common crop practically all over the world, both in open fields and greenhouses. Nowadays hot pepper is one of the most cultivated crops in the world, it represents about 40% of total world spices production in volume terms (FAO), and this data can only give an idea of the commercial importance of this crop. The most economically important species among *Capsicum spp.* are: *C. annuum*, *frutescens* and *chinense* being *C. annuum* the most widespread both in use and culture. World production of hot pepper as estimated by FAO in 2009 was about 28 millions of tons; China is the largest producer in the world for fresh hot pepper, followed by Mexico, Turkey and Indonesia. Spain is the fifth producer in the world and the largest producer in Europe of fresh hot pepper. India is the first world producer for dry hot pepper (Fig. 3) (faostat.fao.org).



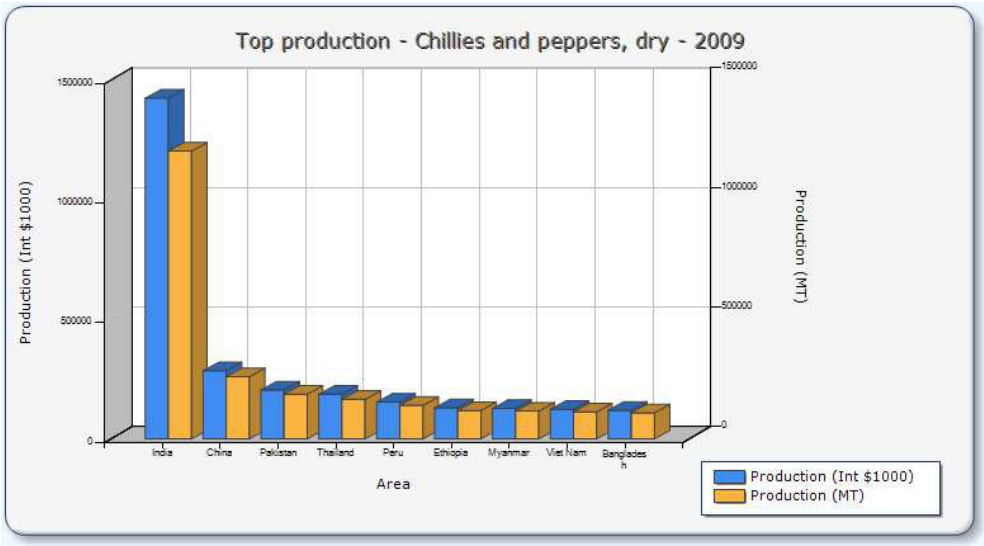


Fig. 3. Top production of dried and fresh hot pepper in 2009 (faostat.fao.org).

In the last 20 years hot pepper production has tripled due to the crescent interest that hot pepper gained in food and pharmaceutical industry. Similar to open field crops, greenhouse hot pepper production has increased significantly, because with greenhouses is possible to produce hot peppers when production in open fields it is not possible and market prices for peppers are highest. The largest greenhouse producing areas in Europe are Spain, Italy, France and Greece. The great amount of the Spanish greenhouse industry centers on Almeria and Murcia along the Mediterranean coast where *Capsicum* production represents the second crop in terms of production after tomato. Additionally to the fresh or dried hot-pepper fruits, it is possible to find an enormous variety of products in the World market derived from hot pepper used in the food, pharmaceutical and cosmetic industries. They have especial importance in the preparation of hot sauces and as coloring agents in food and cosmetics. In pharmaceutical industry hot pepper is used in the preparation of wax acting as insect repellent, spray for self-defense and dermatological balms for the relief of itchy skin, psoriasis, muscle aches, or pain from osteoarthritis and rheumatoid arthritis. The most reliable value of hot pepper fruits is due to the presence of

carotenoids, flavonoids, ascorbic acid, phenolic compounds and capsaicinoids. Capsaicinoids are alkaloids compounds unique from the genus *Capsicum* that are responsible for pungency in hot pepper. The principal alkaloid present in hot pepper fruits is capsaicin (8-methyl-N-vanillyl-6-nonenamide) which is synthesized and accumulated in the epidermal cells of placenta of the fruits (Bosland 1996). Capsaicin is probed to have anti-inflammatory and analgesic activities and is used in creams as a topical analgesic in many commercial formats; capsaicin is also known to have anti-mutagenic and anti-carcinogenic properties. Medicinal use of hot pepper has a very old and documented history, Mayas used hot pepper into medical preparations to treat, coughs, painful throats, infected wounds and fresh burns (Cichewicz and Thorpe 1996). Aztecs used it to alleviate toothaches (Bosland 1996). Capsaicin is probed also as anti-pathogen compound, but its pungent characteristics limit its application, nowadays analogous of capsaicin (capsinoids) are used to make resistant several sweet varieties without altering its non-pungency (Veloso *et al*. 2013).

B-carotenoids and vitamin C are present in hot pepper fruits in high quantity; they are recognized as anti-oxidant compounds, especially vitamin C, isolated for the first time by the Nobel Prize Albert Szent-Gyorgyi from hot pepper fruits and its content is higher than in citrus fruits. Nowadays due to the crescent interest in human diseases, antioxidants have been more and more popular because of their direct or indirect interaction with reactive oxygen species (ROS) which produce oxidative stress in the human body (Percival 1998). For all the above mentioned characteristics hot pepper is gaining more and more impact as crop and a crescent interest from pharmaceutical and seed companies. Furthermore, the increasing demand for early high-quality varieties adapted to the greenhouse production have led seed companies to introduce hot pepper breeding programs to extend the offer in hot pepper varieties.

3. Hot pepper improvement as a target for international agriculture.

Plant breeding starts with the domestication of wild plant by ancient civilizations coinciding with the permanent and semi-nomad way of life. The *Solanaceae* family is composed of 102 genera and nearly 2500 species including some of the most cropped and used vegetables in the world such as potato, tomato and pepper. In the last decades plant breeding has been strongly influenced by the globalization of the production and commercialization of the agricultural products. The increasing interests in hot pepper in particular for food, pharmaceutical and cosmetic industries has lead seed companies to establish extensive hot pepper breeding programs and today all over the world hundreds of new varieties are produced per year. The development and distribution of hybrid pepper seed has led to an explosion of new cultivars during the last 20 years (Crosby 2008). Hot pepper breeding program, as a typical breeding program, follows market trends. Its objectives are to produce new cultivars having disease resistance, earliness, vigor and adaptation to the greenhouse production. Other searched characters are yield, stress tolerance, color and shape.

Hot pepper in particular represents a breeder's challenge because of the diversification in the use of the fruits. Fresh market pepper exigencies are in fact different than those for processing or spice markets. In addition, nowadays consumers and food industries are being more and more exigent not only for color, flavor, shape of the fruits and degree of pungency but also in the quality and quantity of bioactive products present in fruits. That is why breeding programs are beginning to be focused on the genetic control of these compounds (Liu *et al*. 2009). The development and improvement of new cultivars of hot pepper have been achieved by different methods of plant breeding including hybridization followed by traditional selection and molecular marker-assisted selection. The only breeding technique that is not applied, or far from being

employed, in hot pepper is the genetic transformation.

4. Genetic engineering in hot pepper.

Plant genetic transformation is one of the most recently developed biotechnological tools for crop improvement. Transformed plants give scientists and breeders greater insight into the biology and genetics of plants (Ulukan 2009). Some of the problems in conventional plant breeding programs such as sterility or incompatibility can be overcome by the use of genetic engineering techniques (Oldach *et al.* 2001, Doebley *et al.* 2006). The possibility of inserting a gene from wild species in a single step to the selected variety in order to provide resistance or other beneficial characteristics without compromising agricultural quality and without the need for extensive back-crossing makes genetic engineering in plants very attractive (Wilson 1993). Leaving out the legal and social problems regarding the introduction of genetic modified organisms (GMOs), the main problem of the development of transgenic plants is recalcitrance for transformation and regeneration that certain plants and in particular *Capsicum* have. There are mainly tree ways to transfer an engineered gene into a plant chromosome: 1) the transformation by *Agrobacterium tumefaciens* that have the ability to transfer a part of its genetic information into the plant DNA, 2) the electroporation and DNA insertion into protoplasts and 3) the so-called 'gene gun', a technique that allows to shot a specifically engineered gene into plant cells using micro tungsten or gold particles (Manshardt 2004). Many researchers have tried to obtain GM *Capsicum* plants with these techniques but despite their continuous efforts, successful and efficient protocols of pepper transformation are very rare. The first transgenic plant of pepper was developed in 1990 by Liu *et al.*, but the protocol was not reproducible. Transformation have been carried out in *C. annuum*, both hot and sweet pepper (Zhu *et al*. 1996,

Manoharan *et al.* 1998), in *C. frutescens* (Wang *et al.* 1991) and recently in 2010 Arcos-Ortega *et al.* obtained a protocol to transform *C. chinense*, by *A. tumefaciens*, but the transformation efficiencies were very low. Moreover, in many cases the introduction of a gene or a gene pool in the genome of the target plant will have no effect if it is not expressed. Nowadays most of the methods described results in a random insertion on the genome recipient cells and the site of insertion may play an influence on the gene expression making useless all the work (Lupi 1995). Lim *et al.* in 1997 reported a highly efficient regeneration and transformation of hot pepper with a number of genes, including the herbicide resistant gene, *BAR*. They also reported stable inheritance of the gene in F2 progeny of the transgenic plants. In spite of this promising advancement, the accomplishment of transgenic pepper breeding has not yielded other successful results accompanied by scientific documentation (Steinitz *et al.* 1999, Liu *et al*. 2009).

The regeneration remains another big problem. One peculiar aspect of *Capsicum* in fact is the lack of ability to be regenerated from protoplasts, and both transformation and tissue culture are extremely dependent of the genotype and of the type of explant used (Kempken and Jung 2010). Several groups have described advances in plant regeneration by organogenesis and *in vitro* embryogenesis in *Capsicum* (Hyde and Phillips 1996, Binzel *et al.* 1996, Harini *et al.* 1993, Ebida and Hu 1993, Dumas de Vaulx 1981) but still we are far away to accomplish the effectiveness obtained in other key crops. Due to mentioned complex causes genetic engineering is not currently utilized to obtain new varieties in hot pepper.

5. Conventional breeding in hot pepper.

In conventional breeding, two closely related plants are sexually crossed to combine the favorable traits from both parent plants and exclude needless traits in a new and improved plant variety (Lupi 1995, Suslow *et al*. 2002). The scientific bases for plant breeding were provided by G. Mendel in 19's. Therefore it was in 1920 that researchers observed that the self-pollination for several generation of a species cause a decline in its vigor. In the other hand, if two self-pollinated plants are crossed (heterosis) their progeny called F1 hybrids, results often in a better vigor and yield. Heterosis coupled with the fact that the next generation (F2), due to the random assortment of the traits derived from the previous progeny, have a non predictable traits are the basis for the commercial production of hybrid seeds (Suslow *et al*. 2002, Birchler *et al*. 2003). New hot and sweet pepper cultivars have been developed and improved using self-crossing and inter-specific hybridization followed by selection methods. Almost all species of *Capsicum* are self-compatible with few exceptions like *C. cardenasii* (Bermawie and Pickersgill, 1992) and *C. buforum* (Tong and Bosland 2003). Another interesting characteristic of the genus *Capsicum* which can help breeders is the cytoplasmic male sterility, reported in *Capsicum frutescens* for the first time by Martin and Grawford in 1951. The available cytoplasmic male sterile mutants initially were not reliable and controllable so commercial seed production was obtained by hand emasculation and pollination, a very expensive way to obtain new varieties (Poulos 1994). Nowadays the cytoplasmic male sterility in *Capsicum* is largely used and proved to be an effective way of producing F1 hybrid (Yazawa *et al.* 2002, Dhall and Cheema 2010). On the other hand, inter-specific hybridization in breeding of *Capsicum* has encountered some barriers because of unilateral inter-specific incompatibility, and post fertilization seed abortion, present between members of the genus *Capsicum*. Unilateral inter-

specific incompatibility occurs when pollinations between species are successful in one direction but not in the other, for example it happens between *C. pubescens, C. cardenasii, and C. eximium* when pollen tubes reach and fertilize the ovules in one direction but are inhibited in the stigma, style or ovary in the other; it occurs also between other species of *Capsicum* (Bermawie and Pickersgill 1992, Onus and Pickersgill 2004). In *Capsicum* the principal causes of inter-specific incompatibility are embryo abortion and hybrid pollen sterility and nowadays there are few reports on the overcoming of these barriers (Bermawie and Pickersgill 1992, Yoon *et al.* 2006). The other challenging topic of *Capsicum* breeding is represented by post-fertilization seed abortion. Post-fertilization seed abortion is manifesting itself by a very slow development of the endosperm and consequent slow development of the embryo, abnormalities are visible in the endothelium and after about two weeks the embryo sac collapses leading to the death of the seed. Post-fertilization seed abortion is characteristic of several inter-specific crosses in *Capsicum* (Pickersgill 1991, Bermawie 1990). Modern plant breeding combining new biotechnologies and conventional breeding techniques allow to by-pass the previous difficulties for breeding new varieties of *Capsicum.* This modern breeding enables breeders to produce genetically uniform lines within one generation saving the time spent in the process of self-pollination and selection required to produce a breeding plant.

6. Doubled haploid technology as a powerful tool for plant breeding.

Biotechnology offers powerful tools for plant breeding, and tissue culture in particular can help in plant improvement and selection. The induction and regeneration of haploids followed by spontaneous or induced chromosomes doubling, called doubled haploid technology, reduces the generation time and allows producing genetically uniform lines within one generation. Time saving is

significant, mostly in biennial crops and in crops with a long juvenile period, therefore it is a very interesting technique for breeders; it also occurs in nature but at a very low frequency (Suslow *et al.* 2002, Forster *et al.* 2007, Germaná 2011, Murovec and Bohanec 2012). The available methods for haploid production are the *in vitro* culture of male gametophyte (anther and microspore culture), called microspore embryogenesis and the *in vitro* culture of female gametophyte (ovule and ovary culture) called gynogenesis. The number of species responsive to microspore embryogenesis is more or less ten times higher than the number of responding species to gynogenesis being *Compositae* an exception (Chupeau *et al.* 1998). The first natural occurrence of haploids was observed in 1922 by Blakeslee *et al.* in *Datura stramonium*, but it took many years to obtain the first double haploid (DH) crop plant, a cultivar of *Brassica napus* (Thompson 1972, Forster *et al.* 2007). Nowadays, DH technology has been successfully applied to more than 250 plant species most of which are of agricultural interest (Thomas *et al.* 2003, Maluszynski *et al.* 2003). There are crop species with a great ability to regenerate plants from cells, tissues, and organs, but this is not the case of *Capsicum* in which DH technology have not progressed so far. In *Capsicum*, microspore embryogenesis induction, plant regeneration and chromosome doubling are strongly genotype dependent and limit the application of DH technology to small number of genotypes (Forster *et al.* 2007, Chang and Coe 2009). Anther culture and microspore culture are predominantly focused on the development of efficient culture systems to obtain double haploids in recalcitrant crops such as *Capsicum*.

7. Microspore embryogenesis in *Capsicum.*

The success in the obtaining haploid plants by microspore embryogenesis in several *Solanaceae* species (Guha and Maheshwary 1964, Sunderland and

Wicks 1971) led different groups to try this procedure in *Capsicum* and the first haploid plants were obtained by anther culture by Wang *et al.* (1973) and by George and Narayanaswamy (1973). Then a group in France continues this work (Sibi *et al.* 1979) and it was in 1981 when Dumas de Vaulx *et al*. published an efficient protocol to obtain double haploid plants from *Capsicum* (Dumas de Vaulx *et al.* 1981).

Microspore embryogenesis involves the reprogramming of microspore development from the normal gametophytic pathway to the sporophytic pathway to obtain haploid plants from the culture of a single cell that will be used to produce homozygotic double-haploid plants (Raghavan 1986, Maraschin *et al*. 2005, Olmedilla 2010). It has been demonstrated, by many authors, which a stress pretreatment is required for microspore embryogenesis induction; although the mechanisms involved are still not completely understood (Touraev *et al*. 1997, Shariatpanahi *et al*. 2006). Stress pretreatment can be applied directly to the plant, to the excised flower buds, to the anthers or isolated microspores in the *in vitro* culture (Touraev *et al.* 1997, Shariatpanahi *et al.* 2006). The induction of embryogenic microspores after the stress event is associated with structural and biochemical changes in the microspores, being the most common: increase of microspores dimension, cytoskeletal and cytoplasmic rearrangement leading to the formation of star-like structures, chromatin condensation, intine enlargement, and changes in gene expression (Thorpe 1995, Babbar *et al.* 2004, Zoriniants *et al.* 2005, Aionesei *et al.* 2005, Shariatpanahi *et al.* 2006, Olmedilla 2010).

There are different routes leading to microspore embryogenesis identified from different plant genera. In addition, the routes may vary in the same species and depending on the condition of induction (Sunderland 1974). Five of them have been described and imply: A) division of the vegetative nucleus after asymmetrical division, B) initial symmetrical division, C) asymmetrical division followed by fusion of the generative and vegetative nuclei that then divide, D) asymmetrical division followed by division of both nuclei and E) asymmetrical

division followed by the division of the generative cell. (Fig. 4) (Raghavan 1986, Babbar *et al.* 2004).

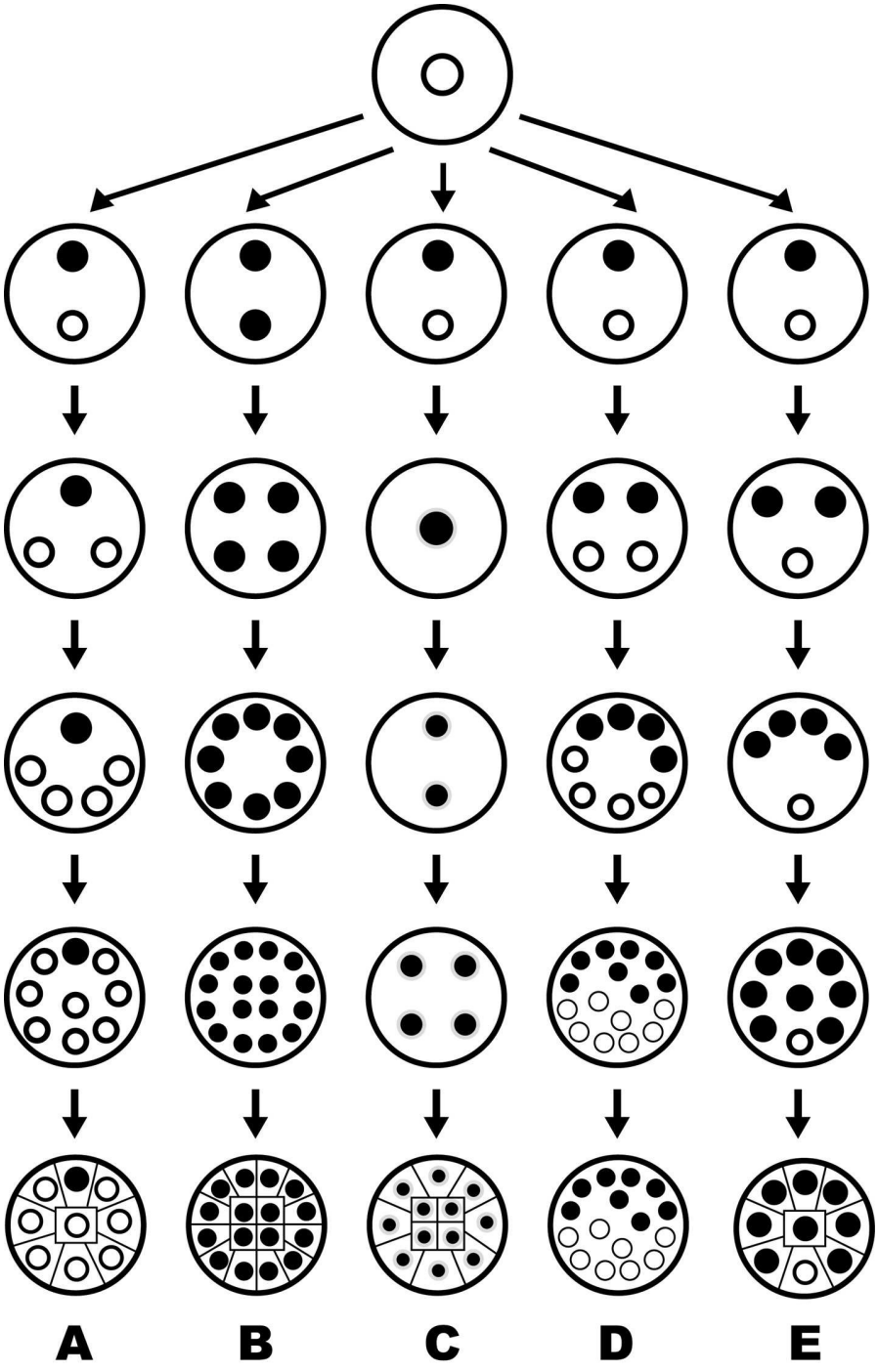


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Fig. 4. Microspore embryogenesis routes followed by immature pollen after embryogenic induction from Raghavan 1986.

The A) pathway where the vegetative cell divide itself to generate an embryo while the generative cell degenerates, is the most common and widely investigated (Raghavan 1986). In *Capsicum* it has been described that both symmetric and asymmetric division can occur after embryogenesis induction in anther culture but the most common is the induction trough asymmetrical division followed by the division of the vegetative cell (Kim and Jang 2000, Kim *et al.* 2004). In the B) pathway where the first mitotic division is a symmetric division, embryo is formed by multiple symmetrical divisions; it was described in *Nicotiana*, *Datura*, *Hordeum* and *Triticum* (Nitsch 1972, Zhou and Yang 1980, Bonet and Olmedilla 2000). The C) pathway was described in *Datura innoxia* and *Hordeum* (Sunderland 1974, Sunderland and Evans 1980) in this pathway the vegetative and generative cells fuse each other. The embryos are formed by repeated divisions of the cells derived from the fusion and are not haploid embryos (Raghavan 1986). The D) pathway was observed in *Hyoscyamus niger*, *Nicotiana tabacum* and also in several cereals (Raghavan 1978, Sunderland *et al.* 1979, Anand *et al.* 1980) this route implies both vegetative and generative nuclei division and there is a contribution of both vegetative and generative cell to embryo formation. The last pathway described is the E) pathway, where the generative cell divide itself to form the embryo while the vegetative cell degenerate directly or sometime after few divisions and it was described only in *Hyosciamus niger* by Raghavan (1978).

Nowadays the pathways followed by microspores during the induction of microspore embryogenesis are known in different species, it is also known that the same species and even the same anther of a plant can follow different pathways, but the reasons of the presence of these different pathways and the reason why an immature pollen can follow one or another pathway during the embryogenic induction are still unknown.

8. Factors affecting microspore embryogenesis.

The main factors involved in microspore embryogenesis induction are: genotype and physiological status of donor plant, stress pretreatment, microspore developmental stage, and media used for culture and regeneration (Dunwell 1979, Raghavan 1986, Touraev *et al*. 1996, Smykal 2000, Olmedilla 2010).

In *Capsicum*, microspore culture effectiveness is strongly influenced by the above mentioned factors; in fact there are different protocols concerning isolated microspore and anther culture to induce microspore embryogenesis on it (Wang *et al*. 1973, George and Narayanaswamy 1973, Dumas de Vaulx *et al*. 1981, Supena *et al*. 2006, Kim *et al*. 2008, Irikova *et al*. 2011).

8.1. Genotype.

The genotype dependency is a critical factor affecting the variability of response in microspore embryogenesis. The genotypes selected for the microspore embryogenesis induction can respond to it in many different ways, even in the same genera or family as it could be *Solanaceae*.

Solanacea members such as *Nicotiana* and *Datura* are responsive to microspore embryogenesis and considered as model plant for this process (Raghavan 1986, Belogradova *et al.* 2009), while *Capsicum* and *Solanum*, members of the same family, are recalcitrant to double haploid technology (Ferrie and Caswell 2011) and even when considering *Capsicum* there is a strong difference between the responsiveness of sweet and hot pepper genotypes. Many hot-pepper genotypes are nowadays practically recalcitrant to microspore embryogenesis induction being the number of successful induction references very limited (Supena *et al*. 2006, Kim *et al.* 2008, Irikova *et al*. 2011). These data indicate the existence of a genetic control on microspore embryogenesis

induction. Genotype of donor plant in *Capsicum*, as in many other species, influences not only the induction of embryogenesis but also the success in plant regeneration (Mityko and Fari 1997, Irikova *et al*. 2011).

8.2. Physiological status and age of the donor plant.

The physiological conditions of donor plants can affect anther development and consequently the number of embryogenic microspores induced (Raghavan 1986, Olmedilla 2010, Irikova *et al*. 2011). Important factors affecting the physiological status of the plant include proper growth conditions of light, temperature, humidity and nutrients.

The optimal growth conditions for *Capsicum* have been tested both in greenhouses and in open field (Dorland and Went 1947). *Capsicum* plants can be damaged by frost and in addition, flowering is drastically affected by changes in temperature. The optimal day temperature range for *Capsicum* is between 24ºC and 30ºC while the optimal night temperature range vary from 15ºC to 18ºC. Night temperatures are one of the most critical factors for *Capsicum* growth and strongly affect flowering time (Dorland and Went 1947).

Furthermore, significant seasonal variations in microspore embryogenesis induction via anther culture have been observed in different species including pepper grown both in open field and greenhouses (Ercan *et al.* 2006, Germaná 2011).

Another important factor affecting microspore embryogenesis is the age of donor plant. Many studies in different species demonstrated that this factor strongly influence the frequency of responding anthers and the number of embryogenic microspores (Raghavan 1986, Germaná 2011, Irikova *et al*. 2011). Maheshwary *et al.* (1982) noticed that embryo yield is higher in anthers from first flowering of many species. In *Capsicum* embryo yield decrease when anthers from older plants are utilized for microspore embryogenesis induction (Kristiansen and Andersen 1993). It has been reported in *Nicotiana* that older

plants at the end of flowering, produce small buds which contain anthers with higher number of abnormal pollen affecting negatively microspore embryogenesis induction (Dunwell and Perry 1973).

All these data confirm the strong influence of the age of donor plant as well as the influence of the flowering period. In general, it is better for induction success to use first flowering from new plants.

8.3. Microspore developmental stage.

Another important aspect for the success of microspore embryogenesis induction is the appropriate selection of the developmental stage of the microspores that are going to be used for the embryogenesis induction. The size and morphology of flower buds and the presence of an anthocyanin color on the anthers can be used as an indirect indicator for determining microspore stage development in *Capsicum* (Sibi *et al*. 1979, Irikova *et al.* 2011). These indirect characteristics have to be determined for each *Capsicum* genotype because in many cases, there are strong differences in bud morphology depending on the different genotypes (Irikova *et al*. 2011). Furthermore, it has been demonstrated that the optimal stages for microspore embryogenesis induction are also highly genotype dependant. The most common stages used for microspore embryogenesis in many different species are late vacuolated microspores and young bicellular pollen (just before or immediately after the first mitosis) (Sangwan and Sangwan-Norrell 1987). In agreement with this general assessment, in *Capsicum*, these stages described as uninucleate stage and early binucleate stages are found to be the most suitable to induce microspore embryogenesis (Dumas de Vaulx *et al.* 1981, González-Melendi *et al.* 1995, Kim *et al.* 2004). The differences in terminology to design these stages are due to the fact that DAPI staining is used for their selection and with this technique only the nuclei are visible.

There is no agreement in the selection of the best stage to obtain the

highest efficiency in microspore embryogenesis induction in hot pepper. There are reports for anther culture in which the best stage was vacuolated microspore (Supena *et al*. 2006) and others reports where the best stage was early bicellular pollen (Kim *et al.* 2004). In the same way, there are reports for isolated microspore culture in which the best results were obtained when the culture was enriched in microspores at vacuolated stage (Lantos *et al.* 2009) and other reports where the best results were obtained when mainly early bicellular pollen was cultured (Kim *et al*. 2008).

8.4. Anther vs isolated microspore culture.

Anther culture is commonly used to obtain double haploids in hot pepper via microspore embryogenesis (Qin and Rotino 1993, Kim *et al.* 2004, Supena *et al.* 2006, Irikova *et al*. 2011) because it requires fewer steps than isolated microspore culture, and therefore it is easier and there are fewer chances to introduce contamination in the culture. In spite of these advantages in anther culture, when a dihaploid embryo is obtained, it is not possible to determine *a priori* if it comes from the sporophytic tissue of anthers or if it is originated from a spontaneously-diploidized microspore. This fact can interfere with the study of the mechanism of microspore embryogenesis induction. Furthermore, in anther culture the nutrients uptake by microspores depends on the anther position in the media which can influence their embryogenic responsiveness (Touraev *et al.* 2001).

Although there are fewer reports to be consulted, isolated microspore culture has also been used for embryogenic induction in hot pepper (Kim *et al*. 2008, Lantos *et al*. 2009). Different techniques have been employed for microspore isolation. In shed microspore culture, microspores are liberated in the medium after dehiscence of the anthers. Sunderland and Roberts (1977) used initially this method for *Nicotiana tabacum* and more recently Supena *et al*.

(2006) use it for *Capsicum*. Lantos *et al.* (2009) use whole anthers in homogenizers to isolate *Capsicum* microspores, their method was based on the one developed by Hunter (1988) for *Hordeum vulgare*. Microspores can be obtained also from whole flowers with a blender with a further elimination of anthers and flower buds debris by progressive filtering and centrifugation, this method has been applied successfully in hot pepper by Kim *et al.* (2008). Although anther culture is a widely used method in microspore embryogenesis induction, isolated microspore culture is acquiring more and more importance in plant breeding and in the microspore-embryogenesis research of mayor crop plants. Isolated microspore culture is needed to study the mechanisms of the embriogenic induction. However, in our knowledge only one procedure for double haploid plants production with high microspore embryogenesis induction efficiency has been published for hot pepper (Kim *et al.* 2008).

8.5. Stress Pretreatment.

The application of stress pretreatment to induce microspore embryogenesis is widely used when both anthers and isolated microspores are used for embryogenesis induction. It has been reported that cold, heat, starvation or a combination of these pretreatments can trigger the induction of microspore embryogenesis (Touraev *et al.* 1996, Shariatpanahi *et al.* 2006). Stress pretreatments can be applied to different types of explants (anthers, isolated microspores, flower buds, spikes) and also to the whole plant. In certain cases, a combination of pretreatments applied to one or more different explants or/and to the whole plant is needed. In different species it has been reported that the combined effect of the stresses mentioned above may positively influence microspore embryogenesis efficiency, and a very efficient procedure of isolated microspore culture using a combination of hot and starvation pretreatment has been applied by Kim *et al*. (2008) to induce microspore embryogenesis in hot

pepper.

8.5.1. Cold stress.

Cold as stress pretreatment was used in *Datura innoxia* by Nitsch and Norreel (1973) and have been used in many species as *Datura metel, Brassica napus, Nicotiana tabacum* both in anthers and microspore cultures (Sunderland 1980). In addition, low temperature regime for donor plants or different regime of temperature (high/low switch) has been reported to increase microspore response to the embryogenic induction in *B. napus* and *B. juncea* (Palmer *et al*. 1996). It has been also suggested that cold pretreatment has the capacity to synchronize the developmental stage of the microspores, allowing a better selection of the right stage to induce microspore embryogenesis and kept the microspores at the optimal stage selected during pretreatment (Touraev *et al*. 1997, Hu and Kasha 1999). Moreover, cold can reduce the degradation processes in the anther tissues and this fact can reduce the presence of toxic compounds in the culture media assuring a higher viability of the embryogenic microspores (Sunderland and Roberts 1979, Shariatpanahi *et al*. 2006). Furthermore, cold increases the total content of free amino acids leading to a better adaptation of microspores to the metabolic changes of the embryogenesis induction (Xie *et al.* 1997, Shariatpanahi *et al.* 2006).

In *Capsicum*, cold pretreatment has been applied to flower buds, anther and directly to isolated microspores leading to different induction results (Sibi *et al.* 1979, Morrison *et al*. 1986, Özkum and Tipirdamaz 2002, Supena *et al.* 2006, Irikova *et al.* 2011). Although for some authors cold shock pretreatment has a positive effect on microspore embryogenesis in *Capsicum* (Sibi et al. 1979, Morrison *et al.* 1986, Supena *et al.* 2006) other authors found that cold pretreatment has no positive effect on microspore embryogenesis (Özkum and Tipirdamaz 2002).

8.5.2. Heat stress.

Heat pretreatment has been used to induce microspore embryogenesis in *Solanaceae* species such as *Nicotiana tabacum* (Touraev *et al.* 1996), *Solanum melanogena* (Miyoshi 1996) and *Capsicum annuum* (Sibi *et al.* 1979, Dumas de Vaulx *et al.* 1981, Kim *et al.* 2004, 2008). It has been reported that during the heat shock pretreatment of anthers, loss of chlorophyll and degeneration of tapetum and anther-wall cells were observed; it was postulated that the death of these cells may liberate signal molecules that are able to activate the switch of the microspores to the sporophytic-development pathway (Wang *et al.* 2000). Heat shock proteins are associated with the acquisition of microspore embryogenic potential, irrespective of the stress pretreatment applied for the induction (Pechan *et al.* 1991, Barany *et al.* 2001). Without the production of these signaling proteins as the results of a stress response, microspore embryogenesis cannot be initiated (Pechan and Smykal 2001). Sibi *et al.* (1979) reported that the application of a heat stress to induce embryogenic microspores in *Capsicum* was more efficient than the application of a cold stress. A highly effective method for embryogenesis induction in *Capsicum* was obtained by Dumax de Vaulx *et al.* (1981) applying a heat shock pretreatment of 35ºC to the anthers. These authors also demonstrated that 8 days was the optimal duration of this heat pretreatment. Since then this heat pretreatment with slight modifications was successfully applied to many genotypes of hot pepper and nowadays is the most used stress pretreatment for microspore embryogenesis induction in sweet and hot pepper, both in anther and microspore culture (Qin and Rotino 1993, Rodeva *et al*. 2004, Kim *et al.* 2004, 2008, Irikova *et al*. 2011).

8.5.3. Starvation pretreatment.

Starvation pretreatment was found to be an efficient stress to induce embryogenesis in tobacco, barley and wheat (Touraev *et al.* 2001). It has been applied not only to many types of explants (anthers, isolated microspores, flower buds, spikes) but also to the whole plant (Heberle-Bors 1989). Starvation pretreatment can be achieved replacing sucrose or maltose in the media by mannitol, a non-assimilable carbohydrate. When mannitol is applied at higher concentrations, it modifies the osmotic pressure of the culture medium causing a combined stress pretreatment (starvation coupled with osmotic stress) (Cistué *et al*. 1994, Zoriniants *et al.* 2005). Starving microspores undergo changes in the respiratory status due to the variation in the rate of the carbohydrates uptake from the culture medium (Zoriniants *et al.* 2005), and they also suffer a decrease of RNA synthesis and protein kinase activities (Zarsky *et al.* 1992). Starvation pretreatment have been successfully used in combination with heat shock pretreatment by Kim *et al*. (2008) in isolated microspore culture of hot pepper, while no starvation pretreatment or a combination of it with other stress pretreatment was reported to be effective until now in hot pepper anther culture.

8.5.4. N-butanol pretreatment.

Different chemical pretreatments such as chemical inducers (Liu *et al*. 2001, Zheng *et al*. 2001) or heavy metals (Zonia and Tupy 1995) have been used to the whole plant or to different explants (excised inflorescences, flower buds or anthers) to induce microspore embryogenesis (Touraev *et al*. 1997). Hause *et al*. (1993) reported that the first steps of microspore embryogenesis induction require cytoskeletal changes, consequently the utilization of chemicals as colchicine or cytochalasin D is adequate to induce microspore embryogenesis (Zhao *et al*. 1996; Gervais *et al*. 2000, Soriano *et al*. 2008). Furthermore it has

been reported that n-butanol pretreatment can improve the production of embryos and green plantlets in *Triticum* (Soriano *et al.* 2008) and in *Zea mais* (Füredi *et al*. 2011). The effect of this primary alcohol seems to be based on its aptitude as antimicrotubule agent (Hirase *et al*. 2006). Antimicrotubule agents, as demonstrated by Barnabás *et al*. (1991) in wheat, are able to enhance microspore embryogenesis. In addition to the stimulation of microspore embryogenesis, n-butanol activity is reversible and has the advantage of non-toxicity. To the date no reports on the use of n-butanol in microspore embryogenesis induction in *Capsicum* have been published.

8.6. Media composition.

Several basal media have been used for the induction of microspore embryogenesis in anther and isolated microspore culture such as MS medium (Murashige and Skoog 1962), NLN medium (Lichter 1982) and Nitsch medium (Nitsch and Nitsch 1969). In *Capsicum*, the most used are C and M media (Dumas de Vaulx *et al.* 1981, Supena *et al.* 2006), MS medium (Ozkum and Tipirdamaz 2002, Kim *et al*. 2004), and NLN medium (Kim *et al.* 2008). Very often, the final medium composition is modified by changing the concentration of the different components or the carbon source, and also by the addition of plant growth regulators or other supplements that could be beneficial for microspore embryogenesis. The most common carbon sources used in culture media of *Capsicum* are sucrose (Dumas de Vaulx *et al*. 1981, Supena *et al.* 2002, Kim *et al.* 2004, 2008) and maltose (Dolcet-Sanjuan and Claveira 1994, Supena *et al.* 2006). In hot pepper, different variations in sucrose and maltose concentration were tested in solid, liquid and double layer media.

Concentrations rate from 3% to 9% of sucrose or maltose have been tested in *Capsicum*, leading to different results (Kim *et al.* 2008, Supena *et al.* 2006).

Although maltose is supposed to have a positive effect on microspore embryogenesis induction, avoiding the inhibitory effect of other sugars on the microspore development during the induction (Dolcet-Sanjuan *et al.* 1997, Irikova *et al.* 2011), so far in hot pepper the best results were obtained using sucrose as carbon source (Kim *et al.* 2004, 2008, Supena *et al*. 2006).

Several growth regulators are used to increase and stimulate microspore embryogenesis induction (Raghavan 1986, Maraschin *et al*. 2005, Olmedilla 2010, Irikova *et al*. 2011). Most of the plants require auxins or cytokinins or a combination of both in the medium for microspore embryogenesis induction while others do not need this external addition in the media because they have endogenous hormones (Raghavan 1986).

In certain species of *Solanaceae*, it was possible to obtain double haploids plants without the addition of hormones (Nitsch 1974, Belogradova *et al*. 2009). In hot pepper, kinetin, zeatin, 2.4-dichlorophenoxyacetic acid (2.4-D), 1-Naphthaleneacetic acid (NAA) and Indole-3-acetic acid (IAA) have been used in different concentrations and combinations to induce microspore embryogenesis (Qin and Rotino 1993, Kim *et al.* 2004, Supena *et al.* 2006) but recently (Kim *et al.* 2008), it has been reported that without using hormones it was possible to induce microspore embryogenesis by isolated microspore culture.

Other components frequently added to the culture media for the induction of microspore embryogenesis, both by anther and isolated microspore culture, are antibiotics and/or activated charcoal (Olmedilla 2010, Irikova *et al*. 2011).

In hot pepper *in vitro* culture, contamination represents a big problem (Supena *et al.* 2006), that can be avoided by adding antibiotics to the media. The addition of rifampicin and timentin has been used in hot pepper microspore culture by Supena *et al.* (2006).

Nevertheless, there are no exhaustive studies on the influence of antibiotics on microspore embriogenic induction.

Activated charcoal has been used in microspore embryogenesis in order to adsorb a wide range of toxic substances produced during the culture such as phenols, agar impurities and hydroxy methyl furfural coming from sugar sterilization (Johansson 1983, Winkle and Pullmann 1996). On the other side, activated charcoal can also adsorb favorable compounds as pyridoxine, biotin, thiamine, folic acid, nicotine acid and endogenous hormones released from the anther tissues during the culture (Johansson *et al.* 1990, Vagera 1990).

The addition of activated charcoal in the culture media has been used successfully in microspore embryogenesis induction in different species of *Capsicum* (Vagera 1984, Morrison *et al*. 1986, Dolcet-Sanjuan and Claveira 1994, Ozkum and Tipirdamaz 2002) including hot pepper (Supena *et al*. 2006).

9. Regeneration media and chromosome doubling.

Regeneration media play a crucial role in double haploid technology because obtaining a high embryo yield does not always mean to regenerate a high number of haploid plants. Furthermore, once haploid plants are obtained it is necessary to diploidize them by an optimized method.

Both regeneration and chromosome doubling rate may depend on the genotype, basal media composition and the physical culture condition (Olmedilla 2010, Irikova *et al*. 2011).

In hot pepper many attempts have been carried out to increase the number of plant regenerants using both media containing hormones (Sibi *et al*. 1979, Dumas de Vaulx *et al*. 1981) and hormone-free media (Kim *et al.* 2004, Supena *et al.* 2006) with different results. Furthermore the obtaining of low quality embryos during microspore embryogenesis induction in pepper represents a very important factor affecting the regeneration rate. Low quality embryos fail to regenerate plantlets in practically the 100% of the cases.

Chromosome doubling of haploids plantlets is another critical step in haploid breeding programs and may be obtained by spontaneous diploidization or by colchicine treatments. Spontaneous chromosome doubling rates among microspore-derived embryos may vary depending on the genotype as reported in wheat by Stober and Hess (1997). Diploidization is needed because haploid plants are sterile. Although spontaneous diploids may appear due to the fusion of nuclei, which can occur during the initial stages of microspore embryogenesis, a chromosome doubling protocol using an anti-mitotic chemical as it could be colchicine is commonly required if there is a low level of spontaneous doubling of haploid material (Barnabas *et al*. 2001). Colchicine may be applied to the plantlets directly or to the roots by immersion, in the regeneration medium or to the induction medium directly (Mityko *et al.* 1998), nevertheless these methods are laborious, time consuming and require relatively large amounts of an expensive chemical.

As colchicine is highly toxic to humans, several attempts have been made to reduce its concentration or to find an alternative method with other chemicals for chromosome doubling, but since now colchicine has been proved to be the most useful one to induce diploidization (Soriano *et al*. 2007). In *Capsicum* it is possible to obtain diploidized plants produced by spontaneous diploidization of haploid plants (Jedrzejczyk and Nowaczyk 2009) and an increase in the frequency of doubled haploid regeneration rate was obtained when a colchicine treatment was applied *in vitro* on young plants (Mityko and Fari 1997, Mityko *et al*. 1998).

Objetives

The present work is focused on microspore embryogenesis induction in hot pepper as a way to shorten its breeding process. Hot pepper is an attractive crop because of its renewed importance in agriculture and in pharmaceutical and cosmetic industry. It represents an interesting species in microspore embryogenesis due to its recalcitrant attitude to respond to the embryogenic induction. The objective of the present work was to develop an efficient and reproducible microspore culture protocol for hot pepper genotypes.

To achieve these goals the following technical objectives will be carried out:

Objective 1) To optimize the conditions for the induction of microspore embryogenesis in hot pepper genotypes using anther culture.

Objective 2) To optimize the conditions for the induction of microspore embryogenesis in hot pepper genotypes using isolated microspore culture.

Objective 3) To analyze cell changes accompanying induction and development of microspore embryogenesis.

Material and methods

1. Plant material.

Fourteen pepper genotypes from Monsanto Company were initially tested for their responsiveness to microspore embryogenesis induction. Nine hot genotypes: *Capsicum annuum* L: Serrano (08AL001), Jalapeño (08AL002), Jalapeño (08AL003), Pusa Jwala (08AL004), CM 334 (08AL005) and Perennial (08AL006); *Capsicum frutescens* L.: Frutescens (08AL007); *Capsicum baccatum* L.: Baccatum (08AL008), *Capsicum chinense* L.: Chinense (08AL009). As a control, four sweet genotypes where also tested: *Capsicum annuum* L.: Lamuyo (08AL010), Block (08AL011), Punt (08AL012), Demonio (08AL013) and Block (08AL014). Donor plants were raised from seeds in soil-filled plastic pots and grown in greenhouses in El Ejido (Almería, Spain) under natural light. The water and nutrients were daily supplied automatically to each pot. Temperature set points in the greenhouse were 24ºC /18ºC, day/night. Flowers were collected randomly from 20 plants for each genotype early in the morning.

2. Identification of microspore developmental stage.

The size of the flower bud, corolla and calyx was measured with scale paper. Anthers were excised and anthocyanin pigmentation was analyzed under a stereomicroscope (Olympus). Squashed fresh anthers were immersed in 4',6-diamidino-2-phenylindole (DAPI) aqueous staining solution (1µg/ml) for 10 min and subsequently observed under a fluorescence microscope to identify the responsive stages: late vacuolated microspores and young bicellular pollen. Correlation between pollen developmental stage and macroscopic characteristics of flower buds and anthers, was established for each genotype to facilitate the

selection of the optimum stage of microspores for the induction of microspore embryogenesis.

3. Anther culture procedures for microspore embryogenesis induction.

3.1 Sterilization of flower buds and media.

Flower buds containing anthers with microspores in the optimum stage of development (late vacuolated microspore/young bicellular pollen) were selected and surface-sterilized by rinsing them in 70% ethanol for few seconds. Then flowers were treated in 2% (w/v) sodium hypochlorite with a few drops of Tween-20 for 15 minutes, and finally washed three times with sterilized distilled water. Anthers were excised after flower buds sterilization and plated in solid medium under sterile conditions. Anthers from 3 flower buds (18 anthers) were plated in a 35 mm Petri dish containing 12 ml of solid medium, previously sterilized at 115ºC for 15 min. Hormones (when utilized) were sterilized by filtering them with a 22 μm filter membrane and added after the sterilization of the media. The pH was adjusted from 5.8 to 6.0 before sterilization. All these steps were followed in all the procedures used for anther culture.

3.2. Anther culture procedures to induce microspore embryogenesis.

For anther culture, 7 different procedures were carried out to induce microspore embryogenesis in this study:

a) Procedures derived from Dumas de Vaulx *et al.* (1981):

1. DDV: procedure adapted from Dumas de Vaulx *et al.* (1981).
2. DDV_{ac}: procedure derived from DDV where activated charcoal was added in the culture media.

b) Procedure according Kim *et al.* (2004):

1. Kim: procedure following exactly the Kim *et al.* (2004) procedure.

c) Procedures derived from McComb and McComb (1977):

1. MC1: procedure derived from McComb and McComb (1977) with the following modifications: 0.3% (w/v) activated charcoal, 0.8% (w/v) plant agar and 3% (w/v) maltose.
2. MC31: procedure derived from MC1 with a reduction on the temperature of the hot stress pretreatment applied to the anthers from 35ºC to 31ºC.
3. MCstarv: procedure derived from MC1 where the pretreatment was changed by a combination of hot (31ºC) and starvation (mannitol) pretreatment applied to the anthers.
4. MC2: procedure derived from MC1 where the pretreatment was changed by a combination of cold (7ºC) pretreatment applied to the flower buds combined with a starvation (mannitol) pretreatment applied to the anthers.

All the procedures used for anther culture are summarized in Table 1 in which embryo formation was studied week after week during at least 2 weeks once the first embryo was visible. The first embryo used to appear 3-4 weeks after culture.

Procedure	Pretreatment	Pretreatment material	Carbon source
DDV	35ºC 8 days	anthers	sucrose
DDV_{ac}	35ºC 8 days	anthers	sucrose
KIM	31ºC 3 days	anthers	sucrose
MC1	35ºC 7 days	anthers	maltose
MC31	31ºC 7 days	anthers	maltose
MCstarv	31ºC 7 days/ 0.7M mannitol 7 days	anthers	maltose
MC2	7ºC 4 days/ 0.7M mannitol 3 days	flower buds/anthers	maltose

Table 1. Summary of the procedures used for anther culture.

3.2.1. DDV procedure derived from Dumas de Vaulx *et al.* (1981) procedure.

In Dumas de Vaulx *et al.* (1981) procedure: anthers were pretreated on solid C medium (Dumas de Vaulx *et al*. 1981) with 0.01 mg/l kinetin and 0.01 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) at 35ºC in the darkness for 8 days. Then, the anthers in the same Petri dishes sealed with Parafilm were transferred to a phytotron and kept for 4 days at 25°C with a 16/8 hours day/night photoperiod. Finally, anthers were sub-cultured on R medium (Dumas de Vaulx *et al*. 1981) and kept in the same conditions in the phytotron until embryos were visible. DDV procedure was based on Dumas de Vaulx *et al.* (1981) with modifications (data not shown).

3.2.2. DDV_{ac} procedure.

DDV_{ac} was a modification of the DDV procedure in which 0.3% (w/v) activated charcoal was added in the R culture medium (Dumas de Vaulx *et al*. 1981).

3.2.3. Kim procedure.

The procedure applied according to Kim *et al*. (2004) was designated as Kim procedure. In this procedure a hot stress pretreatment was applied to the anthers plated in Petri dishes containing MS medium (Table 2), in which it was added 0.1 mg/l NAA and 0.1 mg/l kinetine. Plates were sealed and anthers were kept at 31ºC in darkness for 3 days and at the end of the stress pretreatment, the plates containing the anthers were transferred to a phytotron at 25°C with a 16/8 hour day/night photoperiod.

Compound	Concentration (mg/l)
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
KI	0.83
$MnSO_4 \cdot H_2O$	16.90
$Na_2MoO_4 \cdot 2H_2O$	0.28
$ZnSO_4 \cdot 7H_2O$	8.60
$CaCl_2$	322.02
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
Glycine	2.00
Myo-Inositol	100.00
Nicotinic acid	0.50
Pyroxidine HCl	0.50
Thiamine HCl	0.10
Sucrose	3%
Plant agar	0.8%
pH	5.8

Table 2. Composition of MS medium used for culture and induction in Kim procedure.

3.2.4. MC1 procedure.

MC1 procedure was based on McComb and McComb (1977). In McComb and McComb (1977) procedure anthers were cultured on Nitsch's H medium (Nitsch and Nitsch 1969), containing 1% (w/v) activated charcoal which was solidified with 1% (w/v) agar, no hormones were added.

In MC1 procedure the pretreatment was a hot stress applied directly to the anthers. Anthers were incubated in sealed Petri dishes containing solid medium MC, a modified Nitsch's H medium, (Table 3) and kept at 35ºC in darkness for 7 days. After this pretreatment the same plates were transferred to a phytotron and kept at 25°C with a 16/8 hour day/night photoperiod. No subcultures were done during the whole process.

Compound	Concentration (mg/l)
$CuSO_4 \cdot 5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	10.00
$MnSO_4 \cdot H_2O$	18.94
$Na_2MoO_4 \cdot 2H_2O$	0.25
$ZnSO_4 \cdot 7H_2O$	10.00
$CaCl_2$	166.00
KH_2PO_4	68.00
KNO_3	950.00
$MgSO_4$	90.27
NH_4NO_3	720.00
Biotin	0.05
Folic acid	0.50
Glycine	2.00
Myo-Inositol	100.00
Nicotinic acid	5.00
Pyroxidine HCl	0.50
Thiamine HCl	0.50
Maltose	3%
Activated charcoal	0.3%
Plant agar	0.8%
pH	5.8

Table 3. Composition of MC induction and culture media for MC1 procedure.

3.2.5. MC31 procedure.

MC31 designates a procedure that was a modification of MC1 procedure in which the temperature of the hot stress pretreatment applied to the anthers was reduced from 35ºC to 31ºC. Anthers were excised and incubated on solid medium MC (Table 3) at 31ºC in the darkness for 7 days. After the stress

pretreatment anthers in the same plate were transferred, as in MC1 procedure, to a phytotron at 25ºC with a 16/8 hour day/night photoperiod.

3.2.6. MCstarv procedure.

MCstarv designates a procedure that was a modification of MC1 procedure in which the temperature of the hot stress pretreatment applied to the anthers was reduced from 35ºC to 31ºC. Furthermore, this stress was combined with a starvation pretreatment. Anthers were excised and incubated at 31ºC in the darkness for 7 days on a solid medium MC (Table 3) where 3% (w/v) maltose was substituted by 0.7M mannitol. After the stress pretreatment anthers were transferred to fresh MC medium (Table 3) and kept in a phytotron at 25ºC with a 16/8 hour day/night photoperiod.

3.2.7. MC2 procedure.

MC2 designates a procedure that was a modification of MC1 procedure in which instead of the hot pretreatment, a combination of cold and starvation pretreatments was applied. Flower buds with the appropriate microspore developmental stage were placed in a Petri dish with a wet filter paper and stored at 7°C for 4 days. Then anthers were excised and incubated on the starvation medium, MC medium (Table 3) in which 3% (w/w) maltose was substituted by 0.7M mannitol, at 25ºC in the darkness for 4 days. After the stress pretreatment, anthers were transferred to a fresh MC medium (Table 3) and kept in a phytotron at 25°C with a 16/8 hour day/night photoperiod.

4. Isolated microspore culture procedures for microspore embryogenesis induction.

4.1. Sterilization of flower buds and media.

The flower buds containing microspores at the adequate stage (late vacuolated microspore/young bicellular pollen) were surface-sterilized by rinsing them in 70% ethanol for few seconds. Then, they were treated in 2% (w/v) sodium hypochlorite with a few drops of Tween-20 for 15 minutes, and washed three times with sterilized distilled water. For each microspore isolation thirty five flower buds were used. Culture media were sterilized by sequential filtration through membrane filters of 0.45 and 0.22 μm pore sizes and freshly prepared before each experiment. The pH values were adjusted to 5.8–6.0.

4.2. Isolated microspore culture procedures to induce microspore embryogenesis.

To induce microspore embryogenesis by isolated microspore culture different procedures were used:

a) One procedure according to Kim *et al.* (2008) designated as KIM* procedure in which microspore isolation was carried out using electric blenders to extract microspores from flower buds and the stress pretreatment was applied directly to microspores.

b) Six procedures derived from Kim *et al.* (2008), designated as. MC1*, MC1-P*, MC1-PM*, MC-H*, MC-S* and MC-SH*. In these procedures microspore isolation was carried out as mentioned before in KIM* procedure and the stress pretreatment was applied directly to microspores. The differences in these procedures are described later.

c) Two procedures derived from Soriano *et al.* (2008) designated as MC1[1]* and MC1-B*. In MC1-B* procedure a combination of hot stress and n-butanol pretreatment was applied to the anthers whilst in MC1[1]* procedure, only a hot stress pretreatment was applied. In both procedures, glass rod homogenizers were used to extract microspores from anthers.

All the procedures used for isolated-microspore culture are summarized in Table 4 in which embryo formation was studied week after week during at least 4 weeks once the first embryo was visible. The first embryo used to appear 6-8 weeks after culture in all procedures tested except in MC1[1]* and MC1-B* in which embryos used to appear 4-5 weeks after culture.

	Pretreatment	Pretreatment material	Isolation method	Pretreatment medium	Culture[¥] medium
KIM*	31ºC 3 days	Microspores	Blenders	SM	CM
MC1*	31ºC 3 days	Microspores	Blenders	MCS*	MC
MC1-P*	35ºC 4 days	Microspores	Blenders	MCS*	MC
MC1-PM*	35ºC 4 days	Microspores	Blenders	MCS*	MC
MC1-H*	31ºC 3 days	Microspores	Blenders	MCS*	MC-H
MC1-S*	31ºC 3 days	Microspores	Blenders	MCS*	MC-S
MC1-SH*	31ºC 3 days	Microspores	Blenders	MCS*	MC-SH
MC1[1]*	35ºC 7days	Anthers	Homogenizers	MCS*	MC
MC1-B*	35ºC 7days/ n-butanol 5 hours	Anthers	Homogenizers	MCS*	MC

[¥] All the culture media were double layer medium and only the solid under layer contained activated charcoal.

Table 4. Summary of the procedures used for isolated microspore culture.

4.2.1. KIM* procedure.

KIM* procedure was carried out according to Kim *et al.* (2008). A combination of hot and starvation pretreatment was applied directly to the isolated microspores. Thirty five sterile flower buds were immersed in 10 ml of SM starvation medium (Table 5) and immediately blended at maximum speed for 10 seconds with a blender (Waring). Blended anthers were transferred to 50 ml plastic Falcon centrifuge tubes and placed on a vortex (Fisher) for 20 seconds three times at maximum speed. Somatic tissue debris was removed by sequential filtration of the solution through sieves with mesh sizes of 75 and 38µm to obtain a clear microspore suspension. The resulting suspension was centrifuged at 500 rpm for 5 minutes, and the microspore pellet was washed twice with SM starvation medium. Pellet was resuspended in fresh SM medium and microspore density was set up to $18 \cdot 10^4 – 20 \cdot 10^4$ microspore/ml using a Neubauer counting chamber. Then 10 ml aliquots of resuspended microspores were transferred to 90 mm Petri dish, where a pretreatment consisting in incubation at 31ºC in the darkness for 3 days was applied. After this stress pretreatment, microspore suspension was centrifuged at 500 rpm for 5 minutes and the pellet washed with a culture medium with the same composition of the SM medium (Table 5) but substituting 0.37M mannitol by 10% (w/v) sucrose. Finally, microspores were resuspended in fresh culture medium. Microspore density was adjusted to $8 \cdot 10^4 – 10 \cdot 10^4$ microspores/ ml and 1 ml of this suspension was plated on 30 mm Petri dishes containing a culture medium with the same composition of the SM medium (Table 5), but substituting 0.37M mannitol by 2% (w/v) sucrose and

solidified with 0.4% phytagel. These double-layer culture Petri dishes were sealed with Parafilm, and incubated at 25ºC in the darkness.

Compound	Concentration (mg/l)
$CuSO_4 \cdot 5H_2O$	0.025
$FeSO_4 \cdot 7H_2O$	27.80
Na_2EDTA	37.20
H_3BO_3	6.20
$MnSO_4 \cdot H_2O$	16.90
$Na_2MoO_4 \cdot 2H_2O$	0.25
$ZnSO_4 \cdot 7H_2O$	8.60
$CoCl_2 \cdot 6H_2O$	0.025
KH_2PO_4	125.00
KNO_3	125.00
$MgSO_4 \cdot 7H_2O$	90.27
Biotin	0.05
Folic acid	0.50
Glycine	2.00
Myo-Inositol	100.00
Nicotinic acid	5.00
Pyroxidine HCl	0.50
Thiamine HCl	0.50
Glutamine	800.00
Glutathione	30.00
Serine	100.00
Mannitol	0.37M
pH	5.8-6.00

Table 5. Composition of SM starvation medium used in KIM* procedure.

4.2.2. MC1*procedure.

MC1* procedure was derived from Kim *et al.* (2008). As in KIM* procedure, pretreatment was applied directly to isolated microspores but the composition of both induction and culture media were modified according to that of the MC1 procedure in anther culture.

Thirty five sterile flower buds were immersed in 10 ml of MCS starvation medium (Table 6) and immediately blended at maximum speed for 10 seconds with a blender (Waring). Blended flowers were transferred to 50 ml plastic Falcon centrifuge tubes and placed on a vortex (Fisher) for 20 seconds three times at maximum speed. Somatic tissue debris was removed by sequential filtration of the solution through sieves with mesh sizes of 75 and 38µm to obtain a clear microspore suspension. The resulting suspension was centrifuged at 500 rpm for 5 minutes, and the microspore pellet was washed twice with MCS starvation medium. Pellet was resuspended in fresh MCS starvation medium and microspore density was set up to $18 \cdot 10^4$ – $20 \cdot 10^4$ microspore/ml using a Neubauer counting chamber. Then 10 ml aliquots of resuspended microspores were transferred to 90 mm Petri dish where a pretreatment consisting of an incubation at 35ºC in the darkness for 3 days was applied. After the stress pretreatment, microspore suspension was centrifuged at 500 rpm for 5 minutes and the pellet washed with a culture medium with the same composition of the MCS starvation medium (Table 6) but substituting 0.37M mannitol by 3% (w/v) maltose. Finally, microspores were resuspended in fresh culture medium. Microspore density was adjusted to $8 \cdot 10^4$ – $10 \cdot 10^4$ microspores/ ml and 1 ml of this suspension was plated on 30 mm Petri dishes containing a culture medium with the same composition of the MCS starvation medium (Table 6) where 0.37M mannitol was substituted by 3% (w/v) maltose, 0.3% (w/v) activated

charcoal was added and medium was solidified with 0.8% plant agar. These double-layer culture Petri dishes were sealed with Parafilm, and incubated at 25ºC in the darkness. Medium refreshing by the addition of 1ml of fresh culture medium after 3 days of culture was also tested.

Compound	Concentration (mg/l)
$CuSO_4 \cdot 5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	10.00
$MnSO_4 \cdot H_2O$	18.94
$Na_2MoO_4 \cdot 2H_2O$	0.25
$ZnSO_4 \cdot 7H_2O$	10.00
$CaCl_2$	166.00
KH_2PO_4	68.00
KNO_3	950.00
$MgSO_4$	90.27
NH_4NO_3	720.00
Biotin	0.05
Folic acid	0.50
Glycine	2.00
Myo-Inositol	100.00
Nicotinic acid	5.00
Pyroxidine HCl	0.50
Thiamine HCl	0.50
Mannitol	0.37M
pH	5.8

Table 6. Composition of MCS medium.

4.2.3. MC1-P*procedure.

MC1-P* procedure was derived from MC1*. In this procedure all the conditions and media were kept as in MC1* procedure but the temperature and the length of the hot stress pretreatment were changed. The hot stress pretreatment applied directly to the microspores was 35ºC for 4 days. All the other parameters and were kept as in MC1* procedure.

4.2.4. MC1-PM*procedure.

MC1-PM* procedure was derived from MC1*. In this procedure the hot stress pretreatment applied to the excised anthers was 35ºC for 4 days and the concentration of maltose was increased from 3 to 6% (w/v) both in solid and liquid culture medium. All the other parameters were kept as in MC1* procedure.

4.2.5. MC1-H*procedure.

MC1-H* procedure was derived from MC1* but hormones were added during the culture. The hormones were added in the culture medium both in the liquid upper layer and in the solid under layer. The stress pretreatment and culture conditions were the same of that of MC1* procedure and the composition of the culture medium was the same of MCS starvation medium (Table 6) but substituting 0.37M mannitol by 3% (w/v) maltose and adding 0.01mg/l of

kinetine and 0.01mg/l of 2-4D. 0.3% (w/v) activated charcoal was also added to the solid under layer. The under layer was solidified with 0.8% (w/v) plant agar.

4.2.6. MC1-S*procedure.

MC1-S* procedure was derived from MC1* but a different carbon source was used in the culture medium. The carbon source used in MC1-S* procedure was 3% (w/v) sucrose. The culture conditions were the same of that MC1* procedure and the composition of the double-layer culture medium used in this procedure was that of MCS starvation medium (Table 6) but substituting 0.37M mannitol by 3% (w/v) sucrose in both layers. In the under layer 0.3% (w/v) activated charcoal was added and for its solidification 0.8% (w/v) plant agar was used.

4.2.7. MC1-SH*procedure.

MC1-SH* procedure was derived from MC1* but the culture medium was supplemented with hormones and a different carbon source was used. The carbon source used in MC1-S* procedure was 3% (w/v) sucrose. These modifications were applied both in the liquid upper layer and in the solid under layer of the culture medium. The culture conditions were the same of that in MC1* procedure and the composition of the culture medium used in this procedure was the same of MCS starvation medium (Table 6), but adding 0.01mg/ml of kinetine and 0.01mg/ml of 2-4D and substituting 0.37M mannitol by 3% (w/v) sucrose in both

layers. In the under layer 0.3% (w/v) activated charcoal was added and for its solidification 0.8% (w/v) plant agar (Duchefa) was used.

4.2.8. MC1-B* procedure.

MC1-B* procedure was derived from Soriano *et al.* (2008), where a combination of hot pretreatment (35ºC for 7 days) and n-butanol pretreatment (5 hours after the hot stress pretreatment) was applied directly to the anthers.

Anthers were excised from selected flower buds, plated on solid MC medium (Table 3) and kept at 35ºC in the darkness for 7 days. After this period anthers were immersed in 0.2% n-butanol for 5 hours in the darkness. Microspores were released from the anthers with a glass rod homogenizer in a 0.3M mannitol solution. Somatic tissue debris was removed by sequential filtration of the solution through sieves with mesh sizes of 75 and 38µm to obtain a clear microspore suspension. Fresh 0.3M mannitol solution was added to this microspore suspension to bring the volume up to 15 ml. The solution was centrifuged for 5 min at 1.000 g. After removing the supernatant, the microspores were resuspended in 1.5 ml of 0.3M mannitol solution and gently layered onto a 20% (w/v) maltose solution where a density gradient was obtained by centrifuging for 5 minutes at 100 g. Viable microspores settled at the interface between the mannitol and maltose were collected by a Pasteur pipet and washed in 0.3M of mannitol solution to eliminate maltose. Then the washed microspores were centrifuged for 5 minutes at 100 g and resuspended in a medium with the same composition of MCS (Table 6), but substituting 0.7M mannitol by 3% (w/v) maltose. Microspore density was set up to $3 \cdot 10^4$ microspores/ml by using a Neubauer counting chamber. The resulting solution was plated on 30 mm Petri dishes containing a solid under layer with the same composition of the MCS

medium (Table 6), but substituting 0.37M mannitol by 3% (w/v) maltose and adding 0.3% (w/v) activated charcoal. This layer was solidified with 0.8% (w/v) plant agar. The double layer Petri dishes, sealed with Parafilm, were incubated at 25 ºC in the darkness.

4.2.9. MC1[1]* procedure.

MC1[1]* procedure was derived from Soriano *et al.* (2008). The MC1[1]* procedure had the same conditions and media of MC1-B* procedure but without n-butanol pretreatment. The stress pretreatment was applied to the excised anthers and glass rod homogenizers were used to isolate microspores.
Anthers were excised from selected flower buds, plated on solid MC (Table 3) medium and kept at 35ºC in the darkness for 7 days.

5. Plant regeneration.

Plant regeneration was carried out according to Kim *et al.* (2008). Perfect dicotyledonary embryos raised from microspore and anther culture were transferred under sterile conditions to RG regeneration medium (Table 7), which was a Gamborg B5 medium supplemented with 2% sucrose and solidified 0.4% phytagel. Then they were kept in a growth chamber at 25ºC and 16/8 hour day/night photoperiod. Normal looking green plants were transferred to DeWitt tubes containing 8 ml of RG medium and grown in the growth room at 25ºC and 16/8 hour day/night photoperiod. The ploidy level of the well-rooted plantlets

with 3–4 leaves was determined by flow cytometry by the Company service. The haploid plantlets were spontaneously diploidized and after the acclimatization transferred to the greenhouse. Controls with the regeneration medium routinely used in the Company were also carried out without significant changes (data no shown).

Compound	Concentration (mg/l)
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	3.00
$MnSO_4 \cdot H_2O$	10.00
$Na_2MoO_4 \cdot 2H_2O$	0.25
$ZnSO_4 \cdot 7H_2O$	2.00
$CaCl_2$	113.23
KNO_3	2500.00
$MgSO_4$	121.56
NaH_2PO_4	130.44
$(NH_4)_2SO_4$	134.00
Myo-Inositol	100.00
Nicotinic acid	1.00
Pyroxidine HCl	1.00
Thiamine HCl	10.00
Sucrose	2%
Phytagel	0.4%
pH	5.8

Table 7. Composition of RG regeneration medium.

6. Data analysis.

Statistical analysis was carried out using Microsoft® Excel 2010 software. At least 8 replicates were carried out for each independent experiment. Each replicate represents the results obtained after one week of culture using flowers from at least 20 plants from each genotype. There was a high variability among the results obtained during different weeks, but no clear correlation was found with the use of flowers collected in different flowering times. Data analysis for the different parameters of microspore embryogenesis both for anther and isolated-microspore culture (efficiency in embryo yield, responding anthers, dicotyledonary embryos and abnormal embryos), were analyzed by descriptive statistical analysis. The data for the culture were normalized considering either the number of anthers plated (for anther culture) or the number of anthers from which the microspores were isolated (for isolated microspore culture). Mean and standard errors were calculated and sorted by treatment.

7. Light-microscope procedures to study cellular changes after embryogenic induction.

Cellular changes after embryogenesis induction were studied by light microscopy using different techniques during the first three weeks of culture.

7.1. Direct observation of samples.

For a first analysis of the cellular changes produced in microspores by microspore embryogenesis induction a comparison of anthers at A0 (before

pretreatment) and A7 (after pretreatment) stages was done. Anthers were squashed in culture media and directly observed under Zeiss Axioplan microscope equipped with a CCD camera. For the study of later changes in anther culture, anthers at C7, C14 and C21 stages (cultured during 7, 14 and 21 days respectively) were squashed and observed as described above. For statistical purposes 21 anthers from those of the 35 flowers buds cultured were randomly selected from the different plates. For the study of later changes in isolated microspore culture, plates containing microspores at C7, C14 and C21 stages were directly observed in an inverted LEICA microscope.

7.2. DAPI staining.

To study the developmental stage of microspores in the different flower buds of the selected genotypes 4',6-diamidino-2-phenyl-indole (DAPI) staining (Vergne *et al*. 1987) was used. Fresh anthers and microspores were stained for 10 minutes at room temperature with an aqueous solution of DAPI at a final concentration of 1µg/ml. Squashed material was studied under UV-light with a Zeiss Axioplan microscope.

7.3. FDA staining.

To assess viability of isolated pepper microspores, microspores were stained with fluorescein diacetate (FDA) at a final concentration of 1g/ml. A drop of the culture medium containing isolated microspores or microspores from

squashed anthers was mixed with a drop of FDA solution, preparation were stored for 15 minutes at 4ºC and then observed by an inverted microscope.

7.4. Sample processing for light microscopy.

Due to the fact that the best results for microspore embryogenesis were obtained with the MC1 procedure for anther culture applied to Serrano genotype, for the study of the cytochemical changes taking place after microspore embryogenesis induction we have analyzed samples derived from this procedure applied to Serrano genotype at different stages: A0 before the induction pretreatment, A7 just after the induction pretreatment, C7 seven days in culture after the induction pretreatment, C14 fourteen days in culture after the induction pretreatment and C21 twenty-one days of culture after the induction pretreatment. Lamuyo anthers cultured with MC1 procedure taken at the same stages were also studied to have a sweet genotype for comparison.

For the isolated-microspore-culture MC1* procedure and Jalapeño genotype were selected and C21 stage was analyzed.

Anthers containing microspores at different stages as well as isolated microspores were fixed in 4% (w/v) paraformaldehyde and 0.25% (w/v) glutaraldehyde in 0.05 M cacodylate buffer at pH 7.2 at 4 ºC for four hours and then kept overnight in fresh fixative. The isolated and fixed microspores were then embedded in 1% agarose solution in 0.05 M cacodylate buffer and after solidification washed in the same buffer. The anthers were not embedded in agarose but washed straight away in cacodylate buffer after fixation both anthers and isolated microspores were then dehydrated in ethanol series (50%, 70%, 90% and 100%), embedded in Unicryl and polymerized under UV light at -20 ºC.

Semi-thin sections of 1µm were obtained using an Ultracut E (Reichter-Jung) microtome and after applying the different staining techniques further described they were observed under a Zeiss Axioplan microscope equipped with a Canon digital camera.

7.4.1. Toluidine blue staining.

For general histological observation section were stained with toluidine blue. Toluidine blue staining solution was prepared dissolving 0.5g of borax in 30 ml of distilled water and then 0.25g of methylene blue and 0.25g of toluidine-blue were added to this solution and its volume was set up to 50ml. Semi-thin sections were observed under bright field microscopy.

7.4.2. PAS staining.

To identify insoluble polysaccharides and starch granules, sections were prepared according the procedure reported by Feder and O'Brien (1968) with slight modifications introduced by Serrano *et al*. (2008). The slides were immersed in a 1% (w/v) periodic acid for 10 min, rinsed in running water for 5 min and stained in Schiff's reagent for 10 min in the dark. Then slides were washed three times for 3 min each with 2% (w/v) sodium metabisulfite solution, and in running water for 10 min. Finally, the slides were covered with Merckoglass (Merck).

7.4.3. Coomassie brilliant blue staining.

Protein material was stained according to Fisher's method (1968) with slight modifications introduced by Serrano *et al*. (2008). The staining solution consisted of 0.25% (w/v) Coomassie Brilliant Blue R-250 dye in a solution of methanol: acetic acid: water (MAA, 5:1:4) filtered prior to use. The sections were stained for 30 min at room temperature, following rinsing in MAA which removed excess stain for 15 min at room temperature. The sections were covered in Merckoglass (Merck).

7.4.4. Sudan black staining.

Sudan black staining was used for neutral lipid identification (Bronner 1975), following the method described by Serrano *et al*. (2008). Slides were immersed for 15 min at 60ºC in a 70% ethanol solution of Sudan Black B which was prepared immediately before use. Sections were mounted on slides with a thin film of APTES, a 3% acetone solution of 3-aminopropyl triethoxy-silane and rinsed in running water. Specimens were covered in gelatin-glycerol at room temperature.

Results

I. Anther culture.

1. Genotype selection.

In order to select the hot pepper genotypes of *Capsicum annuum* L. more suitable for this study a prescreening for the microspore embryogenesis induction was carried out with 14 genotypes. For this prescreening an anther culture procedure based on Dumas de Vaulx *et al.* (1981) modified and developed in the Monsanto Company (DDV) was applied. DDV procedure is applied routinely in sweet pepper for double haploid production.

Results obtained for this procedure applied in 14 genotypes were summarized in Table 1 where the efficiency in embryo yield was illustrated counting as embryos only the perfect-dicotyledonary embryos obtained per 100 anthers. Abnormal embryos and calli were not counted. The experiment was repeated 10 times with anthers obtained from the same plants but with flowers collected in 10 different weeks. The results obtained differed substantially from week to week as it was reflected in the values of the standard error.

Genotype	Pungency	Number of cultured anthers	Dicotyledonary embryos obtained	%Efficiency[a] (embryos/100 anthers)
Lamuyo	sweet	1066	156	12.84±4.66
Serrano	hot	2281	49	1.92±0.59
Jalapeño	hot	1493	19	1.56±0.46
CM334	hot	783	7	0.90±0.68
Jalapeño (08AL003)	hot	1365	7	0.82±0.38
Block pepper (08AL011)	sweet	1068	139	10.98±2.29
Punt (08AL012)	sweet	1646	140	8.30±2.20
Pusa Jwala	hot	1429	23	1.48±0.52
Capsicum Chinense	hot	1583	0	0
Capsicum Baccatum	hot	1603	0	0
Demonio	sweet	1178	103	7.73±1.90
Perennial	hot	803	0	0
Gedeonx	sweet	940	30	2.70±0.99
Block pepper (08AL014)	sweet	872	67	7.36±1.65

[a]Values are the means of ten repetitions for each genotype ± Standard Error (SE) (n=10).

Table 1. Efficiency in embryo yield using DDV procedure in anther culture.

As it was expected, the hot genotypes were less responsive than the sweet genotypes.

Lamuyo was selected from the sweet genotypes. Three hot genotypes were selected: CM334, Serrano and Jalapeño. From the last two hot genotypes: Serrano pedigree 08AL001 and Jalapeño pedigree 08AL002 were chosen due to their commercial importance (Fig. 1). From now on the pedigree of the selected genotypes will not be mentioned.

Fig. 1. Plants of *Capsicum annuum* from the four genotypes selected. a) Serrano pedigree 08AL001; b) Jalapeño pedigree 08AL002; c) CM334 genotype; d) Lamuyo genotype. Bars = 15 cm.

2. Identification of the optimal stage of the microspores for microspore embryogenesis induction.

The identification and selection of the microspore developmental stage is one of the key factors involved in microspore embryogenesis induction. For an easier selection of the flower buds which contain microspores at late-vacuolated stage and/or pollen at early-bicellular stage it was necessary to find a correlation between pollen-developmental stage and macroscopic indicators.

To establish the developmental stage DAPI staining of squashed anthers was used. DAPI staining binds to DNA allowing us to determine the position of the nucleus and the condensation stage of the chromatin in the different developmental stages (Fig. 2).

The nucleus of the young microspore presented a central nucleus with decondensed chromatin (Fig. 2 a). Vacuolated microspore just before mitosis showed a nucleus with very condensed chromatin in a peripheral position because of the big vacuole occupying most of the microspore space (Fig. 2 b). In

microspores in mitosis it was possible to distinguish the formation of chromosomes (Fig. 2 c). Early bicellular pollen after the first mitosis showed a vegetative nucleus with decondensed chromatin and a generative nucleus with condensed chromatin at the periphery of the pollen grain (Fig. 2 d). In more advanced pollen grains stages generative nucleus migrated to a more central position close to the vegetative nucleus (Fig. 2 e). It has been observed that in anthers containing late vacuolated microspores, young bicellular pollen grains were also visible (Fig. 2 f).

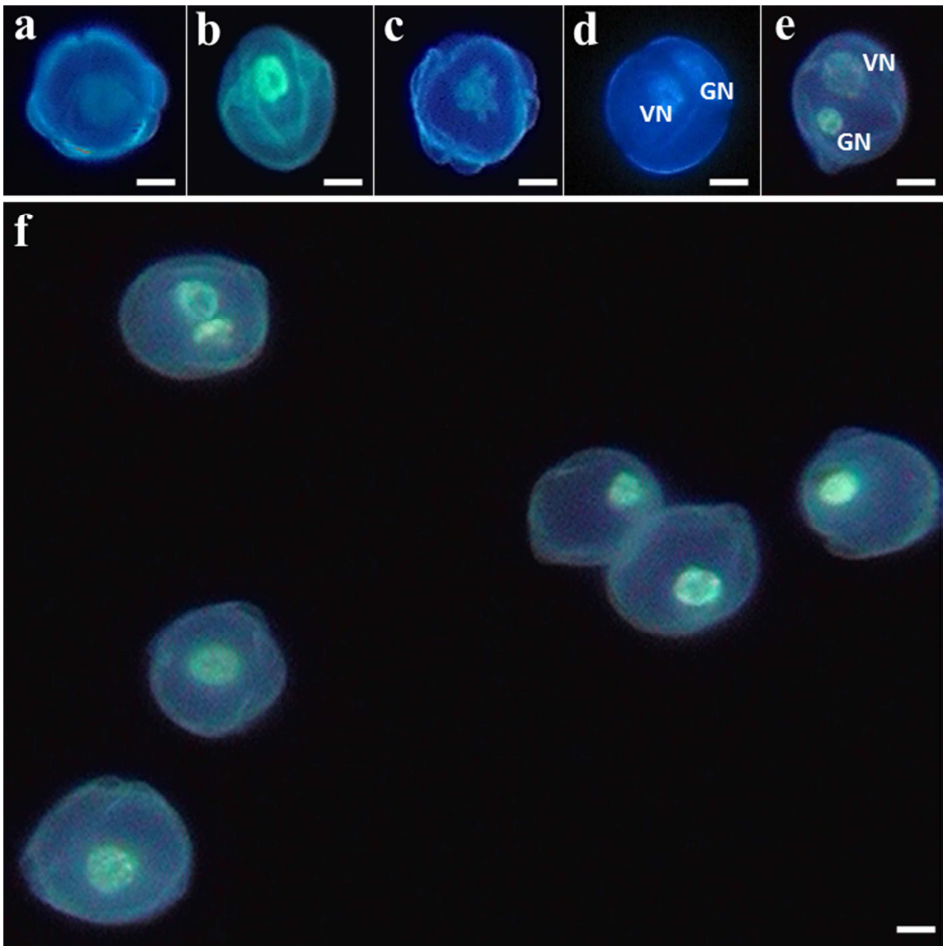

Fig. 2. DAPI staining of Serrano hot pepper squashed anthers. Pollen grains at different developmental stages: a) young microspore; b) vacuolated microspore just before mitosis; c) microspore in mitosis; d) young bicellular pollen with

vegetative nucleus (VN) and generative nucleus (GN) attached to the pollen wall; e) more advanced bicellular pollen with both nuclei centered in the pollen grain presenting the generative nucleus condensed chromatin while the vegetative one showed decondensed chromatin. f) Late vacuolated microspores and early-bicellular pollen extracted from a single anther showing asynchrony of pollen development. Bars = 10 μm.

The macroscopic indicators chosen for the selection of the anthers containing pollen in the adequate developmental stage were the relation between the length of the corolla petals and calyx sepals. Anthocyanin pigmentation of the anthers was also considered for this selection. These indicators were considered for the 4 pepper genotypes selected for this study (Serrano, Jalapeño, CM334 and Lamuyo). Table 2 presents the correlation between macroscopic indicators (corolla and calix length and anther anthocyanin pigmentation) and the corresponding microspore developmental stage. Among the genotypes studied, differences between the dimensions of the flower buds and anthers were observed. The flower bud of the sweet genotype, Lamuyo, was bigger than the flower bud of the hot genotypes; being the length of the one containing late vacuolated microspores about 5.5 mm whilst the length of the flower buds containing microspores at the same stages in the smallest CM334 hot pepper variety 3.5 mm (Table 2). It was found that the blue pigmentation of the tip of the anther due to the presence of anthocyanin increases as the developmental stage of the microspores inside the anthers progress. When microspores were at the most adequate stages for microspore-embryogenesis induction (vacuolated microspores or early bicellular pollen) this blue pigmentation was localized at the tip or the anther or at the most at ¼ of the length of the anther.

Genotype	Calix length (mm)	Corolla length (mm)	Anthocyanin pigmentation	Microspore stage
Lamuyo	5	5.5	anther with purple tip	Late vacuolated microspore/ early bicellular pollen
	5	6	anther with ¼ purple color	early bicellular pollen/mature pollen
	5	7	anther with ½ purple color	Mature pollen
Serrano	4	4	anther with purple tip	Young microspore
	4	5	anther with ¼ purple color	Late vacuolated microspore /early bicellular pollen
	4	6	anther with ½ purple color	Mature pollen
Jalapeño	4	4	anther with purple tip	Young microspore
	4	5	anther with ¼ purple color	Late vacuolated microspore / early bicellular pollen
	4	6	anther with ½ purple color	Mature pollen
CM334	3	3.5	anther with purple tip	Young microspore
	3	4	anther with ¼ purple color	Late vacuolated microspore /early bicellular pollen
	3	5	anther with ½ purple color	Mature pollen

Table 2. Correlation between macroscopic indicators (corolla and calix length and anther anthocyanin pigmentation) and microspore developmental stage.

3. Anther macroscopical changes observed during microspore embryogenesis.

During the prescreening, using DDV procedure, no visible differences were observed in the anthers between hot and sweet genotypes after the induction pretreatment (8 days at 35ºC). However, after the subsequent 3 days of anther culture a noticeable difference was observed: the darkening of the hot pepper anthers. No signs of this oxidation were observed in the sweet pepper anthers cultured in the same conditions. After 2 weeks of culture more than 25% of the anthers from the 3 hot pepper genotypes selected were completely black, while the anthers of the Lamuyo sweet pepper genotype did not show signs of oxidation (Table 3).

Genotype	Number of cultured anthers	Black anthers	% of Black anthers
Lamuyo	1066	12	0.02±0.01
Serrano	2281	519	25.14±3.14
Jalapeño	1493	365	27.38±2.65
CM334	783	193	28.06±3.16

Table 3. Evaluation of black anthers after 2 weeks of culture using DDV procedure.

This behavior was maintained after 3 weeks of culture (Fig. 3). It was also found that non-oxidized anthers underwent size and shape changes during the culture including the formation of callus and microspore-derived embryos. These changes were not observed in black anthers (Fig. 3).

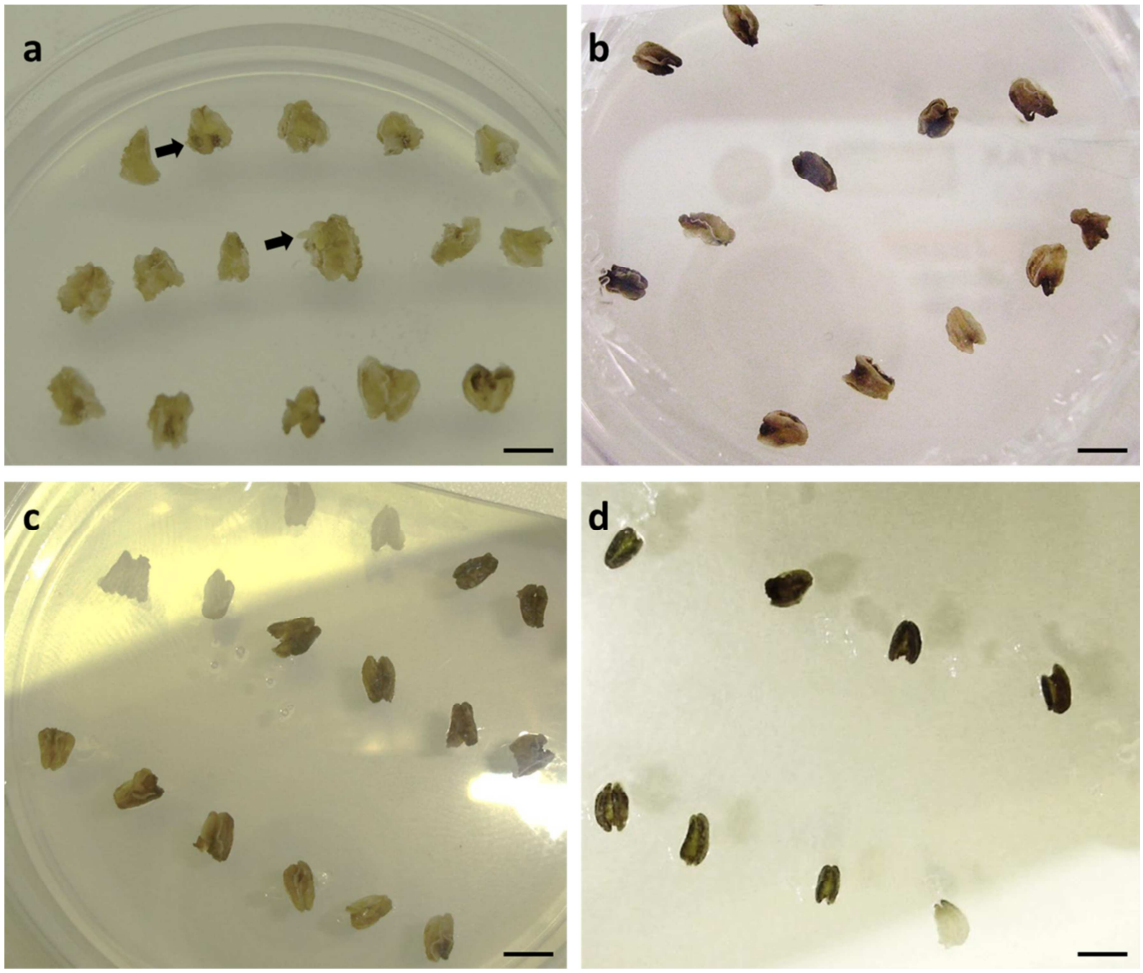

Fig. 3. Signs of anther oxidation of the 4 genotypes studied after 3 weeks of culture using DDV procedure. a) Non-oxidized anthers of Lamuyo sweet pepper showing microspore-derived embryos (arrows), b, c and d) black anthers of hot pepper after. b) Serrano, c) Jalapeño, d) CM334. Bars = 1cm.

4. Procedures assayed to improve microspore embryogenesis in hot pepper by anther culture.

4.1. DDV procedure adding activated charcoal. DDV_{ac} procedure.

To avoid darkening of the anthers and improve embryo yield in hot pepper the addition of activated charcoal in the culture medium was assayed. We designated this modified procedure as DDV_{ac}. Table 4 shows the results obtained after the addition of activated charcoal in the culture media maintaining both the induction pretreatment and culture conditions used in DDV procedure for microspore embryogenesis induction.

Genotype	Number of cultured anthers	Dicotyledonary embryos obtained	%Efficiency[a] (embryos/100 anthers)
Lamuyo	265	72	22.59±9.46
Serrano	756	25	2.00±0.85
Jalapeño	571	7	0.72±0.47
CM334	504	1	0.23±0.24

[a]Values are the means of ten repetitions for each genotype ± SE (n=10).

Table 4. Efficiency in embryo yield using DDV_{ac} procedure.

When the results obtained after DDV procedure were compared to the DDV_{ac} procedure an obvious reduction in the number of black anthers obtained after culture was observed. Furthermore, the quantification of embryo efficiency obtained after the use of DDV_{ac} procedure showed an increase in the efficiency of embryo production in Lamuyo while in hot pepper genotypes practically no differences were observed (Table 5).

	DDV	DDV_{ac}
Genotype	%Efficiency (embryos/100 anthers)	%Efficiency (embryos/100 anthers)
Lamuyo	12.84±4.66	22.59±9.46
Serrano	1.92±0.59	2.00±0.85
Jalapeño	1.56±0.46	0.72±0.47
CM334	0.90±0.68	0.23±0.24

Table 5. Comparison between the efficiency of embryo yield, with and without activated charcoal after anther culture using DDV procedure.

4.2. Procedure derived from that of McComb and McComb (1977). MC1 procedure.

Due to the fact that embryo yield obtained for hot pepper genotypes after DDV procedure was very low we decided to investigate the efficiency in microspore embryogenesis induction using a different anther culture procedure for embryogenesis induction.

We selected the procedure of McComb and McComb (1977) because being a procedure successful in other *Solanaceae* (*Nicotiana silvestris*) it was a relative simple procedure when compared with DDV: it did not include any subcultures after the induction pretreatment and did not include the addition of hormones neither in the media used for the induction pretreatment nor in the media used for culture. Activated charcoal which controls anther oxidation was originally included in this procedure and this was another reason for its selection.

We introduced slight changes in this procedure like a reduction in the concentration of activated charcoal, as well as a change in the carbon source because as it was mentioned before McComb and McComb (1977) procedure was originally described for *Nicotiana silvestris* and our previous results with pepper using less activated charcoal showed a reduction in anther oxidation and an effective increase in embryo production, at least for sweet pepper. Maltose

was used instead of the suggested sucrose because of the results reported in sweet and hot pepper by Dolcet-Sanjuan *et al*. (1997) and Supena *et al*. (2006). We designated this modified procedure as MC1.

The results on embryo yield obtained after MC1 procedure in anther culture are presented in Table 6.

Genotype	Number of cultured anthers	Dicotyledonary embryos obtained	%Efficiency[a] (embryos/100 anthers)
Lamuyo	256	34	8.69±4.52
Serrano	620	52	7.61±3.81
Jalapeño	492	1	0.14±0.13
CM334	596	0	0.00

[a]Values are the means of ten repetitions for each genotype ± SE (n=10).

Table 6. Efficiency in embryo yield after using MC1 procedure in anther culture.

When the results obtained after the procedures applied so far were compared (Table 7) the high dependence on pepper genotype became obvious because no comparable results were obtained in the 4 genotypes tested. Nevertheless, the use of MC1 procedure produced an encouraging increase in the number of dicotiledonary embryos in Serrano genotype (Table 7) accompanied by a reduction in callus formation in the 4 genotypes tested.

	MC1	DDV_{ac}	DDV
Genotype	%Efficiency[a] (embryos/100 anthers) MC1	%Efficiency[a] (embryos/100anthers) DDV**ac**	%Efficiency[a] (embryos/100 anthers) DDV
Lamuyo	8.69±4.52	22.59±9.46	12.84±4.66
Serrano	7.61±3.81	2.00±0.85	1.92±0.59
Jalapeño	0.14±0.13	0.72±0.47	1.56±0.46
CM334	0.00	0.23±0.24	0.90±0.68

[a] Values are the means of ten repetitions for each genotype ± SE (n=10).

Table 7. Comparison of the efficiency in embryo yield using MC1 procedure and DDV procedure with and without activated charcoal.

4.3. Kim *et al.* (2004) procedure. Kim procedure.

We also tested in our genotypes the Kim *et al.* (2004) anther culture procedure which we designated as Kim. With this procedure good results were reported to be obtained in microspore embryogenesis induction in a Korean hot pepper genotype (*Capsicum annuum* L. cv Milyang-jare).

In this procedure as in the DDV one, hormones were used while activated charcoal was avoided. When Kim procedure was applied in the genotypes selected callus formation and darkening of anthers were observed mainly in Jalapeño and CM334. In CM334 these calli appeared in about 30% of the cultured anthers and they were localized on the pollen sac and in anther surface in direct contact with the culture medium. Furthermore, in Jalapeño and CM334 most of the anthers were completely black after 2 weeks while no darkening was observed in Lamuyo and in Serrano this darkening appear some days later. Practically no embryos were obtained with Kim procedure neither in Jalapeño nor in CM334 (Table 8). Embryos were obtained with Lamuyo and Serrano but these results were not better than those obtained with previous procedures tested (Compare Tables 7 and 8).

Genotype	Number of cultured anthers	Dicotyledonary embryos obtained	%Efficiency[a] (embryos/100 anthers)
Lamuyo	669	8	2.80±2.39
Serrano	608	34	5.28±1.93
Jalapeño	320	0	0
CM334	559	1	0.33±0.51

[a]Values are the means of ten repetitions for each genotype ± SE (n=10).

Table 8. Efficiency in embryo yield after using Kim procedure in anther culture.

In the light of these results and comparing the different procedures tested so far in hot pepper genotypes we decided to continue working with MC1 procedure and to test other stress pretreatments in order to obtain a higher efficiency in embryo yield.

4.4. Modifications of the MC1 procedure to improve embryo yield.

To overcome the problem of the low efficiency in embryo yield, several modifications in MC1 procedure were applied. These modifications were focused on the stress pretreatment.

4.4.1. Reduction in the temperature of hot stress pretreatment. MC31 procedure.

A reduction in the temperature from 35ºC to 31ºC was used as stress pretreatment in MC1 anther culture procedure. This procedure was designated as MC31. The presence of a significant number of abnormal embryos together with

the perfect-cotyledonary embryos led us to consider in addition to the efficiency in embryo production, where only perfect embryos were counted, other parameters as the number of abnormal embryos, and the number of responding anthers, in which we included both anthers that form cotyledonary embryos and anthers producing abnormal embryo. The results were summarized in Table 9.

Genotype	Number of cultured anthers	Number of responding anthers	% Responding anthers	Number of abnormal embryos	% Abnormal embryos	Number of dicotyledonary embryos	%Efficiency[a] (embryos/ 100 anthers)
Lamuyo	918	24	9.47±4.46	19	12.44±3.26	9	4.34±2.33
Serrano	649	118	13.56±2.36	77	9.10±1.73	73	8.98±1.63
Jalapeño	415	18	2.82±0.78	24	4.00±1.08	2	0.30±0.19
CM334	220	43	11.98±5.15	48	9.29±3.55	17	5.15±2.88

[a]Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 9. Efficiency in embryo yield after using MC31 procedure in anther culture.

The most relevant effect of the reduction of the temperature in the stress pretreatment was the significant increase in the number of embryos produced in CM334 genotype (from practically 0 to 5%, compare embryo efficiency of Table 7, 8 and 9). Furthermore, there was a slight increase in the efficiency in embryo production in Serrano while no significant changes were observed for Jalapeño genotype when compared with MC1 procedure (Table 7 and 9). There was no improvement for the sweet genotype with MC31 procedure.

The formation of abnormal embryos with MC31 procedure was relatively high in the four genotypes tested but these embryos were not able to continue embryo development. To try to increase the quality and quantity of the embryos obtained we decided to use a combination of 2 stresses in the pretreatment

because it was described that this combination can help to improve microspore embryogenesis in many species including the recalcitrant one (Shariatpanahi *et al*. 2006).

4.4.2. Combination of hot (31ºC) with starvation (mannitol) pretreatment. MCstarv procedure.

A combination of a reduction of the temperature in the pretreatment (from 35ºC to 31ºC) with the concomitant application of a starvation pretreatment (use of mannitol instead of maltose) was used as stress pretreatment maintaining the other conditions of MC1 anther-culture procedure. This procedure was designated as MCstarv.

When the MCstarv procedure was applied there was no response either in sweet or in hot genotypes. Morphology of anthers did not change after the pretreatment and no microspore divisions were observed after it. No embryos were obtained even after 6 weeks of culture (Table 10).

Genotype	Number of cultured anthers	Responding anthers	Dicotyledonary embryos obtained	%Efficiency[a] (embryos/100 anthers)
Lamuyo	209	0	0	0
Serrano	405	0	0	0
Jalapeño	170	0	0	0
CM334	128	0	0	0

[a]Values are the means of five repetitions for each genotype ± SE (n=5).

Table 10. Efficiency in embryo yield using MCstarv procedure.

4.4.3. Combination of cold (7ºC) with starvation (mannitol) pretreatment. MC2 procedure.

A combination of a cold pretreatment (7ºC) applied to the flower buds and a starvation pretreatment, using mannitol instead of maltose to culture the anthers, were used as stress pretreatment maintaining the other conditions of MC1 anther culture procedure. This procedure was designated as MC2.

When the MC2 procedure was applied we observed that at the end of stress pretreatment anthers appear more turgid than in MCstarv procedure but the embryo production was very low (Table 11). The only genotype that seemed to respond to this combined pretreatment was Jalapeño. Furthermore, there was a delay in the formation of embryos when compared to the other procedures. With MC2 embryos started to be visible only after 6 weeks of culture while with previous procedures they were formed after 4 weeks of culture.

Genotype	Number of cultured anthers	Number of responding anthers	Dicotyledonary embryos obtained	%Efficiency[a] (embryos/100 anthers)
Lamuyo	107	0	0	0
Serrano	164	0	0	0
Jalapeño	123	7	4	3.38±2.24
CM334	126	0	0	0

[a]Values are the means of five repetitions for each genotype ± SE (n=5).

Table 11. Efficiency in embryo yield using MC2 procedure.

4.4.4. Comparison using the same plants in all the procedures derived from that of McComb and McComb (1977). MC1, MC31, MCstarv and MC2.

As it was known that physiological status and age of the donor plant can affect microspore embryogenesis induction we decided to apply MC1 procedure in the same plants we used for all the procedures derived from MC1 where stress pretreatments were modified (MC31, MCstarv and MC2) (Table 12). In these new experiments the number of responding anthers and abnormal embryos were also calculated and a high number of abnormal embryos were obtained. This number was similar to that obtained with MC32 procedure (compare Tables 9 and 12). After the application of MC1 procedure it was found that again the best results for hot pepper were obtained with Serrano genotype. The efficiency obtained with these flowers was slightly better with the 3 hot genotypes studied but the opposite occurred with the sweet genotype (compare Tables 6 and 12).

Genotype	Number of cultured anthers	Number of responding anthers	% responding anthers	Number of abnormal embryos	% Abnormal embryos	Number of dicoty-ledonary embryos obtained	% Efficiency[a] (embryos/ 100 anthers)
Lamuyo	197	22	10.89±4.11	32	15.93±4.82	39	3.75±1.38
Serrano	765	72	13.87±4.37	58	10.84±3.05	25	13.55±5.10
Jalapeño	534	10	3.14±1.80	11	6.65±5.44	21	0.20±0.20
CM334	444	8	1.81±1.16	9	1.64±0.92	17	0.49±0.38

[a]Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 12. Efficiency in embryo yield using MC1 procedure.

The results obtained with MC1 procedure and those obtained with procedures in which stress pretreatment were modified were compared and summarized in Table 13.

	MC2	MCstarv	MC31	MC1
Genotype	%Efficiency (embryos/100 anthers)	%Efficiency (embryos/100 anthers)	%Efficiency (embryos/100 anthers)	%Efficiency (embryos/100 anthers)
Lamuyo	0	0	4.34±2.33	3.75±1.38
Serrano	0	0	8.98±1.63	13.55±5.10
Jalapeño	3.38±2.24	0	0.30±0.19	0.20±0.20
CM334	0	0	5.15±2.88	0.49±0.38

Table 13. Efficiency in embryo yield using different stress pretreatments in procedures derived from MC1.

No general improvement in embryo efficiency for hot genotypes was found with the modifications of the stress pretreatment tested. With MC2 procedure embryo production for Jalapeño was increased although the efficiency of this procedure was lower than 5% (Table 13). The highest embryo efficiency for CM334 (around 5%) was obtained with MC31 procedure (Table 13).

Taking into account that the highest efficiency in embryo yield for hot pepper was obtained with MC1 procedure applied to Serrano genotype the repeatability of this procedure was tested again another year. Results are summarized in Table 14.

Genotype	Number of cultured anthers	Responding anthers	%responding anthers	Irregular embryos	% irregular	Dicotyle-donary embryos obtained	%Efficiency[a] (embryos/100 anthers)
Lamuyo	460	27	5.14±2.43	20	3.90±1.35	14	2.63±1.44
Serrano	601	98	16.08±3.46	53	9.13±1.96	61	9.89±2.19
Jalapeño	590	10	1.58±0.53	10	1.59±0.57	1	0.15±0.15
CM334	602	25	4.31±1.44	19	3.49±1.39	12	1.95±0.76

[a] Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 14. Efficiency in embryo yield using MC1 procedure.

These new results confirmed that, for Serrano genotype, MC1 was the best procedure and have efficiency in embryo yield higher than 8%.

5. Plant regeneration from anther culture procedure.

For plant regeneration 2 types of regeneration media were used: RG medium without hormones according to Kim *et al*. (2004) and the modified medium with hormones derived from that of Dumas de Vaulx *et al*. (1981). All plants regenerated from embryos were counted. Then ploidy of the plants that were able to survive until 3-4 leaves were formed was analyzed. Finally, spontaneous-double-haploid plants were counted (Table 15 and 16). The highest number of spontaneous-double-haploids plants was obtained with Serrano genotype with both regeneration media. Regeneration medium containing hormones was especially suitable for CM334 and, as expected, for Lamuyo the sweet genotype used to develop this method.

MC1	Number of regenerated plants	Number of analyzed plants	Number of double haploid plants
Lamuyo	6	0	0
Serrano	44	18	7
Jalapeño	1	1	1
CM334	1	0	0

MC31	Number of regenerated plants	Number of analyzed plants	Number of double haploid plants
Lamuyo	6	0	0
Serrano	38	18	8
Jalapeño	1	1	1
CM334	8	4	1

Table 15. Plant regeneration after MC1 and MC31 procedure using the regeneration medium of Kim *et al*. (2004).

MC1	Number of regenerated plants	Number of analyzed plants	Number of double haploid plants
Lamuyo	8	5	4
Serrano	26	12	6
Jalapeño	0	0	0
CM334	8	8	4

Table 16. Plant regeneration after MC1 procedure using the modified regeneration medium derived from Dumas de Vaulx *et al*. (1981).

The principal goal of this study was the obtaining in Serrano hot pepper genotype of about 1 double haploid plant per 100 anthers both with MC1 and MC31 procedures. These results in anther culture were achieved without adding hormones to the media and by spontaneous diploidization of the regenerated plants; these regenerated plants were growing in the greenhouse and were able to give mature fruits (Fig. 4).

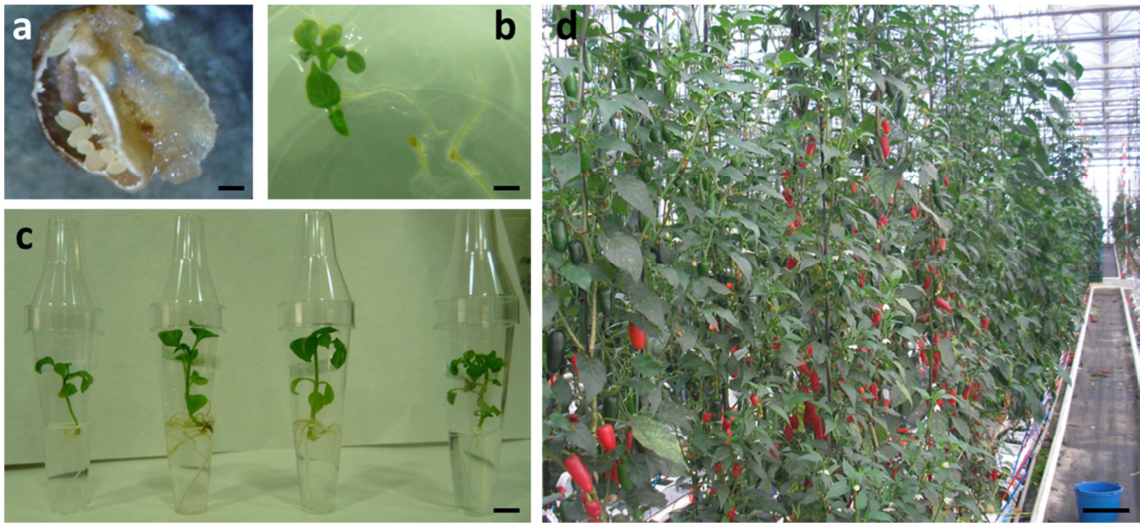

Fig. 4. Obtaining of DH plants from Serrano genotype with MC1 procedure. a) Anther with emerging microspore-derived embryos after 4 weeks of culture; b) microspore-derived plantlets in Kim *et al*. (2004) regeneration medium; c) regenerated DH plants; d) DH plants in the greenhouse. a, b: Bars = 1mm, c: Bar = 1cm and d: Bar = 20 cm.

II. Isolated microspore culture.

1. Procedures assayed to improve microspore embryogenesis in hot pepper by isolated microspore culture.

Different procedures of isolated microspore culture were developed and tested in the genotypes selected.

1.1. Isolated microspore culture according to Kim *et al.* (2008). KIM* procedure.

Due to the effectiveness of induced microspore embryogenesis obtained with a hot pepper genotype using isolated microspore culture by Kim *et al*. (2008) we decided to test this procedure in our material. We named it KIM* procedure. For this procedure flower buds from the four genotypes were collected and microspore isolated by direct blending of the flower buds. Microspore isolations were carried out using 35 flower buds containing 210 anthers. In KIM* procedure stress pretreatment was applied to isolated microspores in liquid medium and the density of this microspores was adjusted to $2 \cdot 10^5$ microspores/ml to obtain the best results.

In order to set up the optimal conditions for microspore isolation by direct blending of the flower buds, different speeds and time of blending were tested for each genotype. The main difficulty was to achieve the optimum microspore density ($2 \cdot 10^5$ microspores/ml) minimizing at the same time the presence of somatic tissue in the culture and the number of successive centrifugations to obtain a clear microspore solution (Fig. 5).

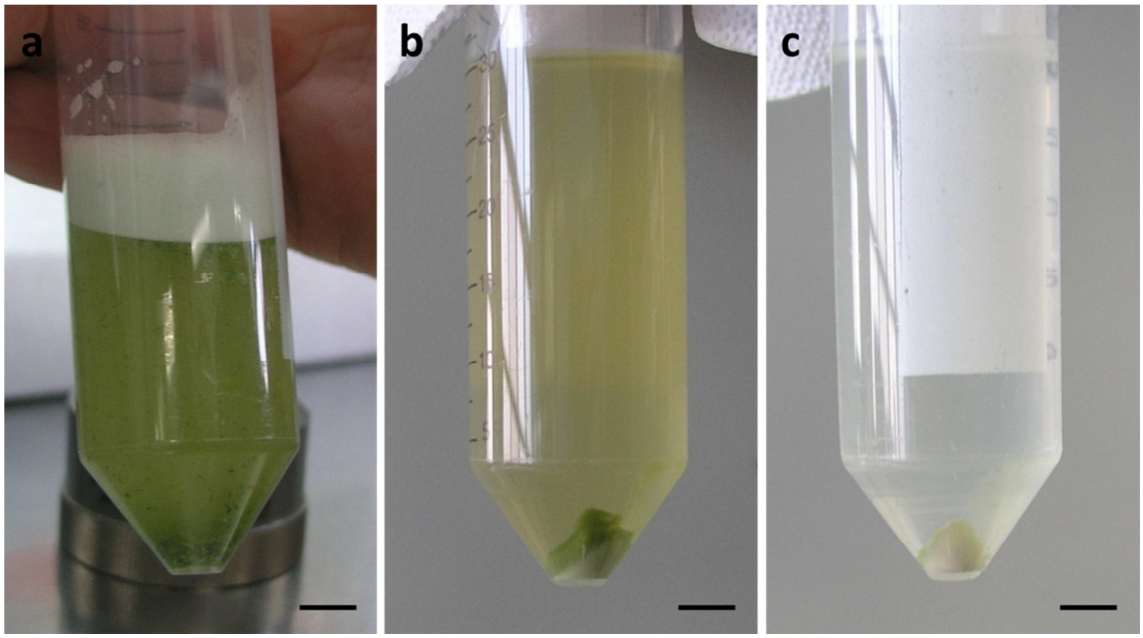

Fig. 5. Progressive cleaning of microspores after successive centrifugations applied after the blending of the flower buds. a) Microspore solution after the first centrifugation; b) microspore pellet after the second centrifugation and c) clean microspore pellet after the third centrifugation. Bars = 1cm.

The optimum speed and time of blending for each genotype are showed in Table 17. Once the speed and time of blending for each genotype were optimized they were used in all the procedures derived from Kim *et al*. (2008).

Genotype	Number of anthers used for microspore isolation	Blender speed	Time of blending	Microspore density ($\cdot 10^4$)	Final volume (ml)
Lamuyo	210	Medium	10'' x 3 times	19 - 20	12 - 15
Serrano	210	Medium	15'' x 3 times	19 - 20	11 - 13
Jalapeño	210	Medium	15'' x 3 times	19 - 20	11 - 13
CM334	210	Maximum	20'' x 3 times	19 - 20	8 - 10

Table 17. Optimization of time and speed of blending to obtain the adequate microspore density ($20 \cdot 10^4$ microspores/ml) to induce microspore embryogenesis in each genotype.

In the results summarized in Table 17 it is possible to see that the final volume with the adequate density ($2 \cdot 10^5$ microspores/ml) of resuspended microspores depended on the genotype and more precisely on the size and volume of the flower buds. In this way Serrano and Jalapeño which have similar dimensions required similar blending conditions while Lamuyo whose flowers are bigger required milder conditions. CM334 whose flowers are smaller requires stronger conditions of blending (Table 17). Surprisingly, it was observed that the flower buds of the sweet genotype suffered a rapid oxidation after the blending that was not observed in the hot genotypes.

When KIM* procedure was applied the number of microspore divisions observed either after the pretreatment or after different weeks of culture was very low for all genotypes tested when compared to the number obtained after anther culture for the same genotype (Table 18).

Genotype	%Microspore divisions after 3 days of stress pretreatment (3 to 5 nuclei)[a]	%Microspore divisions after 1 week of culture (up to 8 nuclei)[a]	%Microspore divisions after 2 weeks of culture (more than 8 nuclei)[a]
Lamuyo	4%	0	0
Serrano	7%	2%	less than 1%
Jalapeño	4%	less than 1%	0
CM334	6%	less than 1%	0

[a] Average values on a total of 360 microspores observed from samples taken at random of a given culture.

Table 18. Microspore divisions observed after KIM* procedure.

The consequence of these low numbers of divisions was reflected in the practical absence of proembryos obtained with this procedure (Table 19). Furthermore, no dicotyledonary embryos were obtained with this procedure in any of the genotypes tested. With Serrano genotype it was possible to obtain abnormal embryos; these embryos did not regenerated plants. Additionally, these abnormal embryos appeared only after 6 weeks of culture, a very long time when compared to the 3 weeks required for obtaining embryos with the hot genotype (*Capsicum annuum* L. cv Milyang-jare) used by Kim *et al*. (2008) in the same conditions.

Genotype	Number of cultured anthers	Number of dicotyledonary embryos	Number of abnormal embryos	%Efficiency[a] (embryos/100 anthers)
Lamuyo	840	0	0	0
Serrano	840	0	15	0
Jalapeño	840	0	0	0
CM334	840	0	0	0

[a] Values are the means of five repetitions for each genotype ± SE (n=5).

Table 19. Efficiency in embryo yield using KIM* procedure**.**

1.2. Isolated microspore culture procedure derived from that of Kim *et al.* (2008). MC1* procedure.

A procedure using the method of microspore isolation described by Kim *et al*. (2008) named MC1* was applied in the four genotypes selected. In the MC1* procedure the conditions of microspore pretreatment were the same of that used in KIM* procedure but the media used derived from MC1 procedure: MCS (Table 6 of Materials and Methods) for the pretreatment. For the culture the same MCS in which 0.37M mannitol was substituted by 3% (w/v) maltose was used. For this procedure microspore isolation was carried out by direct blending of the flower buds using the blending conditions previously optimized for each genotype (Table 17).

The observation of microspore divisions during microspore embryogenesis in the four genotypes revealed that there was a very low rate of divisions during the induction and culture (Table 20).

Genotype	%Microspores division after 3 days of stress pretreatment (3 to 5 nuclei)[a]	%Microspores divisions after 1 week of culture (up to 8 nuclei)[a]	%Microspores division after 2 weeks of culture (more than 8 nuclei)[a]
Lamuyo	3%	0	0
Serrano	8%	3%	less than 1%
Jalapeño	13%	5%	less than 1%
CM334	10%	3%	less than 1%

[a] Average values on a total of 360 microspores observed from random taken samples of a given culture.

Table 20. Microspore divisions observed after MC1* procedure.

Consequently, the number of embryos obtained with MC1* procedure was also reduced. With this procedure dycotiledonary embryos were obtained only with Jalapeño and CM334 genotypes while with Serrano genotype low quality embryos or abnormal embryos were obtained (Table 21).

Genotype	Number of cultured anthers	Number of dicotyledonary embryos	Number of abnormal embryos	%Efficiency[a] (embryos/100 anthers)
Lamuyo	840	0	0	0
Serrano	840	0	11	0
Jalapeño	840	11	18	1.52±1.05
CM334	840	2	1	0

[a] Values are the means of five repetitions for each genotype ± SE (n=5).

Table 21. Efficiency in embryo yield using MC1* procedure.

Plant regeneration was only possible with Jalapeño genotype while no plants were regenerated from the dicotyledonary embryos obtained with CM334 genotype. The Jalapeño plants were not analyzed because they did not survive to reach the adequate stage for that purpose (3-4 leaves) (Table 22).

Genotype	Number of dicotyledonary embryos	Number of regenerated plants	Number of analyzed plants	Number of double haploid plants
Lamuyo	0	0	0	0
Serrano	0	0	0	0
Jalapeño	11	4	0	0
CM334	2	0	0	0

Table 22. Plant regeneration using MC1* procedure.

Due to the high number of abnormal embryos obtained (Table 21) the addition of fresh medium was tested. This addition was carried out after 3 days of culture. The results showed non-significant improvemen in embryo yield in all the genotypes studied (Table 23).

Genotype	Number of cultured anthers	Number of dicotyledonary embryos	Number of abnormal embryos	%Efficiency[a] (embryos/100 anthers)
Lamuyo	1890	0	0	0
Serrano	2100	0	2	0
Jalapeño	2100	1	10	0
CM334	1680	0	0	0

[a] Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 23. Efficiency in embryo yield using MC1* procedure.

The embryo obtained with Jalapeño genotype regenerate a plantlet that did not survive to be analyzed for ploidy (Table 24).

Genotype	Dicotyledonary embryos	Regenerated plants	Analyzed plants	Double haploids
Lamuyo	0	0	0	0
Serrano	0	0	0	0
Jalapeño	1	1	0	0
CM334	0	0	0	0

Table 24. Plant regeneration using MC1* procedure.

1.3. Procedures derived from MC1*.

Modifications on the carbon source, stress pretreatment length and media were introduced in the MC1* procedure in order to obtain a higher efficiency in embryo production. These procedures derived from MC1* were named MC1-P*, MC1-PM*, MC1-H*, MC1-S* and MC1-SH*.

1.3.1. MC1-P* procedure.

In the MC1-P* procedure the temperature of the hot pretreatment and its length were increased from 31ºC to 35ºC and from 3 to 4 days. This stronger stress pretreatment did not improve the number of microspore divisions obtained with MC1*(compare Table 20 and Table 25).

Genotype	%Microspore divisions after 3 days of stress pretreatment (3 to 5 nuclei)[a]	%Microspore divisions after 1 week of culture (up to 8 nuclei) [a]	%Microspore divisions after 2 weeks of culture (more than 8 nuclei)[a]
Lamuyo	3%	1%	0
Serrano	7%	3%	1%
Jalapeño	11%	6%	4%
CM334	9%	0	less than 1%

[a] Average values on a total of 360 microspores observed from random taken samples of a given culture.

Table 25. Microspore divisions observed after MC1-P* procedure.

The results of the application of this procedure to the four genotypes selected are shown in Table 26. No dicotyledonary embryos were obtained in any of the four genotypes. No plants were regenerated from the abnormal embryos obtained with Jalapeño and CM334.

Genotype	Number of cultured anthers	Number of dicotyledonary embryos	Number of abnormal embryos	%Efficiency[a] (embryos/100 anthers)
Lamuyo	1470	0	0	0
Serrano	1470	0	0	0
Jalapeño	1470	0	7	0
CM334	1470	0	1	0

[a] Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 26. Efficiency in embryo yield using MC1-P* procedure.

1.3.2. MC1-PM* procedure.

In the MC1-PM* the length and temperature of the pretreatment were increased as in MC1-P* but in this procedure the concentration of maltose in both solid and liquid medium was increased from 3 to 6% (w/v). The percentage of microspore divisions observed after MC1-PM* procedure was very low (Table 27).

Genotype	%Microspore divisions after 3 days of stress pretreatment (3 to 5 nuclei)[a]	%Microspore divisions after 1 week of culture (up to 8 nuclei)[a]	%Microspore divisions after 2 weeks of culture (more than 8 nuclei) [a]
Lamuyo	4%	Less than 1%	0
Serrano	7%	2%	0
Jalapeño	6%	5%	less than 1%
CM334	10%	3%	less than 1%

[a] Average values on a total of 360 microspores observed from random taken samples of a given culture.

Table 27. Microspore divisions observed after MC1-PM* procedure.

The results summarized in Tables 26 and 28 showed that there was no improvement with this procedure except for the slight increase obtained with CM334 in the number of abnormal and dicotyledonary embryos (Table 28), although no plants were regenerated from these embryos. As in all previous procedures derived from Kim *et al*. (2008) the quality of the embryos obtained was very low being the number of abnormal embryos higher than that of dicotyledonary embryos (Table 28).

Genotype	Number of cultured anthers	Number of dicotyledonary embryos	Number of abnormal embryos	%Efficiency[a] (embryos/100 anthers)
Lamuyo	1680	0	0	0
Serrano	1890	0	0	0
Jalapeño	1680	0	0	0
CM334	1890	3	17	0.16±0.05

[a] Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 28. Efficiency in embryo yield using MC1-PM* procedure.

1.3.3. MC1-H* procedure.

In MC1-H* procedure the pretreatment used was the same of MC1-P* procedure but hormones were added in the culture medium. The addition of hormones to the culture medium did not significantly increase the number of divisions obtained both after the stress pretreatment and after different weeks of culture (Table 29).

Genotype	%Microspore divisions after 3 days of stress pretreatment (3 to 5 nuclei)[a]	%Microspore divisions after 1 week of culture (up to 8 nuclei) [a]	%Microspore divisions after 2 weeks of culture (more than 8 nuclei)[a]
Lamuyo	4%	Less than 1%	0
Serrano	8%	2%	less than 1%
Jalapeño	12%	4%	less than 1%
CM334	8%	2%	less than 1%

[a] Average values on a total of 360 microspores observed from random taken samples of a given culture.

Table 29. Microspore divisions observed after MC1-H* procedure.

A slight but significant increase in the number of abnormal embryos was observed in hot pepper genotypes. In sweet genotype no embryos were obtained either abnormal or dicotyledonary. In CM334 two dicotyledonary embryos were obtained although no plants were regenerated from them (Table 30).

Genotype	Number of cultured anthers	Number of dicotyledonary embryos	Number of abnormal embryos	%Efficiency[a] (embryos/100 anthers)
Lamuyo	1440	0	0	0
Serrano	1800	0	2	0
Jalapeño	1800	0	6	0
CM334	1620	2	12	0

[a] Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 30. Efficiency in embryo yield using MC1-H* procedure.

1.3.4. MC1-S* procedure.

In the MC1-S* procedure the conditions of microspore pretreatment were the same of MC1-P* but maltose was substituted by sucrose in the culture medium. The number of microspore divisions observed at the end of the stress pretreatment was lower than that obtained with MC1-H* and no embryos were produced (Table 31 and 32).

Genotype	%Microspore divisions after 3 days of stress pretreatment (3 to 5 nuclei)[a]	%Microspore divisions after 1 week of culture (up to 8 nuclei) [a]	%Microspore divisions after 2 weeks of culture (more than 8 nuclei)[a]
Lamuyo	3%	less than 1%	0
Serrano	5%	2%	0
Jalapeño	6%	4%	less than 1%
CM334	5%	3%	less than 1%

[a] Average values on a total of 360 microspores observed from random taken samples of a given culture.

Table 31. Microspore divisions observed after MC1-S* procedure.

Genotype	Number of cultured anthers	Number of dicotyledonary embryos	Number of abnormal embryos	%Efficiency[a] (embryos/100 anthers)
Lamuyo	2100	0	0	0
Serrano	2100	0	0	0
Jalapeño	2100	0	0	0
CM334	2100	0	1	0

[a] Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 32. Efficiency in embryo yield using MC1-S* procedure.

1.3.5. MC1-SH* procedure.

In the MC1-SH* procedure the conditions of microspore pretreatment were the same used in MC1-P* but hormones were added to the culture medium and the maltose was substituted by sucrose like in MC1-S* procedure. The number of microspore divisions (Table 33) was very low and no embryos were obtained with this procedure in the four genotypes tested (Table 34).

Genotype	%Microspore divisions after 3 days of stress pretreatment (3 to 5 nuclei)[a]	%Microspore divisions after 1 week of culture (up to 8 nuclei) [a]	%Microspore divisions after 2 weeks of culture (more than 8 nuclei)[a]
Lamuyo	8%	4%	0
Serrano	5%	2%	0
Jalapeño	4%	5%	less than 1%
CM334	5%	4%	less than 1%

[a] Average values on a total of 360 microspores observed from random taken samples of a given culture.

Table 33. Microspore divisions observed after MC1-SH* procedure.

Genotype	Number of cultured anthers	Number of dicotyledonary embryos	Number of abnormal embryos	%Efficiency[a] (embryos/100 anthers)
Lamuyo	2100	0	0	0
Serrano	2100	0	0	0
Jalapeño	2100	0	0	0
CM334	2100	0	0	0

[a] Values are the means of eight repetitions for each genotype ± SE (n=8).

Table 34. Efficiency in embryo yield using MC1-SH* procedure.

1.4. Isolated microspore culture derived from Soriano *et al*. (2008). MC1[1]* and MC1-B* procedures.

It was reported by Soriano *et al*. (2008) that the use of n-butanol can improve the efficiency of embryo and green plant production in isolated microspore culture, that is why two different procedures using the method of microspore isolation from pretreated anthers described by Soriano *et al*. (2008) named MC1[1]* and MC1-B* were applied.

In MC1[1]* procedure microspores were isolated according to Soriano *et al*. (2008). In this procedure MC media was used for pretreatment and culture (Table 3 of Materials and Methods) and the conditions of anther pretreatment of the MC1 anther culture procedure were applied.

In MC1-B* procedure two successive anther pretreatments were applied: first the one applied in MC1 anther procedure (35ºC during 7 days) and then a pretreatment of 5 hours in n-butanol as described in Soriano *et al*. (2008). After the pretreatment microspores were isolated according to Soriano *et al*. (2008) method and culture conditions of MC1 procedure were applied.

Both procedures were tested in the most responsive sweet and hot genotypes: Lamuyo and Serrano respectively.

Due to the fact that when the procedure of Soriano *et al*. (2008) was applied to cereals the most adequate microspore density was around $3 \cdot 10^4$ microspores/ml different attempts were done to reach this density with pepper. A minimum of 35 pepper flower buds containing 210 anthers was used for each of the isolations carried out in order to have a reference to compare these results with those obtained with Kim *et al*. (2008) derived procedures. Two concentrations of maltose were tested for the separation of viable microspores by density gradient centrifugation: 20 and 30% (w/v). After the isolation of microspores from the same number of flower buds using glass rod homogenizers, the microspore density and consequently the number of isolated microspores obtained during one isolation was higher when the sweet genotype was used compared to the hot genotype and we found that for a given genotype the best results were obtained using 30% (w/v) maltose (Table 35).

Genotype	Number of anthers	Maltose band concentration	microspore density 10^3/ml
Lamuyo	210	20%	19 - 22
Lamuyo	210	30%	24 - 27
Serrano	210	20%	15 - 18
Serrano	210	30%	21 - 24

Table 35. Isolated microspores density obtained from 35 flower buds using two different maltose concentrations for density gradient centrifugation.

To be sure that a reasonable number of viable microspores were obtained after microspore isolation FDA staining was used. Results showed that more than 50% viable microspores were obtained after the isolation of both Lamuyo and Serrano genotypes (Table 36).

Genotype	FDA stained microspores counted after isolation without pretreatment	% Viable microspores without pretreatment
Lamuyo	1143	61.4
Serrano	1499	54.2

Table 36. Viable microspores obtained directly after microspore isolation.

FDA staining was also used to study the viability after the pretreatments of 35 and 31ºC applied during 7 days to the anthers of Lamuyo and Serrano. Once again the number of viable microspores obtained after the different pretreatments was higher for the sweet Lamuyo genotype than for the hot Serrano genotype (Table 37). The hottest temperature produced a reduction in the number of viable microspores for both genotypes (Table 37).

Genotype	Pretreatment	% Viable microspores after pretreatment
Lamuyo	35ºC 7 days	27.5
Lamuyo	31ºC 7 days	33.1
Serrano	35ºC 7 days	15.2
Serrano	31ºC 7 days	21.2

Table 37. Viable microspores obtained after two different pretreatments.

The results obtained from isolated microspore culture using MC1[1]* and MC1-B* procedures were summarized in Table 38. With both methods the efficiency in embryo yield was higher when applied to the sweet than to the hot genotype and slightly higher after the application of n-butanol with both genotypes (MC1-B* procedure) (Table 38).

Genotype	Number of anthers used for MC1[1]*	Number of anthers used for MC1-B*	Number of dicotyle-donary embryos MC1[1]*	Number of dicotyle-donary embryos MC1-B*	%Efficiency[a] (embryos/100 anthers) MC1[1]*	%Efficiency[a] (embryos/100 anthers) MC1-B*
Lamuyo	227	175	13	10	7.10±2.41	8.07±4.34
Serrano	262	320	10	12	4.75±2.26	5.30±1.83

[a] Values are the means of five repetitions for each genotype ± SE (n=5).

Table 38. Comparison of the efficiency in embryo yield using MC1[1]* and MC1-B* procedures**.**

For plant regeneration, RG medium without hormones according to Kim *et al*. (2004) was used. In Table 39 and 40 the number of regenerated plants using the RG regeneration medium in the Soriano *et al*. (2008) derived procedures tested are represented.

MC1[1]*	Number of regenerated plants	Number of analyzed plants	Number of double haploid plants
Lamuyo	8	0	0
Serrano	6	0	0

Table 39. Plant regeneration after MC1[1]* procedure using RG regeneration medium.

MC1-B*	Number of regenerated plants	Number of analyzed plants	Number of double haploid plants
Lamuyo	4	0	0
Serrano	9	0	0

Table 40. Plant regeneration after MC1-B* procedure using RG regeneration medium.

III. Cellular changes after embryogenic induction.

To carry out the study of the changes taking place during microspore embryogenesis induction, different stages before and after this induction were selected and first, fresh samples were directly observed under light microscopy to follow changes in size and general aspect. Then, nuclear changes were analyzed after DAPI staining. Finally, both anthers and isolated microspores were processed for microscopy and cytochemical techniques were applied to follow changes in cellular composition.

1. Size changes.

To study size changes take in place during microspore embryogenesis induction, fresh samples were squashed and directly observed under the light microscope. Before the induction (A0 stage), microspores of Lamuyo, Serrano and Jalapeño at the same stage of development showed similar size in spite of the differences observed in the size of the anthers and flowers containing them (Table 2). CM334 genotype showed the smallest size both in anthers and flowers when compared with the other three genotypes studied at the same stage of development.

Whatever the procedure used and the genotype analyzed, an obvious swelling of a certain number of microspores was visible after the embryogenic induction (Fig. 6) except when a combination of hot with starvation pretreatment was applied in anther culture (MCstarv procedure). With MCstarv procedure neither microspore divisions nor embryo production were obtained (Table 10).

No statistical relation was observed between the number of swelled microspores and the number of embryos produced after the different procedures for microspore embryogenesis induction both by anther and isolated microspore culture.

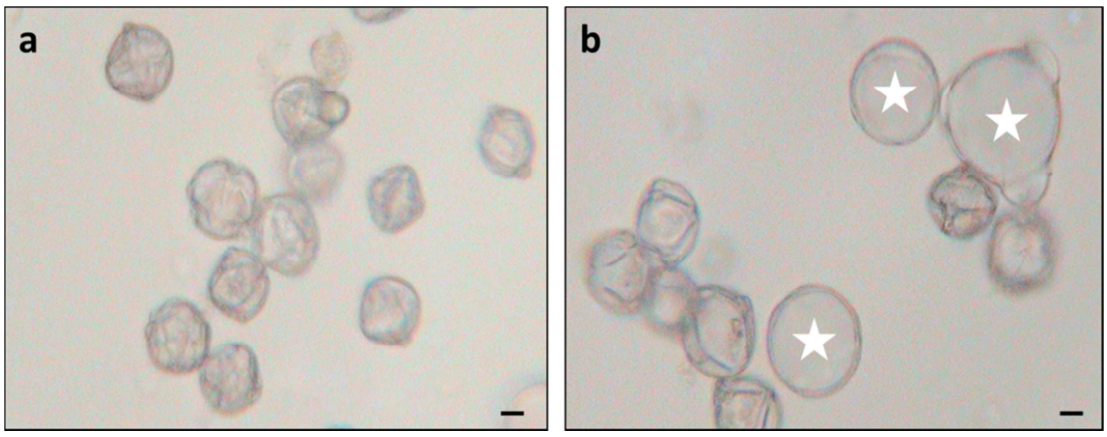

Fig. 6. Changes in microspore size after microspore-embryogenesis induction. Microspores of Serrano genotype (MC1 procedure). Phase-contrast micrographs. a) Microspores before the pretreatment; b) microspores after a 35ºC hot stress pretreatment applied for 7 days including swelled microspores (white stars). Bars = 10 µm.

2. Nuclear changes.

DAPI staining was used to follow nuclear changes after the different pretreatments applied to induce embryogenesis (A7 stage) as well as after different moments of the culture once these pretreatments were applied (C7, C14 and C21 stages) for both anther and isolated microspore culture.

Both symmetrical and asymmetrical divisions were observed after embryogenic induction (A7) whatever the pretreatment or the genotype was studied although symmetrical divisions were generally more frequent. A non-homogeneous response to the embryogenic induction was observed: non-responding microspores together with responsive ones were obtained after the different pretreatments (Fig. 7). Non-synchrony was obtained after the different induction pretreatments applied to the anthers: presence of multinuclear pollen grains with 2, 3, and, less frequently, 6 nuclei was found (Fig. 7 e). Different degree of chromatin condensation was observed in some pollen grains with 3 nuclei, showing after DAPI staining two nuclei with de-condensed chromatin and the third with condensed chromatin (Fig. 7 d, e). Similar results were obtained after the embryogenic induction in isolated microspore culture with respect to asymmetry and asynchrony.

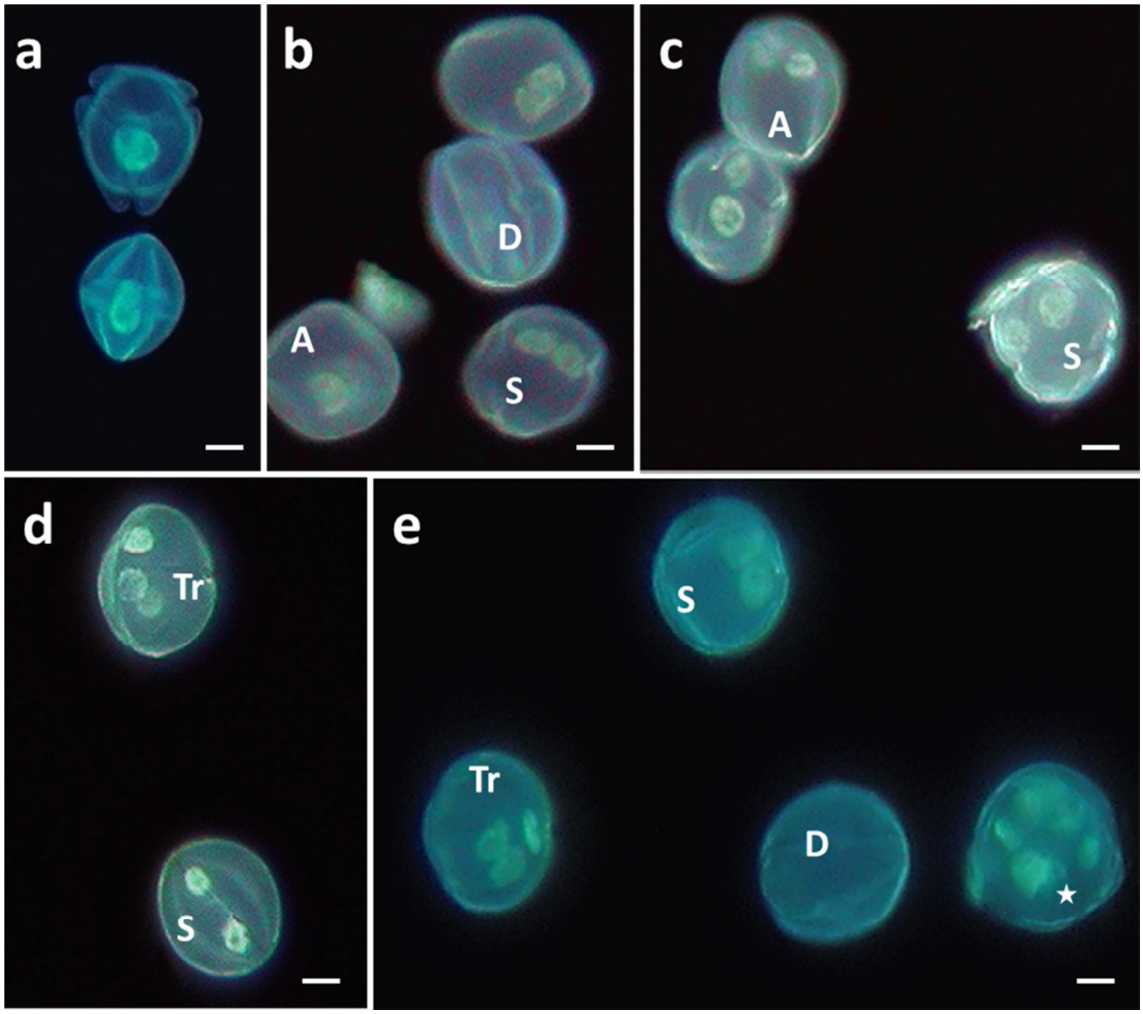

Fig. 7. Nuclear changes after embryogenic induction. MC1 procedure for microspore embryogenesis induction by anther culture. DAPI staining of squashed anthers: a) microspore before pretreatment; b-e) enlarged microspores showing first embryogenic divisions observed after 7 days at 35ºC pretreatment; b and c) asymmetrical (A) and symmetrical divisions (S) observed together with empty-dying microspores (D); d-e) pollen grains with 3 nuclei showing different degrees of chromatin condensation (Tr); e) after pretreatment non-homogenous and asynchronic answer was obtained, including multinuclear pollen grains (star) which were non very frequently found at this stage. Bars = 10 µm.

In anthers cultured for seven days after the induction pretreatment (C7) it was possible to observe multinuclear structures showing the presence of 8-12 nuclei (Fig. 8 a, b). In anthers cultured for fourteen days after the induction pretreatment (C14) embryo-like structures with more than 20 nuclei were found. In these structures the beginning of the exine rupture was observed. In anthers after twenty-one days of culture (C21) it was possible to observe embryo-like structures without exine containing numerous nuclei (Figs. 8, 9).

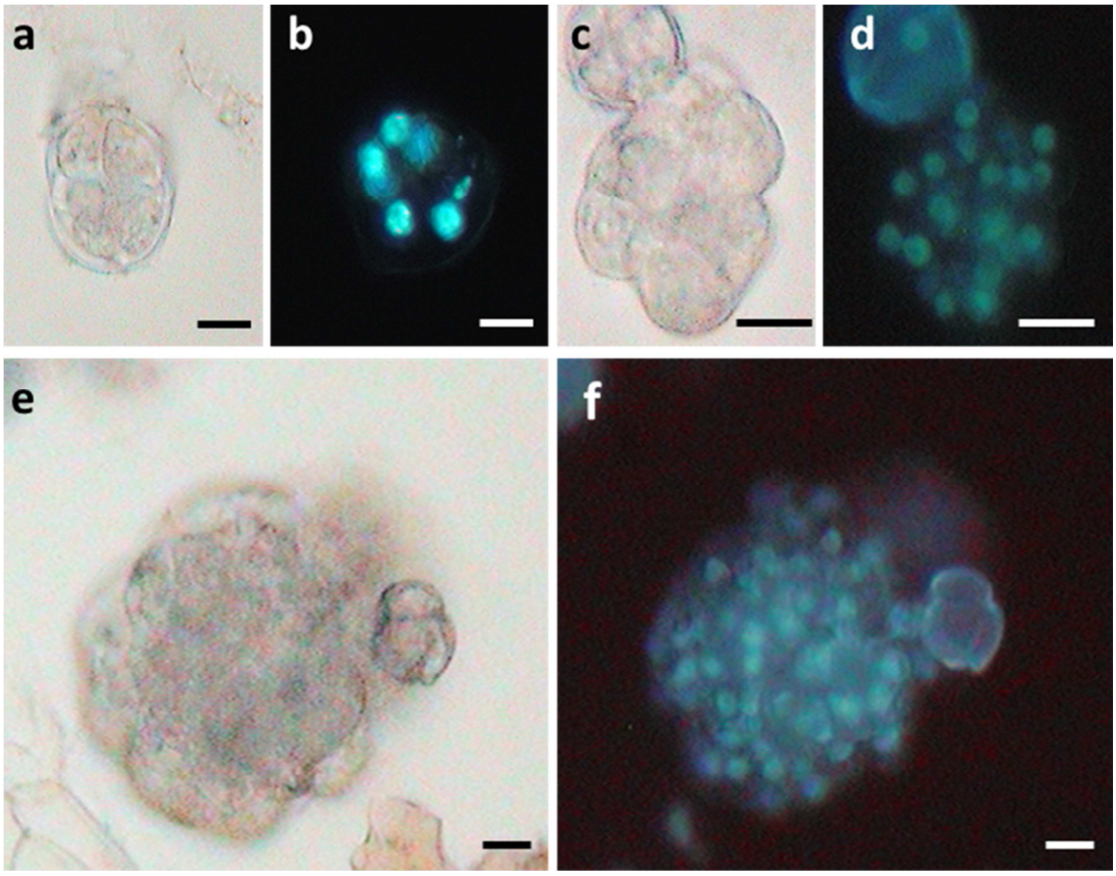

Fig. 8. Nuclear changes after different moments of the anther culture in hot pepper. MC1 procedure. Serrano anthers cultured during 7, 14 and 21 days (C7, C14, C21). a, c and e) Phase contrast images of different multicellular structures and b, d and f) DAPI-stained epifluorescence images of the same structures. a, b) Microspores showing 8 nuclei in anthers at C7; c, d) multicellular structures in anther at C14 showing signs of exine disruption and containing about 20 nuclei; e, f) proembryo at C21 where exine has practically disappeared. Bars = 20 µm.

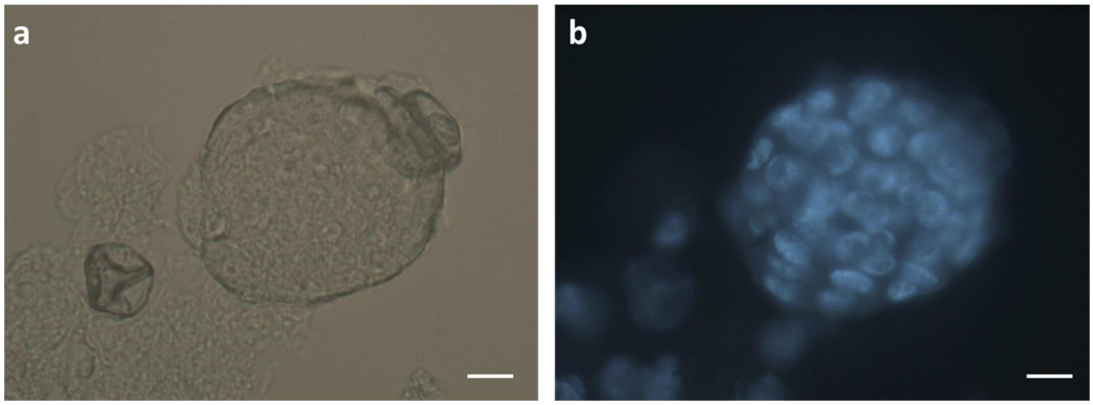

Fig. 9. Nuclear changes after anther culture in sweet pepper. DDV procedure. Lamuyo proembryo obtained after 21 days of culture (C21). a) Phase contrast image and b) DAPI-stained epifluorescence image of the same structure. Bars = 20 µm.

The changes observed in the structures obtained in the sweet genotype were similar to those described for the hot genotypes whatever procedure or stage considered (Figs. 7, 8).

Although similar structures were found for isolated microspore culture, both multicellular and proembryo structures were detected later in isolated microspore culture than in anther culture irrespective of the procedure used.

3. Cytochemical changes.

To carry out the cytochemical study of the changes taking place during microspore embryogenesis induction, different stages before and after this induction were selected (A0, A7, C7, C14 and C21). Samples were processed for

light-microscope observation and cytochemical techniques allowing the detection of carbohydrates, proteins and lipids were applied to the sections obtained from the different samples. To study anther culture MC1 procedure was applied to Lamuyo and Serrano genotypes. To study isolated microspore culture MC1* procedure was applied to Jalapeño.

3.1. Toluidine blue staining.

The toluidine blue staining was used first to verify good sample preservation and then to follow the structural changes taking place during microspore embryogenesis induced both after anther and isolated-microspore culture.

The optimal stages selected in this study for the induction of microspore embryogenesis were: microspores at late vacuolated stage and young bicellular pollen. At late vacuolated stage the nucleus was pushed to the periphery of the cell and the cytoplasm was reduced to a thin layer due to the presence of a big vacuole (Fig 10 a) while at young bicellular pollen the vacuole was fragmented and the generative cell was visible inside the vegetative one (Fig. 10 b).

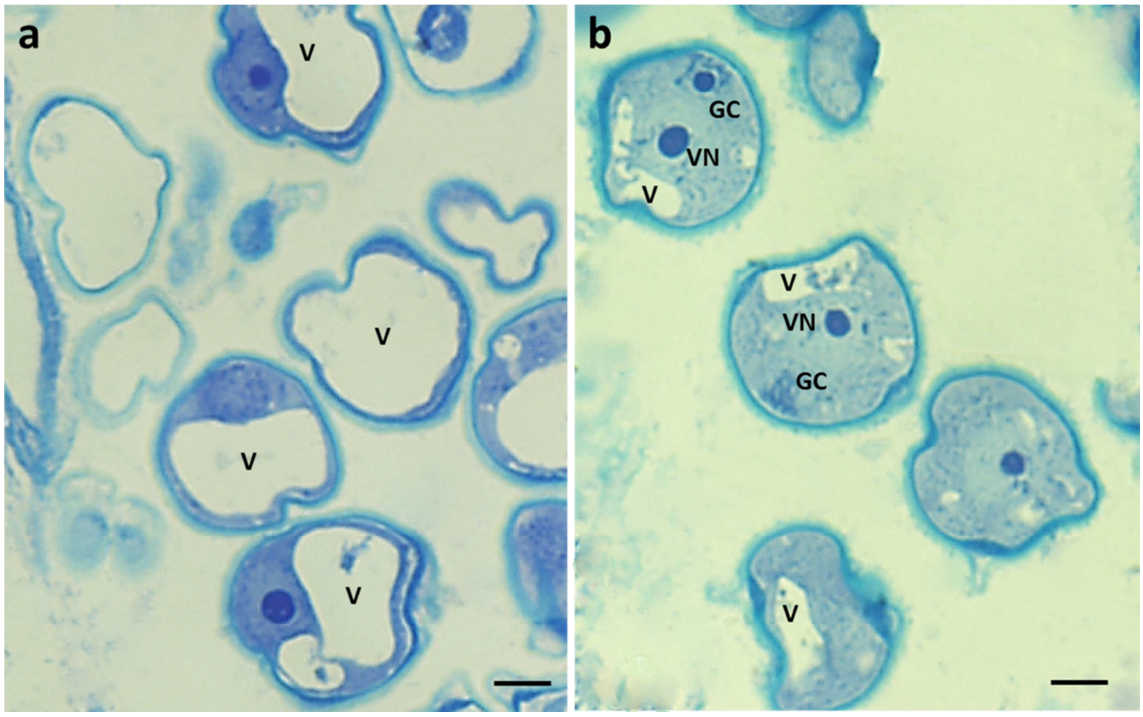

Fig. 10. Micrographs showing semi-thin sections of anthers before embryogenesis induction (A0). Toluidine blue staining. a) Anthers containing microspores at late vacuolated stage showing a big vacuole (V), b) anthers containing young bicellular pollen with fragmented vacuole (V), showing the generative cell (GC) and the vegetative nucleus (VN). Bars = 10µm.

After the induction pretreatment (A7) of MC1 procedure both in Serrano (Fig. 11 a-e) and Lamuyo (Fig. 11 f) genotypes it was possible to observe dividing microspores with two cells which were, in the majority of the cases, apparently equal. In the same anthers, confirming the results obtained after DAPI staining, it was also possible to find: empty, dead or vacuolated microspores practically non-changed from A0 stage (Fig. 11).

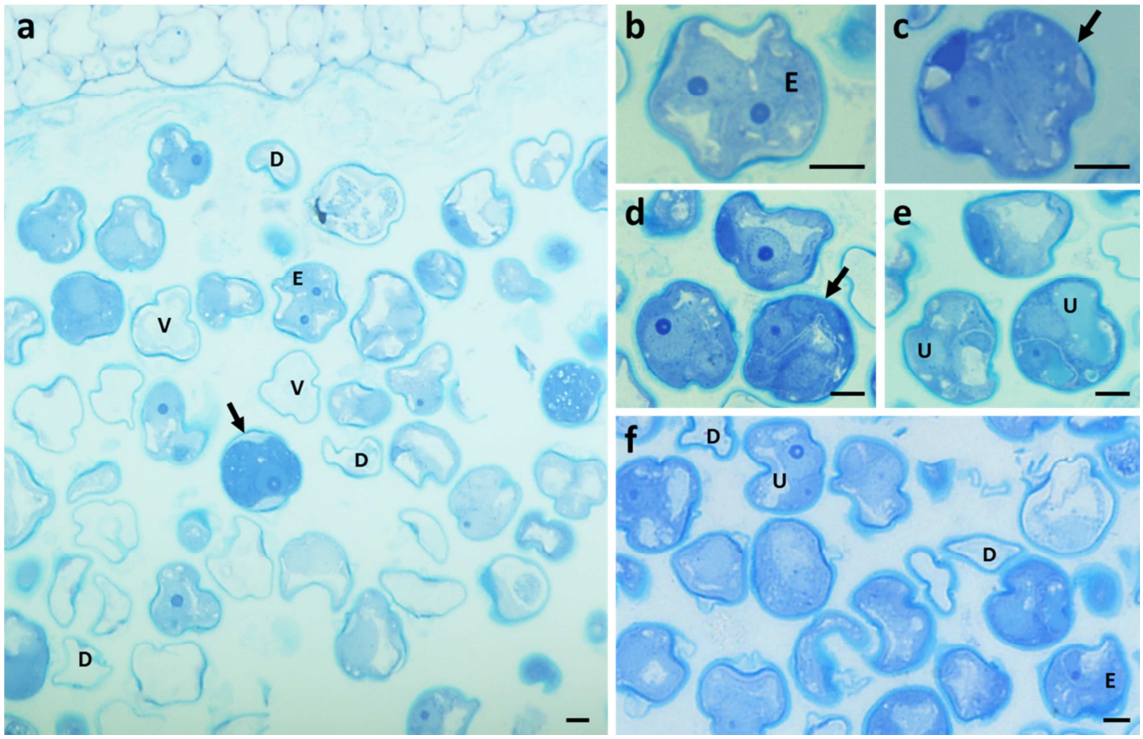

Fig. 11. Micrographs showing semi-thin sections of Serrano and Lamuyo anthers pretreated at 35ºC for 7 days (A7). MC1 procedure. Toluidine blue staining. a-e) Serrano anther showing: two-celled pollen grains with equal cell size (E), two-celled pollen grains with unequal cell size (U), multicellular pollen grains (arrow), vacuolated microspores (V) as well as dead microspores (D). f) Lamuyo anther showing the same heterogeneous and asynchronous answer to microspore embryogenesis induction. Bars = 10µm.

In anthers cultured for seven days after the induction pretreatment (C7) multicellular pollen grains with two and more than two cells were found together with microspores showing signs of death confirming the heterogeneous and asynchronous response found in the previous stage studied (Fig. 12). Although the majority of the multicellular structures showed a complete exine (Fig. 12 d) in this stage we found for the first time some multicellular structures with signs of exine disruption (Fig. 12 e).

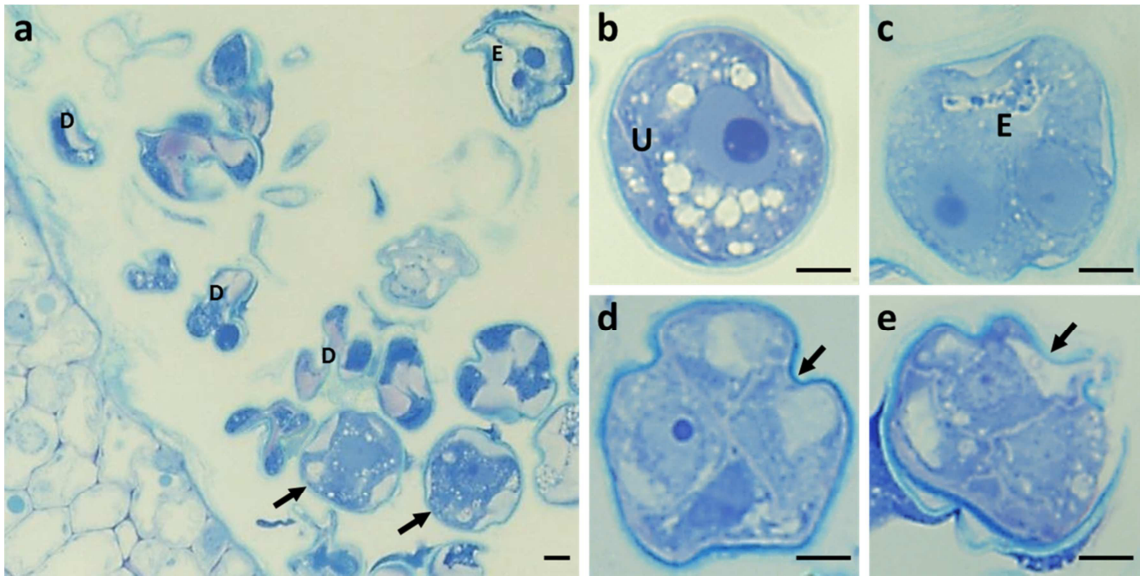

Fig. 12. Micrographs showing semi-thin sections of Serrano anther at C7 stage stained with toluidine blue. MC1 procedure. a) Anther showing neither homogeneous nor synchronic response: multicellular pollen grains with two equal cells (E) or more cells (arrows), dead microspores and empty microspores (D) b, c) multicellular pollen grains with at least two cells coming from an unequal division (U) or from an equal division (E), d) multicellular structure inside the exine, e) multicellular structure showing a ruptured exine. Bars = 10 µm.

In anthers cultured for twenty-one days after the induction pretreatment (C21) different structures were observed in the same anther were it was possible to find: proembryos without exine and proembryos in which the exine was not completely disrupted (Fig. 13 a-d), together with multicellular pollen grains surrounded by a complete exine containing different number of cells (Fig. 13 e, f). Some proembryos, where the exine was not visible, showed suspensor-like structures surrounded by exine (Fig. 13 b, c).

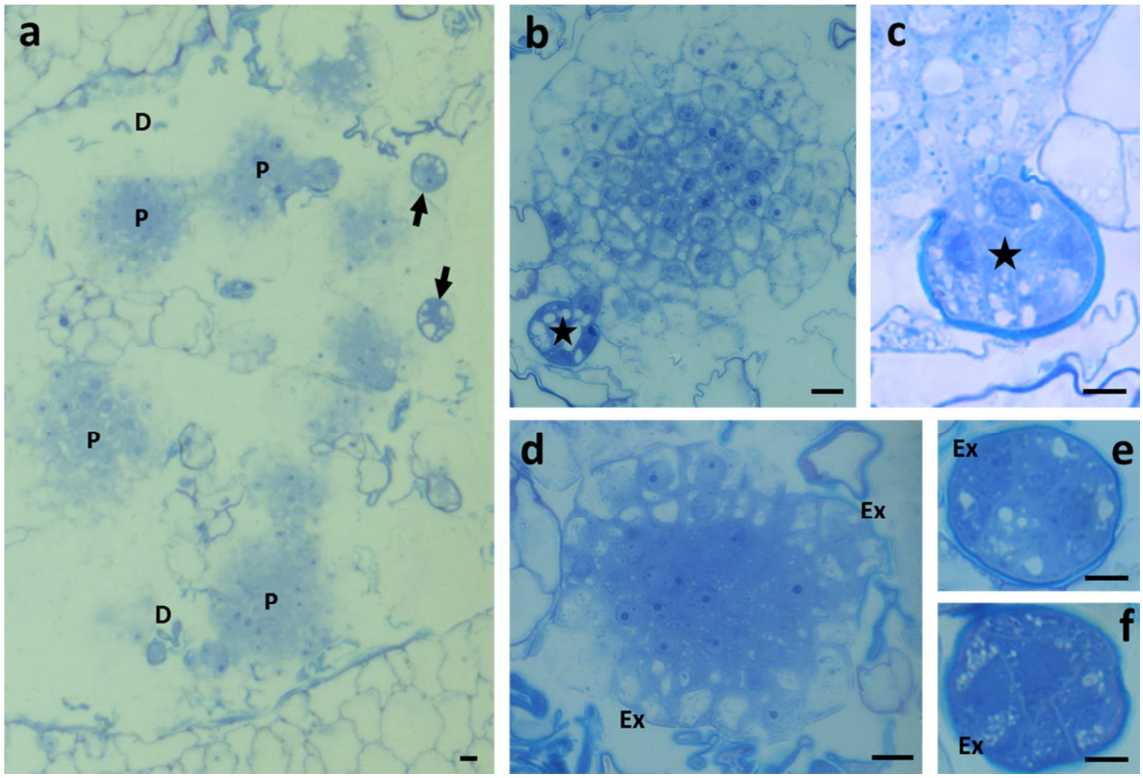

Fig. 13. Micrographs showing semi-thin sections of Lamuyo anther at C21 stage after anther cultured with MC1 procedure stained with toluidine blue. a) Anther showing proembryos (P), multicellular pollen grains (arrows) and empty microspores (D). b, c) Proembryos showing suspensor-like structures surrounded by exine (stars), d) proembryo with some remaining exine (Ex) and e-f) multicellular pollen grains with a complete exine. c, e, f: Bars = 10 µm a, b, d: Bars =20 µm.

In isolated microspore culture of Jalapeño genotype using MC1* procedure when cultured microspores were observed directly with the inverted microscope it was possible to find at C21 stage proembryos showing the formation of a suspensor-like structures (Fig. 14 a, c). In semi-thin sections of these microspores at C21 stage, multicellular pollen grains were observed and in some of them exine appear to be broken. The dehiscence of the exine generally

took place at least in one of the three apertures where the exine was thinner (Fig. 14 d, e). Extrusion of material was observed in the regions where exine was broken (Fig. 14 d, e) but a structure made up of vacuolated cells and clearly corresponding to the suspensor-like structure has not been found in semi-thin sections (Fig. 14 e, f).

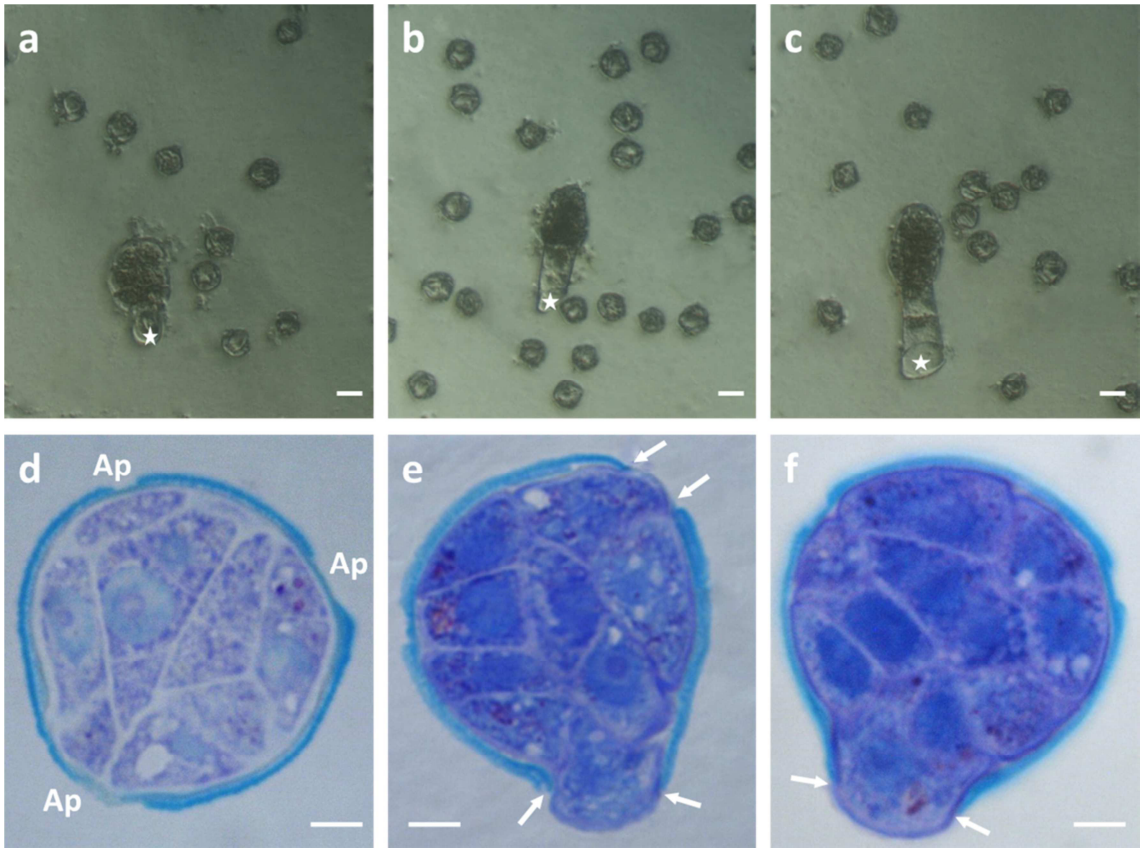

Fig. 14. Suspensor-like-structure in Jalapeño isolated microspore culture after 21 days of culture (C21). MC1* procedure. a-c) Micrographs of the direct observation of different plates of the isolated-microspore culture where proembryos with suspensor-like structures (stars) are visible. d-f) Micrographs of semi-thin sections of proembryo structures stained with toluidine blue. d) Multicellular pollen grain showing thinner exine at the 3 apertures (Ap). e, f) Multicellular pollen grains where exine is broken and some cells are extruded (arrows) at 1 or 2 of the apertures. a, b, c: Bars = 10µm. d, e, f: Bars = 10µm.

3.2. Periodic acid Schiff's reagent (PAS) staining.

To follow changes in carbohydrate distribution during microspore embryogenesis, PAS staining detection technique was used in semi-thin sections of the samples previously described for anther culture following MC1 procedure.

In Serrano and Lamuyo anthers before embryogenesis induction (A0), PAS-positive reaction was found at the walls of the cells forming the anther locule: epidermis, endotecium and middle layer, as well as at the degenerating tapetum cells. PAS-positive reaction was also found at the intine in the vacuolated microspores (Fig 15).

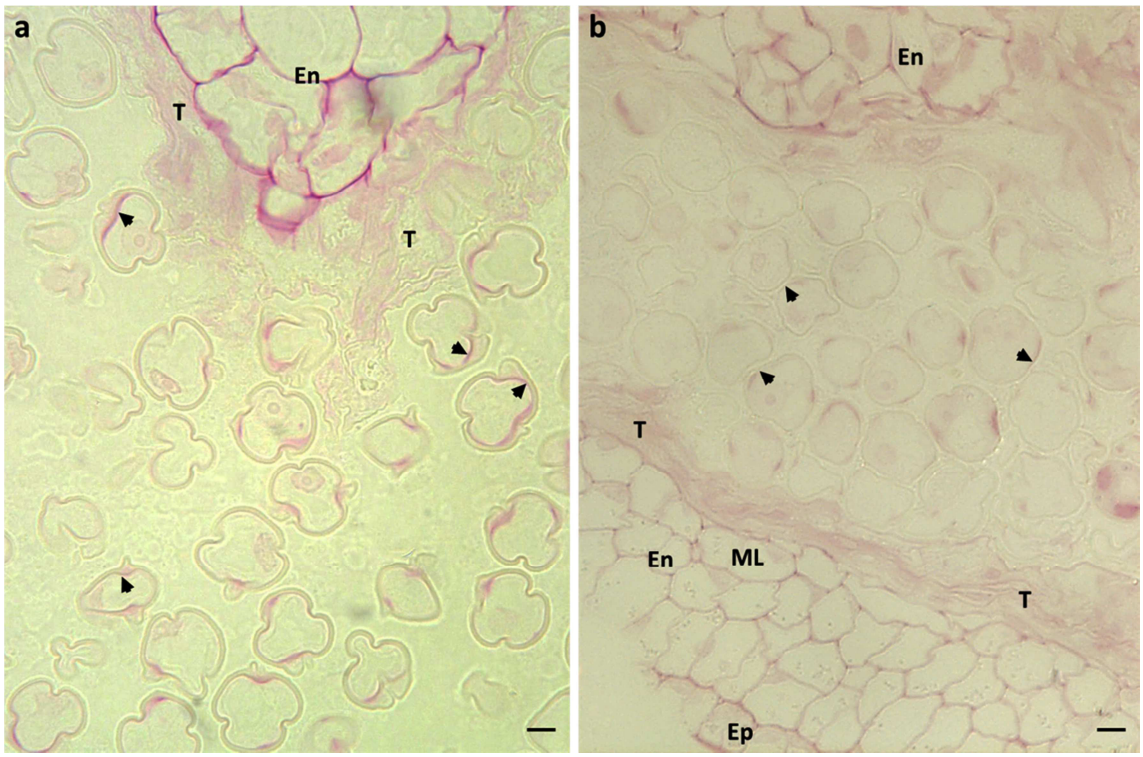

Fig. 15. Micrographs of semi-thin sections of anthers before embryogenesis induction (A0), insoluble polysaccharides stained with PAS. a) Lamuyo and b)

Serrano genotype. Epidermis (Ep). Endotecium (En). Middle layer (ML). Degenerating tapetum (T). Vacuolated microspores with stained intine (arrows). Bars = 10μm.

In Serrano and Lamuyo anthers after the induction pretreatment (A7), PAS-positive reaction was visible at the anther tissue walls as well as at the intine of microspores and at the swollen intine of multicellular pollen grains. Exine pollen wall was not stained. PAS-positive reaction was also observed in the intermediate walls formed between the cells of the multicellular pollen grains and in cytoplasmic granules of different sizes accumulating inside these multicellular pollen grains (Fig. 16). These granules that correspond to starch granules were especially large and abundant in some pollen grains (Fig. 16).

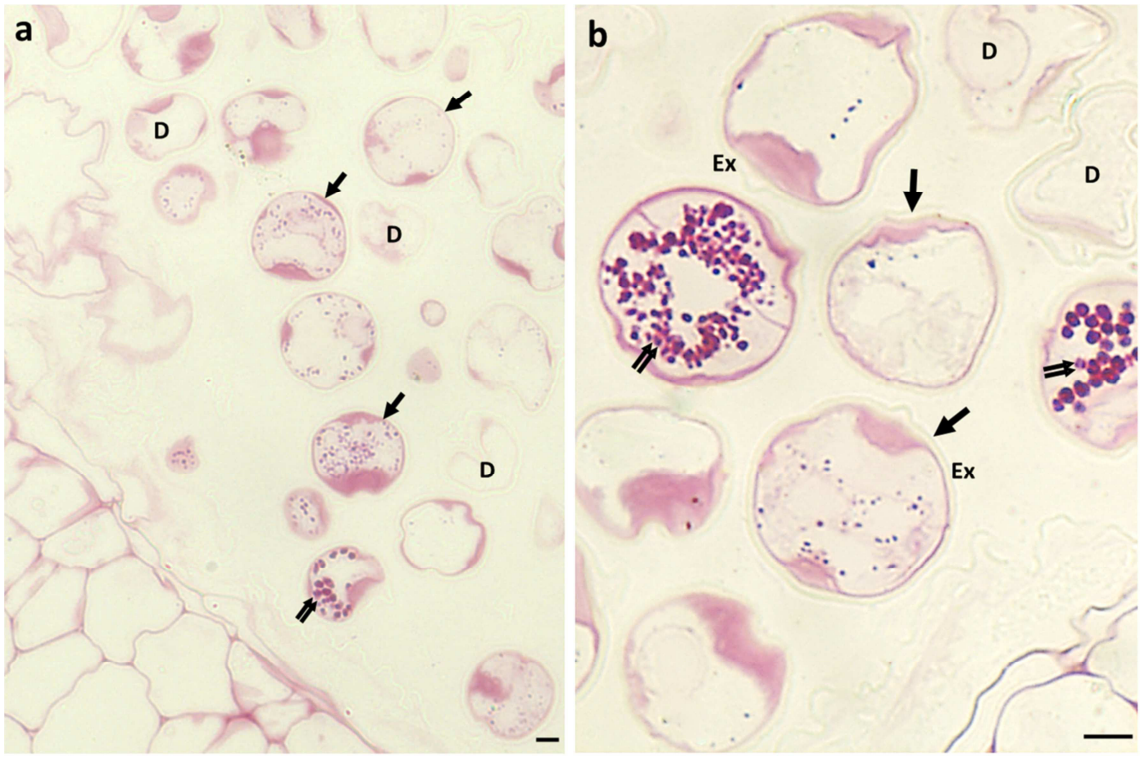

Fig. 16. Micrographs showing semi-thin sections of anthers pretreated at 35ºC for 7 days (A7) insoluble polysaccharides stained with PAS. MC1 procedure. a) Lamuyo and b) Serrano anthers. Multicellular pollen grains (arrows). Dying pollen grains (D). Exine (Ex). Pollen grains with numerous starch cytoplasmic granules (double arrows). Bars = 10µm.

PAS-positive structures found at C7 in Serrano anthers, were the same as those found at A7 but a higher number of multicellular pollen grains made up of more cells were found at C7 (Fig. 17).

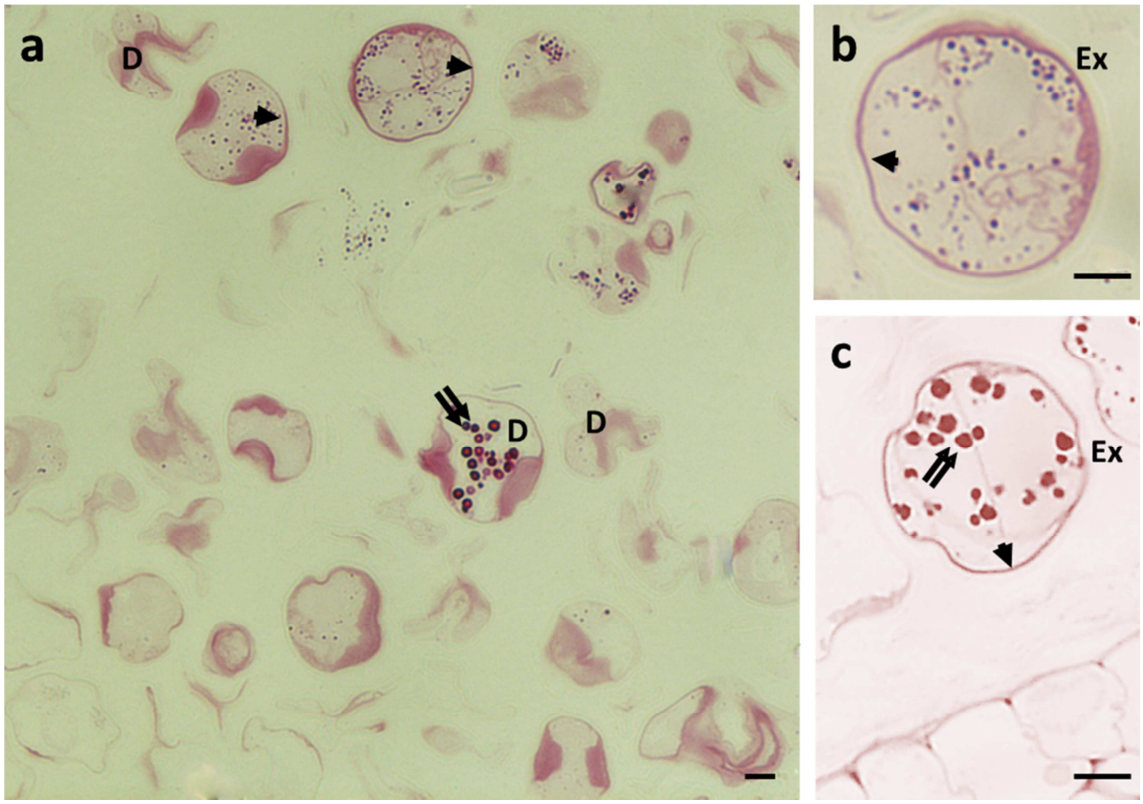

Fig. 17. Micrographs showing semi-thin sections of Serrano anther at C7 stage, insoluble polysaccharides stained with PAS. MC1 procedure. a) Anther containing different types of structures. Detail of: b) multicellular pollen grain, and c) dying multicellular pollen grain (D) with numerous cytoplasmic granules (double arrows). Intine (arrowheads). Exine (Ex). Bars = 10µm.

In Lamuyo anthers cultured for twenty-one days after the induction pretreatment (C21) and stained with PAS, it was possible to observe a PAS-positive reaction in cell walls of anther tissues being the tapetum reduced to a very thin layer (Fig. 18 a). In the proembryos found at this stage it was possible to see an area that could correspond to the suspensor-like structure previously described. In some cases the intine layer surrounding the suspensor-like structure seemed to be thicker than the intermediate walls present between the cells of

these multicellular structures (Fig. 18 b-d). The cells of the suspensor-like structure showed numerous PAS-positive granules. These granules had similar size of those found in the outer cells of the proembryos (Fig. 18 b-d). Together with the proembryos, multicellular pollen grains were visible (Fig. 18 a, b). In these pollen grains thick intine, intermediate walls between the cells as well as numerous cytoplasmic granules showed positive PAS-reaction (Fig. 18 a, b).

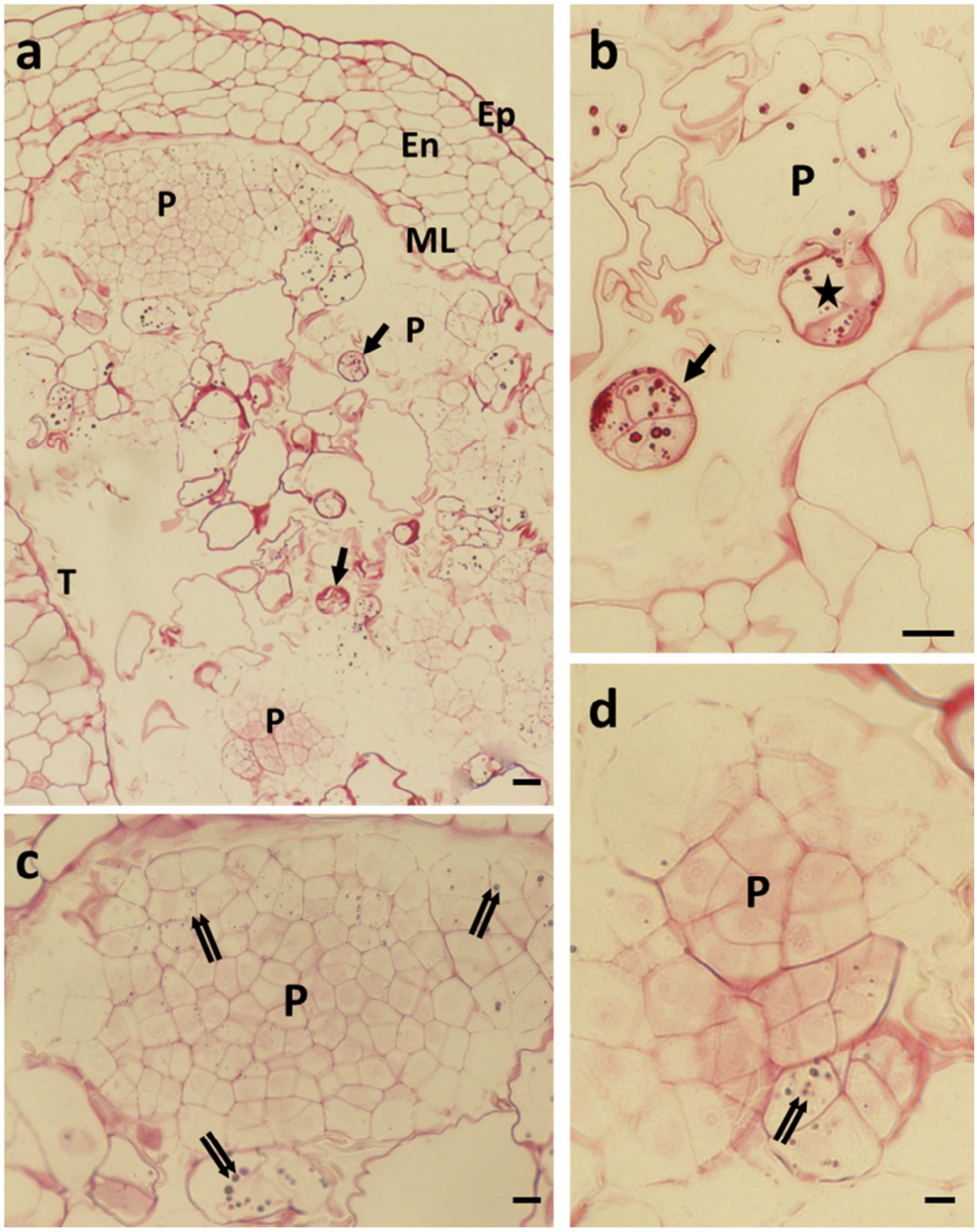

Fig. 18. Micrographs showing semi-thin sections of Lamuyo anther at C21 stage, insoluble polysaccharides stained with PAS. MC1 procedure. a) Anther showing proembryos (P) and multicellular pollen grains (arrows). b) Anther showing a proembryo with an intine-covered suspensor-like structure (star) and a multicellular pollen grain (arrow). c, d) Details of proembryos showing

numerous large PAS-stained granules in the suspensor-like structure and in their outer cells (double arrows). Epidermis (Ep). Endotecium (En). Tapetum (T). Middle layer (ML). a: Bar = 10µm, b, c, d: Bars = 20µm.

3.3. Coomassie brilliant blue staining.

To follow changes in protein distribution during microspore embryogenesis, Coomassie brilliant blue staining was applied on semi-thin sections of Lamuyo and Serrano anthers cultured by MC1 procedure.

In anthers before microspore embryogenesis induction (A0 stage) the locule area was filled with proteinaceous material detected by Coomassie brilliant blue staining both in Lamuyo and Serrano genotype. Tapetal cells were collapsed and their cytoplasm-contain dispersed in the locule (Fig. 19). In vacuolated microspores numerous protein bodies were found in the cytoplasm, and proteinaceous material was also detected inside their vacuoles (Fig. 19).

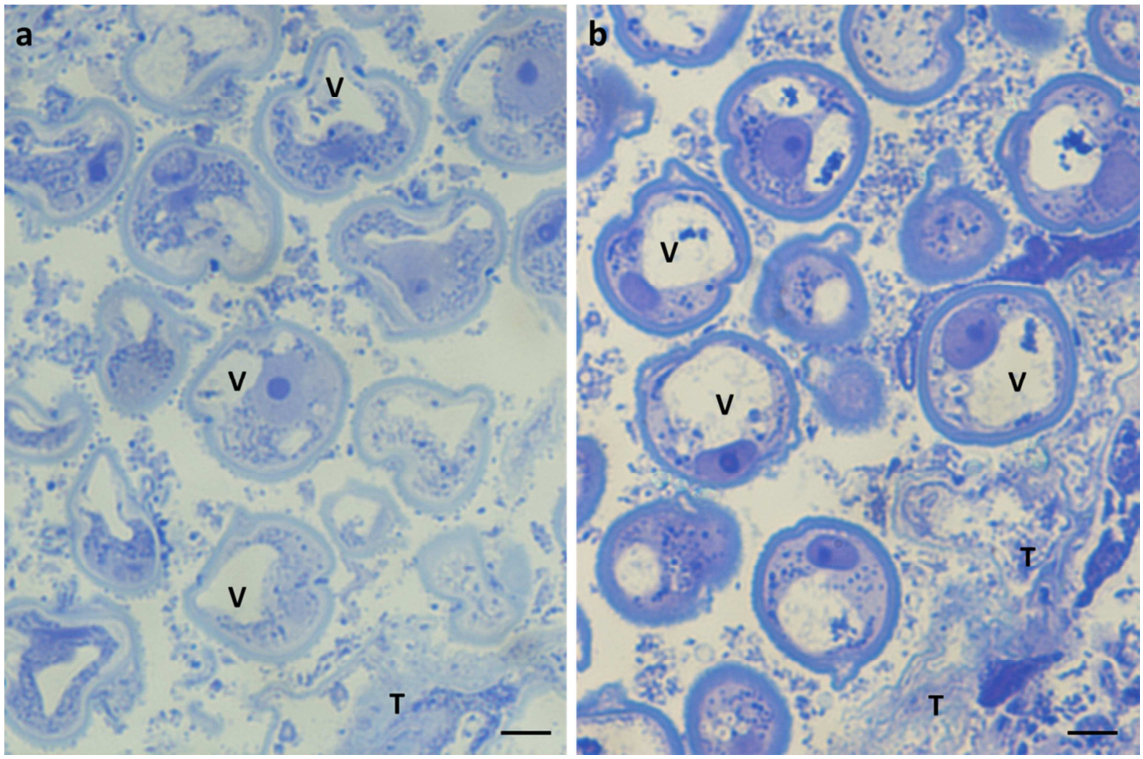

Fig. 19. Micrographs of semi-thin sections of anthers before embryogenesis induction (A0), protein stained with Coomassie brilliant blue. a) Lamuyo and b) Serrano genotype. Tapetum (T). Vacuole (V). Exine (Ex). Bars = 10µm.

At A7 stage, immediately after the induction pretreatment, in Serrano genotype the amount of proteinaceous material found in the locule clearly decreases when compared with that of A0 stage. The degenerating tapetum was still visible. At this stage, multicellular pollen grains with numerous protein bodies in their cytoplasm, two-three celled pollen grains, and dead microspores were visible (Fig. 20).

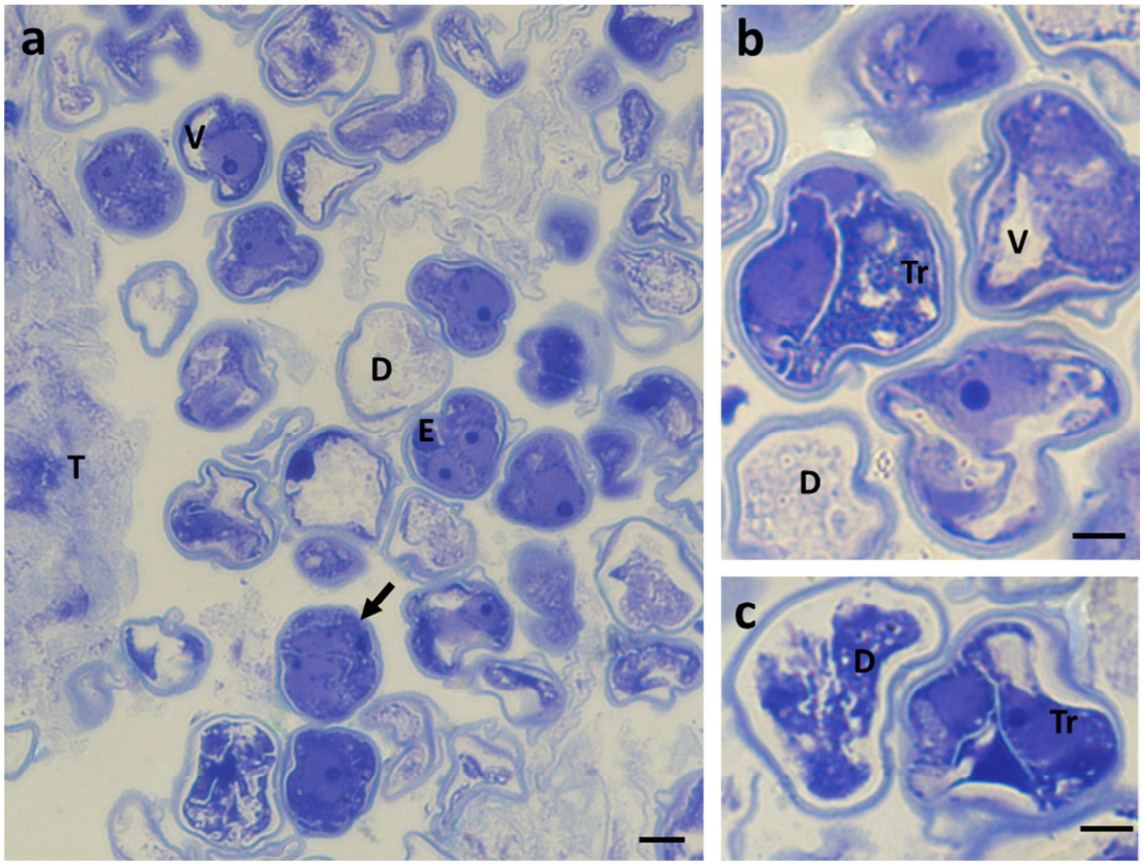

Fig. 20. Micrographs showing semi-thin sections of Serrano anthers pretreated at 35ºC for 7 days (A7), proteins stained with Coomassie brilliant blue. MC1 procedure. a) Anther showing multicellular pollen grains (arrows), two-celled pollen grains coming from an equal division (E), vacuolated microspores (V) and dead microspores (D). b, c) Details of tri-celled pollen grains (Tr) and dead microspores (D). Exine (Ex). a: Bar = 20µm, b, c: Bars = 10µm.

In Serrano anthers cultured for 7 days after the induction pretreatment (C7) and stained with Coomassie brilliant blue stain different structures were found: multicellular pollen grains, two-celled pollen grains coming from asymmetric or symmetric divisions, and dead microspores. In living pollen grains protein bodies were stained in the cytoplasm. Whereas in dead pollen grains

these protein bodies were not distinguishable from the deeply-stained cytoplasm in which it was possible to see large non-stained granules. These latter could correspond to the PAS-positive granules previously described (Fig. 21). Proteinaceous material of the locule observed in previous stages practically disappeared at this stage (Fig. 21).

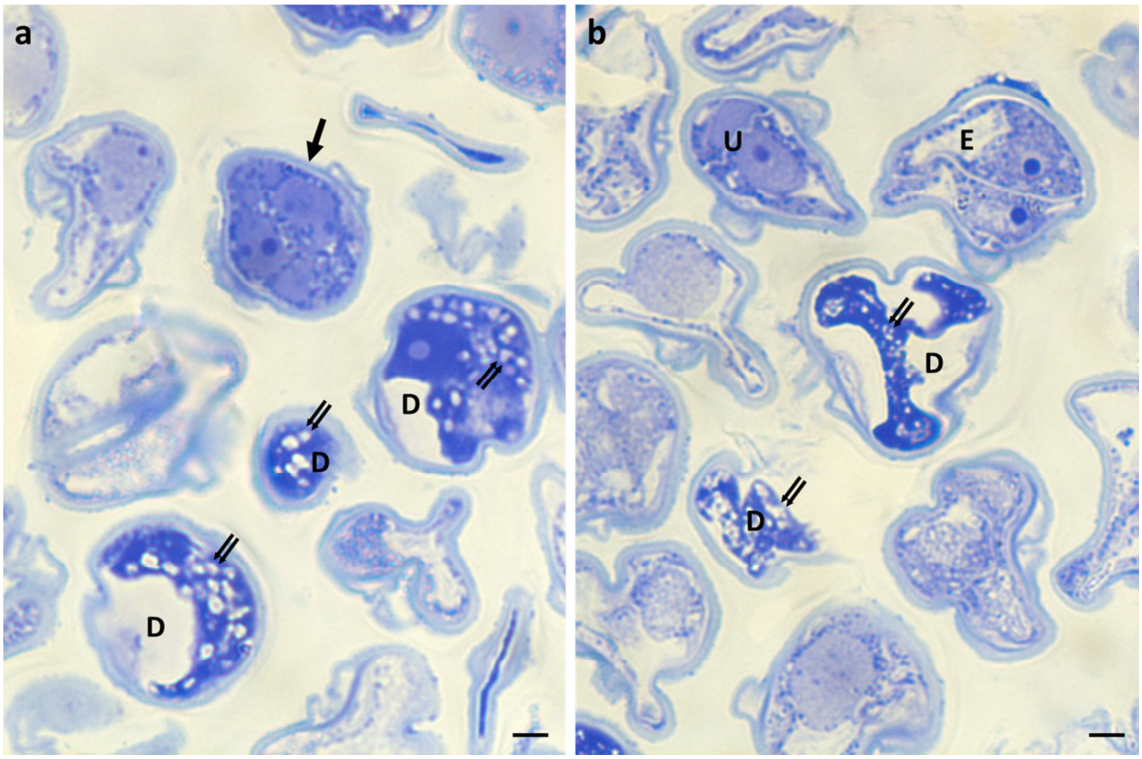

Fig. 21. Micrographs showing semi-thin sections of Serrano anther after 7 days of culture (C7) proteins stained with Coomassie brilliant blue. MC1 procedure. a, b) Protein bodies are visible in multicellular pollen grains (arrows) and two-celled pollen grains with equal cell size (E) and unequal cell size (U), while in dead microspores (D) with very dense cytoplasm non-stained granules (double arrows) are visible. Bars = 10µm.

In Lamuyo anthers cultured for 21 days after the induction pretreatment (C21) and stained with Coomassie brilliant blue, proembryos, multicellular structures and covered-exine dividing cells exhibited protein presence (Fig. 22 a), this was specially visible in advanced proembryo structures where groups of cells forming suspensor-like structures were visible (Fig. 22 b, c). These suspensor-like structures in certain proembryos were surrounded by an exine wall (Fig. 22 b).

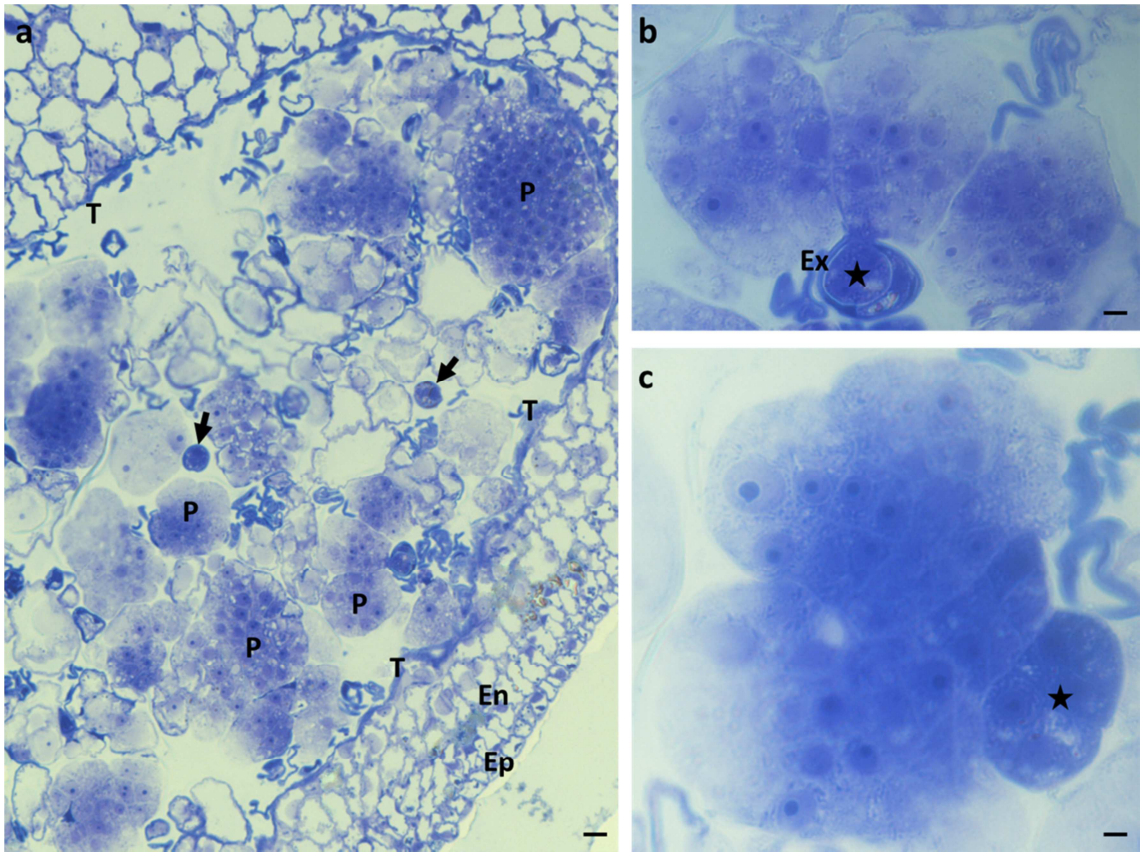

Fig. 22. Micrographs showing semi-thin sections of Lamuyo anther at C21 stage, proteins stained with Coomassie brilliant blue. a) Protein distribution in different structures observed in Lamuyo anthers including proembryos (P) and multicellular pollen grains (arrows). b) Proembryo with exine-covered suspensor-like structure (star). c) Proembryo with suspensor-like structure (star) where the

exine is not visible. Epidermis (Ep). Endotecium (En). Tapetum (T). Exine (Ex). a: Bar = 60µm, b, c: Bars = 20µm.

3.4. Sudan black staining.

To follow changes in lipids distribution during microspore embryogenesis, Sudan black staining was applied on semi-thin sections of Lamuyo and Serrano anthers cultured by MC1 procedure.

In locule of anthers before microspore embryogenesis induction (A0), degenerating tapetum showed the presence of lipoidal material. In the microspores the exine was strongly stained in both Lamuyo and Serrano genotypes (Fig. 23).

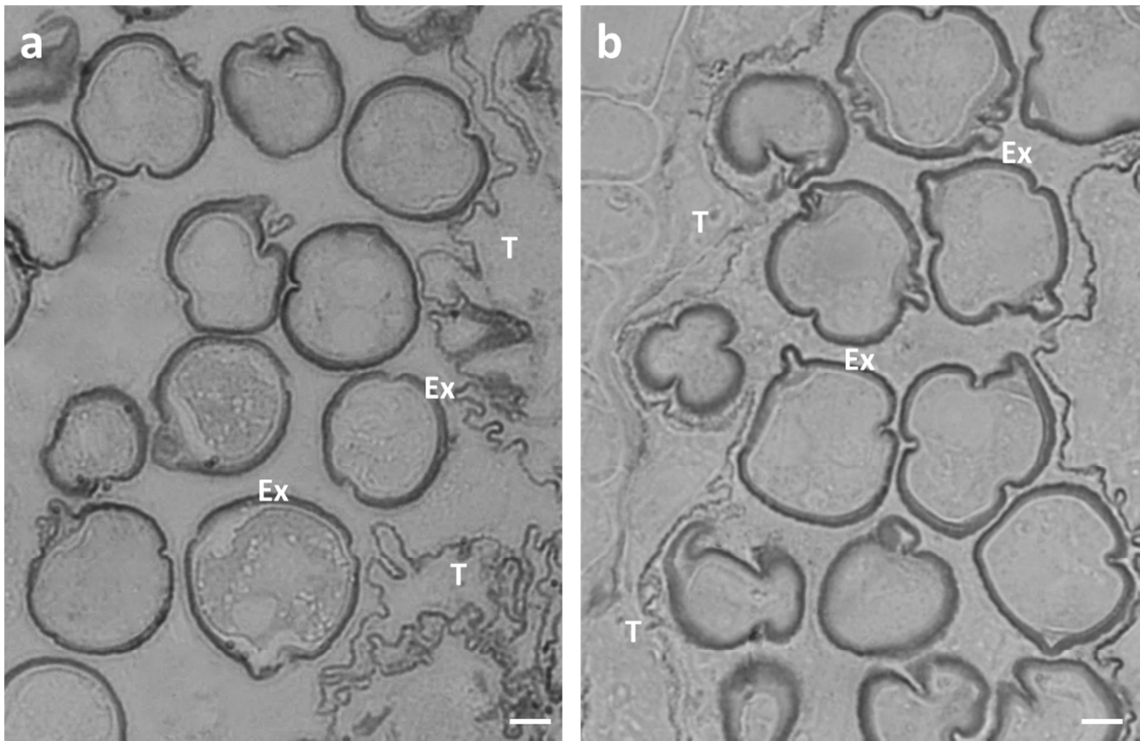

Fig. 23. Micrographs of semi-thin sections of anthers before embryogenesis induction (A0), lipid stained with Sudan black. a) Lamuyo and b) Serrano genotype. Exine (Ex). Tapetum (T). Bars = 10µm.

In Lamuyo anthers cultured for 21 days after the induction pretreatment with MC1 procedure (C21) and stained with Sudan black, exine remnants of proper embryos as well as exine surrounding suspensor-like structures were strongly stained (Fig. 24).

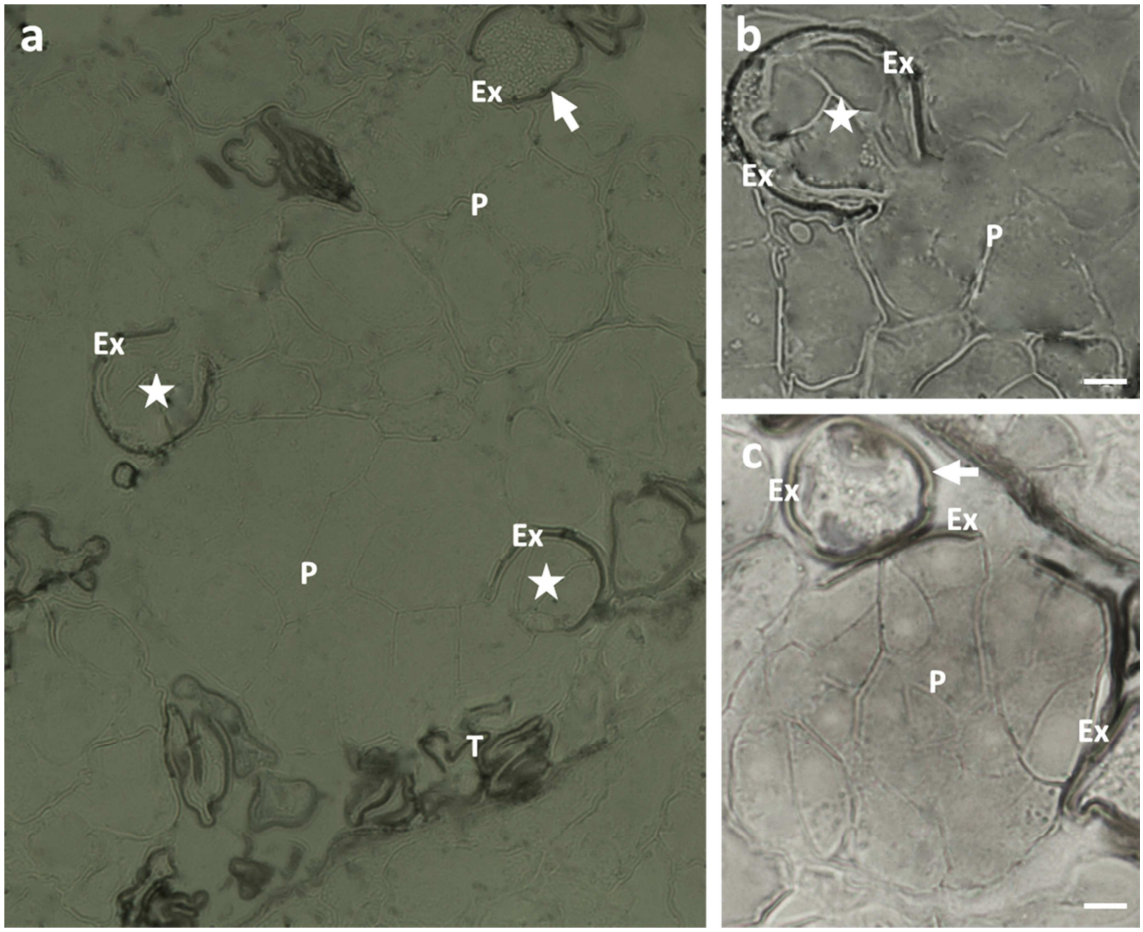

Fig. 24. Micrographs of semi-thin sections of Lamuyo anthers after 21 days of culture (C21), lipid stained with Sudan Black. a) Lipids observed in different structures including proembryos (P), suspensor-like structures (star), multicellular pollen grains (arrows) and dead microspores (D). b) Proembryo

with exine-covered suspensor-like structure and c) proembryos with a residual stained exine (Ex). Bars = 20μm.

Discussion

The development of tissue culture has made a breakthrough in crop plant breeding. Different approaches have been used along the history of agriculture for plant breeding but the development of new varieties with conventional techniques is very time-consuming and this fact considerably increases the production costs. Nowadays the better understanding of the genetic principles, the development of new biotechnologies as well as their application in plant breeding provide powerful tools to raise the efficiency and the speed of these methods (Germaná 2011). In particular, the use of haploid plants represents an attractive biotechnological method that can accelerate plant breeding. Haploid plants through microspore embryogenesis may, in fact, be produced in less than a year (Forster *et al*. 2007, Olmedilla 2010, Islam and Tuteja 2012). Furthermore, the production of these plants followed by chromosome doubling give rise to double haploid plants with a complete homozygous genotype: this means obtaining homozygous plants from heterozygous plants in only one generation (Touraev *et al*. 2001, Maraschin *et al*. 2005).

Since Guha and Maheshwari (1964) discovered that microspores can switch from their gametophytic to a sporophytic development throughout a process called microspore embryogenesis, techniques based on this phenomenon have been tested to raise double haploid plants in more than 250 species, including species of high agricultural importance. This is an important procedure for plant breeding although only in less than thirty species this procedure has been optimized to obtain reproducible and efficient results (Maluszynski *et al*. 2003, Dunwell 2010) and even in these species, the level of success is strongly genotype-dependent (Pechan and Smykal 2001, Touraev *et al*. 2001, Ferrie and Caswell 2011). Another inconvenience is that the knowledge in this field is unfortunately still incomplete and the obtaining of double haploids by microspore embryogenesis is nowadays basically an empirical procedure (Irikova *et al*. 2011).

Two methods have been employed to generate haploid plants by microspore embryogenesis: anther culture and isolated microspore culture (Maeshwari *et al*. 1982, Raghavan 1986, Reynolds 1997, Touraev *et al*. 2001, Maraschin *et al*. 2005, Olmedilla 2010). As mentioned before, microspore embryogenesis has been induced in a considerable number of plant species and there are some, such as tobacco (*Nicotiana tabacum* L.), oilseed rape (*Brassica napus* L.) and barley (*Hordeum vulgare* L.), which, due to great success in terms of the induction and efficient regeneration of haploid plants, are considered to be model plants (Olmedilla 2010). In contrast, there are other species that are considered recalcitrant or at least in which this procedure has not been completely mastered. Among these latter we can include *Capsicum annuum* and especially their hot pepper genotypes (Seguí-Simarro *et al*. 2011).

Breeders are very interested in *Capsicum* not only because of its widespread use all over the world but also because of its nutritional, cosmetic and pharmaceutical beneficial properties (Bosland 1996). Its economic importance drives researchers and companies to invest their efforts in the improvement of this crop. Among the wide range of pepper varieties, hot pepper is gaining importance because this fruit contains capsaicin, which is not present in non-pungent pepper. Capsaicin is responsible for the pungency of hot peppers and has been proved in plants to have an antimicrobial and antifungal activity Veloso *et al*. (2013). Hot pepper was used since pre-Colombian times for the treatment of various human diseases.

Even though hot pepper genotypes are considered to be recalcitrant to microspore embryogenesis induction there is a reduced number of Asiatic hot genotypes in which good results have been obtained (Kim *et al*. 2008, Supena *et al*. 2006) but the procedure that is suitable to obtain haploid plants for these genotypes does not produce good results in all hot pepper genotypes. Nowadays there is an increasing production of hot pepper not only in Spain but also in other Mediterranean countries which made essential to produce new varieties well

adapted to our environmental conditions. In *Capsicum*, as in other recalcitrant species, the main difficulties of this procedure are to reach a reasonable induction-efficiency as well as to obtain embryos that follow a normal developmental pattern which give rise to good quality plants (Supena *et al*. 2006, Seguí-Simarro *et al*. 2011, Irikova *et al*. 2011). This thesis is focused in the improvement of the procedures for microspore embryogenesis induction in hot pepper using both anther and isolated microspore culture and was developed in the frame of a collaboration project between CSIC and Monsanto Company.

I. Anther culture.

Anther culture is at present the most efficient and utilized method to induce microspore embryogenesis in *Capsicum*. Different authors have published variable results in terms of efficiency in embryo yield and plant production for different genotypes. Furthermore, these results are differently expressed: in some cases they talk about embryos per 100 anthers but they do not specify if these embryos are perfect embryos or if they are talking about the total number of embryos produced including abnormal embryos and in other cases they talk about plants per 100 anthers but again they do not specify the ploidy of these plants. For example: for sweet genotypes: Dumas de Vaulx *et al*. (1981), reported to obtain 5 to 50 plants per 100 cultured anthers, Kristiansen and Andersen (1993) 2 embryos per 100 cultured anthers, and Mityko and Fari (1997) 15 embryos per 100 anthers. Using hot genotypes Dolcet-Sanjuan *et al*. (1997), Kim *et al*. (2004), Supena *et al*. (2006) reported to obtain around 40 embryos per 100 anthers. Like in other species, different factors such as: growth conditions of donor plant, genotype, microspore developmental stage, type and duration of stress pretreatment, medium and culture conditions have been reported to affect

the success of microspore embryogenesis induction (Atanassov *et al*. 1995, Smykal 2000, Wang *et al*. 2000).

During the present study, different procedures were tested to induce embryogenesis by anther culture in three hot pepper genotypes. A sweet pepper genotype was also included to test if pungency could represent an extra factor affecting this process.

1. Genotype selection.

The genotype is considered to be the most important factor to microspore embryogenesis induction and consequently, once a procedure for double haploid production is optimized in a given genotype this does not guarantee that it will be suitable for other genotypes. The incidence of this factor was also reported in pepper (Wang and Zhang 2001, Irikova *et al*. 2011, Ferrie and Caswell 2011).

The first step of this research was to select among the fourteen genotypes available at the beginning of this study the most suitable in terms of both economic importance and microspore embryogenesis induction responsiveness. These genotypes were pre-screened including not only hot genotypes, but also some sweet genotypes that would be used as a control. A procedure derived from Dumas de Vaulx *et al*. (1981) was used to test the responsiveness of the fourteen genotypes initially selected because these procedure was found to be appropriate for sweet pepper genotypes. Using this procedure, it was confirmed that the sweet genotype Lamuyo used to set up this procedure was the most responsive but similar results were not obtained with the other sweet or hot genotypes. These data confirmed the idea postulated among others by Ferrie and Caswell

(2011) that the responsiveness of *Capsicum* was strongly genotype-dependent. Bearing these results in mind and to make more feasible this study, three hot genotypes (Serrano, Jalapeño and CM334) as well as a sweet genotype (Lamuyo) were selected. This latter was used to test if this sweet genotype was more responsive to microspore embryogenesis induction than hot genotypes whatever the procedure used.

2. Determination of the optimal microspore developmental stage for microspore embryogenesis induction in *Capsicum*.

The determination of the microspore developmental stage is other key factor for the success of microspore embryogenesis induction. We have found that in all pepper genotypes studied there were bicellular pollen in anthers containing vacuolated microspores. This asynchrony was previously reported by Kim *et al*. (2004).

To save time in the selection of the anthers containing microspores at the adequate stage, the morphological characteristics of the flower buds and anthers containing microspores at this stage were determined. For this purpose calix and corolla size of the flowers containing the appropriate anthers were determined. At the same time we also studied the distribution of the purple pigmentation of these suitable anthers. These characteristics were found to be very different depending on the genotype as it was previously reported by Irikova *et al*. (2011). These characteristics were distinguishable by naked eye in Lamuyo sweet genotype as well as in Serrano and Jalapeño hot genotypes while this was not achievable in CM334 hot genotype, where this distinction was only possible using a stereomicroscope.

In pepper, as in many other species, it has been found that the stages close to the first mitotic division (late vacuolated microspore and early bicellular pollen) are the most suitable for microspore embryogenesis induction as it was reported by several authors (Sibi *et al*. 1979, Kim *et al*. 2004, Irikova *et al*. 2011). Sibi *et al*. (1979) reported that, to get the best results, the proportion of late bicellular pollen should not exceed the 40% of the total population of microspores in the cultured anthers. According to these data, Supena *et al*. (2006) determined that anthers from flower buds containing over 50% of microspores in vacuolated stage were the most successful. Furthermore, Gonzalez-Melendi *et al*. (1995) found that late vacuolated stage is the most appropriate one for embryogenesis induction. Finally, Kim *et al*. (2004) using hot pepper reported that the anther stage optimal for embryo production using anther culture is that containing a large proportion (over 75%) of early-bicellular pollen.

We used anthers containing mainly late-vacuolated microspores in all the procedures tested as it was reported to be routinely used for the induction of microspore embryogenesis in sweet pepper. When we were testing the Kim *et al*. (2004) procedure to the four genotypes selected we initially used these anthers but we tested also anthers containing mainly early bicellular pollen, because this latter was the optimal stage for these authors as we mentioned above, but no significant differences were obtained.

3. Anther macroscopical changes observed during microspore embryogenesis.

The exudation of toxic substances, including phenols, is very common during tissue culture and greatly affects the results obtained. The darkening of the

anthers observed during anther culture, due to this toxic production has been widely reported in different species including *Capsicum* (Irikova *et al*. 2011, Olmedilla 2010). From our results, using the procedure derived from that of Dumas and de Vaulx *et al*. (1981) (DDV procedure) it was clear that the hot pepper genotypes and particularly the three hot genotypes selected for this study were especially sensitive to this problem. When anthers turned black, they underwent an apparent retard in their development that did not lead to the opening of embryo sacs and embryo formation that take place in non-oxidized anthers which means that these toxic substances could affect this process. The use of activated charcoal has been reported to be a good solution to overcome this problem (Thomas 2008). In this way the oxidization of the anthers was avoided and microspore embryogenesis was stimulated by the elimination, among other toxic substances, of abscisic acid inhibitors. In addition, activated charcoal has the characteristic property of high absorptive power for colloidal solids, gases and vapors that can have different toxic and/or inhibitory effects for microspore embryogenesis (Thomas 2008).

4. Procedures assayed to improve microspore embryogenesis in hot pepper by anther culture.

4.1. Procedure derived from Dumas de Vaulx et al. (1981). DDVac procedure.

The addition of activated charcoal in the culture media led to a decrease in the number of black anthers in hot pepper, as well as to an increase of embryo produced in sweet pepper as it was demonstrated in the results obtained with DDV_{ac} procedure. On the basis of these results, we decided to use activated charcoal routinely in our induction and culture media from this point onwards. This modification in the procedure (DDV_{ac}) as well as the original DDV procedure gave significant results for the sweet pepper genotype which was used to set it up but, not comparable results were obtained with the hot pepper genotypes. These results confirmed previous reported data showing that in *Capsicum*, as in many other species, the efficiency of induction is strongly genotype-dependant (Qin and Rotino 1993, Mityko *et al*. 1995).

4.2 Procedure derived from that of Mc Comb and Mc Comb (1977). MC1 procedure.

The procedure of McComb and McComb (1977) that was reported to be successful in a *Solanaceae* species was tested not only because it uses the addition of activated charcoal that we previously found beneficial for microspore-embryogenesis induction but also because in this procedure no hormones were used. The reduction or elimination of hormones used in the anther culture medium could be useful in several aspects: it can reduce the appearance of somatic embryos produced by the anther tissue, it can reduce costs in the production of new varieties by anther culture and it is a new approach to reduce the use of potentially harmful substances in this culture. Furthermore, there are some reports supporting that hormone reduction or elimination increased the embryo production by anther culture (Johansson 1983, Morrison *et al*. 1986, Ozkum and Tipirdamaz 2002).

An additional advantage that made us to test a procedure based on McComb and McComb (1977) (MC1 procedure), in our material, was that this procedure does not implied subculture steps making shorter the time required in the obtaining of results if compared with previous procedures and reducing in this way the risks of culture contamination.

The significant genotype dependence in microspore embryogenesis response is sustained by the results obtained after applying MC1 procedure in the four genotypes selected. The encouraging number of dicotiledonary embryos obtained in the in Serrano genotype prompted us to develop new procedures based on MC1 procedure whose results will be discussed later in section 4.4.

4.3. Kim *et al*. 2004 procedure. Kim procedure.

The successful results in hot pepper reported by Kim *et al*. (2004) led us to test this procedure in our material, despite the fact that this procedure uses hormones and did not use activated charcoal.

The efficiency obtained with the Kim procedure did not give better results with our genotypes than those obtained with MC1 procedure and our results were far from those reported for the Korean genotype in the Kim's group (Kim *et al*. 2004). In our material, the use of hormones caused the induction of somatic embryogenesis, especially in the CM334 genotype, where many calli were observed. Either with the MC1 procedure or Kim procedure, the best result was obtained for Serrano genotype, suggesting maybe, that this is the most responsive of the hot pepper genotypes used in this study.

4.4. Modifications of the MC1 procedure to improve embryo yield.

In the light of these results we decided to continue further experiments with the MC1 procedure in anther culture, modifying the stress pretreatment without changing other conditions of the procedure.

4.4.1. Reduction of the temperature of hot stress pretreatment MC31. MC31 procedure.

The original MC1 procedure included a hot stress pretreatment at 35ºC. This pretreatment was used by other authors for anther culture in pepper both with sweet and hot genotypes (Dumas de Vaulx *et al*. 1981, Qin and Rotino 1993, Supena *et al*. 2006). Kim *et al*. (2004) procedure included a heat pretreatment at 31ºC. In view of the results obtained with MC1 and Kim procedure, we decided to reduce the temperature of the stress pretreatment to 31ºC and with this modified MC1 procedure an improvement in the production of dicotyledonary embryos were obtained in the hot pepper genotypes, being especially noticeable for CM334 genotype.

4.4.2. Combination of hot (31ºC) with starvation (mannitol) pretreatment. MCstarv procedure.

The combination of stress pretreatments has been used in different species to induce microspore embryogenesis (Islam and Tuteja 2012). To our knowledge

the combination of two or more stress pretreatments has not been previously tested for the induction of microspore embryogenesis by anther culture in hot pepper genotypes. In this study we used the combination of both cold and starvation pretreatments as well as the combination of both hot and starvation pretreatments. Due to the high concentration (0.7M) of mannitol used we can say that this pretreatment represent also an osmotic stress (Cistué *et al*. 1994, Zoriniants *et al*. 2005).

As it was mentioned before the best results reported for microspore embryogenesis in *Capsicum* were obtained when a hot stress pretreatment was used (Sibi *et al*. 1979, Dumas de Vaulx *et al*. 1981).

The combination of a hot and starvation stress pretreatment has been proved to be effective to enhance microspore embryogenesis in tobacco (Touraev *et al*. 1996), wheat (Indrianto *et al*. 2001) and barley (Kasha *et al*. 2001) while this combination has been not used before for hot pepper. When this combination was applied (MCstarv procedure) no results were obtained in any of our four genotypes. These results may be due to the fact that these stress conditions were too strong for our material, and that is why, most of the microspores observed immediately after the stress pretreatment in any of the four genotypes tested were non-viable.

4.4.3. Combination of cold and starvation pretreatment. MC2 procedure.

There were several reports (Sunderland and Roberts, 1979, Morrison et *al*. 1986, Supena *et al.* 2006) of the use of a cold pretreatment applied to the flower buds of pepper before anthers were plated into the induction medium. The cold pretreatment at 7 ºC combined with the starvation pretreatment using mannitol, a non-metabolizable sugar, instead of maltose allowed us to improve microspore

embryogenesis induction in the Jalapeño hot pepper genotype whose efficiency was near to zero percent with all the previous procedures tested. These results confirmed once more, the strong-genotype dependence of the efficiency of microspore embryogenesis induction and indicate that this pretreatment although not suitable for the other two hot genotype tested (CM334 and Serrano) was the more suitable from those tested for the induction in Jalapeño. The sweet genotype Lamuyo, again do not respond to combined pretreatments.

4.4.4. Comparison using the same plants in all the procedures derived from that of McComb and McComb (1977). MC1, MC31, MCstarv and MC2.

Growth conditions of the plants from where flowers were excised to extract anthers for culture are an important factor affecting the success of microspore embryogenesis induction whatever the species were considered including *Capsicum* (Irikova *et al*. 2011). In the present study the optimal plant growth environment (temperature, light and humidity) was kept practically constant by the cultivation of plants in greenhouses. The conditions were set and maintained in the greenhouses of the Company to obtain an adequate production of pepper seeds. Furthermore, with the greenhouses plants were not only kept from outdoor conditions changes but also protected from pests. However from time to time to avoid pests it was necessary to apply chemicals against them. From this study it was obvious that sweet were more sensitive than hot pepper genotypes to pest attack. This fact could be explained for the presence of capsaicin which has been suggested to have a possible role in plant defense against pathogens (Veloso *et al*. 2013).

Applying MC1 procedure with plants from different years we found that the results obtained with this procedure were practically the same year after year with donor plants in which the physiological status was maintained. The best results were obtained with Serrano genotype where embryo efficiency was always about 10%. In our knowledge these results were only exceeded by those obtained with the Korean hot pepper genotype by Kim *et al*. (2004).

5. Embryo yield and plant regeneration.

We have found that even with the most suitable procedures not all the embryos produced, after microspore embryogenesis induction, were perfect dicotyledonary embryos. The formation of a high number of abnormal embryos after anther culture pointed out the importance of the embryo quality evaluation because of its future impact on plant regeneration (Supena *et al*. 2006, Kim *et al*. 2008). Practically with all procedures tested the number of abnormal embryos obtained was higher than that of dicotyledonary embryos and these abnormal embryos failed to regenerate plants. This was the reason why we calculated efficiency based on the dicotyledonary embryos produced by 100 anthers.

Cytological studies revealed a non-homogeneous answer of microspores to embryogenic induction and a marked asynchrony among embryogenic microspores during the application of microspore embryogenesis in *Capsicum*. This fact is clearly visible observing microspores after stress pretreatment when microspores non-dividing were found together with microspores suffering a different number of divisions. These observations, found also in other species both in anther and microspore culture (Pulido *et al*. 2001, Testillano *et al*. 2002), reintroduce the question of the possible presence of intrinsic factors determining

the different answer of the microspores to embryogenic induction (Bonet *et al*. 1998).

It has been postulated that seasonal variations could affect anther response during microspore embryogenesis induction in many species (Sunderland 1974, Tiainen 1992, Datta 2005). In our experiments practically no seasonal variations were introduced because the flowers that were used during the four years of this study were produced in plants that were sowed in the same season and grown under stable greenhouse conditions. Furthermore, in our experiments no clear differences were observed in embryo yield when comparing anthers excised from flower buds produced during the first weeks with the embryo yield obtained with anthers excised from older plants although Kristiansen and Andersenn (1993) have observed in *Capsicum* a direct dependence between microspore embryogenesis induction and the donor plant age.

The different results obtained with the different pretreatments tested confirmed the high dependency of microspore embryogenesis induction with genotype, suggesting that a design of a particular procedure is needed practically for each genotype. Among the hot genotypes tested, Serrano genotype seems to be the most responsive. The best results for Serrano genotype were obtained with the MC1 procedure (about 13 perfect embryos per 100 anthers), for CM334 genotype the best results were obtained with the MC32 procedure (about 5 perfect embryos per 100 anthers) and for Jalapeño genotype the best results were obtained with the MC2 procedure (about 3 perfect embryos per 100 anthers). Finally, we have improved the results of the original procedure with the sweet genotype Lamuyo using activated charcoal (DDV_{ac} procedure) (about 22 perfect embryos per 100 anthers).

Plant regeneration is a critical step for hot pepper in embryogenesis induction using both isolated microspore and anther culture (Supena *et al*. 2006).

Two basic factors can affect plant regeneration: the composition of the media and the embryo transferring time to the regeneration media. Regarding the composition, two different regeneration media were used in this study (RG and the regeneration medium according Dumas and de Vaulx *et al*. (1981)) with no significant-different results. Concerning embryo transferring time, in our genotypes and with practically all the procedures tested, embryos were visible after three weeks of anther culture and consequently anthers were checked every week; embryos were immediately transferred to the regeneration media to maximize plant regeneration.

Double haploid production can occur either spontaneously or by the use of different inducers (Jensen 1974, Barnabas *et al*. 2001). In *Capsicum* spontaneous formation of double haploid plants has been reported (Morrison *et al*. 1986) although colchicine has also been used in this species (Supena *et al*. 2006). The spontaneous doubling rate in *Capsicum* like in other crops depends on the genotype (Supena *et al*. 2006). We have detected spontaneous diplodization in all genotypes tested. We were able to regenerate plants from approximately fifty percent of the dycotiledonary embryo obtained and about twenty percent of these plants underwent spontaneous diploidization. Serrano genotype gave the best results in terms of embryo yield, plant regeneration and double haploid production. The MC1 procedure gave the best results for Serrano genotype, although haploids plants and fertile double haploid plants were regenerated from the four tested genotypes.

II. Isolated microspore culture.

1. Procedures assayed to improve microspore embryogenesis in hot pepper by isolated microspore culture.

Isolated microspore culture is technically more complicated than anther culture because it requires an extra step of isolation. This extra step could represent also a higher risk of contamination in comparison with anther culture. But in spite of these disadvantages, in isolated microspores culture factors playing a role in embryogenesis induction can be better controlled and the possible effect of anther tissue can be avoided, giving better results in the obtaining of haploid plants in microspore embryogenesis (Olmedilla 2010). These reasons, together with the fact that a very efficient procedure to induce the formation of haploid plants by isolated microspore culture was published in hot pepper by Kim *et al*. (2008) lead us to try this procedure with our material. In this procedure they isolated microspores by direct blending of the flower buds, avoiding thus the tedious and time-spending step of anther isolation from the flowers. Using *Capsicum annuum* L. cv Milyang-jare, these authors found a noticeable efficiency in embryo yield with this procedure (Kim *et al*. 2008).

In our knowledge there were only two reports on microspore embryogenesis induction by isolated microspore culture in hot pepper: Kim *et al*. (2008) and Lantos *et al*. (2009). Apart from the fact that with Kim *et al*. (2008) procedure higher number of embryos were obtained one of the main differences between these two procedures was the optimal microspore developmental stage selected for microspore embryogenesis induction: Kim *et al*. (2008) reported that the best results were obtained when anthers containing mainly early bicellular

pollen (over 75%) were used for microspore isolation, whilst Lantos *et al*. (2009) found that the anthers containing 80% late vacuolated microspores were the most suitable for embryogenesis induction by isolated microspore culture. With our genotypes we selected anthers containing mainly early bicellular pollen (over 75%) to isolate microspores to carry out procedures based on Kim *et al*. (2008).

1.1. Microspore culture according to Kim *et al.* (2008). KIM* procedure.

We tested the Kim *et al.* (2008) procedure with the four genotypes selected but no results were obtained with this KIM* procedure. Since the only difference with the original Kim *et al*. (2008) procedure was the genotype we can say again that our results support the idea that there is a high influence of the genotype in the success of microspore embryogenesis.

1.2. Microspore culture procedure derived from that of Kim *et al*. (2008). MC1* procedure.

To try to improve these results we decided to develop a new procedure of isolated microspore culture based on Kim *et al*. (2008) procedure but substituting their media and culture conditions by those of MC1 because using this latter promising results were obtained in anther culture with our material. In fact, with this MC1* procedure we improved the production of embryos, but only with hot genotypes. We obtained dicotyledonary embryos from Jalapeño and CM334 that were able to regenerate plants, although these plants were not double haploids. No embryos were obtained either with Serrano or with the sweet genotype

Lamuyo. Another change that we introduce in KIM* procedure was the refreshment of the medium suggested among other authors by Kim *et al*. (2008) to reduce the number of abnormal embryos but it was not successful with our genotypes.

1.3. Procedures derived from MC1*. MC1-P*, MC1-PM*, MC1-H*, MC1-S* and MC1-SH* procedures.

Based on our results as well as on the published results reported for pepper embryogenesis induced by anther culture, and trying to improve our results using isolated microspore culture, we tested the strengthening of the stress pretreatment by increasing both its temperature and length (MC1-P*). In the same set of experiments we applied the stress pretreatments mentioned before and change other culture parameters such as: carbon source concentration (MC1-PM*), hormones addition (MC1-H*), carbon source change (MC1-S*) and a combination of a carbon source change and hormone addition (MC1-SH*). The results obtained showed that the strengthening of the stress alone did not produce embryos, but when combined with an increase in maltose concentration or with hormone addition more embryos, although mainly abnormal, were produced with the hot genotypes. The substitution in the culture medium of maltose by sucrose raised no embryos. When sucrose is combined with hormones again no embryos were obtained. These results agreed with those of Scott *et al*. (1995), obtained in barley (*Hordeum vulgare* L.) embryogenesis, induced by isolated microspore culture, where it was reported that microspores die when they were cultured on media containing sucrose but undergo embryogenesis when maltose was used. Scott *et al*. (1995) based this difference on the fact that metabolism of maltose is slower than that of sucrose. With sucrose large quantities of ethanol are

accumulated within the cell having a hypoxic effect while metabolism of maltose being slower, keep sufficient oxygen available to allow cells to survive in culture (Scott *et al*. 1995). Nevertheless we cannot forget that Kim *et al*. (2008) obtained excellent results using sucrose with their Korean hot pepper genotype.

The high proportion of abnormal embryos that we obtained in isolated microspore culture when compared with the proportion of these embryos obtained after anther culture could be explained by the lack of anther tissue protection in microspores directly cultured for microspore embryogenesis induction.

1.4. Procedures derived from Soriano *et al.* (2008). MC1[1]* and MC1-B* procedures.

The application of n-butanol treatment was successfully used by Soriano *et al*. (2008) to enhance embryo and green plant production after microspore embryogenesis induction in wheat (*Triticum aestivum*). The effect of this alcohol in microspore embryogenesis seems to be based on the alterations in cortical microtubules this data is in accordance with other reports pointing out the stimulation of microspore embryogenesis produced by anti-microtubule agents in cereals (Barnabás *et al.* 1991, Soriano *et al*. 2007) as well as in other species (Herrera *et al*. 2002, Zhou *et al*. 2002). All these reports lead us to include n-butanol in microspore embryogenesis induction in *Capsicum* using the procedure developed by Soriano *et al*. (2008) for microspore isolation.

Apart from the use of n-butanol, the main difference between the method of Soriano *et al*. (2008) and the method of Kim *et al*. (2008) was that in the former they use a glass rod homogenizer to isolate microspores from anthers

instead of the use of blenders to isolate microspores from flower buds. Another difference was that in Soriano *et al*. (2008) the stress pretreatment was applied to the excised anthers while in the Kim *et al*. (2008) procedure the stress pretreatment was directly applied to the isolated microspores. Due to the fact that Soriano *et al*. (2008) procedure was more time-consuming than that of Kim *et al*. (2008), we decided to adapt it to *Capsicum* before testing the effect of n-butanol using two of the genotypes selected: the hot relatively good responsive Serrano genotype and the sweet genotype Lamuyo. This procedure without n-butanol was designated as MC1[1]* while the one adding n-butanol was called MC1-B*. Both MC1[1]* and MC1-B* tested in Serrano and Lamuyo genotypes produced embryos with a higher efficiency compared to that obtained with any of the procedures derived from Kim *et al*. (2008) but no significant difference in embryo yield was obtained when comparing MC1[1]* and MC1-B* procedures. Possible explanations of the increase obtained after the use of MC1[1]* and MC1-B* procedures could be the fact that in the genotypes tested in this study: 1) with the isolation of microspores from anthers there were less chances of introducing in the culture medium toxic compounds derived from sporophytic tissue than in the fastest KIM* and KIM* derived methods; 2) the application of the stress to the anthers could lead to a less intense effect in the viability of microspores than the direct application of the stress to the microspores; and 3) the less intense mechanical force applied with the glass rod homogenizer to the anthers could be less harmful for microspores than the electric blender.

Although for the genotypes tested n-butanol seems have only a slight increase in embryo yield the assays carried out with MC1[1]* and MC1-B* procedures underline the great influence that the method of microspore isolation could have on microspore embryogenesis induction. In the light of the higher embryo production obtained in Lamuyo when compared with Serrano genotype

using these two methods of microspore embryogenesis induction we cannot discard a possible influence of capsaicin in the embryogenesis induction. This observation does not appear obvious with anther culture where, except for the DDV procedure, always better results were obtained with the hot genotypes than with the sweet genotype.

Further studies are needed to improve the results with isolated microspore culture due to the advantages that isolated microspore culture could have for the research in molecular and cellular bases of this process. And although the methods derived from Soriano *et al*. (2008) are more time-consuming they can be interesting also for commercial purposes if the efficiency of embryo production is higher enough.

2. Embryo yield, plant regeneration and double haploids.

Capsicum embryo yield in microspore embryogenesis induced by isolated microspore culture was lower than that obtained by anther culture with the procedures used in this study. Furthermore, the number of abnormal embryos produced after isolated microspore culture was in all the procedures tested higher than the number of dycotiledonary embryos, and although it was tried to be reduced by medium refreshing, no successful results were obtained. As a consequence, the number of regenerated plants was very low and no spontaneous double-haploid plants were obtained after isolated microspore culture. The reason for the non-spontaneous production of double haploids with isolated microspore culture could be related to the low number of regenerated plants obtained with this method.

It has been reported by Kim *et al*. (2004, 2008) that the time required for the obtention of hot pepper embryos (*Capsicum annum* L. cv Milyang-jare) by isolated microspore culture and by anther culture was similar (3-4 weeks), however, in our material with any of the most successful procedures used, we found that by anther culture a shorter period of time was required especially for the procedures derived from Kim *et al*. (2008) (6-8 weeks by isolated microspore culture, 3-4 weeks by anther culture). Additionally, it was observed that the first embryos produced after the application of Soriano *et al*. (2008) derived procedures (MC1[1]* and MC1-B*) appeared after 4 weeks of culture, being shorter than the time needed in the procedures derived from Kim *et al*. (2008). Number of embryos and regenerated plants obtained with MC1[1]* and MC1-B* was higher than that of the Kim *et al*. (2008) derived procedures (MC1*, MC1-P*, MC1-PM*, MC1-H*, MC1-S*, MC1-SH*). These observations surprisingly showed that a procedure developed for a hot pepper genotype can give worse results in terms of microspore embryogenesis induction, than a procedure originally developed for cereals reinforcing the idea that microspore-embryogenesis efficiency is strongly genotype-dependent. Another point that reinforces this idea is that using the sweet Lamuyo genotype we were only able to obtain embryos and plants with the Soriano *et al*. (2008) derived procedures.

Further studies to improve isolated microspore culture procedure are needed in our material. The new procedures could be based: 1) in the microspore extraction directly from flower buds, which is faster than the extraction from anthers; 2) the use of maltose gradient for microspore purification, which will avoid the sporophytic tissue influence and 3) the use of different stress pretreatments applied in flower buds, anthers or directly to isolated microspores because stress pretreatment is a factor which needs to be investigated in isolated microspore culture in our material.

The development of an optimum procedure for isolated microspore culture will give us the possibility to undertake cellular and molecular studies that will help in the understanding of the basis of this process.

III. Cellular changes after embryogenic induction.

The main problem to study cellular changes in microspore embryogenesis in hot pepper was that these genotypes were, independently of the procedure used, low responsive. Another inconvenience was the slowness of this process in *Capsicum*. Actually, with the exception of the results published by Kim *et al*. (2008) and Supena *et al*. (2006) working with a Korean and an Indonesian hot-pepper genotype respectively, in our knowledge, there was no other reports of good responsiveness of hot pepper. Furthermore, all the reports about microspore embryogenesis in *Capsicum* pointed out that at least 3 weeks after the embryogenic pretreatment were needed to obtain proembryos (Barany *et al*. 2005, Kim *et al*. 2007) whilst in other species like *Brassica* only 6 days were needed to obtain globular embryos and 16 days to obtain heart-shaped embryos (Supena *et al*. 2008). Nevertheless these species needs short pretreatments when comparing with *Capsicum*, in *Brassica* for example, Pechan *et al*. (1991) reported that 8 hours of pretreatment at 32ºC were sufficient to induce microspore embryogenesis while in *Capsicum* at least 3 days of pretreatment at 31ºC were needed.

One of the first signs of microspore embryogenesis induction we observed, just by the direct observation of microspores, using the most responsive genotype with the best procedure, was the swelling of a certain number of microspores both inside the anther and in the isolated microspore

culture population. This feature has been reported as a sign of embryogenic potential acquisition by several authors in different species including *Capsicum* (Hoekstra *et al*. 1992, Touraev *et al*. 1996, Barany *et al*. 2005, Malik *et al*. 2007).

By DAPI staining, we found that another common feature in microspore embryogenesis in hot pepper, induced both by anther and isolated microspore culture, was the neither synchronous nor homogeneous response observed after the embryogenic induction. This response was found independently of the genotype and pretreatment used for the induction and occurs also in different species of monocots as *Hordeum vulgare* (Pulido *et al*. 2005) and dicots as *Capsicum annuum* (Barany *et al*. 2005), although Cordewener *et al*. (1994) reported that microspores of *Brassica napus* 'become committed to sporophytic development synchronously, within 8 h after initiation of the culture'. These observations lead us to think that the responsiveness was a genetically controlled as it was previously suggested by Bonet *et al.* (1998).

The nuclear staining with DAPI, also allowed us to see that the initial embryogenic divisions were both symmetrical an asymmetrical being the first more common, these observations were also reported by other authors in pepper (Kim *et al*. 2004) as well as in many other species like for example wheat (Hu and Kasha 1999) and barley (Kasha *et al*. 2001), these authors also reported the influence of the type of stress pretreatment on the pathways followed in the first embryogenic division by these species.

The studies carried out on semi-thin sections allowed us to confirm the features described above as well as to analyze in more detail the formation of a suspensor-like structure found during the direct observation of the cultured anthers and isolated microspores. This structure was similar to the suspensor found in *Capsicum chinense* during somatic embryogenesis and described by Santana-Buzzy *et al*. (2009).

In our study, we have found the formation of this suspensor-like structure in hot stress pretreatment applied both to Jalapeño in isolated microspore culture using MC1* procedure and to Lamuyo anther culture using MC1 procedure. The formation of this suspensor-like structure in isolated microspore culture was not previously described in pepper with the exception of the work published by Chun-ling *et al.* (2008) but the details of this work are only available in Chinese language.

The formation of suspensor-like structures during microspore embryogenesis induction has been described in *Brassica* (Custer *et al.* 2001, Supena *et al.* 2004, 2008) where it is possible to induce embryos containing these structures by microspore embryogenesis under very specific conditions, in *Brassica* the application of a hot stress pretreatment of 32ºC for 24 h is enough to obtain a high yield of proembryos with these suspensor-like structures (Supena *et al*. 2008). We found that exine covered frequently this suspensor-like structure when formed in pepper. Similar exine-covered suspensor-like structures where observed in *Brassica* (Tang *et al*. 2013). Nevertheless, this structure was formed in Brassica from the very beginning of embryo formation (first asymmetrical division) while in *Capsicum* we have found these structures only once the globular embryo is clearly discernible. In isolated microspore culture of different tomato (*Solanum lycopersicum* L.) cultivars Seguí-Simarro and Nuez (2007) observed suspensor-like structures emerging from the exine, these structures were very similar to those observed in isolated microspore culture of Jalapeño genotype.

To determine possible differences in the cytoplasm composition in the microspores during embryogenic induction, we applied different cytochemical techniques that allowed us to localize insoluble carbohydrates, proteins and lipids. Sangwan and Sangwan-Norreell (1987) after a comparative study of the

distribution of the cytoplasmic organules of different species which were responsive and non-responsive to microspore embryogenesis induction propose that, plastids containing starch could be used as marker of responsiveness because they found that starch accumulation at the gametophytic stages where embryogenesis induction was carried out was not compatible with good responsiveness. In our study, we were not able to find any differences in starch accumulation in microspores at vacuolated stage in any of the genotypes selected. Sangwan and Sangwan-Norreell (1987) found that in *Nicotiana tabacum*, amyloplasts did not appear until the bicellular-pollen stage was reached while in *Lycopersicum esculentum*, another *Solanaceae* non-responsive to embryogenesis induction, the starch was accumulated in vacuolated microspores. They related these data with those obtained in tomato by Gresshoff and Doy (1972) in which they demonstrated that the best results were obtained when microspore embryogenesis induction was applied at stages before this gametophytic stage.

The cell wall ontogeny and its changes are central for growth and plant development and in particular for embryo development (Raghavan 1986). Many authors described the differentiation processes during microspore embryogenesis induction as imitating that of the zygotic development, Zhang *et al*. (2009) described similar changes in cell organization affecting plastids, cell wall and nucleus after embryogenesis induction in *Capsicum*.

We have found that first embryogenic divisions took place inside the microspore wall and that after 7 days of culture, exine started to be broken and later after 21 days of culture formation of proembryos took place. This time-lapse was similar to that required for another *Solanaceae* like tobacco (Reinert *et al*. 1975) and in barley another model species for microspore embryogenesis induction (Pulido *et al*. 2005).

Another significant change that we have found in multicellular pollen grains was the intine enlargement that some authors described as the formation of a fibrillar wall in the exine, this feature was also described in many other species including sweet pepper (Rodriguez-García *et al*. 2000, Bonet and Olmedilla 2000, Barany *et al*. 2005). This feature appears in all embryogenic multicellular pollen grains while the accumulation of a great number of big starch grains was restricted to dying microspores. Once microspore embryogenesis proceeded multicellular structures were liberated from the exine and proembryos were formed. The prodermis formation in these proembryos indicated that embryogenic process progressed successfully. With PAS staining we have found that cells forming prodermis contain numerous large starch granules. These granules were also presents in the vacuolated cells forming the suspensor-like structure. Exine wall was present surrounding the suspensor-like structure at the initial stages of its formation while the cells constituting the proper embryo were not completely surrounded by this wall. This exine was visible with Coomassie brilliant blue and with Sudan black staining showing its proteinaceous and lipidic composition while the insoluble carbohydrate composition of the intine wall was evident after PAS-staining. Coomassie brilliant blue staining pointed out how proteins in the anther locule were metabolized during the embryogenic process.

Studies like the present, focused on species which are non-responsive, are necessary to better understand the mechanisms controlling the morphogenetic changes involved in this complex process and in this way broaden its application as a source of homozygous-lines production.

Conclusions

1. The efficiency of procedures for microspore embryogenesis is highly genotype-dependent in *Capsicum* and given a genotype, the results obtained strongly depend on the initial explant material (anthers or isolated microspores). Among the genotypes studied and with the procedures tested the most responsive hot pepper genotype is Serrano.

2. Hot pepper genotypes unlike sweet pepper genotypes undergo noticeable oxidation during anther culture, which affects embryogenesis induction. This oxidation is reduced with the addition of activated charcoal in the culture medium. Although sweet pepper genotypes do not undergo oxidation, the addition of activated charcoal to the culture medium stimulates the production of their embryos.

3. The use of hormones was not necessary for microspore embryogenesis induction either using anther or isolated microspore culture and both for hot and sweet pepper.

4. Among the anther-culture procedures evaluated for microspore embryogenesis induction the most efficient for the hot genotypes tested was the MC1 procedure, which is a reproducible, one step method that do not use hormones.

5. There is a strong influence of the type of stress pretreatment and of the material in which this stress is applied in *Capsicum* embryo production by microspore embryogenesis. The combination of stress pretreatments was successful for the induction of microspore embryogenesis in hot pepper using isolated microspore culture but not when anther culture was employed.

6. *In Capsicum* high proportion of abnormal embryos are obtained after microspore embryogenesis induction using both anther and isolated microspore culture which has a strong influence on the efficiency in plant production.

7. The method of isolation of microspores is crucial for the success of microspore embryogenesis in *Capsicum*. The isolation from excised anthers with a glass rod homogeneizer is much more effective than that of the isolation by electric blender of microspore from the whole flower buds.

8. One of the first sign of microspore embryogenesis induction in Capsicum is the presence of swelled microspores but the presence of these microspores alone does not guarantee the final embryo production.

9. Symmetric and asymmetric divisions are induced after microspore embryogenesis induction with all the pretreatments assayed in this study, being symmetric divisions more frequent. Nuclear fusion after this first symmetric division occurs because spontaneous double haploid production is frequently detected in anther culture.

10. Suspensor-like structures were formed in hot and sweet pepper during microspore embryogenesis induced both by anther and isolated microspore culture using different procedures but their formation was not correlated with embryo yield efficiency.

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