Universitat Politècnica de València

Degree in Biotechnology

Relationship between osmolality and *in vitro* viability in pepper (*Capsicum annuum*) microspores

Final's Degree Dissertation

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Title: Relationship between osmolality and *in vitro* viability in pepper microspores (*Capsicum annuum*).

Keywords: embryogenesis, microspores, *Capsicum annuum*, androgenesis, osmolality, viability.

Abstract:

Production of hybrids is the basis of the current seed production system for conventional agriculture. In order to obtain them, it is previously necessary to generate pure lines, which is expensive and time-consuming when conventional approaches based on self-fertilization and selection are used. As an alternative to this, production of androgenic doubled haploids by *in vitro* culture is increasingly demanded in order to reduce costs and speed up the process of pure line production.

As a result of this, the interest in studying the factors that influence the induction of androgenesis and production of doubled haploids by *in vitro* culture has increased considerably. It is a complex procedure very influenced by external and internal conditions that still has to be optimized for some species of agronomic interest.

In this way, interesting results have been obtained regarding the factors that affect the correct development and induction of microspores such as the genetic background of the donor plants they come from, as well as the growth conditions of the donor plants, the stage of development of the microspores or the conditions in which the *in vitro* culture was carried out. Nevertheless, the osmotic context of the culture is a factor that, despite being part of the culture conditions, and being able to influence this process significantly, has been barely studied.

For this reason, the main objective of this work was to study the relationship between the viability of microspores when cultivated *in vitro,* the internal osmolality of these microspores and the external osmolality, that is, the osmolality of the *in vitro* culture medium in which microspores are cultivated.

The results of this work suggest a relationship between osmolality and viability, since the osmolality of microspores increases as viability decreases. In this way, it is possible to carry out a study on the conditions affecting the cultures of microspores of *Capsicum annuum* in order to be able, later on, to elaborate optimized protocols for their induction towards embryogenesis.

Título: Relación entre osmolalidad y viabilidad *in vitro* en microsporas de pimiento (*Capsicum annuum*).

Palabras clave: embriogénesis, microsporas, *Capsicum annuum*, androgénesis, osmolalidad, viabilidad.

Resumen:

La obtención de híbridos es la base del actual sistema de producción de semillas para la agricultura convencional. Para conseguir híbridos, anteriormente se necesita generar líneas puras, lo que es muy costoso en tiempo y recursos utilizando los abordajes convencionales basados en la autofecundación y selección. Como alternativa a esto, la obtención de dobles haploides androgénicos por cultivo *in vitro* es cada vez más buscada para abaratar costes y acelerar los procesos de producción de diversas líneas vegetales.

Debido a ello, el interés por estudiar los factores que influyen en la inducción de androgénesis y la obtención de dobles haploides mediante cultivo *in vitro* ha aumentado considerablemente ya que se trata de un proceso nada sencillo y muy influenciado por las condiciones tanto externas como internas, que todavía debe ser optimizado para algunas especies de interés agronómico.

De esta forma, se han obtenido resultados interesantes en cuanto a los factores que afectan al correcto desarrollo e inducción de las microsporas, como puede ser el fondo genético de las plantas donantes de las que provienen, así como las condiciones de crecimiento de las plantas donantes, el estadio de desarrollo de las microsporas o las condiciones en las cuales se lleve a cabo el cultivo *in vitro*. No obstante, el contexto osmótico del cultivo es un factor que, pese a formar parte de las condiciones de cultivo y que podría influir significativamente en la androgénesis, apenas ha sido estudiado.

Es por ello por lo que el objetivo principal de este trabajo es estudiar la relación existente entre la viabilidad de las microsporas cuando son cultivadas *in vitro*, la osmolalidad interna de dichas microsporas, y la osmolalidad externa, esto es, la osmolalidad del medio de cultivo *in vitro* en el que son cultivadas las microsporas.

Los resultados de este trabajo sugieren una relación entre la osmolalidad y la viabilidad, ya que según aumenta la osmolalidad de las microsporas, la viabilidad disminuye. De esta forma, se está en condiciones de plantear un estudio sobre las condiciones que afectan los cultivos de microsporas de *Capsicum annuum* para poder, más tarde, elaborar protocolos optimizados para su inducción de la embriogénesis.

Títol: Relació entre osmolaritat i viabilitat *in vitro* en microespores de pimentó (Capsicum annuum).

Paraules clau: embriogènesi, microspores, *Capsicum annuum*, androgènesi, osmolalitat, viabilitat.

Resum:

L'obtenció d'híbrids és la base de l'actual sistema de producció de llavors per a l'agricultura convencional. Per a aconseguir híbrids, anteriorment es necessita generar línies pures, la qual cosa és molt costosa en temps i recursos utilitzant els abordatges convencionals basats en l'autofecundació i selecció. Com a alternativa a açò, l'obtenció de dobles haploides androgénicos per cultiu in vitro és cada vegada més buscada per a abaratir costos i accelerar els processos de producció de diverses línies vegetals.

A causa d'això, l'interés per estudiar els factors que influïxen en la inducció d'androgénesis i l'obtenció de dobles haploides per mitjà de cultiu in vitro ha augmentat considerablement ja que es tracta d'un procés res senzill i molt influenciat per les condicions tant externes com internes, que encara ha de ser optimitzat per a algunes espècies d'interés agronòmic.

D'esta manera, s'han obtingut resultats interessants quant als factors que afecten el correcte desenrotllament i inducció de les microsporas, com pot ser el fons genètic de les plantes donants de què provenen, així com les condicions de creixement de les plantes donants, l'estadi de desenrotllament de les microsporas o les condicions en les quals es duga a terme el cultiu in vitro. No obstant això, el context osmòtic del cultiu és un factor que, a pesar de formar part de les condicions de cultiu i que podria influir significativament en l'androgénesis, a penes ha sigut estudiat.

És per això pel que l'objectiu principal d'este treball és estudiar la relació existent entre la viabilitat de les microsporas quan són cultivades in vitro, l'osmolalitat interna d'aquestes microspores, i l'osmolalitat externa, açò és, l'osmolalitat del medi de cultiu *in vitro* en el que són cultivades les microspores.

Els resultats d'este treball hem vist que clarament hi ha una relació entre l'osmolalitat i la viabilitat, ja que segons augmenta l'osmolalitat de les microspores, la viabilitat disminuïx. D'aquesta manera, s'està en condicions de plantejar un estudi sobre les condicions que afecten els cultius de microspores de *Capsicum annuum* per a poder, més tard, elaborar protocols optimitzats per a la seua inducció de l'embriogènesi.

Acknowledgements:

In this brief section of my dissertation I would like to dedicate a few words to all those who have accompanied me during this stage of my life, where everything has not been as easy and beautiful as it may seem.

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Contents

1. Introduction

1.1. About pepper (*Capsicum annuum*)

1.1.1. Taxonomy

Pepper (*Capsicum annuum*) is a plant that belongs to the *Solanaceae* family. It is cultivated in temperate and tropical regions around the world. It is an autogamous species with a reported percentage of cross-pollinization between 7 - 90% and whose taxonomic tree (CABI, 2018) is the following:

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Solanales

Family: Solanaceae

Genus: *Capsicum*

Species: *Capsicum annuum*

The genus name *Capsicum* has been said to have its origin from the latin word '*kapto'*, a Greek-based derivative, that means 'to bite' due to the pungency of its fruit. Nonetheless, the Latin word '*capsa'* -meaning 'a box' in reference to the species' fruit- has also been speculated to be the origin of the genus name. Be that as it may, the Latin American common name '*chile'* comes from the Nahuatl Aztec dialect as a variation of 'chilli'.

On the other hand, *Annuum* -meaning 'annual'- leads to the misleading fact that the species is annual when it is really perennial under optimum growing conditions. However, due to its sensitivity to frost and cooling damage *Capsicum annuum* is normally cultivated annually hence its name.

1.1.2. Origin and domestication

All the species of the genus *Capsicum* were originated in the 'New World', in the tropical and subtropical regions of America (Bolivia, Mexico and Peru) and nowadays embraces about 25 wild species plus 5 domesticated ones: *C. annuum*, *C.* baccatum var. *pendulum*, *C. chinense*, *C. frutescens* and *C. pubescens.* While *C. annuum* and *C. pubescens* are believed to have their origin and centre of diversity in Mexico, the other three seem to have originated in other areas of South America but it was not until the XV century that was introduced in Europe and from there, to the rest of the world (CABI, 2018).

Regarding the domestication process of the different species of *Capsicum*, it occurred around 5200 and 3400 b.C, probably in a different manner for the different wild species spread around all the territory (Andrews, 1984).

Thanks to archaeological and genetic evidence plus the actual distribution of the crops researchers have been able to suggest that *Capsicum annuum* was initially domesticated in Mexico and that already in the very first of the Columbus it was introduced into the actual Spain. It was thanks to its easy germination process and the versatility of its fruits that soon led the crop to become widely known and spread around Europe and the world hence becoming one of the first domesticated and cultivated plants in the western hemisphere (Wang and Bosland, 2006). As a consequence, nowadays pepper crops are present around the globe (Figure 1) becoming so an outstanding cultivar in the European agroalimentary sector (Figure 2).

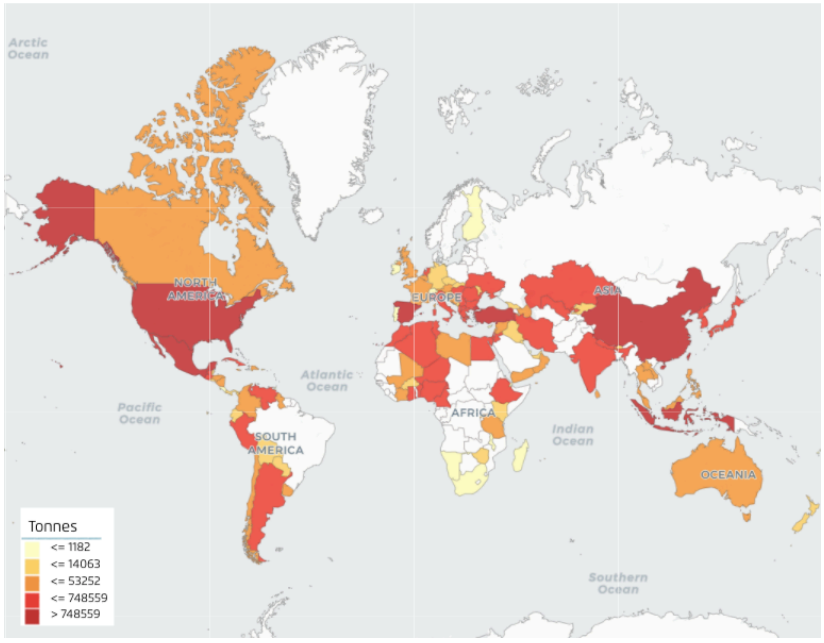

***Figure 1**: Production quantities of Chillies and peppers, green by country. (Source: FAO)*

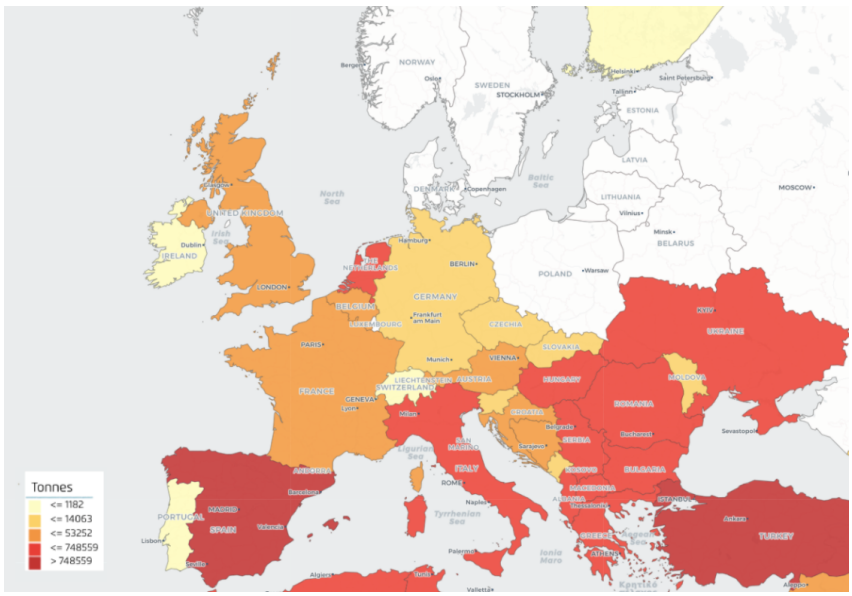

***Figure 2**: Production quantities of Chillies and peppers, green by European country. (Source: FAO)*

1.1.3. Cytogenetics

The genus *Capsicum* is mostly diploid (2n=2x=24). Although there is a small number of wild species that have a chromosomic basis of x=13 (2n=2x=26, such as *C. campylopodium* Sendt. and *C. rhomboideum*) yet the majority present a chromosomic basis of x=12 (2n=2x=24).

Besides, the *Capsicum annuum* genome is quite big with around 3.69 pg/nucleus, being its genome about three to four times bigger than that of tomato (*Solanum lycopersicum* L.)

1.1.4. Economic importance

In terms of production and cultivated area, pepper is one of the most important horticultural crops in the whole world and this is mainly due to the value of its fruits. Pepper is rich in vitamins (A,C and B complex) plus in minerals, essential oils and carotenoids between others components of interest (Barroso et al., 2015). 1.987.059 ha is the area harvested for *Capsicum annuum* in the whole world in 2017 -according to the Food and Agriculture Organization of the United Nations (FAO)- with a total production of 36.092.631 tonnes (Figure 3).

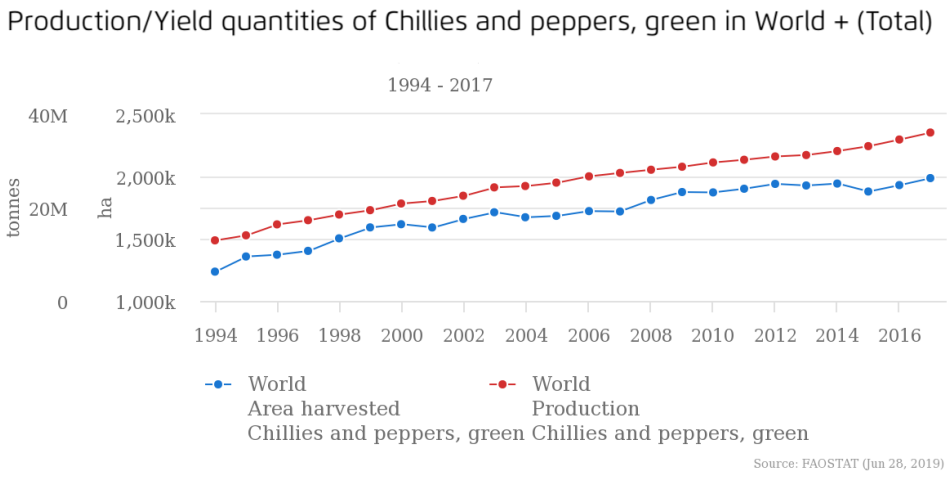

Figure 3. *Total production and area harvested of C.annuum in the World between 1994 and 2016. (Source: FAO)*

When it comes to Europe, Capsicum annuum's production is higher in the Southern region, highlighting Spain -which is the world's 5th largest producer (Figure 4)-. Besides, Italy and The Netherlands are also remarkable producers being the 12th and 11th world largest pepper producers in the World.

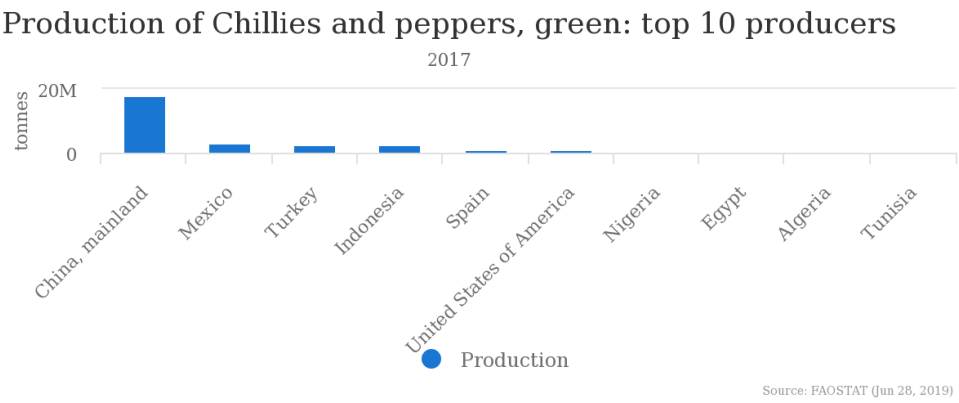

Figure 4: Top 10 pepper producers in the World in 2017 (Source: FAO)

In fact, Spain, not only is the 5th largest producer of pepper in the World being pepper one of its top 10 produced products (Figure 5), but also is worth mentioning the large income generated by pepper production in the country due to its export (Figure 6), thus constituting one of the main sources of wealth (FAO, 2002).

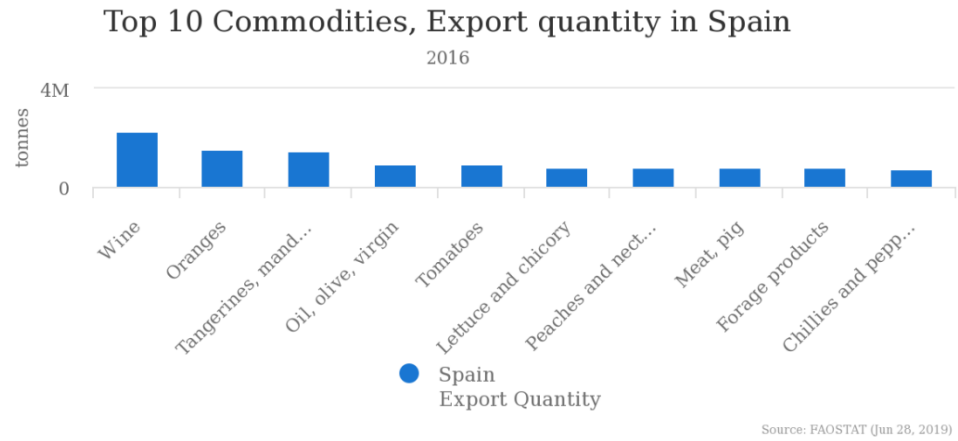

Figure 5: Top 10 products exported in Spain by quantity in 2016. (Source: FAO)

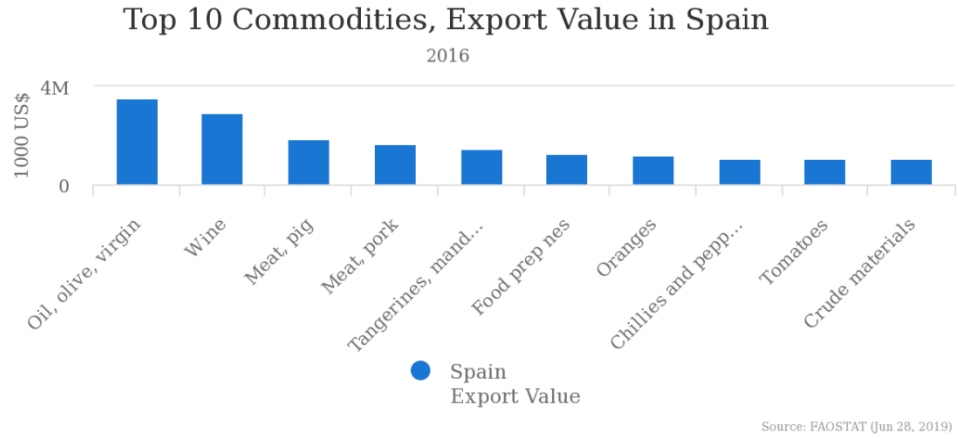

Figure 6: Top 10 products exported in Spain by revenue in 2016. (Source: FAO)

1.2. Pepper and Biotechnology

1.2.1. Biotechnology and genetic improvement

Biotechnology is defined as '*the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services*' (OECD, 2005). A broad definition that encompasses processes carried out for thousands of years (production of cheese, bread, wine, beer…).

Human beings, in their struggle for survival, have been taking advantage of biological processes to improve their quality of life for around 10,000 years, starting with the first farming communities. However, what is understood as biotechnology today is not the production of daily consumer goods, but the new molecular and cellular techniques that began to emerge in the sixties and seventies of the last century.

Despite the fact that this branch of science has a multitude of applications that have led it to differentiate by colours (Table 1), perhaps plant biotechnology is the one that has been applied in practice the longest and, to this day, continues to surprise with its potential.

***Table 1**: The eleven colours of Biotechnology (Source: Mariscal Abogados, 2019)*

Color Type	Area of Biotech Activities
Red	Health, Medical, Diagnostics
Yellow	Food Biotechnology, Nutrition Science
Blue	Aquaculture, Coastal and Marine Biotech
Green	Agricultural, Environmental Biotechnology: biofuels, biofertilizers, bioremediation, geomicrobiology
Brown	Arid Zone and Dessert Biotechnology
Dark	Bioterrorism, Biowarfare, Biocrime, Anticrop warfare
Purple	Patents, Publications, Inventions, IPRs
Orange	Biotechnology divulgation
White	Gene-based Bioindustries
Gold	Bioinformatics, Nanobiotechnology
Grey	Classical Fermentation and Bioprocess Technology

It is through early biotechnology that the first farmers selected and bred the most suitable crops, the ones with the highest yields, to produce food to maintain a growing population. Throughout the history of agriculture, farmers have inadvertently altered the genetics of their crops by introducing them into new environments and breeding them with other varieties, creating one of the first examples of biotechnology (Seguí Simarro, 2016).

In this aspect, and thanks to many different developments including *in* vitro culture, genetic engineering and, most recently, gene edition, plant biotechnology has grown enormously, multiplying its potential. Plant breeding one of these multiple applications.

Plant breeding is understood as the process comprising all the science-based technologies used for the genetic manipulation of plant species aimed at economic objectives. Today, this definition has been broadened because the application of biotechnology and its tools in genetic manipulation, understood as 'plant breeding', applies beyond purely industrial and economic purposes. Currently, plant biotechnology has a broad scope and serves also for social and humanitarian purposes.

According to data from the United Nations, the world population is expected to increase by more than 1,000 million over the next 15 years, reaching 8,500 million in 2030, 9,700 million in 2050 and 11,200 million by 2100 (United Nations, 2019). As a result of this remarkable increase in the world's population, resource scarcity and limited food will become even greater problems. Thanks to plant improvement, it has been possible to obtain plant species with nwly improved traits. From drought-resistant plants for cropping in arid areas to biofortified varieties to tackle malnutrition -staple crops such as rice, maize and wheat- or even the use of plants as biofactories for the production of vaccines of other therapeutic proteins (Hefferon, 2015). Thus, plant breeding is n an instrument with great potential to provide real solutions to complex problems.

1.2.2. Production of hybrids

Among one of the many and varied options offered by plant biotechnology in the field of genetic improvement, we find the production of hybrids.

In its simplest terms, a hybrid is a cross between two individuals of different groups, varieties or species and with different characteristics of agronomic interest. To hybridize, then, is to fertilize two individuals of different genetic constitution. By combining the peculiarities of these individuals into one, we will be able to obtain a mixture of traits which, by selecting and combining them again, allows us to obtain an organism with the most interesting particularities of the parental ones (Seguí Simarro, 2015).

Hybrids are based on a biological concept named heterosis. Heterosis -or hybrid vigour- is a tool that has been exploited in order to develop new plant varieties from genetically different parentals to obtain offspring with physiological and functional characteristics superior to those of his parents.

The interest in obtaining hybrids lies in the fact that they have greater adaptability, uniformity and tolerance to both abiotic and biotic stress due to hybrid vigour. As a

result, the agri-food industry has great interest in producing hybrids in order to increase yields among other advantages offered by hybrids. To obtain hybrids, pure lines must be developed in first place.

1.2.3. Pure lines

Pure lines are homozygous varieties for all loci in their genome. If we cross two pure lines, we will obtain a hybrid.

Due to the multiple advantages of hybrids discussed above, the vast majority of plant breeding programmes today are based on the production of pure lines, as pure varieties are needed to subsequently achieve hybrid vigour.

Pure lines are the result of multiple generations of self-fertilization. After this self-fertilization cycles, they must go through an analysis in which their characteristics as well as their general and/or specific combinatorial aptitude with other genotypes of interest will be analyzed. This means that not all the pure lines obtained after the self-fertilization process necessary to reach total homozygosis will be considered optimal varieties for their application in industry. Only a small percentage gathers the desired genotypes and, in addition, it is interesting in its combination with other genotypes of interest.

All this results in a process that requires a lot of time, between seven and eight cycles of self-fertilization. High economic costs and multitude of resources are then needed for obtaining pure lines.

However, with the development of protocols for obtaining double haploids (DHs), the possibility of obtaining totally homozygous lines in a single generation has been opened up.

1.2.4. Doubled haploids

Doubled haploids (DHs) come from haploid individuals who have had a duplication of their genome. Haploid individuals, in turn, are sporophytic plants that possess the chromosomal number corresponding to the gamete of the species, that is, they have half the chromosomes of a normal individual of the species (Germanà, 2011).

The main application and interest of DHs lies in the speed and economy with which pure lines can be obtained, although they are also used in basic research: genetic cartography studies, linkage studies and estimation of recombination fractions. (Seguí-Simarro, 2010a)

In addition, the development of doubled haploids is of special interest in breeding programs since, because they have the same alleles for each gene loci, which implies that they are not subjected to dominance/recessivity. This facilitates the selection of characters determined by recessive genes as well as the identification of recessive mutants (Barroso et al., 2015).

Thanks to biotechnology and the development of increasingly complete and detailed protocols for obtaining DHs, the development of homozygous lines for all their loci in a single generation is now possible, at least for some species, eliminating in a relatively

simple way the main inconveniences raised by the conventional methods of production of pure lines: time and money.

1.2.5. Methods to obtain haploids and doubled haploids

The obtention of haploids and doubled haploids is based on totipotency: the ability of a cell to express any part of its genome. This totipotency is possessed by plant organisms and would mean their ability to reproduce asexually.

The totipotency of plants allows their already differentiated plant cells to deviate from their original developmental pathway under certain conditions, being able again to express any part of their genome. Among the routes these cells can take are organogenesis - the ability to regenerate new individuals from vegetative organs - and embryogenesis - the way in which a cell divides symmetrically to develop as a sexual embryo would -. These processes that occur in somatic cells can also occur in both female and male gametophytes and even in their precursor cells, such as microspores. In this case, the organisms generated will be DHs if they have duplicated their genome or haploids if they have not. This duplication can be induced with reagents such as colchicine, which acts by inhibiting the polymerization of the microtubules of the mitotic spindle, preventing the correct separation of the chromosomes during mitosis (Seguí-Simarro, 2010b).

Two different routes can be used to obtain haploid organisms: gynogenesis and androgenesis.

In gynogenesis, haploid individuals are obtained from an unfertilized female gametophyte. This route, despite having the same usefulness as androgenesis, is less studied than androgenesis and is used less. Also, the number of female sex cells is much lower than that of male cells, decreasing the effectiveness of the process. Gynogenesis is used only used in species in which androgenesis does not work, such as sugar beet or onion (Seguí-Simarro, 2016).

Androgenesis, on the other hand, is the process by which an individual develops from a nucleus of male origin. The male gametophyte in the masculine stage deviates from the original gametophytic route and initiates embryogenic development, and can be reprogrammed towards the formation of calluses or embryos, from which new plants can be obtained (Seguí-Simarro, 2010a).

Androgenesis can occur through different routes: the inactivation of the female nucleus in a unicellular zygote after fertilization, the induction of callogenesis from meiocytes, and the regeneration of embryos or calluses from vacuolated microspores or young bicellular pollen. Among them, the latter, commonly known as microspore embryogenesis, is the most studied and used. In this route, a stress treatment is needed to induce the onset of embryogenesis and the consequent abandonment of the gametophytic route. Technically, microspore embryogenesis can be induced either through anther culture or through culture of isolated microspores (Seguí-Simarro, 2010a).

1.2.6. Advantages of androgenesis

Among the different alternative pathways to produce DHs, induction of androgenesis is the most convenient (Parra-Vega, 2015), due to the following reasons:

- There are protocols developed for the induction of embryogenesis in more plant species than with other methods.
- Each flower bud used contains a high number of microspores, so the probability of obtaining DHs is higher than with other methods.
- In androgenesis a high number of individuals spontaneously duplicate their genome, so additional treatments for genome doubling such as colchicine are less necessary to induce genome duplication.

However, there are some species that unfortunately, still lack an optimized protocol. This is the case of some solanaceae such as tomato, eggplant or pepper (Seguí-Simarro et al., 2010c).

1.2.7. Methods to induce embryogenesis from microspores

To induce embryogenesis from microspores, there are two techniques: anther culture and culture of isolated microspores (Seguí-Simarro, 2010a).

Despite the fact that anther culture is technically simpler, it has the disadvantage that proliferation can be induced from the somatic tissue of the anther, giving rise to callus and/or embryos genetically identical to the donor plants. This means that they will not be DHs, and therefore, they must be discarded. To avoid this inconvenience, while being a more complex technique, microspore embryogenesis may be carried out by *in vitro* culture of isolated microspores. In addition, this technique provides better results than anther culture in species where the protocol is well developed.

However, because microspores are in direct contact with the culture medium, there are a number of factors that must be taken into account as they notably affect the induction of embryogenesis (Seguí-Simarro, 2010b).

1.2.8. Factors affecting microspore cultures

There are several factors that considerably affect the induction of embryogenesis and must be taken into account and these can be differentiated into three groups (Seguí-Simarro, 2010a):

- The conditions of the donor plant, among which we highlight the role of the genotype in the induction of androgenesis. Likewise, the genetic endowment of the species, its variety (there are high and low-response cultivars), age (as the plant ages, it loses androgenic potential) and the influence of development conditions such as temperature or photoperiod would be factors to be taken into account.
- The isolation and induction conditions of the microspore: the stage of development in which the microspore was when it was isolated and put into culture. Among the stages of development, vacuolated microspore and young

bicellular pollen are the most likely to carry out embryogenesis, between the end of microsporogenesis and the beginning of microgametogenesis.

- The culture conditions: components of the culture medium (micronutrients, macronutrients, phytohormones...), light conditions, temperature and density of the microspores are some of the key conditions for the induction of embryogenesis. In addition, it has recently been observed that the osmotic pressure of the medium may also play a key role in androgenesis.

According to Dunwell (2010), the submission of flower buds to a cold period before microspore isolation, the modification of osmotic pressure of the medium during initial culture and electroporation are the three stress agents with more impact on the androgenic proliferation efficiency and its subsequent differentiation from microspores.

1.2.9. Role of osmolality in microspore cultures

Temperature, medium pH changes or osmotic stress are treatments that have been studied and used to induce androgenesis (Asif, 2013). However, few studies have focused on the study of the effects of medium osmolality.

Osmolality is defined as the number of osmoles per kg of solvent in a solution, with an osmol being the amount of undissociated solute whose osmotic pressure corresponds to one mole. Osmolality is proportional to osmotic pressure (the pressure required to interrupt the passive movement of water through a semi-permeable membrane in favour of its concentration gradient) and is expressed in milliosmoles per kilogram ($mOsm \cdot kg^{-1}$). In no case should osmolality be confused with osmolarity, which is defined in $mOsm \cdot l^{-1}$ (Erstad, 2003).

In nature, the internal osmolality of plant cells is usually higher than that of the environment, causing a flow of water from the outside to the inside of the cell. Thus, the cell wall acts as a crucial structure preventing water traffic from breaking up the cell. This accumulation of water in the cellular interior is responsible for the turgidity of the plant cells. If a plant cell were to be introduced, on the other hand, into a hypertonic medium, the intracellular content of water would be released to the outside, causing dehydration and consequent cellular death. As a result, the application of medium with high osmolality values is used as pre-treatment and stress agents for the induction of androgenesis, both in anther culture and isolated microspores (Zhou et al., 1991; Hoekstra et al., 1993).

Both in *in vivo* and in *in vitro* cultured cells, osmolality may affect the normal cell homeostasis, mostly when cells are in osmotically unbalanced environments. Studies have been carried out in different species evaluating the effect of osmotic stress pretreatments in isolated microspore cultures with positive results. For example, it has been observed that callous structures increase with the addition of polyethylene glycol (Ayed et al., 2010) or mannitol (Asif et al., 2014) to *in vitro* wheat crops. Also, in leguminous plants a higher yield in the development of callus from microspores of *Medicago truncatula* has been observed when osmotic stresses are applied in combination with electric shocks (Ochatt et al., 2009). Nonetheless, very little is known about the effect of the evolution of the internal osmolality of microspores and the medium on isolated microspore cultures, and how it affects microspore viability.

Recently, a study has been published in which they have observed the existence of a relationship between changes in both internal and external osmolality and the induction of embryogenesis in *in vitro* cultures of *Brassica napus* microspores (Rivas-Sendra et al., 2019). However, nothing similar has been done in *Capsicum annuum*, a recalcitrant species where induction and *in vitro* culture conditions still need serious improvements (Seguí-Simarro et al., 2010 c). In this context, the present work aims to focus on the study of the relationship between osmolality and viability during the induction of embryogenesis in *Capsicum annuum* microspores.

2. Objectives

The aim of this work is to study for the first time the evolution of the internal osmolality of microspores and the medium in *in vitro* cultures of pepper microspores (*Capsicum annuum*), and their relationship with the viability of these microspores. All this in order to verify to what extent the changes in osmolality, if any, would affect the viability and possible induction of androgenesis in *in vitro* cultures of pepper microspores.

3. Materials and methods

3.1. Materials

3.1.1. Plant Material

Commercial F1 hybrids of 'Herminio' pepper (*Capsicum annuum*) were used for this experiment.

The plants were grown in greenhouses belonging to the Institute for the Preservation and Improvement of Valencian Agro-diversity (COMAV) of Universitat Politècnica de València, continuously monitored to ensure their optimal conditions and good survival.

3.1.2. Culture medium

For *in vitro* culture of pepper two different media were needed: a culture medium (Table 2) and a fasting medium (Table 3). For each medium, jars of 0.25 L were prepared. Throughout the experiment it was necessary to prepare several jars of medium to have enough material for all cultures. No larger medium were prepared to ensure the media was fresh and in good conditions.

***Table 2**: Components and quantities of Capsicum annuum culture medium for different volumes.*

***Capsicum annuum* culture medium**				
Components	**Concentration**		**0,5**	**L**
NLN Salt Mixture	0,39	g/L	0,195	g
NLN Vitamin Mixture	1,04	g/L	0,52	g
Cefotaxim	0,3	g/L	0,15	g
Sacarose	100	g/L	50	g
KI	0,83	mg/L	500	uL

***Table 3:** Components and quantities of Capsicum annuum fasting medium for different volumes.*

***Capsicum annuum* fasting medium**				
Components	**Concentration**		**0,5**	**L**
Manitol	67,4	g/L	33,7	g
CaCL2 · 2H2O	1,47	g/L	0,735	g
MgSO4 · 7H2O	0,246	g/L	0,123	g
KNO3	0,101	g/L	0,0505	g
Cefotaxim	0,3	g/L	0,15	g
KH2PO4	0,0272	g/L	500	uL
KI	1,65E-04	g/L	500	uL
CuSO4 · 5H2O	2,50E-05	g/L	50	uL

Both cultures were crucial for the developing experiment as the fasting medium was the one used after extracting the microspores and the one in charge of inducing the osmolality shock while the culture medium was the one needed to stimulate the growth and development of the induced microspores.

3.1.3. Instruments for pepper microspore cultures

For carrying out microspore cultures there was a series of material that was required in order to proceed in an appropriate manner. The material can be divided into sterile material and non-sterile material as shown in Table 4.

***Table 4**: Material needed for Capsicum annuum microspore extraction*

Sterile material	Non-sterile material
- Whatmann paper - Beaker 50 ml - Beaker 100 ml or bigger - Forceps - Erlenmeyer with funnel and nylon filter 41 µm (Millipore) and 11µm (Millipore) - 15 ml tubes - 50 ml tubes - Pasteur pipettes 3 ml - 10 ml pipettes - Petri plates 1 cm, 6 cm and 10 cm of ∅ - 3 glass jars with cold sterile distilled water - Cold distilled water 250 ml	- Centrifugue - Laminar flow hood - Scalpel - Glass jar with 7% of commercial bleach (cold) - Glass jar with ethanol 70% (cold) - Tea sieve - Timer - 50 ml syringe piston stored in 70% ethanol - Parafilm - Pipette pump - 31ªC incubator - 25ªC incubator - Cardboard box (for darkness) - Fluorescein diacetate (FDA)

3.1.4. Instruments for osmolality measurement

For osmolality measurement, both internally and externally, the microsmometer Model 3320 Osmometer of Advanced Instruments, INC. was the one used (Figure 7). This measuring instrument allows to obtain the osmolality in mOsm·kg-1 of 20 µl of sample.

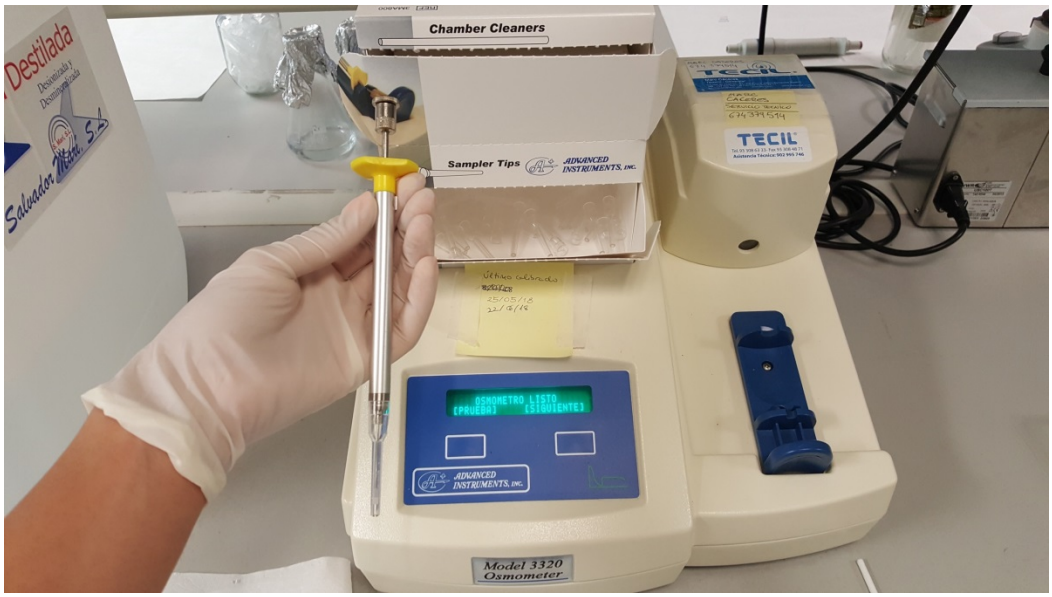

Figure 7: *Microsmometer Model 3320 of Advanced Instruments, INC.*

The osmolality calculation system used by the tool is based on the freezing temperature of the solution to be analyzed. Based on this specific temperature for each solution, the concentration of solutes present in the sample is inferred.

3.2. Methods

3.2.1. Optimal bud selection

Microspore extraction was carried out following the extraction and cultivation protocol described by Parra-Vega et al. (2012) with slight modifications. In the same way, the morphological selection criteria of the appropriate buds for the induction of the same protocol was used.

For each experiment, approximately 50 pepper buds with their anthers in optimal stage were needed. The estimation of the optimal buds was made using the morphological selection criteria described in Parra-Vega et al. (2012): calyx/bud size ratio. The calyx, in this case, should be approximately 80% of the total bud size as shown in Figure 8.

Figure 8: *Optimal bud following the morphological criteria for microspore isolation: calix is 80% of the total bud size.*

With the morphological selection criteria established, buds must be collected from the pepper plants in the greenhouse. For this, a bag with ice and a container was necessary to store the buds in cold from their collection to their processing in the laboratory.

However, it should be mentioned that the calix/bud ratio is not a completely precise selection method. A second criterion needs to be used: analysis of anther pigmentation. This other selection process consists of visualizing the purple pigmentation - whether null, partial or complete - of the apical area of the anther. The pigmentation must only be in the apical zone. If the pigmentation was null, the optimal stage for androgenesis induction had not arrived yet, whereas if the anther was completely pigmented, meant that the optimal stage of embryogenesis induction had passed.

Thus, once in the laboratory, a Petri dish of 6 cm in diameter or similar was prepared on ice so as not to subject the anthers to stress (Figure 9). With a scalpel the buds were opened. First a transversal cut at the base of the anthers was made to facilitate their subsequent opening and removal. Then a slight incision was made in the vertical direction to extract the anthers, just in case they have apical pigmentation, with the help of tweezers.

***Figure 9**: Material needed for anther extraction.*

3.2.2. Isolation of microspores

With the selection of the appropriate buds and subsequent extraction of their anthers (50 buds x approximately 6 anthers/bud = 300 anthers), the extraction of the microspores was carried out. For it, all the anthers that previously were in the petri plate were put inside a tea sieve above ice to continue maintaining a cold atmosphere. Then, the material was taken to the laminar flow cabin, where the whole procedure could be carried out in sterile conditions.

It was essential to ensure the axenia of the materials used along the experimental procedure. *In vitro* cultures of pepper microspores are very prone to contamination, which significantly affects the culture and thus, the performance of the experiment. The growth of unwanted organisms alters the properties of the culture medium. Medium osmolality plus microspore survival and induction would be altered and the results obtained would not be valid.

Before homogenizing the anthers to remove the microspores, the anthers of *Capsicum annuum* were sterilized with the following sterilizing or washing solutions (Figure 10):

- 30 seconds on 70% cold ethanol.
- 5 minutes in cold sodium hypochlorite (bleach) 7g/L.
- 2 minutes in sterile and cold distilled water, three times, in different jars each time.

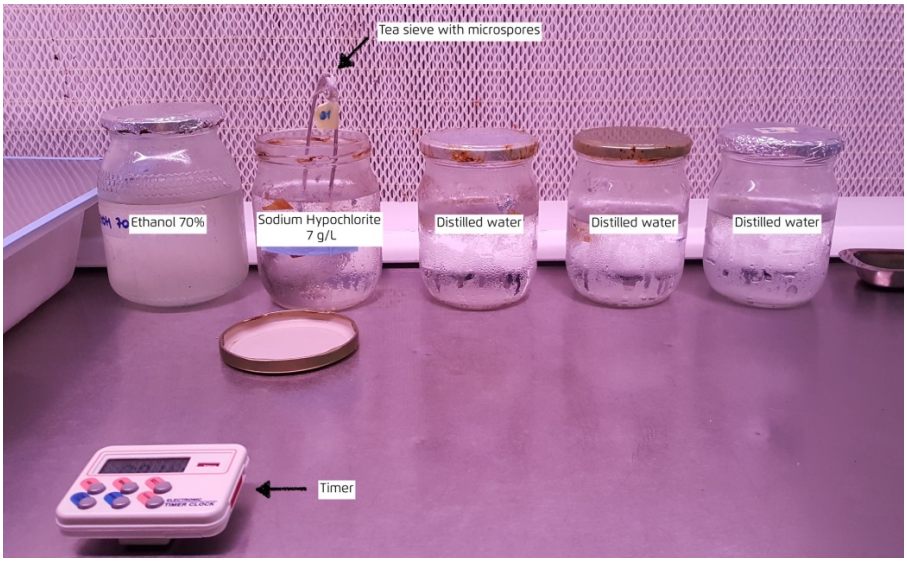

***Figure 10**:Material needed for sterilizing Capsicum annuum anthers.*

While the anthers were in the last jar with distilled water being washed, a sterile syringe piston of 50 mL previously stored in 70% ethanol was prepared. The piston was introduced in one of the distilled water jars where anthers were previously rinsed out, to remove the ethanol. Once the last anther rinse was completed and the piston was washed thoroughly, both the tea sieve and the syringe piston were removed and left on sterile Whatmann paper to dry during one or two minutes.

During this process of microspores isolation was important to carry out the whole procedure in cold conditions to keep the microspores metabolically stable. For this, a tray full of ice and covered with a metal plate was prepared in sterile conditions inside the laminar flow cabin, where the following steps were carried out.

A small beaker of 50 mL with 2 mL of sterile distilled water on top of the cold metal tray was prepared. Once both the piston and the anthers were dry, the anthers of the tea sieve were transferred to the beaker with the help of sterile forceps. With the piston the anthers were smashed in the beaker until they were all well crushed. To avoid losing plant material, the piston was washed with sterile distilled water and the water was let to fall back into the beaker where the anthers had been homogenized.

The crushed anthers in solution were passed through a 41 µm nylon filter (Figure 11). To ensure that no microspore remains in the previous beaker the edges of the container were washed thoroughly with sterile distilled water and passed through the nylon filter again to ensure that there was no material left in the container where the anthers had been smashed.

Figure 11: *Crushed anthers in fasting medium solution being passed through a 41 µm nylon filter.*

Once the solution was filtered, the suspension that the filter had allowed to flow through was collected in four sterile tubes of 15 mL and centrifuged at 870 rpm for 5 minutes at 4ºC.

After centrifugation, a pellet was formed at the bottom of the tubes (Figure 12), in which the microspores were found. In order to concentrate this material, the supernatant was removed and with Pasteur pipettes, the pellet was collected from each tube and put it all together in a single tube of 15 mL.

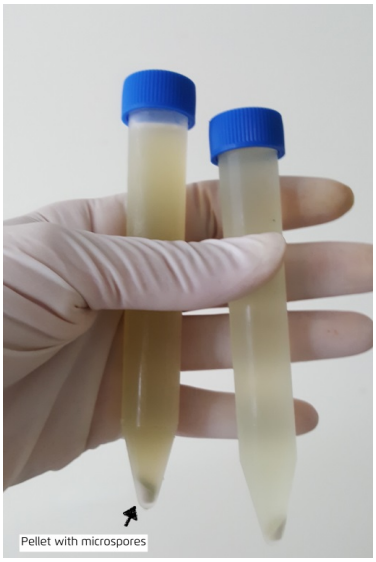

Figure 12*: Microspore pellet in 15 mL tubes after centrifugation.*

The resulting suspension was filtered again, but in this case through an 11 µm nylon filter. In this occasion the material collected was the one that had retained the filter. With the help of two forceps, the filter was removed from the funnel carefully so as not to lose the experimental material and the filter was placed on the side of a large beaker of 100 mL. There, the surface of the nylon could be cleaned with sterile distilled water to recover the microspores at the base of the container. In this procedure it must be avoided that the filter dries since this would cause the consequent drying of the microspores, altering the results of the experiment.

Being the nylon cleaned, at the bottom of the beaker it was possible to find the suspension of microspores which needed to be concentrated by centrifugation. To do so, the suspension was transferred from the container to as many 15 mL tubes as necessary and centrifuged at 870 rpm for 5 minutes at 4ºC twice -removing the supernatant and resuspending and joining the pellet material in the same tube between the first and second centrifugation-.

After the last centrifugation, the supernatant was removed remaining just the pellet. Up to here would be the steps to follow for the cultures of microspores to day zero.

In the case of the three and seven days cultures, the purified microspores were resuspended in 3 mL of induction culture medium (also fasting medium) and the amount of medium lacking for the optimum concentration was calculated.

To do this, an aliquot was taken from the final sample and introduced into an eppendorf. This aliquots were necessary to know the concentration of microspores and thus allow to calculate the final volume of fasting medium necessary to induce microspores and analyze their viability, all taking into account that the optimum density of microspores in the medium was 100.000 microspores per milliliter. With the help of a Neubauer camera, the amount of microspores of the sample was counted, obtaining the actual concentration, and so it was calculated the amount of volume needed to have the optimal concentration of cells.

Having calculated the final volume of induction medium necessary for optimum concentration, the difference between the final volume obtained and the 3 mL from before (fasting medium plus pellet) was introduced. The new volume was introduced together with the previous one if the container was big enough or it was distributed in 2 tubes of 15 mL each taking into account that the suspension of the pellet must be distributed in an equivalent way in both tubes to maintain the optimal concentration.

Next, the total volume was distributed in 1, 3 and 10 mL Petri dishes, always trying to use at least two 1 mL plates that allow to make observations throughout the cycle without losing plant material.

When the plates were filled and closed with Parafilm, they were introduced in an incubator in the dark and at 31ºC for 3 days to induce embryogenesis by heat shock. So far, the steps to follow for the culture of microspores to day three.

In the case of seven days cultures, there were still a few more final steps to follow. Once the 3 days of the culture had passed at 31ºC in darkness, the Petri dishes were collected and, in the laminar flow cabinets in sterile conditions, with the help of a Pasteur pipette, the suspension of each one of the dishes (reserving one of 1 mL to measure viability) was transferred to different 15 mL tubes. There were used as many tubes as dishes to

avoid possible contaminations between the different plates. These tubes were centrifuged at 870 rpm for 5 minutes at room temperature. After, the supernatant was eliminated and the pellet resuspended in the same volume than before but now instead of fasting medium culture medium was used. Each plant material was returned to its original plate to avoid cross contamination.

Once the microspores were in the new culture, the Petri dishes were sealed with Parafilm and the cultures were kept in darkness in an incubator at 25ºC until four more days of culture, seven days since the beginning of the experiment, were completed.

3.2.3. Day zero post-culture proceeding

The microspores that were considered within the 'Day zero culture' did not need to be in fasting medium like the rest (day three or seven culture) since they were the control of the experiment.

Once extracted the microspores from the anthers and the plant material was concentrated in a pellet, it was passed into an Eppendorf of 1.5 mL, which underwent a spin at the centrifuge at 8,000 rpm. The excess of water was eliminated with a pipette and quickly the sample of microspores was conserved in liquid nitrogen at -80ºC until the moment in which the microspores were processed.

3.2.4. Day three post-culture proceeding

The microspores obtained from day 3 cultivation were obtained by collecting all the volumes of the different plates (except the 1 mL plate needed to measure viability), after having spent 3 days in darkness at 31 º C. The suspensions were collected with the help of a Pasteur pipette and placed in as many 15 mL tubes as necessary. Once centrifuged (870 rpm, 5 minutes, ambient temperature), the supernatant was thrown away (although an aliquot of the fasting medium was saved for measuring medium osmolality) and the pellet transferred into an Eppendorf of 1.5 mL that, as in day zero culture, underwent a spin in the centrifuge and after, with a pipette the water was removed so that the sample could be conserved in liquid nitrogen.

3.2.5. Day seven post-culture proceeding

The method of proceeding in day seven culture was the same as day three culture but taking into account that the microspores were collected after seven days of culture: 3 in fasting medium and 4 in culture medium. An aliquot of the culture medium, which was the supernatant after centrifugation, was saved for culture medium measurement.

3.2.6. **Viability measurement**

For measuring viability of the cultures at the different stages (day 0, 3 and 7) it was used fluorescein diacetate, hereandafter FDA, a cell-permanent esterase substrate that serves as a vibility probe as measures enzymatic activity -required to activate the fluorescence of the dye- and cell-membrane integrity -essential for intracellular retention of the FDA- (Thermo Fisher Scientific, 2019).

Thus, viable microspores were those that stained and could be observed with a green fluorescence under the inverted fluorescence microscope.

Once the FDA was used in the sample, the sample could no longer be used in the experiment as FDA is toxic. Viability could only be measured once per plate. For this reason was important to prepare as many 1 mL plates as viability measures were needed throughout the experiment. With small Petri dishes the loss of experimental material was avoided.

Viability was measured at day zero as well as in the third and seventh day in the different cultures. Thus, from day zero culture it was obtained a single viability measure corresponding to the day of isolation and storage of microspores. At day three culture the viability both at day zero and three was obtained. Finally, at day seven cultures, viabilities from day zero, three and seven were obtained. This allowed the study of the evolution of viability in the different samples as well as helped discard contaminated crops.

In all cases, it was followed the same procedure: a small sample of microspores was taken, 5 µL of FDA was added, 30 minutes of waiting in darkness (samples were left under a cardboard box) were needed for the dye to penetrate the cell and finally the sample could be visualized in the inverted microscope. All this procedure was done in the dark as the light alters the fluorescence of the product.

Viability was calculated by counting the viable microspores - the stained ones - on the total number of microspores in the field in a minimum of 3 fields and having counted a minimum of 150 total microspores.

Despite the fact that the procedure was the same in all crops, it should be noted that:

- In the measure of viability of day zero culture, when all the sample of microspores was collected from the 15 mL tubes after centrifugation, with the same Pasteur pipette that had been used to resuspend the microspores of the tubes and to pass them to the epperdorf of 1.5 mL, 1 mL of sterile distilled water was collected to clean the inner part of the pipette. This avoided losing part of the plant matrial as some microspores had been adhered in the lateral surfaces of the pipette. This material was deposited on a plate of 1 mL and was used to measure the viability of day zero culture.

- In the viability of day three culture, just one plate of 1 mL with fasting medium (it was not necessary to change the culture of this plate) was needed. Direct stain with FDA was applied.

- On day seven culture, as when measuring viability of the third day, a plate of 1 mL with the suspension of microspores in culture medium was taken and the amount of viable microspores could be counted after staining with FDA.

3.2.7. Microspore and medium osmolality measurement

The main objective of this experiment was to analyze the relationship between viability and osmolality of *Capsicum annuum* cultures. For this reason, and in order to study how osmolality evolves both in the medium and in the microspores themselves over time, three cultures were used and collected on different days: a day zero culture, a day three culture once the thermal stress had ended and a day seven culture subjected to three days of thermal stress (35ºC) and four on optimal culture conditions under 25ºC.

For all samples it was measured both medium osmolality and microspore osmolality. However, just as viability was measured at days zero, three and seven, osmolality could only be measured at the end of the experiment as there was not enough sample of microspores to allow a detailed study throughout the experiment of the evolution of the microspores osmolality. For this reason, it only made sense to measure the osmolality of the medium on the same days as that of the microspores.

In this way, in day zero cultures it was measured the osmolality of the fasting medium even if the microspores had not been put into culture and the osmolality of the microspores just isolated from the anthers. From day three culture, the osmolality of the fasting medium and microspores was measured once the time of culture under thermal stress had passed. Finally, from day seven culture, it was measured the osmolality of the culture medium and the microspores osmolality at the end of the experiment.

3.2.7.1. *Tissue homogenization for osmolality measurement*

After storing the microspore samples in liquid nitrogen as described in sections 3.2.3, 3.2.4 and 3.2.5, the frozen microspore samples were subjected to a cold/heat cycle homogenisation process.

A Stuart SBH130D block heater was used for heat and cold cycle homogenization. Samples that had previously been stored in liquid nitrogen for preservation were subjected to five cycles of cold/heat of 10 minutes each with the following pattern: heat-cold-heat-cold-heat. The temperature for the heat cycles was set at 55°C for the thermoblock while for the cold cycles a refrigerator was set at -20°C.

After subjecting the samples to the corresponding cycles, the eppendorfs were centrifuged at 14,000 rpm 5 minutes and the supernatant was conserved, which corresponded to the sample analyzed in the osmometer. Osmolality was measured 3 times to take the average and obtain a more accurate result.

In the case where the supernatant obtained after homogenisation did not reach the minimum 20 μL needed for analysis in the microsmeter, dilutions needed to be carried out. These dilutions consisted on adding distilled water to the volume of supernatant that was known until completing the 20 μL necessary for the osmolality analysis. This was taken into account when analyzing the osmolality measurements, the corresponding dilution factor was applied for each case.

*3.2.7.2. **Medium osmolality measurement***

To measure the osmolality of the culture medium, all what was needed to do was tune the osmometer and analyze the aliquots of the medium of each of the cultures, as described above.

4. Results and discussion

4.1. *Capsicum annuum* microspore culture

In vitro microspore cultures are heterogeneous. In the same environment co-exist microspores that have been induced and will initiate embryogenesis, microspores that have survived but have not been sensitive to the inductive treatment so follow a gametophytic-like program developing gametophytic-like structures and dying or dead microspores.

Throughout the experiment it was observed the induction capacity of the microspores and how they were able, altering their normal ontogenetic route, to develop androgenic potential.

Microspores sensitive to the inductive treatment were reprogrammed into embryogenesis, reabsorbing the vacuole and dividing symmetrically. About 7 days after inoculation and of being the microspores in culture it could be observed that some had already experienced the first cell division (Figure 13A), obtaining two symmetrical cells. Once this first division took place, a short time later, on the following day, the asymmetric division typical of totipotent stem cells (Figure 13B) was observed. Multiple divisions began to occur, until eight cells were found in what was previously only one (Figure 13C). The embryogenesis, the development of a new individual, began to become evident when multicellular structures developed after 12 days in culture, being able to observe globular structures (Figure 13D) between 15 and 20 days of culture. The multicellular embryo, was able to develop spatially along the Petri dish.

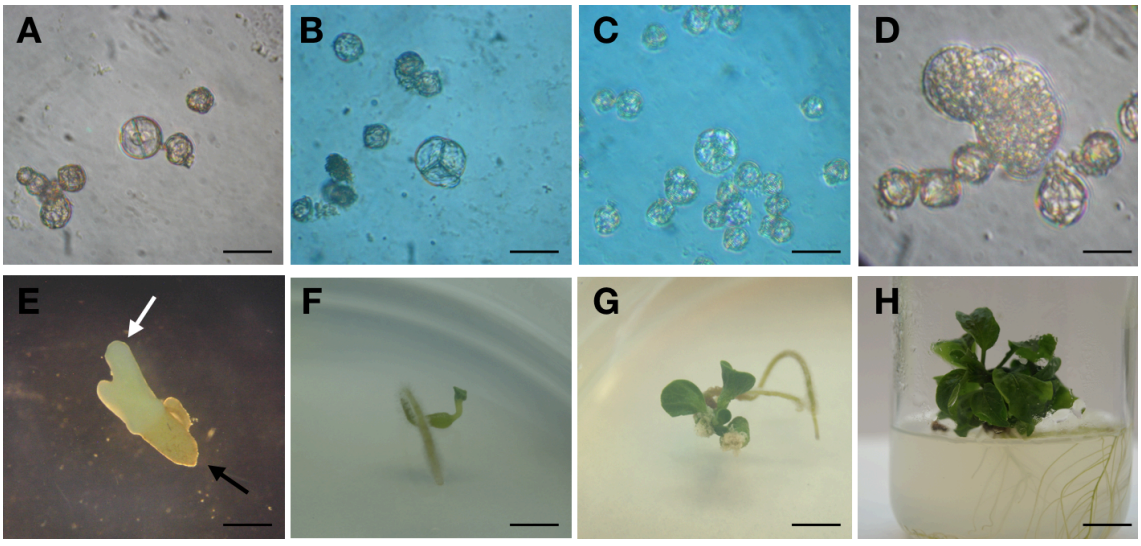

***Figure 13**: Microspore culture of 'Herminio' pepper. **A, B, C**. Dividing, embryogenic microspore. **D**. Globular microspore-derived embryo. **E**. Torpedo-shaped microspore-derived embryo. **F, G, H**. In vitro regenerated plantlet. Bars in **A-D** 10um; **E** 500um; **F,G** 5mm; **H** 1cm*

The correct development of the embryo lead to the formation of identifiable structures typical of the embryogenesis process, as is the case of image E of the figure, where it can clearly be observed that the embryo was between the heart and torpedo phases of embryogenesis.

Due to cell elongation in the longitudinal axis of the embryo, there was expansion of the cotyledons and development of the apical meristem (black arrow in Figure 13E). It could be seen that cotyledons did not developed simultaneously and symmetrically as they would in their somatic embryogenesis development, as marks the white arrow of image E of Figure 13. This slightly abnormal development of cotyledons is a characteristic of microspore cultures when are induced to embryogenesis. Perhaps with more refined protocols we could avoid it. Whatever the case, it was not a serious limitation for the development of a new individual since, the embryo was in a culture medium, this was the main precursor of nutrients. This way, the problem that could arise from the abnormal development of cotyledons was solved with an external nutrients supply.

With the nutrients assured and good external conditions, the embryo continued dividing and developing until a plantlet could be observed in the Petri dish with the naked eye (Figure 13F). Just a few more days were needed to observe a clearly developed plant: with its first leaves at the apical end, the hypocotyl and the radicle (Figure 13G). With the continuous supply of nutrients and good conditions for the correct development, a complete and functional plant of *Capsicum annuum* was obtained, ready for acclimation (Figure 13H).

4.2. Osmolality analysis

Throughout the experiment, the osmolality values for both the medium (starvation and culture medium) and the microspores at zero, third and seventh day were obtained (Figure 14). At the zero and third day, the results obtained corresponded to induction medium, while those obtained at the seventh day corresponded to culture medium. In practical terms, the osmolality of these two media was not very different since the compounds that contributed the most to medium osmolality -mannitol in induction medium and sacarose in culture medium- were in similar amounts. At day seven, osmolality slightly increased, but an ANOVA analysis followed by a Fisher's least significant difference (LSD) procedure showed that there were no significant differences among the three osmolality values.

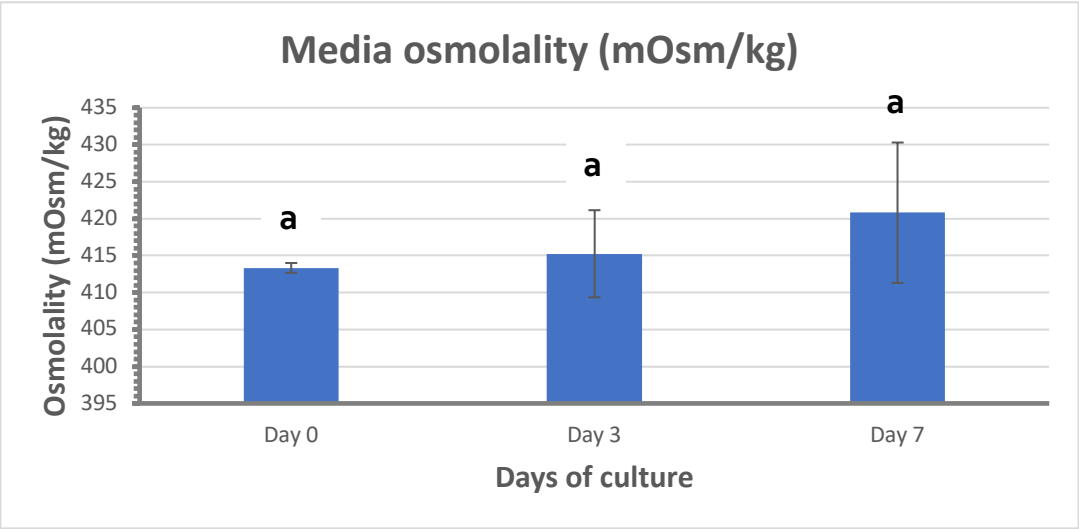

***Figure 14**:Medium osmolality at zero, three and seven days of Capsicum annuum microspore culture. Different letters indicate significant differences (p≤0.05)*

On the other hand, regarding the osmolality of microspores, there was an evolution upon time. While medium osmolality remained balanced, microspore osmolality increased as the experiment progressed (Figure 15).

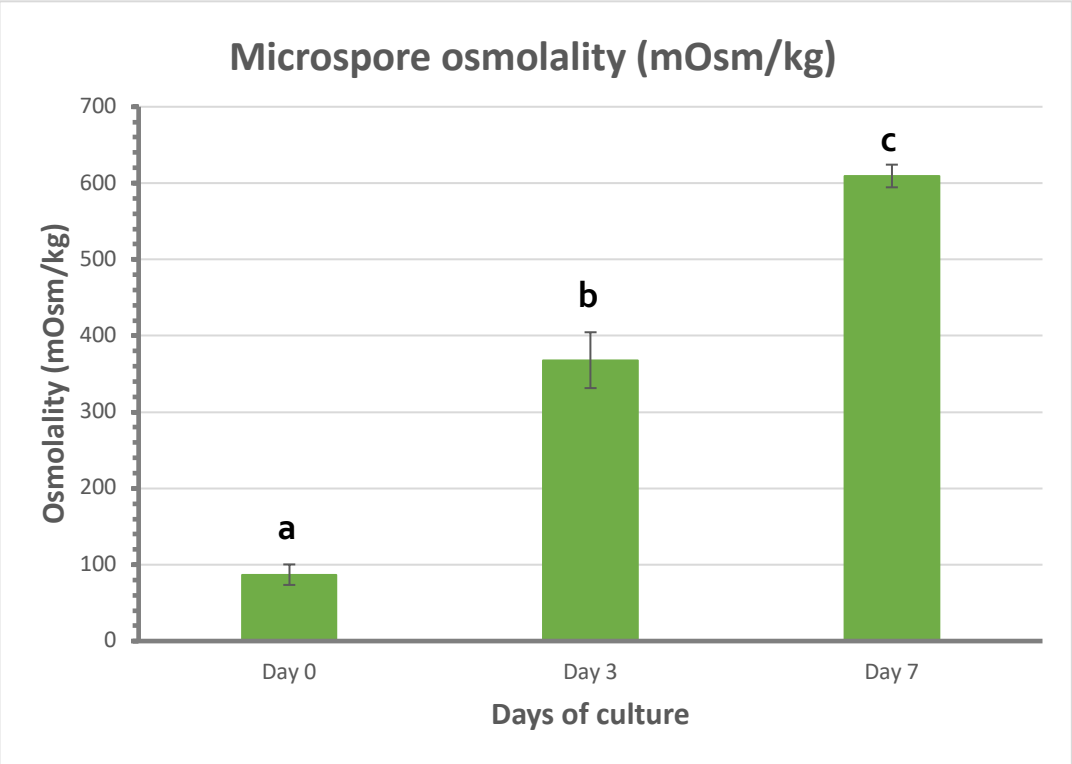

***Figure 15**: Microspore osmolality at zero, three and seven days of Capsicum annuum microspore culture. Different letters indicate significant differences (p≤0.05)*

At the very beginning, microspore osmolality was lower (around 100 mOsm/Kg) than that of the induction medium, which would be hypertonic for microspores. As culture progressed and microspores grew, osmolality increased to reach around 400 mOsm/Kg at day three, just after the end of the induction treatment, and around 600 mOsm/Kg at day seven. An ANOVA analysis followed by a Fisher's least significant difference (LSD) procedure revealed significant differences among the three osmolality values.

4.3. Viability analysis

Viability of microspores decreased considerably with the passage of days in culture (Figure 16). At the beginning, at day zero, the viability of freshly isolated microspores rounded 25%, the highest value. After the induction treatment, viability dramatically decreased to values around 10%. An ANOVA analysis followed by a Fisher's LSD test (p≤0.05) revealed significant differences between viability at days zero and three. As commented before, at the beginning of 'Results and discussion', within microspore cultures not all of them are induced. Among the initially viable microspores, many of them cannot cope with the stress treatments applied and die or become arrested. This is the main reason of the viability loss observed after induction. At day seven, viability

was slightly lower than at day three. However, the difference was not statistically significant (p≤0.05), which confirmed that stress treatments may explain the loss in viability after day three. The cells that were counted as viable coincide with those that seemed to be sensitive to the induction process.

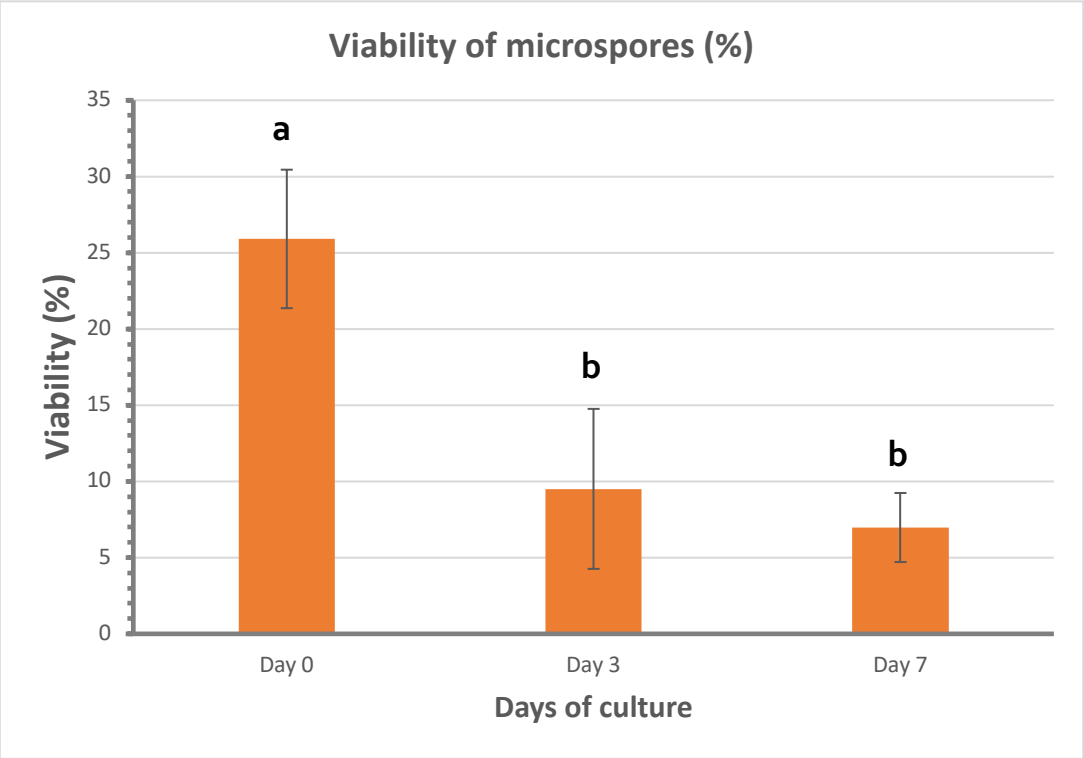

***Figure 16**: Viability at zero, three and seven days of Capsicum annuum microspore culture. Different letters indicate significant differences (p≤0.05)*

4.4.Relationship between osmolality and viability

We represented together the results shown above (Figure 17), for an easy overall visualization of them, and in order to find possible relationships among the osmolality of the medium, the osmolality of the microspores and the viability of the microspores after isolation and culture.

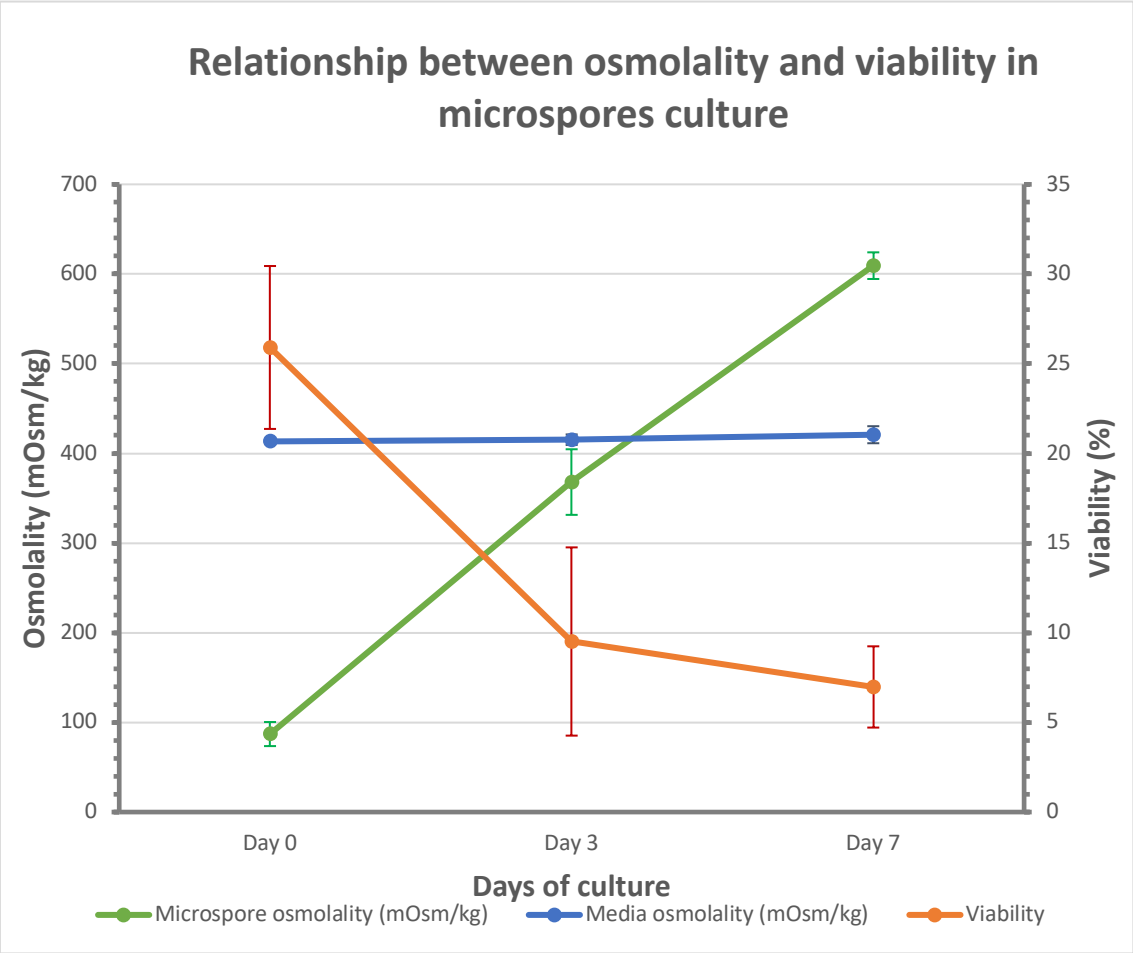

Figure 17: *Relationship between osmolality and viability in microspores culture.*

As mentioned in Section '4.2 Osmolality analysis', the osmolality of the medium remained stable throughout the experiment and independent from the changes experienced by microspores. This seems reasonable since at all stages, the volume of culture medium was largely in excess over the volume of intracellular fluid of microspores. Therefore, the effect on medium osmolality of any possible change in microspore osmolality would be negligible.

On the contrary, the osmolality of cultured microspores steadily increased during these three days. At day zero, microspore osmolality was markedly lower that that of medium. After the heat shock treatment it increased to almost match that of the medium. This may be due to osmotic shock after putting the microspores in culture in a hypertonic medium. To try to compensate this osmolality difference, the microspores undergo a dehydration process that leads them to increase their osmolality until it is

balanced with that of the medium. This phenomenon indicated that the cellular mechanisms to control cell homeostasis were not fully functional, and that a fraction of the microspore population lost them. With no osmotic protection, the logical scenario is to approach both internal and external osmolalities to reach a balanced state. In parallel, the most reasonable consequence of the massive loss of cell homeostasis would be a massive cellular death. This hypothesis is supported by the dramatic drop in viability from day zero to day three. The drop from 25% to 10% represents the death of 60% of the microspores living at day zero. This large percentage may easily account for the osmolality increase observed.

Interestingly, this is not the only species where the heat shock treatment may lead to a massive loss of cell homeostasis and eventually cell death. In our research group, we recently published (Rivas-Sendra et al. 2019) a series of experiments in *Brassica napus* designed to track the changes in viability, embryogenic response, and osmolality of embryogenic microspores and culture medium at different culture stages. To exclude the possible influences of genotype and culture conditions, instead of comparing different lines, we used microspores/pollen from a high-response DH line (DH4079) isolated from the same donor plants, exposed to identical culture conditions, but at different developmental stages. We isolated microspores from 3.0-3.2, 3.3-3.4, and 3.5-3.6 mm buds, which contained mixed populations with predominance of young microspores in the 3.0-3.2 interval, mid-vacuolate microspores in the 3.3-3.4 interval, and vacuolate microspores/young pollen grains in the 3.5-3.6 interval. Viability of the three microspore intervals revolved around 40-60% at day zero and underwent a dramatic decrease at day three. The 3.0-3.2 interval showed ~0% viability, whereas the 3.3-3.4 viability was ~2%. As opposed to this, the 3.5-3.6 interval showed a remarkably higher 14%. As expected according to their composition, the most responsive interval was 3.5-3.6 mm. Indeed, there was a high correlation between viability and divisions at day 3. Next, we measured internal and medium osmolality of cultures from each interval at days zero (just after isolation), three and seven, as in this work, and found that medium osmolality remained nearly constant at all times, and the initial osmolality of microspores of all intervals was remarkably similar, always above medium. However, this pattern diverged among intervals upon culture progression. At day 3, the 3.0-3.2 and 3.3-3.4 intervals dropped to values lower than at day zero, and finally converged at day seven with those of culture medium. However, the dynamics of the 3.5-3.6 interval was remarkably different. Internal osmolality was always significantly higher than medium. All individual values and internal-vs-medium differences were constant at all culture times.

In summary, the interval with highest viability and embryogenic response was also the only interval where microspores kept their osmolality values constant, always above those of culture medium. In other words, we found a relationship between viability, embryogenic response and protection against osmotically unbalanced media. The results shown hereby would support this notion, and in this particular case, would be indicating that pepper microspores do not present the protective mechanisms described for *B. napus* embryogenic microspores, or they are not as effective as in *B. napus*, since they are not able to maintain microspore osmolality from being balanced with medium osmolality.

At day seven, microspore osmolality kept increasing, even beyond the values of media osmolality. According to the notion above mentioned, both osmolality values should

approach and maintain, as it occurred in other species (Rivas-Sendra et al. 2019). Pepper microspores moved from a hyperosmotic environment at day zero to a hyposmotic scenario at day seven. Actually, it is difficult to speculate how this could be produced. Perhaps, the increase in the osmolality of microspores on the seventh day of culture, while viability continues decreasing, may be due to the increase in osmotically active compounds produced during the process of cell death. Nonetheless, it can be observed that a larger decrease in viability is produced from day zero to day three than from day three to day seven. This may be due to the effect of the starvation media and heat shock. Once microspores have survived to the external abiotic stress and have been induced, their probabilities of survival and carrying out embryogenesis are higher. Nonetheless, there is a high decrease in viability that shows that very few microspores manage to induce themselves and survive in culture. Therefore, these living microspores influence to a very small extent the values obtained for osmolality. What would be expected over time is that the osmolality of the microspores decreases, matching that of the medium. The balance between the osmolality of the medium and that of the microspores would be due to the entry of the culture medium into the interior of the cell through the pores of the cell and the degradation of the substances produced during the process of cell death that had caused a temporary increase in the internal osmolality of the microspores.

In (Rivas-Sendra et al. 2019) we confirmed the hypothesis raised in other, previous reports (Parra-Vega et al. 2015) regarding the possible role of the subintinal layer (a cell wall layer beneath the intine produced in induced microspores) on the extra protection against osmotic pressure that induced microspores exhibit. Although we did not do any study about the putative presence (or not) of a subintinal layer in pepper microspores, other authors have previously suggested similar structures in pepper (González-Melendi et al., 1995). However, the results shown in this work indicate that in case a similar cell wall layer exists in pepper microspores, their role as a barrier to prevent osmotic shocks would be very deficient.

It is interesting to note that in the species were we best observed, described and studied the subintinal layer was *B. napus*, a model species where induction of embryogenesis is relatively easy (Segui- Simarro 2010), and microspores are osmotically protected by this layer (Rivas-Sendra et al. 2019). However, species recalcitrant to induction of microspore embryogenesis such as eggplant (Rivas-Sendra et al. 2019) or pepper (this work) presented a very limited protection against osmotic shock, and the subintinal layer is either absent or poorly developed. As a conclusion of this, it is tempting to speculate that the presence of this layer is directly related to embryogenic competence by increasing microspore viability.

Nevertheless, in order to shed light to this and other pending issues, this work should be completed in the future by extending the measurement of osmolality to further stages, beyond day seven, including other stages between days zero and three, and between days three and seven, increasing the sample size at each stage, and conducting cell biology-based studies to try to find the subintinal layer in induced pepper microspores, and to study their structure, thickness, and predicted role by culturing microspores in different experimental conditions of osmotic pressure and of promotion/inhibition of the growth of the subintinal layer. Together, these studies would contribute to a better understanding of the effect of osmolality in pepper microspores induced *in vitro* to embryogenesis.

5. Conclusions

From the results obtained in the present work it can be concluded that:

- There is a clear and statistically significant inverse relationship between the evolution of the osmolality of microspores and their viability in *in vitro* culture of *Capsicum annuum* microspores.

- This may be due to a deficient mechanism of maintenance of cellular homeostasis in an *in vitro* environment, as it happens in other microspore culture systems.

- In order to study this process deeply, it would be necessary to extend this work by increasing the days of study of the culture, as well as increasing the experimental sampling, and by performing cell biology-based studies of the putative subintinal layer of pepper microspores.

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