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In vitro germination of immature embryos for accelerating generation advancement in peppers (*Capsicum annuum* L.)

J.P. Manzur, M. Oliva-Alarcón, A. Rodríguez-Burruezo*

Instituto de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain

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ABSTRACT

Capsicum peppers are one of the most important vegetables in the world and continuous breeding efforts are required to improve yield, resistances, or fruit traits. In this sense, breeding programs usually last many years because many generations each with several months are needed. Therefore, the isolation and *in vitro* germination of immature embryos might be helpful to shorten breeding cycles and accelerate breeding programs. Here, we evaluated the efficiency of this strategy in *Capsicum annuum* under both Autumn–Winter (AW) and Spring–Summer (SS) growing conditions. Five accessions, representing different varietal types, were included in this experiment and immature advanced embryos (torpedo-early cotyledonary) were used because of their high *in vitro* germination aptitude. Conventional breeding cycles (control) ranged between 148 and 184 days in AW and between 117 and 154 days in SS, indicating that no more than two generations per year are possible in peppers. By contrast, the *in vitro* strategy reduced the cycle length by 33–70 days in the AW season and by 13–56 days in the SS season, with California accessions showing the highest shortenings. These findings show that this strategy will allow *Capsicum* breeders to obtain three generations per year in California peppers, and up to four generations in cayenne peppers. Furthermore, compared to controls, *in vitro*-germinated plantlets showed the same high pollen fertility, and no deleterious effects were observed in their subsequent development (plant height and biomass). Therefore, these plants can be integrated safely in breeding programs.

1. Introduction

Paprika and chile peppers (*Capsicum* spp.) are one of the most important vegetables in the world for both fresh and spice consumption, encompassing a worldwide production of 30 million t and an acreage of 4 million ha (FAOSTAT, 2011). Furthermore, among the cultivated species of genus *Capsicum*, *Capsicum annuum* L. is the most diverse and economically important species (DeWitt and Bosland, 2009; Rodríguez-Burruezo et al., 2010). In this regard, Spain is the largest producer and exporter of *C. annuum* in Europe, and the Mediterranean coast of the country is the major producing region (FAOSTAT, 2011; MAGRAMA, 2012).

As in other cultivated species, peppers require continuous breeding to improve different traits like stable resistances against pests, diseases, and/or abiotic stress (*e.g.* water stress, salt stress), as well as fruit quality (*e.g.* content in antioxidants, coloring power, pungency and aroma components) (Moury et al., 2000; Gnayfeed et al., 2001; Lee et al., 2005; Topuz and Ozdemir, 2007; Monroy-Barbosa and Bosland, 2008; Rodríguez-Burruezo et al., 2009, 2010). In this respect, the most usual breeding methods in peppers are: (i) pedigree – selection of individual plants combined with controlled self-pollination – (ii) backcross – particularly for traits controlled by one or few genes, which involves selection of individual plants and successive crosses to a recurrent parent – and (iii) recurrent selection – which involves selecting individuals from a population followed by intercrossing to form a new population – (Bosland and Votava, 2000). Additionally, the single seed descent method, which does not need selection during the breeding process, is also utilized in the development of recombinant inbred lines (RILs) (*e.g.* Sy et al., 2008).

All these methods require a considerable number of successive generations. Moreover, the minimum time to complete each generation is also an important factor in the total length of a breeding program. In the case of *C. annuum*, there are no detailed data about the length of breeding generations, although it is known that the development of peppers is slower than other related crops like tomato, which is due to their higher temperature requirements

* Corresponding author at: Instituto de Conservación y Mejora de la Agrodiversidad Valenciana, Edificio 8E, acceso J. Ciudad Politécnica de la Innovación, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain.
Tel.: +34 963879383; fax: +34 963879422.
E-mail address: adrodbur@upvnet.upv.es (A. Rodríguez-Burruezo).

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(Bosland and Votava, 2000). In addition, differences among genotypes could be expected for this trait and also climate conditions may have a dramatic effect on the growth of these plants.

As observed in other vegetables, the lapse of time between seed sowing and germination in *C. annuum* is very short considering the whole cycle (Bosland and Votava, 2000; Nuez et al., 2003). By contrast, the longest phases of the breeding cycle in peppers are plant growth and fruit development. Furthermore, *Capsicum* pods must reach the fully ripe stage to ensure the viability of seeds, which technically increases the length of the breeding cycle. Otherwise, seeds from unripe fruits show very low or nil germination rates (Sánchez et al., 1993; Nuez et al., 2003). As a result, the completion of a breeding program using this conventional methodology may take several years.

In this regard, shortening the breeding cycle in *C. annuum* would be very helpful for two reasons: (i) to accelerate breeding programs and, indirectly, (ii) to decrease the costs of growing plant materials (*i.e.* less time using greenhouses, heating and cooling systems, workers, *etc.*). To achieve this objective, the excision and *in vitro* cultivation of zygotic embryos from developing fruits may be considered an alternative to the conventional development and ripening of fruits. Furthermore, this breeding procedure could include marker assisted selection for specific traits of interest, which might allow breeders avoiding time-consuming evaluations and use the first fruits set in each generation (Bhattarai et al., 2009).

This *in vitro* technique consists of germinating and obtaining plantlets from immature embryos, grown in a defined nutrient medium under aseptic conditions (Cravero and Cointry, 2007). Since the first studies performed in *Iris* sp. (Randolph, 1945), it has been applied mainly to overcome postzygotic barriers in interespecific crosses in different species, including peppers (Ochatt et al., 2002; Hossain et al., 2003; Liu et al., 2004; Leng and Yamamura, 2006; Yoon et al., 2006; Cravero and Cointry, 2007; Bhattarai et al., 2009; Dagustu et al., 2010; Gebologlu et al., 2011; Wang et al., 2011). However, reports dealing with the shortening of breeding cycles are very scarce in vegetables, apart from very few studies in artichoke and tomato (Cravero and Cointry, 2007; Bhattarai et al., 2009; Gebologlu et al., 2011).

In the present experiment, we have evaluated the efficiency of the *in vitro* culture of immature embryos to shorten the length of breeding generations in *C. annuum*. Moreover, our study encompassed the response of a range of *C. annuum* varietal types and the two most usual growing seasons in the Mediterranean coast of Spain. Also, the subsequent development of plants was monitored to check potential deleterious effects of precocious germination. To our knowledge, this is the first report about this subject in peppers and the derived results will provide useful information to optimize the efforts of *Capsicum* breeders.

2. Materials and methods

2.1. Plant materials and growing conditions

Five accessions, representing different varietal types of *C. annuum*, which were obtained from the germplasm bank of the COMAV, were used in the present study. They encompassed a range of geographical origins and fruit morphological traits (Table 1).

To study the effect of environmental conditions on the breeding cycle of peppers, two separate experiments were performed considering the most common and opposite growing seasons for peppers in the Mediterranean coast of Spain: (i) Autumn–Winter (AW) and (ii) Spring–Summer (SS) (Nuez et al., 2003). Both experiments were carried out under greenhouse and the usual conditions for the commercial production of peppers in Spain. Thus, light conditions were limited to natural illumination, while temperatures were controlled to ensure a minimum of 15 °C and a maximum of 35 °C. Plants were drip irrigated (4 L/h) every 12 h for 2 min in AW and every 8 h for 2 min in SS. Fertilizer was applied with the irrigation water, at a rate of 1 g/L of a commercial 15N–2.2P–24.9K water soluble fertilizer (BASF, Barcelona, Spain).

A total of ten plants per accession and growing season were transplanted at the 4-leaf stage to the glasshouses of the Universitat Politècnica de València (Valencia, Spain) in September 2011 and March 2012 to start the AW and SS experiments, respectively.

2.2. Studied traits

The main topic of the present work was to estimate the efficiency provided by the *in vitro* culture of immature embryos to shorten the breeding cycle in *C. annuum* peppers. To that end, the breeding cycle of peppers was monitored considering the completion of an entire generation, according to an embryo-to-embryo model, from pollination to flowering (*e.g.* Gebologlu et al., 2011). Thus, the cycle was divided into the following phases: (i) phase 1, from pollination (controlled selfings) to seed sowing (conventional strategy) or, alternatively, to embryo isolation and culture (*in vitro* strategy), (ii) phase 2, from seed sowing or embryo culture to the 4-leaf stage, when plantlets are usually transplanted, and finally (iii) phase 3, from the 4-leaf stage to the initiation of the anthesis (recorded at the anthesis of the first three flowers), which was considered the stage at which breeders could perform selfings for a new generation and, therefore, the completion of the cycle.

Cycle monitoring started once flowers were selfed and labeled with the corresponding date, from November 2011 for the AW and April 2012 for the SS experiments, respectively. Selfings were repeated twice a week until obtaining a minimum of 20 fruits per accession and growing season. Half of the fruits were then utilized to provide immature embryos for the *in vitro* technique, while the other half remained in the plants as controls of the conventional methodology, being harvested at the fully ripe stage. The length of the breeding cycles of both strategies was then compared on the basis of the number of days after pollination (DAP), according to the established phases.

Finally, the efficiency of the *in vitro* technique was also evaluated by comparing the germination rates (%) of both *in vitro* cultured embryos and control mature seeds. In addition, to check potential deleterious effects of precocious germination (Bhojwani and Razdan, 1996), we studied other traits related to plant development and fertility at the end of the experiments: plant height (cm), plant biomass (g), and pollen viability (%), which was tested using a 1% (w/v) thiazolyl blue tetrazolium bromide (MTT) dye (M-2128, Sigma–Aldrich) (Rodriguez-Riaño and Dafni, 2000).

2.3. Procedure for embryo isolation and culture and subsequent development

Only immature advanced embryos, at torpedo or early cotyledonary stages, were excised and cultured for the *in vitro* strategy because of their higher response to *in vitro* germination and development (Yoon et al., 2006; Manzur et al., 2013). To achieve that, immature developing fruits were harvested at the most suitable phenological stage of each genotype, according to our expertise (Manzur et al., 2010, 2013). Thus, depending on the growing season and the genotype, immature fruits were harvested between 20 and 40 DAP.

After being harvested, the surface of the fruit was washed with liquid detergent (liquinox® at 5%) and rinsed with tap water. Once in the lab, the whole fruit was surface-sterilized with ethanol (96%) under laminar flow cabinet conditions (model AH-100, Telstar, Terrassa, Spain). Then, immature seeds were removed and sterilized using a 1% dilution of commercial bleach (4% sodium hypochlorite)