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Growth regulators and darkness increase efficiency in *in vitro* culture of immature embryos from peppers

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ABSTRACT: Common pepper (*Capsicum annuum* L.) is one of the most important vegetables in the world, and extensive breeding efforts are being made to develop new improved strains of this species. In this regard, in vitro culture of immature embryos may help breeders accelerate breeding cycles and overcome interspecific barriers, among other applications. In this study, we have optimized a protocol for in vitro culture of immature embryos of *C. annuum*. Levels of indole-3-acetic acid (IAA) and zeatin have been tested to improve the efficiency (germination rates) of this technique in *C. annuum* embryos at the four main immature stages (i.e. globular, heart, torpedo, and early cotyledonary) from four varietal types of this species (California Wonder, Piquillo, Guindilla, and Bola). The effect of 5-day initial incubation in the dark was also tested on the most efficient hormone formulation. On average, relatively low levels of both IAA and zeatin (0.01 mg L^{-1} each) (M_1) provided the highest germination rates, particularly in the advanced stages (torpedo and cotyledonary). To a lesser extent, the lack of these growth regulators (M_0) or high IAA (0.2 mg L^{-1})/low zeatin (0.01 mg L^{-1}) (M_2) combination also had a positive response. On the contrary, high zeatin levels (0.2 mg L^{-1}) produced very low germination rates or callus development (efficiency 0-7 %). Different responses were also found between genotypes. Thus, considering the best media (M_0, M_1, M_2), Bola embryos had the highest rates. M_1 plus 5-days of initial dark incubation (M_1-D) improved the efficiency rates at all embryo stages, particularly in the earliest (globular) embryos which increased from 3 % to > 20 %.
Keywords: *Capsicum* peppers, embryo stage, genotypic diversity, indole-3-acetic acid, zeatin

Introduction

Peppers (*Capsicum annuum* L.) are cultivated throughout the world and are one of the most important vegetables (FAOSTAT, 2011). Because of that, this crop is subjected to huge breeding efforts to ensure yield stability and fruit quality (Blat et al., 2005; Crosby, 2008; de Sousa and Maluf, 2003). In this regard, *in vitro* culture of immature embryos can be useful for shortening generation cycles, producing haploids and double haploids, or rescuing potentially abortive interspecific embryos, which enables the introgression of genes between related species (Bhattarai et al., 2009; Guzzo et al., 2004; Hossain et al., 2003; Lotfi et al., 2003; Yoon et al., 2006).

Embryo culture efficiency depends mostly on: i) genotype: different accessions may show different responses (Kothari et al., 2010), ii) embryo stage (Manzur et al., 2013; Raghavan, 2003), iii) media formulation: mineral salts, carbohydrates, growth regulators, nitrogen compounds, vitamins, etc (Monnier, 1995), and; iv) incubation conditions (i.e. temperature, light/dark conditions) (Razdan, 2003). However, knowledge of the response of *Capsicum* zygotic embryos to *in vitro* culture is still scarce, with few reports available. Hossain et al. (2003) found a positive effect of casein and coconut water and also that sucrose provides better results than fructose or glucose. Yoon et al. (2006) rescued interspecific *C. annuum* × *C. baccatum* embryos, but at low rates and at the most advanced immature stages. Recently, we found that relatively low levels of sucrose and Murashige-Skoog salts (MS) considerably increase the efficiency of embryo germination in most *Capsicum* genotypes and embryo stages, including globular embryos (Manzur et al., 2010, 2013).

The role of factors like growth regulators or darkness has still not been studied. In this respect, low levels of gibberellic acid (GA_3) promote embryo growth in several species (Moshkov et al., 2008) and replace the role of the suspensor, which is lost during excision (Haslam and Yeung, 2011; Monnier, 1995). Additionally, auxins (usually indole-3-acetic acid, IAA) may promote the growth of primary roots, hypocotyls and cotyledons when administered in low doses (Machakova et al., 2008), while cytokinins usually show favorable effects on early stages at high levels (Raghavan, 2006). Furthermore, combinations of auxins and cytokinins have showed favorable effects on early embryos (Salamma and Ravi Prasad Rao, 2013). Moreover, initial dark incubation improved germination rates and embryo growth in several species (Razdan, 2003). The response to these factors is highly dependent on the species (Monnier, 1995) and, therefore, our main objective was to assess the effect of IAA, zeatin and dark incubation on *C. annuum* immature embryos. This study provides useful information for the optimization of *in vitro* culture protocols in immature embryos of peppers.

Materials and Methods

Plant material and growing conditions

A total of four accessions from *C. annuum* were

utilized (Table 1). Plants were transplanted to a glasshouse in Valencia (39°29'00" N, 0°20'28" W) (Spain) in Feb 2011 and grown during the spring-summer season. Natural illumination and a temperature range of 18/25 °C were used for this experiment. Plants were drip irrigated every 8 h for 3 min. (4 L h^{-1}) and fertilizer was applied with irrigation water at a rate of 1 g L^{-1} in the form of the commercial fertilizer 15N-2.2P-24.9K

Evaluated media and conditions

To study the role of auxins and cytokinins a formulation previously optimized by Manzur et al. (2013), consisting of $^1/_2$MS dose (2.2 g L^{-1} of commercial formulation), sucrose at 40 g L^{-1}, gibberellic acid (GA_3) at 0.01 mg L^{-1}, agar at 7 g L^{-1}, and pH 5.7 was utilized as control (M_0). Then, on the basis of M_0, relatively low (0.01 mg L^{-1}) and/or high (0.2 mg L^{-1}) levels of IAA and zeatin were combined to assess the effect of these growth regulators, while GA_3 at 0.01 mg L^{-1} was kept in all formulations as we considered it essential for replacing the suspensor (Kawashima and Goldberg, 2009; Moshkov et al., 2008). As a result, the formulations studied were as follows: M_0 or control, which lacked both IAA and zeatin, M_1 (0.01 mg L^{-1} IAA and 0.01 mg L^{-1} zeatin), M_2 (0.2 mg L^{-1} IAA and 0.01 mg L^{-1} zeatin), M_3 (0.01 mg L^{-1} IAA and 0.2 mg L^{-1} zeatin) and M_4 (0.2 mg L^{-1} IAA and 0.2 mg L^{-1} zeatin). Finally, once the best IAA/zeatin combination had been established, the effect of darkness was tested on embryo cultures in accordance with this formulation plus 5 days of initial incubation in darkness. The medium without hormones was sterilized by autoclave (121 °C for 20 min), while hormones were sterilized separately by microfiltration (0.20 µm filters) to avoid denaturation and, then, added to the warm (35-40 °C) autoclaved media before solidifying.

Isolation and *in vitro* culture of embryos

To achieve the required number of immature seeds for the experiment, plants were self-pollinated repeatedly beginning Apr 2011 (Figure 1A). Subse-

Table 1 – Origin and fruit traits of the accessions utilized in the present experiment.

Accession	Abbreviation	Origin	Color	Weight	Length	Width
				g	cm	
California Wonder	California	Breeding line. UPV-COMAV germplasm bank	Pale red	90-150	8-11	6-10
Pimiento del Piquillo	Piquillo	Cons. Reg. IGP Piquillo Lodosa Navarra. Spain	Red	20-33	7-9	4-6
Guindilla de Ibarra	Guindilla	Neiker. Ibarra. Spain	Deep red	7-12	7-12	1-2
Pimiento de Bola	Bola	Cons. Reg. DOP Pimentón Murcia. Murcia. Spain	Red	10-14	3-5	4-6

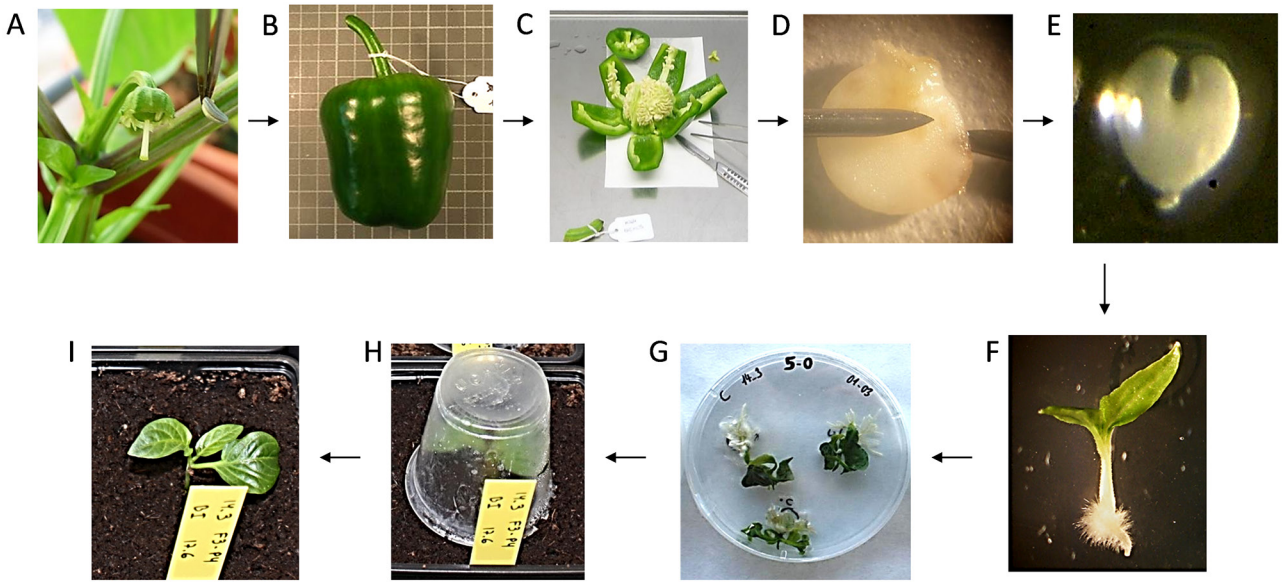

Figure 1 – Diagram of the *in vitro* culture technique in California Wonder embryos: A) self-pollination, B) immature fruit just harvested opening, C) fruit and seed sterilization, D-E) embryo excision, stage identification and *in vitro* sowing, F) embryo development and germination, G) plantlet evaluation and H-I) acclimation.

quently, fruits were harvested 15-30 days after pollination (DAP) (Figure 1B). Once in the lab, the whole fruit was surface-sterilized with ethanol (96 %) under laminar flow cabinet conditions. Then, immature seeds were removed (Figure 1C) and dissected under stereomicroscope (×24), using hypodermic sterilized needles (Figure 1D). Embryos were excised carefully, recording the embryo stage, and immediately cultured in 90 × 15 mm Petri dishes containing the corresponding medium (Figure 1E). These Petri dishes were then sealed with self-sealing film and incubated in a growth chamber (25 ± 1 °C; 70 % HR; 16 h/8 h; light/dark). The only exception corresponded to the dark-incubated embryos. In this case the Petri dishes were wrapped with aluminum foil for five days.

The efficiency of *in vitro* culture was evaluated in terms of the percentage of embryos which evolved to plantlets by showing an early development of root and shoot (Figure 1F-G) and, subsequently, a satisfactory response to acclimatization (Figure 1H-I), which has been found to be correlated with normal development in the adult stage (Manzur et al., 2013). Embryos or plantlets with abnormalities or callus development were considered not germinated and/or not viable.

Experimental design and statistical analysis

The experimental design was set up as a complete factorial design (4 × 4 × 6) with five replicates, including four accessions, four embryo developmental stages (globular, heart, torpedo and cotyledonary), and six *in vitro* media/conditions (M_0, M_1, M_2, M_3, M_4, and M_1 plus 5 days of initial dark incubation = M_1-D). Each replicate consisted of one Petri dish with three embryos each. Thus, 15 embryos were cultured for each accession × stage × media/condition combination and, therefore, the present experiment required the excision, *in vitro* culture, and evaluation of a total of 1,440 embryos. Data of *in vitro* culture efficiency (percentage) were subjected to analysis of variance to assess differences between means. Since original data were recorded as a percentage, they were transformed by arcsine square root. Transformed data were then tested, but differences between the results obtained with transformed and non-transformed data were negligible. Therefore, the ANOVA presented in this work was performed on the original non-transformed data.

Results and Discussion

Effect of growth regulators

On average, the more advanced the embryo, the higher the germination rate. Mean germination rates ranged from 2 % in globular embryos to 28 % in cotyledonary (Table 2). In this regard, embryos are highly dependent in terms of nutrition and draw upon the endosperm, the suspensor and the surrounding maternal tissues during the early development phases (heterotrophic phase), while, later, they are metabolically capable of synthesizing substances required for their growth (autotrophic phase) from mineral salts and carbohydrates (Bhojwani and Razdan, 1996; Ramming, 1990), which explains the higher response observed in the advanced stages.

Table 2 – Culture efficiency (% germination) of immature *C. annuum* embryos depending on the *in vitro* medium (M_0 (control): 0 mg L^{-1} IAA and 0 mg L^{-1} zeatin; M_1: 0.01 mg L^{-1} IAA and 0.01 mg L^{-1} zeatin; M_2: 0.2 mg L^{-1} IAA and 0.01 mg L^{-1} zeatin; M_3: 0.01 mg L^{-1} IAA and 0.2 mg L^{-1} zeatin; M_4: 0.2 mg L^{-1} IAA and 0.2 mg L^{-1} zeatin) and developmental stage. Each value represents the mean of the four accessions for each medium × stage combination.

	Globular	Heart	Torpedo	Cotyledonary	Mean
M_0	3.3 a[a]	26.7 b	28.3 b	36.6 b	23.7 b
M_1	3.3 a	16.7 b	51.7 c	60.0 c	32.9 c
M_2	1.7 a	15.0 ab	40.0 bc	40.0 b	24.2 b
M_3	0.0 a	1.7 a	3.3 a	3.3 a	2.1 a
M_4	1.7 a	1.7 a	1.7 a	1.7 a	1.7 a
Mean	2.0 A[b]	12.3 B	25.0 C	28.3 C	

[a]Different lowercase letters within the same column indicates differences between *in vitro* media at $p < 0.05$ (Fisher's least significant difference, LSD). [b]Different capital letters within the row indicates differences between developmental stages at $p < 0.05$ (Fisher's least significant difference, LSD).

Considering the interaction between *in vitro* media composition and the embryo stage, low levels (0.01 mg L^{-1}) of both IAA and zeatin provided in the M_1 formulation improved ($p < 0.05$) the results observed in our previously optimized medium (M_0). Thus, on average M_1 yielded the highest germination rates (32.9 % mean *versus* 23.7 % in M_0) and at most stages, with the exception of the earliest embryos (Table 2). In comparison, the lack of these growth regulators in M_0 or the combination of high IAA levels and low zeatin in M_2 provoked different responses. Thus, M_0 allowed the highest rates in heart embryos, although no differences were found in comparison to M_1 and M_2. However, M_0 efficiency barely increased in torpedo and cotyledonary embryos, being much lower than that of M_1 (Table 2). In the case of M_2, germination rates of the earliest embryos were similar to those observed in M_1, although rates in the advanced stages were lower. These findings suggest that the lack of hormones may improve embryo germination at the earliest stages, while low levels of both IAA and zeatin provide better results in advanced immature embryos.

By contrast, M_3 and M_4 had the lowest germination rates, which was mainly due to abnormal plantlets or callus development. Thus, regardless of the embryo stage, the efficiency of these culture media was lower than 5 % (Table 2). These findings and those observed in M_2 indicate that high levels of zeatin dramatically decrease the survival rates of *Capsicum* embryos at all stages, while the detrimental effect of similar levels of IAA (M_2) is relatively low (compared to M_1) and only noticeable in cotyledonary embryos (Table 2).

Exogenously supplied hormones are not required in many cases for embryo culture as embryos can be considered as plants with their own endogenous hormones (Monnier, 1996). When growth substances are added to the medium, they can modify the ontogenic pattern of embryos, including suppression of root growth, precocious leaf expansion, and longer thinner embryos, among others. Nevertheless, there are some cases, in which low concentrations of hormones in the medium have facilitated embryo culture.

It seems that in certain species, natural endosperms contain hormones and, therefore, the culture medium may be supplemented with hormones at a very low level that will reproduce the conditions of the *in ovulo* environment (Monnier, 1995). In this regard, auxins have a key regulatory function and are essential to axis establishment at the proembryo stage (Hamann, 2001; Haslam and Yeung, 2011). In fact, alteration of auxin transport may change embryo symmetry from bilateral to radial or provoke the abortion of shoot apical meristem (Liu et al., 1993; Ramesar-Fortner and Yeung, 2006). Thus, exogenous auxins at low concentrations have favored normal embryo growth, while higher levels have either proved inhibitory or favored unorganized callus growth. Moreover, cytokinins have usually resulted in growth inhibition, although low levels have been reported to stimulate embryo growth in some cases (Sharma et al., 1996).

The effect of the media composition on the response of the genotypes was quite similar to the findings for embryo stages. Thus, all the genotypes germinated at very low rates when cultured under M_3 or M_4 and, in fact, only California Wonder embryos cultured in M_3, showed efficiency rates slightly higher than 5 %. Therefore, our zeatin at relatively high levels should be not used to germinate any kind of embryos in peppers, although other authors have reported that levels of zeatin up to 0.2 mg L^{-1} may favor embryo growth, particularly at the earliest stages (Raghavan, 2006; Troncoso et al., 2003). By contrast, the rates observed in the media with low or zero levels of zeatin (M_0, M_1 and M_2) were considerably higher (Table 3).

As observed at the embryo stage, M_1 also provided the highest germination rates in most genotypes (Table 3), although some examples of genotype × medium composition interaction were also found. Guindilla embryos were the only exception for which M_0 gave higher efficiency rates than M_1, suggesting that some genotypes might prefer the lack of IAA and zeatin rather than low/minimum levels of these regulators. Another example of this interaction can be found by comparing M_0 and M_2, as their rates were quite similar in California and Piquillo embryos, while M_0 provided higher rates than M_2 in Guindilla; the contrary being true in Bola (Table 3).

Table 3 – Culture efficiency (% germination) of immature *C. annuum* embryos depending on the *in vitro* medium and accession utilized. Each value represents the mean of the four embryo stages for each medium × accession combination.

	California	Piquillo	Guindilla	Bola
M_0	20.0 b[a]	15.0 b	28.3 d	31.7 b
M_1	38.3 c	28.3 c	16.7 cd	48.3 c
M_2	18.3 b	20.0 bc	13.3 bc	45.0 bc
M_3	6.7 ab	0.0 a	0.0 a	1.7 a
M_4	0.0 a	1.7 a	1.7 ab	3.3 a

[a]Different lowercase letters within the same column indicates differences between medium at $p < 0.05$ (Fisher's least significant difference

Although the effect of growth regulators in embryo culture is quite variable, depending on the species/genotype and embryo stage (Davey and Anthony, 2010; Sharma et al., 1996), we can conclude that, in the case of *C. annuum* immature embryos, a minimum content (0.01 mg L^{-1}) of IAA and zeatin provides the best results. These findings are in agreement with the reports of other authors in several species such as barley, *Phaseolus coccineus* or *Capsella bursa-pastoris*, among others (Monnier, 1995; Raghavan, 2003; Umbeck and Norstog, 1979) and, therefore, medium M_1 was chosen to assess the effect of initial dark incubation on *in vitro* embryo germination.

Effect of initial dark incubation

The combination of M_1 and darkness (M_1-D) in the first five days of embryo culture increased considerably embryo germination in comparison to the application of M_1 alone, suggesting a positive effect of this factor, although the highest differences were mainly found in the earliest stages (Figure 2). Thus, globular embryos showed a mean germination rate > 20 % under M_1-D combination, which was considerably higher than the mean rate of M_1 for these embryos, and even higher than the mean values reported by Manzur et al. (2013) in a wide collection of *Capsicum* spp. M_1-D also provided higher germination rates in heart and torpedo

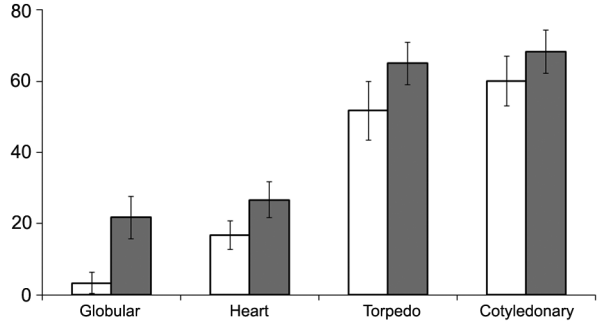

Figure 2 – Culture efficiency (% germination, mean for all genotypes in each stage) of immature *C. annuum* embryos, under M1 medium (0.01 mg L^{-1} IAA and 0.01 mg L^{-1} zeatin) with (grey) or without (white) 5 days of initial dark incubation. Lines on bars represent the mean ± SE.

stages than M_1, although differences were lower than those observed in globular embryos, particularly at the cotyledonary stage (Figure 2), which may be due to the strong autotrophic nature of these embryos, being less dependent on culture conditions. The action of cytokinins and auxins is light-dependent and they show remarkable degradation when exposed to the light, particularly auxins (George et al., 2008; Neumann et al., 2009). Probably, this fact explains the higher response to dark incubation observed in globular embryos, which are more dependent on media components, including growth regulators and, consequently, their degradation.

Considering the genotypes, a positive effect was found for M_1-D, which provided germination rates similar or higher than those of M_1 (Figure 3). M_1 plus initial cultivation under darkness improved the average efficiency in Guindilla, from < 20 % to > 50 %, and to a lesser extent in Piquillo, from < 30 % to 40 %, while this strategy did not improve mean rates in California and Bola embryos (Figure 3). In this regard, the first studies on this subject suggested that light was not critical for *in vitro* growth of immature embryos (Matsubara and Nakahira, 1965; Narayanaswamy and Norstog, 1964). However, more recent studies have revealed that a few days of incubation in darkness may favor subsequent chlorophyll formation in the immature embryos of, among others, barley, flax, and *Aegilops* × *Hordeum* and interspecific *Allium* hybrids (Razdan, 2003).

Comparing M_1-D rates to the values recorded for M_0-M_4 (Tables 2 and 3) we can conclude that, in general, the former provides higher responses at any embryo stage and genotype, suggesting that low levels of IAA and zeatin (0.01 mg L^{-1}) and relatively low levels of sucrose (40 g L^{-1}) and MS salts (2.2 g L^{-1}), combined with darkness during the first five days of embryo culture, is the best alternative for *C. annuum* embryos.

The protocol optimized in the present study can be directly applied to breeding programs to shorten the length of breeding cycles in *Capsicum annuum* as embryos can be germinated at very early stages, instead of using seeds from fully ripe fruits.

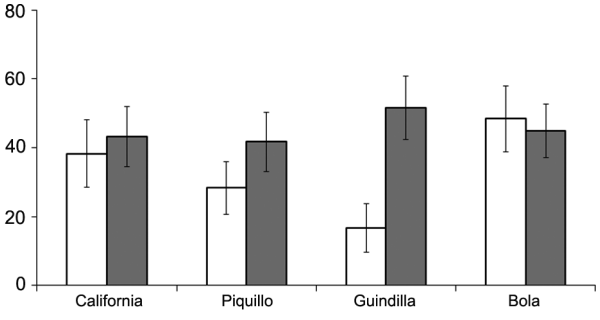

Figure 3 – Culture efficiency (% germination) of immature *C. annuum* embryos, under M1 medium with (grey) or without (white) 5 days of initial dark incubation. Lines on bars represent the mean ± SE.

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