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GENETICS AND PLANT BREEDING

Cultivation of immature *Capsicum* spp. embryos for incompatible-crossing embryo rescue

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ABSTRACT. The *in vitro* culture of embryos is an important technique to enable the rescue of embryos from incompatible crosses. Studies analyzing the factors that affect the *in vitro* culture of zygotic embryos are scarce. This study evaluated the effect of the genotype (*Capsicum baccatum* and *C. frutescens*), the composition of the culture medium (MS and ½ MS medium with different concentrations of sucrose, IAA and GA_3) and the stage of development (globular, cordiform, torpedo and cotyledonary) in the *in vitro* culture of immature embryos. Embryo germination was influenced primarily by the stage of development of the embryo and the composition of the culture medium. Regardless of the species, the most suitable culture medium for the germination of globular and cordiform embryos was ½ MS with 0.05 mg L^{-1} of GA_3 and IAA and 40 g L^{-1} of sucrose. For torpedo and cotyledonary embryos, ½ MS culture medium with 20 g L^{-1} of sucrose without phytoregulators is recommended for germination. These results were better than those described in the literature for all development stages in the two species. The results in the present study will be useful for geneticists and genetic enhancers interested in applying germination techniques conducted *in vitro* for immature *Capsicum* embryos.

Keywords: rescue embryo; peppers; embryo stage; sucrose; mineral salts.

Cultivo *in vitro* de embriões imaturos de *Capsicum* spp. para o resgate de embriões de cruzamentos incompatíveis

RESUMO. O cultivo *in vitro* de embriões é uma importante técnica para viabilizar o resgate de embriões de cruzamentos incompatíveis. Trabalhos analisando os fatores que afetam o cultivo *in vitro* de embriões zigóticos são escassos. Desta forma este trabalho avaliou o efeito do genótipo (*Capsicum baccatum* e *C. frutescens*), da composição do meio de cultura (meio MS e ½ MS com diferentes concentrações de sacarose, IAA e GA_3) e do estádio de desenvolvimento (globular, cordiforme, torpedo e cotiledonar) no cultivo *in vitro* de embriões imaturos. A germinação dos embriões foi influenciada principalmente pelo estádio de desenvolvimento do embrião e pela composição do meio de cultura. Independente da espécie o meio de cultura mais indicado para a germinação de embriões globulares e cordiformes é ½ MS com 0,05 mg L^{-1} de GA_3, 0,05 mg L^{-1} de IAA e 40 g L^{-1} de sacarose. Para os embriões torpedo e cotiledonares, recomenda-se a germinação em meio de cultura ½ MS com 20 g L^{-1} de sacarose sem fitorreguladores. Estes resultados foram superiores aos descritos na literatura para todos os estádios de desenvolvimento das duas espécies. Os resultados deste trabalho são úteis para geneticistas e melhoristas interessados em aplicar a técnica de germinação *in vitro* de embriões imaturos em *Capsicum*.

Palavras-chave: resgate de embriões; pimentas; estádio do embrião; sacarose; sais minerais.

Introduction

Pepper species domestication led to changes in the fruits, primarily in those that were small, erect, deciduous and red, because they became bigger, pendant, non-deciduous and with a great variety of color shades. The plants lost the genes responsible for their rustic features during this process (Pickersgill, Heiser, & McNeill, 1979; Perry et al., 2007; Carvalho & Bianchetti, 2008). Domestication resulted primarily from the necessity of finding safe food sources.

Pepper species (*Capsicum* spp.) present great genetic diversity (Martins et al., 2013), and Brazil is an important diversity center for the genus, as host for domesticated species and semi-domesticated and wild ones (Moscone et al., 2007; Neitzke, Barbieri, Heiden, & Castro, 2008; Nascimento, Rêgo, Rêgo,

Nascimento, & Alves, 2012; Nascimento et al., 2015). This great diversity is useful for *Capsicum* genetic enhancement programs (Blat, Braz, & Arruda, 2007). The genus includes 38 identified species and only five domesticated ones: *C. annuum* and its botanical forms, *C. chinense*, *C. frutescens*, *C. baccatum* and their botanical forms, and *C. pubescens* (Barboza, Agra, Romero, Scaldaferro, & Moscone, 2011).

Many crossings involving *Capsicum* were conducted to enhance genes that presented features adapted to trading purposes. However, in many cases, the use of other species is necessary, including wild ones, to achieve the introgression of genes such as those responsible for resistance to diseases and pests. Hybridization is the instrument used in genetic enhancement programs focused on transferring genes between species or between genotypes of a single species. However, incompatibilities between genotypes may result in lack of seed formation due to pre- and post-zygotic barriers (Charlo, Botelho, Silva, Castoldi, & Braz, 2009; Nascimento et al., 2012; Martins, Martins, Pereira, Souza, Rodrigues, & Amaral Junior, 2015).

Pre-zygotic barriers may derive from lack of pollen grain germination and from the growth delay or inhibition of the pollen tube. The primary post-zygotic barriers are endosperm degeneration and total or partial hybrid plant sterility (Monteiro, Pereira, & Campos, 2011).

Embryo abortion is the most frequent hybridization problem, which stops the embryo from reaching the cotyledonary advanced stage of development. Immature zygotic embryo cultivation, the so-called embryo rescue, is a technique that can be used to solve this problem (Manzur, Penella, & Rodríguez-Burruezo, 2013).

Embryo development stage and culture medium composition are factors of great relevance for seedling germination and growth *in vitro* during embryo rescue (Haslam & Yeung, 2011; Manzur et al., 2013).

Another issue found within the genus *Capsicum* is that the plants are considered recalcitrant for cultivation *in vitro*, i.e., the explants cannot present a morphogenic response when they are cultivated *in vitro* (Benson, 2000; Kothari, Joshi, Kachhwaha, & Ochoa-Alejo, 2010).

Moreover, few studies on *Capsicum* embryos cultivated *in vitro*, such as those by Hossain, Minami, and Nemoto (2003), Yoon, Yang, Do, and Park (2006), and Manzur et al. (2013), report the establishment of rescued immature *Capsicum* embryos, primarily reporting on protocols specific to each stage. Such studies use media containing enriched nutritional compositions such as those in the study by Hossain et al. (2003) who used hydrolyzed casein, coconut water, GA_3 and ANA. However, embryo germination in these studies remained low, which hindered adoption of these protocols as tools in plant enhancement programs.

Thus, the germination rates in immature *Capsicum* embryos must be improved, primarily when the focus is the effective implementation of these techniques in plant genetic enhancement programs. Accordingly, the aim of the present study was to study the influence of (I) genotype, (II) culture medium composition, and (III) embryo development stage on the *in vitro* cultivation of immature *Capsicum baccatum* and *C. frutescens* embryos.

Material and method

Seeds of *Capsicum baccatum* L. var. *pendulum* (UENF 1624, "dedo de moça") and *C. frutescens* (UENF 1636, "malagueta") accessions from the germplasm collection of Darcy Ribeiro North Fluminense State University were used in the current study. The study was developed in the Horticulture Sector of the Phytotechny Laboratory (LFIT) of the Agricultural Sciences and Technologies Center at Darcy Ribeiro North Fluminense State University.

The experiments were based on the studies of Hossain et al. (2003), Yoon et al. (2006) and Manzur et al. (2013). The *C. baccatum* and *C. frutescens* fruits were collected from the 15th day to the 45th day after self-pollination to identify four embryo development stages (globular, cordiform, torpedo, and cotyledonary). These embryos were extracted in a laboratory environment using a stereoscopic microscope (Tecnival®), tweezers, scalpel and sterilized hypodermic needles.

The fruits were initially washed in commercial liquid-detergent and rinsed in running water. The fruits were immersed in 70% alcohol for 5 minutes and in 1.0% NaClO with two drops of Tween 20 added in an 80 mL volume for 15 minutes in an aseptic environment. Subsequently, fruits were rinsed in deionized and autoclaved water (three times autoclaved for 5, 10, and 10 minutes). Subsequently, the immature seeds were removed from the fruits for embryo excision.

Two experiments were conducted according to the development stage of the embryo; all phytoregulators were added to all tested media after autoclaving. Globular and cordiform embryos were used in the first experiment. The basic culture medium was composed of MS mineral salts or of ½ MS mineral salts (½ MS), White vitamin complex (Murashige & Skoog, 1962),

and 100 mg L^{-1} of myo-inositol solidified in 2 g L^{-1} of Phytagel Sigma® and 40 g L^{-1} of sucrose, with pH adjusted to 5.7± 0.1. The experiment was conducted in a factorial scheme of 6 x 2 x 2, with six cultivation media, two genotypes and two embryo development stages. Two concentrations of MS medium salts were tested, in addition to three concentrations of the phytoregulators 3-indoleacetic acid (IAA) and gibberellic acid (GA_3), to generate six culture media: M1 (GA_3 and IAA: 0.01 mg L^{-1}; ½ MS), M2 (GA_3 and IAA: 0.05 mg L^{-1}; ½ MS), M3 (GA_3 and IAA: 0.10 mg L^{-1}; ½ MS), M4 (GA_3 and IAA: 0.01 mg L^{-1}; MS), M5 (GA_3 and IAA: 0.05 mg L^{-1}; MS) and M6 (GA_3 and IAA: 0.10 mg L^{-1}; MS).

The experiment followed a completely randomized design with four repetitions; each repetition used one Petri dish (90 x 15 mm) with four embryos. The germination rate was assessed for 50 cultivation days *in vitro*.

Torpedo and cotyledonary embryos were counted in the second experiment. The basic culture medium was composed of half of the MS mineral salts concentration (½ MS), White vitamin complex, and 100 mg L^{-1} of myo-inositol solidified in 2 g L^{-1} of Phytagel Sigma®, with pH adjusted to 5.7 ± 0.1. The experiment was conducted in a factorial scheme of 5 x 2 x 2, with five culture media, two genotypes and two embryo development stages. Effects of sucrose and the phytoregulators IAA and GA_3 were studied in the second experiment. The culture media were the following: C1 (basic culture medium without sucrose and phytoregulators), C2 (sucrose: 20 g L^{-1}), C3 (sucrose: 40 g L^{-1}), C4 (sucrose: 20 g L^{-1}; GA_3 and IAA: 0.1 mg L^{-1}) and C5 (sucrose: 40 g L^{-1}; GA_3 and IAA: 0.01 mg L^{-1}).

The experiment followed a completely randomized design with four repetitions; each repetition used one Petri dish (90 x 15 mm) with four embryos. The germination rate was assessed for 50 cultivation days *in vitro*.

In the third experiment, cotyledonary advanced embryos from both species were isolated and placed on Petri dishes containing mineral salts of the MS medium (½ MS), White vitamin complex, and 100 mg L^{-1} of myo-inositol solidified in 2 g L^{-1} of Phytagel Sigma®, with pH adjusted to 5.7± 0.1 and autoclaved for 15 minutes at 121ºC and 1.1 atm pressure. The experiment followed a completely randomized design, using a factorial scheme of 2 x 2 (Genotypes x Media), with two sucrose concentrations (0 and 40 g L^{-1}) and four repetitions. Each repetition was prepared on a Petri dish (90 x 15 mm) containing 25 mL of culture medium with 10 embryos. The germination rate was assessed for 15 cultivation days *in vitro*. The Petri dishes were maintained in a cultivation room at 27 ± 2ºC for seven days in the dark and subsequently under a 16h light: dark photoperiod with irradiance of 50 µmol m^{-2} s^{-1} provided by OSRAM® day-light fluorescence lamps. Data were subjected to analysis of variance and then to LSD (fisher's least significant difference) or DMS (significant difference) tests up to $P<0.01$. The parameters were assessed in SISVAR software version 5.4 (Ferreira, 2011).

Result and discussion

Experiment 1

The analysis of variance showed that the embryo development stage and the culture medium composition significantly influenced ($p < 0.01$) the differences in the recorded germination rates of globular and cordiform *Capsicum* embryos (Table 1).

Table 1. Summary of the analysis of variance with mean squares for the effects of genotype (G), development stage (S), culture medium (M) and their interactions on the efficiency of globular and cordiform *Capsicum baccatum* and *C. frutescens* embryo germination *in vitro*.

Effects	DF	Mean squares
Genotype (G)	1	319.0104^{ns}
Development stage (S)	1	3444.0104**
Culture medium (M)	5	1084.6354**
	Interactions	
G x S	1	58.5937^{ns}
G x M	5	22.1354^{ns}
S x M	5	240.8854^{ns}
G x S x M	5	11.7187^{ns}
Error	72	145.3993
C.V. %		72.492

ns and ** indicate differences that are non-significant or significant at $p < 0.01$, respectively.

Based on the mean squares, the greatest contributions for the recorded variation in globular and cordiform embryos regarded the embryo development stage (S), followed by medium composition (M), and the genotype did not present a significant effect (G) (Table 1). Accordingly, although genotype is one of the factors of primary relevance for cultivation *in vitro*, primarily in *Capsicum* (Kothari et al., 2010), the results of the current study indicated that the factors embryo development stage and culture medium had stronger influence than that of genotype on *C. baccatum* and *C. frutescens* embryo cultivation *in vitro*. No interaction effects were detected between the three factors in the experiment with globular and cordiform embryos (Table 1).

In this work, the best germination rates of the globular embryos were obtained in media M1, M2, M4, and M5 for *C. baccatum* reaching germination of 12.50% and in M1, M2, and M5 for *C. frutescens* with a maximum of 18.75%. The highest concentration of phytoregulators (0.10 mg L^{-1}) presented the lowest

germination or did not promote germination (Table 2). This rate is higher than the one recorded by Manzur et al. (2013) who worked with ½ MS medium containing 0.01 mg L^{-1} of IAA and GA_3, and their study is the only one in the literature on the germination of globular embryos in *Capsicum*. The current results are 50.6% and 202% higher for *C. baccatum* and *C. frutescens*, respectively. Such a fact might justify the absence of studies in the literature, in addition to the low germination rate herein recorded. Therefore, embryo removal in more advanced maturation stages is recommended whenever possible.

The M1, M2, and M5 media for *C. baccatum* and M1 and M2 for *C. frutescens* led to the highest germination rates in cordiform embryos (Table 2). The results in the present study are similar to those recorded by Manzur et al. (2013) for *C. baccatum* and slightly higher for *C. frutescens*.

Table 2. Percentage of globular and cordiform *Capsicum baccatum* and *C. frutescens* embryo germination.

Medium	Globular	
	C. baccatum	*C. frutescens*
M1	6.25 ab	12.50 ab
M2	12.50 a	18.75 a
M3	0.00 b	6.25 b
M4	6.25 ab	6.25 b
M5	6.25 ab	12.50 ab
M6	0.00 b	6.25 b
Mean	5.21	10.42
Medium	Cordiform	
	C. baccatum	*C. frutescens*
M1	31.25 a	31.25 ab
M2	31.25 a	37.50 a
M3	6.25 b	12.50 c
M4	12.50 b	12.50 c
M5	25.00 a	25.00 b
M6	6.25 b	6.25 c
Mean	18.75	20.83

Means followed by the same letter in a column did not differ from one another in the DMS test, $p < 0.05$. Medium: M1 = ½ MS + 0.01 mg L^{-1} of IAA and GA_3; M2 = ½ MS + 0.05 mg L^{-1} of IAA and GA_3; M3 = ½ MS + 0.1 mg L^{-1} of IAA and GA_3; M4 = MS + 0.01 mg L^{-1} of IAA and GA_3; M5 = MS + 0.05 mg L^{-1} of IAA and GA_3; M6 = MS + 0.1 mg L^{-1} of IAA and GA_3.

According to ANOVA results, culture medium composition was an essential factor for globular and cordiform embryo germination, regardless of the species (Table 1). More immature embryos (globular and cordiform) germinated in culture media with lower mineral salt and GA_3 and ANA concentrations (Figure 1). The highest germination rates in globular and cordiform embryos were in M1 and M2 media (Figure 1).

The comparison between culture media and concentration of phytoregulators showed that ½ MS and low concentration of phytoregulators increased the efficiency of globular and cordiform embryo germination in *Capsicum* (M1 and M2; Table 2, Figure 1). Monnier (1995) reported that high mineral salt concentrations are toxic to embryos and consequently decrease the germination rate. In that study, the germination rate of *Capsella bursapastoris* embryos increased by diminishing the concentration of some components of the original MS medium formulation, with a remarkable effect due to reduction of iron (added with Fe-EDTA) and nitrate. Therefore, nitrate and Fe-EDTA concentrations and/or other mineral salts included in the MS medium formulation are assumed to be toxic for globular and cordiform *Capsicum* embryos, because these components diminish the embryo survival rates.

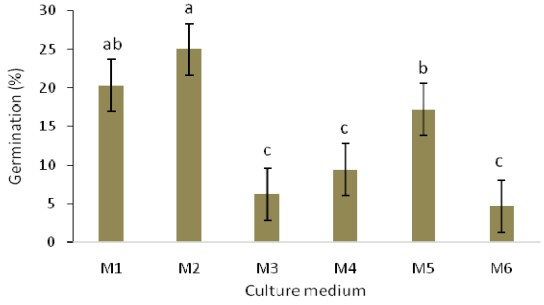

Figure 1. Efficiency of globular and cordiform *Capsicum baccatum* and *Capsicum frutescens* embryo cultivation *in vitro* in the following media: M1 = ½ MS + 0.01 mg L^{-1} of IAA and GA_3; M2 = ½ MS + 0.05 mg L^{-1} of IAA and GA_3; M3 = ½ MS + 0.1 mg L^{-1} of IAA and GA_3; M4 = MS + 0.01 mg L^{-1} of IAA and GA_3; M5 = MS + 0.05 mg L^{-1} of IAA and GA_3; M6 = MS + 0.1 mg L^{-1} of IAA and GA_3. Lines over the bars show the standard error of the mean. Columns with the same letter did not differ from one another in the DMS test, $p < 0.01$.

Regardless of the genotype, the germination rate of cordiform embryos was higher than that of globular ones. Accordingly, the germination rate *in vitro* showed a remarkable increase in the transition from the globular to the cordiform stage (Figure 2).

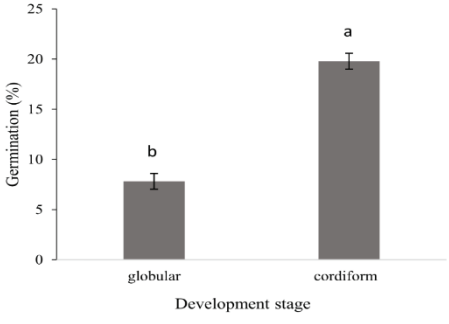

Figure 2. Efficiency of *in vitro* cultivation for globular and cordiform *Capsicum baccatum* and *Capsicum frutescens* embryo germination regardless of the medium used. Lines over the bars show the standard error of the mean. The columns were different from one another based on the F-test, $p < 0.01$.

Experiment 2

The analysis of variance showed that all factors significantly influenced the torpedo and cotyledonary embryos ($p < 0.01$; Table 3). The embryos in the torpedo and cotyledonary stages most benefited from medium composition (M), which was followed by development stage (S) and then by genotype (G). Although the magnitudes were lower than those of the main factors for the torpedo and cotyledonary embryos, a strong contribution from all the assessed interaction factors, except for the triple interaction (G x S x M), was detected (Table 3).

Table 3. Summary of the analysis of variance with mean squares for the effects of genotype (G), development stage (S), culture medium (M) and their interactions on the efficiency of torpedo and cotyledonary *C. baccatum* and *C. frutescens* embryo germination *in vitro*.

Effects	DF	Mean squares
Genotype (G)	1	5695.3125**
Development stage (S)	1	6570.3125**
Culture medium (M)	4	20253.9063**
Interactions		
G x S	1	2257.8125*
G x M	4	1339.8437**
S x M	4	925.7813**
G x S x M	4	636.7187^{ns}

ns indicates non-significant difference; * and ** indicate significant difference at $p < 0.05$ and 0.01, respectively.

The media containing 20 g L^{-1} of sucrose (C2) and 40 g L^{-1} of sucrose with 0.01 mg L^{-1} of IAA and GA_3 (C5) for *C. baccatum* and 20 g L^{-1} of sucrose with 0.01 mg L^{-1} of IAA and GA_3 (C4), C2 and C5 for *C. frutescens* provided the highest germination rates (Table 4).

Table 4. Effect of the culture medium and genotype on the germination percentage of torpedo and cotyledonary *Capsicum baccatum* and *C. frutescens* embryos.

Medium	Germination (%)	Germination (%)
	C. baccatum	*C. baccatum*
C1	0.00 dA	0.00 cA
C2	71.87 aB	93.75 aA
C3	31.25 cA	28.13 bA
C4	46.87 bB	87.50 aA
C5	65.63 aB	90.63 aA
C.V. (%)	18.77	

Means followed by the same capital letter in a row and by the same lowercase letter in a column did not differ from one another in the LSD test, $p < 0.05$. Medium: C1 = without sucrose and without phytoregulators; C2 = 20 g L^{-1} of sucrose; C3 = 40 g L^{-1} of sucrose; C4 = 20 g L^{-1} of sucrose with 0.01 mg L^{-1} of IAA and GA_3; C5 = 40 g L^{-1} of sucrose with 0.01 mg L^{-1} of IAA and GA_3.

The best germination rates of torpedo embryos were recorded in the C2, C4, and C5 media, for both *C. baccatum* and *C. frutescens*. For cotyledonary embryos, the highest germination rates were recorded in C2 and C5 media for *C. baccatum* and in C2, C4, and C5 for *C. frutescens* (Table 5).

No embryo germination occurred in the absence of sucrose (C1), regardless of species and development stage. The medium containing 40 g L^{-1} of sucrose (C3) caused the lowest germination rate; however, when associated with the phytoregulators IAA and GA_3 (C5), embryo germination increased in both species, although the increase did not differ from that in C2 (Tables 4 and 5). Thus, the current study showed no requirement for using high sucrose concentrations with phytoregulators for the germination of *C. baccatum* and *C. frutescens* embryos in torpedo and cotyledonary stages.

Table 5. Efficiency of *in vitro* cultivation on the germination percentage of torpedo and cotyledonary *Capsicum baccatum* and *C. frutescens* embryos.

Medium	Torpedo	
	C. baccatum	*C. frutescens*
C1	0.00 bA	0.00 bA
C2	50.00 aB	93.75 aA
C3	12.50 bA	6.25 bA
C4	37.50 aB	87.50 aA
C5	43.75 aB	93.75 aA
Mean	28.75	56.25
Medium	Cotyledonary	
	C. baccatum	*C. frutescens*
C1	0.00 cA	0.00 cA
C2	93.75 aA	93.75 aA
C3	50.00 bA	50.00 bA
C4	56.25 bB	87.50 aA
C5	87.50 aA	87.50 aA
Mean	57.5	63.75

Means followed by the same capital letter in a row and by the same lowercase letter in a column did not differ from one another in the DMS test, $p < 0.05$. Medium: C1 = without sucrose and without phytoregulators; C2 = 20 g L^{-1} of sucrose; C3 = 40 g L^{-1} of sucrose; C4 = 20 g L^{-1} of sucrose with 0.01 mg L^{-1} of IAA and GA_3; C5 = 40 g L^{-1} of sucrose with 0.01 mg L^{-1} of IAA and GA_3.

The response from the torpedo and cotyledonary embryos was associated with the genotype; the germination response of *C. frutescens* was higher than that of *C. baccatum*. Similarly, germination rates of cotyledonary embryos were higher than those of the torpedo embryos, because they were physiologically more cotyledonary advanced and therefore more capable of germination (Table 6).

Table 6. Effect of genotype and development stage on the germination percentage of torpedo and cotyledonary embryos of *Capsicum baccatum* and *Capsicum frutescens*.

Development stage	Germination (%)		Mean
	C. baccatum	*C. frutescens*	
Torpedo	28.75 bB	56.25 bA	42.50 b
Cotyledonary	57.50 aA	63.75 aA	60.63 a
Mean	43.13 B	60.00 A	51.56
C.V. (%)	18.77		

Means followed by the same capital letter in a row and by the same lowercase letter in a column did not differ from one another in the F-test, $p < 0.01$.

In the interaction between the medium and the development stage of torpedo and cotyledonary embryos, C2, C4, and C5 media led to the highest germination rates for embryos of the torpedo type. The C2 and C5 media were most effective for embryos of the cotyledonary type. Moreover, the

germination of cotyledonary embryos was higher than 50% in all media with sucrose, and this rate was fundamental for the germination either in cotyledonary or in torpedo embryos (Table 7).

Table 7. Effect of the cultivation medium and development stage on the germination of torpedo and cotyledonary *Capsicum baccatum* and *Capsicum frutescens* embryos.

Medium	Germination (%)	
	Torpedo embryos	Cotyledonary embryos
C1	0.00 cA	0.00 dA
C2	71.87 aB	93.75 aA
C3	9.37 bB	50.00 cA
C4	62.50 aA	71.87 bA
C5	68.75 aB	87.50 abA
C.V. (%)	18.77	

Means followed by the same capital letter in a row and by the same lowercase letter in a column did not differ from one another in the LSD test, $p < 0.05$. Medium: C1 = without sucrose and without phytoregulators; C2 = 20 g L^{-1} of sucrose; C3 = 40 g L^{-1} of sucrose; C4 = 20 g L^{-1} of sucrose with 0.01 mg L^{-1} of IAA and GA_3; C5 = 40 g L^{-1} of sucrose with 0.01 mg L^{-1} of IAA and GA_3.

Different from the cotyledonary advanced embryos, which had good germination without the addition of sucrose and phytoregulators, immature embryos likely required sucrose as an exogenous carbohydrate source, because in absence of sucrose, no germination of torpedo and cotyledonary embryos occurred. The use of GA_3 and IAA (C4 and C5) for torpedo and cotyledonary embryo germination led to germination rates similar to those of the treatment without these phytoregulators and 20 g L^{-1} of sucrose (C2). Thus, good efficiency could be achieved rescuing these embryos without GA_3 and IAA (C2; Table 7).

Many authors report that embryos have good growth in the presence of low sucrose content (20 to 30 g L^{-1}) in many species. By contrast, immature embryos (globular, cordiform, torpedo and cotyledonary) often require high sucrose levels (80 to 120 g L^{-1}) as an energy source because they are heterotrophic and/or to maintain a proper osmotic balance (Monnier, 1995). According to Manzur et al. (2013), the use of 40 g L^{-1} of sucrose was more efficient than the use of 80 g L^{-1} for the germination of immature *Capsicum* embryos. The current study demonstrated that low sucrose levels such as 20 g L^{-1} (C2) were as efficient (Table 7). The GA_3 is a vegetal hormone synthesized in seeds in the development phase that is responsible for germination induction through the activation of hydrolytic enzymes, for increasing membrane permeability between cells in the aleurone layer and the endosperm, and for increasing shoot elongation through cell division and amplification (Zimmermann, Sakai, & Hochholdinger, 2010). This effect also depends on the hormonal balance in which gibberellic acid acts to promote germination (Yamaguchi & Kamiya, 2002).

The GA_3 concentration of 0.1 mg L^{-1} used in the M3 and M6 media diminished the germination rate in globular and cordiform embryos (Figure 1). Thus, high concentrations had a negative effect, as also recorded by Pinheiro, Medeiros, Macêdo, and Alloufa (2001) in the lower seed germination percentage of *Hancornia speciosa* (Mangaba) seeds subjected to GA_3 concentrations equal to or higher than 0.3 mg L^{-1} added to the culture medium. Sabá, Lameira, Luz, Gomes, and Innecco (2002) also reported the harmful effect of high GA_3 concentration rates applied to *Pilocarpus jaborandi* (Jaborandi) treatments.

The germination of torpedo and cotyledonary embryos in the absence of the phytoregulators IAA and GA_3 might be associated with the endogenous levels of these phytoregulators in immature seeds. This endogenous concentration would be sufficient to provide metabolic support to the development of these embryos. The addition of the phytoregulators to the two *Capsicum* species in the most advanced stages of immature embryo (torpedo and cotyledonary) was not a requirement. The few studies found in the literature on the cultivation of immature *Capsicum* embryos, such as those by Manzur et al. (2013) and Manzur, Oliva-Alarcón, and Rodríguez-Burruezo (2014), do not test culture media without the addition of phytoregulators.

Experiment 3

Cotyledonary advanced embryos do not require phytoregulators. When the experiment with cotyledonary advanced embryos was observed, the medium without sucrose led to germination higher than or equal to that of the medium with 40 g L^{-1}, which demonstrated that the use sucrose with *C. baccatum* and *C. frutescens* was not required (Table 8). The current study demonstrated that sucrose was the determining factor for immature embryo germination (globular, cordiform, torpedo, and cotyledonary) in culture medium rather than the phytoregulators IAA and GA_3, although these phytoregulators, with the concentration of mineral salts, influenced immature embryos. Sucrose was essential for the germination of immature embryos in any stage of development.

Table 8. Effect of sucrose concentration on the *in vitro* germination of cotyledonary advanced *Capsicum baccatum* and *Capsicum frutescens* embryos.

Sucrose	Germination (%)	
(g L^{-1})	*C. baccatum*	*C. frutescens*
0	100.00 aA	100.00 aA
40	100.00 aA	66.00 bB
Mean	100.00 A	83.00 B
C.V. (%)	4.99	

Means followed by the same capital letter in a row and by the same lowercase letter in a column did not differ from one another in the F-test, $p < 0.01$.

The germination of *C. baccatum* and *C. frutescens* in the present study was higher than that described in the literature regarding all development stages; therefore, the germination was satisfactory in comparison with other studies such as those by Hossain et al. (2003), Yoon et al. (2006), Manzur et al. (2013), and Manzur et al. (2014).

Conclusion

For the germination of immature *Capsicum* embryos, the development stage of the embryo and the composition of the culture medium are the primary influences. Regardless of the species, the most appropriate culture medium for globular and cordiform embryo germination is the ½ MS containing 0.05 mg L^{-1} of GA_3 and IAA with 40 g L^{-1} of sucrose. Germination in the culture medium containing 20 g L^{-1} of sucrose is recommended for torpedo and cotyledonary embryos. The results of the present study will be useful for geneticists and genetic enhancers interested in amplifying germination techniques applied to isolated embryos in *Capsicum*.

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