Embryogenesis in the anthers of different ornamental pepper (*Capsicum annuum* L.) genotypes

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ABSTRACT. The aim of this study was to relate flower bud size with microspore developmental stages and the induction of embryos in the anthers of different ornamental pepper (*Capsicum annuum* L.) genotypes. Flower buds were randomly collected and visually divided into three classes based on both petal and sepal size. The length and diameter of the bud as well as the length of the petal, sepal, and anther were then measured. The microspore stage was also determined for each anther of the bud where it was found. The data were subjected to analysis of variance (P ≤ 0.01), and the means were separated by Tukey's test (P ≤ 0.01). The broad sense heritability, the CVg/CVe relation, and the Pearson correlation between characters were also determined. Anthers from 10 *C. annuum* genotypes were cultivated in four culture media types for the induction of embryos. The data were transformed by Arcsin (x) and subjected to analysis of variance (P ≤ 0.01), and the means were separated by Tukey's test (P ≤ 0.01). The majority of anthers in the second class had uninucleate microspores. No correlation was observed between bud size and the number of uninucleate microspores. Genotype 9 specimens grown in M2 medium induced the highest number of embryos (16) compared to the

other treatments, which indicates a significant interaction effect between culture media and genotypes.

Key words: Bud morphology; Androgenesis; Genotypes; Culture Media; Breeding

INTRODUCTION

Pepper (*Capsicum* spp) is widely cultivated in temperate and tropical regions around the world, and this is mainly because of the value of the fruit [high contents of capsaicin, dry matter, vitamins (A, C, and B complex), minerals, essential oils, carotenoids, etc.] and its various uses in cooking, industry, and ornamentation (Stommel and Bosland, 2006; Irikova et al., 2011a; Rêgo et al., 2012a,b). Among the cultivated species, *Capsicum annuum* is best known, and it has the greatest economic importance and world distribution because of greater genetic variability relative to other domesticated species (Pickersgill, 1997).

Pepper breeding is usually conducted during long-term periods. Currently, *Capsicum* breeding programs have introduced biotechnological methods to reduce the time needed to obtain varieties that exhibit characteristics desired by the consumer market, including those that are more productive and resistant to disease, plague, and transportation. The use of biotechnology stands out as an important tool in obtaining homozygotic double-haploid lines, including the development of new cultivars through techniques such as anther culture as well as microspore and unfertilized egg cultures (Maraschin et al., 2005; Germanà, 2011; Irikova et al., 2011a).

The development of homozygotic double-haploid lines is of great interest to breeding programs because they are free of dominance and recessive issues due to equal allele numbers at gene loci, which facilitates the selection of recessive mutants and the identification of characters determined by recessive genes (Seguí-Simarro, 2010). However, the development of these lines using conventional self-fertilization systems may take between seven and eight cycles in autogamous plants such as the pepper. This time can be reduced using haploid plants, with the subsequent production of homozygous plants, through chromosomal number doubling (Seguí-Simarro, 2010). In addition to time, another major problem associated with obtaining homozygous lines through conventional breeding is the high rate of cross-pollination observed in the species (2-90%) (Tanksley, 1984; Pickergsgill, 1997). Thus, one has to take precautions during breeding to prevent uncontrolled cross-pollination (Tanksley, 1984), which makes the process more laborious and costly.

Somatic embryogenesis from microspores or pollen *in vitro*, also known as androgen, refers to the development of gametic embryos and plants with half the number of chromosomes, which originate from original cell meiotic events (Germanà, 2011). The androgen is considered one of the most striking examples of cell totipotency (Reynolds, 1997). Currently, anther culture through androgenesisis the most employed technique used to obtainhaploids owing to its simplicity and the range of genotypes to which it can be applied (Germanà, 2011).

The development of an efficient protocol involves double haploid evaluation factors that influence the induction of embryos and regenerating plants (Ferrie and Caswell,2011). Studies indicate that external factors can make microspores found in the anther androgenesis-compatible (Reynolds 1997). The composition of the culture medium and associated growth regulators present are two primary factors that determine the regeneration of haploid embryos

(Nowaczyk and Kisiala, 2006; Irikova et al., 2011a). Moreover, the response to external factors is also quite dependent on the genotype of the donor plant (Ferrie and Caswell, 2011; Irikova et al., 2011a; Ferrie et al., 2014). However, many agronomically important crops (e.g., solanaceous crops) are recalcitrant to androgenesis (Wang et al., 2000; Seguí-Simarro et al., 2011), which emphasizes the importance of the study of the genotypes present in breeding programs seeking androgen-responsive plants.

In addition to these factors, the developmental stage of the microspore donor plant also influences the androgen response (Ferrie and Caswell, 2011; Ferrie et al., 2014). Several studies indicate that the best phase in anther development occurs during the period in which the microspore is newly released from the meiotic tetrad up until it reaches its binucleate state, at the latest. At thisphase, the microspore retains sporophytic features that allow pollen differentiation in the embryo (Mityko and Fari, 1997; Lantos et al., 2009; Rêgo et al., 2012c; Olszewska et al., 2013). Some authors, in an effort to monitor the process of meiotic division, attempted to associate floral bud size with different meiotic phases or pollen grain developmental stages (Lauxen et al., 2003; Lantos et al., 2009), and such information is important for the success of research involving anther culture techniques.

The goal of this study was to relate floral bud size to the microspore developmental stage and to test different methods in the induction of haploid embryos from genotypes of ornamental pepper anthers (*Capsicum annuum* L.).

MATERIAL AND METHODS

Plant material

The seeds used in the experiment came from the parents UFPB 134 and UFPB 390 as well as from eight F_2 ornamental pepper plants (*C. annuum* L.). These F_2 plants were resultants from the self-pollination of the F_1 generation, which was derived from the manual crossing between accessions UFPB 134 and UFPB 390. All genotypes belong to the Vegetal Germplasm Bank located in the Plant Biotechnology Laboratory at the Center of Agricultural Sciences of Federal University of Paraíba (CCA/UFPB). These parents and hybrids were selected based on the values of gi and sij (gi = effect of general combining ability of the *i-th* parent and sij = effect of specific combining ability of the F_1 hybrid of the *i-th* and *j-th* parents) from a previous diallel analysis (Nascimento et al., 2011). The seeds were planted in trays with 200 cells filled with Plantmax® substrate. Subsequently, seedlings with six leaves were transplanted to 900 mL pots containing the same substrate, and were maintained in the greenhouse until the flowering period where the flower buds were collected for execution of the experiment.

Correlation between bud size and microspore development stage

To determine the correlation between flower bud size and microspore development stage, flower buds of different sizes were randomly collected from five plants of parent 390, and were then visually divided into three classes, consisting of three buds, according to petal and sepal sizes (Table 1). The buds were then taken into the lab, and the length and diameter of the flower buds, the petal and sepal length, and the length of the five anthers in each bud were measured (mm) using a paquimeter.

Table 1. Description of *Capsicum annuum* L. flower buds for the establishment of classes used to determine the microspore development stage.

Class	Class description
Class 1	Flower buds with petals smaller than sepals
Class 2	Flower buds with petals and sepals of approximately the same size
Class 3	Flower buds with petals larger than sepals

To determine the microspore development stage, all anthers from each previously described flower bud were analyzed. They were removed and then placed on a clean microscopic slide, and 20 µL acetic-orcein dye at 0.5% was added. Pressure was then applied to the anthers with the aid of a glass rod in order to burst the anther and release the microspores. The content was collected with an automatic pipette, and it was then transferred to a Neubauer chamber where the cells were analyzed under an optical microscope with 400X magnification. The number of microspore mother cells (MC), the number of microspores in dyad (DY), the number of microspores in tetrad (TE), the number of uninucleate microspores (UN), and the number of pollen grains (PG) were determined. The number of cells/anther was obtained via the ratio between the total number of cells found at each stage of development (counted in five fields) and the 20-µL volume.

The heritability estimates in the broad sense (h^2) were calculated using the following estimator:

$$h^2 = \frac{\hat{\sigma}_g^2}{\hat{\sigma}_p^2} \times 100 \qquad \text{(Equation 1)}$$

where: $\hat{\sigma}_g^2$ = genetic variance; $\hat{\sigma}_p^2$ = phenotypical variance.

The genetic variance was calculated using the following estimator:

$$\hat{\sigma}_g^2 = \frac{MStreat - MSres}{J} \qquad \text{(Equation 2)}$$

where: MStreat = mean square of the treatment; MSres = mean square of the residue; J = number of replicates.

The ratio of the genetic coefficient of variation by the environmental coefficient of variation (CVg/CVe) was calculated, and the genetic variance coefficient (CVg) was obtained as follows:

$$CVg\ (\%) = \left(\frac{\sqrt{\sigma_g^2}}{M}\right) \times 100 \qquad \text{(Equation 3)}$$

where: σ^2 = genetic variance; M = experimental mean.

The environmental variance coefficient (CVe) was obtained as follows:

$$CVe\ (\%) = \left(\frac{\sqrt{MS_{RES}}}{M}\right) \times 100 \qquad \text{(Equation 4)}$$

where: MSres = mean square of the residue; M = experimental mean.

Additionally, a Pearson correlation analysis was performed using the averages obtained in the five anthers analyzed from each bud, with three buds per class, which totaled to 15 anthers per class. The significance was verified through the Student *t*-test at 5 and 1% probability.

Induction of somatic embryos via anther culture

In the induction of haploid embryos, 200 anthers were used from each genotype: the parents (G1 = 390 and G2 = 134) and eight genotypes from the F_2 generation containing the most microspores in the uninucleate stage. In the laboratory laminar flow chamber, the flower buds were disinfected by immersion in a 100 mL solution of sodium hypochlorite (2% active chlorine), and three drops of Tween 20 were added before they were immersed for 10 minutes. Subsequently, the flower buds were washed three times in distilled, deionized, and autoclaved water (DDA).

After disinfection, the anthers were carefully removed from the flower buds with previously autoclaved tweezers under a stereoscopic microscope (model Quimis-Motic SMZ 168) with 10Xmagnification. The anthers were inoculated in Petri dishes containing 20 mL of embryo induction media (Table 2).

Table 2. Media used in the induction of somatic embryos in anthers from ornamental peppers (*C. annuum* L.).

Treatment	Culture medium	Reference
M1	MS + 4.65 µM kinetin + 5.73 µM NAA	Wang et al. (1973)
M2	MS + 4.65 µM kinetin + 4.52 µM 2,4-D	Wang et al. (1973)
M3	C + 23.25 µM of kinetin + 22.6 µM 2,4-D R + 4.65 µM kinetin	Dumas de Valux et al. (1981)
M4	C + 21.48 µM NAA + 4.44 µM BA R + 4.65 µM kinetin	Dumas de Valux et al. (1981)

A total of 30 g/L sucrose and 8 g/L agar were added to M1 and M2 media (Wang et al., 1973), and the dishes were kept in a growth room under fluorescent light (irradiance of 90 µmol x m^{-2} x s^{-1}) with photoperiod of 16h at 26° ± 2°C for 60 days. A total of 30 g/L sucrose and 8 g/L agar were also added to media M3 and M4.However, the dishes were initially placed in B.O.D. (Biochemical Oxygen Demand) with a controlled temperature of 35° ± 2°C for eight days in the dark, and the temperature was then changed to 25° ± 2°C with a photoperiod of 12 h under an irradiance of 90 µmol · m^{-2} · s^{-1} for another four days. Subsequently, the anthers were transferred to regeneration medium R (Dumas de Valux et al., 1981) supplemented with kinetin (4.65 µM), and the dishes were placed in a growth chamber at 25° ± 2°C with a photoperiod 12 h for 60 days. After 60 days, the percentage of embryogenic calluses and the number of embryos/genotype were evaluated, and the percentage of responsive anthers/genotype was calculated.

Statistical analysis

The experiment was conducted in a completely randomized design in a factorial 10 x 4 scheme (genotypes x media), in which the parents and eight F_2 genotypes were considered (with five replicates). Each replicate consisted of one Petri dish with 10 anthers each. The data for embryogenic callus induction were transformed by Arcsin (x) and subjected to variance analysis (P ≤ 0.01) followed by Tukey's test (P ≤ 0.01). All analyses were performed using the computer

software Genes (Cruz 2006). A descriptive analysis of flower bud morphology in relation to the microspore stage and the number of induced embryos was also performed.

RESULTS AND DISCUSSION

Differentiation of morphological classes and correlation between bud size and microspore development stage

Significant differences were observed by F test (P ≤ 0.01) for seven of the 10 traits related to flower bud morphology and microspore stage. Only the sepal length (SL), number of dyads (DY), and the number of tetrads (TE) did not show significant differences (Table 3). With the exception of SL (69.99%), DY (72.04%), and TE (8.21%), the observed heritability values were greater than 90.00%, which is considered high. According to Vencovsky and Barriga (1992), these values indicate that most of the total observed variability for these characters is due to genetic differences between them. The highest value for heritability was observed in a number of pollen grains (99.81%).

Table 3. Summary of analysis of variance for bud morphology and microspore stage in ornamental peppers (*C. annuum* L.).

S.V	Mean square									
	BL	BD	PL	SL	AL	MC	DY	TE	UN	PG
Treatment	0.5833*	0.6049*	0.8610*	0.1190^{ns}	0.3747*	3559511.1*	1033.3^{ns}	56011.1^{ns}	4937677.7*	51600277.7*
h² (%)	99.51	99.53	99.78	69.99	99.40	99.00	72.04	8.21	94.4	99.81
CVg/CVe	8.26	8.44	12.44	0.88	7.45	5.77	0.93	0.17	2.37	13.54
C.V (%)	2.66	3.05	3.25	13.66	5.58	29.851	84.98	287.41	68.68	12.78

SV-source of variation; BL-Bud Length; BD-Bud Diameter; PL-Petal Length; SL-Sepal Length; AL-Anther Length; MC-Number of microspore mother cells; DY-number of microspores in dyad; TE-Number of microspores in tetrad; UN-Number of uninucleate microspores; PG-Number of pollen grains.

The genetic and environmental variation coefficients ratio (CVg/CVe) was greater than 1.0 for most variables, with the exception of SL, DY, and TE, which obtained CVg/CVe values of 0.88, 0.93, and 0.17, respectively; therefore, indicating an unfavorable situation for selection (Vencovsky, 1978). A ratio greater than 1.0 indicates that genetic variation exceeds environmental variation (Nascimento et al., 2012).

All bud morphology traits differed statistically based on Tukey's test except for the sepal length (Table 4). Smaller buds forming the first class were an average of 1.61 mm in length and 1.26 mm in diameter with smaller petals than sepals. In this class, the anthers were 0.48 mm long and presented the highest number of microspore mother-cells, which was not observed in the other classes (Figure 1A, D, and G). In the second class, buds an of average 1.92-mm length and 1.79 mm diameter, and petals and sepals of similar sizes (1.45 mm and 1.5 mm, respectively) were found. The anthers of these buds were on average 0.87mm, containing mostly uninucleate microspores (Figure 1B, E, and H), reaching a count of approximately 2247 microspores/anther. Moreover, small amounts of dyads and tetrads were found at this developmental stage. Although not statistically significant, this presence confirms a common occurrence in peppers that is referred to as asynchrony of microsporogenesis. Kim et al. (2004) found microspores in uninucleate and binucleate stages in a single anther of *C. annuum*. Furthermore, Rêgo et al. (2012c) demonstrated

the existence of microspores at tetrad and uninucleate stages in *Capsicum* spp. anthers, thus corroborating the findings in this experiment.

Table 4. Means for bud morphology and microspore developmental stage in ornamental pepper flowerbuds (*C. annuum* L.) that were divided into three classes.

Class	BL	BD	PL	SL	AL	MC	DY	TE	UN	PG
1	1.61[c]	1.26[c]	0.73[c]	1.15[a]	0.48[c]	1886.67[a]	36.67[a]	0.00[a]	0.00[b]	0.00[b]
2	1.92[b]	1.79[b]	1.45[b]	1.50[a]	0.87[b]	0.00[b]	23.33[a]	236.67[a]	2246.66[a]	0.00[b]
3	2.48[a]	2.16[a]	1.77[a]	1.49[a]	1.18[a]	0.00[b]	0.00[a]	0.00[a]	50.00[b]	7183.33[a]

BL-Bud Length; BD-Bud Diameter; PL-Petal Length; SL-Sepal Length; AL-Anther Length; MC-Number of microspore mother cells; DY-number of microspores in dyad; TE-Number of microspores in tetrad; UN-Number of uninucleate microspores; PG-Number of pollen grains. *Means followed by the same letter, per column, do not differ based on Tukey's test.
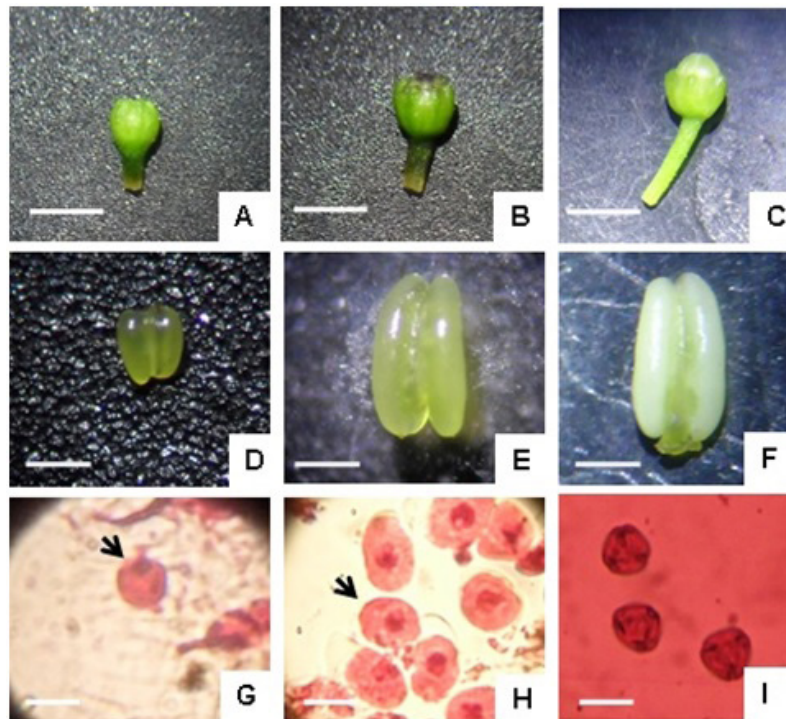
Figure 1. Relationship between flower bud morphologicaltraitsand microspore developmental stage in ornamental pepper plants (*C. annuum* L.) A, B, C-Bud appearence (bar = 2 mm); D, E, F-Anther Appearence (bar = 500 μm); G-Microspore mother cell; H-Microspore in the tetrad stage; I-Uninucleate microspores (bar = 25 μm).

The highest averages were found in the third class, which had flower buds measuring an average of 2.48 mm in length and 2.16 mm in diameter. Unlike the other classes, these buds presented larger petals than sepals and anthers with high quantities of formed pollen grains (approximately 7184 pollen grains/anther). Moreover, a small amount of microspores in the uninucleate stage (50 microspores/anther) were present, which characterized the asynchronous

division of microspores (Figure 1C, F, and I). Thus, the use of buds with petals larger than sepals (as found in class 3) is not recommended, since they contain a large number of pollen grains already formed, which are not desirable for anther culture.

As expected, it was observed that the flower bud morphological traits are highly positively correlated with bud growth, and this demonstrates that the parameters used to separate classes was efficient (Table 5). In other words, there is a growing relationship between the classes and the morphological traits, so the buds in the most advanced classes present greater length, diameter, and petal size. The sepal length size was the only trait that was not correlated with the established classes and the bud length, and a lack of correlation between this trait and microspore developmental stage was also observed. Therefore, because it is more present in buds at stage 2, this trait should not be used to relate to microspore developmental stages.

Table 5. Correlation between variables related to bud morphology and microspore developmental stage of ornamental pepper flower buds (*C. annuum* L.).

	Class	BL	BD	PL	SL	AL	MC	DY	TE	UN	PG
Class	1.0	0.9794**	0.9878**	0.9734**	0.6252ns	0.9891**	-0.8534**	-0.7285*	0ns	0.0180ns	0.8637**
BL		1.0	0.9542**	0.9234**	0.5977ns	0.9600**	-0.7508*	-0.7620*	-0.1501ns	-0.0953ns	0.9250**
BD			1.0	0.9886**	0.6765*	0.9717**	-0.8888**	-0.6972*	0.0107ns	0.1172ns	0.7982**
PL				1.0	0.6877*	0.9777**	-0.9359**	-0.7101**	0.0772ns	0.2372ns	0.7308*
SL					1.0	0.6237ns	-0.6503ns	-0.2991ns	0.0512ns	0.4161ns	0.3746ns
AL						1.0	-0.8813**	-0.7612*	0.0486ns	0.0977ns	0.8249**
MC							1.0	0.6450ns	-0.2543ns	-0.4712ns	-0.4914ns
DY								1.0	0.3352ns	-0.0855ns	-0.6864*
TE									1.0	0.2210ns	-0.2574ns
UN										1.0	-0.4463ns
PG											1.0

BL-Bud Length; BD-Bud Diameter; PL-Petal Length; SL-Sepal Length; AL-Anther Length; MC-Number of microspore mother cells; DY-number of microspores in dyad; TE-Number of microspores in tetrad; UN-Number of uninucleate microspores; PG-Number of pollen grains. ns,*, and **: not significant, significant at 5 and 1% of probability by *t*-test, respectively.

We observed a negative correlation among the number of microspore mother-cells and dyads with flower bud size at this stage (i.e., as flower bud size increases, the number of microspores decreases). Regarding the number of cells at the uninucleate stage, no correlation to any bud morphological traits were observed, which indicated a concentration of cells in a particular class. The mean test (Table 4) confirms the greater presence of uninucleate microspores in class 2, which can be found in small quantities in class 3 and not at all in class 1. In several plant species, the cultivation of uninucleate microspores is recommended, as these are more suitable for embryo induction and formation from anther culture (Pretova et al., 1993; Mityko and Fari, 1997; Kim et al., 2004; Lantos et al., 2009, Irikova et al., 2011a). These studies showed that the redirection of the normal gametophytic pathway to androgenesis requires a signal-stimulus. According to Grando and Moraes-Fernandes (1997), there are two moments in which a microspore could have its normal ontogenetic pathway altered, leading to the acquisition of androgenetic potential. The first point of change would be the beginning of meiosis, and the second is during the pollen uninucleate stage. The synthesis of mRNA and proteins that are believed to be responsible for altering the ontogenetic pathway via microspores was identified during these stages (Wang et al., 2000). Therefore, it is inferred that uninucleate microspores are preferred for embryogenesis induction from anther culture, so it is of the utmost importance to determine the particular stage at which the buds contain these cells.

Since no correlation was found, and because there is a statistical difference between the means of uninucleate cells/established size classes, it is possible to determine the ideal bud size in order to find the greatest amount of microspores in the uninucleate stage. To do so, approximate petal and sepal sizes may be used as standards for the determination of classes. Several authors describe the existence of a relationship between the size of petals and sepals and the microspore stage. For instance, Sibi et al. (1979) and Nowaczyk and Kisiala (2006) describe buds with petals of equal length or slightly larger than the sepals that contained microspores at the early binucleate stage and uninucleate microspores in their anthers. The same was reported by Lantos et al. (2009), in which the culturing of *C. annuum* anthers identified on average 80.0% uninucleate microspores in anthers with petal lengths slightly greater than sepal lengths. *C. chinense* flower buds, with petals measuring 1.9 mm and sepals measuring 2.1 mm, presented uninucleate microspores (Rêgo et al., 2012c). Thus, as there was no correlation between morphological bud size variables and the number of uninucleate microspores, it can be concluded that the optimal flower bud size for obtaining uninucleate microspores is found in class 2. A situation reversed of the one found for mother cells and dyads was observed for amount of pollen grain, wherein a positive correlation can be described with the increase . As expected, flower buds at more advanced developmental stages belonging to class 3 contained large amounts of formed pollen grains.

Induction of embryogenic calli and embryos

In this experiment, the induction of somatic embryos followed the indirect induction model, in which the embryos form from a callus. Presence of friable regions was observed in the calli, and these regions were usually white and translucent, which characterized them as embryogenic calli (Figure 2). There was a significant interaction between the environment and genotypes for the *F* test (P ≤ 0.01) for number of induced calli, indicating additional effects due to the joint action of the two factors (Table 6).

Genotypes 1, 2, 3, 4, and 5 behaved similarly regardless of the medium used, and this differed from what was observed in genotypes 6, 7, 8, 9, and 10, which were significantly influenced by the culture media (Table 6). Within genotypes, there were significant differences among almost all media with the exception of the M3 medium (Table 6). Genotypes 6 and 9 presented higher percentages of callus induction when cultured in media M1, M2, and M4. For both genotypes only 2.40% of anthers induced calli in medium M3 (Table 7). This medium contained the highest concentration of cytokinin (kinetin 23.25 µM) among the four media used. Chée and Cantliffe (1990) reported that cytokinins favor the production of embryogenic calli. However, in this experiment, there was an opposite effect, and the high concentration of cytokinin in the M3 medium inhibited callus production in the genotypes (Table 7). This effect may have been further enhanced by the concentrations of these endogenous regulators. Irikova and Rodeva (2004) concluded that the absence and the lowest level of growth regulators were sufficient to induce callus formation in different genotypes.

M4 media was more efficient in callus induction for genotype 7, and 36.0% of anthers presented embryogenic calli. However, different behaviors were observed in genotypes 8 and 10, which had lower performances in M4 medium, and both only exhibited 0.5% of embryogenic calli. In latter ones, media M1 and M2 were more efficient. Wang et al. (2004) observed significant callus formation when using cytokinins combined with naphthalene acetic acid (NAA), and both were present in M1 and M4 media tested in this experiment.

Table 6. Summary of analysis of variance for the percentage of embryogenic calli induced in anthers from ornamental peppers (*C. annuum* L.).

S.V	Mean square Percentage of embryogenic calli
Media (M)	**1444.82****
Genotypes (G)	**405.06****
Media x Genotypes	**193.84****
Medium/G1 (P1)	194.15^{ns}
Medium/G2 (P2)	132.75^{ns}
Medium/G3 (F$_2$)	89.40^{ns}
Medium/G4 (F$_2$)	42.05^{ns}
Medium/G5 (F$_2$)	67.24^{ns}
Medium/G6 (F$_2$)	417.48**
Medium/G7 (F$_2$)	1093.30**
Medium/G8 (F$_2$)	487.30**
Medium/G9 (F$_2$)	326.15**
Medium/G10 (F$_2$)	1033.90**
Genotype/M1	204.58**
Genotype/M2	172.65**
Genotype/M3	31.96^{ns}
Genotype/M4	577.40**
CV (%)	**90.98**

SV = source of variation. ^{**,ns}Significant and not significant, respectively, at 1% probability based on *F* test. G1-parent UFPB 390; G2-parent UFPB 134; G3-F$_2$ 18; G4-F$_2$ 20; G5-F$_2$ 26; G6-F$_2$ 32; G7-F$_2$ 28; G8-F$_2$ 19; G9-F$_2$ 1; G10-F$_2$ 5.

Table 7. Percentage (%) of embryogenic calli/50 anthers from ornamental pepper plants (*C. annuum* L.) at 60 days of cultivation.

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
M1	12.2^{ABa}	12.1^{ABa}	12.1^{ABa}	0.5^{Ba}	6.3^{ABa}	16.1^{ABab}	12.3^{ABbc}	20.0^{Aa}	22.0^{Aa}	18.0^{ABa}
M2	14.1^{ABa}	20.0^{Aa}	10.3^{ABa}	0.5^{Ba}	8.3^{ABa}	18.1^{ABa}	18.0^{ABb}	14.2^{ABab}	14.1^{ABab}	8.2^{ABab}
M3	0.5^{Aa}	8.2^{Aa}	4.3^{Aa}	0.5^{Aa}	0.5^{Aa}	2.4^{Ab}	0.5^{Ac}	0.5^{Ab}	2.4^{Ab}	0.5^{Ab}
M4	12.2^{BCa}	10.3^{BCa}	14.1^{BCa}	6.3^{BCa}	8.2^{BCa}	24.0^{ABa}	36.0^{Aa}	0.5^{Cb}	14.1^{BCab}	0.5^{Cb}

G1-parent UFPB 390; G2-parent UFPB 134; G3-F$_2$ 18; G4-F$_2$ 20; G5-F$_2$ 26; G6-F$_2$ 32; G7-F$_2$ 28; G8-F$_2$ 19; G9-F$_2$ 1; G10-F$_2$ 5. Means followed by the same upper case letter, per line, do not differ statistically based on Tukey's test (P ≤ 0.01). Means followed by the same lower case, per column, do not differ statistically by Tukey's test (P ≤ 0.01).
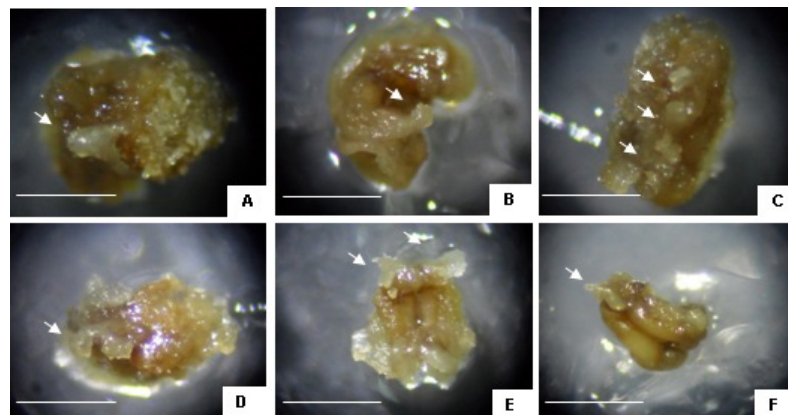
Figure 2. Embryogenic calli and embryos induced from anther culture of *C. annuum* L. A-Genotype 1 (Medium 1); B-Genotype 1 (Medium 3); C-Genotype 2 (Medium 2); D-Genotype 6 (Medium 4); E-Genotype 8 (Medium 2); F-Genotype 9 (Medium 2). *Bar* = 2mm. Arrowheads indicate pro-embryos and embryos.

There was considerable variation in callus induction when comparing genotypes in the same culture medium (M1, M2, and M4), showing the effect of genotypes. The genotype is the most important and often the most limiting factor in the response of *Capsicum* to androgenesis, and this factor was studied by several authors (Rodeva et al., 2004; Koleva-Gudeva et al., 2007; Irikova et al., 2011b). In the M1 medium (Table 7), genotypes 8 and 9 presented the highest averages for callus induction (20 and 22%, respectively). However, they did not differ statistically from genotypes 1, 2, 3, 5, 6, 7, and 10. Only genotype 4 was less responsive, and it only obtained 0.5% of anthers regenerated into calli. This downward trend in callus induction was also observed in M2 medium, which was the least responsive genotype among all studied for this two media. Regarding M2 medium, genotypes 1, 2, 3, 5, 6, 7, 8, 9, and 10 again did not differ in the percentage of callus induction, and averages varied from 8.2% (observed in the genotype 10) to 20.0% (found in genotype 2).

The biggest differences in genotypes were observed when they were grown in M4 medium. Genotypes 6 and 7 (Table 7) developed well and obtained the highest averages among all treatments: 24.0 and 36.0% of calli induced, respectively. Genotypes 8 and 10 had the lowest performances in this medium, both with only 0.5% of embryogenic calli, which was similar to genotypes 1, 2, 3, 4, 5, and 9.

Genotypes that presented high percentages of callus induction in other media, such as genotypes 8 and 10, showed low induction in M4 medium, which indicated a significant effect of the genotype x medium interaction. An important factor that may have influenced these differences in callus induction in M4 is the time of exposure of the explants to the induction medium, which includes concentrations of growth regulators auxin and cytokinin that are required for callus development. According to the methodology proposed by Dumas de Valux et al. (1981), after 12 days, the explants are transferred to a regeneration medium (R + 4.65 µM) that has a low concentration of cytokinin and an absence of auxin.

Irikova et al. (2011b) studied the response of *C. annuum* anthers to different culture media and exposure time, and realized that genotypes that seemed non-responsive to the induction of embryos and embryogenic calli by the original protocol of Dumas de Valux et al. (1981) showed embryogenic potential when cultured for 40 days in medium C, followed by transfer to medium R. This was the case withgenotype 1957, which presented 2.3% of induced calli in the original protocol with a subsequent increase to 13.7%. Possibly, the cells of the latter genotypes need more time to acquire the necessary competence for callogenesis/embryogenesis and to respond to stimuli induced by growth regulators present in the induction medium. Olszewska et al. (2013) also describe these results.

The genotype effect was also observed by Rodeva et al. (2004) in a study examining the response of different *C. annuum* genotypes and F_1 hybrids that were cultured according to the protocol of Dumas de Valux et al. (1981). The results indicated wide variation in callus formation between genotypes that showed no callus formation (F_1: 1647 x 1962) to those that induced up to 16.6% of calli (Genotype 146). Koleva-Gudeva et al. (2007) also describe the influence of genotypes and culture media on the formation of embryogenic calli in *C.* annuum. For instance, 58.9% of the Feferona variety of anther induces calli when cultured in medium N (Nitcsh, 1969) supplemented with 1 mg/L KIN + 0.001 mg/L IAA. Whereas, the anthers cultured in medium C of Dumas de Valux et al. (1981) induced no calli. In contrast, the Venice Fight variety responded better when the anthers were cultured in medium C (27.9%) compared to those grown in medium N (9.8%). These results corroborate the influence of the media and genotypes on embryogenic callus induction, especially the interaction between the two factors, which are indicated in the results of this experiment.

The differentiation of somatic embryos occurred on the calli surface. Of the ten studied genotypes, only genotype 4 presented no formation of embryos in any of the media used, and it is thus considered non-responsive to androgenesis under the conditions of this experiment (Table 8). The results suggest that the M3 medium is inefficient at inducing embryos, because only genotypes 2, 3, 5, and 10 showed androgenesis. However, it was considered low in that all mentioned genotypes only showed 2.0% responsive anthers.

Table 8. Number of embryos per genotype, percentage of responsive anthers, total and percentage of induced embryos per genotype of ornamental pepper plants (*C. annuum* L.) at 60 days of cultivation.

	Medium 1		Medium 2		Medium 3		Medium 4		Total	
	Number of embryos	Responsive anthers (%)	Number of embryos	Responsive anthers (%)	Number of embryos	Responsive anthers (%)	Number of embryos	Responsive anthers (%)	Number of embryos	Embryos/ genotype(%)
G1	1	2	4	6	0	0	4	3	9	7.03
G2	7	8	4	4	1	2	0	0	12	9.37
G3	1	2	3	6	3	2	1	2	8	6.25
G4	0	0	0	0	0	0	0	0	0	0
G5	0	0	2	2	1	2	2	2	5	3.90
G6	4	8	9	12	0	0	11	16	24	18.75
G7	6	8	8	10	0	0	3	6	17	13.29
G8	10	12	2	4	0	0	0	0	12	9.37
G9	6	6	16	12	0	0	5	8	27	21.10
G10	12	16	0	0	2	2	0	0	14	10.94
Total	47	62	48	56	7	8	26	35	128	100.00

G1-parent UFPB 390; G2-parent UFPB 134; G3-F_2 18; G4-F_2 20; G5-F_2 26; G6-F_2 32; G7-F_2 28; G8-F_2 19; G9-F_2 1; G10-F_2 5.

M1 and M2 media proved to be the most efficient at embryo induction. In M1, only genotypes 4 and 5 were not responsive. Under this condition, the culture of genotype 10 induced the largest number of embryos (12) in 16.0% of inoculated anthers. However, this result was not observed in M2 medium where genotype 10 did not respond to embryogenesis. In the latter medium, genotype 9 presented the largest quantity of embryos among all treatments, in which 12.0% of responsive anthers induced 16 embryos (Figure 2). This variation in the response demonstrates that there is a best-suited combination of medium culture and growth regulators for each genotype.

Four evaluated genotypes (2, 4, 8, and 10) did not induce embryos in M4 medium. Genotype 6 responded best to embryogenesis when cultured in this medium, forming 11 embryos in 16.0% of inoculated anthers (Figure 2). The other responsive genotypes obtained low embryogenesis with an average of three embryos per genotype. Koleva-Gudeva et al. (2007), examined the culture of anthers from nine varieties of pepper (*C. annuum*) in MS medium that was supplemented with kinetin (1.0 mg/L), 2,4-D (0.01 mg/L), and IAA (0.001 mg/L). However, the presence of embryos was not verified (only calli were present), so embryogenesis induction was only achieved when these anthers were cultured in C medium. Luitel and Kang (2013) also obtained inferior results in three genotypes of minipaprika (*C. annuum* L.) cultured in MS medium as compared to C medium. These results differ from the findings of this experiment where a larger quantity of embryos was found in MS media corresponding to M1 and M2 media.

Differences were observed regarding the performance of both parents (G1 and G2) in the evaluated culture media. Genotype 1 was more responsive to the induction of embryos when cultured in media M2 or M4, but genotype 2 developed best when grown in medium M1. Genotype 2 (parent 134) showed a higher number of embryos (12 total) in relation to genotype 1 (parent

390), which exhibited nine total embryos (Table 8). It is noteworthy that for the percentage of embryogenic calli there was no difference in the performance of the parents (Table 7).

Genotypes that induced a number of embryos outside the parental range were observed in the F_2 generation, which characterizes transgressive segregation. Genotypes G3, G4, and G5 presented a number of embryos inferior to the lowest parent (G1), varying from 0 to 8 embryos. On the other hand, genotypes G6, G7, G9, and G10 presented a number of embryos higher than parent 134 (G2). For instance, genotype 9 induced a total of 27 embryos (21.1%), which was more than twice the number of embryos found in parent 134 (G2) (12 total embryos). The most likely causes for this phenomenon in segregating generations are overdominance and epistasis (Rieseberg et al., 1999).

The total number of embryos formed from anther culture in this experiment ranged from 0 to 27 embryos in 200 anthers, regardless of the media used. These results agree with those obtained by Nowaczyk et al. (2009) who examined the reaction of F_2 *C. annuum* genotypes and observed the effect of genotypes in the response to embryo induction, and variations ranged from 0 to 39 embryos in 100 anthers. Similar results were previously described by Rodeva et al. (2004) and Nowaczyk and Kisiala (2006).

The low induction of *in vitro* androgenesis in pepper may have been influenced by several factors, and the most important among them is the genetic predetermination of plants, which is explained by the presence of genes involved in receptivity of growth regulators (Close and Gallagher-Ludeman, 1989; Rêgo et al., 2012c) that are usually required for the determination and differentiation of cells. Another determining factor for the success of androgenesis is the way the microspore develops, which happens asynchronously (Rego et al., 2012c). Furthermore, the results were also verified in this experiment with the presence of cells at different stages of development in anthers of the same class. This fact can be seen in the work of Kim et al. (2004), in which anthers at development stage 3 presented 75.0% of binucleate pollen grains, indicating the asynchronous development of microspores.

Other accessions of *C. annuum* should be studied to search for genotypes that are more responsive. An alternative to overcome genotype non-responsiveness is the use of hybridization with other highly responsive genotypes, wherein genes determining the androgenic response can be transferred to accessions that are of interest to breeding programs. The protocol should also be perfected by the study of other variables such as photoperiod and levels of growth regulators, the development and testing of protocols for microspore culture, and histological studies for a better understanding of somatic embryogenesis in ornamental peppers.

ACKNOWLEDGMENTS

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