



# Impact of genotype, plant growth regulators and activated charcoal on embryogenesis induction in microspore culture of pepper (*Capsicum annuum* L.)<sup>☆</sup>



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## ABSTRACT

The production of double haploids through androgenesis is used by breeders to produce homozygous lines in a single generation. Androgenesis can be achieved by isolated microspore culture, which, however, allows the production of embryogenesis with a very low efficiency. In order to improve the overall embryogenesis in pepper, we study the differences of microspore embryogenesis in different genotypes of pepper, and also document the effect of growth regulators in pretreatment media, and activated charcoal (AC) on embryogenesis induction. Fifty different pepper genotypes were evaluated, and the swollen rate of microspores from different genotypes varied from 3.11% to 29.56% with the mean value of 13.13%. Microspores from genotype '36' had the highest swollen rate, and the lowest swollen rate of microspores was observed in genotype '26'. It was concluded from the statistical results of  $L_9(3^3)$  orthogonal test that changes in the level of BA influenced the swollen rate of microspores more significantly, and the combination of  $0 \text{ mg l}^{-1}$  6-benzyladenine (BA),  $0.2 \text{ mg l}^{-1}$   $\alpha$ -naphthaleneacetic acid (NAA) and  $0.5 \text{ mg l}^{-1}$  kinetin (Kin) was best. AC at a concentration of 0.05% could act as a promoter of embryogenesis in the microspore culture of different pepper genotypes, while the more significant effect was observed with the low responsive genotypes.

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## 1. Introduction

Pepper (*Capsicum annuum* L.) is one of the most variable genera among horticultural species, and its fruits range from tiny hot chillies to long cayenne chillies (Derera et al., 2005). And pepper is a widely grown crop all over the world, which can be used as a vegetable, an ingredient of sauces, a coloring and pungent agent of foods, or in pharmaceutical applications. At present, people mainly adopt the method of continuous selfing to purify parent in heterosis breeding. Therefore, it usually needs 5–6 generations or even more generations of continuous selfing and selection to cultivate the excellent inbred line from heterozygous breeding materials. The major advantage of doubled haploids in

plant breeding is the immediate achievement of complete homozygosity. Desired genotypes are thus fixed in one generation, reducing time and cost for cultivar or inbred development. Among the different technologies to produce doubled haploids, microspore embryogenesis is by far the most common.

For some vegetable species, such as *Brassica rapa* (Cao et al., 1994), *Brassica oleracea* (Dias, 1999), and *Rapanus sativus* (Lichter, 1989), establishing isolated microspore cultures has been readily well set up. In pepper, Dumas de Vaulx et al. (1981) was the first to publish a reproducible in vitro anther culture method which is still the basis of any other modified techniques used in all over the world. Now, private seed companies and certain biotechnology laboratories in Europe show significant practical activity on production of doubled haploid pepper lines via in vitro anther-culture. Supena et al. (2006) published the first microspore culture-derived haploids in Indonesian hot pepper by a shed-microspore culture protocol. Later, Kim et al. (2008) reported on establishing haploids from the hot pepper 'Milyang-jare' using microspore culture, and optimized plating density of microspores ( $8 \times 10^4$ – $10 \times 10^4$ ) as well as source and amount of added carbon (9% w/v sucrose) in the medium. Recently, isolated microspore cultures of Hungarian and Spanish spice pepper genotypes were improved by using the wheat ovary co-culture method (Lantos et al., 2009). Moreover, experiments conducted with sweet pepper aimed at improving

Abbreviations: AC, Activated charcoal; BA, 6-Benzyladenine; NAA,  $\alpha$ -Naphthaleneacetic acid; Kin, Kinetin; ELSs, Embryoid-like structures.

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the efficiency of microspore cultures (Gémes Juhász et al., 2009; Seguí-Simarro et al., 2011; Lantos et al., 2012). Whereas, the genotype is still the most important and often limiting factor in the pepper androgenic reaction.

In those pioneer experiments, androgenesis was induced in growth regulator-free medium (Kim et al., 2008) as well as in media supplemented with 2.5 µM zeatin and 5 µM indole-3-acetic acid (IAA) (Supena et al., 2006, 2011), or in the induction media of 0.5 mg·l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg·l<sup>-1</sup> kinetin (Kin) (Lantos et al., 2009). A clear understanding of the effects of growth regulators in microspore culture of pepper requires further studies.

Activated charcoal (AC) could absorb substances, including phenols and abscisic acid inhibitory to microspore embryogenesis from the environment of culture vessel and is often added to tissue culture media. In *B. oleracea* Dias (1999) observed that AC seems to have a stimulatory effect on microspore embryogenesis of the different accessions and, in contrast to other factors, seems not to be genotype dependent. In shed-microspore culture of Indonesian hot pepper 'Tombak', Supena et al. (2006) described a method using a double layer culture medium containing AC which was needed, as embryo formation almost completely failed without its addition.

The objective of the present research was to study the differences on microspore embryogenesis in different genotypes of pepper, and also document the effect of growth regulators in pretreatment media, and AC on embryogenesis induction of pepper.

## 2. Material and methods

### 2.1. Plant material

Fifty pepper genotypes (obtained from Chinese seed companies or Institute of Vegetable Research, Shanxi Academy of Agricultural Sciences, China) were used. Seedlings were cultivated in cold-bed in March, and eighteen plants of each genotype were planted in the field in May 2011. Plants were grown with two main stems, and young fruits were removed and plants were severely pruned to stimulate the development of new young side shoots for flower bud production.

### 2.2. Donor bud collection and pretreatment of anthers

Flower buds of the desired size (petals equal or slightly longer than sepals and anthers with a faint purple tip), which contained more than 50% of microspores in the late unicellular stage, with amounts of mid unicellular microspores and of bicellular pollen not exceeding 20% and 50% respectively, were harvested from young inflorescences in the morning.

After being rinsed under tap water for 30 min, flower buds were sprayed with 70% ethanol, then surface-sterilized in a 0.1% (w/v) mercuric chloride containing 0.05% (v/v) Tween 20 and periodically agitated for 12 min before three rinses in sterile distilled water for 5 min each. Anthers isolated from one bud were placed on one Petri dish (60 mm × 15 mm), with their inner part facing the sucrose-starvation medium containing 0.37 M mannitol, 10 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 mM KNO<sub>3</sub>, 200 µM KH<sub>2</sub>PO<sub>4</sub>, 1 µM KI, 100 nM CuSO<sub>4</sub>·5H<sub>2</sub>O, and 0.5% agar (pH 5.8). For the first seven days anthers were incubated in the darkness at the temperature of 33 °C.

### 2.3. Isolation protocol and culture of microspores

For both isolation and culture of the microspores, a filter sterilized Nitsch and Nitsch (1967) medium, as modified by Lichter (1981) and by Takahata and Keller (1991) with 9% sucrose (NLN-9), at pH 5.8 was used. After heat treatment for seven days, the swollen anthers were chosen, mixed with NLN-9 medium and immediately extrusioned with glass rod to isolate microspores. Microspore suspension was obtained by filtration through 150 µm nylon screens. This suspension

was centrifuged 3 times at 500 rpm for 5 min and resuspended in NLN-9 medium. The microspore density was adjusted to 8 × 10<sup>4</sup>–10 × 10<sup>4</sup> per ml. Isolated microspores were cultured in 60 mm Petri dishes containing 2 ml microspore suspension.

The cultures were incubated at 28 °C with 80% air humidity and maintained in the dark for three weeks, and then transferred to a gyratory shaker agitating at 60 rpm in darkness at 28 °C.

### 2.4. Testing of the embryogenic potential of different genotypes

Only the swollen microspores had the possibility to develop into embryo-like structures (ELSs), so in the first part of our experiments the swollen rate of microspores (mean fraction of the number of swollen microspores and the number of total microspores in five fields of microscope) for each genotype was analyzed by isolating microspores of 6 swollen anthers in 1 ml NLN-9 medium after anther pretreatment.

### 2.5. Testing of the effects of growth regulators and activated charcoal

In order to find the optimum ratio of BA (0 mg·l<sup>-1</sup>, 0.2 mg·l<sup>-1</sup>, 0.4 mg·l<sup>-1</sup>), NAA (0 mg·l<sup>-1</sup>, 0.1 mg·l<sup>-1</sup>, 0.2 mg·l<sup>-1</sup>) and Kin (0 mg·l<sup>-1</sup>, 0.2 mg·l<sup>-1</sup>, 0.5 mg·l<sup>-1</sup>) in pretreatment medium, each factor was set three levels according to L<sub>9</sub> (3<sup>3</sup>) orthogonal design and the total were 9 level combinations.

Activated charcoal suspension preparation and addition of a 0.1 ml drop to the culture dishes in charcoal treatments were made following Dias' (1999) procedure: autoclaved suspension of 1 g of activated charcoal, 0.5 g of agarose and 100 ml of bi-distilled water. It is important to associate the charcoal with agarose because suspended charcoal without agarose adheres to the microspores and hampers embryogenesis.

### 2.6. Data collection and statistical analysis

At least ten dishes of each treatment were made with three replications, giving a total of thirty dishes per single treatment. Statistical analyses were performed using SPSS 17.0 for Windows software, and means were compared according to pairwise t tests. The number of microspore-derived ELSs was visually counted after six weeks of culture.

## 3. Results and discussion

### 3.1. Embryogenic potential of different genotypes

After 7 d anther pretreatments in sucrose-starvation medium, significant differences on the swollen rate of microspores were recorded among the fifty genotypes in this experiment (Fig. 1). The swollen rate of microspores from different genotypes varied from 3.11% to 29.56% with the mean value of 13.13%. Microspores from genotype '36' had the highest swollen rate. While, the least swollen rate was observed in genotype '26', which were in accordance with the results of subsequent culture of ELSs (data not shown). Thus, genotype '36' was selected as an experimental material to study the effects of growth regulators.

Based on the present results, there was demonstrated a visible heterogeneity in the effectiveness of androgenesis in respective plants of the genotypes researched. Some genotypes naturally respond better than others to pre-treatment applied, and the high and low responding genotypes have different requirements for optimal pre-treatment conditions in the same species. Supena et al. (2006) used ten varieties of Indonesian hot pepper and the quantity and quality of embryos obtained depended on the variety used for the same pretreatment. Similarly, Nowaczyk et al. (2009) showed that high temperature (35 °C) of anther pretreatment, which is used frequently in pepper and which has improved the embryogenesis and regeneration of green plants, proved ineffective in certain recalcitrant pepper genotypes. In our work, although genotype '36' and '26' do belong to the same type,

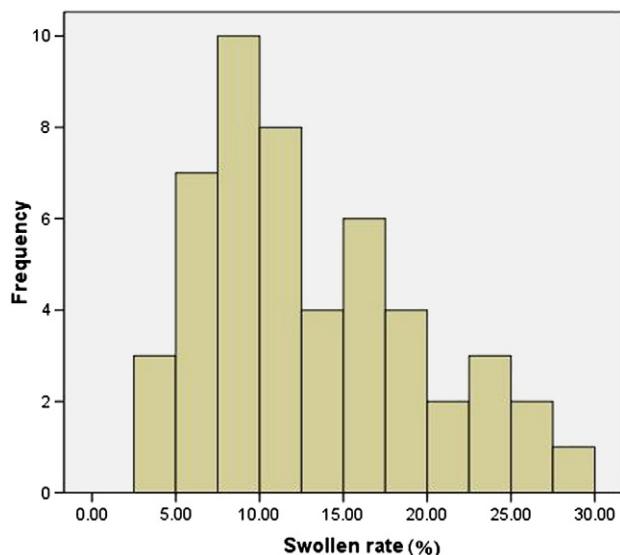


Fig. 1. Frequency distribution of the swollen rate of microspores in fifty different genotypes of pepper.

they have different fruit character. So it is not surprising to see the huge difference of microspore embryogenesis between them.

Whereas, the possibility of applying different types of inductive protocols allows for the choice of the most convenient for each variety. As an example, the variety 'Piquillo' shows a null response to the method described by Dumas de Vaulx (Mityko et al., 1995), and a positive response to the biphasic method described by Dolcet-Sanjuan et al. (1997). Therefore, before starting a breeding program based on doubled haploid (DH) production it is advisable to assess the response of each variety to the different protocols available.

### 3.2. Effects of different combination of plant growth regulators on embryogenesis

Different combinations of BA, NAA and Kin were tested in genotype "36" which had the highest swollen rate of microspores. Data of the swollen rate of microspores were collected to analyze the combined effect of the three growth regulators in the pretreatment medium. The statistical results of  $L_9$  ( $3^3$ ) orthogonal test were listed in Tables 1 and 2. It was concluded from square sum of growth regulator that the influences on the swollen rate of microspores were listed in the following order BA > NAA > Kin (Table 2). The influences of BA and NAA on the swollen rate of microspores were at 1% or 5% significant level, while the influence of Kin was not at significant level. Average swollen rate of microspores could reflect the effects of different combination of plant growth regulators on embryogenesis at different levels of the same factor. Range of average swollen rate of microspores could reflect the effects of changes in the level of various factors on embryogenesis.

Table 1  
Variance of plant growth regulator on the swollen rate of microspore in genotype "36".

Growth regulator	F value	P value
BA	32.852 <sup>**</sup>	<0.01
NAA	17.189 <sup>*</sup>	<0.05
Kin	2.721	

\* means square sum of grows regulator significant at 0.05.

\*\* means square sum of grows regulator significant at 0.01.

Table 2

Effect of different concentration of plant growth regulators on the swollen rate of microspore in genotype "36".

Code	Error term	BA (A, mg·l <sup>-1</sup> )	NAA (B, mg·l <sup>-1</sup> )	Kin (C, mg·l <sup>-1</sup> )	Swollen rate of microspores (%)
1	1	0	0	0	29.56
2	1	0.2	0.1	0.2	22.72
3	1	0.4	0.2	0.5	26.33
4	2	0	0.1	0.5	29.17
5	2	0.2	0.2	0	28.83
6	2	0.4	0	0.2	18.25
7	3	0	0.2	0.2	34.16
8	3	0.2	0	0.5	29.94
9	3	0.4	0.1	0	18.75
$K_1$	78.61	92.89	77.75	77.14	
$K_2$	76.25	81.49	70.64	75.13	
$K_3$	82.85	63.33	89.32	85.44	
$\bar{K}_1$		30.96	25.92	25.71	
$\bar{K}_2$		27.16	23.55	25.04	
$\bar{K}_3$		21.11	29.77	28.48	
R		9.85	6.22	3.44	

$K_1$ ,  $K_2$  and  $K_3$  are the sums of swollen rate of microspores for each factors and levels respectively.

$\bar{K}_1$ ,  $\bar{K}_2$  and  $\bar{K}_3$  are the average swollen rates of microspores for each factors and levels respectively.

R is the range of average swollen rate of microspores at different levels of the same factor.

Changes in the level of BA influenced the swollen rate of microspores more significantly, and the combination of plant growth regulators A<sub>1</sub>B<sub>3</sub>C<sub>3</sub> was best (Table 2). However, the terms and conditions of the test did not appear in the 9 combinations of the orthogonal table. So additional combination of 0 mg·l<sup>-1</sup> BA, 0.2 mg·l<sup>-1</sup> NAA and 0.5 mg·l<sup>-1</sup> Kin was tested, and the highest swollen rate of microspores at 38.47% was got.

In embryogenesis of microspore, an appropriate concentration and ratio of auxin and cytokinin plays an important role in early embryogenesis processes. In the present study, when exogenous growth regulators were omitted from the induction media, the basic media can not only ensure the survival and physiological activities of cultures, but also induce more embryogenesis than some combination of growth regulator (Table 2). The different androgenic responsiveness is due to genetic pre-determination of the genotype and the ratio between endogenous and exogenous plant growth regulators (Irikova et al., 2011). Only with proper addition of plant growth regulator, it can induce the proper change of start-up of cell division and callus growth.

Table 3

Effect of activated charcoal (AC) on embryogenesis of microspore culture in different genotypes of pepper.

Genotype	Treatments	No. of ELSs per dish
04	NLN-9	0.73 ± 0.1 b <sup>y</sup>
	NLN-9 + AC	1.98 ± 0.2 a
10	NLN-9	4.83 ± 0.7 b
	NLN-9 + AC	7.97 ± 1.1 a
33	NLN-9	1.60 ± 0.3 b
	NLN-9 + AC	4.13 ± 0.4 a
36	NLN-9	7.20 ± 0.8 b
	NLN-9 + AC	9.97 ± 1.1 a
40	NLN-9	1.33 ± 0.2 b
	NLN-9 + AC	3.20 ± 0.5 a
46	NLN-9	0.12 ± 0.06 b
	NLN-9 + AC	0.37 ± 0.1 a

ELSs = embryoid-like structures.

<sup>y</sup> Values represent means ± SEM. The means followed by the same letter do not significantly differ at  $p = 0.05$  according to pairwise t-tests.

### 3.3. Effect of activated charcoal on embryogenesis

ELS yields were significantly increased in all of the genotypes by the addition of a 0.1 ml drop of AC to the microspore culture media (Table 3). In no instances did the addition of AC have any detrimental effect on ELSs yield. Therefore AC at a concentration of 0.05% seems to act as a promoter of embryogenesis in the microspore culture of different pepper genotypes. Although the more significant effect was observed with the low responsive genotypes '04' and '46', it was observed that even the high responsive '36' responded positively to the addition of AC. The magnitude of the response to the addition of AC varied with the different genotypes. The best results were obtained with '46' with increases in ELSs yield of 208.3%. For all the other accessions, the increasing efficiency of AC on ELSs yield varied from 38.5% in '36' to 171.2% in '04'.

The present results are in accordance with the observations of Supena et al. (2006) who saw a significant increase in the in vitro androgenesis of pepper on 1% AC medium no matter the low and high responsive microspore culture genotypes.

The explanation for the promotion of embryogenesis by AC is not yet well clarified. AC possesses strong adsorptive properties and is usually used in chemistry to absorb both gases and dissolved solids. When added to tissue culture media, AC is commonly thought to remove growth inhibitory substances exuded by tissues or present in the ingredients of the medium, but it is now clear that promontory substances can also be absorbed and made unavailable to the plant. The effects of increasing concentrations of charcoal on the total embryo yield and the yield of normal-looking embryos did not run in parallel, and 2% charcoal impaired embryonic shoot development (Supena et al., 2006).

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.sajb.2013.08.012>.

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