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Studies on isolated microspore embryoid induction of *C. annuum* L.

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Abstract A system to improve isolated microspore embryoid induction rate of pepper (*Capsicum annuum* L.) was studied in this paper. The results showed that low-temperature pretreatment, growth regulators combinations, activated carbon concentrations, and preculture temperatures were critical factors affecting embryoid formation of isolated pepper microspores *in vitro*. One day after pretreatment of the buds at 4°C, the anthers that differentiated and developed into embryos were cultured in double-layer medium system for one week at 7°C and then went on culturing at 28°C in darkness. All the seven genotypes of the tested pepper responded to this protocol. The embryoid induction rate of the best genotype increased from 81.11% to 147.22%. This protocol can be used as a potential tool for producing doubled haploid plants for pepper breeding.

Keywords *Capsicum annuum* L., isolated-microspore culture, embryoids, embryoid induction rate, double-layer medium system

1 Introduction

Pepper (*C. annuum* L.) is an important vegetable crop in Europe, Asia, and Central and South America. Pepper haploid breeding is more efficient than conventional breeding. Generally, to obtain a true breeding line from a hybrid, it at least needs five or six generations by self fertilization, while it only needs a single generation by

haploid breeding. The most common method for producing haploid plants is anther culture (Dumas De Vaulx et al., 1981; Mitykó et al., 1995; Ramon et al., 1997; Wang et al., 2004a; 2004b; Zhang et al., 2005). Compared with anther culture, microspore culture is now becoming a realistic approach for haploid production. Liu et al. (2007) cultured microspores and derived multicells in a modified liquid CP medium after a 15-d preculture of anther in a solidified medium. Thirty days later in suspension culture, at 28°C dark condition, embryoids at different developmental stages were formed. After a heat shock treatment ($32\pm 1^\circ\text{C}$) in sucrose-starvation medium for 3 d, Kim et al. (2008) cultured microspores with modified NLNS medium for 4 weeks, and various sized embryos were observed. Studies on pepper isolated microspore culture also have some successful reports, but the rate of embryoid formation is still very low. Therefore, improving the embryoid induction rate is very important for this technique applying to pepper haploid breeding. It is known that the efficiency of microspore culture may be affected by a number of factors, including growth conditions of donor plants, type of stress treatment, composition of culture medium, carbon source, and so on. In this paper, we study the effects of some factors, such as genotypes, low-temperature pretreatment, growth regulator combinations, activated carbon concentration, and temperature preculture, on the pepper microspore embryoid formation, aiming to improve the embryoid induction rate and lay a foundation for pepper haploid breeding.

2 Materials and methods

2.1 Materials

Four hot pepper varieties, 08-200, 08-204, 08-234, and 08-264, and three sweet pepper varieties, 08-488, 08-490, and 08-491, as materials, were provided by the vegetable breeding laboratory in the Agricultural University of Hebei, Hebei Province, China.

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2.2 Isolated microspore culture

2.2.1 Plant material

Plants were transferred to green house in April. The young fruits were removed from the plants to stimulate the new branch and more floral bud formation.

2.2.2 Selection and disinfection of the buds

At the beginning of flowering and full flowering stage, flower buds (petals equal to or slightly longer than sepals) were chosen in the morning, disinfected in a 70% alcohol solution for 30 s and in a 2% NaClO solution for 10–12 min, and then rinsed three times with sterilized distilled water. The anthers with a faint purple tip isolated from the buds were used for isolated microspore culture.

2.2.3 Preparation of medium

A double-layer medium was used, in which the under layer consisted of NLN-H components (Nitsch and Nitsch, 1969) with 2% maltose and was solidified with 0.6% Plant Agar, while the liquid top layer contained NLN-H components plus 2% maltose. The solid media was sterilized by autoclaving and the liquid media was sterilized by filtration through membrane filters of successive pore sizes 0.45 and 0.22 μ m, prior to which the pH was adjusted to 5.8. Commonly, 6 cm Petri dishes were used, with approximately 5 mL solid medium and 3 mL liquid top layer.

2.2.4 Induction of embryoids

Anthers were transferred to the Petri dishes with double layer medium, 12 anthers in each dish, and 15 dishes for each genotype. The anthers were incubated at 4°C in the dark for the first week and then cultured at 28°C in the dark for 2–3 weeks. The microspores were naturally released from the anthers and then removed the dehiscent anthers, culturing was continued after. After 8 weeks, the embryoids were counted under the microscope and determined the frequencies of embryoid formation for each genotype, respectively. The rate (%) = the total number of induced embryoids / cultured anthers $\times$ 100%.

2.2.5 Plant regeneration

The cotyledonary embryos were removed from the double layer media and transferred to NLN-H solid media with 2% sucrose and 0.9% agar to culture at 28°C and 16 h illumination for plantlet formation (4000–5000 lx).

2.3 Experiments performed

Genotypes 08-488 was used for studying the effects of low-temperature pretreatment period (0–4 d),

preculture-induced temperature (4°C, 7°C, 32°C, and 35°C), activated carbon concentration (0, 0.25%, 0.5%, and 1%), growth regulators combinations (Table 1) on the embryoid formation.

Table 1 Combinations of growth regulators

serial number	1	2	3	4
ZT/mg·L ⁻¹	0.5	0.5	1.0	1.0
IAA/mg·L ⁻¹	0.5	1.0	0.5	1.0

3 Results

3.1 Establishment of the system for isolated microspore culture and effect of genotypes

When the uninucleate microspores (Fig. 1(a)) were inoculated on the double layer NLN-H medium at 28°C in dark for a week, some microspores had divided several times (Fig. 1(b)), and after about two or three weeks, embryoids at different state of development were detected (Figs. 1(c), (d)). The rates of embryoid formation of the seven pepper varieties were listed in Table 2. It revealed that the percentages of embryoid formation in the seven genotypes varied from 9.44% to 81.11%, and the genotype 08-488 had the highest frequency. The cotyledonary embryoids were selected from the Petri dishes, transferred into the solid medium, and cultured in 4000–5000 lx illumination for a few days; most of them could normally grow and form normal plantlets (Figs. 1(e), (f), (g)).

3.2 Factors affecting microspore embryoid induction rate

3.2.1 Effect of low-temperature pretreatment on the microspore embryoid induction rate

Flower buds of the genotype 08-488 were treated at 4°C for 1, 2, 3, and 4 d, and then, the microspores were removed by natural anther dehiscence to incubate at 28°C. The result showed that with lower temperature treatment, the pepper flower buds for one day were most available for the microspore culture; the embryoid induction rate was 1.3 times that of the control. With a period of treatment delay, the rates began to decline, until the fourth day, the frequency was only 45.37% (Table 3), which is about 57% of the control. Thus, the period of treatment at 4°C for the pepper floral buds should be done in the first 24 h.

3.2.2 Effect of preculture temperature treatment on the microspore embryoid induction rate

The effect of different temperature treatment prior to culture at the normal temperature (28°C) on the embryoid induction was researched. The results showed that the microspores were incubated at higher or lower temperature

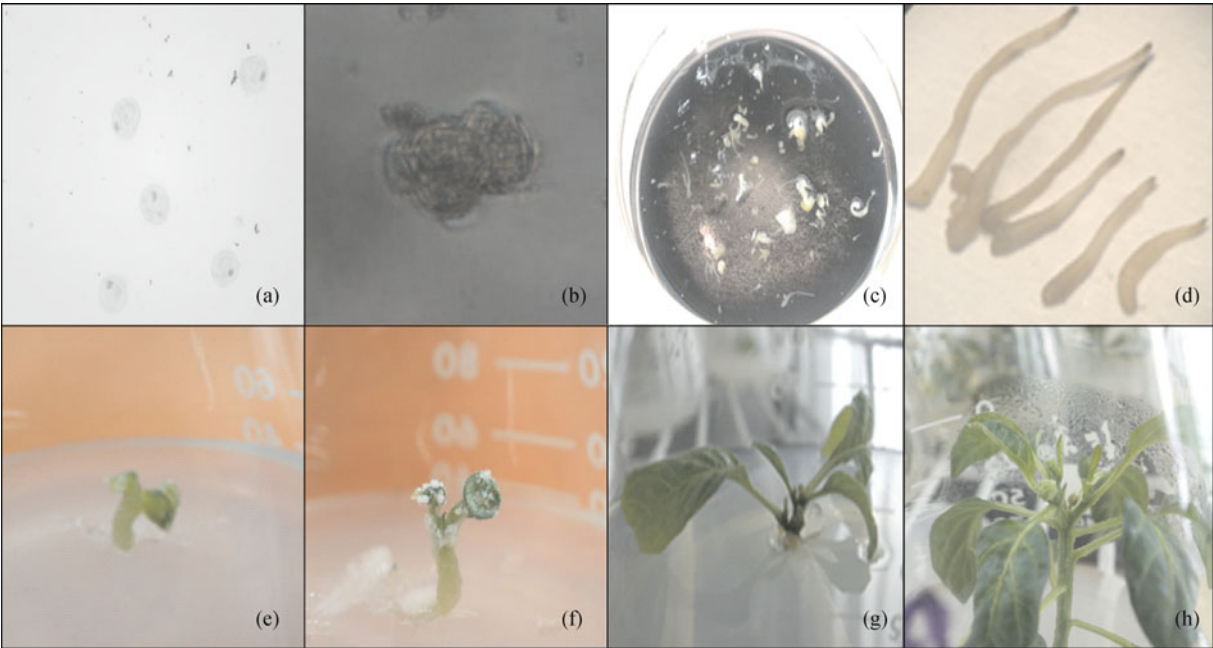


Fig. 1 Time-course of events during isolated microspore culture of pepper

Note: (a): late-unicellular microspores; (b): cell clusters after 3 weeks of microspore culture; (c) and (d): categories of embryos obtained from microspore culture after 7 weeks; (e) and (f): differentiated embryoid of microspore culture; (g) and (h): regenerated plantlets.

Table 2 Embryos induced from different genotypes under the same cultural conditions

serial number	genotypes	embryos induced	embryoid induction rate /%
1	08-200	23	19.44cd
2	08-204	22	10.56d
3	08-234	99	42.78b
4	08-264	82	35.56b
5	08-488	192	81.11a
6	08-490	50	9.44d
7	08-491	44	23.89c

Note: Values in the same column followed by different letters are significant at 0.05 probability levels (same as below).

for a week, the embryoid induction rate was much higher than those continuously cultured at 28°C, and the lower temperature treatments were generally better than higher ones, in which the 7°C treatment gave the highest embryoid induction rate (Table 4).

3.2.3 Effect of growth regulators on the microspore embryoid induction rate

The effect of the addition of ZT (0.5 to 1.0 mg·L⁻¹) or IAA (0.5 to 1.0 mg·L⁻¹) to the liquid media (upper layer) on

isolated microspore culture was studied. The results were presented in Table 4. It showed that the embryoid induction rates were significantly different in different growth regulators combinations and concentrations. The combination of 0.5 mg·L⁻¹ ZT and 1.0 mg·L⁻¹ IAA was very promising; the embryoid induction rate was increased up to 147.22%, which is significantly higher than that of control treatment. It can be seen that growth regulators play an important role on the embryoid induction rate in isolated microspore culture of pepper. The appropriate combination and concentration can increase the rate of embryo induction, but it is not the key to success.

3.2.4 Effect of activated carbon on the microspore embryoid induction rate

The embryoid induction rate significantly increased with increasing concentrations of activated charcoal from 0 to 2%. The embryoids induction rate of the control treatment (without activated carbon) was 37.2%, while for those with the activated carbon, the concentrations were 0.25% and 0.5%, and the rates were 48.15% and 85.16%, respectively. When the concentration of carbon increased to 1.0%, the embryoids induction rate increased up to 93.25%.

Table 3 Effect of low-temperature pretreatment of the flower buds for the embryoids induction rate

serial number	CK	1	2	3	4
different periods of 4°C cold pretreatment of the flower buds/d	0	1	2	3	4
induction rate of embryos induced/%	79.63b	106.48a	87.96b	64.81c	45.37d

Table 4 Effect of preculture temperature during the first week and the combinations and concentrations of growth regulators on embryoids induction rate

serial number	temperature during the first week/°C	embryoids induction rate /%	combination of growth regulators/mg·L ⁻¹	embryoids induction rate /%
CK	28	19.44e	0	82.41b
1	4	70.37b	0.5ZT + 0.5IAA	57.41c
2	7	100.93a	0.5ZT + 1.0IAA	147.22a
3	32	52.85c	1.0ZT + 0.5IAA	42.59c
4	35	35.18 d	1.0ZT + 1.0IAA	25.93d

4 Discussion

4.1 Pretreatment temperature

As for the pretreatment temperature on isolated microspore culture in pepper, the high temperatures were often used (Dumas De Vaulx et al., 1981; Zhuang et al., 2001; Kim et al., 2008). Ramon et al. (1997) and Supena et al. (2006b) proved that low temperature could also induce the formation of embryoid and had a higher embryoids induction rate. In this study, low and high temperatures were used for anther preculture, and it showed that the effect of low temperature treatment was better than heat treatment. The possible cause of promoting the microspore embryoid formation by higher or lower temperature treatment was that the adversity might change the normal development of gametes microspore pathway (Liu et al., 1995; Shen et al., 1999; Zhao et al., 2007).

4.2 The system of culturing

There are many media that can be used for anther and isolated microspore culture in pepper, such as MS, B5, H, NLN, C, R, NLNS, CP, and so on. The main systems of culture are solid culture and liquid culture. Dumas De Vaulx et al. (1981) used two kinds of solid medium to induce pepper embryoid formation and increased the induction rate from 5% to 40%. Kim et al. (2008) obtained over 54 embryos on individual plate in two kinds of liquid media to culture. Using a double-layer medium, Ramon et al. (1997) obtained 3 to 750 embryos per 100 flowers, and Supena et al. (2006a; 2006b) produced four to seven plants per original flower bud with best genotypes. In this study, we used the double-layer medium to culture the pepper microspores and obtained a frequency of 147.22%. The reason is that the activated carbon in the solid medium (under layer) can effectively absorb the harmful material, and the liquid medium (upper layer) can provide adequate nutrients that are required by microspore development.

4.3 Growth regulators

Growth regulators play important roles in pepper microspore culture. Ramon et al. (1997) reported that they

induced embryoid successfully without any growth regulators, but supplement of growth regulators generally can promote embryo formation. In the present study, the combination of 0.5 mg·L⁻¹ ZT and 1.0 mg·L⁻¹ IAA was very promising, and the rate of embryoid induction was as high as 147.22%, which has exceeded the growth regulators concentration of 0.01–0.5 mg·L⁻¹ of the common range (Zhang et al., 2006). It indicated that the different genotypes of pepper required different combinations and concentrations of growth regulators.

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