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Improved efficiency of microspore culture of *Brassica campestris* ssp. *pekinensis* (Chinese cabbage)

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We selected two cultivars of *Brassica campestris* ssp. *pekinensis* (Chinese cabbage), 'Futian 50' and 'Changkuai,' to investigate the factors that influence microspore embryogenesis and plantlet regeneration. We have also discussed some protocols for the induction culture of microspore-derived embryos and for rooting and transplantation of microspore-derived plantlets. We obtained the following findings in our study. Although NAA promotes the development of embryo and reduces the percentage of abnormal embryos, it inhibits the formation of microspore-derived embryos. 2,4-D also inhibits the formation of microspore-derived embryos. Low concentrations of cytokinins facilitate embryogenesis, while high concentrations inhibit embryogenesis; BA has a stronger influence than zeatin (Z) on embryo induction. The combined effects of auxin and cytokinin are synergistic. Organic compounds increase the rate of formation of microspore-derived embryos. However, activated charcoal (AC) inhibits embryo development. The better the development of the embryos, the higher is the plantlet regeneration rate. The plant regeneration rate increased significantly on the MS culture medium supplemented with 200 mg·L^{-1} AC. The MS medium s suitable for the subculture of the regenerated plantlets. MS medium containing 0.1 mg·L^{-1} NAA is the optimal medium for rooting of microspore-derived plantlets.

Key words: Chinese cabbage, microspore culture, embryogenesis, plant regeneration.

INTRODUCTION

Doubled haploids (DHs) are now being commonly used in plant breeding as an important means of accelerating the development of new cultivars (Yao et al., 2008) or as one of the favored mapping populations for constructing linkage maps to identify quantitative trait loci (Pilet et al., 2001; Zhao et al., 2005; Yang et al., 2008). In recent years, isolated microspore culture has attracted a lot of interest because of its great potential for DH production. A microspore-derived embryo is also a suitable system for gene mapping (Graner, 1996; Zhang et al. 2005; Geng et al., 2007; Yu et al., 2008), genetic transformation (Stöger et al., 1995) and selection of dominant and recessive traits (Polsoni et al., 1988; Swanson, 1989; Liu et al., 2003; Wang et al., 2008; Zou et al., 2009). Since Lichter (1982) first reported the embryogenesis in microspore cultures of *Brassica napus*, embryogenesis has been induced in microspore cultures of different *Brassica* species (Babbar et al., 2004). Since the first report on

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successful regeneration of haploid plants from isolated microspores of *Brassica campestris* ssp. *pekinensis* (Chinese cabbage) (Sato et al., 1989), the technique of isolated microspore culture has been considerably improved and embryogenesis can be induced effectively (Cao et al., 1992; Li et al., 1993; Liu et al., 1997; Shen et al., 1999; Zhao et al., 2008; Fang et al., 2009). However, the effective use of the technique in terms of breeding progress is impeded by limitations such as low embryo yield and poor plant regeneration from microspore-derived embryos.

Plant hormones play an important role in embryogenesis and organogenesis. However, only few studies have shown the role of phytohormones in the induction of microspore and embryonic development in Chinese cabbage. Moreover, no study has compared the effects of different types of auxins and cytokinins on microspore embryogenesis. Therefore, our study was designed to investigate the effects of different types of auxins and cytokinins on the microspore embryogenesis in Chinese cabbage. In addition, we determined the concentration of the hormone that was the most suitable for enhancing the rates of induction and germination of microspore embryos and which would thereby improve the efficiency of microspore culture. Therefore, we systematically studied the optimal conditions required for the induction of embryogenesis and germination of microspore-derived embryos, growth of these embryos, and the rooting and transplantation of the regenerated plantlets. Our findings may lead to considerable improvement in the efficiency of the microspore culture and increase its application in the breeding program of Chinese cabbage.

MATERIALS AND METHODS

Plant material for culture

B. campestris ssp. *pekinensis* 'Futian 50' and *B. campestris* ssp. *pekinensis* 'Changkuai' were used as the experimental materials. The seeds were vernalized at 4°C for 26 days and planted in 10 cm pots. At the 6-leaf stage, the young plants were transferred to a 3 L flower pot. The plants were cultivated in a greenhouse under controlled conditions (25°C/18°C, 16 h photoperiod). Fluorescent lamps were used as the light source, and the light intensity was approximately 300 $\mu E \cdot m^{-2} \cdot s^{-1}$. The plants were supplied with nitrogen-phosphorus-potassium (NPK) compound fertilizer every 2~3 days. The plants flowered by the end of May, and the flower buds were harvested for microspore culture.

Microspore culture

The procedures for microspore isolation and culture were based on the methods established by Sato et al. (1989) ith some modifications. When most of microspores reached the uninucleate stage (determined on the basis of bud size; usually 2.5~3.5 mm in length), 30 flower buds were harvested. The buds were washed under running water for 30 min, then surface-sterilized in 70% ethanol for 30 s, and then in 0.1% $HgCl_2$ for 8 min; this procedure was followed by 3 rinses for 3 min in sterile distilled water. The flower buds were dried and held with a glass rod in B5 medium (Gamborg et al., 1968) supplemented with 13% (w/v) sucrose (B5-13); subsequently, the microspores were squeezed out. The fluid containing the microspores was filtered through a 50 µm-mesh nylon screen. Then, the filtrates were collected in a 10 ml centrifuge tube and centrifuged at 1000 rpm for 3 min. After discarding the supernatant, the pellets were resuspended in 5 ml of B5-13 and centrifuged at 500 rpm for 3 min; this procedure was repeated twice. The supernatant was discarded, and a pure preparation of microspores was obtained.

This preparation was suspended in the Nitsch and Nitsch (NLN) medium (Lichter, 1982) supplemented with 13% (w/v) sucrose (NLN-13) and cultured in 6 cm petri dishes. Each dish contained 4 ml suspension of microspores at a concentration of $1\sim2 \times 10^5$ microspores/ml. The petri dishes were sealed with double layers of parafilm, and the microspores were cultured in the dark at 33°C for 1 day and later cultured at 25°C in the dark.

Experiment 1: Effects of auxin and cytokinin on the rate of embryo formation

To investigate the effects of auxin and cytokinin on microspore embryogenesis, we cultured the microspores in the NLN-13 culture media with different concentrations of BA, Z, NAA, 2, 4-D (Sigma, USA). The NLN-13 culture medium is control group. After 3 weeks of microspore culture, the yields of the various developmental stages of the embryo were examined.

Experiment 2: Effect of organic compounds on microspore embryogenesis

To investigate the effect of organic compounds on microspore embryogenesis, we cultured the microspores of *B. campestris* ssp. *perkensis* 'Futian 50' in NLN-13 without glutathione, serine, and glutamine. The NLN-13 culture medium is control group. After 3 weeks of culture, the embryo yields were determined.

Experiment 3: Effect of activated charcoal on microspore embryogenesis and plantlet regeneration

To investigate the effect of AC on microspore embryogenesis, we cultured the microspores in NLN-13 supplemented with 100 or 200 $mg \cdot L^{-1}$ AC. The NLN-13 culture medium is control group. After 3 weeks of culture, the embryo yields were determined. Four weeks after the microspores were cultured in the induction medium, the cotyledonous embryos that emerged from the microspores were transferred onto solid MS medium (supplemented with AC 100, 200, or 400 $mg \cdot L^{-1}$) and incubated at 25°C with a 16 h photoperiod regime and a light intensity of 200 $\mu E \cdot m^{-2} \cdot s^{-1}$. After 3 weeks, the regenerated plantlets were counted.

Experiment 4: Subculture and transplantation of the regenerated plantlet

The large leaves and roots of the plantlets were excised, the 2~3-cm-long microspore-derived plantlets were transferred to the MS subculture medium and cultured at 25°C under a 16 h photoperiod regime and a light intensity of 200 $\mu E \cdot m^{-2} \cdot s^{-1}$. The 2~3-mm-long seedlings were transferred to MS or MS + 0.1 $mg \cdot L^{-1}$ NAA for rooting. After 3 weeks of culturing in these media, the rooting rate was determined. We selected 3 cm-tall microspore-derived plantlets with well-developed roots for transfer to the soil. The culture bottle was opened 1~2 days before the plantlets were transferred. After washing the agar deposits on the roots, we transferred the

Table 1. Effect of auxins on the rate of formation of microspore-derived embryos.

NAA content (mgL^{-1})	2,4-D content (mgL^{-1})	No. of embryos /30 buds	
		Futian50	Changkuai
0 (CK)	0	136	61
0.1	0	107	43
0.2	0	112	43
0.5	0	98	40
1.0	0	81	25
0	0.2	0	0
0	0.5	0	0

Table 2. Effect of cytokinins on the rate of formation of microspore-derived embryos.

BA concentration ($mg·L^{-1}$)	Z concentration ($mg·L^{-1}$)	No. of embryos/30 buds	
		Futian 50	Changkuai
0 (CK)	0	136	61
0.05	0	137	67
0.1	0	162	73
0.2	0	151	90
0.4	0	183	112
0.6	0	118	93
0	0.05	136	73
0	0.1	155	81
0	0.2	160	96
0	0.4	118	75
0	0.6	98	71

regenerated plantlets to a loam-vermiculite mixture (loam:vermiculite = 1:1 mixture sterilized by high temperature treatment) and were maintained in a culture room (25°C/18°C, natural light). For the first week, the plantlets were covered with plastic sheets. Three weeks after the transfer, the survival rate of the plantlets was calculated. The above procedure was repeated at least 3 times with 3~5 replicates per treatment.

RESULTS

Effect of auxin on the rate of formation of microspore-derived embryos

Addition of NAA to the media reduced the yield of embryos (Table 1). When 'Futian 50' microspores were cultured in MS medium with NAA concentrations ranging from 0.1 to 1.0 $mg·L^{-1}$, the average percentage of microspore-derived embryos was 26.84%, which was lower than that of microspore-derived embryos in the control medium.

Similarly, when 'Changkuai' microspores were cultured in MS medium with 0.2 and 0.5 $mg·L^{-1}$ of NAA, the percentage of embryos was low, at 29.51 and 34.43%, respectively.

In contrast, when the microspores were cultured on MS medium containing either only 2,4-D (0.2 or 0.5 $mg·L^{-1}$) or 2,4-D in combination with BA and Z, few cell clusters developed and no embryos developed. These results indicated that 2,4-D strongly inhibited the formation of microspore-derived embryos.

Effect of cytokinin on the rate of formation of microspore-derived embryos

When the concentrations of BA and Z in the medium were increased, the rate of formation of microspore embryogenesis increased at first and then decreased later (Table 2). This finding suggested that lower concentrations of BA and Z promoted embryogenesis, while higher concentrations of BA and Z inhibited embryogenesis (effective concentrations: BA, 0.4 $mg·L^{-1}$; Z, 0.2 $mg·L^{-1}$). Although both BA and Z had the same mechanism of embryo induction, the magnitude of their effect was different. In this experiment, the effect of BA was stronger than that of Z, regardless of whether the effect was the promotion or the inhibition of microspore embryogenesis.

Table 3. Combined effect of the auxins and cytokinins on the rate of formation of microspore-derived embryos of *Brassica campestris* ssp. *pekinensis* 'Futian 50'.

Hormone concentration (mg·L^{-1})			No. of embryos/30 buds
BA	Z	NAA	
0 (CK)	0	0	136
0.05	0	0.2	118
0.2	0	0.2	133
0	0.05	0.2	89
0	0.2	0.2	129

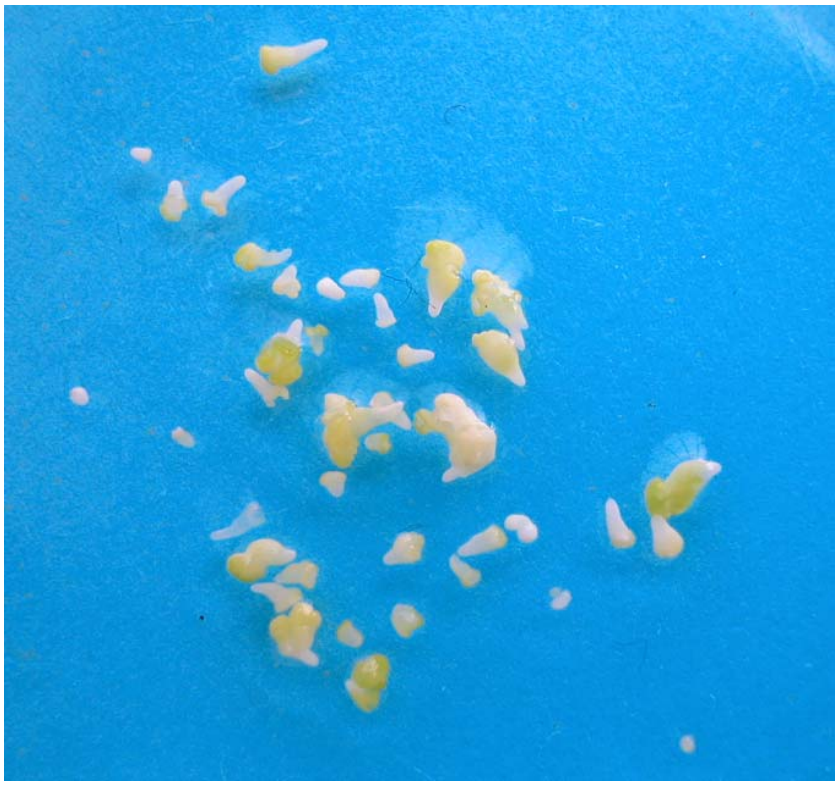

Figure 1. Embryos developed from microspores after 3 weeks in culture.

Combined effects of auxin and cytokinin

A set of experiments was performed to evaluate the combined effects of auxin and cytokinin (Table 3). The rate of induction of microspore-derived embryos in the induction medium containing BA or Z together with 0.2 mg·L^{-1} NAA was lower than that of embryos in media supplemented with the same concentration of only BA or only Z. These were also lower than that in the control MS medium but were higher than that in the medium containing only 0.2 mg·L^{-1} NAA. These results indicated that the combined effect of NAA and BA or Z was synergistic.

Effect of phytohormones on the development of microspore-derived embryos

Four weeks after the microspores were cultured in the induction media, various developmental stages of the embryos existed simultaneously (Figure 1), and these stages were cotyledonary embryo, torpedo embryo, heart embryo, globular embryo, abnormal embryo, and germinated embryo. Although NAA inhibited the formation of microspore-derived embryos, it promoted the development of the embryos. After 4 weeks of microspore culture in the medium with NAA, the percentage of cotyledonary and germinated microspore-derived embryos

Table 4. Effect of developmental stages of embryos on the plantlet regeneration rate.

Developmental stages of embryos	Number of embryo	Number of regenerated plantlet	Rate of regenerated plantlet (%)
Cotylednary embryo	360	199	55.28[a]
Torpedo embryo	165	7	4.2[b]
Heart and globular embryo	300	0	0[c]

The differences of the values followed by different letters in the column are significant at $p \leq 0.05$.

increased on an average from 84.42 to 88.75%. Concurrently, the percentage of abnormal embryos dropped from 7.35 to 4.94%. We observed that higher the concentration of NAA, the more obvious was the effect. The most favorable effects of NAA were observed at a concentration of 1.0 mg·L^{-1}: the percentage of cotyledonary and germinated embryos was 90.86%, but the percentage of abnormal embryos was only 3.70%.

When cytokinin was added to the medium, the percentage of cotyledonary and germinated embryos decreased while the percentages of heart and globular embryos increased. The percentage of abnormal embryos greatly increased, especially when the induction medium contained higher concentrations (0.4~0.6 mg·L^{-1}) of Z or BA. The yield of abnormal embryos could be up to 19.39 and 26.27% when the media contained 0.6 mg·L^{-1} Z and BA, respectively. The above results suggested that the cytokinin exerted adverse effects on the development of microspore-derived embryos and inhibited embryo development to a certain extent. High concentrations of cytokinin could promote abnormal development of microspore-derived embryos.

Effect of organic compounds on the rate of formation of microspore-derived embryos

In this experiment, only the microspores of 'Futian 50' were used. We separated and purified the microspores and then cultured them in NLN-13 without glutathione, serine, and glutamine to determine the effect of the organic compounds on the rate of formation of microspore-derived embryos. Our results showed that the rate of embryo induction was 80%, which was lower than that observed in the control. This indicated that glutathione, serine, and glutamine were essential for microspore embryogenesis in Chinese cabbage.

Effect of the developmental stage of embryo on the plantlet regeneration rate

The embryo development was asynchronous. After 3 weeks of culture, in addition to cotyledonary embryos and torpedo embryos, small globular embryos and heart embryos were observed. The rate of embryo development decreased after 4 weeks of culture, regardless of the embryo size, probably because of the nutrient depletion in the medium. The cotyledonary, torpedo, heart, and globular embryos were inoculated separately in the MS regeneration medium, and after 3 weeks, the plantlet regeneration rate was analyzed, as shown in Table 4. Some microspore-derived embryos germinated normally in the regeneration media. They continued to grow into normal plantlets (Figure 2). Table 5 shows that the plantlet regeneration rate was closely associated with the developmental stage of embryos. The rate of regeneration from mature cotyledonary embryos was 55.28%, which was higher than the percentage of incomplete embryos. Culturing of early embryos in the regeneration media was difficult. The heart and globular embryos could not regenerate into plantlets.

Effect of AC on microspore embryogenesis and plantlet regeneration

The microspores were cultured in NLN-13 supplemented with 100 or 200 mg·L^{-1} AC. Our results indicated that AC did not promote embryogenesis (data not shown). The effect of AC on plantlet regeneration is shown in Table 5. The regeneration rate was higher in the regeneration medium supplemented with AC than it was in the control medium. A maximum regeneration rate of 44.5% green plants was observed on the medium supplemented with 200 mg·L^{-1} AC. Our results showed that AC promotes plant regeneration.

Subculture and transplantation of microspore-derived plantlets

The initially obtained microspore-derived plantlets were weak. The slender seedlings grew much stronger after 2~3 subcultures. The leaves of plantlets become larger as their color changed from yellow to green; concurrently, their petioles became thick. MS + 3% sucrose + 0.8% agar" was the optimal medium for subculture. The seedlings were transplanted to the rooting media. The rooting rate was 100% in MS + 0.1 mg·L^{-1} NAA medium and 70% in only MS medium. After 20 days of culture in the rooting medium, 10~15 adventitious roots developed.

Figure 2. Normal germination from a microspore embryo.

Table 5. Effect of activated charcoal on plantlet regeneration rate.

AC concentration ($mg \cdot L^{-1}$)	No. of embryos	No. of regenerated lantlets	Regeneration rate of the plantlets (%)
0 (CK)	180	36	20.0[a]
100	135	34	25.2[a]
200	180	80	44.5[b]
400	150	36	24.0[a]

The differences of the values followed by different letters in the column are significant at $p \leq 0.05$.

The type of rooting media affected the quality of the plantlets. Plants grown in media without NAA had a larger number of roots than those grown in media with NAA. The root growth rate was different in different media. The roots were delicate when plantlets were grown in media without NAA, while the root system was strong when plantlets were grown in media containing 0.1 $mg \cdot L^{-1}$ NAA. The root growth rate and the number of plantlet leaves were not different in the media with or without NAA.

Although a strong root system was observed in the medium supplemented with NAA, the number of roots was relatively low, while the survival rate of the plantlet was high during transplantation. The average survival rate of the plants rooting on MS + 0.1 $mg \cdot L^{-1}$ NAA was 90%. A weak root system was observed in the seedlings grown in the medium without NAA, and the survival rate during transplantation was 73.33%, which was lower than that of the plantlets growing on medium with 0.1 $mg \cdot L^{-1}$ NAA. The average survival rate of plantlets was 73.33% (Figure 3).

DISCUSSION

The addition of auxins and/or cytokinins to the induction media has been shown to influence microspore

Figure 3. A regenerated plant that was transferred to the pot.

embryogenesis in *Brassica* species (Lichter, 1981; Charne and Beversdorf, 1988; Li, 2004). There are a few studies on the effect of phytohormones on the formation of microspore-derived embryos in Chinese cabbage, and their results have been quite inconsistent. According to Sato et al. (1989), the effect of 0.5 $mg \cdot L^{-1}$ NAA + 0.05 $mg \cdot L^{-1}$ BA (basal medium, NLN) on induction of embryo formation was the same as that of the medium without plant growth regulators. Many researchers have also used the culture media without plant growth regulators. However, Xu et al. (2001) and Zhang et al. (2009) reported BA at a concentration of 0.2 $mg \cdot L^{-1}$ promoted embryo development. The results of our study were not completely consistent with the above mentioned reports. Our results showed that auxins inhibited microspore embryogenesis, and lower concentrations of BA or Z promoted embryogenesis, while high concentrations inhibited embryogenesis.

Our study is the first that describes the effect of phytohormones on the development of microspore-derived embryos in Chinese cabbage. The mechanism of how phytohormones regulate the development of microspore-derived embryos is unknown and will be studied in future. Shen et al. (1999) reported that addition of 0.05~0.10 $mg \cdot ml^{-1}$ AC increased the embryo yield of Chinese cabbage more than 2 times. Jiang et al. (2008) reported that addition of 0.50 $mg \cdot ml^{-1}$ AC increased the embryo yield by 4.8 or 16.1 times in the microspore cultures of the Y536 and Y535 of Chinese cabbage. However, in our study, the addition of AC did not increase the induction frequency of microspore-derived embryos.

The success of haploid technology ensures the successful conversion of microspore-derived embryos into plants. Direct germination of microspore-derived embryos resulted in a plumular shoot and a root that was expected in microspore cultures. Generally, the rate of direct germination of embryos in microspore cultures in *Brassica* species has been significantly low. A few reports have described the difficulties in the germination of microspore-derived embryos in the microspore culture of Chinese cabbage (Liu et al., 1997; shen et al., 1999; Jiang et al., 2008). Our results indicate that AC was helpful for obtaining high rate of direct plantlet regeneration.

The formation of abnormal embryos reduced the regeneration rate of microspore-derived plantlets. Although this is a common occurrence in microspore culture, the mechanism of abnormal embryo formation has not been discussed much. On the basis of the results of this experiment, we assume that cytokinins influence the development of microspore-derived embryos. High concentrations of cytokinin can result in the abnormal development of microspore-derived embryos of Chinese cabbage.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Abbreviations: DH, doubled haploid; **NAA,** naphthalene acetic acid; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **BA,** 6-benzylaminopurine; **Z,** zeatin; **AC,** activated charcoal; **MS,** Murashige and Skoog Stock Medium.

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