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Efficient doubled haploid production in microspore culture of Zengcheng flowering Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* [L.] Makino var. *utilis* Tsen et Lee)

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ABSTRACT

Microspore culture has been implemented in breeding programs to produce doubled haploids (DH) and thus accelerate the breeding process. This study aimed to establish an efficient microspore culture protocol for Zengcheng flowering Chinese cabbage, a highly nutritious and valued vegetable, that would be suitable for heterosis breeding. Microspores of three genotypes, 17AY010, 17AY011, and 17AY012 were successfully induced to produce embryos in NLN-13 medium. Two genotypes, 17AY011 and 17AY012 were cultivated in NLN-13 medium supplemented with different concentrations (0, 0.2, 1, 5 or 25 μM) of L-ascorbic acid sodium salt (Vitamin C Sodium Salt, VcNa) to enhance microspore embryogenesis and plant regeneration without an intervening callus phase. In both genotypes, the addition of 1 μM and 0.2 μM VcNa significantly increased the frequency of embryogenesis, 6.55-fold and 10.33-fold as compared with the control, respectively. The optimum concentration of VcNa application that would improve the rates of direct plant regeneration in the two tested genotypes was 1 μM. Moreover, the doubled haploid rates of regenerated plants of the three genotypes were above 60%. Five DH lines of 17AY012 with high yield were obtained. According to the measurement of the affinity index, they were prospective self-incompatible lines for hybrid breeding.

1. Introduction

Zengcheng flowering Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* [L.] Makino var. *utilis* Tsen et Lee) is a variety of *Brassica* vegetable crop with edible flower stalk typical for Zengcheng City in Guangdong Province, China. It is appreciated for its fresh, crisp, sweet flavor, and high nutritional value (Liu et al., 2009). As more people are attracted to this variety, its market demand and cultivation area continue to increase steadily. Zengcheng flowering Chinese cabbage is a typical cross-pollinated plant with significant levels of heterosis, and therefore homozygous parental materials play an important role in the breeding of this hybrid.

Compared with conventional breeding methods, microspore culture is an effective technology for rapid development of doubled haploid lines, which can significantly accelerate the breeding process (Zhou et al., 2002; Ferrie and Caswell, 2011). Since Lichter (1982) successfully created embryos of *Brassica napus* L. by using microspore culture, this technique has been used in many plant species. For example, Cao et al. (1992) produced 200 Chinese cabbage regenerated plants by isolated microspore culture, and the method has been successfully implemented in many other *Brassica* crops such as broccoli (*B. oleracea* L. var. *italica*, Takahata and Keller, 1991; Duijs et al., 1992), tronchuda cabbage (*B. oleracea* L. var. *costata*, Vicente and Dias, 1996), ornamental kale (*B. oleracea* L. var. *acephala*, Zhang et al., 2008), and sauerkraut cabbage (*B. oleracea* L. var. *capitata*, Cao et al., 1990).

The induction frequency of microspore-derived embryos is influenced by many factors including donor plant genotype, the developmental stage of microspores, culture conditions, and culture medium (Babbar et al., 2004; Bhowmik et al., 2011; Ferrie and Caswell, 2011; Winarto and Teixeira da Silva, 2011). Many studies have shown that the addition of appropriate amount of chemical reagents to the medium can increase microspore embryogenesis. For instance, adding 1 μM of aminoethoxyvinylglycine to the NLN-13 medium can significantly increase embryo yield of Brussels sprouts (Zeng et al., 2015a), whereas addition of trichostatin A (0.05 μM), suberoylanilide hydroxamic acid (0.05 μM and 0.10 μM), and sodium butyrate (2 μM) effectively increased the

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frequency of microspore embryogenesis and plant regeneration in pakchoi (Zhang et al., 2016). In addition, Zeng et al. (2017) reported that the addition of 10 mg/L ascorbate and 20 mg/L glutathione in ½ NLN medium increased the rate of embryo induction of broccoli. Adding organelle antioxidants to the microspore induction medium of *Brassica napus* and white cabbage allows mitochondria to maintain normal content of reactive oxygen species (ROS), which ensures normal development of microspores, and increases the frequency of microspore-derived embryogenesis (Hoseini et al., 2014; Zeng et al., 2015b).

To date, the application of microspore culture in Zengcheng flowering Chinese cabbage has not been reported. Given its limited success in *Brassica rapa* L., we investigated the effects of different genotypes and addition of organelle antioxidant VcNa on microspore embryogenesis and plant regeneration. The ploidy level of regenerated plants, the morphological characters and plot yield of DH lines were identified. The main purpose of this study is to establish an efficient microspore culture protocol in Zengcheng flowering Chinese cabbage and get DH lines that can be applied to the practice of heterosis breeding.

2. Material and methods

2.1. Plant material

The heterozygous, self-incompatible donor plants were three genotypes of Zengcheng flowering Chinese cabbage that were imported from Guangdong Province, China. In order to guarantee the uniformity of the microspores for genotype and VcNa treatment, buds from all plants were selected and mixed into groups equally. The codes and basic characteristics of the three genotypes are shown in Table 1. The seeds were sown in the greenhouse at the genetic breeding experimental base of Shenyang Agricultural University in Liaoning Province. When the plants developed four leaves, about 12 cm height, they were transplanted into flowerpots with a diameter of 20 cm and cultivated in the greenhouse under a photoperiod of 16 h and at a day/night temperature of 25/15 °C. Once first flowers developed, the top inflorescences were removed, and after the lateral branches blossomed again, the buds were harvested for microspore culture.

2.2. Microspore culture

This experiment followed the microspore culture methods described by Sato et al. (1989) and Hoseini et al. (2014). The pest- and disease-free inflorescences were harvested on sunny days at 10:00 and pretreated at 4 °C for 24 h. Flower buds with the ratio of petal length to anther length of 1/3–4/5 were selected for microspore culture. The buds were sterilized in 75% ethanol (30 s), and 0.1% mercuric chloride solution (6 min) and rinsed with sterilized distilled water three times for 5 min each. The buds were macerated in 10–15 mL B5 liquid medium with 13% (m/v) sucrose, pH 5.84 (Gamborg et al., 1968) using sterile glass rods to release the microspores. The microspores were filtered into a 100 mL beaker through a 74 μm stainless steel cell sieve. The obtained microspore suspension was filtered into 50 mL centrifuge tubes through a 40 μm cell sieve and centrifuged at 2000 rpm for 3 min. The supernatant was removed and 30 mL of B5 medium was added to the pellet and the suspension was centrifuged for additional 3 min. The supernatant was discarded and the pellet was resuspended in NLN-13 medium (pH 5.84) at a cell density of 1×10^5–2×10^5 microspores·mL^{-1}. The microspore suspension was divided into sterile plastic culture dishes (60 mm × 15 mm), 5 mL per dish, and 100 μL activated carbon solution (0.05 g agarose and 1 g activated carbon suspended in 100 mL double-distilled water) were added to each dish. Finally, the Petri dishes were sealed with Parafilm membranes and incubated at 33 °C for 24 h, and then transferred to an incubator, cultured at 25 °C in the dark. Once the embryoids were visualized to the naked eye (about 0.5 cm long, about 10 days after microspore culture), the Petri dishes were transferred to a rotary shaker (25 °C, 50 rpm).

Table 2
Effect of three genotypes on microspore embryogenesis of Zengcheng flowering Chinese cabbage.

Code	No. of embryos per bud ± SD
17AY010	0.16 ± 0.04b
17AY011	0.42 ± 0.02a
17AY012	0.21 ± 0.02b

Note: Means followed by different lowercase letters are significantly different at $P = 0.05$ level.

2.3. Effect of genotypes on microspore embryogenesis

After 21 days, the number of embryos of the three genotypes 17AY010, 17AY011, and 17AY012 was recorded and the difference in microspore embryogenesis among the genotypes was analyzed.

2.4. VcNa treatments

Two varieties, 17AY011 and 17AY012, were used for VcNa treatments. The VcNa stock solution with a concentration of 200 μM was dissolved in double-distilled water. The stock solution was filter-sterilized. After dispensing 5 mL microspore suspension into the Petri dish, different volumes of VcNa were added. The final concentration in the NLN-13 medium was 0, 0.2, 1, 5 or 25 μM.

2.5. Plant regeneration and ploidy level of the regenerated plants

The cotyledonary embryos with a length of 3–4 mm from different VcNa concentrations in the NLN medium were inoculated into the conventional solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (m/v) sucrose, 0.55% (m/v) agar, and 0.1% (m/v) activated carbon with pH adjusted to 5.84. After 3 weeks of incubation at 25 ± 1 °C and 16 h/8 h photoperiod, the rates of embryos directly converting to plants, formation of callus, and mortality were recorded. Flow cytometry was used to identify ploidy of plants regenerated by microspore culture for each genotype (Niu et al., 2015).

2.6. Morphological traits identification and plot yield analysis of DH lines

Eleven DH lines of 17AY012 were sown in 32-hole seedling trays on 1 st August 2017, in the plastic tunnel, and after a month they were planted in the field. Complete random design was used with three

Table 1
Donor plants used for microspore embryogenesis of *Brassica campestris* ssp. *Chinensis* var. *utilis*.

Genotype code	Variety	Characters
17AY010	sweet Zengcheng flowering Chinese cabbage	medium-maturing, fewer lateral tillers, bright green leaf; main stalk height 18–26 cm, main stalk width 2–4 cm
17AY011	late-maturing Zengcheng flowering Chinese cabbage	late-maturing, more lateral tillers, light green leaf; main stalk height 20–25 cm, main stalk width 2–4 cm
17AY012	late-maturing Zengcheng flowering Chinese cabbage, extra-sweet	late-maturing, more lateral tillers, light green leaf; main stalk height 20–28 cm, main stalk width 2–3 cm