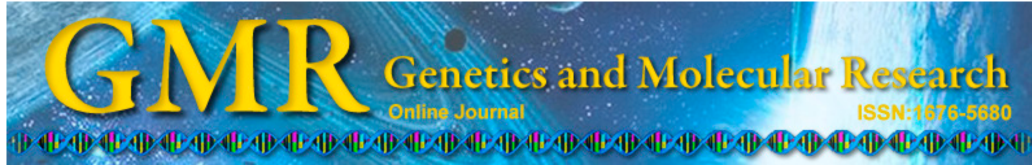

Effects of *p*-chlorophenoxyisobutyric acid, arabinogalactan, and activated charcoal on microspore embryogenesis in kale

R.Q. Niu, Y. Zhang, Y. Tong, Z.Y. Liu, Y.H. Wang and H. Feng

College of Horticulture, Shenyang Agricultural University, Shenyang, China

Corresponding author: H. Feng
E-mail: fenghuiaaa@263.net

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ABSTRACT. To improve embryogenesis in microspore cultures of kale (*Brassica oleracea* L. var. *acephala* DC.), 6-benzylaminopurine (6-BA), naphthaleneacetic acid (NAA), arabinogalactan (AG), *p*-chlorophenoxyisobutyric acid (PCIB), and activated charcoal (AC) were added to the medium using four varieties of kale. The results showed that the addition of AG (0.1-0.2 g/L), AC (0.1-0.2 g/L) or a combination of 6-BA (0.1-0.2 mg/L) and NAA (0.1-0.2 mg/L) promoted embryogenesis. Adding 40 μM PCIB or a combination of 40 μM PCIB and 0.2 g/L AC to NLN-13 medium at pH 5.8 effectively enhanced embryogenesis. Treatment with a combination of 40 μM PCIB and 10 mg/L AG gave the highest rate of embryonic induction, especially in genotype "Y007," which showed a twelve-fold increase in yield.

Key words: Kale; Embryogenesis; *p*-Chlorophenoxyisobutyric acid; Arabinogalactan; Activated charcoal

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INTRODUCTION

Ornamental kale (*Brassica oleracea* var. *acephala*) is an important decorative foliage plant that displays a wide range of leaf colors and shapes and is thus widely used as a potted ornamental plant or a cut flower. Most of the commercial cultivars of kale are F_1 hybrids, which ensure high uniformity and yield as well as protection of plant breeders' rights and the markets of seed companies. The production of hybrid cultivars requires the use of homozygous parental lines, and thus, a simple and fast method to accelerate breeding programs is needed to meet the market's demands.

Kale is a 2-year-old cross-pollinated herb, and obtaining inbred lines requires at least 6-8 years using traditional breeding methods of self-pollination. However, inbred lines can be obtained in only 2 years by applying isolated microspore culture technology. Compared with the traditional production of genetically stable homozygous lines, which is labor-intensive and time-consuming, microspore culture is effective in accelerating the breeding process and facilitating the selection of fine recessive traits (Henderson and Pauls, 1992).

Lichter (1982) first reported the successful application of microspore culture techniques to *B. napus*, thereby establishing the basis for isolated microspore cultures for other *Brassica* crops. Subsequently, successful microspore cultures in some Ethiopian mustard (*B. carinata*), black mustard (*B. nigra*), cabbage (*B. campestris* ssp. *pekinensis*), broccoli (*B. oleracea* var. *italica*), and condiment mustard (*B. juncea*) were described by Chuong and Beversdorf (1985), Hetz and Schieder (1989), Sato et al. (1989), Takahate and Keller (1991), and Lionneton (2001), respectively.

Lichter (1989) first reported the successful application of microspore culture techniques to kale. Since then, some progress in developing this system has occurred, and considerable research effort has been devoted to increasing the frequency of embryogenesis in kale. Previous studies have shown that some factors such as the growth conditions of donor plants, developmental stages of microspores, and pretreatments influence microspore embryogenesis (Lichter, 1989; Jiang et al., 2005a; Jiang and Feng, 2006; Feng et al., 2007, 2009; Wang et al., 2011). In some studies, stress pretreatments, such as cold and heat shocks, were required to induce microspore embryogenesis (Wang et al., 2011), and the addition of naphthaleneacetic acid (NAA), 6-benzylaminopurine (6-BA; Lichter, 1989), 2,4-epibrassinolide (EBR; Jiang et al., 2006), activated charcoal (AC; Jiang et al., 2005b), or glutamine to the medium have increased embryogenesis in kale. Despite these efforts, however, reports published on microspore cultures of kale show low embryogenesis yields compared to other *Brassica* species. As reported by Jiang et al. (2005b), two genotypes of kale did not produce any embryos, and the frequency of embryogenesis in three other genotypes ranged from 0.10 to 0.58 embryos per bud. In the study by Feng et al. (2007), the highest frequency of embryogenesis was 0.73 embryos per bud in kale. Wang et al. (2011) stated that 'Zhouyehongxin' has the highest frequency of embryogenesis at 1.91 embryos per bud. Thus, the low frequency of embryogenesis has limited the application of this technology in hybrid breeding.

The present study was undertaken with the aim of increasing the frequency of embryogenesis in microspore cultures of kale. The study examined the effects of different concentrations of NAA, 6-BA, *p*-chlorophenoxyisobutyric acid (PCIB), arabinogalactan (AG), AC, and their combinations on kale embryogenesis.

MATERIAL AND METHODS

Donor plants and their growth conditions

Four F_1 hybrids of kale introduced by the Sakata Seed Corporation (Yokohama, Japan), ‘Bolangyehongxin’ (Y007; Figure 1a), ‘Zhouyehongxin’ (Y009; Figure 1b), ‘Zhouyebaixin’ (Y010; Figure 1c), and ‘Baikongque’ (Y020; Figure 1d), were used in the present study. Seeds were sown in plastic containers with a mixture of soil and sand in the middle of August 2011 and 2012. At the four-leaf stage in early September, the seedlings were transplanted to 15-cm-diameter pots and then transferred to a cold shed for cold treatment. At the beginning of November, the seedlings were moved to a greenhouse and the temperature was controlled at 25°C/10°C (day/night) with a 16-h photoperiod.

Figure 1. Donor plant materials for the microspore culture. a:Y007; b:Y009; c:Y010; d:Y020.

Microspore culture

The microspore culture procedure was based on the protocols of Lichter (1982) and Jiang et al. (2005a), with minor modifications. Flower buds were picked in the full bloom stage, and buds with a petal length to anther length ratio (P/A) of about 0.5-0.75 were selected to isolate microspores. The buds were pretreated at 4°C for 24 h and then surface-sterilized with 70% (v/v) ethanol for 30 s and treated with a 0.1% (w/v) chlorinated mercury solution for 5-8 min. The buds were rinsed three times using sterile, distilled water with shaking (5 min each time). After disinfection, 20 buds were crushed gently in 10 mL B5-13 medium (pH 5.8) with a sterilized glass rod to squeeze out microspores. The ho-

mogenate was then filtered through a 40-μm nylon filter into a 10-mL centrifuge tube. The microspore suspension collected was centrifuged for 3 min at 1100 rpm three times. The final pellet was then resuspended in filter-sterilized NLN medium (Licher, 1982) with 13% (w/v) sucrose. A 5-mL volume of microspore suspension was transferred to a sterilized 60 x 15-mm Petri dish and sealed with Parafilm. Petri dishes were incubated at 33°C for 24 h and then transferred to a 25°C incubator in the dark. Embryos formed after 18-22 days, at which time visible embryos were cultured with oscillation in a swing bed at low speed (70 rpm) at 25°C. One week later, large and healthy-looking embryos (Figure 2) were used for plantlet regeneration.

Figure 2. Embryos derived from Y007 by isolated microspore culture.

6-BA and NAA treatments

6-BA (10 mg/L) and NAA (10 mg/L) stock solutions were prepared in distilled water, filter sterilized, and stored at 4°C. As shown in Table 1, different volumes of NAA and 6-BA stock solution were added to NLN-13 liquid medium when preparing it. We then set the volume and adjusted the pH to 5.8. Other steps were as previously indicated (REFS). Y007, Y009, and Y010 were used to test the effects of NAA and 6-BA on microspore embryogenesis.

AG treatments

AG was dissolved in distilled water with gentle shaking at room temperature. The pH was adjusted to 5.8 with 1 N NaOH and 1 N HCl, and the solution was maintained in a refrigerator at 4°C until needed. AG (0, 5, 10, 15, or 20 mg/L) was added to Petri dishes after the step in which the microspore suspension was transferred to a sterilized 60 x 15-mm Petri dish. The other steps were as previously indicated (REFS). Y007, Y009, and Y010 were used as materials for this experiment.

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PCIB treatments

Y007, Y009, and Y020 were used as materials, and PCIB at concentrations of 0, 10, 20, 40, or 80 µM was added to the NLN-13 liquid medium (pH 5.8).

AC treatments

An AC suspension was prepared according to da Silva Dias (1999) procedure by combining an autoclaved suspension of 1 g AC, 0.5 g agarose, and 100 mL distilled water. To investigate the effect of AC on microspore embryogenesis in ornamental kale, samples of NLN-13 liquid media containing AC (0, 5, 10, 15, or 20 mg per Petri dish) were tested.

Plant regeneration

All embryos obtained from previous experiments were used for plant regeneration. When the embryos reached the cotyledon stage, they were transferred to solid hormone-free Murashige and Skoog (MS) medium supplemented with 3% sucrose, 0.1 g/L AC, and 7.5 g/L agar; the pH was adjusted to 5.8-5.84, and they were cultured in conical flasks at 25°C with a 16-h photoperiod under a photosynthetic photon flux density of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ emitted by cool-white tubular florescent lamps. When shoots developed from the embryos, they were excised from the callus or hypocotyl tissues and transferred to MS solid differentiation medium. This procedure was repeated until morphologically normal plantlets with roots were obtained. Seedlings were transferred to pots to continue growth (Figure 3).

Figure 3. Microspore-derived plantlets **a** = Callus; **b** = Subculture plant of Y009; **c** = Root of regeneration plant.; **d** = Regenerated plantlet of Y009 after planting.

Ploidy identification

The ploidy of the plantlets was identified by flow cytometry. First, 1- to 2-cm sections of fresh leaves were placed on a Petri dish. Next, 1.5-2.0 mL chopping buffer solution [15 mM Tris, 2 mM EDTA-Na_2, 0.5 mM spermine, 80 mM KCl, 20 mM NaC1, 0.1% (v/v) TritonX-100, and 15 mM *β*-mercaptoethanol, at pH 7.5] were added to the leaf pieces. The leaves were cut into pieces using medical scissors. The homogenate was filtered through a 300-mesh screen filter and transferred to a centrifuge tube. The sample was then centrifuged at 1000 rpm for 10 min. The supernatant was removed after centrifugation and 1 mL PI dye was added to the pellet, which was then mixed and allowed to stand for 15 min in the dark. The sample was filtered through a 500-mesh screen, placed in a tube, and examined in a FACSCalibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The DNA absorption peak of the wild type (diploid) was at approximately the 200 channel (on the *x*-axis); each plant was measured three times. Samples with DNA absorption peaks at the 200 channel were considered diploid, while samples with peaks at the 100 and 400 channels were haploid and tetraploid, respectively (Figure 4).

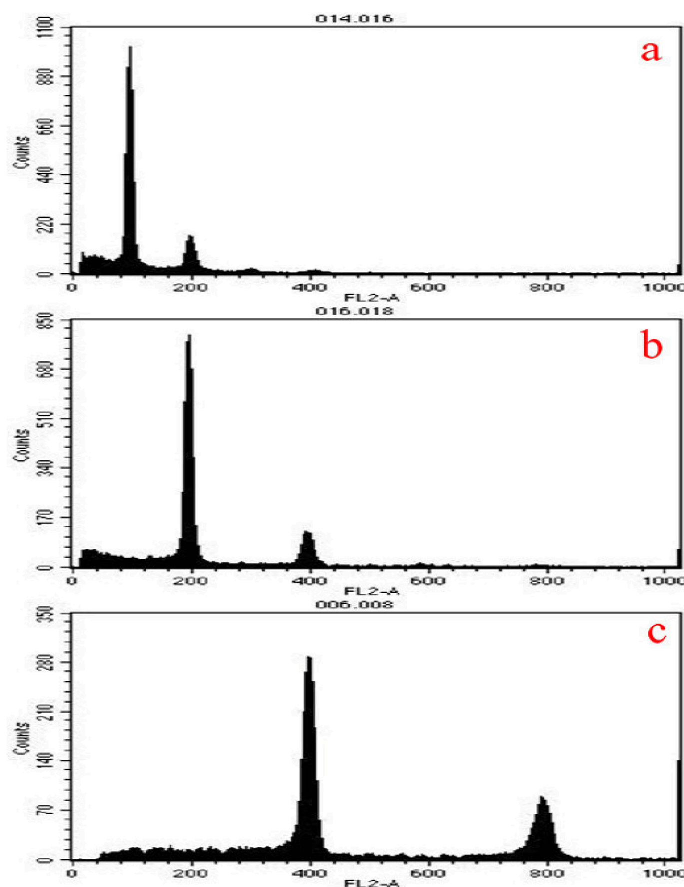

Figure 4. Ploidy identification by Flow Cytometer a: Haploid; b: Double haploid; c: Tetraploid.

Data analysis

This experiment had six treatments, and each treatment had three replications. The embryos were counted after 30 days of culturing in NLN liquid medium, and the frequency of embryogenesis was expressed in terms of the number of embryos per bud. Data were analyzed using the SPSS 12.0.1 statistical system for Windows (SPSS, 2007). ANOVA was performed, and the Duncan least significant difference (LSD) test (P = 0.05) was used to determine the significance of differences between the treatments.

RESULTS

Effects of 6-BA and NAA on microspore embryogenesis

Microspores were extracted from the Y007, Y009, and Y010 varieties and transferred to six kinds of NLN-13 medium with varying combinations of 6-BA and NAA, along with a control containing no 6-BA or NAA (Table 1). The combination of 6-BA and NAA that was most effective at increasing the rate of embryogenesis induction differed between the varieties. The highest rates were obtained with 0.1 mg/L 6-BA for Y007, 0.2 mg/L 6-BA and 0.2 mg/L NAA for Y009, and 0.1 mg/L 6-BA and 0.1 mg/L NAA for Y010. Embryogenesis was increased by the addition of hormones in all three varieties relative to the control. The addition of both 6-BA and NAA to the NLN-13 medium gave the highest rate of embryonic induction for the Y009 and Y010 varieties, but the addition of 6-BA alone produced the highest rate for the Y007 variety.

Table 1. Effects of 6-BA and NAA on the microspore embryogenesis of kale.

6-BA $mg \cdot L^{-1}$	NAA $mg \cdot L^{-1}$	No. of embryos per bud ± SD		
		Y007	Y009	Y010
0	0	0.783 ± 0.104^{e}	0.817 ± 0.104^{e}	0.217 ± 0.076^{d}
0.1	0	1.233 ± 0.104^{a}	1.017 ± 0.029^{d}	0.283 ± 0.029^{bcd}
0.1	0.1	1.100 ± 0.100^{b}	1.250 ± 0.087^{bc}	0.433 ± 0.076^{a}
0.1	0.2	1.017 ± 0.029^{bcd}	1.383 ± 0.029^{ab}	0.367 ± 0.029^{ab}
0.2	0	1.067 ± 0.029^{bc}	1.200 ± 0.050^{c}	0.250 ± 0.050^{cd}
0.2	0.1	0.950 ± 0.050^{cd}	1.333 ± 0.029^{bc}	0.350 ± 0.050^{ab}
0.2	0.2	0.900 ± 0.050^{de}	1.517 ± 0.176^{a}	0.317 ± 0.083^{bc}

Means followed by different letters are significantly different at P = 0.05 level.

Effect of AG on microspore embryogenesis

Microspores extracted from Y007, Y009, and Y010 were transferred to five different kinds of NLN-13 medium with AG concentrations of 0, 5, 10, 15, and 20 mg/L. The frequency of embryogenesis was recorded after 30 days (Table 2). Adding AG to the NLN-13 medium increased the embryonic induction rate, and the most effective AG concentration was 10 mg/L. Compared with the control (0 mg/L AG), the embryo yields of Y007, Y009, and Y010 increased 1.89-, 2.38-, and 3.18-fold, respectively.

Table 2. Effect of AG on the microspore embryogenesis of kale.

AG mg·L^{-1}	No. of embryos per bud ± SD		
	Y007	Y009	Y010
0	0.783 ± 0.076^{c}	0.800 ± 0.312^{bc}	0.183 ± 0.104^{d}
5	1.033 ± 0.104^{b}	1.083 ± 0.236^{bc}	0.467 ± 0.029^{ab}
10	1.483 ± 0.225^{a}	1.900 ± 0.218^{a}	0.583 ± 0.104^{a}
15	0.867 ± 0.076^{bc}	1.150 ± 0.132^{b}	0.367 ± 0.029^{bc}
20	0.467 ± 0.067^{d}	0.733 ± 0.076^{d}	0.233 ± 0.168^{cd}

Means followed by different letters are significantly different at P = 0.05 level.

Effect of PCIB on microspore embryogenesis

Three genotypes of kale were evaluated for their microspore embryogenesis response to four concentrations of PCIB (10, 20, 40, and 80 μM). All genotypes tested responded positively to PCIB, and embryogenesis increased significantly with the addition of 40 μM PCIB (Table 3). In this experiment, Y007 was the most responsive genotype, producing an average of 8.18 embryos per bud. In the absence of PCIB, embryogenesis in the three genotypes ranged from 0 to 0.75 embryos per bud; however, the addition of PCIB caused a 1.27- to 11.16-fold increase in the number of embryos. The frequency of embryogenesis decreased when the concentration of PCIB was greater than 40 μM.

In this experiment, the optimal concentration of PCIB could successfully induce embryos in the recalcitrant genotype Y020. No embryos were produced in NLN-13 liquid medium containing 0 or 80 μM PCIB. With the addition of 10, 20, or 40 μM PCIB, Y020 successfully induced embryos, and the best PCIB concentration was 40 μM. Although the recalcitrant genotype Y020 produced some embryos, the embryo yield was still low.

Table 3. Effect of PCIB on the microspore embryogenesis of kale.

PCIB μM	No. of embryos per bud ± SD		
	Y007	Y009	Y020
0	0.733 ± 0.076^{d}	0.750 ± 0.328^{c}	0.000 ± 0.000^{c}
10	1.533 ± 0.379^{c}	1.717 ± 0.501^{b}	0.117 ± 0.029^{c}
20	5.683 ± 0.375^{b}	2.400 ± 0.229^{b}	0.500 ± 0.132^{b}
40	8.183 ± 0.715^{a}	3.183 ± 0.651^{a}	1.267 ± 0.076^{a}
80	0.700 ± 0.132^{d}	0.950 ± 0.050^{c}	0.000 ± 0.000^{c}

Means followed by different letters are significantly different at P = 0.05 level.

Effect of AC on microspore embryogenesis

AC treatment had a significant effect on microspore embryogenesis in the Y007, Y009, and Y010 genotypes. Compared with the control, embryo yields increased when 0.1 or 0.2 g/L AC was added to the microspore culture medium, but when the concentration of AC was greater than 0.2 g/L, the embryo yield decreased (Table 4).

These results indicated that AC had an effect on microspore embryogenesis in kale. For Y007, the best result obtained was a 1.76-fold increase in the frequency of embryogenesis in medium with 0.2 g/L AC compared to medium without AC. However, when 0.1 g/L AC was added to the medium, the embryo yields in Y009 and Y010 were the highest, and even increased three-fold relative to the embryo yields without AC in Y010.

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Table 4. Effect of AC on the microspore embryogenesis of kale.

AC g·L^{-1}	No. of embryos per bud ± SD		
	Y007	Y009	Y010
0	0.717 ± 0.076^{b}	0.800 ± 0.087^{c}	0.283 ± 0.076^{c}
0.1	0.917 ± 0.104^{b}	1.333 ± 0.058^{a}	0.850 ± 0.100^{a}
0.2	1.267 ± 0.176^{a}	1.150 ± 0.087^{b}	0.567 ± 0.161^{b}
0.4	0.467 ± 0.104^{c}	0.733 ± 0.076^{c}	0.283 ± 0.126^{c}
0.6	0.050 ± 0.050^{d}	0.033 ± 0.058^{d}	0.017 ± 0.029^{d}

Means followed by different letters are significantly different at P = 0.05 level.

Effects of PCIB and AG on microspore embryogenesis

NLN-13 liquid medium without PCIB and AG was used as a control (Table 5). Adding 10 mg/L AG alone gave an improvement compared to the control in Y007 and Y009 (Table 2). In Table 3, 40 µM PCIB appeared to produce the greatest response. Comparing the AG treatment with PCIB, 40 µM PCIB seems to have induced more embryos compared to 10 mg/L AG. Therefore, we attempted to determine the impact of combining 40 µM PCIB and 10 mg/L AG on kale microspore embryogenesis. In this treatment, embryogenesis efficiency in microspore cultures improved significantly with the combination of 40 µM PCIB and 10 mg/L AG compared to 40 µM PCIB or 10 mg/L AG alone. The combination of 40 µM PCIB and 10 mg/L AG was more effective in all genotypes tested, especially Y007. This combination significantly increased the embryo frequency in the high-response genotype Y007 by about twelve-fold, and a five-fold increase was observed in the low-response genotype Y009 (Table 4).

Table 5. Effects of PCIB and AG on the microspore embryogenesis of kale.

PCIB µM	AG mg·L^{-1}	No. of embryos per bud ± SD	
		Y007	Y009
0	0	0.767 ± 0.161^{b}	0.817 ± 0.202^{d}
0	10	1.367 ± 0.425^{b}	1.850 ± 0.361^{c}
40	0	7.933 ± 0.889^{a}	3.117 ± 0.388^{b}
40	10	8.800 ± 0.200^{a}	4.033 ± 0.275^{a}

Means followed by different letters are significantly different at P = 0.05 level.

Effects of PCIB and AC on microspore embryogenesis

The effects of PCIB and AC on the rate of embryonic induction were tested using Y007 and Y009. Microspores were grown in NLN-13 liquid medium with combinations of different concentrations of PCIB and AC. NLN-13 liquid medium without PCIB and AC was used as a control (Table 6). The addition of 40 µM PCIB or 0.2 g/L AC to the medium promoted increased embryogenesis, and the highest rate was observed with the combination of 40 µM PCIB and 0.2 g/L AC. However, no significant difference was detected compared with the treatment that only included 40 µM PCIB.

Table 6. Effects of PCIB and AC on the microspore embryogenesis of kale.

PCIB μM	AC g·L^{-1}	No. of embryos per bud ± SD	
		Y007	Y009
0	0	0.750 ± 0.087[b]	0.933 ± 0.189[b]
0	0.2	1.283 ± 0.126[b]	1.133 ± 0.153[b]
40	0	7.550 ± 1.331[a]	2.716 ± 0.584[a]
40	0.2	8.667 ± 1.428[a]	3.533 ± 0.732[a]

Means followed by different letters are significantly different at P = 0.05 level.

DISCUSSION

Microspore culture techniques are effective technologies for the production of double haploid (DH) plants in *Brassica* species. Because the current culture established system, with its low rate of microspore embryogenesis, cannot satisfy the needs for crop improvement in breeding programs, research has been conducted to identify methods to increase the frequency of embryogenesis in kale (Lichter, 1989; Jiang et al., 2005a; Jiang and Feng, 2006; Feng et al., 2007, 2009; Dai et al., 2009; Wang et al., 2011).

Our experiment showed that several factors may influence microspore embryogenesis. The results not only showed that AG, AC, or the combination of 6-BA and NAA added separately to the induction medium could increase embryo production to some extent, but also showed that PCIB, the combination of PCIB and AG, or the combination of PCIB and AC added to NLN-13 liquid medium could greatly enhance microspore embryogenesis.

We tested seven different combinations of 6-BA and NAA concentrations in NLN-13 medium and found that the optimal combination of 6-BA and NAA for embryogenesis differed between kale varieties, consistent with prior research (Jiang et al., 2005b). Differences in responses may be related to the specific genotypes; 6-BA may promote the development of Chinese cabbage microspores in some genotypes (Xu et al., 2001), and medium containing 6-BA and NAA may increase embryogenesis in pakchoi (Li et al., 1993), while adding 6-BA and NAA has no influence on embryo induction (Sato et al., 1989). Genotype-specific testing will likely be necessary to determine optimal hormone concentration combinations for different genotypes.

We also found that increases in the AG concentration up to 10 mg/L in NLN-13 medium resulted in higher rates of embryogenesis; higher concentrations of AG may inhibit the induction of embryogenesis. The observed effect of AG is consistent with the promotion of embryo formation by AG in wheat (Letarte et al., 2006). The most striking effect of AG was to prevent the death of microspores and to induce them to divide (Toonen et al., 1997; Kasha and Simion, 2004; Zheng et al., 2002; Letarte et al., 2006). AG may increase microspore embryogenesis by triggering signaling to help cells recover from pretreatment and isolation stress, or it could provide a carbon source that is similar to those complex polysaccharides naturally present *in vivo* in pollen to prevent the death of microspores and to induce them to divide.

The present study showed that adding PCIB to the induction medium greatly enhance microspore embryogenesis, similar to the results of many previous studies. Find et al. (2002) found that PCIB promoted the development of numerous high-quality mature embryos in embryogenic cultures of Nordmann fir (*Abies nordmanniana*). Agarwal et al. (2006) indicated that 20 μM PCIB led to a five-fold increase in the frequency of microspore embryogenesis in cultures of *B. juncea*, while Zhang et al. (2011) reported that 40 μM PCIB resulted in a

3.4- to 6.2-fold increase in the number of embryos in *Brassica rapa*. Behzad et al. (2012) also reported that treatment with 4 mg/L PCIB gave the highest embryo yield. The reason may be interpreted as follows. First, PCIB is an antiauxin or an auxin antagonist; it inhibits the action of auxin by binding to auxin receptor sites and thus inhibits many auxin-induced physiological effects (Foster et al., 1995; Heupel and Stange, 1995; Kim et al., 2000; Xie et al., 2000). Second, microspore cultures of different plants produce different concentrations of endogenous hormones (Baillie et al., 1992; Ferrie et al., 1999), so when PCIB and endogenous hormones work together, different plants have different optimal PCIB concentrations.

Adding 0.1-0.2 g/L AC to the microspore suspension resulted in higher embryo yields in kale. Kott et al. (1988) postulated that some toxic compounds could be released from non-embryonic microspores into the medium, which would harm potential embryogenic microspores and reduce the embryogenic frequency. So far, AC has been used with *B. campestris* (Guo and Pulli, 1996), *B. napus* (Gland et al., 1988), *B. oleracea* (da Silva Dias, 1999), and pepper (Supena et al., 2006; Cheng et al., 2013) to improve embryogenesis. In *B. campestris*, 150 mg/L AC improved embryo quality as well as the quantity of embryos by two- to five-fold. The enhancing effect of AC could be due to the adsorption of toxic or polyphenolic compounds produced by microspores (Guo and Pulli, 1996).

In this study, 10 mg/L AG or 0.1-0.2 g/L AC alone was able to increase kale microspore embryogenesis, but embryo yield still was low (Tables 2 and 4). The findings showed that 40 µM PCIB was more effective for embryogenesis in kale, especially for the recalcitrant genotype Y020. Furthermore, the combination of 40 µM PCIB and 0.2 g/L AC greatly increased embryo yield, but the effect was not better than the combination of 40 µM PCIB and 10 mg/L AG. Remarkable improvements in embryogenesis were achieved with the combination of 40 µM PCIB and 10 mg/L AG in NLN-13 liquid medium. This was especially true for the genotype Y007, in which embryo yield increased about twelve-fold. With its substantial potential to improve kale microspore embryogenesis, this novel combination could have wide application for efficient DH production in kale breeding.

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