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ORIGINAL PAPER

PCIB an antiauxin enhances microspore embryogenesis in microspore culture of *Brassica juncea*

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Abstract An efficient protocol to improve microspore embryogenesis is established in an important oleiferous crop, *Brassica juncea* (Indian mustard). Colchicine was used for enhancing microspore embryogenesis and also to obtain doubled haploid embryos. Colchicine at high concentrations (>10 mg l^{-1}), for 24 h, proved convenient for direct recovery of diploid embryos. Higher temperature treatment and an antiauxin PCIB (*p*-chlorophenoxyisobutyric acid) enhanced microspore embryogenesis significantly as compared to colchicine. An increase in temperature from 32°C to 35°C proved very efficient in increasing embryogenesis by 10-fold. The highest embryogenesis rate was obtained when PCIB was added at 35°C in the culture after 1 day of culture initiation. 20 μM PCIB could enhance microspore embryogenesis by 5-fold. Different abnormal shapes of embryos like lemon, banana, flask and fused cotyledons were observed. Both normal and fused cotyledonous embryos showed normal germination when transferred on the B_5 basal medium.

Keywords Antiauxin · *Brassica juncea* · High temperature · Microspore embryogenesis

Abbreviations
DAPI 4,6-Diamidino-2-phenylindole
PCIB *p*-Chlorophenoxyisobutyric acid

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Introduction

Haploid technology has been developed successfully among wide variety of crop species. This technique has become quite popular in breeding programmes, as it reduces considerably the time period required in developing homozygous lines. Since its discovery in *Datura innoxia* (Guha and

Maheshwari 1964), androgenesis in anther culture has been achieved in a number of *Brassica* species but the yield of embryos has been rather low. Lichter (1982) first reported the embryogenesis in microspore cultures of *Brassica napus*. Since then it has been possible to induce embryogenesis in microspore culture of other *Brassica* species (Babbar et al. 2004). Various factors such as donor plant genotype (Guo and Pulli 1996; Wong et al. 1996; Lionneton et al. 2001), physiology of the donor plant (Pechan et al. 1991; Binarova et al. 1993; Zhou and Scarch 1995), developmental stage of microspore (Kott et al. 1988a; Telmer et al. 1992; Guo and Pulli 1996), culture medium composition and culture conditions have been observed to affect the yield profoundly. Temperature pre-treatment, specially an initial exposure to high temperature, influences the fate of microspore culture decisively in the Brassica crop (Babbar et al. 2004). Cold pre-treatment also showed higher embryogenesis in *B. napus* (Gu et al. 2004).

Microspore culture in *B. napus* has been developed as an efficient tool to produce haploid plants for developmental studies (Zaki and Dickinson 1995; Telmer et al. 1993; 1995), mutagenesis, gene transfer (Swanson et al. 1989) and for development of doubled haploid homozygous lines (Zhou et al. 2002a, b). However, the microspore culture of *B. juncea* has not been very successful. Anther culture of this oilseed has met with limited success (George and Rao 1982; Sharma and Bhojwani 1985; Agarwal and Bhojwani 1993; Malik et al. 2001). The published reports of microspore culture of *B. juncea* (Katiyar 1994; Hiramatsu et al. 1995; Purnima and Rawat 1997; Lionneton et al. 2001; Chanana et al. 2005), showed low embryogenic yield as compared to other *Brassica* species. Therefore, the present study was undertaken with an aim to enhance embryo yield in microspore culture of Indian mustard, *B. juncea*. The effect of different temperatures and *p*-chlorophenoxyisobutyric acid (PCIB) on microspore culture response was studied. PCIB is also called α-(4-chlorophenoxy) isobutyric acid, 2-(*p*-chlorophenoxy)-2-methylpropionic acid, or clofibric acid is described as an antiauxin or an auxin antagonist because it inhibits auxin action (Foster et al. 1995; Heupel and Stange 1995; Kim et al. 2000; Xie et al. 2000). The PCIB inhibits the auxin action by binding at the auxin receptor site (MacRae and Bonner 1953). PCIB inhibits many auxin-induced physiological effects, it antagonizes IAA-induced inhibition of wheat (*Triticum aestivum*) root growth (Burström 1950) and competitively inhibits IAA and 2,4-D induced growth of *Avena* sp. coleoptile (MacRae and Bonner 1953). PCIB also promoted the development of numerous high-quality mature embryos in *Abies nordmanniana* (Find et al. 2002). Hadfi et al. (1998) have observed the effect of PCIB and other hormones on zygotic embryo development in *B. juncea*. We first time report that PCIB has a promotional effect on microspore embryogenesis in combination with high temperature, no report on the role of PCIB on microspore embryogenesis is published hitherto.

Materials and methods

Isolation of microspore culture

Plants of *B. juncea* cv. PR-45 were grown in green house at 25°C with natural day light condition and then transferred at 10°C/5°C day/night temperatures before start of bolting. After 2 weeks, young and green inflorescences were harvested and taken to the laboratory. Flower buds were sorted in two categories on the basis of length 2.6–2.8 and 2.8–3.0 mm with the help of vernier callipers. Microspore stage was confirmed by staining with DAPI solution (4,6-diamidino-2-phenylindole) and observed under UV light with the help of fluorescence photomicroscope (Nikon Optiphot). Buds were surface sterilized in 2% NaOCl solution for 10 min followed by washing in sterilized distilled water for 1, 4 and 10 min. After washing, buds were transferred to sterilized 50 ml beaker and homogenised in 3 ml cold ½B_5-13 medium (Gamborg et al. 1968) containing 13% sucrose with the syringe piston by turning pressure movement, piston was washed and total suspension was made to 10 ml. Microspore suspension was filtered through a 55 μm nylon screen in a 10 ml sterilized tube. Microspores were washed two times with ½B_5-13 medium by pelleting at

100 × *g* (800 rpm) at 4°C for 4 min and then third time the pellet was washed with NLN-13 medium (Lichter 1982) containing 0.83 mg l^{-1} KI (Potassium Iodide); NLN-13 KI. Finally, the pellet was resuspended in 1 ml of NLN-13 KI medium and microspore density was adjusted to 40,000 microspores per ml using hemocytometer. Three milliliter of microspore suspension was dispensed into 60 × 15 mm petri dishes. The petri dishes were sealed by Parafilm and incubated in dark at different temperatures. For each treatment two petri dishes were cultured. Each experiment was repeated two times.

Colchicine treatment

Colchicine stock (10 mg ml^{-1}) solution was prepared in distilled water, filter sterilized and stored at –20°C. It was added at various concentrations (0, 1, 10, 20, 30, 40 and 50 mg l^{-1}) to the petri dishes containing microspore culture immediately after isolation and subjected to 32°C for 2 days in dark. After 6 and 24 h colchicine was removed by centrifugation, microspores were washed two times with NLN-13 KI medium and again incubated at 32°C in dark. After high temperature treatment the petri dishes were incubated at 25 ± 2°C in dark.

Assessment of diploidization by the Flow Cytometer

Three weeks old embryos were analyzed by Coulter Epics XL-MCL (Beckman-Coulter, USA) flow cytometer, to check the ploidy status of colchicine treated and untreated embryos, according to the protocol described (Lanteri et al. 2000). Each individual embryo was nicely chopped in A&E buffer (10 mM $MgSO_4$, 50 mM KCl, 5 mM $C_8H_{18}N_2O_4S$ pH 8.0 and stored at 4°C) containing PVP (429 ml A&E buffer, 450 mg DTT, 11.25 ml Triton X-100 and 4.5 gm PVP) and filtered through 88 μm nylon screen. 10 μl propidium iodide (1 mg ml^{-1}) was added to filtrate and incubated for 15 min in dark. The ploidy level was measured by flow cytometer. Flow cytometer was standardized using the control diploid plant leaves.

Temperature treatment

Microspore suspension culture was incubated at different temperature regimes (32, 33 and 35°C) for 2 and 4 days in dark condition. After 2 days of incubation in dark, cultures were refreshed with NLN-13 KI medium. The microspore suspension were collected from the petri dish into 10 ml tube and centrifuged at 100 × *g* for 4 min then the pellet was resuspended in NLN-13 KI medium. After refreshement the petri dishes were transferred to 25 ± 2°C in dark.

Addition of *p*-chlorophenoxyisobutyric acid (PCIB)

PCIB (Sigma, St. Louis) an antiauxin was tested at different concentrations (0, 5, 10, 20, 40, 80 μM) at different period of culture incubation viz. 0 day, 1 day and 2 days. In total these cultures were provided higher temperature treatment (35°C) for 2 days (Table 3). In first two cases (0 day and 1 day), cultures were refreshed after 2 days of culture initiation as mentioned above in microspore isolation protocol and incubated with the same concentration of PCIB at 25°C in dark. In the third case (2 days) PCIB was added after refreshment and cultures were transferred in dark at 25°C.

PCIB (20 μM) was also tested in microspore culture raised from two bud sizes and cultures were provided different temperature treatment for 2 days (32, 33 and 35°C, Table 4).

Germination of microspore embryos

For the conversion of microspore embryos in to plantlets the fully developed dicotyledonous embryos and fused cotyledonous embryos were picked up and transferred directly to B_5 medium containing 1% sucrose. The cultures were incubated under normal culture room conditions and the germination frequency was observed after 3 weeks. The plantlets with proper shoot and root growth were considered as normal germination.

Data collection and result analysis

In each experiment number of embryos except globular and heart shaped were counted after 3 weeks of culture initiation. The results are presented as mean number of embryos per ml (40,000 microspores) of culture and standard error of the mean (indicated by ±) is calculated over two replicates. For each experiment two-factor ANOVA with replication was carried out using Microsoft Excel. The CD values were calculated at $P = 0.05$ level to find the significant difference between the means over different treatments.

Results

Microspore culture provides very convenient and efficient tool for producing haploid embryos. This system gives an opportunity to make the time-course study of embryo development. The embryogenesis is entirely dependent on high temperature treatment for switching the gametophytic pathway to sporophytic pathway. At 32°C many microspores showed 4–8 cells after 48 h that developed to heart or torpedo shaped embryos after 10–12 days. To standardize the microspore culture protocol different colchicine concentrations, temperature regime, hormones and antiauxin treatments were used.

Effect of colchicine

Different concentrations of colchicine were used during the microspore culture at 32°C in order to increase the percentage of diploids. At all the concentrations from 1 mg l^{-1} to 50 mg l^{-1}, colchicine induced 6-fold increase in the number of embryogenesis, when applied for 6 h (Table 1). However, none of these treatments improved diploidization status over the control. Longer treatment (24 h) of colchicine improved the productivity of diploid embryos but was significantly detrimental for embryo yield (Table 1) except at the lowest level (1 mg l^{-1}).

Temperature treatment

The microspore cultures from 2.8 to 3.0 mm bud size incubated at 32°C for 2 days yielded 10–21 embryos per ml. Therefore, the microspore cultures from two different sizes of the buds (2.6–2.8 and 2.8–3.0 mm) were raised and incubated at three temperature conditions (32, 33 and 35°C). The smaller and larger buds showed 51.7% and 16.5% early to mid uninucleate and 47.8% and 77.4% late uninucleate microspores, respectively. The late uninucleate microspore had enlarged nucleus compared to mid uninucleate microspores. Microscopic observations showed that most of the microspores subjected to 32°C temperature treatment formed oval shaped pollen grains after 2 days (Fig. 1a), which subsequently bursted after 4 days (Fig. 1d). The 33°C treated cultures were quite similar to the 32°C treated cultures (Fig. 1b and e). The high temperature treatment at 35°C proved to be the best, as it showed a complete arrest of the pollen development. Oval shaped bodies were almost absent and spherical bodies with dense cyto-

Table 1 Effect of colchicine on embryo yield and percentage of diploid embryos in microspore culture *of B. juncea* cv. PR-45

Colchicine concentration (mg l^{-1})	Duration of colchicine treatment			
	6 h		24 h	
	Embryos per ml	% diploidy	Embryos per ml	% diploidy
0	6.0 ± 1.0[a]	14	8.0 ± 2.0[a]	24
1	32.0 ± 4.0[b]	10	17.0 ± 2.0[f]	30
10	29.0 ± 2.0[c]	19	7.0 ± 1.0[a]	60
20	38.0 ± 2.0[d]	9	5.0 ± 2.0[ah]	80
30	26.0 ± 1.0[e]	14	2.0 ± 1.0[g]	67
40	36.5 ± 3.5[d]	20	3.0 ± 2.0[gh]	86
50	26.0 ± 6.0[e]	20	1.0 ± 1.0[g]	–

Bud size 2.8–3.0 mm. High temperature of 32°C for 2 days was provided in all treatments, Number of embryos are mentioned as mean ± SE, means followed by the same letters are not significant at the 5% level

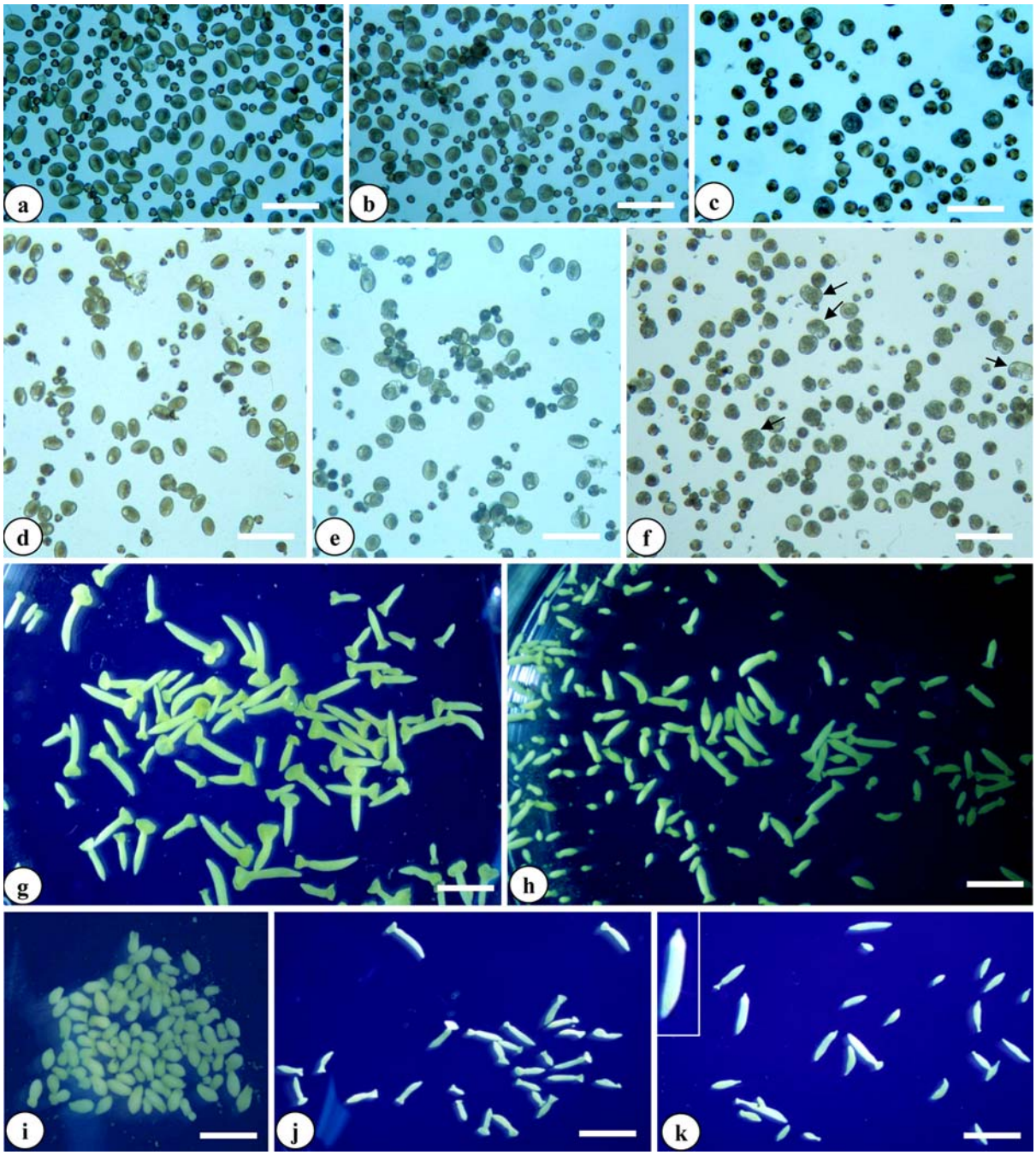

Fig. 1 A 2-day-old microspore cultures of *B juncea* at 32, 33 and 35°C (a–c, bar = 200 μm). The same cultures after 4 days at 32°C (d, bar = 200 μm) and 33 °C (e, bar = 200 μM) showing oval shaped bodies whereas at 35°C (f, bar = 200 μM) many microspores showing embryo induction (arrow marked). Control culture (g, bar = 6 mm) showing less number of embryos compared to the 20 μM PCIB culture (h, bar = 5 mm). Different shapes of embryos like lemon (i, bar = 3 mm), round cotyledon (j, bar = 5 mm) and banana (k, bar = 5 mm) were obtained in PCIB added cultures. Banana shaped embryos were lacking cotyledons completely (see inset)

Table 2 Effect of bud size and different temperature pre-treatment on microspore embryogenesis in B. juncea cv. PR-45

Bud size (mm)	Treatment for the days	Number of embryos per ml at different temperature		
		32°C	33°C	35°C
2.6–2.8	2 days	4.5 ± 2.5[a]	10.5 ± 1.5[b]	12.0 ± 1.0[bd]
	4 days	3.5 ± 1.5[a]	20.5 ± 5.5[c]	28.0 ± 3.0[e]
2.8–3.0	2 days	10.5 ± 0.5[b]	14.5 ± 2.12[d]	139.0 ± 8.48[f]
	4 days	21.0 ± 4.0[c]	13.0 ± 2.0[bd]	166.0 ± 4.5[g]

Medium was refreshed after 2 days, Number of embryos are mentioned as mean ± SE, means followed by the same letters are not significant at the 5% level

plasm were observed after 2 days (Fig. 1c). Multicellular grains resembling globular embryos protruded out from the microspore wall at 4 days (Fig. 1f, arrow marked). Embryo yield was significantly higher at 35°C (Table 2). The microspores from 2.8 to 3.0 mm buds at 35°C exhibits 10–15-fold increase in embryo yield over 32°C and 33°C. Normal and fused cotyledonous embryos produced at 35°C showed 70% normal germination on B_5 medium. Following the strong increase in embryo yield more thorough studies were performed. At 35°C many multicellular grains without progressive embryo formation were seen, which seems to be arrested at early developmental stages. This developmental arrest may be due to the loss of polarity signal because of higher amount of auxin or some other medium component.

Effect of PCIB

Higher temperature increased overall frequency of microspore embryogenesis. In addition, large numbers of multicellular grains were also seen. To overcome this problem cytokinins such as kinetin, zeatin, and TDZ (thidiazuron) and an antiauxin PCIB were tested. A remarkable increase in embryo production was obtained with the addition of PCIB in the medium. Other hormones did not outperform the embryogenic yield over the control (data not presented).

PCIB was added in the cultures immediately after isolation or after 1 or 2 days of the culture initiation as mentioned in materials and methods (Table 3). PCIB showed the best results when it was incorporated in the cultures immediately or after 1 day of incubation. In both the cases embryo yield was improved 4–5-fold, application of the antiauxin delayed by 2 days was not as effective (Table 3). The best results were obtained with 20 μM PCIB. Further increase in the concentration proved detrimental. Control cultures formed ca. 119 embryos (70 normal and 20 fused cotyledonous, Fig. 1g) whereas cultures with 20 μM PCIB produced 586 embryos (102 normal and 167 fused cotyledonous, Fig. 1h). PCIB treated cultures also showed different abnormal morphological shapes like lemon (Fig. 1i), flask, fused cotyledons (Fig. 1j) and

Table 3 Effect of PCIB on the embryogenesis in microspore culture of *B. juncea* cv. PR-45 at 35°C

PCIB Concentration (μM)	Number of embryos		
	PCIB added after		
	0 day	1 day	2 days
0	101 ± 11.0[a]	119.5 ± 19.5[a]	109 ± 29.5[a]
5	146 ± 21.0[b]	296.5 ± 16.5[c]	154 ± 31.0[b]
10	299 ± 63.0[c]	551.5 ± 51.5[g]	165 ± 41.0[bl]
20	509.5 ± 38.5[d]	586.5 ± 38.5[h]	224 ± 24.0[k]
40	75 ± 5.0[e]	100 ± 10.0[a]	184 ± 4.0[l]
80	13 ± 5.0[f]	41.5 ± 1.5[i]	37 ± 8.0[i]

Bud size 2.8–3.0 mm, High temperature was provided at 35°C for 2 days, medium was refreshed after 2 days. Number of embryos are mentioned as mean ± SE, means followed by the same letters are not significant at the 5% level

banana (Fig. 1k). Banana shaped embryos showed complete loss of cotyledons, this kind of embryos were only 15–20% of the total population. To investigate, whether PCIB was promoting embryogenesis by affecting the uninucleate microspore or it acts by overcoming the retarded growth of multicellular grains, microspores from buds of two different sizes (2.6–2.8 and 2.8–3.0 mm) were used to initiate cultures at three different temperature treatments (32, 33 and 35°C). For all the sizes, PCIB showed significantly higher embryogenesis irrespective of the bud size, but the best results were obtained with microspores from 2.8 to 3.0 mm bud and at 35°C (Table 4).

Discussion

Homozygous diploid plants are highly important for screening high yielding lines (Babbar et al. 2004). Spontaneous diploidization is an inherent tendency of several species. In *B. napus* the colchicine added immediately after culture for short duration induced 70–90% diploidization (Chen et al. 1994; Zhao et al. 1996; Zhou et al. 2002a, b). In anther culture of *B. juncea* the spontaneous diploidization is reported to be 18.7%, however colchicine treatment increased diploidization to 43.2% (Agarwal and Bhojwani 2004). In the present study colchicine was applied for the induction as well as direct diploidization of microspore embryos. A 6-fold increase in embryogenic efficiency was observed at 6 h colchicine treatment, however it was not effective for diploidization. Longer treatment of colchicine for 24 h improved the diploidization rate but reduced the embryogenic frequency significantly. Similarly, in *B. napus* longer period of colchicine treatment for 30 h revealed less positive effect on embryo development and yield, however the 15 h treatment proved best (Zhou et al. 2002a).

In most of the protocols, microspore cultures of Brassicas are incubated for 12–72 h at 30–35°C before being transferred to 25°C (Babbar et al. 2004). The *B. juncea* microspore cultures were pre-treated at 32°C for various durations by Lionneton et al. (2001) and Chanana et al. (2005). However, in the present study 35°C was found to be best for embryogenic yield. At 32°C microscopic observation revealed that lots of microspore proceeded to pollen formation and subsequently bursted in the medium. At 35° C large bud size showed 10-fold higher embryogenesis, which is quite higher compared to earlier reports (Lionneton et al. 2001; Chanana et al. 2005). Thus, it reflects that higher temperature treatment is better for *B. juncea.*

Refreshement of the culture after 2 days of culture initiation proved better for higher embryogenic yield in *B. juncea.* Earlier groups have carried detailed studies in this regard. Kott et al. (1988b) postulated that some toxic compounds released from the non-embryonic microspore in the medium, affect potential embryogenic microspores thus reducing the embryogenic frequency. Replacement of culture medium after 24 h of microspore culture reduced the toxicity level and allowed embryos to grow normally. A similar problem was faced by others, necessitating the need for refreshment of medium after few hours of incubation in *B. napus* (Lichter

Table 4 Effect of PCIB on the embryogenesis in microspore culture at different temperature pre-treatment in *B. juncea* cv. PR-45

Bud size (mm)	Concentration of PCIB (μM)	Number of embryos per ml at different temperature		
		32°C	33°C	35°C
2.6–2.8	0	6.0 ± 2.0^{a}	21.5 ± 6.5^{ab}	58.5 ± 8.5^{c}
	20	31.5 ± 6.5^{b}	56.5 ± 8.5^{c}	206.5 ± 26.5^{e}
2.8–3.0	0	10.5 ± 0.5^{a}	32.0 ± 6.0^{b}	91.0 ± 9.0^{d}
	20	39.0 ± 9.0^{bc}	108.0 ± 12.0^{d}	548.0 ± 77.0^{f}

PCIB was added after 1 day of culture initiation, medium was refreshed after 2 days, Number of embryos are mentioned as mean ± SE, means followed by the same letters are not significant at the 5% level

1989; Hansen and Svinnset 1993). In this context it is pertinent that activated charcoal (AC) was used in *B. campasteris* (Guo and Pulli 1996), *B. napus* (Gland et al. 1988) and *B. oleracea* (da Silva Dias 1999) to improve embryogenesis. In *B. campasteris* 150 mg l^{-1} AC improved quality as well as quantity of the embryos by 2–5-fold. The promotory effect of AC could be due to adsorption of toxic or poly-phenolic compound produced by the microspores (Guo and Pulli 1996). To date, there is no report on the precise nature of the toxic compounds or other factors responsible for retardation of embryonic growth of multicellular grains. Thus, it can be hypothesized that cell polarity is not properly established or gets disturbed due to presence of some compounds or because of high amount of auxin from microspore (Stead 1992).

In the present study higher temperature treatment improved embryogenesis quite efficiently. But the observation under the inverted microscope revealed several multicellular grains, which did not proceed further to fully developed embryos. PCIB proved effective in converting these multicellular grains to embryos thus increasing embryogenic yield. This is the first attempt, where an antiauxin promoted microspore embryogenesis remarkably. PCIB was more effective in combination with 35°C, where the number of multicellular grains or pre-globular embryos was higher. Similarly, Hadfi et al. (1998) have reported that PCIB when used at globular embryo stage increases the chance of further zygotic embryo development in *B. juncea.* When the IAA was used at 10–40 μM concentrations the embryo development gets arrested at ball shaped stage. Similarly Michalczuk et al. (1992) have explained that during the carrot somatic embryo study, lower level of free and total auxin in post globular embryo allows the establishment of internal auxin gradient, which is quite important in establishment of normal embryo development. PCIB is also reported to promote the normal embryo development in *Abies nordmanniana* (Find et al. 2002). Therefore, it suggests that PCIB is probably involved in promoting embryo development from multicellular grains. This effect could be due to overcoming the inhibitory effect of high auxin concentration in the multicellular grains.

Various treatments like ABA, GA_3 application and stress treatment such as chilling, desiccation, cotyledon excision were used to escalate the normal germination (Senaratna et al. 1991; Takahata et al. 1993; Wakui et al. 1994; Hansen 2000; Zhang et al. 2006). During present study we have tested normal embryos and fused cotyledonous embryos on B_5 medium. Both type of the embryos showed normal germination frequency up to 70%. Similar germination frequency was reported from the microspore embryos in *B. juncea* (Chanana et al. 2005). The fused cotyledons embryos showed normal germination having proper roots and formed the normal shoots from the cotyledon base as mentioned by Liu et al. (1993).

The present study highlights the use of PCIB for high frequency embryogenesis and further conversion of the embryos to full plantlets successfully for utilization in breeding programme.

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