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Somatic cell genetic studies in *Brassica* species

I. High frequency production of haploid plants in *Brassica alba* (L.) H. f. & T.

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ABSTRACT

In vitro culture of Brassica alba anthers on a growth medium containing inorganics of KB5 and organics, iron, sucrose and hormones of B5 resulted in a very high response of anthers (93.75%) towards callus induction. All the calli transferred to regeneration media responded favourably even after six months of callus induction. Numerous torpedo-shaped embryoids developed in clusters at many sites from each callus mass. Secondary embryogenesis and multiple shoot formation was also observed in many cases. The number of embryoids and plantlets produced by one embryogenic anther were as high as 169.8 and 17 respectively. 87% of the regenerated plants were haploids.

ABBREVIATIONS

SH - Schenk and Hildebrandt, 1972; B5 - Gamborg et al. 1968; NN - Nitsch and Nitsch, 1969; N6 - Chu et al. 1975; MS - Murashige and Skoog, 1962; BL - Blaydes, 1966; DBM II - Gresshoff and Doy, 1972; KB5 - Keller et al. 1975; BAP - 6-benzyladenine; 2,4-D - 2, 4-dichlorophenoxyacetic acid; p-CPA - p-chlorophenoxyacetic acid; Kin - Kinetin; NAA - naphthaleneacetic acid; IAA - indoleacetic acid.

KEY WORDS

Anther culture, Brassica alba, primary and secondary embryoids.

INTRODUCTION

The utility in plant breeding of haploid plant production through immature microspores has been well conceived. In fact, considerable successes have been already achieved in adopting the technique of haploid plant production in Brassica which comprises a number of oil, vegetable, condiment and fodder yielding crop plants. Brassica alba (L.) H.f. & T., also known as B. hirta Moench or Sinapis alba L., is a commercially important crop, seeds of which yield oil, condiment and protein rich meal. Recently limited success has been achieved in production of haploids in this crop (Klimaszewska and Keller 1983). We report in this paper production of haploids at a very high frequency from cultured anthers.

MATERIALS AND METHODS

Anther donor plants of B. alba were grown in the Experimental Field of Bose Institute during the months of October-February without any application of fertilizer. The average day/night temperature ranged from 25-34°C/14-22°C during this period. Buds were collected from the main inflorescence before anthesis. Buds of appropriate size (when the length of petal was 1/4 - 1/2 of the length of the anther) containing uninucleate microspores were dissected and the anthers free from filaments were placed on agar slants. All operations were carried out under sterile conditions.

Induction Media

Eight established media, SH, B5, NN, N6, MS, BL, DBM II and KB5 were tried individually as well as in combination with organics, iron, sucrose and growth hormones of B5. All cultures were incubated in dark at 24 ± 1°C and were examined after 20 to 60 days.

Regeneration Media

Anther derived calli lines were transferred to regeneration media three months after their induction. Subsequent transfers were made after every one month. Calli were initially grown on R1: MS + BAP 1.0 mg l^{-1} or R2: SH + 2,4-D 0.2 mg l^{-1} + p-CPA 2.0 mg l^{-1} + Kin 0.1 mg l^{-1} growth media for 2 weeks and then transferred to R3: MS + NAA + 2,4-D 0.1 mg l^{-1} each. Depending upon the developmental status of the callus, they were transferred to MS medium with the following hormonal combinations - R4: MS + no hormones; R5: MS + NAA 0.2 mg l^{-1} + kin 0.5 mg l^{-1}; R6: MS + NAA 0.2 mg l^{-1} + BAP 0.5 mg l^{-1}. All cultures transferred to regeneration media received continuous light (4000 lux).

Roots were induced in MS medium containing IAA 1.0 mg l^{-1} and the regenerates were maintained on the basal medium devoid of hormones until they were transferred to pots.

Counts were made of the number of anthers

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producing callus and the number of primary and secondary embryoids produced by each embryogenic anther (since each callus was derived from a single anther). An average number of embryoids (anther productivity) and plantlets (plantlet yield) produced by one embryogenic anther were calculated. Procedures followed for chromosomal analyses in root tip cells of regenerates were as described by Satyanarayana and Sen (1973).

RESULTS

All the media except NN and KB5 favoured callus growth from anther cells (Table 1).

Table 1. Response of B. alba anthers towards callus induction in various culture media.

Culture media[a]	No. of anthers cultured	No. of anthers producing callus	% of anthers producing callus
SH	92	8	8.69
B5	96	41	42.70
NN	96	-	-
N6	96	8	8.33
MS	96	6	6.25
BL	96	4	4.17
DBM II	96	34	35.41
KB5	96	-	-
SH[b] + B5[c]	96	68	70.83
NN[b] + B5[c]	96	48	50.00
N6[b] + B5[c]	94	56	59.57
MS[b] + B5[c]	96	62	64.58
BL[b] + B5[c]	96	58	60.41
DBM II[b] + B5[c]	84	46	54.76
KB5[b] + B5[c]	96	90	93.75

a All media were solidified with 0.8% agar and pH (5.8) adjusted prior to autoclaving
b inorganics
c other components (organics, iron, sucrose and hormones).

Better response (42.7%) was obtained on B5 medium compared to other established media. Organic constituents, iron, sucrose and hormones of B5 when combined with inorganics of the other media, favoured profuse callus formation. In fact, the highest response (93.75%) could be derived from the anthers which were grown on medium containing inorganics of KB5 and the remaining components of B5. 232 calli lines were transferred to regeneration media. Within two weeks these calli masses became light green colour with dark green sopts on R1 and R2. However, no further development occurred in such cultures until they were transferred to R3. On R3, numerous torpedo-shaped green embryoids emerged from the calli mass (Fig. 1a). Embryoids developed in clusters of 20 to 30 (Fig. 1b,c) in most cases. Individual embryoids were separated from the clusters (Fig. 1d), and were plated on R4. Most of such embryoids developed abnormal hypocotyl like structures (Fig. 1e). The primary embryoids and/or hypocotyl like structures developed secondary embryoids when the calli were maintained on R4. However, when subcultured either on R5 or R6 these hypocotyl like structures developed in to plantlets.

Growth of anther derived primary calli on R1 or R2 was found to be essential for differentiation into torpedo-shaped embryoids on R3. This was realised from the fact that when calli were exposed to R3 straight away, the callus failed to turn green and differentiate into embryoids. Furthermore, if the clusters of primary embryoids developed on R3 medium were not separated, their cotyledonary leaves eventually fused resulting in a big mass of abnormal structures (Fig. 1f). These structures, nevertheless, maintain high potentiality to form shoots and secondary embryoids (Fig. 1g).

In hormone free MS medium, embryoids developed into plantlets and shoots were usually devoid of root system. Rooting could be induced in such cases by culturing them in MS containing IAA.

All the calli lines (100%) transferred in four batches to regeneration media produced embryoids . The calli tested for regeneration after six months of their induction also responded favourably. The number of primary embryoids produced by each embryogenic anther ranged from 136.26 to 122.67 (Table 2). The number of secondary embryoids produced by abnormal structures on R4 increased with culture age. Thus the highest number (43.65) of secondary embryoids formation was recorded in the first batch of transferred calli to regeneration media. From Table 2 it would also be evident that the maximum anther productivity and that of plantlet yield could be realised as 169.8 and 17 respectively. 33785 embryoids were regenerated from 232 embryogenic anther derived calli. We raised 2448 plants from such embryoids. The cytological analysis revealed that the primary embryoids were gave rise to haploid (n = 12) plants (Fig. 1h). On the other hand, frequency of haploid plants originated from secondary embryoids are ca.80% and from shoots ca.60%. No hyperploids could be observed.

DISCUSSION

Induction of *in vitro* formation of embryos from microspores of several species of *Brassica* has been successfully achieved in the past (Thomas and Wenzel 1975; Wenzel et al. 1977; Keller et al. 1975; Keller and Armstrong 1977; Keller and Stringam 1978; Hansson 1978; Jain et al. 1980; Renard and Dosba 1980; Lichter 1981,1982; Loh and Ingram 1982; Hoffmann et al. 1982; George and Rao 1982; Klimaszewska and Keller 1983). Regeneration of plants from cultured anthers has also been earlier reported in *Brassica* (Kameya and Hinata 1970; Anonymous 1975; Chung et al. 1977; Yang et al. 1979; Quazi 1979). The frequency of haploid plant production in these previous studies was low. Keller et al. (1983) achieved 16.95 as the maximum anther response and 3.57 as the

Table 2. Production of embryoids and plantlets in B. alba anther cultures

Age of calli (in months)	No. of calli transferred	No. of embryoids obtained					Anther productivity[c]	No. of plantlets obtained			Plantlet yield[d]
		Primary embryoids		Secondary embryoids		Total		From primary embryoids	From abnormal structures		
		No.	Efficiency[a]	No.	Efficiency[b]				Through secondary embryoids	Through shoots	Total
3	26	3280	126.15	1135	43.65	4415	169.80	184	235	23	442	17.00
4	75	10220	136.26	1780	23.73	12000	160.00	518	221	51	790	10.53
5	75	9660	128.80	710	6.13	10370	138.26	688	125	28	841	11.21
6	56	6870	122.67	130	2.32	7000	125.00	362	8	5	375	6.69
Total	232	30030	129.43	3755	16.18	33785	145.65	1752	589	107	2448	10.55

a no. of primary embryoids per embryogenic anther; b no. of secondary embryoids per embryogenic anther;
c average number of total embryoids per embryogenic anther; d average number of plantlets per embryogenic anther.

maximum anther productivity. We obtained a very large number of embryoids (maximum anther response 93.75, regeneration ability 100% and maximum anther productivity 169.8) by a simple modification of nutrients.

The phenomenon of secondary embryogenesis in Brassica anther cultures has been observed in the past (Thomas et al. 1976; Keller and Armstrong 1977,1981; Loh and Ingram 1982). In our cultures, 232 calli lines had produced 3755 secondary embryoids out of which 589 plantlets were recovered. Multiple shoot formation was observed in many abnormal structures when transferred to hormone containing media. However secondary embryoid formation could be achieved at very low level in such cases.

The high anther response (93.75%) in our studies is primarily due to the changes in the constituents of the growth medium evolved essentially by combining KB5 and B5. The suitability of KB5 for anther culture of various species of Brassica has been adequately demonstrated earlier by Keller and his colleagues. In our studies anther derived calli showed 100 % regeneration ability even after six months of their induction. Regeneration of high frequency haploids from four times subcultured calli indicated the stability of haploidy in the system.

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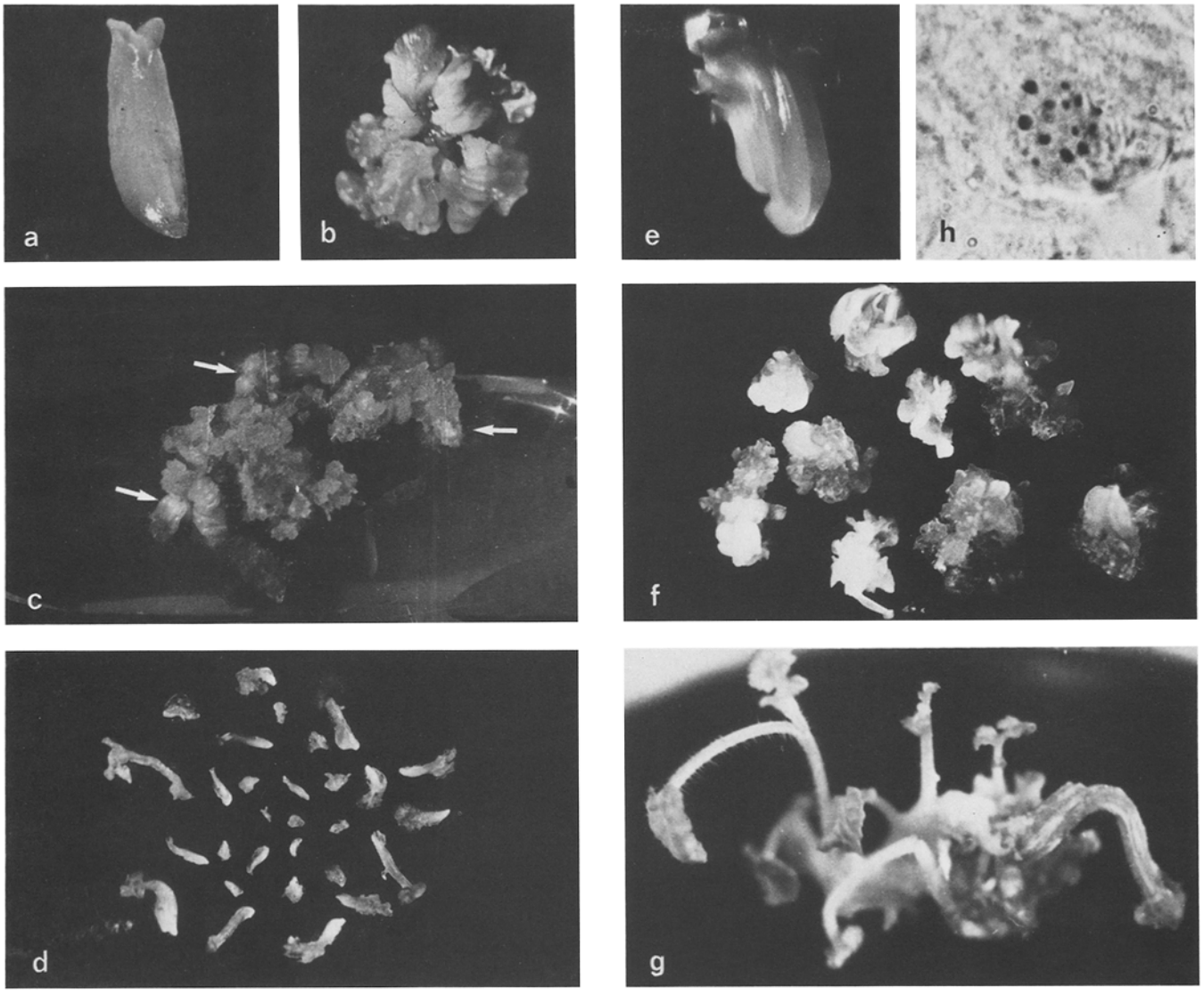

Fig. 1a-h: Anther cultures of Brassica alba and regeneration of haploid plants.

a) Torpedo-shaped embryoid developed from regenerating callus; b) single cluster isolated from differenting callus showing 20 to 30 embryoids; c) differentiation of anther derived callus into clusters of embryoids; d) isolated embryoids from clusters; e) abnormal growth of an embryoid; f) embryoids in clusters showing fused cotyledonary leaves; g) multiple shoots arising from primary embryoid; h) a root tip cell of regenerated plant showing haploid chromosome number (n = 12).

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