EVALUATION OF PROTOCLONAL VARIATION VERSUS CHEMICALLY INDUCED MUTAGENESIS IN *BRASSICA NAPUS* L.

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ABSTRACT

Brassica napus L. protoclones were evaluated for seed shape and colour in the R_1 generation and for flowering time in the R_2 and R_3 generations. Yellow and shrivelled seeds were recovered in the R_1 generation of EMS mutagenized protoclones and plants. The flowering times of R_1 families did not change significantly compared to control plants in the R_2 and R_3 generations. However, protoclone No. 19 (R_1 family) flowered a week earlier than the control plants in the R_2 generation and did not differ significantly with control in the R_3 generation. The frequency of seed quality and germination rate was poor in mutagenized protoclones versus non-mutagenized protoclones. The application of mutagenesis has not induced any useful variation and hence protoclonal variation can be used in crop improvement.

INTRODUCTION

A reliable system for plant regeneration from protoplasts is very important for the success of gene transfer, somatic cell fusion, organelle uptake, and mutation, which are essential in plant improvement[1]. Murashige and Nakano[2] recognized the phenomenon of genetic variability associated with cultured plant cells.

Somaclonal variation has been attributed to: single gene mutation[3-5], aneuploidy[6], and transposable elements[7]. Quantitative traits[8-11] are also affected by tissue culture. Ryan *et al*[12] found significant increase in wheat kernel weight, hardiness and protein content and a significant reduction in yellow pigmentation. The potential application of somaclonal variation has been widely recognized in plant improvement[13, 14]. However, the specific use of protoclonal variation in crop improvement has been limited[15, 16]. Recently, single gene mutants were recovered in *Brassica napus* L. protoclones resistant to the herbicides phenmedipham and atrazine[17], and in tomato protoclones resistant to fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici*[18].

In this article, our aim was to evaluate protoclonal variation versus chemically induced mutagenesis in *B. napus* L.

MATERIALS AND METHODS

Three-week-old seedlings of *Brassica napus* L. var. Westar, amphidiploid were used for protoplast work.

Protoplasts and Mutagenesis

Brassica napus leaf mesophyll protoplasts were isolated, cultured and regenerated according to the procedure of Jain and Newton[17]. Filter sterilized ethylmethane sulfonate (EMS) (0.0005%) was added to the protoplast cultures for 48 h[17]. Protoplasts were washed with the washing medium 3–4 times and cultured on $1.5 \times B_5$ medium[19]. Similarly, *B. napus* L. seeds were mutagenized with 1% EMS for 6 h[17] and were grown in the green house. Mutagenized plants were self-pollinated and all seeds were collected from each plant and screened for variation to compare with protoclones and mutagenized protoclones.

Evaluation of protoclonal variation

All the regenerants (R_0) were self-pollinated and seeds of each plant were collected in the R_1 generation and evaluated in the subsequent generations in the green house. Variation in seed colour and shape was recorded in self-pollinated EMS-treated and untreated protoclones in the R_1 generation. Seeds of each randomly selected R_1 family [derivatives of (R_0) single regenerant plant], replicated five times, were sown in the pots filled with sterile soil in the greenhouse. Flowering time (days to first-flowering) of 24 R_1 families was recorded in the R_2 generation. Seeds of 12 self-pollinated R_2 families were selected on the basis of their extreme flowering times and were tested for genetic stability in the R_3 regeneration.

The mean and standard deviation of flowering

times of R_1 families were calculated in the R_2 and R_3 generations. The comparison of flowering times of R_1 families with control plants was done by selecting early flowering families in the R_2 and R_3 generations and analysing the differences between their means using the t test for comparing two treatment means at the $t = 0.05$ level of significance.

RESULTS AND DISCUSSION

The efficiency of protoplast division (50–70%) and growth was dependent upon having a low number of broken protoplasts in the culture medium and addition of fresh medium to the protoplast cultures. This is probably due to release of inhibitors by broken protoplasts into the culture medium.

After 4–5 days, 30–40% of the 3-week-old (2–3 mm) proto-calli (p-calli) formed caulogenesis on B_5 shoot regeneration medium[17]. Several days later shoot buds were formed and they (25–33%) later developed into shoots. Well developed shoots (95–100%) were rooted directly in the soil under 100% relative humidity[17]. Some of the p-calli were continuously grown on liquid medium without shaking for 5–6 weeks and the old medium was changed 2–3 times with a fresh medium. Proto-calli (7–8 mm size) were cultured on solidified B_5 shoot induction medium[17] and were kept under low light intensity (1000 lux). After 10–12 days, somatic embryoids were observed in p-calli. Induction of somatic embryoids was due to osmotic shock.

Evaluation of protoclones

A total of 182 self-pollinated protoclones, 66 mutagenized protoclones, 73 mutagenized plants from seeds, and 69 control plants were evaluated for seed shape and colour in the R_1 generation. Variation in seed shape was observed in all the plants tested. The total percentage of shrivelled seeds was highest (79%) in mutagenized protoclones as compared to 28% in protoclones (table 1). Similarly, a total of 78% shrivelled seeds were recovered in mutagenized plants and their germination rate was very poor. There have been reports on considerable variation in protoclones[16, 20]. On the other hand, genetic stability of the protoclones has been reported in several plants[21–23]. Furthermore, Vasil[24] explained that the plants regenerated via somatic embryogenesis were genetically stable. However, regeneration of protoclones via organogenesis from well developed calli has shown phenotypic variability[16, 20]. Variation of seed shape in our experiments may be due to regeneration of plants via organogenesis and differences in the age of shoots and their rooting time in R_0 generation.

Table 1 *Variation in seed shape of B. napus L. protoclones and mutagenized protoclones and plants in the R_1 generation. Numbers in parentheses indicate percentage*

	No. of protoclones		No. of plants from seeds	
Seed shape	(+)EMS	(−)EMS	(+)EMS	(−)EMS
Round (100%)	--	15(08)	--	44(64)
Shrivelled (100%)	17(26)	18(10)	21(29)	--
Round > 90%	--	89(49)	--	25(36)
Shrivelled > 90%	35(53)	32(18)	36(49)	--
Round and Shrivelled (50% each)	14(21)	28(15)	16(22)	--

-- indicate 0(0).

A range of variation in seed colour was displayed in protoclones and in mutagenized protoclones and plants. Over 30% yellow seeds were recovered from mutagenized protoclones as compared to 5% from protoclones and 43% from mutagenized plants (table 2). Yellow seeds were shrivelled and their germination rate was very poor. Therefore, genetic stability of yellow seed colour could not be tested in

Table 2 *Seed colour variation in B. napus L. protoclones, mutagenized protoclones and plants in the R_1 generation. Numbers in parentheses indicate percentage*

	No. of protoclones		No. of plants from seeds	
Seed colour	(+)EMS	(−)EMS	(+)EMS	(−)EMS
Black and brown (50% each)	--	30(20)	--	51(74)
Black > 90% + brown	--	48(25)	--	18(26)
Black (100%)	--	15(8)	--	--
Brown > 90% and Black	30(45)	42(22)	21(29)	--
Light brown (100%)	--	4(2)	--	--
Brown (100%)	--	35(18)	--	--
Yellow (100%)	4(6)	4(2)	--	--
Brown > 90% + yellow	15(23)	--	20(27)	--
Yellow > 90% + brown	14(21)	2(1)	28(38)	--
Yellow > 90% + black	2(3)	1(1)	4(5)	--
Yellow + black (50% each)	1(2)	1(1)	--	--

-- indicates 0(0).

the subsequent generations. George and Rao[25] also obtained yellow seeded variants in *B. juncea* through tissue culture which were stable during the succeeding generations. Jain and Newton[17] reported variation in total seed weight per plant of self-pollinated *B. napus* protoclones and mutagenized protoclones in the R_1 generation. The protoclones and mutagenized protoclones produced yellow-black, yellow-brown and brown-black seeds which might be the result of outcrossing. Similarly, blackish-seeded variants of *B. juncea*, obtained through tissue culture, were due to outcrossing[25]. A comparative study of somaclonal variation versus indueed mutagenesis in tomato indicated that the two sources of variability differ in their effect, changing the spectrum and frequency of mutants as well as in some cases, their pattern of segregation[26]. Somaclonal variation occurred at a higher frequency than chemical induced mutagenesis[26]. Our results show a higher frequency of variability in EMS-treated protoclones and plants than protoclones. But mutagenized protoclones produced mostly shrivelled seeds and their germination rate was poor. The seed quality of protoclones was better and their germination rate was over 95%. Ethylmethane sulfonate treatment has caused considerable damage to the plant cells, which has resulted in more useless variation. Therefore, mutagenesis may be avoided and exploit protoclonal variation for the selection of desirable traits[17]. Protoclonal variation may be dependent upon the manner of which plants are regenerated including organogenesis, somatic embryogenesis, culture conditions and genotype.

Protoclonal variation in R_2 and R_3 generations

Brassica napus seeds of randomly selected 24 non-mutagenized R_1 families were evaluated for early flowering in the R_2 generation in the greenhouse. In control plants, flowering began after 40–45 days. In R_1 families, flowering started as early as 32 days and as late as 52 days (table 3). However, in most of the

Table 3 *Variation in flowering time of B. napus protoclones in the R_2 and R_3 generations. Five replications of R_1 and R_2 families*

	Generation					
	R_2			R_3		
R_1 family No.	Flowering time in days	Mean $\bar{X}$	SD	Flowering time in days	Mean $\bar{X}$	SD
Control	40–45	42.0	1.87	35–42	38.5	2.22
1	38[a]–44	41.0	2.29	35–38	36.0	1.22
2	36–49[a]	43.0	4 87	35–39	36.2	1.78
3	36–42	38.6	5.21			
4	37–47	42.4	3.97			
5	38–45	41.0	3.31			
6	36[a]–41	39.0	2.12	35–38	36.0	1.22
7	36–47[a]	41.0	4.50	37–40	36 8	1 92
8	37–41	39.4	1.51			
9	36–45	40.8	3.83			
10	37–39	37.6	0.89			
11	37–45	41.0	3.04			
12	36–42	39.6	2.60			
13	32[a]–41	35.0	3 67	35–38	35.8	1.30
14	37[a]–39	37.6	3.20	36–39	37.0	1.22
15	37–39	37.8	1.90			
16	37–45[a]	40.4	3.57	37–41	38.0	2.00
17	37–43[a]	39.8	2.28	35–39	36.8	1.64
18	37–47	42.2	3 96			
19	33[a]–36	34.4*	1.34	33–37	35.4[ns]	1 67
20	34–52[a]	44.6	6.87	34–39	36.8	2.16
21	36–42	39.6	2.50			
22	35[a]–40	38.2	2.16	36–37	36.4	0.54
23	36–39	37.4	1.14			
24	36–45[a]	41.2	3.42	35–40	37.0	2.34

[a]Plants with extreme flowering time; *Significance of t test (0.05) control vs protoclones; SD, Standard deviation; ns, Non significant value of t test at 0.05 level.

R_1 families, flowering commenced as early as 36–37 days and as late as 41–47 days. Protoclone No. 19 (R_1 family) was the only significantly early flowering type (X 34.4 days) (table 3). The flowering times of other R_1 families although less than the control, were non significant. These results indicate that early and late flowering protoclones could be recovered in the R_2 segregating population. Differences in vine morphology, tuber yield and composition, maturity and flowering were observed in potato protoclones[15].

The genetic stability of the flowering time was tested in the R_3 generation by selecting 12 R_2 families with extreme flowering times. In control plants, flowering began 3–5 days earlier in the R_3 generation compared to the R_2 generation. The flowering time ($\bar{X}$ 35.4 days) of protoclone No. 19 (R_2 family) was unstable in the R_3 generation and was not significantly different from the control plants (table 3). These results indicate that the genetic instability in flowering time may be due to continuous segregation of genes during the succeeding generations. Ryder[27] suggested that more than one gene may govern flowering time in *Lactuca sativa*. It is also influenced by day length and temperature. Extreme flowering times of R_1 family No. 13 (figures 1, 2) and 20 (figure 3) were 32 and 52 days, respectively in the R_2 generation and their flowering times in the R_3 generation were in the range of 35–38 (X 35.8) and 34–39 (X 36.8) days, respectively (table 3). The flowering times of other families did not differ significantly from the control plants. These results suggest that protoclonal variation in flowering time is somehow checked under the controlled conditions in the greenhouse. Further investigation is needed for the evaluation of protoclones in the field.

Figures 1–3. **1.** First-flowering began in R_1 family No. 13 after 32 days; **2.** Pod formation in R_1 family No. 13, and **3.** First-flowering started in R_1 family No. 20 after 52 days.

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