Effects of mutagenic treatments of isolated microspores and microspore-derived embryos on embryogenesis and plant regeneration in oilseed rape

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Abstract

Ultraviolet (UV) irradiation, mutagenic agents ethylmethane sulphonate (EMS) and sodium azide (NaN_3) were applied to isolated microspores and microspore-derived embryos of four *Brassica napus* genotypes M9, h28, h57 and h58 *in vitro*. In the UV light irradiation, the isolated microspores and microspore-derived embryos were exposed for 10, 30, 60 and 120 s. Embryo yield showed a sharp decrease with increasing UV exposure and only 10 embryos were developed following an exposure of 120 s in genotype M9. Embryo germination was also decreased when isolated microspores and microspore-derived embryos were treated by UV irradiation. The highest callus induction (77.78 %) and plant regeneration (55.56 %) were observed in genotype h57 after treated with UV radiation for 10 s. Mutagenic treatments, NaN_3 (1, 10, 100 μM) and EMS (0.001, 0.01, 0.1 %), were applied to the isolated microspores and embryos at early cotyledon stage for various time intervals (1, 5, 15 h). When the isolated microspores were treated, chemical mutagens with low concentration promoted the embryo yield. With increasing of the mutagen concentrations and prolonging of the exposure time, embryo yield reduced gradually. Rates of survival embryos and embryo germination decreased with the increase in EMS concentrations and treatment intervals. Interestingly, when the embryos were treated 0.01% EMS for 5 h, better results of embryo survival were achieved, with the higher rates of embryo germination and plant regeneration. The application of low concentration NaN_3 had a promotive effect on embryogenesis and plant regeneration in most genotypes studied. When the isolated microspores were treated by 10 μM NaN_3 for 1 h, rate of plant regeneration of genotypes M9, h57 and h58 reached 11.11 %, 15.79 % and 22.22 % respectively. In genotype h28, when the microspore-derived embryos were treated 10 μM NaN_3 for 1 h, higher rate of plant regeneration (19.05 %) was reached. But when the concentration of NaN_3 reached 100 μM, no plant was regenerated in all four genotypes. Thus it is very important to use the appropriate concentration of NaN_3 in the *in vitro* mutagenesis.

Key words: Rapeseed (*Brassica napus*), *in vitro* mutagenesis, microspore culture, embryogenesis, plant regeneration

Introduction

Mutation techniques have been extensively used to improve yield, quality and disease and pest resistance in crops (Barro et al., 2003), and have already proven their potential for generating useful variability in plant breeding. Mutant cultivars of cereals are the most abundant, followed by legumes and oil crops (Maluszynski et al., 1995). The new genetic variability from cultured haploid cells will be increased by the application of physical or chemical mutagens. The major advantage of haploid tissue mutagenesis is that all traits are expressed, including recessive traits and mutations, both before and after chromosome doubling. Fortunately, haploid cells and plants can be successfully obtained by microspore or anther culture for many plant species (Maluszynski et al., 1995). Microspore culture technique has its potential applications in plant genetic research, gene transformation and breeding program because of its relative simplicity and efficiency in haploid production compared to other methods (Zhou et al., 2002a). In the last two decades, remarkable progress in isolated microspore culture technique has been made in all major *Brassica* species, and especially in oilseed rape (*Brassica napus*) (Dias, 2001; Zhou et al., 2002b; Gu et al., 2003b; Zhang et al., 2006).

The application of mutation techniques to haploid systems has been limited to the few species for which there is an efficient protocol for obtaining green plants regenerated from haploid cells. Among crops, the *Brassica* microspore culture system has been used, together with mutagenic treatments, to generate variation in important agronomic and quality traits, as well as resistance to herbicides such as chlorsulfuron, glyphosate and imidazolinones. The objectives of the present experiments were to develop an efficient protocol of *in vitro* mutagenesis and to create some novel germplasms for further selection in rapeseed breeding.

Materials and Methods

Plant material: The donor plants include four genotypes of oilseed rape (*Brassica napus* L.), which had relatively good response to microspore culture among several genotypes tested. The donor plants were grown under growth cabinet condition with a 16 h photoperiod (approximately 350 $\mu E/m^2/s$), a day/night temperature of 20/15 ^oC and with a high nutritive status. A couple of days prior to microspore isolation the temperature were adjusted to 12/10 ^oC day /night regime. Five plants were grown for each genotype.

Microspore culture: The microspore culture protocol was based on Fletcher et al. (1998) and Zhou et al. (2002a) with

modifications. Ten flower buds at the late uninucleate stage of pollen development were collected randomly from racemes based on bud size (3-4 mm). After surface sterilization in 5% sodium hypochlorite for 18 min and washing 3 times in sterile water the buds were then macerated in cold NLN-13 medium with 13 % sucrose and buffered at pH 6.0, and filtered through a 40-µm nylon mesh into a centrifuge tube. After adding 2 % (w/v) activated charcoal, the microspore suspension was dispensed into 60× 15 mm Petri dishes with 4 ml per plate. A microspore density of about 2×10^4 per ml was used, i.e. 10 flower buds equaled 10 Petri dishes. The dishes were sealed with double layers of Parafilm and incubated in the dark at 32 °C for 2 days, before moved to 24 °C.

Mutagenic treatments: UV-irradiation was applied to isolated microspores and microspore-derived embryos *in vitro*. Microspores and microspore-derived embryos were placed in open Petri dishes on a laminar flow bench at a distance of 25 cm from a UV light source. Major emission was at a 254 nm with an incident dose rate of 3.0 J $m^{-2}s^{-1}$. EMS and NaN_3 were applied to the isolated microspores and microspore-derived embryos at early cotyledon stage for various time intervals (1, 5, 15 h). When isolated microspores were treated, EMS and NaN_3 of different concentrations in NLN-13 medium (13 % sucrose, pH 6.0) were added to the centrifuge tubes immediately after microspore isolation and cultured for different time intervals at 32 °C in the dark. Microspores were then washed twice with fresh NLN-13 medium and re-suspended in 40 ml of NLN-13 medium and cultured as described above. Where microspore-derived embryos were large enough, the two mutagenic agents were added to the 60 × 15 mm Petri dishes for different time intervals. Embryos were then washed twice with NLN-13 medium and finally cultured in NLN-13 medium at 24 °C in the dark.

Embryogenesis and plant regeneration: Once embryos were visible to the naked eye, cultures were transferred to a slow rotary shaker, in the dark at 24°C. At the late torpedo stage, large embryos were transferred to the solid induction medium (2.0% sucrose, 0.9% agar, 1/2 MS macronutrients and 2.0 mg/l BA, pH 5.8). After sterilization, culture media were poured into growth containers. The cultures were incubated under a 16-h photoperiod (100 µE/m^2/s) at 24°C. When shoots developed they were cut free of any callus or hypocotyl and were transferred to larger growth vessels with solid MS medium where rooting took place. Plantlets were transferred to a soil-perlite mixture and kept for 2 weeks in a nursing room. Gradual adaptation to glasshouse conditions followed.

Results

The effect of UV irradiation on embryo yield was determined when isolated microspores were treated (Fig. 1). One week after treatment all embryogenesis decreased sharply with only half of embryos developed from the control following an exposure of 10 sec of UV irradiation. With the increase of exposure intervals of UV treatments, further decrease in embryo production was observed and only 10 embryos were induced in genotype M9 when the radiation time reached 120 s. However, there was variation among genotypes in the sensitivity to the UV irradiation. For instance, in h28 the highest embryogenesis was obtained when microspores were exposed for 30 s except for the control. In fact, obvious difference existed among genotypes in the response to microspore embryogenesis, as significant difference of embryogenesis was observed in the control (Fig. 1). Furthermore, callus induction and plant regeneration also decreased along with the increase of UV exposures in most genotypes. The highest callus induction (77.78 %) and plant regeneration (55.56 %) were observed in h57 (also higher than that of the control) after treated with UV radiation for 10 s (data not shown because of the limited space). Similarly, the rate of survival embryos treated by UV irradiation at early stage of embryo development was also decreased along with the increase of UV exposures in all genotypes. The rate of survival embryos of genotype h57 was only 27.27 % when the radiation time reached 120 s, compared with the control of 97.5 % (Fig. 2).

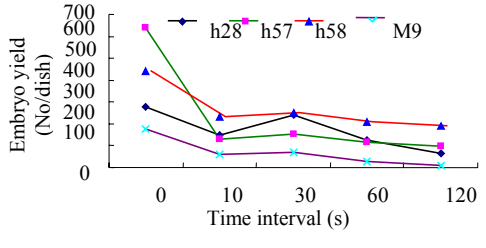

Fig. 1. Embryo yield of isolated microspores by UV treatment (sec) in 4 genotypes of *B. napus*.

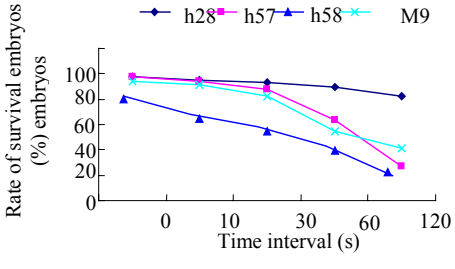

Fig. 2. Rate of survival embryos by UV treatment (sec) at early stage of embryo development in *B. napus*.

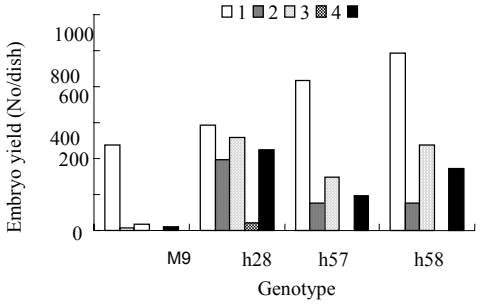

Fig. 3. Embryo yield of isolated microspores by EMS treatment in 4 genotypes of *B. napus*.

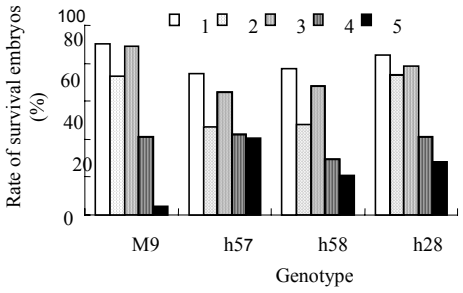

Fig. 4. Rate of survival embryos by EMS treatment at early stage of embryo development in 4 genotypes of *B. napus*.

EMS treatment: 1 = 0% EMS (CK); 2 = 0.001% EMS for 15 h; 3 = 0.01% EMS for 5 h; 4 = 0.01% EMS for 15 h; 5 = 0.1% EMS for 5 h.

Rate of embryo germination and survival embryos decreased when the isolated microspores and microspore-derived embryos were treated by the mutagen EMS. The lowest embryo production was observed in the fourth treatment (0.01 % EMS for 15 h), with no embryo generated in M9, h57 and h58 (Fig. 3). Relatively high embryo yield was achieved by EMS mutagenesis except the fourth treatment in h28. Many fertile microspore-derived embryos died after the applying of EMS. Rates of survival embryos and embryo germination decreased with the increase in EMS concentrations and treatment intervals, and the lowest level was observed when the maximum concentration (0.1 %) was applied (Fig. 4). Interestingly, when the embryos were treated 0.01% EMS for 5 h, better results of embryo survival were achieved. Furthermore, their rates of embryo germination and plant regeneration all exceeded that of the control (data not shown).

In this study it was found that the application of low concentration NaN_3 had a promotive effect on embryogenesis and plant regeneration in most genotypes studied. When the isolated microspores were treated by 10 μM NaN_3 for 1 h, rate of plant regeneration of genotypes M9, h57 and h58 reached 11.11 %, 15.79 % and 22.22 % respectively, which all exceeded over the controls. When the same treatment (10 μM NaN_3) was prolonged to 5 h, the rates of embryo germination and plant regeneration started falling. The lowest embryo germination was observed when the NaN_3 concentration was increased to 100 μM, without the regeneration of any plants (Table 1). Similarly, low concentration of NaN_3 had a stimulant effect on microspore-derived embryos. In genotype h28, when the microspore-derived embryos were treated 10 μM NaN_3 for 1 h, the rate of plant regeneration reached 19.05 %, which was a significant increase over the control. But when the concentration of NaN_3 reached 100 μM, no plant was regenerated in all four genotypes (data not shown). Therefore, it is very important to use the appropriate concentration of NaN_3 in the *in vitro* mutagenesis.

Table 1. Embryo germination and plant regeneration from isolated microspores by NaN_3 treatment in *B. napus*.

Genotype	NaN_3 (μM)	Duration (h)	No. of embryos transferred to solid medium	No. of germinated embryos	Rate of germinated embryos (%)*	No. of regenerated plants	Rate of regenerated plant (%)*
M9	0	0	15	4	26.67 b	1	6.67 b
	1	5	36	17	47.22 a	2	5.56 b
	10	1	36	18	50.00 a	4	11.11 a
	10	5	21	5	23.81 b	1	4.76 b
	100	1	7	1	14.29 b	0	0.00 c
h57	0	0	59	45	76.27 a	9	15.25 a
	1	5	40	33	82.50 a	3	7.50 ab
	10	1	57	41	71.93 a	9	15.79 a
	10	5	21	2	9.52 b	1	4.76 b
	100	1	14	1	7.14 b	0	0.00 b
h58	0	0	42	11	26.19 c	4	11.90 b
	1	5	105	85	80.95 a	23	21.90 a
	10	1	99	82	82.83 a	22	22.22 a
	10	5	63	31	49.21 b	3	4.76 bc
	100	1	0	0	0.00 d	0	0.00 c
h28	0	0	72	59	81.94 a	23	31.94 a
	1	5	21	6	28.57 b	2	9.52 bc
	10	1	36	23	63.89 a	6	16.67 b
	10	5	21	2	9.52 c	1	4.76 c
	100	1	0	0	0.00 d	0	0.00 d

* Within columns for each genotype, means followed by the same letter are not significantly different at the 0.05 level of probability.

Discussion

Every organism contains variations that may be inherited and can be used as the bases in breeding, this kind of variations usually come from the spontaneous and induced mutations and the latter is one of the important methods in breeding (An et al., 2003). Microspore culture provides the opportunity to produce haploid embryos and homozygous lines at high frequency in *Brassica* species (Zhou et al., 2002a, b). A doubled haploid population obtained from heterozygous starting material provides a broad spectrum of genetic recombinants from which selection can be made easily. For this purpose, microspore culture provides an ideal method for mutation because the mutated traits can be fixed in homozygous condition by chromosome doubling. Thus mutant gene in doubled haploid is a purely homogeneous, and recessive mutant gene can be expressed and easily selected (Shi et al., 2004).

However, there are still lots of obstacles to the success of the microspore mutagenesis. Apart from an efficient *in vitro* mutagenesis protocol especially for the recalcitrant crops and genotypes (Gu et al., 2004; Zhang et al., 2006), many chemical and physical mutagens are harmful to the microspore embryogenesis and plant regeneration (Gu et al., 2003a). Our present results also confirmed this phenomenon especially applied at higher concentrations and prolonged intervals. In addition, some agronomic traits of mutant plants may deteriorate, leading to the low yield (Ma & Zhao, 1998). With this improved protocol, we were able to achieve some satisfactory results with the regeneration of the mutant plants derived from the isolated microspores and embryos. For instance, low concentration NaN3 (10 μM for 1 h) could promote the embryo germination, plant regeneration, and improve shoot growth. Therefore, it is very critical to use the appropriate concentration and duration in the process of the microspore mutagenesis. Mutant plants derived from this *in vitro* mutagenesis are currently under the field investigation for their physiological and genetic studies as well as breeding utilization.

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