Mutagenic treatments of cotyledons for *in vitro* 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 cotyledons of two *Brassica napus* cultivars N1 and N6 *in vitro*. Cotyledons were exposed to the UV radiation for different time intervals (30, 60, 120, 180, 240 s). The results showed that UV had some promotive effect on cotyledon regenerations. The highest rates of callus induction (85.71 and 83.33 %) and plant regeneration (67.86 and 50.00 %) were observed from UV treatment of 120 and 60 sec in genotypes N1 and N6 respectively. With the increase of UV radiation intervals, the rate of plant regeneration decreased gradually although the rate of callus induction was not much affected. There was no difference in the rates of callus induction and plant regeneration between the treatment of lower EMS concentration (0.01 % for 5 h) and the control. However, with the increase in concentrations and prolongation of treatment intervals, the rate of induced calli and the rate of regenerated plants decreased progressively in both genotypes. No plant was regenerated when the EMS concentration reached 1 % and the exposure time lasted for 25 h. After NaN_3 treatment, the rate of callus induction was not much affected or even higher than that of the control. As for the plant regeneration, there was no obvious difference between the treatment of lower NaN_3 concentration (10 μM) and the control in both genotypes. Higher concentrations of NaN_3 (100 and 1000 μM) inhibited the plant regeneration, and no plant was produced when its concentration reached 1000 μM and the exposure time lasted for 25 h.

Key words: Rapeseed (*Brassica napus* L.), *in vitro* mutagenesis, cotyledon, plant regeneration

Introduction

Mutagenesis technology has been applied to plant breeding comprehensively, which made crops to produce beneficial varieties with good traits (Maluszynski et al., 1995; Joong & Seung, 2002; Gu et al., 2003). Mutant Varieties Database reveals that 2541 varieties derived from mutagenesis were currently registered online, among them the oilseed crops and oilseed rape being 63 and 25 varieties respectively (FAO/IAEA, 2006). Recent years, *in vitro* mutagenesis technology was increasingly applied to the development of quality and resistance traits, which speeded up crop improvement and germplasm innovation (Song et al., 2003). The plant regeneration system of cotyledonous explants is one of the best *in vitro* regeneration system, which could yield lots of sterile explants in a short time. Moreover, the experiment will not be limited to the factors such as growing season and site. Therefore, under the *in vitro* condition, the mutageneses of cotyledonous explants have substantial potential. However, only a few researchers have been dedicated to these studies. Based on our previous results (Tang and Zhou, 2001; Zhou et al., 2002; Li et al., 2003; Tang et al., 2003), further studies of *in vitro* mutagenesis of cotyledonous explants on the plant regeneration were conducted in two varieties of oilseed rape, with the objectives of establishing an efficient mutagenesis system and creating novel plant germplasm for rapeseed breeding.

Materials and methods

Plant material: The donor plants include two genotypes (N1 and N6) of oilseed rape (*Brassica napus* L.), which had relatively good response to cotyledon culture among several tested genotypes.

Sterile shoot culture: After surface sterilization in 0.1% $HgCl_2$ for 15 min and washing 5 times with sterile water, rape seeds were transplanted to 1/2 MS culture medium. Seedlings were grown in tissue culture room with a 16-h photoperiod (approximately 100 $\mu E/m^2/s$), at 24 °C and cultured for 6 days before transferred to the following secondary culture medium.

Callus induction and shoot differentiation: Cotyledons with the petiole (1-2 mm) were cut by scalpel and transplanted to MS medium containing 1.5 mg/L 2,4-D. Ten explants per Petri dish and 3 replicates per treatment were used for secondary culture medium. After 3 days pre-culture, they were transplanted to differentiation culture medium, which contained 3 mg/L BA, 0.15 mg/L NAA and 2.5 mg/L $AgNO_3$.

UV treatment: UV-irradiation was applied to the cotyledons. Cotyledons 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 and an incident dose rate of 3.0 $J/m^2/s$. Time intervals were 30, 60, 120, 180 and 240 s, respectively. After irradiated, cotyledons were transplanted to fresh differentiation culture medium. The rate of callus induction and shoot regeneration was recorded after several days of culturing.

EMS and NaN_3 treatments: EMS and NaN_3 were applied to cotyledons for various time intervals (30, 60, 120, 180 and 240 s). The concentration of EMS included three levels (0.01, 0.1 and 1%), and that of NaN_3 (10, 100 and 1000 μM). Pre-cultured cotyledons were taken out, and immersed into different concentrations of mutagen. After treatment of 5, 15 and

25 h, the cotyledons were washed 3 times by sterile water and removed to fresh differentiation culture medium. After several days, the rate of callus induction and shoot regeneration was observed. Ten explants per Petri dish and 3 replicates per treatment were used for UV and chemical mutagenesis.

Plant regeneration: Approximately 3 weeks later, when shoots developed from the cotyledons, they were then cut free off any callus or hypocotyl tissue and transferred to plastic growth vessels with fresh solid MS medium, where rooting took place. Rooted plantlets were transferred to a solid-perlite mixture and kept for 2 weeks in a nursing room (24 °C, 16 h photoperiod, low light intensity and high humidity), followed by gradual adaptation to greenhouse condition.

Table 1. Plant regeneration from cotyledons by various UV treatments in *B. napus*

Genotype	Duration (s)	Total No. of treated cotyledons	No. of germinated cotyledons	Rate of induced callus (%)*	No. of regenerated plants	Rate of regenerated plants (%)*
N1	0	30	15	50.00 b	9	30.00 bc
	30	30	19	63.33 b	12	40.00 b
	60	30	21	70.00 ab	13	43.33 ab
	120	28	24	85.71 a	19	67.86 a
	180	28	19	67.86 ab	7	25.00 bc
	240	30	16	53.33 b	4	13.33 c
N6	0	30	12	40.00 b	7	23.33 b
	30	28	17	60.71 ab	10	35.71 ab
	60	30	25	83.33 a	15	50.00 a
	120	30	20	66.67 ab	8	26.67 b
	180	28	16	57.14 ab	5	17.86 bc
	240	28	13	46.43 b	2	7.14 c

* Within columns for each genotype, means followed by the same letter are not significantly different at the 0.05 level of probability. The same is true for Tables 2 and 3.

Table 2. Plant regeneration from cotyledons by various EMS treatments in *B. napus*

Genotype	EMS (%)	Duration (h)	Total No. of treated cotyledons	No. of germinated cotyledons	Rate of induced callus (%)*	No. of regenerated plants	Rate of regenerated plants (%)*
N1	0	0	28	22	78.57 a	14	50.00 a
	0.01	5	30	19	63.33 ab	10	33.33 ab
	0.01	15	30	15	50.00 bc	6	20 bcd
	0.01	25	30	8	26.67 d	4	13.33 cd
	0.1	5	30	15	50.00 bc	8	26.67 abc
	0.1	15	30	9	30.00 cd	4	13.33 cd
	0.1	25	30	6	20.00 d	2	6.67 de
	1	5	30	10	33.33 cd	5	16.67 bcd
	1	15	30	8	26.67 d	2	6.67 de
	1	25	28	4	14.29 d	0	0.00 e
N6	0	0	28	20	71.43 a	11	39.29 a
	0.01	5	30	17	56.67 ab	9	30 ab
	0.01	15	30	11	36.67 bcd	7	23.33 ab
	0.01	25	30	7	23.33 cde	5	16.67 b
	0.1	5	28	12	42.86 bc	6	21.43 ab
	0.1	15	30	7	23.33 cde	4	13.33 b
	0.1	25	28	5	17.86 de	1	3.57 c
	1	5	30	8	26.67 ce	4	13.33 b
	1	15	30	5	16.67 de	1	3.33 c
	1	25	30	4	13.33 e	0	0.00 c

Results

UV effect: The result showed that UV had some promotive effect on cotyledon regenerations. Significant difference in the callus induction and plant regeneration was found between various UV irradiation intervals (F=3.96**, F=9.04**). The

highest rates of callus induction (85.71 and 83.33 %) and plant regeneration (67.86 and 50.00 %) were observed from UV treatment of 120 and 60 sec in genotypes N1 and N6 respectively, which were significant higher than that of the control (Table 1). With the increase of UV radiation intervals, the rate of plant regeneration decreased gradually although the rate of callus induction was not much affected. Significant difference in the plant regeneration was also observed between two genotypes in response to various UV irradiation intervals (F=5.69*).

EMS effect: No obvious difference in the rates of callus induction and plant regeneration was observed between the two varieties (F=3.51, F=1.49), while significant difference was found among EMS concentrations and treatment durations in the rates of callus induction and plant regeneration (F=11.86**, F=18.70**). The results indicated that there was no difference in the rates of callus induction and plant regeneration between the treatment of lower EMS concentration (0.01 % for 5 h) and the control. However, with the increase in concentrations and prolongation of treatment intervals, the rate of induced calli and rate of regenerated plants decreased progressively in both genotypes (Table 2). No plant was regenerated when the EMS concentration reached 1 % and the exposure time lasted for 25 h.

NaN_3 effect: Significant difference was observed among NaN_3 concentrations and treatment durations in the rates of callus induction (F=14.71**, F=5.72**) and plant regeneration (F=16.45**, F=7.35**), respectively. After NaN_3 treatment, the rate of callus induction was not much affected or even higher than that of the control. As for the plant regeneration, there was no obvious difference between the treatment of lower NaN_3 concentration (10 μM) and the control in both genotypes (Table 3). Higher concentrations of NaN_3 (100 and 1000 μM) inhibited the plant regeneration, and no plant was produced when its concentration reached 1000 μM and the exposure time lasted for 25 h.

Table 3. Plant regeneration from cotyledons by various NaN_3 treatments in *B. napus*

Genotype	NaN_3 (μM)	Duration(h)	Total No. of treated cotyledons	No. of germinated cotyledons	Rate of induced callus (%)*	No. of regenerated plants	Rate of regenerated plants (%)*
N1	0	0	30	15	50.00 bcd	9	30.00 a
	10	5	30	17	56.67 bcd	10	33.33 a
	10	15	30	11	36.67 cd	6	20.00 ab
	10	25	30	8	26.67 d	3	10.00 abc
	100	5	30	19	36.33 cd	4	13.33 abc
	100	15	28	16	57.14 bcd	2	7.14 bcd
	100	25	28	15	53.57 bcd	1	3.57 cd
	1000	5	28	25	89.29 a	2	7.14 bcd
	1000	15	28	23	82.14 ab	1	3.57 cd
	1000	25	30	20	66.67 abc	0	0.00 d
N6	0	0	30	12	40.00 bc	7	23.33 a
	10	5	30	15	50.00 bc	8	26.67 a
	10	15	30	10	33.33 bc	5	16.67 ab
	10	25	30	7	23.33 c	2	6.67 bcd
	100	5	30	17	56.67 ab	3	10.00 abc
	100	15	30	13	43.33 bc	2	6.67 bcd
	100	25	28	11	39.29 bc	1	3.57 cd
	1000	5	30	24	80.00 a	2	6.67 bcd
	1000	15	30	17	56.67 ab	1	3.33 cd
	1000	25	28	14	50.00 bc	0	0.00 d

Discussion

This article discussed mutagenic effects on in vitro plant regeneration from cotyledon explants in rapeseed. Because of the special merit in crop mutagenesis technology such as relatively high mutagenic rate and useful mutants developed from the basically unchanged genetic background, *in vitro* mutagenesis have been developed fast (Ma & Zhao, 1998; Castillo et al., 2001; Barro et al., 2001, 2003). In this experiment, three mutagens were applied separately to induce cotyledon explants, and among them NaN_3 and UV had the stimulating effects. However, there were some adverse effects such as low mutation rate, the random mutation, tremendous works of assessment in offspring, and the harmfulness and toxic residue of the mutagens (An et al., 2003).

New nontoxic mutagens and efficient mutation approaches need to be explored so that mutation breeding will become more efficient and safer. Moreover, with the further development of reliable tissue culture technique and modern molecular biotechnology, *in vitro* mutagenesis may be able to directly act on pollen, anther or single haploid cell, or induce mutation directly on DNA and analyze the offspring with sophisticated marker techniques, leading to the efficient target and directional

mutations as well as the genetic studies of mutation characters (Liu & Cheng, 1997).

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