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*Full Length Research Paper*

# Effect of ethyl methane sulfonate (EMS) in *in vitro* mutation on anther-derived embryos in loquat (*Eriobotrya japonica* Lindl.)

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**A series of experiments were carried out to investigate the effects of ethyl methane sulfonate (EMS) as an *in vitro* mutation mutagen using the anther-derived embryos of loquat (*Eriobotrya japonica* Lindl) which had been newly developed in the laboratory for the first time in the world. The results showed that EMS treatment caused changes in vitality and conformation of the anther-derived embryos. All EMS treatments employed in the study resulted in lower vitality to various extents depending on the EMS concentration and duration. The death rate of loquat anther-derived embryos was over 50% while the EMS concentration ranged from 0.1 to 0.9% and the exposure time of treatment was 0.5, 1 and 2 h, respectively. Maximal survival rate (46.2%) was obtained in the treatment of 0.3% EMS for 0.5 h and minimal survival rate (2%) was obtained in the treatment of 0.7% EMS for 2 h. The induction ability of secondary embryos decreased after the EMS treatment. Both the maximal percentage (46.2%) of embryos producing new embryos and the maximal number (5.4) of newly formed embryos per embryo producing new embryos were obtained in the treatment of 0.3% EMS for 0.5 h. Minimal percentage (2.8%) of embryos producing new embryos was obtained in the treatment of 0.7% EMS for 2 h. Minimal number (2.75) of newly formed embryos per embryo producing new embryos was obtained in the treatment of 0.9% EMS for 2 h. EMS treatment caused more morphological malformations in the secondary anther-derived embryos. Changes were detected with RAPD amplification profiles in the secondary anther-derived embryos from EMS treatment. There were diversities on the RAPD profiles in the EMS treated embryos, and 0.3% EMS for 2 h was the most effective in causing changes detected by RAPD in all treatments.**

**Key words:** *In vitro* mutation, ethyl methane sulfonate (EMS), anther-derived embryos, *Eriobotrya japonica* Lindl.

## INTRODUCTION

As an important economic fruit crop, loquat (*Eriobotrya japonica* Lindl) is widely cultivated between latitude 20° and 35° including China, Japan, India, Pakistan, Madagascar, Reunion, Island, Mauritius Island, the Mediterranean countries (Spain, Turkey, Italy, Greece, Israel), the United States (mainly California and Florida), Brazil, Venezuela and Australia (Badenes et al., 2000; Vilanova et al., 2001). Ethyl methane sulfonate (EMS) is a mutagen with the characteristic of high mutation rate and has been successfully used in many crops (Zhu, 1997). It has already been used to induce mutation in shoot tips of *in vitro* plants of loquat (*E. japonica* L. 'Dawuxing') (Wang, 2007).

Anther culture has become one of the major sources of haploid plant production used to develop homozygous

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**Abbreviations: EMS,** Ethyl methane sulfonate; **RAPD,** random amplified polymorphic DNA; **DNA,** deoxyribonucleic acid; **NAA,** naphthalene acetic acid; **IBA,** indole butyric acid; **CTAB,** cetyl trimethylammonium bromide; **PCR,** polymerase chain reaction.

**Table 1.** The primers used in the RAPD analysis and their base sequences.

| Primer | Base sequence (5'→3') | Primer | Base sequence (5'→3') |
|---|---|---|---|
| S55 | CATCCGTGCT | S232 | ACCCCCCACT |
| S362 | GTCTCCGCAA | S377 | CCCAGCCTGTG |
| S369 | CCCTACCGAC | S373 | GGTTGTACCC |

**Table 2.** The reaction system of PCR amplification used in the study.

| Reaction component | Concentration | Dosage (μL) |
|---|---|---|
| Taq | 2.5 $U.\mu l^{-1}$ | 0.5 |
| 10×PCR buffer($Mg^{2+}$ free) * | --- | 2.5 |
| $MgCl_2$ | 25 mmol/L | 2.0 |
| dNTPs | 2.5 mmol/L | 2.0 |
| Primer | 2 μmol/L | 2.5 |
| Template | — | 1.0 |
| $ddH_2O$ | — | 14.5 |
| Total | — | 25.0 |

10×PCR buffer ($Mg^{2+}$ free) =Tris-HCl (pH8.3)100mM and KCl, 500 mM.

diploids in plant breeding programs (Bajaj, 1983). Anther culture in loquat (*E. japonica* L.) has been reported successfully (Li, 2008). The anther-derived embryos in *in vitro* are good materials for mutagenic breeding. In this paper, the effects of ethyl methane sulfonate (EMS) as an *in vitro* mutation mutagen using the anther-derived embryos of loquat was investigated.

## MATERIALS AND METHODS

The anther-derived embryos *in vitro* of loquat has been newly developed in the laboratory for the first time in the world. An anther-derived embryo with strong acidity was selected as the experimental materials for the investigation.

### Mutation method

The anther-derived embryos (about 0.5 cm in size) were immersed in filtered sterilized EMS solutions of various concentrations for various durations. The concentrations of EMS were 0.1, 0.3, 0.5, 0.7 and 0.9%, and treatment duration was 0.5, 1.0 or 2 h.

### Analyses of the materials after EMS treatment

After immersing in EMS solution, anther-derived embryos were rinsed five times in sterile water and then cultured in MS + sucrose 3% +gelrite 2.5% + ZT 0.05 mg $L^{-1}$ + NAA 0.02 mg $L^{-1}$ +IBA 0.02 mg $L^{-1}$. The responses of loquat anther-derived embryos to EMS treatment and effects of EMS treatment on anther embryo production in loquat were surveyed and statistics were made.

RAPD analysis was performed according to the method described by Maliga et al. (1995), with CTAB method for DNA extraction (Doyle and Doyle, 1990). The primers used in RAPD analysis are listed in Table 1. The PCR amplification reaction system is summarized in Table 2. The amplification cycle consist of: 94°C pre-denatured at 5 min; 94°C denatured at 1 min, 38°C annealing 1 min and 72°C extension 1 min for 40 cycles; 72°C extension 5 min and stored at 4°C. The amplified products were separated in 1.5% agarose gel, visualized by ethidium (EB) staining and photographed.

## RESULTS

### Vitality changes caused by EMS treatment

All EMS treatments employed in the study resulted in lower vitality to various extents depending on the EMS concentration and duration (Table 3). Most of the loquat anther-derived embryos were black-surfaced and dead. At the same treatment duration, with the concentrations of EMS increasing, the death rate increased. The death rate of loquat anther-derived embryos was over 50% when the EMS concentration ranged from 0.1 to 0.9% and the exposure time of treatment were 0.5, 1 and 2 h, respectively. Maximal survival rate was obtained in the treatment of 0.3% EMS for 0.5 h and minimal survival rate was obtained in the treatment of 0.7%.

### Induction ability of secondary embryos changes caused by EMS treatment

The induction ability of secondary embryos decreased after the EMS treatment (Table 4). Only a small number of these EMS treatments anther-derived embryos had induction ability of secondary embryos. Most of these secondary embryos were at the globular stage. Both the

**Table 3.** The responses of loquat anther-derived embryos to EMS treatment.

| EMS concentration (%) | Time of treatment (h) | Number of loquat anther-derived embryo before treatment | Number of loquat anther-derived embryo after treatment | Livability (%) |
|---|---|---|---|---|
| 0.1 | 0.5 | 130 | 50 | 38.5 |
| | 1 | 114 | 22 | 19.3 |
| | 2 | 131 | 11 | 8.4 |
| 0.3 | 0.5 | 124 | 58 | 46.8 |
| | 1 | 104 | 24 | 23.1 |
| | 2 | 122 | 22 | 18.0 |
| 0.5 | 0.5 | 111 | 36 | 32.4 |
| | 1 | 94 | 14 | 14.9 |
| | 2 | 123 | 17 | 13.8 |
| 0.7 | 0.5 | 90 | 15 | 16.7 |
| | 1 | 149 | 20 | 13.4 |
| | 2 | 106 | 3 | 2.8 |
| 0.9 | 0.5 | 141 | 37 | 26.2 |
| | 1 | 120 | 19 | 15.8 |
| | 2 | 100 | 4 | 4 |
| CK | 0 | 50 | 48 | 96 |

**Table 4.** Effects of EMS treatment on anther embryo reproduction in loquat.

| EMS concentration (%) | Time of treatment (h) | Loquat anther-derived embryo before treatment | Number of embryo producing new embryo | Percentage of embryo producing new embryo | Number of newly formed embryo per embryo producing new embryo |
|---|---|---|---|---|---|
| 0.1 | 0.5 | 130 | 48 | 36.9 | 3.85 |
| | 1 | 114 | 22 | 19.3 | 2.95 |
| | 2 | 131 | 11 | 8.4 | 3.64 |
| 0.3 | 0.5 | 124 | 58 | 46.8 | 5.41 |
| | 1 | 104 | 24 | 23.1 | 4.67 |
| | 2 | 122 | 22 | 18.0 | 4.27 |
| 0.5 | 0.5 | 111 | 36 | 32.4 | 3.89 |

**Table 4.** Contd.

| | | | | | |
|---|---|---|---|---|---|
| | 1 | 94 | 14 | 14.9 | 3.71 |
| | 2 | 123 | 17 | 13.8 | 4.53 |
| | 0.5 | 90 | 15 | 16.7 | 3.80 |
| 0.7 | 1 | 149 | 20 | 13.4 | 3.85 |
| | 2 | 106 | 3 | 2.8 | 3.67 |
| | 0.5 | 141 | 37 | 26.2 | 4.70 |
| 0.9 | 1 | 120 | 19 | 15.8 | 5.05 |
| | 2 | 100 | 4 | 4.0 | 2.75 |
| CK | 0 | 50 | 44 | 88.0 | 5.83 |

**Table 5.** RAPD amplificaqtions with 4 primers on loquat anther-derived embryos.

| EMS concentration (%) | Treatment duration (h) | Number of variation by PCR using s55 as primer | Number of variation by PCR using s232 as primer | Number of variation by PCR using s377as primer | Number of variation by PCR using s362 as primer | Number of average variation |
|---|---|---|---|---|---|---|
| | 0.5 | 2 | 2 | 1 | 2 | 1.75$^{b}$ |
| 0.1 | 1 | 1 | 4 | 1 | 0 | 1.5$^{b}$ |
| | 2 | 3 | 2 | 1 | 0 | 1.5$^{b}$ |
| | 0.5 | 1 | 3 | 1 | 3 | 2.0$^{b}$ |
| 0.3 | 1 | 1 | 3 | 1 | 0 | 1.25$^{b}$ |
| | 2 | 3 | 4 | 3 | 7 | 4.25$^{a}$ |
| | 0.5 | 1 | 0 | 2 | 0 | 0.75$^{b}$ |
| 0.5 | 1 | 1 | 1 | 1 | 0 | 0.75$^{b}$ |
| | 0.5 | 1 | 0 | 1 | 0 | 0.5$^{b}$ |
| 0.7 | 1 | 1 | 1 | 5 | 0 | 1.75$^{b}$ |
| | 0.5 | 0 | 1 | 1 | 0 | 0.5$^{b}$ |
| 0.9 | 1 | 0 | 1 | 1 | 0 | 0.5$^{b}$ |
| | 2 | 1 | 1 | 3 | 0 | 1.25$^{b}$ |

Data were statistically analyzed by Duncan’s multiple range test (a，b＝0.05) (Means having the same letter in the columns were not significantly different according to Duncan ’s multiple range test (P = 0.05)).

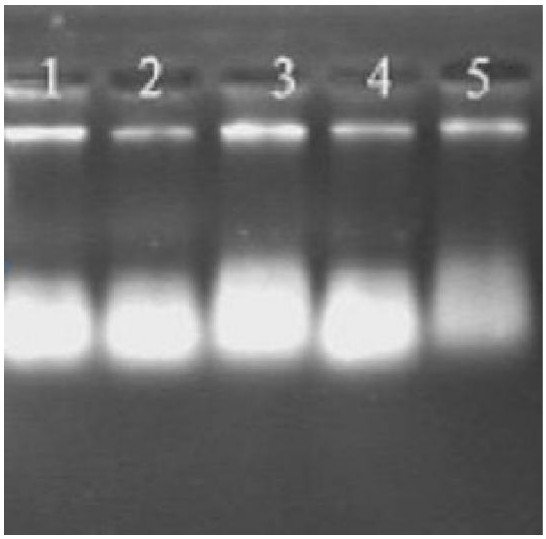


**Figure 1.** Results of DNA electrophoretic scan.

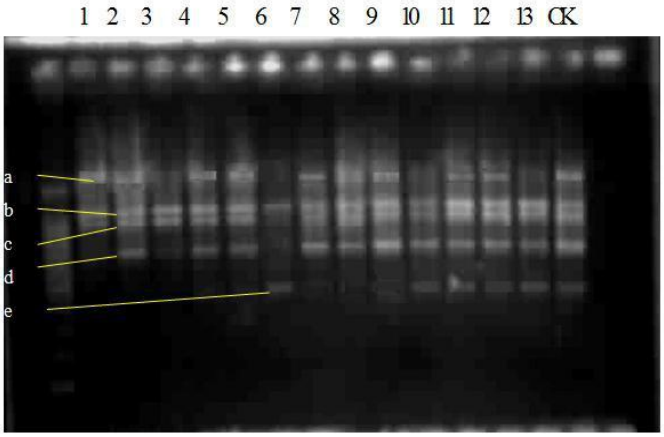


**Figure 2.** The RAPD profiles of different materials (s55 primer).

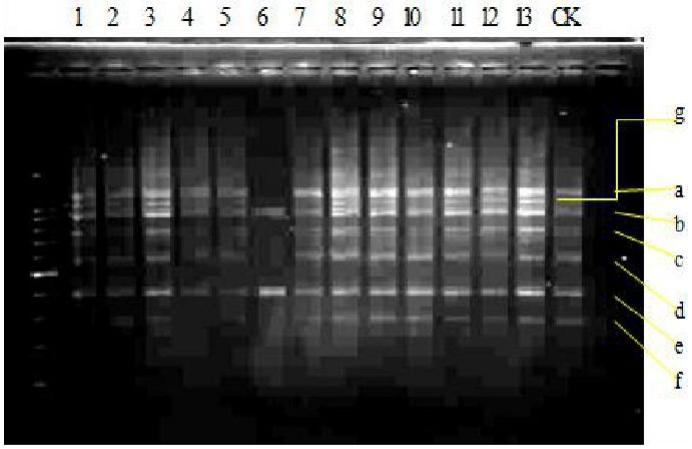


**Figure 3.** The RAPD profiles of different materials (s232 primer).

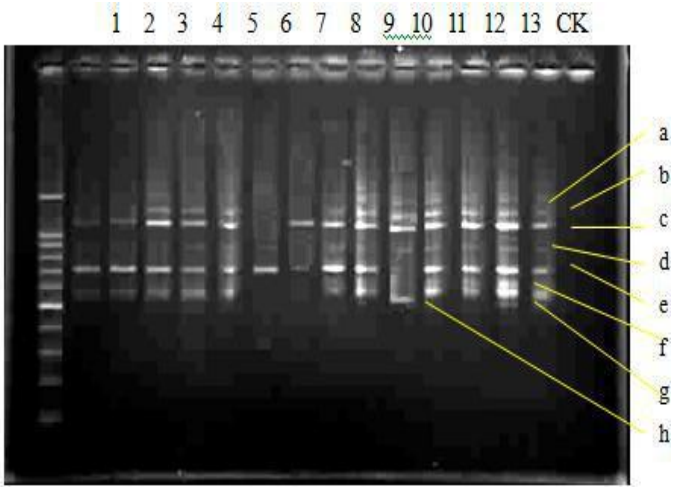


**Figure 4**. The RAPD profiles of different materials (s377 primer).

maximal percentage of embryos producing new embryos and the maximal number of newly formed embryos per embryo producing new embryos were obtained in the treatment of 0.3% EMS for 0.5 h. Minimal percentage of embryos producing new embryos was obtained in the treatment of 0.7% EMS for 2 h. Minimal number of newly formed embryos per embryo producing new embryos was obtained in the treatment of 0.9% EMS for 2 h.

### RAPD profiles of the anther embryo from EMS treatment

There were more morphological malformations in the EMS treatment secondary anther-derived embryos including one embryo with three or more cotyledons and embryos with malformed cotyledons. Changes were detected with RAPD amplification profiles in the secondary anther-derived embryos from EMS treatment (Table 5; Figures 1, 2, 3, 4 and 5). There were 11 treatment differences from the contrast when using s55 as the primer, 11 treatment differences when using s232 as the primer, 13 treatment differences when using s377 as the primer, and 3 treatment differences when using s362 as the primer. 0.3% EMS for 2 h was the most effective in all treatments.

## DISCUSSION

The results of this study indicated that EMS was double to induce variations in anther-derived embryos in Loquat. For different material, the best concentrations and treatment duration of EMS maybe different. When treatment of 0.3% EMS for 2 h was employed, the

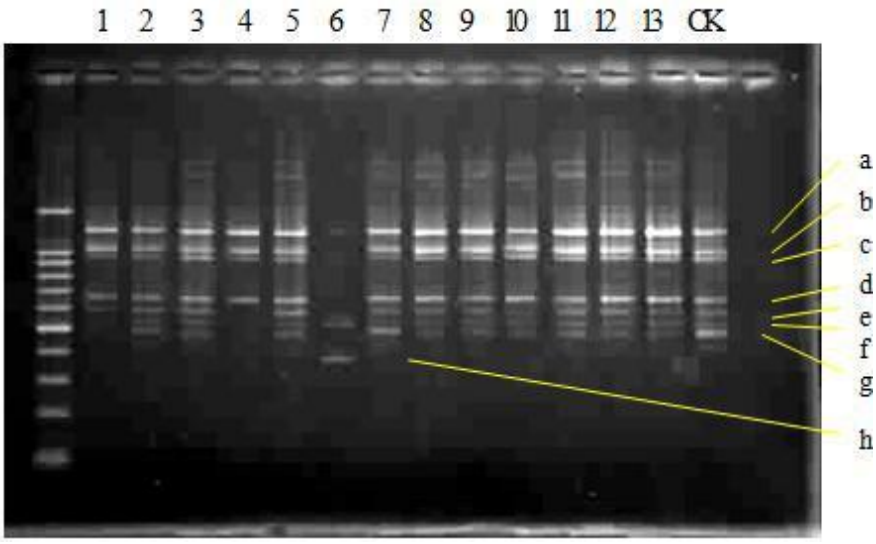


**Figure 5.** The RAPD profiles of different materials (s362 primer).

anther-derived embryos in loquat could keep higher livability and their secondary embryos could keep higher induction ability with other treatments. RAPD assays proved genetic variation in all treatments, and treatment of 0.3% EMS for 2 h was the best. Mutagens applied to regenerating strawberry anther have resulted in production of plants with wide variation in chromosome number (Zimmerman, 1994). It suggested that these EMS treatment embryos could produce plants with wide variation in chromosome number once budded successfully.

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