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ORIGINAL PAPER

Mutation induced by ethylmethanesulphonate (EMS), in vitro screening for salt tolerance and plant regeneration of sweet potato (*Ipomoea batatas* L.)

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Abstract Salt tolerant cultivars of sweet potato (*Ipomoea batatas* L.) can be obtained from induced mutation. The objective of the present study was to induce mutation for salt tolerance using ethylmethanesulphonate (EMS) in calli of sweet potato, followed by cell line selection and subsequent plant regeneration. Calli initiated from leaf explants were treated with 0.5% EMS for 0, 1, 1.5, 2, 2.5 and 3 h, followed by rinsing with sterile distilled water for four times. Preliminary experiments showed that 200 mM NaCl could be used as selection pressure. Salt tolerant calli were sub-cultured on medium supplemented with 200 mM NaCl for selection of mutant cell lines and this process repeated 5 times (20 days each). The selected calli were transferred onto somatic embryo formation medium, which was Murashige and Skoog (MS) medium supplemented with 4 mg l^{-1} abscisic acid (ABA), 10 mg l^{-1} gibberellic acid (GA). After 15 days, somatic embryos were transferred onto MS medium supplemented with 0.05 mg l^{-1} ABA, 0.2 mg l^{-1} zeatin (ZT) for regeneration. Plants designated as ML1, ML2 and ML3 were regenerated from the somatic embryos formed by calli treated with 0.5% EMS for 2 and 2.5 h. After propagation, salt tolerance of these mutants was investigated. Data suggested the mutants were more salt tolerant than control plants.

Keywords Sweet potato · Calli · Mutagenesis · Mutant · Salt tolerance

Abbreviations

EMS	Ethylmethanesulphonate
MS	Murashige and Skoog
ABA	Abscisic acid
GA	Gibberellic acid
ZT	Zeatin
4FA	4-fluorophenoxyacetic acid

Sweet potato is an important food crop and ranks seventh in terms of worldwide staple food production (Ekanayake et al. 1993). In addition to its nutritional value, its stems, leaves and roots have the anticancer attributes (Huang et al. 2004). Its roots also serve as a rich source of starch for industrial uses. However, soil salinity limits high productivity and expansion of sweet potato cultivation.

The most effective means that can deal with soil salinity is to grow cultivars that can establish and be productive on such soils. Salt tolerant sweet potato can be derived from induced muta-

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tion with physical or chemical mutagens. Compared with physical agents, chemical mutagens are perhaps more capable of leading to specific and predictable mutations. Bhagwat and Duncan (1998) treated the shoot apices of in vitro grown cultures of banana with various concentrations of mutagens sodium azide, diethyl sulphate, and ethylmethanesulphonate. There were no significant differences between the numbers of variations induced by the mutagens and between different mutagen treatment durations. Regenerated plants were screened for tolerance to the fungus under greenhouse conditions. The selected plants were tolerant even under the field conditions. EMS is a chemical mutagen of the alkylating group and has been commonly used in plant breeding because it can cause high frequency of gene mutations and low frequency of chromosome aberrations (Van Harten 1998). Recently this mutagen has been used to treat in vitro explants of many species (Duron 1992) and efficient mutagenesis of plant cell culture is possible, as the cells can be uniformly treated.

In vitro culture is an ideal system for screening of salt tolerant mutants, as it can be carried out under controlled conditions with limited space and time. Moreover, unlike the whole plant, a very large number of cells can be screened at one time for a desired trait. Because the cells are grown in a uniform cultural environment, reproducible selection protocol can be employed. Hence, plant tissue culture techniques have been used for the isolation of spontaneous or induced mutant cell lines that have direct application in agriculture as well as in basic research (Flick 1983). In addition, cell line selection and subsequent plant regeneration have been used extensively to develop salt tolerant plants (Kirti et al. 1991; Sumaryati et al. 1992). There is no report of EMS or other chemical mutagens use on calli of sweet potato for salt tolerance and cell line selection. The objective of the present study was to obtain salt tolerant mutant via application of EMS on calli of sweet potato followed by plant regeneration.

The sweet potato cultivar Lu8 was used in the experiment. Though widely grown in China, this cultivar was sensitive to salt stress.

Axillary buds from healthy shoots were collected, washed in running tap water, treated with 75% ethanol for 30 s and 0.1% mercuric solution for 8 min, then rinsed in sterile distilled water for four times. The explants were then cultured on growth regulator free MS (Murashige and Skoog 1962) medium. After 3 weeks of culture, leaf segments were collected and cut into small pieces of about 5×5 mm for further experiment. The MS medium supplemented with 3% (w/v) sucrose was used as a basal medium. The medium was solidified with 0.6% (w/v) agar. 1 mg l^{-1} 4 FA was added to the medium as growth regulator, pH was adjusted to 5.8 and autoclaved at 121°C for 20 min. To culture callus, small pieces of leaves were incubated in flasks containing 30 ml medium in the dark at the temperature 28 ± 1°C and transferred to fresh medium every 15 days. After 30 days, calli were cut into 2–3 mm^3 clumps. Afterwards, the clumps were dipped in MS medium supplemented with 1 mg l^{-1} 4 FA, 3% (w/v) sucrose and 0.5% EMS for 0, 1, 1.5, 2, 2.5 and 3 h and there were 60 clumps for each dose.

After 0.5% EMS treatment, the clumps were rinsed in sterile distilled water for three to four times. Preliminary experiments showed that growth of sweet potato cell cultures was inhibited in 200 mM NaCl. Therefore, this concentration of NaCl was used as selection pressure. In the experiment, we found that long time of subculture can reduce the regeneration rate. So in the selection procedure, we subcultured the calli on callus culture medium supplemented with 200 mM NaCl for only 5 times (20 days each).The salt tolerant calli were regarded as mutant cell lines.

The viable calli grown under the selective pressure were selected out and transferred onto somatic embryo formation medium which was MS medium containing 4 mg l^{-1} ABA, 10 mg l^{-1} GA. After 15 days, these somatic embryos were transferred onto regeneration medium, which was MS medium supplemented with 0.05 mg l^{-1} ABA, 0.2 mg l^{-1} ZT. The regenerated mutants were propagated on the growth regulator free 1/2 MS medium in the absence of salt for 3–4 weeks.

To evaluate salt tolerance in mutants, 1/2 MS medium amended with various concentrations (0, 50, 100, 200 and 400 mM) of NaCl was used in experiment. The plantlets regenerated from the

calli that were not exposed to EMS and NaCl were used as control. Mutants and control plants were transferred as single node cuttings to fresh solidified 1/2 MS medium supplemented with various concentrations of NaCl for 60 days. After stress treatment, the following parameters were measured: shoot length, shoot fresh weight, chlorophyll content and membrane permeability. All data were subjected to statistical analysis using Duncan's Multiple Range Test. Each treatment consisted of five replicates.

Both EMS treatment and ion toxic effect can result in death of a large number of calli. Under the selection pressure (200 mM NaCl), the growth of almost all the calli treated by 0.5% EMS for 0, 1, 1.5 and 3 h were inhibited. The results suggest that 0, 1, and 1.5 h of 0.5% EMS treatment might be too short to cause mutagenesis in improving salt tolerance. However, this concentration for 3 h was highly toxic and result in adverse effect on proliferation of calli. 30 and 32% of calli treated by EMS for 2 and 2.5 h respectively were viable, so these salt tolerant ones were selected for the subsequent experiment.

Sweet potato is considered as recalcitrant species regarding plant regeneration (Sihachakr et al. 1997). Besides, 100 days of subculture reduced the regeneration rate. So 16 days after inoculating in regeneration medium, there were only three mutants designed as ML 1, ML 2 and ML 3 were regenerated from two somatic embryos (Fig. 1). ML 1and ML 2were regenerated from the somatic embryo that was formed by the calli treated with 0.5% EMS for 2 h. ML 3 was regenerated from the somatic embryo that formed by the calli treated with 0.5% EMS for 2.5 h.

In the 1/2 MS medium containing 0 mM NaCl, both mutants and control grew well and there was no significant difference between them. However, in the medium supplemented with various concentrations of NaCl, it is obvious that the mutants showed a better growth status than the control. In the medium supplemented with 200 mM NaCl, control plants showed a yellow color when inoculated for 7 days, and gradually died. This change was visible in ML1 and ML2 10 days after inoculating. However, ML3 remained alive during a 60 days period. Tiny buds emerged, but no roots formed. It was a good indicator of salinity tolerance. When the concentration of NaCl was increased to 400 mM, no survival was recorded 5 days after inoculation. The growth status showed that mutants exhibited stronger salt tolerance than control.

Table 1 illustrates the data obtained in the experiment on the performance of the regenerated plants (both control plants and mutants). The results also showed that mutants expressed stronger salt tolerance than control. At each stress level, the mutants had higher shoot length, shoot fresh weight and chlorophyll content, lower membrane permeability than control plants. Although when grown in the 1/2 MS medium supplemented with 50 mM NaCl , there was no significant difference in shoot length between control and mutants, the different was significant when the concentration of NaCl reached 100 mM. Obviously, the injurious effect of salinity was more striking in control than in mutants. Among the mutant lines, the ML3 exhibited the strongest

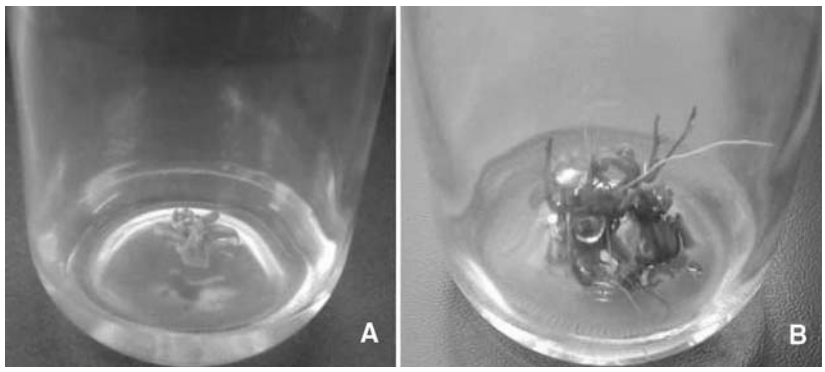

Fig. 1 The mutant plantlets regenerated from the somatic embryos. Sixteen days after transferring onto the regeneration medium, three mutants were regenerated from two somatic embryos (A) 5 days after regeneration (B) 15 days after regeneration

Table 1 The performance of mutants and control in 1/2 MS medium containing different concentrations of NaCl (inoculated for 60 days)

NaCl conc. (mM)	Plantlet	Shoot length[a](mm)	Shoot fresh weight (g)	Chlorophyll content (mg/g)	Membrane permeability
50	ML1	34.2 ± 2.857 a	0.287 ± 0.035 ab	1.463 ± 0.110 ab	0.317 ± 0.019 ab
	ML2	34.6 ± 2.728 a	0.260 ± 0.051 ab	1.402 ± 0.112 ab	0.332 ± 0.029 ab
	ML3	35.0 ± 3.162 a	0.323 ± 0.057 a	1.591 ± 0.171 a	0.285 ± 0.028 b
	Control	35.4 ± 2.871 a	0.200 ± 0.039 b	1.288 ± 0.133 b	0.361 ± 0.051 a
100	ML1	14.6 ± 1.200 a	0.271 ± 0.038 ab	1.463 ± 0.110 ab	0.377 ± 0.018 b
	ML2	4.6 ± 2.245 a	0.246 ± 0.050 b	1.288 ± 0.112 b	0.389 ± 0.015 b
	ML3	16.4 ± 2.728 a	0.317 ± 0.057 a	1.505 ± 0.158 a	0.330 ± 0.036 c
	Control	11.2 ± 1.720 b	0.171 ± 0.042 c	1.076 ± 0.067 c	0.476 ± 0.021 a

[a] Value is mean ± SD

salt tolerance. It might suggest that 2.5 h was the optimum duration of 0.5% EMS treatment, which could induce the highest frequency of mutagenesis for salt tolerance.

Mutation breeding in crop plants is an effective tool in plant breeders especially in crops as they have narrow genetic base. Mutagenesis has been popular over past decades because it is simple, cheap to perform, applicable to all plant species and usable at small or large scale. By varying mutagen dose, the frequency of induced mutations can be regulated and saturation can be readily achieved (Robbie et al. 2006). As a chemical mutagen, EMS has been most commonly used. Carlson (1970) selected auxotrophic mutants in haploid cells using EMS as the mutagen, and microbiological techniques for screening. Mirza et al. (1997) isolated a spermine-resistant mutant of *Arabidopsis thaliana* L. Heynh. from M2 population of ethylmethanesulphonate–mutagenized seeds. The results of using EMS have been in fact mixed. While some improvement in the recovery of variants has been reported for salt. Nabors et al. (1975) selected NaCl-resistant cell lines from *Nicotiana tabacum* L. cell suspension culture treated by the mutagen EMS and grown in a medium containing 0.03 MNaCl. Cells derived from these lines even resisted concentrations as high as 0.09 MNaCl in the medium. The mutant was resistant to seed germination inhibition by spermine. Lu et al. (1994) treated embryogenic calli of millet with 0.5% EMS for 2.5 h, the regenerate plants exhibited strong resistance to NaCl stress. Though the cell culture technique itself causes variation, it may be desirable to enhance the variability by additional physical or chemical mutagenic treatments. In the present study, despite the fact that only three mutants regenerated, the improved salt tolerance of mutants suggests mutagenesis with EMS in sweet potato is significative. This system may be useful for mutation breeding and for the development of salt tolerant sweet potato plants.

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