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# Selection of Acid Tolerant Red Clover Cell Line on the Cellular Level

## Ⅱ. Effect of some factors affecting suspension culture and acid tolerant cell selection

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# 耐酸性 레드 클로-버의 培養細胞水準에서의 選拔

## Ⅱ. 레드 클로-버의 현탁배양 및 내산성 세포의 선발

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### 摘 要

레드 클로-버의 액체배양세포로부터 내산성 세포선발에 대한 몇가지 요인들의 영향을 규명하기 위한 실험으로 부터 다음과 같은 결과들을 얻었다.

액체배양세포의 유도에는 auxin원으로 2,4-D 2 mg/ℓ 와 cytokinin으로 BAP 0.5 mg/ℓ 를 혼합처리한 것이 가장 좋았으며, 여러가지 기본배지중 PC배지가 액체배양세포의 유도에 가장 효과적이었다. 내산성 세포를 선발하기 위하여 EMS를 여러가지 농도에서 4시간 처리했을 때, 가장 효율적인 EMS농도는 1%였으며 이 때 plate당 평균 27개의 colony가 선발되었다. 1%의 EMS를 처리한 결과 내산성 세포 발생빈도는 $8.9\times10^{-5}$으로서 EMS 무처리구의 100배나 높은 수준으로 나타났으며 최초에 선발된 변이주는 총 176주였으나 선택배지에서 4주간격으로 2회 계대배양 후 살아남은 colony수는 44주였다.


## Ⅰ. INTRODUCTION

It is very expensive, time consuming and laborious work to select an environmental tolerant plant from $10^5$-$10^6$ plants in the field. On the other hand it is a very simple work to select a tolerant cell from about $10^8$ cells in the laboratory if the technique is established. The history of a mutant selection on the cellular level has not been so long. Researches in selection of cells tolerant to unfavorable environments are mostly concentrated on the tolerance to salt, disease or to herbicide. Many scientists reported the results of selecting mutant cell lines tolerant to salt in rice(Flowers et al., 1981, Oono et al., 1978, Yoshida et al., 1983) and tobacco (Dix et al., 1975, Nabors et al., 1975, Nabors et al., 1980), to $CuSO_4$(Oono 1978), valine analog(bourgin 1978), para-flourophenylalanine(Flick 1980) and cyclohexamide in carrot(Gresshoff 1979). Although in vitro legume culture technique was first established in the 1950's, somatic cell culture techniques on the several legume species were applied only recently (Phillips et al., 1978). The reports on the in vitro culture of red clover have been limited to plant regeneration from callus culture and from cell suspension cultures by Phillips et al.

This experiment was conducted to select acid tolerant cell lines of red clover at the cellular levels.

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## Ⅱ. MATERIALS AND METHODS

### 1. Suspension culture of red clover

Fortyfive days old friable calli subcultured twice with 10 day-intervals derived from Kenland red clover were used to establish cell suspension cultures. One gram of fresh calli were placed in a conical flask with 20 ml of plant regulator free PC liquid medium and agitated on gyratory shaker with 120 rpm at 28℃ for 7 days. Cells were screened with a 80 ㎛ sieve for single cells and 1 ml of sieved cells as packed cell volume were distributed to every 100 ml conical flask containing 19 ml PC liquid medium for plant regulators treatment. Several concentrations of 2,4-D and picloram as auxin source and BAP as cytokinin source were combinated for optimal conditions of suspension culture. Basal media for optimum suspension culture conditions were investigated among PC, SH, $B_5$, SL and BO. The proliferated cells were counted with packed cell volume every other day for 12 days.

### 2. Selection of acid tolerant cell lines

Kenland red clover was used for the selection of acid tolerant cell lines. Single cells on the exponential phase were collected by centrifugation at 1,000 rpm for 3 minutes and were treated with EMS (ethyl methane sulfonate, $CH_3SO_3C_2HS$) as a mutagen. PC medium containing 2 mg/ *l* 2,4-D and 0.5 mg/ *l* BAP were adjusted to pH 5.8 and bacterial cells were eliminated by filtration with a 0.2 ㎛ membrane filter. Various concentrations of EMS were treated to $3 \times 10^6$ cells/ml for 8 hours with 2-hour intervals. Survived cells after 2 days EMS treatment were stained by Evan's blue and counted under a microscope. Suspension cultured cells of $3 \times 10^6$ cells/ml in the exponential phase were inoculated to medium containing 2 mg/ *l* 2,4-D, 0.5 mg/ *l* BAP and 0.5% agar and were acidified at the range of pH 5.8-3.0 in order to determine the least lethal concentration of acidity. Colonies formed after 6 weeks of inoculation on the plate were counted. In order to select acid tolerant cell lines, a semisolid agar medium containing 2 mg/ *l* 2,4-D, 0.5 mg/ *l* BAP was adjusted the pH lower than the least lethal concentration of acidity and cells were inoculated to the medium. After 12 weeks incubation, the survived colonies were selected and transfered to a neutral medium of pH 5.8. Colonies were subcultured twice at the neutral medium for 8 weeks, transfered to the medium adjusted to pH 3, and subcultured twice for another 8 weeks. The survived colonies were selected as acid tolerant cell lines and subcultured twice at the neutral medium for 8 weeks.

## Ⅲ. RESULTS AND DISCUSSION

### 1. Suspension culture

Picloram and 2,4-D as auxin source and BAP as cytokinin source were used for suspension culture from callus of Kenland red clover. Concentrations and combinations of plant regulators were compared and the results are shown in Fig. 1. Growth are denoted as the percentage of packed cell volume. Packed cell volume was determined by measuring the cell volume after centrifugation of the suspension cultured cells at 1,000 rpm for 3 minutes. The average growth was better in the treatment of picloram than that of 2,4-D as auxin source but the highest yield was obtained in the 2,4-D treatment. In the 2 mg/ *l* 2,4-D + 0.5 mg/ *l* BAP treatment, the growth rate was 90% which was the highest yield. The highest yield in the picloram treatment was achieved in the 0.5 mg/ *l* picloram + 0.1 mg/ *l* BAP with the 80% growth rate which was different tendency in the callus formation where picloram resulted more callus formation than 2,4-D did (Son et al., 1992 in contribution). The cells treated with picloram only turned brown more rapidly than those treated with 2,4-D only. Phillips et al. (1980) used 0.06 mg/ *l* picloram + 0.1 mg/ *l* BAP as

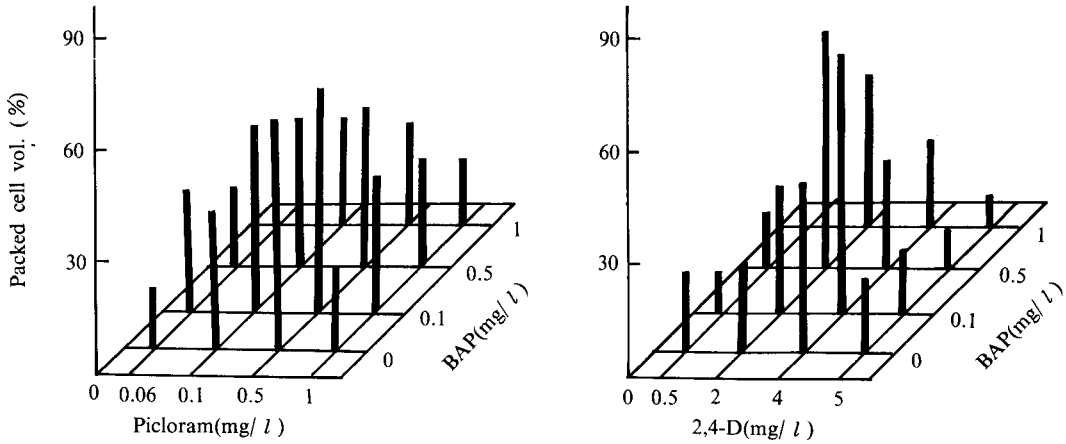


Fig. 1. Effect of growth regulators interaction on the cell growth of Kenland red clover.

plant growth regulator. The growth pattern according to media after 12 days culture showed similar tendency among treatments as shown in Fig. 2. The growth rate in the PC medium was 75% after 8 days from incubation start but decreased thereafter. Phillips et al. (1980) used SL medium. SL medium did not showed better result than PC medium in this experiment and cells grown in the PC medium was used for the suspension culture of Kenland red clover by subculturing 8 days old cells.

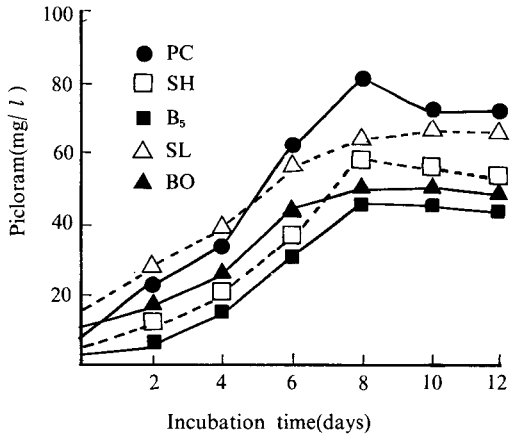


Fig. 2. Effect of various midia on the cell growth of Kenland red clover.

**2. Treatment of Ethyl Methane Sulfonate(EMS)**

EMS treatment experiment was conducted to get the information of optimum range of EMS concentration and treatment duration. Cells were treated with various periods and concentrations of EMS and survived cells after EMS treatment were counted by staining with Evan's blue. Viable cells decreased with the increased EMS concentrations and with the treated time as shown in Fig. 3. At 2.0% and 8 hours EMS treatment, survived cell decreased to nearly 0%, on the other hand at 0.1% and 8 hours treatment about 70% of the cell survived after 2 days incubation. The purpose of EMS treatment is to obtain as many mutated cells as possible. Too high concentration of EMS resulted in all dead cells whereas too low concetration brought no mutated cells. So, the 1.0% and 4 hours of EMS treatment was selected for the purpose, because at that treatment about 50% of the treated cells were alive after 2 days incubation.

**3. The least lethal pH**

Medium pH was adjusted to the six steps at the range of 5.0 to 3.0 in order to investigate the least lethal pH of Kenland red clover cells when EMS was free from the medium. Colonies survived

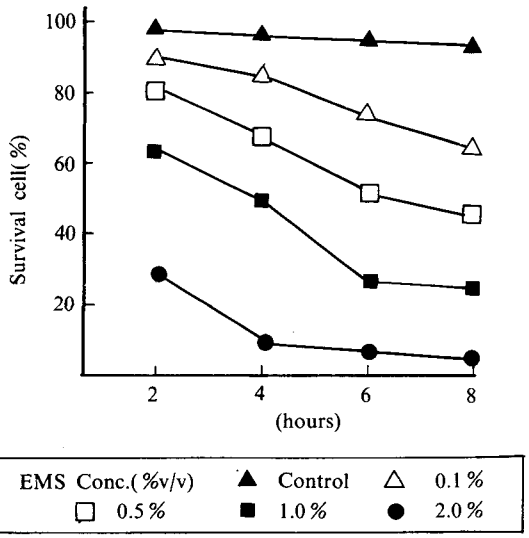


Fig. 3. Effect of EMS on the survival of Kenland red clover cells with time.

were counted after six weeks incubation in each treatment. After six weeks incubation, average 172 colonies/plate were formed in the control treatment of pH 5.8 when $3 \times 10^6$ cells/plate were applied initially. The number of colonies were decreased with the decreased medium pH and when pH was lowered to 3, average only one colony was alived as shown in Table 1. So the medium pH 3 was selected as the least lethal pH.

#### 4. Selection of acid tolerant cell line

The EMS concentrations were designed to 0.0, 0.1, 0.5, 1.0 and 2.0% on the basis of the previous experiment. In each EMS concentration, cells were treated for 4 hours. The percentages of viable cells were decreased with increasing of EMS

Table 1. Number of pH-resistant colonies after 6 weeks cultured in various pH concentration treatments.

| pH concentration | No. of resistant colonies | | | Total No. of resistant colonies | Mean±SD[a)] |
|---|---|---|---|---|---|
| | Rep. 1 | Rep. 2 | Rep. 3 | | |
| 0.0 | 180 | 170 | 165 | 515 | 171.7 ± 7.64 |
| 5.0 | 110 | 90 | 100 | 300 | 100.0 ±10.00 |
| 4.5 | 80 | 70 | 65 | 215 | 71.77± 7.63 |
| 4.0 | 30 | 40 | 33 | 103 | 34.3 ± 5.13 |
| 3.5 | 12 | 15 | 8 | 35 | 11.7 ± 3.57 |
| 3.0 | 1 | 0 | 2 | 3 | 1.0 ± 1.73 |

[a)] Standard deviation.

concentrations (Table 2). The survived cells after 4 hours incubation in EMS were plated every $3 \times 10^6$ cells/plate in PC semisolid agar medium adjusted to the least lethal pH, the pH 3. After 6 weeks incubation in each treatment, the colonies formed were increased with increasing EMS concentration until 1.0% but at 2.0%, the number decreased drastically. It seemed that the cells treated with 2 % EMS were damaged seriously before acquiring acid tolerance. The 1% EMS treatment was the most suitable in Kenland red clover cells for mutation in which average 27 colonies/plate aquired acid tolerance. The average frequency of mutation, which was calculated by dividing the number of acid tolerant colonies by $3 \times 10^6$, the initially plated cell number, was $8.9 \times 10^{-5} \pm 5.1 \times 10^{-6}$ in 1.0% EMS treatment. The mutated colonies at the first selection was total 176, but the survived colo-

Table 2. Effect of EMS concentration on the % viable cells, the number of resistant colonies to pH and frequency of colony formation.

| Number cells plated (cells/plate) | EMS concentration (%v/v) | % viable cells | Total[a] number of cells | Mean±SD[b] | Average frequency of colony formation ± SD[c] |
|---|---|---|---|---|---|
| | 0.0 | 95 | 4 | 1.3±1.66 | $4.3 \times 10^{-7} \pm 5.5 \times 10^{-7}$ |
| | 0.1 | 85 | 37 | 12.3±1.07 | $4.1 \times 10^{-6} \pm 3.5 \times 10^{-7}$ |
| $3 \times 10^6$ | 0.5 | 65 | 43 | 14.3±3.57 | $4.8 \times 10^{-6} \pm 1.2 \times 10^{-6}$ |
| | 1.0 | 50 | 80 | 26.7±1.53 | $8.9 \times 10^{-5} \pm 5.1 \times 10^{-6}$ |
| | 2.0 | 10 | 12 | 4.0±1.00 | $1.3 \times 10^{-6} \pm 3.3 \times 10^{-7}$ |

[a] Total number from 3 seperate experiments.
[b] Mean number of colonies ± standard deviation.
[c] The frequency was calculated by dividing the number of resistant colonies by the total number of plated viable cells.

nies after two subcultures with 4-week intervals were decreased to 44.

## Ⅳ. SUMMARY

This experiment was conducted to select acid tolerant cells from red clover suspension culture and obtained following results. The most effective condition of plant growth regulators for suspension culture of red clover cells was 2 mg/ *l* 2,4-D as auxin source plus 0.5 mg/ *l* BAP as cytokinin source. Among serveral basal media, PC medium brought the best result. The most suitable EMS concentration for the mutated cells tolerant to acid was 1 % with fours treatment and average 27 colonies/plate were selected. The frequency of the occurence of mutants tolerant to acid in 1 % EMS with four hours treatment was $8.9 \times 10^{-5}$ which was 100 times larger than that of control. The total colonies selected initially were 176 but the survived colonies after two subcultures with 4 weeks interval in a selection medium were 44.

## Ⅴ. LITERATURE CITED

1. Flowers, T.J., and A. Yeo. 1982. Variability in the resistance of sodium chloride salinity within rice variety, New phytol 88:363-373.
2. Oono, K., and Sakaguchi. 1978. Induction of mutation in tissue culture and their use for plant breeding. Jpn. J. Breed. 28(Suppl. 2):122-125.
3. Yoshida, M. Shouichi, M. Ogawa, K. Suenage and C.Y. He. 1983. Induction and selection of salt-tolerant rices by tissue culture, in "Recent progress at IRRI in cell and tissue culture technics for cereal crop improvement" pp. 215-218.
4. Dix, P., and H.E. Street. 1975. Sodium chloride-resistant cultured cell lines from Nictiana sylvestris and Capsicum annuum. Plant Sci. Lett. 5:231-237.
5. Nabors, M.W., A. Daniels., Nadolnu and C. Brown. 1975. Sodium chloride tolerant lines of tobacco cells. Plant Sci. Lett. 4:155-159.
6. Nabors, M.W., S.E. Gibbs, C.S. Bernstien and M.L.E. Meis. 1980. NaCl-tolerant tobacco plants from cultured cells. Z. Pflanzenphysiol. 97:13-17.
7. Bourgin, J.P. 1978. Valine-resistant plant from in vitro selected tobacco cells. Mol. Gen. Genet. 161:225-230.
8. Flick, C.E., R.A. Jensen and J.A. Evans. 1980.

Isolation of parafluorophenyl alanine resistant mutants of Nicotiana tabacum. *In vitro* 16 : 213-219.

9. Gresshoff, P.M. 1979. Cyclohexamide resistance in Daucus carota cell culture. Theor. Appl. Genet. 54:142-143.
10. Phillips, G.C. and G.B. Collins. 1979. In vitro tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop Sci. 19:59-64.