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Selection of Salt Tolerant Cell Lines From Cell Suspension Cultures of alfalfa (*Medicago sativa*)

Amini, F., and Ehsanpour, A.A.
Dept. of Biology, Esfahan University, Esfahan, Iran
E-mail: ehsanpou@yahoo.com
Fax: 311-7932456

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Abstract

Plant breeding improvement and using conventional methods is difficult and relatively time consuming. Biotechnological methods, especially tissue culture and cell suspension culture can be used to transfer desirable traits such as salt resistance to forage plants such as *Medicago*. Stem, leaf and root explants belonging to three Medicago species: rigidula, *scutellata* and sativa, were initially cultured on MS (Murashige and Skoog, 1962) medium containing 2 mg/L yeast extract, 2mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg/L a-naphthaleneacetic acid (NAA), 1.5 mg/L benzyladenine (BA), 0.5 mg/L Thiodizuron (TDZ) and 2 mg/L Kinetin. Only M. sativa produced callus and was able to produce cell suspension in liquid medium. Viable cells were then transferred to the same liquid medium with 0, 30, 60, 90, 120 mM NaCl and finally, tolerant cell lines were selected at 90 mM NaCl according to packed cell volume (PCV).

Keywords: *cell suspension, in vitro, Medicago sativa, Medicago rigidula, Medicago scutellata, salt tolerance.*

Introduction

Abiotic stresses which plants are subjected to are salt and heavy iones in the soil and water, oxidative, UV, and temperature (cold and heat) stresses. For plants, any level of these factors that differ significantly (more or less) from levels to which they are usually adapted to can be considered as a stressful factor. The loss of yield due to such stresses can be tremendous. Thus, it would be desirable to obtain crop plants that can tolerate specific stresses, and yet maintain high yield. Since the conventional plant breeding methods are in some cases difficult or

even impossible to carry out, therefore using plant cell and tissue culture technology can be beneficial in achieving plants with desirable characteristics such as salt tolerance (Dracup, 1997, Arcioni *et al.,* 1990). Several cases of isolation of cell lines with higher tolerance to salt concentration in the nutrient solutions have been reported, e.g., in rice, mustard and Nicotiana plumbaginifolia (Naborsm *et al.,* 1975).

Medicago species with high economically important value and a world wide range of cultivation are relatively salt tolerant (Leone *et al.,* 1994). Many attempts have been made using in vitro culture including plant regeneration and somatic embryogenesis of M. littoralis and M. suffruticosa (Xiu-Qing and Demarly, 1996; Zafar *et al.,* 1995). Thus obtaining plants that can tolerate hard conditions and at the same time retain their high cop yield would be much desirable. This study is aimed to: 1- set up an in vitro system for callus production and cell suspension culture for M. sativa, M. rigidula and M. scutellata. 2- selecting the salt tolerant cell lines from cell suspension belonging to the above mentioned species.

Materials and Methods

Callus initiation was carried out using seeds of three Medicago species. Seeds were surface sterilized with 70% ethanol for 1min followed by 20% (v/v) sodium hypochlorite solution for 20min, and then rinsed three times in sterile distilled water. Seedlings were grown from seeds on a MS medium (Murashige and Skoog, 1962) solidified with 0.8% agar at pH=5.8. All cultures were kept in the culture room under 16 h light (approx. 2000 Lux) and 8 h dark photoperiod at 25°C. To break the dormancy of seeds and promote germination, seeds of M. rigidula and M. scutellata were pre-treated with absolute sulfuric acid for 2-3 h before sterilizing. For callus production from in vitro grown seedling, segments of leaf, stem and roots were cultured on different MS media consisting of A: 2 mg/L Yeast extract, 2 mg/L Kinetin, 2 mg/L NAA and 2mg/L 2,4-D, B: 2mg/L NAA, 1.5 mg/L BA and C: 2mg/L NAA, 0.5 mg/L TDZ. In each experiment 10 explants with 10 replications were used and calli were subcultured at 2 weeks intervals. To establish cell suspension calli were transferred to 250 ml of the same liquid medium, using shaker with 120 rpm. For selection of salt tolerant cell line suspensions were transferred to the liquid medium

containing 30 mM NaCl. Then at the end of the 3rd subcultures with 12 days intervals, relative growth according to packed cell volume (PCV) (Ehsanpour and Jones, 2001) was measured and compared with the control (medium without salt). This protocol was applied for selection of the cell lines in medium with 60,90 and 120 mM NaCl respectively.

Results

Results from in vitro culture of stem, leaf and root segments of three species of Medicago showed that only the stem explants of M. sativa were able to produce callus at higher frequency (88%) (Table 1) than other two species. The percentage of responsive stem explants was 3% and that of root explants was 25% on medium B in M. rigidula and M. *scutellata* respectively. The next step of experiments (cell suspension culture) was carried out by transferring the calluses into the same liquid medium. Since the only calluses from M. sativa continued to grow and produced cell suspension, selection experiments for salt tolerance was concentrated on M. sativa.

Table 1- Percentage of explants produced callus in three species of *Medicago*.

Species	*medium*	*stem*	*leaf*	*root*
M. sativa	**A**	88^{a}	35^{b}	12^{e}
	B	38^{b}	29^{b}	8.7^{c}
	C	27^{b}	17^{d}	6.5^{c}
M. rigidula	**A**	19^{b}	2.2^{c}	1.6^{c}
	B	37^{a}	1^{c}	1.5^{c}
	C	12^{b}	1.8^{c}	1.2^{c}
M. scutellata	**A**	12^{c}	3.4^{d}	4.2^{d}
	B	25^{b}	3^{d}	4^{d}
	C	17^{c}	4^{d}	6.7^{d}

Uncommon letters are significantly different at $p<0.05$.

Treatment of cell suspension with different salt concentration (0, 30, 60, 90, 120 mM NaCl) resulted in selection of cell lines with high

tolerance up to 90 mM NaCl according to PCV measurement. The viability of these cells was 80-85%. To confirm the stability of the salt tolerance of the selected cell lines, they were transferred to the same medium without salt and after three subcultures, transferred to the medium with different concentrations of salt again (Fig. 1). As a control, normal suspension (non-pretreated cells with salt) also treated with medium having NaCl. In this step the selected cell line continued to growth but non-selected did not.

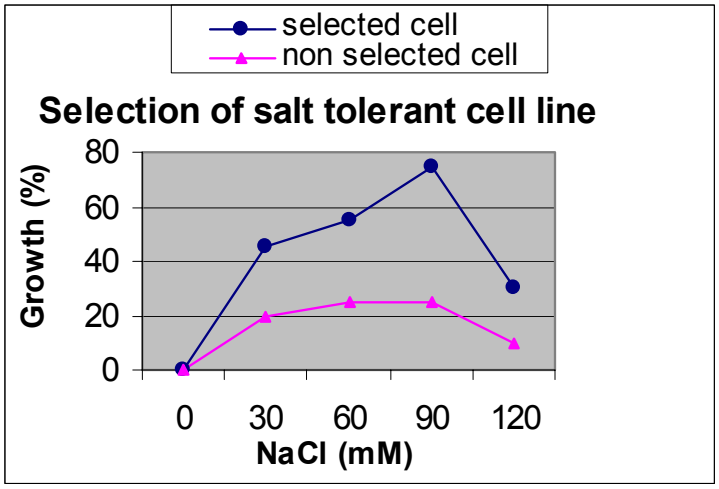

Figure 1- Relative growth of selected cell lines for salt tolerance in different NaCl concentrations

Discussion

Because of the economic impact of stresses and the large amount of energy required to alter the environment to suit the plant, it is becoming increasingly important to utilize existing techniques to breed plants better adapted to stress. In the present study to obtain salt tolerant cell lines, the first step was callus production. Stem explants of M. sativa were able to produce callus, whereas, the other two species (M. *rigidula* and M. *scutellata*) were not responsive. These results indicated that callus production and development are highly dependent to genotype. In addition, the response of different explants even from the same species was different, for example, stem explants in M. sativa were much responsive than leaf and root explants. When calli from M. sativa were transferred to the same liquid medium, cell suspension was produced easily. It could be resulted from cell wall

structure and component which are different in three species (Lerner, 1985). Cell suspension technique has been used for selection of tolerant cell line for a number of traits in many plant species. Zenk (1974) reported the isolation of a resistant cell line from haploid cells in Nicotiana sylvestris. At 1% NaCl the resistant cells grew at 50%, the rate of that of control without salt. No growth occurred at 1% NaCl for the non-selected cells. This method has also been applied to Medicago species (Arcioni *et al.,* 1990). In our study, selected cell lines of M. sativa were able to grow on the medium with 90 mM NaCl and seems that they behave like a halophyte (Croughan *et al.,* 1987). However, at 120 mM and higher concentrations of salt, cells did not grow (data did not show). One of the important points in these experiments was the growth of viable cells at 90mM salt after being cultured in salt free medium for three subcultures and returned back to the medium with high concentration of NaCl. This can be interpreted possibly by different types of genetic and chromosomal variation or somaclonal variation mechanisms which are likely to be most relevant for salt tolerant breeding (Chudhary *et al.,* 1997; Dracup 1997; Hanson, 1984).

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