Bull. Tokyo Univ. For., 102, 143–150 (1999)

Growth of Suspension Cultured Cell of *Populus euphratica*, *Populus alba* cv. Pyramidalis and *Populus maximowiczii* × *Populus plantierensis* in NaCl Containing Medium

Hailong SHEN*, ***, Shin WATANABE** and Yuji IDE*

Introduction

Salinity stress affects significantly survival and growth of trees in semi-arid and arid regions (Allen *et al.*, 1993; Fung *et al.*, 1998). Fortunately, there are several tree species tolerant for salinized soil. To understand the tolerance and the adaptive mechanism of those plants will be very helpful to breed new varieties with high tolerance to salinity. This idea will be beneficial to the ecological and economic development of regions with salinized soil.

Populus euphratica Oliv. naturally distributed in semi-arid regions from Europe to Central Asia (FAO, 1979) is one of such tolerant species for salinity (Wei, 1988) and is a relatively fast-growing species (Wang *et al.*, 1996).

Efforts have been made for *P. euphratica* on analyzing its tolerance mechanism for salinity using potted seedlings (Luo and Zhou, 1991; Ma *et al.*, 1997; Zhao *et al.*, 1997), and tissue cultured materials (Kang *et al.*, 1996). Mature trees of *P. euphratica* grew prosperously on the soil contained 2.25% salt in natural (Wang *et al.*, 1996). Cuttings of *P. euphratica* could develop new roots in the media containing 1.1% (about 200 mM) NaCl by hydroponics (Luo and Zhou, 1991). *In vitro* cultured shoot of *P. euphratica* could grew on a media containing 100 mM NaCl with only 28% reduction of the growth to the control (Kang *et al.*, 1996). These results showed high tolerance ability of *P. euphratica* for salinity at an intact plant level.

For further investigation of the tolerance mechanism, it is necessary to confirm the tolerance not only at an intact plant but also at a cell level. However, there is no report evaluating its tolerance at a cell level.

In this paper we report the growth responses of suspension cultured cells of three poplar species *i.e. P. euphratica*, *Populus alba* cv. Pyramidalis and *Populus maximowiczii* × *Populus plantierensis* after about one years evaluation in the liquid media supplemented by NaCl. *P. alba* cv. Pyramidalis is a fast-growing poplar commonly planted in the semi arid regions of Northwest China and has certain tolerance for salinity (Xu, 1988; Zhang and Du, 1997). *P. maximowiczii* × *P. plantierensis* is a first-growing hybrid poplar for Temperate Zone (FAO, 1979) and called as *FS51* in Japan (Inokuma, 1958).

Materials and Methods

Suspension cultured cells of *P. euphratica* (*PE*), *P. alba* cv. Pyramidalis (*PP*) and *P. maximowiczii* × *P. plantierensis* (*FS51*) were used.

Well-grown calli were selected from callus cultures of these three *Populus* species, which had been derived from plantlets grown *in vitro* (Shen *et al.*, 1998). After three times

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of subculture, the calli were transplanted in the MS liquid medium.

MS medium (Murashige and Skoog, 1962) supplemented with 20 g/*l* sucrose, 0.2 mg/*l* BAP (6-benzylaminopurine) and 1.0 mg/*l* 2,4-D (2,4-dichlorophenoxyacetic acid) was used throughout the experiment and cultures were kept at 25℃. All the media were adjusted to pH 5.6 and autoclaved at 120℃ for 20 minutes. Cell suspension culture was conducted in 50 ml Erlenmeyer flasks filled with 30 ml of the liquid medium. The flasks were shaken on a reciprocal shaker with 110 turns/min under dark condition. A cell line for each poplar was transplanted in the same liquid medium at an interval of about four weeks successively.

After about one-year cultivation, about 1–2 grams of cells from these lines were transplanted into the liquid medium supplemented with various concentrations of NaCl. The cells were subcultured nine times at an interval of six weeks.

MS media containing with 0, 100, 150, 200 and 250 mM NaCl were prepared to evaluate initial tolerance for salinity stress. The acclimation test of cells to salinity stress was then followed. In this case, concentrations of NaCl in the media were gradually enriched in the sequence of subculture with nine different patterns (Table 1).

Cell growth was measured in the first stress culture and in the 6th subculture. Before the inoculation, fresh weight and dry weight of sample cells were weighed and their ratio was calculated. The actual dry weight of inoculated cells for each flask was calculated by the ratio. At the end of each subculture, cells in flask was put onto a filter paper (Whatman Co. No. 1) and absorbed the liquid medium with a vacuum pump. Then the fresh weight of cells was weighed. The dry weight of cells on a filter paper was weighed after 12 hours drying in a oven at 60–70℃. Relative cell growth in dry weight was calculated as increment of cell dry weight/dry weight of inoculated cells. Three flasks were used as samples for each NaCl level respectively. The survival and division of cells were observed under a microscope at the end of 9th subculture with FDA (Fluorescein Diacetate) staining method (Larkin, 1976). To know the statistical difference among cell growth in different culture pattern series, Duncan's new multiple range tests were conducted.

A part of cells obtained from 5th subculture of CP1 and CP2 series and from 6th subculture of CP3, CP4, CP5, CP6 and CP7 series were selected and transferred onto 20 m*l* of MS-agar medium in 50 m*l* Erlenmeyer flasks for callus regeneration. The medium was supplemented with 0.2 mg/*l* BAP and 1.0 mg/*l* 2,4-D. They were kept under dark at 25℃.

A part of cultured cells obtained from 7th subculture of CP1, CP2, CP3, CP4, CP5, CP6 and CP7 series were selected and transferred MS-agar medium for callus regeneration. The

Table 1. Changing patterns of NaCl concentrations in the medium during the acclimation culture

Culture pattern	NaCl concentration at each subculture (mM)								
	1	2	3	4	5	6	7	8	9
CP1	50	100	100	100	100	100	100	100	100
CP2	100	100	100	100	100	100	100	100	100
CP3	50	100	100	150	150	150	150	150	150
CP4	100	100	100	150	150	150	150	150	150
CP5	50	100	200	200	200	200	200	200	200
CP6	100	200	200	200	200	200	200	200	200
CP7	50	100	100	250	250	250	250	250	250
CP8	100	200	300	300	300	300	300	300	300
CP9	50	100	200	400	400	400	400	400	400

medium used for *PE*, *PP* and *FS51* was supplemented with 0.4 mg/*l* BAP and 0.8 mg/*l* NAA, 0.1 mg/*l* BAP and 0.5 mg/*l* NAA and 0.2 mg/*l* BAP and 1.0 mg/*l* NAA, respectively. They were kept under scattered light about 1,500 lux.

NaCl contents in callus-regeneration medium were shown in Table 3 and Table 4. Their agar contents were 0.6% for the media without NaCl and 0.7% for the media supplemented with NaCl. NaCl containing medium solidified by 0.6% agar was softer than the medium without NaCl, then agar was enriched to 0.7% in NaCl containing medium.

Results and Discussion

Survival and growth of cells under different NaCl concentrations

Cells cultured in the media containing 300 mM and 400 mM NaCl did not increase and seemed to be dead by microscopic observation with FDA staining method (data not shown) in *PP* and *FS51*. Then they were eliminated from the measurement.

The growth of *PE* and *FS51* cells was significantly different between the medium containing 150 mM NaCl and the medium containing 200 mM NaCl at the first stress subculture. On the other hand, there was significant difference in cell growth of *PP* between the medium containing 100 mM NaCl and the medium containing 150 mM NaCl (Fig. 1).

Growth of cells after the 6th subculture in NaCl containing medium was shown in Figure 2. While growth of *PE* cells in CP3 and CP4 series was considerably bad, the same cells grew well in CP5 and CP6 series. While the reason of the recessive growth in CP3 and CP4 series was not clear, *PE* cells can grow well even in the medium containing 200 mM NaCl in CP5 and CP6 series. There was significant difference between CP4 and CP5 series in the growth of *PP* cells. Then *PP* cells can grow in the medium containing 150 mM NaCl. Significant difference was observed between CP2 and CP3 series in the growth of *FS51* cells. This means that *FS51* cells can grow only in the medium containing less than 100 mM NaCl.

During the acclimation of the cultures, *PE* and *PP* cells developed their adaptability for

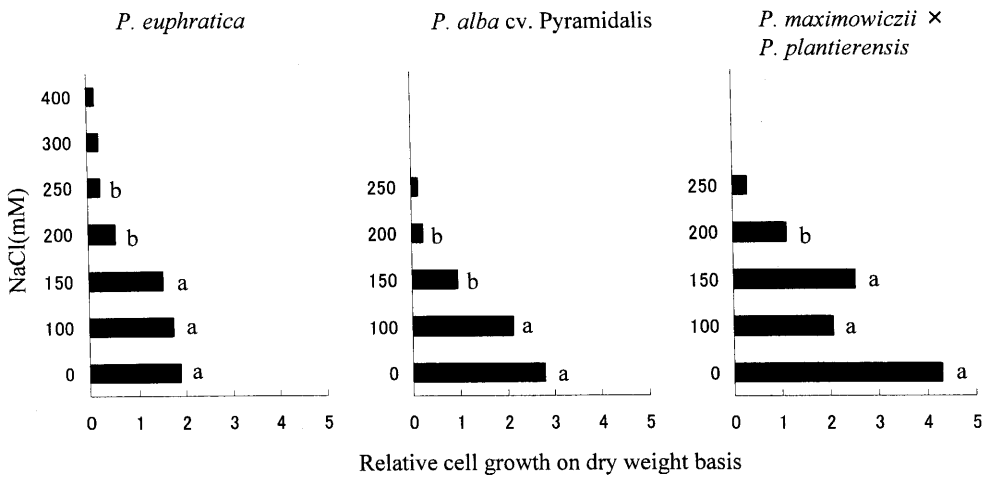

Fig. 1. Cell growth at first stress culture in the media containing NaCl.
The letters "a" and "b" indicate the results of Duncan's new multiple range test. There is no significant difference within the same letters but significant difference between different letters. Data without the letters are data which sample number was not sufficient for the statistical test.

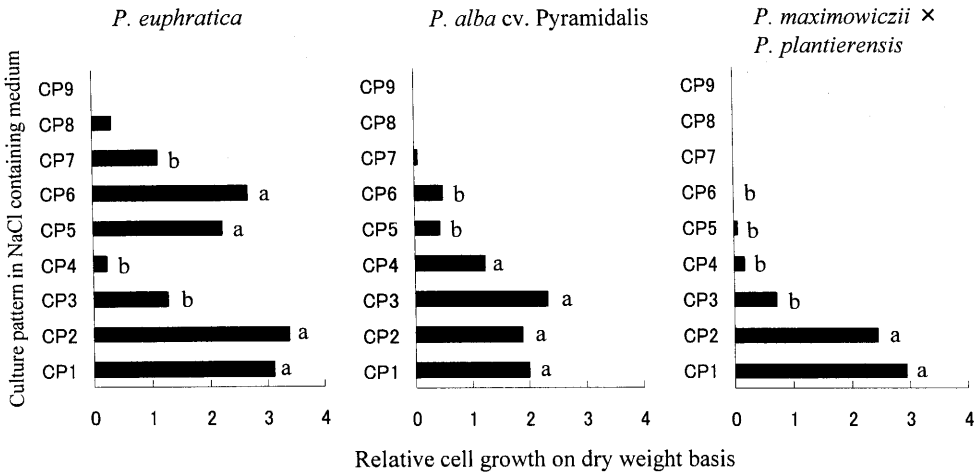

Fig. 2. Cell growth after sixth subculture in the media containing NaCl.
The letters "a" and "b" indicate the results of Duncan's new multiple range test. There is no significant difference within the same letters but significant difference between different letters. Data without the letters are data which sample number was not sufficient for the statistical test.

Table 2. Status of cells in 9th subculture by microscopic obsevation*

Culture pattern of the acclimation culture	NaCl in 9th subculture medium (mM)	*Populus euphratica*	*Populus alba* cv. Pyramidalis	*Populus maximowiczii* × *Populus plantierensis*
CP1	100	+	+	+
CP2	100	+	+	+
CP3	150	+	+	+
CP4	150	+	+	+
CP5	200	+	±	±
CP6	200	+	±	±
CP7	250	+	−	−
CP8	300	−		
CP9	400	−		

* Cell status was observed under a microscope by FDA staining method.
+: Dividing cells and fluorescent cells were observed, ±: No dividing cell was observed but some fluorescent cell was exist, −: No fluorescent cell was observed.

NaCl concentrations. NaCl concentrations, which allowed the cell growth of these two species, were increased by 50 mM at the 6th subculture in comparison with the first stress culture. On the other hand, *FS51* cells reduced their adaptability for NaCl concentration by 50 mM.

Survived cells were observed in the medium containing less than 200 mM NaCl for all the poplar tested after 9th subculture (Table 2). While remarkable growth of *PE* cells was not observed in the medium containing 250 mM NaCl at 6th subculture, the cells could survive and divide even in the 250 mM NaCl medium after 9th subculture. *PP* and *FS51* cells could survive in the medium containing 200 mM NaCl. However they could not grow

in the medium containing such high NaCl at 6th subculture. These results showed that these poplars kept their viability even at higher salinity condition after long term repeated subcultures.

NaCl concentration where one to two-year-old *PE* cuttings could develop new roots, could raised from 0.1% to 1.1% (about 200 mM) by gradual enrichment of NaCl in the hydroponic medium (Luo and Zhou, 1991). This means that *PE* cells have obtained adaptability for salinity stress as long as the level of the intact plant. While cells of *PP* and *FS51* cells have positive changes on adaptability for stress level, their abilities are less than those of *PE* cells.

Callus regeneration from suspension cultured cell

The results of callus regeneration were shown in Table 3 and Table 4. All the cells cultured on the NaCl free medium could regenerate callus under dark condition. This means that the cells, which did not grow well in high NaCl suspension culture, still kept viability for regeneration. Callus was also regenerated on the NaCl containing medium. Highest NaCl concentration which allowed callus regeneration were 250 mM, 150 mM and 150 mM, for *PE*, *PP*, and *FS51* cells, respectively.

It took longer incubation to regenerate callus from the cells cultured in higher NaCl concentrations than that cultured in lower NaCl concentrations under scattered light. Incubation period required for the callus regeneration tended to be shorter under scattered light than under dark. However it is not concluded as the effect of light condition, because there were hormonal differences in the medium among callus regeneration cultures.

PE shoots could grow well on the medium containing 100 mM NaCl, but shoot growths of *P. alba* cv. Pyramidalis × *P. tomentosa* and *FS51* were arrested (Kang *et al.*, 1996). Moreover, *PE* plantlets could grow well and develope new leaves on the liquid medium contain-

Table 3. Callus regeneration under dark from the cells cultured in NaCl containing medium*

Culture pattern of the acclimation culture**	NaCl in the callus regeneration medium (mM)	Days needed for callus regeneration***		
		Populus euphratica	*Populus alba* cv. Pyramidalis	*Populus maximowiczii* × *Populus plantierensis*
CP1 and CP2	0	30	30	30
	100	80	80	80
CP3 and CP4	0	40	40	40
	100	65	65	65
	150	65	—	—
CP5 and CP6	0	40	40	40
	100	65	65	65
	150	65		
	200	105	—	—
CP7	0	65		
	250	—		

* After 5th subculture in NaCl containing medium.

** Cells from the same NaCl containing medium were mixed.

*** Callus regeneration was checked every 5 days. —: No callus regeneration was observed until 110 days of culture. Blank: Test was not conducted.

Table 4. Callus regeneration under scattered light from the cells cultured in NaCl containing medium*

Culture pattern of the acclimation culture**	NaCl in the callus regeneration medium (mM)	Days needed for callus regeneration***		
		Populus euphratica	*Populus alba* cv. Pyramidalis	*Populus maximowiczii* × *Populus plantierensis*
CP1 and CP2	100	30	30	30
	150	30	30	30
	200	30	—	—
CP3 and CP4	100	30	30	30
	150	30	30	30
	200	65	—	—
CP5 and CP6	100	30	30	—
	150	30	30	—
	200	30	—	—
CP7	250	30		

* After 5th subculture in NaCl containing medium.
** Cells from the same NaCl containing medium were mixed.
*** Callus regeneration was checked every 5 days. —: No callus regeneration was observed until 80 days of culture. Blank: Test was not conducted.

ing 250 mM NaCl (Watanabe, unpublished). These results showed that *P. euphratica* has the highest tolerance for salinity among tested poplars at an intact plant and a cell level. However, cells of all the poplars tested in this experiment could survive during the course of repeated subculture in considerably high NaCl concentrations and still have the ability for callus regeneration.

Acknowledgement

This study was supported by the Grant-in Aid for Creative Basic Research from the Ministry of Education, Science, Sports and Culture Japan.

Summary

Tolerance for gradually increasing NaCl stress was evaluated for the suspension cultured cells of *Populus euphratica*, *Populus alba* cv. Pyramidalis and *Populus maximowiczii* × *Populus plantierensis*. In the first culture in a medium containing more than 200 mM NaCl, growth of *P. euphratica* and *P. maximowiczii* × *P. plantierensis* cells was remarkably suppressed. Growth of *P. alba* cv. Pyramidalis cells was suppressed in a medium containing more than 150 mM NaCl. During the subculture, *P. euphratica* and in *P. alba* cv. Pyramidalis cells developed their adaptability for NaCl stress. At the end of the sixth subculture, *P. euphratica* could grow well in a medium containing 200 mM NaCl and *P. alba* cv. Pyramidalis could grow well in a medium containing 150 mM NaCl. On the other hand, *P. maximowiczii* × *P. plantierensis* reduced its adaptability for NaCl concentration to 100 mM. Although there were differences between species in their ability of cell growth under high NaCl concentration, cells could survive in 200 mM NaCl after the ninth subculture in every species. Particularly *P. euphratica* could divide even in a medium containing 250 mM NaCl. Such surviving cells still have the ability for callus regeneration after the fifth or sixth

subculture.

Key words: NaCl stress, Cell suspension culture, Callus regeneration, *Populus euphratica*, *Populus alba* cv. Pyramidalis, *Populus maximowiczii* × *Populus plantierensis*

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(Received Apr. 30, 1999)
(Accepted Sep. 14, 1999)

NaCl を含む培地における *Populus euphratica*, *Populus alba* cv. Pyramidalis, *Populus maximowiczii* × *Populus plantierensis* の培養細胞の成長

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要　旨

Populus euphratica, *Populus alba* cv. Pyramidalis, *Populus maximowiczii* × *Populus plantierensis* の3種のポプラの培養細胞について，培地への NaCl の添加量を徐々に増加させた場合の耐性を評価した。NaCl を添加した最初の培養では，*P. euphratica*, *P. maximowiczii* × *P. plantierensis* の細胞は，200 mM の NaCl 添加では成長が著しく抑制された。また，*P. alba* cv. Pyramidalis の細胞では 150 mM の NaCl 添加で成長が抑制された。しかし，培養を繰り返すうちに細胞は NaCl に対する適応性を発達させ，6回目の継代培養終了時には，*P. euphratica* の細胞は 200 mM, *P. alba* cv. Pyramidalis の細胞は 150 mM の NaCl 添加培地でも良好な成長が可能になった。しかし，*P. maximowiczii* × *P. plantierensis* の細胞は，100 mM までしか良好な成長を示さなくなった。高 NaCl 条件下において種ごとの細胞成長に違いが見られたが，すべての種において9回目の継代培養において 200 mM の NaCl 条件下での細胞の生存が確認された。また，NaCl 200 mM で培養された細胞は，5回から6回目の継代培養後でもカルスの再生能力を有していた。特に，*P. euphratica* では NaCl 250 mM の条件で培養した細胞からもカルスが再生した。

キーワード: NaCl ストレス，培養細胞，カルス再生，*Populus euphratica*, *Populus alba* cv. Pyramidalis, *Populus maximowiczii* × *Populus plantierensis*

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Tolerance for gradually enriched NaCl concentration was evaluated for the suspension cultured cells of *Populus euphratica*, *Populus alba* cv. Pyramidalis and *Populus maximowiczii* × *Populus plantierensis*. During the subculture, cells developed their adaptability for NaCl stress in *P. euphratica* and in *P. alba* cv. Pyramidalis. At the end of the sixth subculture, *P. euphratica* could grow in a medium containing 200 mM NaCl and *P. alba* cv. Pyramidalis could grow in 150 mM NaCl. Cells of each species could survive in 200 mM NaCl after ninth subculture in every species. Such surviving cells still have the ability for callus regeneration after the fifth or sixth subculture.

The Discussion for the Reduction of The Tokyo University Forests in the Prewar Period —The Response of The University of Tokyo to the Project for Rearranging National Property—

Yoichiro OKUYAMA

The Tokyo University owned experimental forests of a very large area in Hokkaido, Sakhalin, the Korean Peninsula, Taiwan in the period before the Firse World War. But, in the Project for Rearranging National Property, that began in 1929, reduction of the University's experimental forests was planned. The experimental forests in Hokkaido and overseas colonies were plamed to be reduced to 1000 ha. The Ministry of Education and The University of Tokyo objected to this. As a result the reduction plan was stopped.