INVITRO SELECTION OF SALT OR WATER STRESS MUTANTS AND PROLINE OVER PRODUCTION IN *NICOTIANA PLUMBAGINIFOLIA* (Viviani)

Sumaryati Syukur* and M. Jacobs**

*Laboratory of Biochemistry/Biotechnology, Department of Chemistry, Andalas University, Padang
**Plant Genetics, Institute Molecular Biology, Vrije Universitiet Brussel, Paardenstraat 65, B-1640 St Genesius-Rode, Belgium

ABSTRACT

Several mutants have been selected and derived from protoplast colonies of haploid *N. plumbaginifolia* leaves. The mutants were characterized resistance against NaCl, KCl (200 mM) or polyetylene glycol (PEG) 6000 (25%). The selection was started at single cell culture of fresly isolated protoplasts. The frequency of resistant colonies ranged from 10^{-5} to 10^{-6}. All resistant callus were regenerated into plants and produce 10-25 times more proline than the wild type when grown on a-non-selective medium. Clear genetic basis was found as single dominant nuclear gene after progeny F2 generation crosse between resistant wild type plants.

INTRODUCTION

There is considerable potential in the application of cell culture techniques in plant biotechnology and genetic engineering for future plant improvement. Cellular tolerance from single cell culture is appropriate method to isolate mutant resistant to salt and water stress. Plant cell cultures and biotechnology is a fertile area for basic research, with possible application for breeding proposes (Sumaryati et. al, 1992).

The specific mechanisms conferring tolerance is not yet understood and traditional breeding approaches to salt tolerance have generally failed to identify a clear genetic basis for the trait. Several report on salt-tolerant cell lines obtained by invitro selection methods are available and NaCl-tolerant tobacco plant has been regenerated. In a few cases, relatively stable variants have been obtained and tolerance has been retained in the progeny of regenerated plants (Bhaskaran et. al., 1986). However, the genetic basis concerning tolerant trait was not clearly established in any of these investigations.

The development of sophisticated cellular invitro techniques based on single cell protoplast culture combined with mutagenesis and appropriate selection methods have resulted in wide spectrum of biochemical variants of mutant resistant salt and water stress condition (Sumaryati, et. al., 1992; Negrutiu, 1990). Such mutants have proved to be fruiful for studying metabolic pathways and gene regulation in plants. In important point is that the resistant gene has to expressed at cell and whole plant level transfer to next generation.

The aim of the investigation described in this paper is to select mutant resistant to salt and water stress from haploid protoplasts *N. plumbaginifolia*. The biochemical characterization as praline over-production from the resistant mutants is also determined.

MATERIALS AND METHODS

Selection Methods and Protoplast Culture

Protoplats from haploid *N. plumbaginifolia* were isolated and medium cultures as

described (Negratiu, et. al., 1981; Jacobs, et. al., 1987). The mutagenic treatment was performed 1 day after protoplast isolation. The were exposed to UV light at dose of 25 erg/mm2/sec for 20 seconds and then placed in the dark for 2 days. One week later the surviving colonies were washed by sedimentation and transferred at final density in MDS medium containing the selection agents at a concentration of 200 mM NaCl or KCl and 25% PEG 6000. These concentration were chosen on the basis of lethality curve established for wild type cells growing on media containing such compounds (Sumaryati, et. al., 1992). Actively growing callus lines were used for free amino acids analysis.

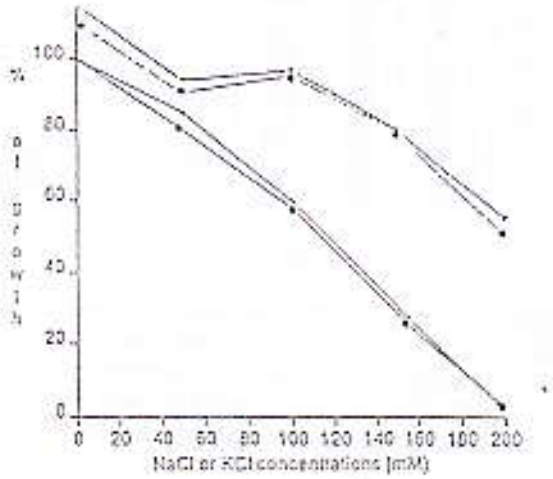
Amino Acids Analysis

Free amino acids in callus and plantlet were analyzed by homogenizing 1 g of callus with a mixture of methanol, chloroform and water (12 :5 : 1) (Bieleski and Turner, 1966). Amino acid determination in callus required an addition all step: the material was boiled in 70% ethanol for 10 min. chlorophyll was recovered by adding two parts of chloroform plus one part of water to the extract. The aqueous layer was taken and completely evaporated. The residu was redissolved in concentrated HCl and hydrolyzed under vacuum at 110 Celcius degree. The extracts were subsequently resuspended in the loading buffer (Na-citrate, pH 2.2) and analyzerd by amino acids analyzer Biotronik LC 5001.

RESULT AND DISCUSSION

Characterization of Resistant Mutans

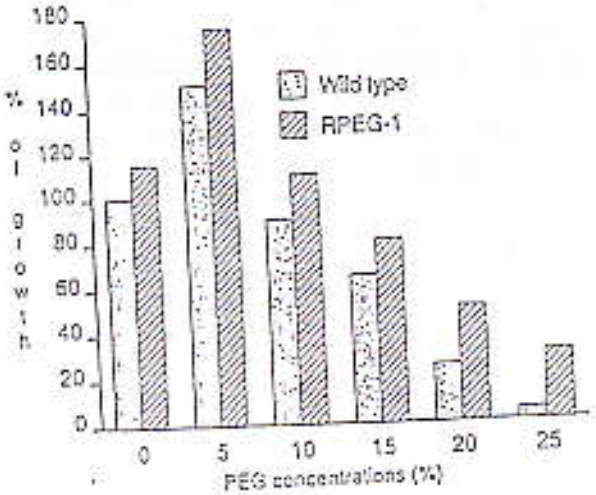
Inhibiton curves in the basis of (LD > 90%) of callus growth to the selection agents NaCl or KCl 200 mM and PEG 25% resulted three different resistant mutant, designated (RNaCl-1, RKCl-1) as salt resistant and RPEG-1 as water stress. The salt resistant line grew better than the wild type in the presence of selection agent, while a 100 mM salt concentration reduced callus fresh weight by 50% of the control value in the wild type, both salt resistant mutants grew better only slightly affected. Doubling the salt concentration to 200 mM the wild type cells were blocked completely, while the mutants fresh weight only reduced by 50% (Figur 1).



Figur 1. Growth of salt-resistant lines and wild-type callus in the presence of increasing concentrations of NaCl of KCl. Mean fresh weights were determined after 4 weeks of culture on control and supplemented media. The values are means of five replicates (25 calli per plate). Wild type (—), RNaCl-1 (...), RKCl-1 (--) on NaCl (•) or KCl (γ).

The addition of 5% PEG in the culture medium resulted in a fresh weight increase or both the wild type and the resistants line (Figur 2). The inhibitory effect of PEG was only noticeable at concentrations above 10%. Under these conditions the RPEG-1 line was grew significantly faster than the control line and maintained a 30% growth rate on 25% PEG while the wild type was completely inhibited.

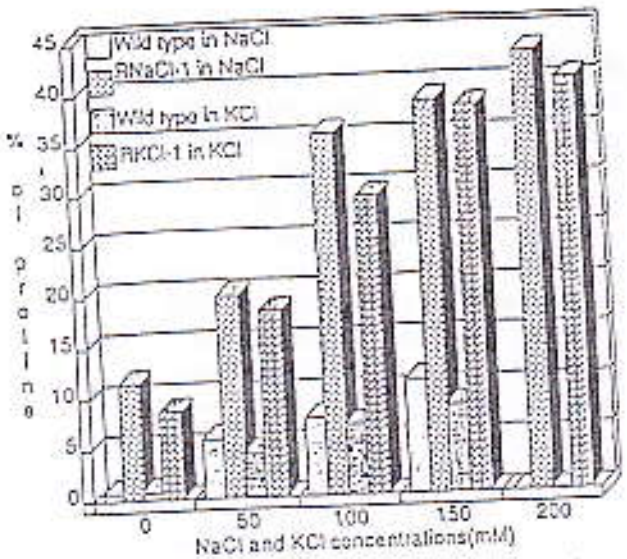
ISSN : 0853-8018



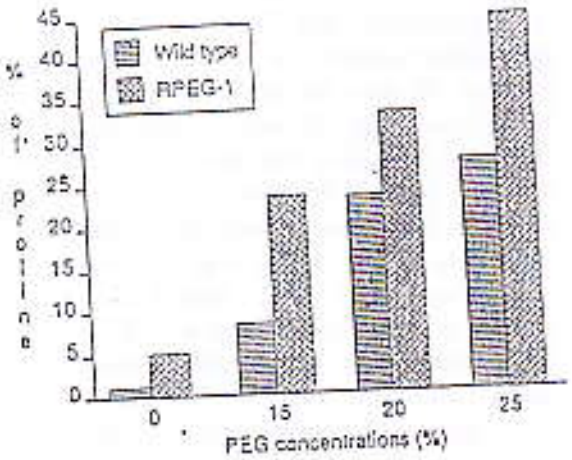
Figur 2. Growt of PEG-resistant and wild-type callus in the presence of increasing concentrations of PEG. Mean fresh weights were determined after 4 weeks of culture on control and PEG-suplemented media. The values are means of five replicates (25 calli per plate).

Proline Content

Wild type callus cultures grown in the presence of salt concentration showed a parallel increase in their free proline content, which on 150 mM salt reached 6 to 8 fold the values determined on the non-saline medium (Figur 3). The same response could be observed for the tolerant lines, however they accumulated 10 to 15 times more proline than the wild type callus on non saline medium (Table 1). Very high proline content values were obtained for resistant line growing on 20 to 25% PEG (Figur 4).



Figur 3. Proline content as percentage of total free amino acids in callus from wild type, RNaCl-1 and RKCl-1. Callus were subcultured for 4 weeks in the presence of increasing salt concentration.



Figur 4. Proline content as percentage of total free amino acids in callus from wild type and RPEG-1. Callus cultures were subcultured for 4 weeks in the presence of increasing PEG concentration.

Table 1. Free amino acid analysis of wild-type and resistant callus from the lines RNaCl-1, RKCl-1 and RPEG-1 grown on non-selective medium. Absolute values (in nmol g^{-1} fresh weight) and percentages.

Amino Acids	Wild Type		RNaCl-1		RKCl-1		RPEG-1	
	nmol/g	(%)	nmol/g	(%)	nmol/g	(%)	nmol/g	(%)
Aspartale	956	10.5	676	7.2	709	7.7	1.236	13.0
Threonine	630	6.9	660	7.1	630	6.8	121	1.2
Serine	529	5.8	343	3.7	330	3.6	292	3.0
Glutamate	3.699	40.9	3.844	41.4	3.800	41.5	3.742	39.3
Proline	69	0.8	950	10.2	784	8.6	460	5.0
Glycine	158	1.8	70	0.7	72	0.7	197	2.0
Alanine	140	1.5	147	1.5	195	2.1	480	5.0
Cysteine	ND	ND	10	0.1	10	0.1	94	0.9
Valine	679	7.5	590	6.3	580	6.3	179	1.8
Methionine	ND	ND	10	0.1	9	0.1	9	0.1
Isoleucine	187	2.0	21	0.2	20	0.2	71	0.7
Leucine	316	3.5	94	1.0	140	1.0	58	0.6
Tyrosine	211	2.3	147	1.5	140	1.5	117	1.2
Phenylalanine	134	1.5	199	2.1	230	2.5	356	3.7
Histidine	1.057	11.7	1.094	11.8	1.050	11.5	978	10.2
Lysine	57	0.6	39	0.4	35	0.3	502	6.1
Arginine	196	2.1	375	4.0	410	4.4	489	5.1
Total	9.018		9.269		9.144		9.512	

Stability of Trait in the Resistant Lines

When selected callus lines were transferred from the selective medium to a non-selective one for the three successive subcultures of 4 weeks each and then put back on a medium with the original concentration of salt or PEG, they maintained an active growt comparable to the one observd originally. These data was published (Sumaryati, et. al., 1992). The genetic basis concerning clear mutation was also reported as a dominant gene segregate as single mandelian gene. Seeds from second generation showing resistant against very high concentration of salt (200 mM) and 20% PEG.

CONCLUSION

Selection from single cell protoplast and mutagenic treatment created useful salt and water stress mutanta of *N. plumbaginifolia*. This method of selection mutant could be important to apply in other economic plants.

ACKNOWLEDGEMENTS

MUCIA-INDONESIAN World Bank XVII and Belgium Government (ABOS Program) supported this experiment. I appreciate very much to Prof. Yu Gleba for your suggestion and communicated first publication on *Journal Theory Applied Genetics* (1992) **83**: 613-619.

REFERENCES

1. Bieleski, RL, Turner NA (1996). Separation and Estimation of Amino Acids in Crude Plant Extracts By Chromatography. *Anal. Biochem.* **17**: 278-293.
2. Bhaskaran, S. Smith RH, Schertz KF (1986). Progeny Screening of Sorghum Plants Regenerated From Sodium Chlorida-Selected Callus for Salt Tolerance. *J. Plant Physiol.* **122**: 205-210
3. Jacobs, M., Negrutiu, I Dirks, R., Cammaerts, D. (1987). Selection Programs for Isolation and Analysis of Mutants in Plant Cell Cultures. Plant Tissue Cult. 243-264.
4. Negrutiu, I (1981). Improve Conditiona for Large-Scale Culture, Mutagenesis and Selection of Haploid Protoplast of *N. plumbanginifolia*, Pflanzenphysiol **104**: 413-442.
5. Negrutiu, I (1990). Selection and Choice of Genetic Markers, In Dix. PJ (ed) Plant Cell Line Selection. Weinheim, New York Basel Cambridge, pp 217-229.
6. Sumaryati, S., Negrutiu, I and Jacobs, M. (1992). Characterization and Regeneration of Salt and Water Stress Mutants From Protoplast Culture of *N. plumbanginifolia* (Viviani) **83**: 613-619.