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Functions of Exogenously Proline against Negative Effects of Salt Stress in Onion (*Allium cepa* L.)

Dilek Çavuşoğlu[1*], Kürşat Çavuşoğlu[2]

[1*] Department of Plant and Animal Production, Isparta University of Applied Sciences, Isparta, Turkey.
[2]Department of Biology, Süleyman Demirel University, Isparta, Turkey.

Abstract— *The effects of proline on the seed germination, seedling growth (radicle lenght, radicle number and fresh weight), mitotic index, micronucleus frequency and chromosome aberrations of Allium cepa L. germinated under saline conditions were examined in this study. Salt stress markedly inhibited the seed germination and seedling growth of A. cepa L. Moreover, it reduced the mitotic index in the root-meristem cells of the seeds and fairly increased the number of chromosome aberrations and micronucleus frequency which is the simplest indicator, the most effective of cytological damage. On the other, the inhibitive effect of salt stress on the seed germination, fresh weight and mitotic index was significantly decreased with proline application. However, this amino acid was ineffective in reducing of salt damage on the radicle lenght, radicle number, micronucleus frequency and chromosome aberrations.*

Keywords— ***Cytogenetical parameters, onion, proline, physiological parameters, salt stress.***

I. INTRODUCTION

Increasing salinity of agricultural irrigation water together with progressive salinization of agricultural land is of increasing importance to agriculture because it limits the distribution of plants in certain natural habitats and induces a wide range of adverse metabolic responses in higher plants. Salinity stress is one of the most common abiotic factors that inhibit crop growth and productivity by reducing the photosynthetic capacity of plants [1]. High salinity increases the levels of reactive oxygen species (ROS) in plants, such as superoxide radicals, hydrogen peroxide, singlet oxygen and hydroxyl radicals [2]. ROS damage normal metabolism via oxidation of membrane lipids, proteins and plant nucleic acids [3]. Plants develop various defensive mechanisms to cope with salinity-induced damage by compatible solutes as proline and glycinebetaine, and by up-regulating antioxidant enzymes and Na^+/H^+ antiporters [4].

Proline has been known to be involved in the response to a number of environmental stresses such as salt, temperatures, drought, chilling, sorbitol, radiation, heavy metals stress for many years. Proline is an important ozmoregulator that provides of protein integrity and activates of antioxidative enzymes in plants exposed to especially NaCl stress. It is generally accepted that under conditions environmental stresses, proline accumulation serves as a defence against osmotic challenge by acting as a compatible solute. Stress factors, the amount of internal proline in plants cause an increase. Exogenously proline applications are also known to have a positive effect on salt stress tolerance. Plants develop various defensive mechanisms to cope with salinity-induced damage by accumulating such compatible solutes as proline [5, 6]. Proline has been found to protect cell membranes of onion against salt injury [7].

Allium cepa L. (2n=16, chromosomes), the common onion, constitutes a very convenient test system for estimating the harmful effects of chemicals on biological materials. *Allium cepa* test, which is called Levan's test, is one of the most frequently used plant bio-assays. The *Allium* test has been used since it was introduced to evaluate mutagenic effects in the root tips of onions. Additionally, the most important advantage of *A. cepa* bioassay is supported by the very similar of the mutagenic activity of numerous compounds on mammalian cells and *Allium* test cells. This test is now frequently used for laboratory studies [8]. The present study was designed to examine the influences of proline in the reducing of detrimental effects of salt stress on the seed germination, seedling growth, mitotic activity, micronucleus frequency and chromosomal aberrations of *Allium cepa* L.

II. MATERIALS AND METHODS

In this study, *Allium cepa* L. seeds were used. Salt (NaCl) concentration used was 0.125 M. L-proline concentration used in the experiments was 75 mg L^{-1}. L-

proline were obtained from Merck. By a preliminary investigation carried out, firstly it was determined as 0.125 M salt concentration (tried out concentrations of 0.10, 0.125, 0.15, 0.175, 0.20, 0.225, 0.25, 0.275, 0.30 M) which largely preventing the germination of *A. cepa* L. Then it was designated as 75 mg L^{-1} L-proline concentration (tried out concentrations of 1, 5, 15, 25, 35, 45, 55, 65, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mg L^{-1} dose of L-proline) alleviating the adverse effects of this salt concentration (0.125 M) on the seed germination and seedling growth. The present study has realized in Plant Physiology and Cytogenetic Laboratories of Biology Department in Süleyman Demirel University.

Germination of seeds of *Allium cepa* L. was carried out at a constant temperature (20°C), in the dark in an incubator. Healthy and approximately equal-sized *A. cepa* seeds were selected. Twenty seeds from each treatment group were placed into the plastic containers. The seeds were divided into four groups: Group I (control) was treated with distilled water for 7 consecutive days. Group II was treated with 0.125 M NaCl alone for 7 consecutive days. Group III was treated with a 75 mg L^{-1} dose of proline for 7 consecutive days. Group IV was treated with a 75 mg L^{-1} dose of proline + 0.125 M NaCl for 7 consecutive days. Plastic containers were placed into an incubator for germination. It was assumed that the radicle should be 10 mm long for germination. At the end of the 7th day, after determination of the final germination percentages, radicle numbers were also recorded, and radicle lengths of the seedlings were measured in mm. In addition, the fresh weights in g/seedling were determined. All experiments were repeated 3 times.

After several days, root tips of germinated *A. cepa* were excised (1-1.5 cm segment) for cytogenetic analysis. Then, they were pretreated with saturated para-dichlorobenzene for 4 hrs, fixed in solution of ethanol: acetic acid (3:1) overnight at room temperature and stored at 4ºC in 70% ethanol until used. The root tips were hydrolysed in 1 N HCl at 60°C for 15 min, were stained with Feulgen for 1-1.5 hrs, smashed in a drop of 45% acetic acid and squashed [9]. 24 hrs later, microscopic slides were made permanent by mounting in balsame. The representative of mitotic phases and mitotic aberrations were photographed (500X) with a digital camera (Olympus C-5060) mounted on an Olympus CX41 microscope. Mitotic index, i.e. percentage of dividing cells scored was evaluated by analysing at least 9.000 cells per treatment (approx. 3.000 per slide). Statistical analyses of all parameters were performed by using SPSS program according to DMRT.

III. RESULTS AND DISCUSSIONS

As shown in Table 1, the germination percentage and radicle length of the group III seeds treated with proline statistically showed the same values as the group I (control) seeds germinated in distilled water medium while their radicle numbers and fresh weight partly increased according to ones of the group I seeds. Yan et al. [10] reported that 0.2 mM exogenous proline application increased the fresh weight of two melon cultivars under normal condition. This result is in agreement with the present findings. Deuschle et al. [11] showed that 100 mM exogenous proline application inhibited growth of tobacco cell under normal condition. The discrepany in the findings indicated that the effect of proline may dependent on the differences in treatment times, concentrations used and plant species.

Salt stress showed the restrictive effect on all examined growth parameters. For instance, the group I (control) seeds germinated in distilled water medium displayed germination 100% on the 7th day while this value became 27% in the group II seeds germinated in 0.125 M salinity. In other words, NaCl prevented 73% the germination of *A. cepa* seeds. Salt stress can perform its preventive effect in many ways. It may interfere with seed germination by changing the water status of the seed so that water uptake is inhibited [12]. The present results showing the decrease in the fresh weight and water content of the seedlings in saline medium may be explained by the failure of the roots to receive sufficient water due to the high osmotic pressure of the medium. The inhibitive effect of salt on the radicle length and radicle number may result from reducing cell division, nucleic acid and protein synthesis [13].

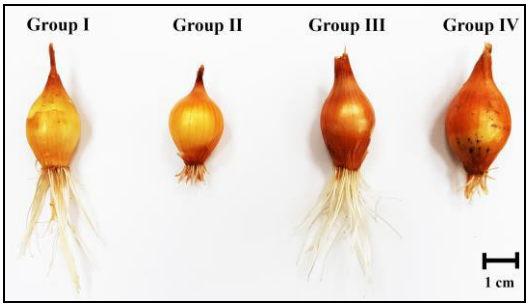

Fig. 1: Root tip cells of Allium cepa showing germination situations at the end of 7 day. Group I (control): distilled water, Group II: 0.175 M NaCl alone, Group III: 75 mg L^{-1} proline and Group IV: 75 mg L^{-1} proline + 0.125 M NaCl. Scale bar = 1 cm

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Table 1: Effects of proline on some growth parameters of Allium cepa L.

Groups	Growth parameters			
	Germination percentage (%)	Radicle length (mm)	Radicle number	Fresh weight (g/seedling)
Group I	*100 ± 0.0^{c}	58.7 ± 0.7^{b}	45.1 ± 0.7^{b}	10.5 ± 0.3^{b}
Group II	27 ± 2.8^{a}	13.5 ± 1.2^{a}	18.4 ± 1.4^{a}	7.1 ± 0.2^{a}
Group III	100 ± 0.0^{c}	59.3 ± 1.0^{b}	50.1 ± 0.6^{c}	14.0 ± 0.8^{c}
Group IV	70 ± 0.0^{b}	15.0 ± 0.1^{a}	17.6 ± 0.3^{a}	10.1 ± 0.7^{b}

*At the level 0.05 (±SD), the difference between values with the same letter in each column is not significant. Group I (control) treated distilled water, Group II treated 0.125 M NaCl alone, Group III treated 75 mg L^{-1} dose of proline, Group IV treated 75 mg L^{-1} dose of proline+0.125 M NaCl.

Proline application markedly mitigated the inhibitive effect of salt stress on the seed germination. The group IV The fresh weight of the group II seeds grown in 0.125 M salinity was 7.1 g, respectively while this value was 10.1 g in the group IV seedlings treated with proline (Tab. 1). But, the mentioned application was unsuccessful in alleviation of the inhibitive effect of salt stress on the radicle length and radicle number of the seedlings. There are many published studies about the effects of proline on the seed germination and seedling growth under salt conditions until now. However, from these studies, no conclusion could be reached. Thus, proline has been reported to promote [10, 14, 15, 16, 17] or inhibit [11, 18, 19] the germination and growth under salt stress conditions. That proline alleviates salt stress on the seed germination and seedling growth can be understood from the decrease in the salt's osmotic effects. For example, at 0.125 M NaCl medium, proline application partly increased the fresh weights of the seedlings in comparison with the control indicates this probability (Table 1). It reduced the preventive effect of salt on the seed germination and seedling growth by stimulating mitotic activity of the embryo (Table 2). In addition to all these, proline might have been successful in decreasing the inhibitive effect of salt stress on the seed germination and seedling growth by increasing nucleic acid and protein synthesis, by providing stabilization of cell membranes or by raising antioxidant enzyme activities viz. catalase (CAT), peroxidase (POX), superoxide dismutase (SOD) and reactive oxygen species [6, 20, 21].

As a result of our literature studies, although there are a limited number of studies relating to effects of proline on the mitotic index under non-stress and salt stress conditions, no studies were found on the micronucleus frequency and chromosomal aberrations. Therefore, in the present study was carried out to find whether proline is affecting these parameters in normal and saline conditions. The data obtained in this work indicated that seeds treated with proline showed 70% germination (Fig. 1). Proline also continued its success on the fresh weight. the mitotic index in root tip meristems of *A. cepa* germinated in the media containing 0.75 M proline alone increased 96% as compared with group I seeds germinated in distilled water medium. And their micronucleus frequency (11 fold) and chromosomal aberrations (approximately 58 fold) excessively increased according to ones of the group I seeds. In this case, it may be said that some aberrations may result from this imino acid. Mitotic activity expressed as mitotic index decreased at 0.125 M salt concentration (group II) as compared to those of group I (control) samples germinated in distilled water. At the same time, the salt concentration caused an increase on the micronucleus frequency and chromosomal aberrations in root tips of *A. cepa*. For instance, while mitotic index, micronucleus frequency and chromosomal aberrations were 8.2%, 0.0% and 0.0% at control (group I), respectively, they were 0.8%, 9.2% and 12.4% respectively, at 0.125 M NaCl concentration. The inhibitory and cytotoxic effects of salt stress on the mitotic activity are known for a long time [22]. According to some researchers, high salt concentration causes to total inhibition of mitotic activity, micronucleus frequency and chromosomal abnormalities in root-tip cells [23, 24]. On the other hand, proline+NaCl application (Group IV) showed a perfectly good performance in ameliorating the negative effects of salinity on the mitotic index (4.8%). However, the mentioned imino acid application was ineffective in reducing of salt damage on the micronucleus frequency (8.6%) and chromosome aberrations (54.7%). Statistically, all values mentioned here are substantially significant (Table 2).

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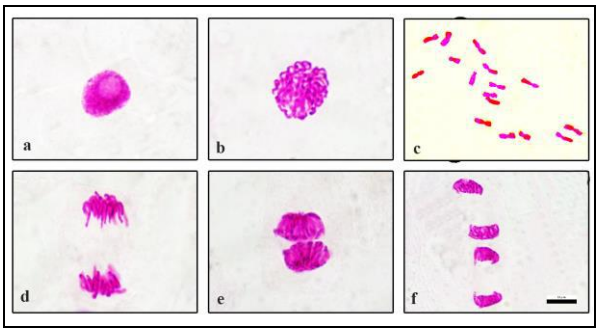

Fig. 2: Normal stages in mitosis a- interphase b- prophase c- metaphase (2n = 16, chromosomes) d- anaphase e- early telophase f- telophases, Scale bar = 10 µm

The normal mitosis phases observed during the microscopic examination of *A. cepa* root tip meristematic cells are shown in Fig. 2 and abnormal mitosis phases are shown in Fig. 3. The chromosomal aberrations noticed in this study were majorly binucleolars in this study. Other chromosomal damages observed in the cells are as follows: micronucleus, irregular prophase, vagrant chromosomes, diagonal at anaphase/telophase, metaphase with chromosome loss, nucleus with nuclear bud, multiple bridge formation in anaphase, adhered chromosomes in metaphase, alignment anaphase, giant cell, chromosomal distrubtions in metaphase, early ball metaphase, ring formation (Fig. 3).

Chromosomal abnormalities (CAs) are changes in chromosomal material or exchange in the structure of the chromosome resulting from breakage. Most of the CAs could affect the fertility, vigour, competitive or yield ability of the exposed plants [25]. Excess proteins and nucleic acids production induced by cytotoxicans result in nuclear buds (Fig. 3b) [26]. As a result of spindle dysfunction, micronucleus (Fig. 3c, o) formation occurs as a result of chromosomal breaks and all chromosomes that do not migrate during the anaphase. Chromosome loss (Fig. 3j) are typically associated with mitotic spindle malfunction. Bridge formation (Fig. 3l) consist of chromatid and/or chromosome breaks, which is indicative the clastogenic effect [27]. İrregular prophase failure (Fig. 3f) might cause chromosome loss when they can not bind to the spindle and therefore are not separated [28]. Ring chromosome (Fig. 3ı) can spontaneously occur after breakage of the chromosomal ends and after the joining of the raw ends of the chromosomes [29]. Diagonal orientation at anaphase / telophase (Fig. 3m, p) was caused by a slight tilt in the spindle apparatus [30].

IV. CONCLUSION

As a result of our literature studies, although there are published studies about the effects of proline application on the seed germination, seedling growth and mitotic index under non-stress and salt stress conditions, current literature data related to the effects of proline application in both normal and saline conditions on the micronucleus frequency and chromosomal aberrations from the cytogenetical studied here have not been encountered. Therefore, this present work was carried out to find whether proline is affecting these parameters in saline conditions or not. As a result, this study showed that proline can significantly increase the activations like the seed germination, fresh weight and mitotic activity under saline conditions. But the mechanisms by which salt inhibits growth are controversial and complex, also they might vary according to cultivar and species. An universal mechanism has still not been established. While the reasons of saltinity have been determined, it is still very poor to understand the mechanisms by which salty prevents plant growth. Therefore, further investigation should be done to learn more about the effect of proline on cell division, cell cycle and germination molecular metabolism. For designing salinity tolerance hypotheses in plants, this literature study can serve to present new conceptual tools.

Table 2: Effect of proline on some cytogenetical parameters of Allium cepa L.

Groups	Mitotic index (%)	Micronucleus frequency (%)	Chromosome aberration (%)
Group I	*8.2 ± 0.3^{c}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Group II	0.8 ± 0.0^{a}	9.2 ± 0.5^{b}	12.4 ± 0.6^{b}
Group III	16.1 ± 0.7^{d}	11.0 ± 1.0^{c}	58.3 ± 1.2^{d}
Group IV	4.8 ± 0.5^{b}	8.6 ± 0.5^{b}	54.7 ± 0.8^{c}

*Shows values with insignificant difference ($P < 0.05$) for each column shown with same letters. Group I (control) treated distilled water, Group II treated 0.125 M NaCl alone, Group III treated 75 mg L^{-1} dose of proline, Group IV treated 75 mg L^{-1} dose of proline+0.125 M NaCl.

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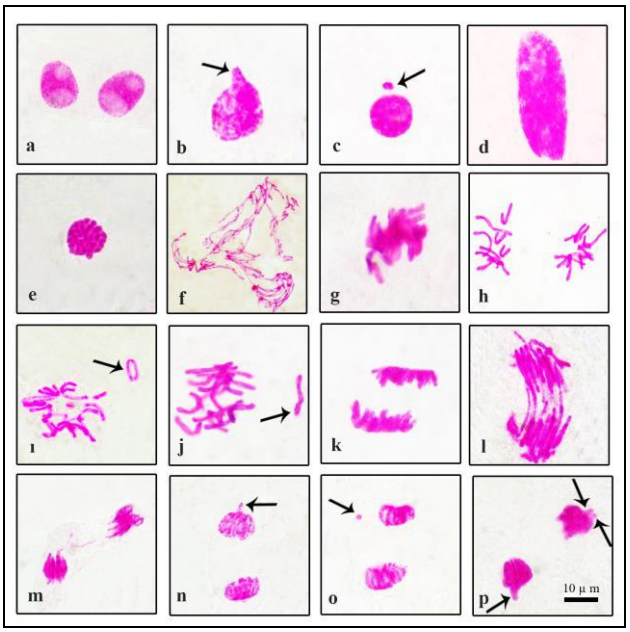

Fig. 3: Main chromosomal aberrations observations in Allium cepa L. meristematic cells, scale bar=10 µm; a: binucleolars b: nucleus with nuclear bud=arrow c: micronucleus=arrow d: giant cell e: early ball metaphase f: irregular prophase g: adhered chromosomes in metaphase h: chromosomal distrubtions in metaphase ı: ring formation=arrow j: metaphase with chromosome loss=arrow k: alignment anaphase l: multiple bridge formation in anaphase m: diagonal at anaphase n: telophase with vagrant chromosome=arrow o: telophase with micronucleus=arrow p: diagonal at telophase with vagrant chromosomes=arrows.

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