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Cytogenetical and Physiological Effects of β-alanine Treatment in Alleviation of Salt-Induced Stress in *Allium cepa* L.

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Abstract: In this study, β-alanine effects on the seedling growth (radicle number, radicle length and fresh weight), seed germination, chromosomal aberrations, mitotic activity and micronucleus frequency in *Allium cepa* L. germinated in both salt stress and normal conditions investigated. In only β-alanine medium, the fresh weight, radicle number and radicle length of the seeds partially reduced compared to the control seeds germinated in distilled water medium but the germination percentage indicate statistically the same value as the control. Besides, while the micronucleus formation and mitotic index in root tip meristems of *A. cepa* seeds germinated in alone β-alanine medium showed the same values as the control seeds germinated in distilled water medium, the chromosomal abnormalities exhibited an increase compared to the control. On the other hand, salt stress significantly inhibited the seedling growth and seed germination of *A. cepa*. What's more, it majorly reduced the mitotic index in meristem cells of the seeds and increased the number of chromosomal abnormalities and micronuclei formation, which is the simplest indicator and the most effective of cytological damage. Althought the harmful affects of saltness on the chromosomal aberrations, seed germination, seedling growth and mitotic activity alleviated in varying degrees by β-alanine treatment, this application was inefficient in damage reducing of salt on the micronucleus formation.

***Key words**:* β-alanine, Chromosomal aberrations, Seedling growth, Seed germination, Salt stress, Mitotic index

Allium cepa L.'da Tuz Teşvikli Stresin Hafifletilmesinde β-alanin Uygulamasının Sitogenetik ve Fizyolojik Etkileri

Özet: Bu çalışmada, hem normal hem de tuz stresi koşullarında çimlendirilen *Allium cepa* L.'da fide büyümesi (radikula sayısı, radikula uzunluğu ve taze ağırlık), tohum çimlenmesi, kromozomal anormallikler, mitotik aktivite ve mikronükleus sıklığı üzerine β-alaninin etkileri incelenmiştir. Tek başına β-alanin ortamında, tohumların taze ağırlığı, radikula sayısı ve radikula uzunluğu distile su ortamında çimlendirilen kontrol tohumlarına kıyasla kısmen azalmış ancak çimlenme yüzdesi istatistiki olarak kontrolle aynı değeri göstermiştir. Bununla birlikte, tek başına β-alanin ortamında çimlendirilen *A. cepa* tohumlarının kök ucu meristem hücrelerindeki mitotik indeks ve mikronükleus oluşumu distile su ortamında çimlendirilen kontrol tohumlarıyla aynı değerleri gösterirken, kromozomal anormallikler kontrole kıyasla bir artış sergilemiştir. Diğer taraftan, tuz stresi *A. cepa*'nın tohum çimlenmesi ve fide büyümesini önemli ölçüde engellemiştir. Dahası, tuz tohumların kök ucu hücrelerindeki mitotik indeksi önemli ölçüde azaltmış ve sitolojik hasarın en basit ve en etkili göstergesi olan mikronükleus oluşumu ve kromozomal anormalliklerin sayısını arttırmıştır. Kromozomal anormallikler, tohum çimlenmesi, fide büyümesi ve mitotik aktivite üzerindeki tuzun zararlı etkileri β-alanin uygulamasıyla çeşitli derecelerde azalmasına rağmen bu uygulama mikronükleus oluşumu üzerindeki tuz hasarının azaltılmasında etkisiz olmuştur.

***Anahtar kelimeler**:* β-alanin, Kromozomal anormallik, Fide büyümesi, Tohum çimlenmesi, Tuz stresi, Mitotik indeks

1. Introduction

Predominantly our world is a salty planet and most of its water contains about 3% salt. The soil is very salty due to this salt concentration. Since salt represents a serious threat to agricultural productivity, it projected that around 900 Mha land affected. The existing salinity is a major challenge for food safety as most agricultural crops will not grow in conditions where salted concentration is high. Innately occurring salination is mainly due to the rise of subsequent evaporation and the capillary water level of the saline groundwater. But man-made salinization is very common. Irrigated soils in arid regions are particularly susceptible to salting. Irrigation-practices lead to an increase in groundwater level and an increase in evaporation. High salt levels do not only increase the pH-level of soil but also lead to damaging effects on plants. So under high pH-levels, most crop plants do not grow well. It hinders the desired air-water balance required for biological processes occurring in the plant roots as well. In consequence of all harmful effects of salinisation, the product yield decreased, the cultivatable area irreversibly lost. Salinity stress brings about diverse effects in plant physiology like ion toxicity, increased photosynthetic rate, mineral distribution and changes in plant growth, membrane permeability, membrane instability on account of calcium displacement by sodium. NaCl stress effects plant physiology in the whole plant at cellular levels by ionic and osmotic stress. Although it causes ionic and osmotic stress, salinity brings about ionic imbalances that may cause potassium deficiency and impairs the selectivity of root membranes [1–5].

Alanine (Ala) also referred to as 2-aminopropanoic acid. Alanin is an important source obtained by major muscle proteins. Approximately 7.8% of the proteins composed of alanine building blocks. Because the methyl group in the alanine is chemically highly inactive, it may only indirectly contribute to protein function [6].

Allium cepa has been found not only to be an excellent indicator target with many privileges like low cost, ease of use, short test duration and large size chromosome for the study of chromosomal abnormalities but has validated also as an effective test for environmental monitoring in international collaborative studies under World Health Organization (WHO), US Environmental Protection Agency (USEPA) and the United Nations Environmental Program (UNEP) [7–10]. In this study, therefore, *A. cepa* used as a test material. There are no published studies on role of β-alanine on the seedling growth, seed germination, micronucleus frequency, mitotic activity and chromosomal aberrations under both saline and normal conditions. For this reason, this is the first study designed to investigate on the effects of β-alanine in reducing of the harmful influences of NaCl stress on some cytogenetic and physiological parameters in *A. cepa* L.

2. Material and Methods

2.1. Seed, β-alanine and salt concentrations

Approximately equal-sized and healthy *Allium cepa* L. seeds (2n=16) were used as test material in this laboratory bioassay. By a preliminary investigation carried out in the

present study, tried out concentrations of 1, 5, 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mg L^{-1} dose of β-alanine. However, concentration of β-alanine, which the best promotes seedling growth and the seed germination against the inhibitory impact of salinity, was determined as 200 mg L^{-1}. On the other hand, salt (NaCl) concentration was determined as 0.175 M. Thus these concentrations were used in the study.

2.2. Seed germination

Experiments of seed germination performed under dark to 20°C incubator. Approximately equal-sized and healthy onion seeds have selected. *A. cepa* L. seeds have sterilized by sodium hypochlorite solution (2.5%) for 10′ and washed with ultra-pure water for 24 hrs. 20 seeds selected from each application group placed in plastic containers. The bulbs have split in four groups:

- Group I (control) during 7 sequential days have treated by distilled water
- Group II during 7 sequential days have treated by 0.175 M NaCl alone
- Group III during 7 sequential days have treated by 200 mg L^{-1} dose of β-alanine
- Group IV during 7 sequential days have treated by 200 mg L^{-1} dose of β-alanine+0.175 M salt

It is assumed that the seeds in plastic containers placed in the incubator for germination should have a length of 10 mm. After 7 days, the final germination percentage taken, the radicle numbers recorded, the radicle lengths of onions measured in mm, the fresh weights also determined in g/seed. All experiments repeated 3 times.

2.3. Statistical and cytological analysis

After a few days for cytogenetic analysis, 1-1.5 cm segment of germinated *A. cepa*'s root tips excised. Initially, these have pretreated using saturated para-dichlorobenzene for four hours, fixed in 3ethanol/1:acetic acid solution for 24 hours at room temperature and stock up in 70 % ethanol at 4^{o}C until making the microscopic slides. *A. cepa* rootlets hydrolysed for 45 minutes in 5 N HCl, dyed in Feulgen for 1-1.5 hours and lysed with a drop of 45 % CH_3COOH. Squashes have prepared as suggested by Sharma and Gupta [11]. At the end of 24 hours, microscopic preparations made permanent by means of balsame. These preparations inspected using an Olympus CX41 microscope then photographed using 100X objective.

Cell division frequency in these preparations analyzed by calculating the mitotic index (%) (MI) was calculated by analysing at least 30000 cells per sample (about 10000 per preparation). Chromosomal abnormalities were evaluated for each concentration as the percentage of 2000 dividing cells counted. The latter designated as a percentage between the total number of cells analyzed (N) and the number of dividing cells (N') according to formula: MI (%) = (N'/N) x 100. Statistical analysis performed utilizing SPSS program [12].

2.4. Micronucleus (MN) assay

1000 cells per slide scored for micronucleus analyses. Micronuclei inspected using a binocular light microscope. For the scoring of micronucleated cells, Fenench et al. [13] used the protocol they followed. These; ı- the micronucleus diameter should be a tenth

of the main nucleus ıı- The micronucleus should be separated from marginally overlapped from the main nucleus, provided that the nucleus boundary is clearly defined ııı- the micronucleus staining should be similar to that of the main nucleus.

3. Results and Discussion

3.1. Influences of β-alanine on the seedling growth and seed germination

The results from Table 1 clearly demonstrate that while the germination percentage of group III showed statistically the same value as control (group I), their fresh weight, the radicle length and the radicle number partly decreased according to control seeds germinated under normal condition.

On all growth parameters examined, NaCl showed preventive affects. For instance, group I seeds germinated in normal condition after 7 days showed 100% germination, whereas this value was 23 % in group II seeds germinated at 0.175 M saltness. That is to say, NaCl prevented 77 % seeds germination of *A. cepa*. The β-alanine application significantly mitigated the inhibitory impact of salinity stress on seed germination. Group IV seeds treated with β-alanine at said salt level showed 70 % germination. Eventually, *A.* seeds displayed a performance as if they germinated in normal conditions, but this was not the case for salt conditions (Figure 1). In addition, β-alanine went on its success on seedling growth parameters like the fresh weight, the radicle length and the radicle number. The radicle length, the radicle number and the fresh weight of group II seeds grown in 0.175 M salted were 10.3, 12.7 and 7.0 g, respectively while these values became 14.7, 17.7 and 13.8 g in group four (Table 1).

Table 1. Affects of β-alanine 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}	63.5 ± 0.5^{d}	63.2 ± 0.6^{d}	14.2 ± 0.8^{b}
Group II	23 ± 2.8^{a}	10.3 ± 0.3^{a}	12.7 ± 0.5^{a}	7.0 ± 0.5^{a}
Group III	100 ± 0.0^{c}	31.0 ± 0.6^{c}	31.6 ± 1.2^{c}	15.9 ± 0.8^{c}
Group IV	70 ± 5.0^{b}	14.7 ± 0.6^{b}	17.7 ± 0.8^{b}	13.8 ± 0.8^{b}

*Indicated difference between values with the same letter in each column is not significant at the level 0.05 (±SD). Group I (control): distilled water, Group II: 0.175 M NaCl alone, Group III: 200 mg L^{-1} dose of β-alanine and Group IV: 200 mg L^{-1} dose of β-alanine + 0.175 M NaCl

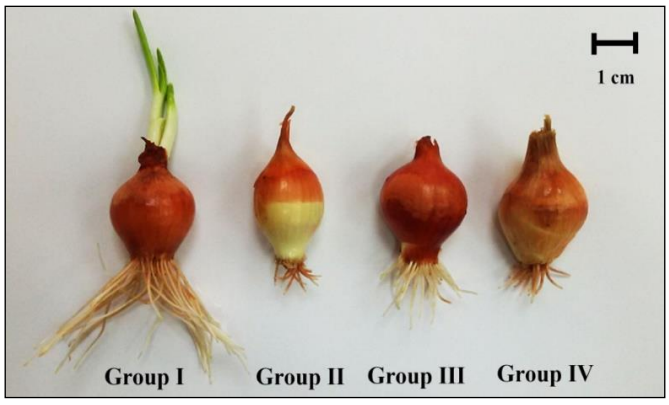

Figure 1. *Allium cepa* root tip cells showing germination situations at the end of the seventh day (Scale bar = 1 cm). Control (Group I) seeds treated by distilled water, Group II seeds treated by 0.175 M NaCl alone, Group III seeds treated by a 200 mg L^{-1} dose of β-alanine and Group IV seeds treated by a 200 mg L^{-1} dose of β-alanine + 0.175 M NaCl

3.2. Effects of β-alanine on micronucleus formation, chromosomal aberrations and mitotic activity

While the mitotic index and the micronucleus frequency of group III seeds germinated in only β-alanine medium showed the same values compared to group I (control) samples, the chromosomal aberrations partially increased (Table 2). Exposure to 0.175 M salinity results in significant inhibition in mitotic index. In another words, MI of the root tip meristems of germinated seeds in 0.175 M saline media compared to the group I seeds (control, distilled water) decreased by 89% and significantly increased the chromosome aberrations and the frequency of micronucleus. Simultaneously, β-alanine+NaCl treatment (group IV) may successful in improving adverse impacts of saltinity on the mitotic activity and the chromosomal aberrations except the micronucleus formation. Statistically, all values mentioned here are highly significant. Table 2 summarizes all cytogenetic parameters obtained from the control and other treated seeds. The normal and abnormal stages of mitotic cell division in course of microscopic examination of *A. cepa* root tip are shown in figure 2 and 3. Micronuclei and disturbed anaphase were the most frequent abnormalities induced by β-alanine and its salt constituents. Some other aberrations also observed in cells with the frequency of occurrence as: ring chromosome > vacuolated nucleus at prophase > spindle disturbance > chromosome stickiness > C-metaphase > metaphase with chromosome losses > chromosome breaks at metaphase > anaphase with chromosomal loss > formation of an anaphasic bridges > alignment anaphase > telophase with chromosome loss > telophase with lagging chromosome > telophase with forward chromosomes > polar slip in telophase > bridge in telophase.

Table 2. Influence of β-alanine on some cytogenetic parameters of *Allium cepa* L.

Groups	Mitotic index (%)	Micronucleus frequency (%)	Chromosome aberration (%)
Group I	*11.6 ± 1.0^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Group II	1.2 ± 0.2^{a}	13.0±1.0^{b}	17.0 ± 0.4^{d}
Group III	10.4 ± 0.5^{b}	0.0±0.0^{a}	5.3 ± 0.4^{b}
Group IV	11.6 ± 0.6^{b}	19 ± 1.7^{c}	10.7 ± 0.9^{c}

*Indicated difference between values with the same letter in each column is not significant at the level 0.05 (±SD). Group I (control): distilled water, Group II: 0.175 M NaCl alone, Group III: 200 mg L^{-1} dose of β-alanine and Group IV: 200 mg L^{-1} dose of β-alanine + 0.175 M NaCl

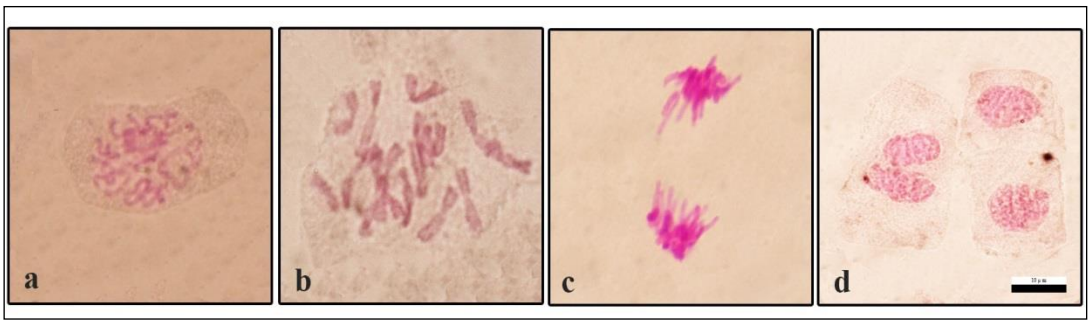

Figure 2. Normal stages of chromosome in *A. cepa* L. root meristematic cells; a- prophase b- metaphase c- anaphase d- early and late telophase, Scale bar = 10 µm

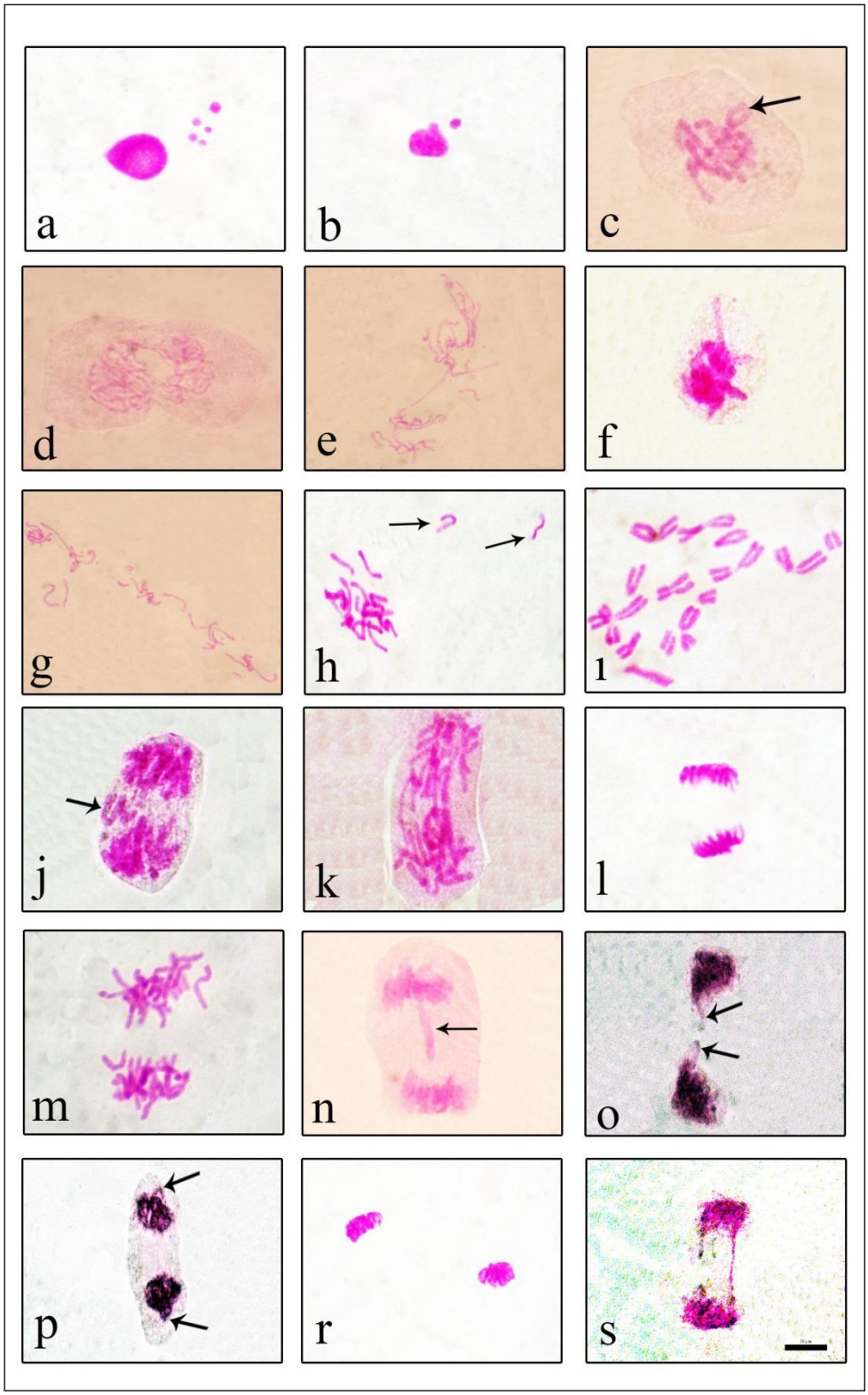

Figure 3. Alterations observed by the *A. cepa* test system analysis; a- accumulation of micronuclei in cell, b- nuclear bud with micronucleus, c- ring chromosome, d- vacuolated nucleus at prophase, e- spindle disturbance, f-chromosome stickiness, g-C-metaphase, h-metaphase with chromosome losses=arrows, ı-chromosome breaks at metaphase, j- anaphase with chromosomal loss=arrow, k- formation of an anaphasic bridges, l- alignment anaphase, m- disturbed anaphase, n- telophase with chromosome loss=arrow, o- telophase with lagging chromosome=arrows, p- telophase with forward chromosomes=arrows, r- polar slip at telophase, s- bridge at telophase, Scale bar = 10 µm

4. Discussion

4.1. Cytogenetical and physiological effects of exogenous β-alanine under normal conditions

If stress conditions are present in the environment, any plant growth regulator should be added as exogenous in the germination process. The addition of a plant growth regulator exogenously under stress-free conditions can have positive or negative effect on seedling growth and seed germination [14, 15, 16]. However, there is no available literatüre data respecting influences of β-alanine on the seedling growth and seed germination. Therefore, the laboratory study has firstly investigated the influences of β-alanine application on the mitotic activity, the chromosomal aberrations, the micronucleus frequency, the seedling growth and seed germination under normal conditions requested to be tested. The laboratory study's results revealed that the germination percentage, the mitotic index and the micronucleus frequency of the seeds germinated in only β-alanine medium showed statistically the same values as the group I seeds germinated in normal condition, whereas their fresh weight, radicle number and radicle length decreased slightly compared to control (Table 1).

Effects of β-alanine application on chromosomal aberrations, mitotic activity and micronucleus frequency under normal conditions still unknown. When the mitotic index, the micronucleus frequency and the chromosomal abnormality compared with the control in applied of 200 mg L^{-1} β-alanine, the chromosomal abnormality statistically increased. In this case, some abnormalities can said to be caused by this stimulator (Table 2). Micronucleus and disturbed anaphase were the most frequent abnormalities in this study. The formation of micronucleus (Figure 3a, b) could obtainded from the elimination of exceeding genetic material promoted by genetic content amplification, which are eventually expelled in ‘‘mini cells’’ form from the original cell. Disturbed anaphases (Figure 3m) might occur due to disturbance of spindle apparatus that lets the chromosomes to irregularly spread on cell [17]. Nuclear buds (Figure 3b) may be associated with micronucleus and constraints in the salt may be closely correlated to a of genetic material loss. Salt might be induced vacuolated nucleus at prophase (Figure 3d).

4.2. Physiological and cytogenetical effects of β-alanine exposed to saline conditions

Almost half of the all irrigated areas and twenty percent of the cultivated of the world area affected currently from saltness. High saltness conditions could result in reduced productivity or plant death at the whole plant level. Undergo high salness could effect all of the major plant processes, including lipid metabolism, photosynthesis, energy and protein synthesis. NaCl stress effects plants by causing hyperosmotic stress and ion imbalance. As a result of these primary impacts in plants, secondary stresses like oxidative damage often occur [18, 19]. The results from Table 1 clearly demonstrate that as expected the seedling growth and germination of *A.* seeds restricted in salinity conditions. Indicates the presence of high concentrations of soluble salts in the soil moisture of the root zone indicative of soil salinity in agriculture. Due to their high osmotic pressures, the concentrations of these soluble salts effect plant growth by limiting water uptake of the roots [20, 21]. Results in these statements are consistent with the results of the present study in terms of displaying the decrease in water content and fresh weight of the seedlings in salted conditions.

In contrast with, by application of the amino acid β-alanine, the inhibitory effect of salinity stress on parameters such as seedling growth and the seed germination significantly eliminated (Table 1). But till date, there are no studies investigating influences of β-alanine on seedling growth and seed germination in saline conditions. As can be seen in Table 1, β-alanine can be understood from decrease in osmotic impacts of the salt, which relieves the salt stress on seedling growth and seed germination. For example, in 0.175 M NaCl medium, it is observed that the fresh weight of the seedlings significantly increased by β-alanine application compared to Group I indicates this probability. Also, it may a counter-attack on ABA, an increased germination inhibitor, probably amount increases due to the presence of NaCl.

The increased number of chromosomal abnormality and micronucleus frequency in the *A. cepa* assay are strong evidence for analysis of these parameters and mutagenicity of the substance evaluated are succeded way to assess the mutagenic effect promoted by the chemical(s) of interest and a simple [22–26]. The mitotic index can used to determine root growth rate and as a reflection of cell proliferation. More interestingly, this study results showed that the salt caused a decrease in mitotic activity and this decrease was achieved by decreasing the number of cells entering mitotic division. This decrease in the number of divided cells suggests that the salt may have mitodepressive effects on *A. cepa* L. cell division. With this study, it should noted that salinity adversely affects chromosome behaviors and the mitotic activity of *A. cepa* meristematic cells. Results of this study display that saltness decreased MI by 89% compared to the group I and showed an increase in the number of micronucleus and chromosomal abnormality. For example, while MN and CA in the root tip meristems in group I were 0.0 and 0.0, respectively these values became 13.0 and 17.0 in 0.175 M salt (Table 2). Furthermore, β-alanine+NaCl may effective in alleviating harmful effect of NaCl on mitotic index and chromosomal abnormality but was observed increased micronucleus frequency. So, the chromosomal abnormality with the application of simultaneously β-alanine+NaCl decreased by 37%. This result shows β-alanine repair role against salt injuries during *A. cepa*'s mitosis.

Chemical and physical agents could induce chromosomal abnormalities, which carried out by different mechanisms including aneugenic and clastogenic actions. While aneugenic effect involves inactivation of a cell structure like mitotic spindles leading to chromosomal losses, clastogenic effect characterized by induction of chromosomal break during cell division. The chromosomal breaks (Figure 3ı) and bridges (Figure 3k, s) indicate to consist of clastogenic influence. The chromosomal losses, breaks and excess material, promoted by the DNA replication, can induce micronucleus, which can eliminate from the cell in mini cells form, like small cytoplasm portions with a reduced fraction of nuclear material. C-metaphase (Figure 3g) which might arise as a consequence of in mitotic spindle (spindle dysfunction disturbances) and forward chromosomes (Figure 3p) were categorized as indicators of aneugenic influence. The polar slip (Figure 3r) condition where the poles of the mitotic spindle has been shifted to the corners of the cell which can prevent the normal cell division leading to aneugenicity. Chromosome loss (Figure 3h, j, n) and the lagging segregation (Figure 3o) typically alterations associated to the malfunction of the mitotic spindle [27]. Indiate to stickiness (Figure 3f), which is considered to be a physiological effect caused by the affected proteins of the chromosome, might cause daughter chromosomes not to be completely separated by the cross-linking of chromoproteins [28]. Ring chromosome (Figure 3c) is one consequence of chromosome losses in the telomer domain [29].

5. Conclusions and Comment

There are no literature data on effects of β-alanine application on physiological and cytogenetic parameters examined in saline conditions. Therefore, the results of this study have particularly reported for the first time in salt conditions. As a conclusion, this study shows that β-alanine can significantly increase activations like seedling growth and seed germination in alone or saline conditions. But, mechanisms in which salinity inhibits growth controversial and complex. They can also vary by species and cultivar. Up till now, an universal mechanism has not established. In spite of characterized salinity causes, the understanding of the mechanisms by which saltness prevents plant growth remains weak. It is for this reason with further work needed to learn more knowledge about the effect of β-alanine on the cell cycle, cell division and molecular metabolism of germination. In summary, this study to design salt tolerance hypotheses in plants can serve to provide new conceptual tools.

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