Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

**Citation:** Köseoğlu, E., & Aytürk, Ö. (2023). Microtubule response to salt stress. *Caryologia* 76(3): 51-62. doi: 10.36253/caryologia-2229

**Received:** July 5, 2023

**Accepted:** January 19, 2024

**Published:** February 29, 2024



**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Competing Interests:** The Author(s) declare(s) no conflict of interest.

**ORCID**

ÖA: 0000-0002-8652-7545

# Microtubule response to salt stress

Emre Köseoğlu, Özlem Aytürk*

*Department of Gastronomy and Culinary, University of Maltepe, Istanbul, Turkey*
*Corresponding author. E-mail: ozlemayturk@maltepe.edu.tr

**Abstract.** This study has aimed to investigate the relationship between salt stress, programmed cell death (PCD) and microtubule distribution in terms of duration and stress dose. PCD is an important mechanism that benefits living organisms throughout their lives. On the other hand, PCD is an indirect effect that reduces efficiency when it occurs under stress. In this research The maize (*Zea mays*) roots were exposed to salt stress with 0, 50, 100, 300 and 500 mM NaCl. The prepared paraffin sections of these five groups were subjected to DAPI (4-6-diamidino-2-phenylindole) and TUNEL analysis to study the morphological changes caused by stress-induced nuclear degeneration. PCD was determined. Microtubule labeling analysis was performed on the tissues to determine whether there were stress-induced microtubule changes in these cells and disturbances were found; they exhibited aggregation, regional thickening, and random distribution around the nucleus and vacuole and under the cell wall. When all groups were evaluated, cells exposed to a salt concentration of 50 mM (even after 24 hours) were significantly less damaged than cells at other concentrations (100, 300, and 500 mM) at each time point. The rate of progression and spread to the whole tissue was significantly higher at 300 and 500 mM salt concentrations compared to the other groups. To reduce economic losses in salty soils, it is of great importance to fully investigate stress. The data that will emerge from our research, which is the subject of a small number of studies, will help to understand the mechanism of stress, microtubule and PCD.

**Keywords:** maize, microtubules, programmed cell death, salt stress, TUNEL.

## INTRODUCTION

Due to their stability in the soil, plants come in for abiotic stress factors along their lifetime that affect their physiological and biochemical mechanisms (Salika and Riffat 2021; Koyro et al. 2012). This effect leads to a decrease in plant yield (Yadav et al 2020) or has more serious consequences. Since stress conditions reduce the productivity of plants and the number of people in the world are growing day by day, it is necessary to reverse the negative situations in plants that people consume as food, increase the quality of plants and minimize their losses. With this planned study, one of the impacts of salt stress on maize was investigated and support was provided to solve the stress-related problems for which people are sought as food.

Maize belongs to the Poaceae family. It is generally grown in hot and humid areas. (Rouf et al 2016) The maize plant is used worldwide for both

consumption and industrial purposes. Its seed is one of the cold-tolerant seeds that can be kept a few years under optimal conditions and has low moisture content (Macar 2017). Its kernel contains 70% starch, 10% protein, 5% fat, 2% sugar, and a lot of vitamin A. Environmental factors (drought, salinity, high temperatures, etc.) are important factors affecting the maize yield worldwide and Turkey. (Tollenaar and Lee 2002) Our literature research of maize plants revealed that research on stress-programmed cell death and microtubules in maize is insufficient.

We chose salt, an element that stresses plants, as a research topic. In an earlier study, the salt tolerance of Maize *(Zea mays* L.) plants was determined to be 50-75 mM in sensitive genotypes and 125-150 mM in resistant genotypes. It was found that 17 different maize varieties were seriously affected at all levels after 150 mM. (Aydınoğlu and Akgül 2021). The time at which the maize is exposed to salt stress is also important. In short-term exposure, growth is affected by osmotic stress. There is an accumulation of sodium in the roots of the plant, the seed cannot absorb enough water, and germination does not occur in time (Farooq et al. 2016). Akay et al. (2019) showed that by increasing salinity, root length is negatively affected, and its elongation decreases by 82%, as well as, germination rate, seedling vigor and root to stem ratio decreases. These studies show that plants undergo some morphological changes under stress conditions. Salt stress, which is our research topic, causes many negative conditions for the maize plant. For example, the stress factors salinity, UV radiation, temperature, water, light, etc. can trigger the Programmed cell death (PCD) process (Sychta et al. 2021; Petrov et al. 2015).

PCD is a controllable mechanism. PCD is observed as long as the plant life cycle continues (Rocha and Hernandez, 2017). Characteristic features indicating the presence of PCD have been identified in plant tissue studies. DNA fragmentation, increased vacuolization, chromatin condensation are some of the most obvious (Sychta et al 2021). Internucleosomal fragmentation, which is a common consequence of PCD, is detected by the TUNEL assay (Petrov et al. 2015). The PCD process occupies an important place in the developmental stages of the plant. All these stages take place regularly. For example, the formation of unisexual flowers, the degradation of root tissue, the opening of anthers (Gunawardena 2008). Although PCD is important for the continuation of the life cycle of the plant, it is a process that seriously damages the maize plant under stress (Choudhury et al. 2017; Rocha and Hernandez 2017), there is almost no information on how this process affects microtubules. Here, the relation between PCD and microtubule distribution is investigated in detail.

Microtubules, which are dynamic structures, are perfect transformation devices (Mollinedo and Gajate 2003). Microtubules play a role in various cellular tasks such as intracellular movement, intracellular transport, and proliferation. (Borowiak et al. 2015). A microtubule has an average diameter of 21 nm and can be up to 10 µm long. Microtubules appear as bundles around chromatin concentrated in the nuclei of newly formed plant cells. (Lü et al. 2007). Plant cortical microtubules create a non-centrosomal arrangement that anchors laterally to the plasma membrane (Gutierrez et al., 2009; Crowell et al., 2011; Sampathkumar et al., 2013). This arrangement's course is directed by cellulose synthase-interacting proteins (Bringmann et al., 2012; Li et al., 2012), which act on the complex (Paredez et al., 2006).

Many different factors, including stress, can affect microtubule organization. For example, in the studies of Blancaflor et al. (1998), it was observed that when roots are exposed to aluminum stress, microtubule sensitivity decreases, their arrangement is disrupted, they are randomly organized, and their reorganization is prevented in the inner cortex, outer cortex, and epidermis. In addition, symbiotic and non-pathogenic fungi are known to alter the sequence of microtubules in plant cells (Uetake and Peterson 1998).

These findings contribute to the latest research in the field. Based on the lack of parts of literature, our study aimed to investigate the effect of salt concentrations (50 mM, 100 mM, 300 mM, and 500 mM) on maize roots. The study utilized TUNEL analysis to examine whether programmed cell death (PCD) was induced, and also analyzed the microtubule organization under PCD stress.

## MATERIAL AND METHOD

In this project, monoecious maize, which belongs to the Poaccea family, was selected as the study material. The research was conducted on five groups under laboratory conditions using maize plant seeds, with three replicates for each group (Table 1).

### *Fluorescence microscopic studies*

Fluorescent staining applications, DAPI (4-6-diamidino-2-phenylindole) and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling: in situ end labeling) used to detect PCD and microtubule proteins used for imaging. 1.5-2.0 cm long roots under salt stress

**Table 1.** Salinity of the solutions used to generate salt stress.

| | groups | | | | |
|---|---|---|---|---|---|
| | 1. group | 2. group | 3. group | 4. group | 5. group |
| salt concentration | control-0 mM | 50 mM | 100 mM | 300 mM | 500 mM |
| salt treatment time | 0 | 15min<br>30min<br>1h<br>2h<br>6h<br>12h<br>24h | | | |

were fixed with FAA (formalin-acetic-alcohol). Then, 3 µm sections were taken using the paraffin embedding method. Fluorescence microscopic observations using Olympus BX -51 fluorescence microscopy were made by staining the obtained sections with dyes suitable for the method to be used. To detect changes in nuclear morphology caused by salt concentration, we performed staining with 4',6-diamidine-2'phenylindole dihydrochloride (DAPI), taking into account the studies conducted by Schweizer (1976). We stained our samples with 1 µg ml-1 DAPI for 1 hour in the dark. After staining, we washed the samples 4-6 times with PBS so that no residual dye was present. We performed our TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling: in situ end labeling) analysis using the ApopTag® Plus Fluorescein In situ Apoptosis Detection Kit according to the kit manufacturer's instructions (Chemicon, Temecula, CA, USA).

*Microtubule labeling analysis*

The characteristics of the intracellular skeletal system of maize cells were examined. Microtubules were labeled with FITC (fluorescein isothiocyanate) and their changes were followed, and data were obtained on the change (relationship, location) of microtubules in the cytoplasm, nucleus, and cell wall. The microtubule labeling study performed on our thin-sectioned samples was carried out using a method obtained by modifying the data from the studies of Kumagai et al. (2001). The sectioned samples were placed on slides coated with poly-L-lysine. First, the samples were washed thoroughly in 50 mM PBS (pH 6.8) for 30 min. To destroy the cell wall, the preparations were treated with enzymes dissolved in 0.4 M mannitol: 0.5% cellulose for 25 minutes, 2% driselase for 15 minutes, and finally 1% cellulysin for 16 minutes. The air-dried slides were permeabilized in methanol cooled at -20°C for 10 minutes. Then they were rehydrated with these chemicals: 1% Triton X-100, 0.1 M PIPES, 2 mM EGTA, 1 mM MgSO4, and 0.4 M mannitol (pH 6.9) buffer for 30 minutes. Then the labeling step with antibodies was started. Samples were incubated with anti-a-tubulin antibody (IgG, Sigma-Aldrich)/buffer (ratio 1/50) for 1 hour at 37°C in a humidified oven in the dark. After incubation, samples were washed 1-2 times with a buffer. Fluorescent Goat Anti-Rabbit IgG (secondary antibody, Sigma-Aldrich) / buffer (ratio 1/24) was incubated in an oven at 37°C for 30 minutes. After incubation, slides were washed 3 times with a buffer for 2 minutes. To prevent the loss of fluorescence radiation, the samples were bound with 1,4-diazabicyclo-(2,2,2) (DABCO) and observations were started in a short time. A KAMERAM fluorescence camera and an Olympus BX -51 fluorescence microscope (wavelength of 420-490 nm) were used for imaging, analyzes were performed using KAMERAM software, and photographs were taken.

## RESULTS

Here, root tissue was first exposed to different salt concentrations considering the results of literature studies. Five groups were formed, namely the control group (group 1) and 50 Mm (group 2), followed by 100 Mm (group 3), 300 Mm (group 4) and 500 Mm (group 5). The preparations of these five groups were subjected to DAPI (4-6-diamidino-2-phenylindole) and TUNEL analysis to study in detail the morphological changes caused by stress-induced nuclear degeneration. It was determined that the tissue cells were subjected to PCD. Microtubule labeling analysis was performed on the tissues to determine whether there were stress-induced microtubule changes in these cells, and all results were discussed below.

*Findings of DAPI Staining*

Findings of the group 1 (control)

In this group, which we determined to be the control, the plant roots were treated with pure water only. Fluorescence microscopy observations of DAPI staining in the cells showed that the nuclear morphologies were mostly smooth and spherical (Figure 1a-d).

Findings of the group 2

The cells of root tissue stressed with 50 mM salt for 15 minutes, chromatin condensation disorder was rare, seen in a few cells at the edge of the root tissue (figure 1e, arrows). When the time was increased to 30 minutes, chromatin condensation of cells in the edge region was almost twice as high as after 15 minutes. In case of extension to 1 hour, the number of cells with chromatin condensation increased rapidly in the central region, but their numbers were relatively small compared with the peripheral region. In case of extension to 2 hour (figure 1f, arrows), and then up to 6 hours, the cells with chromatin condensation increased in almost the whole tissue, while it has not been observed that the cells in the lower part of the root tissue have not changed up to this stage, additionally the cells whose nuclei were fragmented in 6 hours began to increase. When the duration was extended to 12 h (figure 1g, arrows) and 24 h, there wasn't a crucial difference between them. It was shown that the nuclei in relatively many cells of the 1.5-2.0 cm tissue lost their spherical shape, except for the region of the root tip, and the tissue was extremely damaged.

Findings of the group 3

By examining the DAPI staining and fluorescence microscopy images of the cells of root tissue stressed with 100 mM salt for 15. minute, it was found that these cells that showed chromatin condensation in the root tissue were regional and had a similar degree of deterioration as the 15 min and 30 min of 2. groups. In contrast to the 15. minute of the 2. group, a few cells in the middle region showed deterioration in the form of chromatin condensation (figure 1h, arrows). When the time of salt concentration was increased toward 24 h, 30 min in the form of 1, 2h (figure 1i, arrows), 6, 12 (figure 1j, arrows), and 24 h, a similar tissue change was observed as in the 2. group, but with faster and more intense chromatin condensation and further nuclear fragmentation.

Findings of the group 4

Examination of fluorescence microscopy images of the cells of root tissue stressed with 300 mM salt for 15 minutes shows that they are in a similar state to cells of root tissue exposed to a salt concentration of 100 mM for 2 hours, and that damage has and that damage has progressed to a very early stage observed. It was found that when the time was increased from 15 minutes (figure 1k, arrows) to 30 minutes, 1, 2 (figure 1l, arrows), 6, 12 (figure 1m, arrows), and 24 hours, the damage increased greatly in the marginal and middle regions, while it was less advanced in the cells of the root tip tissue. It was observed that chromatin was not properly distributed in the degraded cells, but partially condensed and the cells lost their spherical shape . In contrast to the 2nd and 3rd groups, it was observed that the staining was intense at all stages. While there was a high level of stress induced degradation of the cells, particularly after the 1st hour, it was observed that the integrity of the tissue was not compromised, and the pseudo-blinding was more intense than in the previous two groups.

Findings of the group 5

Examination of the fluorescence microscopy images of the cells of root tissue stressed with 500 mM salt for 15 minutes showed a similar picture to the cells exposed to a salt concentration of 300 mM for 30 minutes and 1 hour and cellular defects were seen in more than half of the tissue (figure 1n, arrows). In case of extension to 30 minutes, 1, 2 (figure 1o, arrows), 6, 12 (figure 1p, arrows), and 24 hours, it was found that the defects started in the marginal and middle region as in all previous groups, but spread very early and rapidly to more than half of the tissue. It was found that the pseudo glare became more intense from the 1st hour and the integrity of the tissue was severely damaged.

When all groups were evaluated, cells exposed to a salt concentration of 50 mM (even after 24 hours) were significantly less damaged than cells at other concentrations (100, 300, and 500 mM) at each time point. The rate of progression and spread to the whole tissue was higher at 300 and 500 mM salt concentrations compared to the other groups.

*Findings of TUNEL analysis*

Starting from the control group, fluorescence microscopy images of DAPI staining root tissues

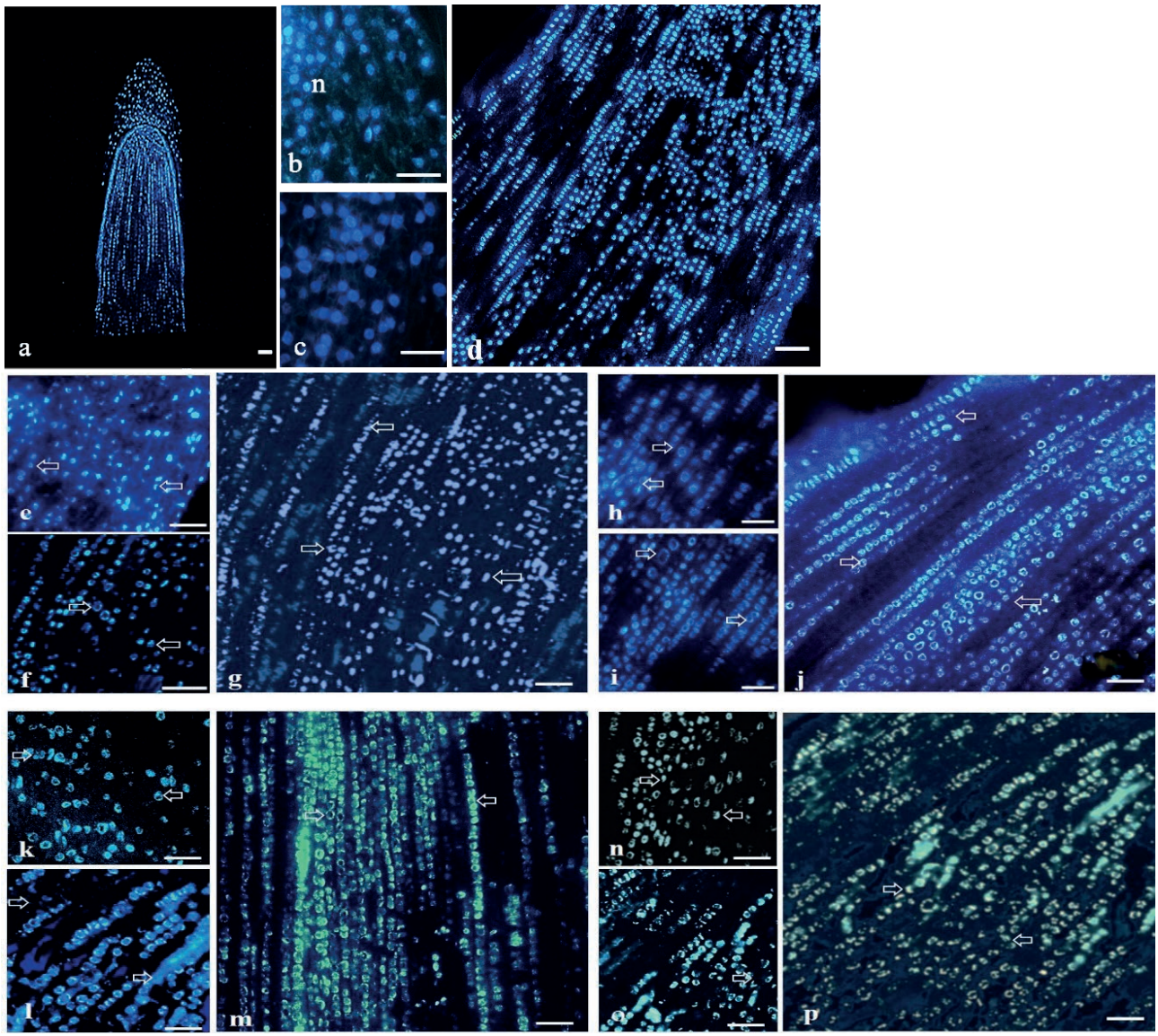


**Figure 1a-p.** Fluorescence micrographs showing DAPI staining in the cells of maize root tissue (arrow). a-d. group (control): **a.** overall view of root tissue, **b, c.** image of nuclei, d. magnified view of texture. e-p*. Condition of the cells exposed to salt concentration. **e-g.** at 50 mM; e. 15min, f. 2h, g. 12h. **h-j.** at 100 mM, h. 15min, i. 2h, j. 12h. **k-m.** at 300 mM, k. 15min, l. 2h, m. 12h. **n-p.** at 500 mM, n. 15min, o. 2h, p. 12h. Bars, a. 50 µm, b,c.10 µm and d-p.50 µm. * To avoid repetition in similar phases, the situation is presented with the images of only 3 groups.

exposed to salt stress in the 50, 100, 300, and 500 mM groups respectively for 15 and 30 minutes and for 1, 2, 6, 12, and 24 hours were examined (Figure 2a-d7). Unusual differences in the tissue cells, such as rapid vacuolization, enlargement and elongation of the cells, as well as increased staining, irregularities in nuclear shape, and changes in chromatin condensation, indicated that stress-induced apoptosis might occur in these tissues. To confirm this assumption, TUNEL analysis was performed on the tissues and a detailed study of cell death was continued. The TUNEL reaction confirmed the formation of DNA breaks and the appearance of PCD in these cells by fluorescent labeling.

## Findings of the group 1 (control)

When the fluorescence microscopy image of the TUNEL staining of the maize plant was examined, the TUNEL staining in the cells of the control group showed negative results.

Findings of the group 2

When the fluorescence microscopy images of TUNEL analysis of the cells of root tissue stressed with 50 mM salt for 15 minutes were examined, it was found that the labeled cells were located at the edges of the tissue, and a slightly positive result was obtained. In the case of extension to 30 minutes, it was found that the labeled cells formed at the edges and at the center of the tissue, and the labeled cells increased relatively compared to 15 minutes at the edge of the tissue. When the salt stress was applied for 1 hour by doubling the time, it was determined that the cells labeled at both the edge and at the center of the tissue increased rapidly. When the time is continuously increased to 2, 6, 12 and 24 hours, it is seen that the signs spread to the entire root tissue from the second hour, that is, the number of positive cells increases up to the 24 hour group. In each group, relatively less radiating cells were observed in the root tip cells than in the middle and marginal cells of the root tissue. At the stage when the time was increased to 24 hours, some of these radiations might be pseudo-radiation, because the number of radiating cells increased greatly, but the nuclei lost their structure due to the damage caused by the stress in the tissue. No completely obvious auto fluorescence was found in the root tissue at any stage (Figure 2a-a7).

Findings of the group 3

Examination of fluorescence microscope images of TUNEL analysis of stem tissue cells stressed with 100 mM salt for 15 minutes, as in group 2, revealed that there was a slight positive labeling in the peripheral parts of this tissue, which was more pronounced than in the central parts.In case of extension to 30 minutes and then to 1 hour, a significantly rapid increase in positive reaction was observed in the edge region. When the time was continuously increased up to 2, 6, 12, and 24 hours : after 1 hour, the positive reaction spread to the entire maize root tissue, unlike the 2. group, i.e., marks formed at the edge and center, at the root tip, and at the upper parts of the root. Both in the 12 and 24-hour

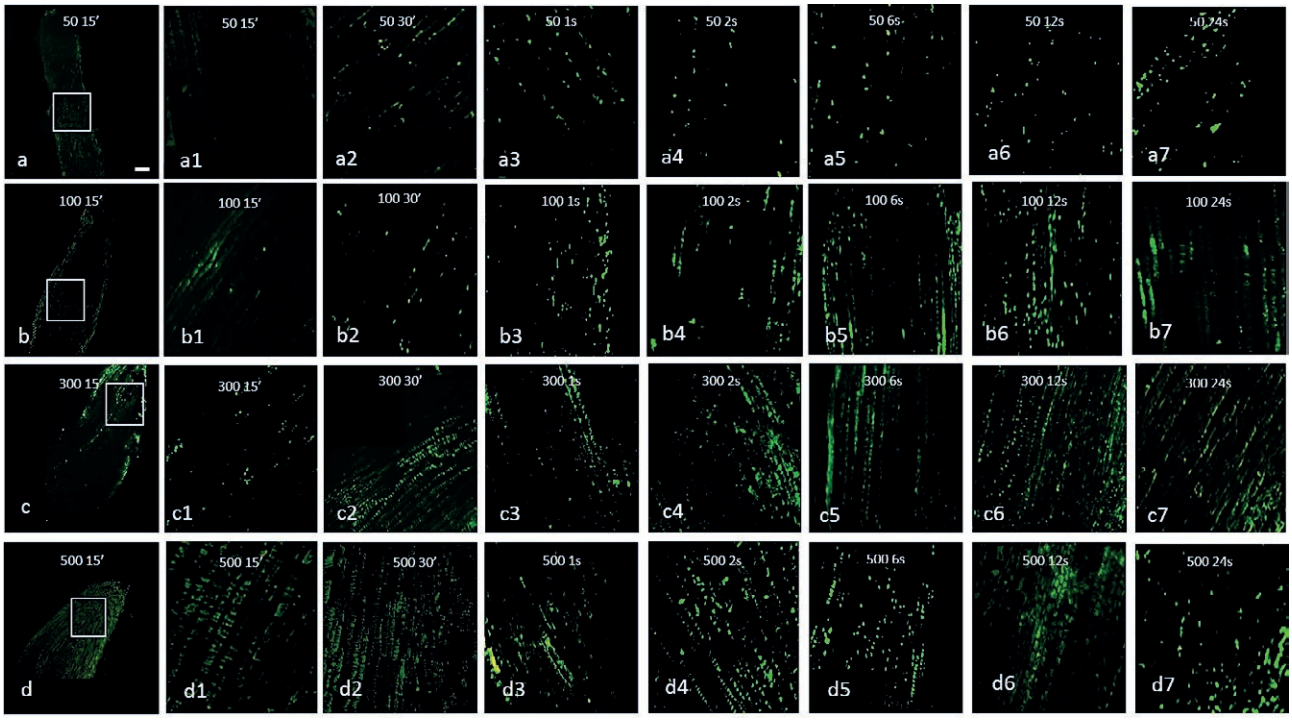


**Figure 2a-d7.** Fluorescence micrographs with TUNEL staining in cells of root tissue exposed from 50 to 500 mM salt concentration. The a'-g' photos show an enlarged view of the marked area (square) in the a-g photos. Bars 10 µm (a'-g'), 50 µm (a-g). 7a-g'. Fluorescence micrographs with TUNEL staining in cells of root tissue exposed to 100 mM salt concentration. The a'-g' photos show an enlarged view of the marked area (square) in the a-g photos. Bars 10 µm (a'-g'), 50 µm (a-g).8a-g'. Fluorescence micrographs with TUNEL staining in cells of root tissue exposed to 300 mM salt concentration. The a'-g' photos show an enlarged view of the marked area (square) in the a-g photos. Bars 10 µm (a'-g'), 50 µm (a-g).9a-g'. Fluorescence micrographs with TUNEL staining in cells of root tissue exposed to 500 mM salt concentration. The a'-g' photos show an enlarged view of the marked area (square) in the a-g photos. bars 10 µm (a'-g'), 50 µm (a-g).

groups, as well as in the 24-hour group of the 2. group, it was observed that the structures of the cells deteriorated due to the damage caused by the stress, so some of the radiations formed in the tissues were considered as pseudo-radiations. Thus, it was found that the deterioration caused by 100 mM salt concentration occurred in a shorter time than the deterioration caused by 50 mM salt concentration, but the degree of damage was similar in these two groups (Figure 2b-b7)

Findings of the group 4

When we applied the TUNEL assay to cells of root tissue stressed with 300 mM salt for 15 minutes, we saw that the result was different from the results of the 2. and 3. groups for 15 minutes: the marking was more pronounced in the peripheral region. At 30 minutes, the irradiation rapidly increased in both the middle and marginal sections, and the markings in the nuclei exposed to PCD were more frequent than in the 2. and 3. groups. At 1 hour, the irradiation spread to all parts of the root, as in the 3. group, but the markings in the nuclei exposed to PCD increased more. When the time was gradually increased to 2, 6, 12, and 24, it was determined that the density of the positive in the root tissue was similar in all these periods, the nuclei lost their structure, and the markings spread throughout the root. As a result the positive reaction formed by the 300 mM salt concentration was much more intense from the 1st hour, in contrast to the positive reactions formed by the 50 and 100 mM salt concentrations. (Figure 2c-c7)

Findings of the group 5

Examination of fluorescence microscopy images of TUNEL analysis of the cells of root tissue stressed with 500 mM salt for 15 minutes revealed that the tissue showed a positive response of low intensity, with flash spreading to the entire root tissue compared to the previous groups, but the intensity was weaker. The situation seen at 15 min continued at 30 min, with most cells in the edge and middle parts of the root tissue being positive. When the time was doubled, i.e., to 1 hour, it was observed that false glow occurred everywhere in the root and their structures could not be determined, i.e., there were cells with compromised integrity. This situation was observed in the last stages in groups 2 and 3. In the 4. group, it was observed later, almost 12 hours, in contrast to the 5. group. When the time was gradually increased to 2, 6, 12, and 24 hours, the false positive reactions were more evident at the edge and middle of the root, they were intense in the nuclei exposed to PCD, and these positive reactions were strongly positive. The intensity of the positive response persisted after 24 hours, as well as the presence of amorphous cells and nuclei, and that the positive reaction in most roots were false radiations. The structure of the root tissue after 12 and 24 hours was similar to that of the 4. group (Figure 2d-d7)

*Findings of microtubule labeling*

In our study, the characteristics of microtubule changes associated with stress and PCD of cells of root tissue were determined. For this purpose, root tissue sections were collected. Microtubule proteins belonging to these tissue cells were labeled with FITC (fluorescein isothiocyanate) and visualized by fluorescence staining analysis, and data were obtained on the changes (relationship, location) of microtubules in the cell wall, nucleus and cytoplasm, which we have described below against salt stress.

Findings of the group 1 (control)

In the first group, which we determined to be the control, it was observed that the microtubules in the cells of the root tissue were mostly concentrated around the nucleus and under the cell wall (anchors laterally to the plasma membrane) and rarely formed short extensions from the nucleus to the cell wall. It was observed that the microtubules under the cell wall formed a thin layer in a parallel arrangement, while the microtubules around the nucleus tightly surrounded the nucleus. They were also found to be located around the vacuoles and generally homogeneously distributed in the cytoplasm (Figure 3a-c).

Findings of the group 2

When the cells of root tissue stressed with 50 mM salt for 15 minutes were examined, it was observed that the regular state of microtubules under the cell wall was disturbed at a low level and very weak clusters were formed at this stage, in which PCD was weakly positive. It was observed that the density of microtubules in the cytoplasm and around the nucleus increased slightly. When the duration was extended to 30 minutes, a situation similar to that observed at 15 minutes was observed, except for the increase in microtubule density. However, when the time was advanced to 1 and 2 hours,

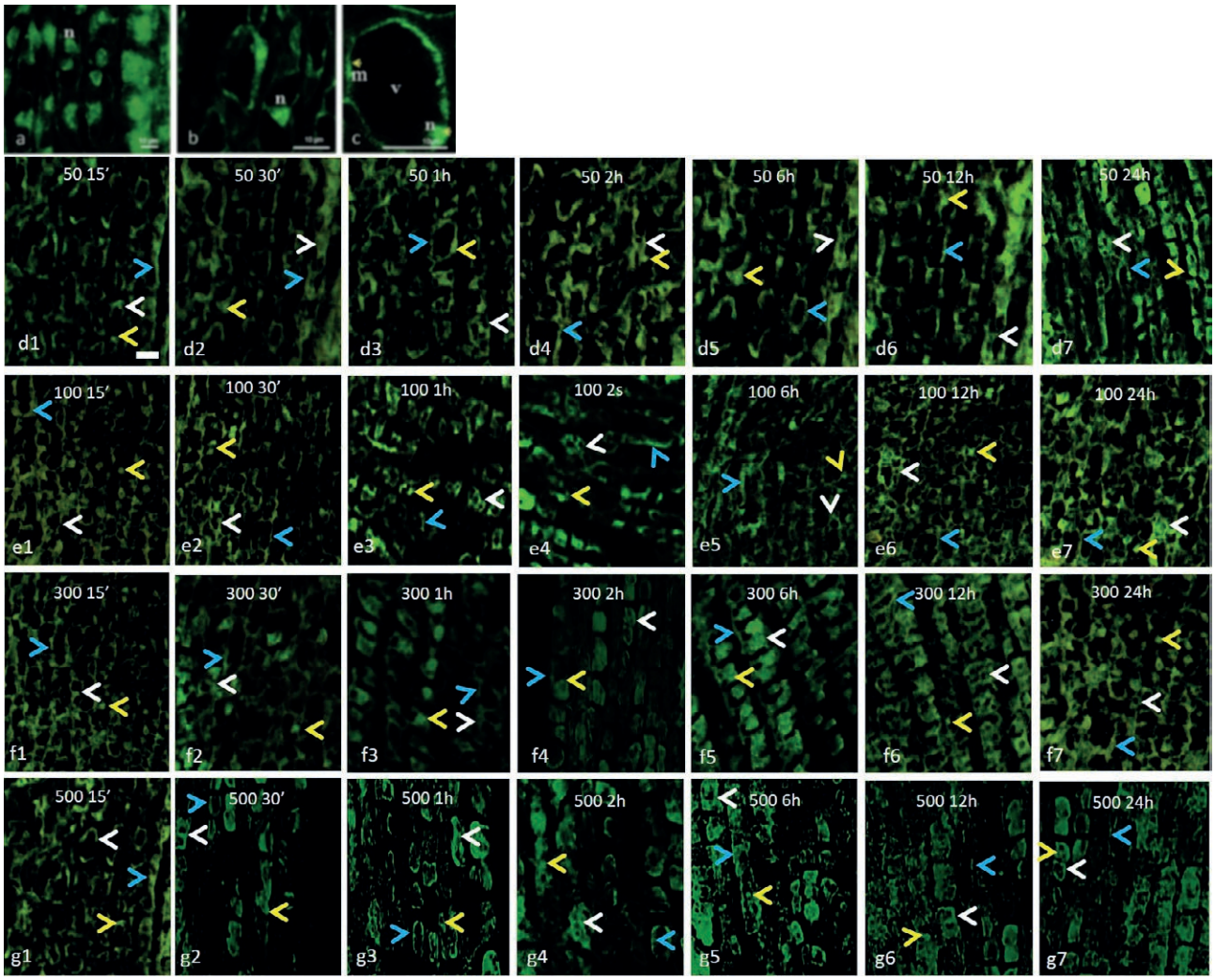


**Figure 3a-g7.** Analysis of fluorescence staining of microtubule proteins labeled with FITC (flurecein isothiocyanate) in root tissue cells exposed to different salt concentrations. hungry. Control, d1-d7. 50mm, e1-e7. 100mm, f1-f7. 300mM, g1-g7. 500mM. Arrows show where microtubules were examined: under the cell wall (blue arrow), around the nucleus (yellow arrow), around the vacuole (white arrow). Bars, 50 µm (a,b), 10 µm (c-g7).

it was determined that microtubule irregularities, aggregation, and condensation showed a more significant increase. At this stage, it was observed that the regularity of microtubules around the nucleus began to give way to random organization. When the time was extended to 2 hours and then to 6 hours, the microtubule changes observed in the first hour progressed at a moderate rate. It was found that after 6 hours, the change occurred mainly in the form of microtubule condensation, and the increase in irregularities and aggregations was similar to the situation at 2 hours. After 12 and 24 hours, it was observed that the appearance of microtubules changed into a band in which the nucleus and vacuoles were wrapped. At these stages, when PCD was strongly positive, the microtubule bundles were dense and irregularly shaped in many places, and the increase in aggregation was evident (Figure 3d1-d7).

## Findings of the group 3

Examination of fluorescence microscopy images of microtubule labeling analyzes of the cells of root tissue stressed with 100 mM salt for 15 minutes revealed that at this stage, when PCD was observed as a weak positive intensity, the regular state of microtubules under the cell wall was disturbed at a relatively low level and aggregates were formed. In the cytoplasm and micro-

tubules around the nucleus, it was observed that the density increased slightly as under the cell wall. It was observed that the situation did not change significantly when the time was extended to 30 minutes, and when it was extended to 1 hour, microtubule density and microtubule disruption increased significantly in all regions. After 2 and 6 hours, microtubule irregularity, aggregation, and condensation had slightly increased. In addition to density, it was observed that random order began to deteriorate, especially for microtubules around the nucleus. When time was extended to 12 hours, microtubule condensation, increased reticular appearance, and irregular shape changed relatively little. After 24 hours, the irregularity was more pronounced than expected. The density around the nuclei and vacuoles covered a quarter of the cell (Figure 3e1-e7).

Findings of the group 4

When the fluorescence microscopy images of microtubule labeling analysis of the cells of root tissue stressed with 300 mM salt for 15 minutes were examined, It was observed that the microtubules under the cell wall, normally in the form of parallel extensions, were rearranged by 50% and these parallel arrays began to aggregate. The aggregation increased at this stage where PCD was observed to be moderately positive. A rapid increase in the density of microtubules in the cytoplasm and around the nucleus was observed. When the time was extended to 30 minutes and then to 1 hour, it was observed that, in contrast to the salt concentrations of 50 and 100 mM, high aggregations occurred in all regions at these stages and the regularity of microtubules was severely disturbed. When the time was gradually prolonged to 2, 6, 12, and 24 hours, it was observed that the appearance of microtubules changed into a dense band, and at these stages, when PCD was strongly positive, the microtubule bundles were irregularly shaped in many places and the increase in aggregation was obvious (Figure 3f1-f7).

Findings of the group 5

Examination of fluorescence microscopy images of microtubule labeling analysis of the cells of root tissue stressed with 500 mM salt for 15 min revealed a rapid increase in microtubule density in the cytoplasm and around the nucleus, where PCD was observed to be moderately positive. When the time was extended to 30 minutes, it was observed that their regular state under the cell wall was relatively disturbed and an intense increase in their aggregation was observed. It was observed that after these stages at 1, 2 and 6 hours, the microtubule bundles formed densely and irregularly in many places, and the clusters increased even more. After 12 and 24 hours, they had a similar appearance. The microtubules were difficult to observe because most of the cells of the root tissue were destroyed. At these stages, almost the entire cell was covered with irregular microtubule clusters and pseudo-staining (Figure 3g1-g7).

In this study, a fluorescence microscope was used to examine microtubule changes in cellular changes that maize plants undergo when exposed to salt stress. In this study, which is one of the few studies, the relationship between PCD and microtubule distribution was investigated in detail. If we evaluate the results of increased salinity (50, 100, 300 and 500 Mm), we can say that: The results of 15-minute, 30-minute, 1-hour and 2-hour applications are similar to each other, and the results of 6-hour, 12-hour and 24-hour applications are similar. In the PCD process seen as a result of the response of root tissue cells to salt stress of 50 mM, microtubule organization showed clustering, regional thickening, and random distribution around the nucleus and vacuole.

## DISCUSSION

In our study, it was found that low and high salt stress can cause programmed cell death (PCD). A review of the literature shows that some studies support our findings, for example, in *Halopyrum mucronatum* at salt stress of 0, 90, 180 and 360 mM, it was found that growth was inhibited with increasing salinity, followed by plant death (Khan and Ungar 2001). TUNEL analysis was applied to determine the damage that occurred when 200, 300, 400, and 500 mM salt stress acted on the cells of the root tissue of the rice plant. TUNEL analysis gave positive results and it was found that the plant underwent PCD. As a result of 500 mM salt stress, chromatin condensation and cellular deformation were observed in the cells of the root tissue; DNA agarose gel electrophoresis was performed and breaks were observed (Yazdani and Mahdieh 2012). Another study discovered that the high salt concentrations augmented the quantity of cells exhibiting TUNEL-positive nuclei and DNA ladders in roots of both Barley and Arabidopsis. (Huh et al. 2002; Katsuhara and Shibasaka 2000; Li and Dickman 2004). Thus, when the plant is under salt stress, it can induce PCD (Williams and Dickman 2008) As shown in previous studies, stress causes PCD. Cell death can occur at different salt levels. All of these studies support our findings. In addition to salt stress, there are also studies that have found PCD in other types of stress. For

example, when water stress affected root tissue pea cells for 6, 12, and 24 hours, chromatin condensation and clusters were detected in the cells, TUNEL analyzes and DNA agarose gel electrophoresis experiments were performed, and apoptosis-like findings were noted (Gladish et al. 2006). Wang et al. (1996) demonstrated positivity by performing TUNEL analysis to investigate PCD in a toxin-treated tomato plant and found that 50% of the cells were subjected to PCD by the TUNEL assay. The root tissue of the *Vicia faba* plant was exposed to caffeine stress and the differentiations in the cells indicated the formation of PCD. COMET and TUNEL assays were applied and positive PCD results were obtained (Rybaczek et al. 2015).

In our study, it was observed that TUNEL positivity did not increase proportionally with increasing stress. According to the literature, the plant can achieve a certain level of recovery in the early stages of PCD. Cells that have undergone chromatin condensation can perform healing studies by activating early repair mechanisms (Ciniglia et al. 2010). As can be seen from our results, there are also PCD outcomes that do not parallel the dose or duration of stress.

Plant microtubules have been the subject of numerous studies (Zhao et al. 2021; Rui and Dinneny 2020; Hashimoto 2015). The subject of our study, the relationship between stress, PCD, and microtubules, is one of the few studies (Smertenko and Franklin-Tong 2011; Yanık et al. 2017). Based on the observations of DAPI staining, our studies have shown that prolonged salt can cause stress-induced damage even at low doses. The initial dose of stress-induced cell death was slightly higher and the time of onset was slightly longer. Microtubule change in stressed tissue progressed more slowly than stress-induced damage. Studies have been performed, some of which are consistent with our results. Some of the results are: in the stress study with bisphenol in maize root tissue, it was found that bisphenol disrupted microtubule arrangement and mitosis. It was observed that the microtubule arrangement became disorganized and cell division was disrupted or completely stopped. As the amount of bisphenol increased, the shapes and directions of microtubules changed (Stavropoulou et al. 2018). Cortical microtubules are located under the cell wall (anchors laterally to the plasma membrane). Microtubules were found to affect the shape of the cell as a result of the application of mechanical stress in the maize plant (Landrein and Hamant 2013). It was also observed that microtubules changed when light, auxin, and mechanical stress were applied to the coleoptile tissue of the maize plant. (Fischer and Schopfer, 1997). As additionally to literature in this study, in the PCD process seen as a result of the response of root tissue cells to salt stress of microtubule organization showed clustering, regional thickening, and random distribution around the nucleus and vacuole. It was thought that the rapid vacuolization that occurred during the PCD process triggered the condensation around the nucleus and in the cytoplasm.

## ACKNOWLEDGEMENTS

Our investigation was supported by MUKKAM (Maltepe University Cancer and Stem Cell Research Centre). We thank MUKKAM for providing laboratory resources.

## FUNDING

This project was supported by Maltepe University Research Foundation (grant number FEN -13571271-604).

## REFERENCES

1. Akay HE, Öztürk İ, Sezer M, Bahadır C. 2019. Effects of different salt concentrations on germination and early seedling growth in sugar maize (*Zea mays* L.) cultivars. Turk J Agric For. 7(2):103-108. https://doi.org/10.24925/turjaf.v7isp2.103-108.3160
2. Aydınoğlu F, Akgül B. 2021. Mısır (*Zea mays* L.) bitkisinin üşüme stresine toleransı sırasında yaprak büyüme bölgelerinde mikroRNA aracılıklı redoks regülasyonunun incelenmesi. Anadolu Tarım Dergisi. 2019:1–11. https://doi.org/10.7161/omuanajas.482710
3. Blancaflor EBD, Jones L, Gilroy S. 1998. Alterations in the cytoskeleton accompany aluminum-induced growth inhibition and morphological changes in primary roots of maize. Plant Physiol. 118(1):159–172. https://doi.org/10.1104/pp.118.1.159
4. Borowiak MW, Nahaboo RM, Nekolla K, Jalinot P, Hasserodt J, Rehberg M, Delattre M, Zahler S, Vollmar A, Trauner D, Thorn-Seshold O.. 2015. Photo Switchable Inhibitors of Microtubule Dynamics Optically Control Mitosis and Cell Death. Cell. 162(2):403–411. https://doi.org/10.1016/j.cell.2015.06.049
5. Bringmann, M., Li, E., Sampathkumar, A., Kocabek, T., Hauser, M. T., and Persson, S. 2012. POM-POM2/cellulose synthase interacting1 is essential for the functional association of cellulose synthase and

microtubules in Arabidopsis. Plant Cell 24:163–177. https://doi.org/10.1105/tpc.111.093575
6. Choudhury FKR, Rivero M, Blumwald E, Mittler R. 2017. Reactive oxygen species abiotic stress and stress combination. Plant J. 90(5):856–867. https://doi.org/10.1111/tpj.13299
7. Ciniglia C, Pinto G, Sansone C, Pollio A. 2010. Acridine orange/Ethidium bromide double staining test: A simple In-vitro assay to detect apoptosis induced by phenolic compounds in plant cells. Allelopathy J. 26(2):301–308.
8. Crowell, E. F., Timpano, H., Desprez, T., Franssen-Verheijen, T., Emons, A. M., Hofte, H., et al. 2011. Differential regulation of cellulose orientation at the inner and outer face of epidermal cells in the Arabidopsis hypocotyl. Plant Cell 23:2592–2605. https://doi.org/10.1105/tpc.111.08733
9. Farooq M, Hussain M, Wakeel A, Kadambot HMM, Farooq Hussain M, Wakeel A, Siddique K, HM, Farooq M, Hussain M, Wakeel A. 2016. Salt stress in maize:effects resistance mechanisms and management Agron. Sustain. Dev. 35:461–481. https://doi.org/10.1007/s13593-015-0287-0
10. Fischer K, Schopfer P. 1997. Interaction of auxin light and mechanical stress in orienting microtubules in relation to tropic curvature in the epidermis of maize coleoptiles. Protoplasma. 196(1–2):108–116. https://doi.org/10.1007/BF01281064
11. Gladish D, Xu KJ, Niki T. 2006. Apoptosis-like programmed cell death occurs in procambium and ground meristem of pea (*Pisum sativum*) root tips exposed to sudden flooding. Ann Bot-London. 97(5):895–902. https://doi.org/10.1093/aob/mcl040
12. Gunawardena AH. 2008. Programmed cell death and tissue remodeling in plants. 59(3):445–451. https://doi.org/10.1093/jxb/erm189
13. Gutierrez, R., Lindeboom, J. J., Paredez, A. R., Emons, A. M., and Ehrhardt, D. W. 2009. Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat. Cell Biol. 11:797–806. doi: https://doi.org/10.1038/ncb1886
14. Hashimoto, T. 2015. Microtubules in plants. The Arabidopsis Book/American Society of Plant Biologists, 13. https://doi.org/10.1023/B:BILE.0000012896.76432.ba
15. Huh G, Damsz HB, Matsumoto TK, Reddy MP, Rus AM, Ibeas JI, Narasimhan ML, Bressan RA, Hasegawa PM. 2002. Salt causes ion disequilibrium-induced Programmed cell death in yeast and plants. Plant J. 29:649–659. https://doi.org/10.1046/j.0960-7412.2001.01247.x
16. Katsuhara M, Shibasaka M. 2000 Cell death and growth recovery of barley after transient salt stress. J. Plant Res. 113:239–243. https://doi.org/10.1007/PL00013934
17. Khan MA, Ungar IA. 2001. Alleviation of salinity stress and the response to temperature in two seed morphs of *Halopyrum mucronatum* (Poaceae). Aust J Bot. 49(6):777-783. https://doi.org/10.1007/PL00013934
18. Koyro HW, Ahmad P, Geissler N. 2012. Abiotic stress responses in plants: an overview. Environmental adaptations and stress tolerance of plants in the era of climate change, 1-28. https://doi.org/10.1071/BT01014
19. Kumagai F, Yoneda NA, Tomida T, Sano T, Nagata T, Hasezawa S. 2001. Fate of nascent microtubules organized at the M/G1 interface, as visualized by synchronized tobacco BY-2 cells stably expressing GFP-tubulin: time-sequence observations of the reorganization of cortical microtubules in living plant cells. Plant and Cell Physiol. 42(7):723-732. https://doi.org/10.1093/pcp/pce091
20. Landrein B, Hamant O. 2013. How mechanical stress controls microtubule behavior and morphogenesis in plants: History experiments and revisited theories. Plant J. 75(2):324–338. https://doi.org/10.1111/tpj.12188
21. Li W, Dickman MB. 2004 Abiotic stress induces apoptotic-like features in tobacco that is inhibited by expression of human Bcl-2. Biotechnol. Lett. 26:87–95. https://doi.org/10.1023/B:BILE.0000012896.76432.ba
22. Li S, Lei L, Somerville CR, and Gu Y. 2012. Cellulose synthase interactive protein 1 (CSI1) links microtubules and cellulose synthase complexes. Proc. Natl. Acad. Sci. U.S.A. 109:185–190. https://doi.org/10.1073/pnas.1118560109
23. Lü B, Gong Z, Wang J, Zhang J, Liang J. 2007. Microtubule dynamics in relation to osmotic stress-induced ABA accumulation in *Zea mays* roots. J.Exp. Bot. 58(10):2565-2572.
24. Macar T. 2017. Genetiği değiştirilmiş (Transgenik) mısır (*Zea mays* L.) tohumlarında bazı biyokimyasal ve fizyolojik parametrelerin araştırılması. Doktora Tezi. Giresun Üniversitesi.
25. Mollinedo F, Gajate C. 2003. Microtubules microtubule-interfering agents and apoptosis. Apoptosis 8(5):413–450. https://doi.org/10.1023/A:1025513106330
26. Paredez AR. Somerville CR. and Ehrhardt DW. 2006. Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312:1491–1495. https://doi.org/10.1126/science.1126551

27. Petrov V, Hille J, Mueller-Roeber B, Gechev TS. 2015. ROS-mediated abiotic stress-induced programmed cell death in plants. Frontiers in Plant Science, 6:69. https://doi.org/10.3389/fpls.2015.00069
28. Rocha GL, Hernandez J. 2017. Programmed Cell Death-Related Proteases in Plants. Enzyme Inhibitors and Activators. book. https://dx.doi.org/10.5772/65938
29. Rouf ST, Prasad K, Kumar P. 2016. Maize—A potential source of human nutrition and health: A review. Cogent Food & Agriculture, 2(1):1166995. https://doi.org/10.1080/23311932.2016.1166995
30. Rui Y and Dinneny JR. 2020. A wall with integrity: surveillance and maintenance of the plant cell wall under stress. New Phytologist, 225(4):1428-1439. https://doi.org/10.1111/nph.16166
31. Rybaczek D, Musialek MW, Balcerczyk A. 2015. Caffeine-induced premature chromosome condensation results in the apoptosis-like programmed cell death in root meristems of *Vicia faba*. PLoS ONE 10(11):1–33. https://doi.org/10.1371/journal.pone.0142307
32. Salika R, Riffat J. 2021. Abiotic stress responses in maize: a review. Acta Physiologica Plantarum, 43(9):130.
33. Sampathkumar A, Gutierrez R, Mcfarlane H, Bringmann M, Lindeboom J, Emons AM. 2013. Patterning and life-time of plasma membrane localized cellulose synthase is dependent on actin organization in Arabidopsis interphase cells. Plant Physiol. 162:675–688. https://doi.org/10.1104/pp.113.215277
34. Schweizer D. 1976. Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma. 58(4):307–324. https://doi.org/10.1007/BF00292840
35. Smertenko A, and Franklin-Tong VE. 2011. Organisation and regulation of the cytoskeleton in plant programmed cell death. Cell Death & Differentiation, 18(8):1263-1270. http://dx.doi.org/10.1038/cdd.2011.39
36. Stavropoulou K, Adamakis IDS, Panteris E, Arseni EM, Eleftheriou EP. 2018. Disruption of actin filaments in *Zea mays* by bisphenol A depends on their crosstalk with microtubules. Chemosphere 195:653–665. https://doi.org/10.1016/j.chemosphere.2017.12.099
37. Sychta K, Słomka A, Kuta E. 2021. Insights into Plant Programmed Cell Death Induced by Heavy Metals-Discovering a Terra Incognita. Cells. 10(1):65. https://doi.org/10.3390/cells10010065
38. Tollenaar M, Lee EA. 2002. Yield potential, yield stability and stress tolerance in maize. Field crops research, 75(2-3):161-169. https://doi.org/10.1016/S0378-4290(02)00024-2
39. Uetake YR, Peterson L. 1998. Association between microtubules and symbiotic fungal hyphae in protocorm cells of the orchid species *Spiranthes sinensis*. New Phyto. 140(4):715–722. https://doi.org/10.1046/j.1469-8137.1998.00310.x
40. Wang H, Li J, Bostock RM, Gilchrist DG. 1996. Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. The Plant Cell. 8(3):375-391. https://doi.org/10.1105/tpc.8.3.375
41. Williams B, Dickman M. 2008. Plant programmed cell death: Can't live with it; Can't live without it. Mol. Plant. Path. 9(4):531–544. https://doi.org/10.1111/j.1364-3703.2008.00473.x
42. Yadav S, Modi P, Dave A, Vijapura A, Patel D, Patel M. 2020. Effect of abiotic stress on crops. Sustainable crop production, 3.
43. Yanık F, Aytürk Ö, Vardar F. (2017). Programmed cell death evidence in wheat (*Triticum aestivum* L.) roots induced by aluminum oxide (Al2O3) nanoparticles. Caryologia, 70(2):112-119.https://doi.org/10.1080/00087114.2017.1286126
44. Yazdani M, Mahdieh M. 2012. Salinity Induced Apoptosis in Root Meristematic Cells of Rice. IJBBB. 40–43. https://doi.org/10.7763/IJBBB.2012.V2.66
45. Zhao S, Zhang Q, Liu M, Zhou H, Ma C, Wang P. 2021. Regulation of plant responses to salt stress. International Journal of Molecular Sciences, 22(9):4609. https://doi.org/10.3390/ijms22094609