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# Role of humic acid against salt-induced cytotoxicity in *Hordeum vulgare* L.

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**Abstract.** The effect of humic acid, which is an replace by a biostimulant on mitotic activity and chromosome behaviors in meristem cells of *Hordeum vulgare* L. germinated under different salt concentrations were investigated. In the parallel to increasing salt concentrations, mitotic index partly decreased and observed the higher number of chromosomal abnormalities as compared to control. Also, it was determined that the mitotic index of seeds pretreated with only humic acid increased by 30% according to control and by 42% of mitotic aberrations. Whereas, humic acid along with salt significantly inhibited to mitotic index with parallel to increasing salt concentrations. Moreover, the frequency of chromosomal aberrations in seeds germinated in humic acid and salty medium significantly decreased according to its own control. Humic acid revealed to a successful performance in ameliorating of the detrimental effect of salinity in the all concentrations studied. Humic acid application at 0.35 M salinity displayed perfectly successful by reaching to the same abnormality percentage of control.

**Keywords.** Barley, chromosomal aberrations, cytotoxicity, humic acid, mitotic activity, salinity.

## INTRODUCTION

It is a known fact to affect of abiotic stresses on plant growth and development. One of the most common environmental stress factors is salinity, which an increasing problem of many arid and semiarid areas of the World. Approximately 20% of the world's cultivated land and accounts for over 6% of the world total area is threatened by salinity (FAO 2015). Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and the natural status of the environment. Increased salinization of all arable land is expected to have devastating global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang et al. 2003).

It has been known for a long time that salt adversely affects plant growth and development, hindering seed germination, seedling growth (Çavuşoğlu and Ergin 2015), enzyme activity (Dash and Panda 2001), DNA, RNA and protein synthesis (Anuradha and Rao 2001) and mitosis (Tabur and Demir 2010 a, b; Çavuşoğlu et al. 2016). However, recent investigations are focus-

ing more on the mechanisms of salt tolerance in plants (Munns and Tester 2008).The most efficient way to minimize the detrimental effects of salinity on plant breeding is the development of varieties with high salinity tolerance. Hence, researchers have used various plant growth regulators and leaf extracts to reduce or eradicate negative effects of salinity on seed germination, seedling growth (Çavuşoğlu and Ergin 2015), and mitotic activity (Tabur and Demir 2010 a, b; Çavuşoğlu et al. 2016). However, in spite of substantial efforts, the outcome is still disappointingly poor due to the physiological and genetic complexity of this trait, the lack of reliable screening tools, and most importantly, the lack of a comprehensive understanding of the mechanisms behind salinity tolerance (Zhu et al. 2016). The data relating to mitotic activity are also mostly paradox.

The main ingredient of organic substances in the soil is humus. The most active biochemical substance of humus is humic acid. Humic substances have been known that the germination and growth of plants has stimulated. Humic substances can pass through micropores of biological or artificial membrane systems, facilitate the transport of trace elements in plant roots and behave like growth hormones in plants (Chen et al. 2004). Therefore, humic substances are evaluated as a biostimulant by du Jardin (2012) who conducted a bibliographic analysis of plant biostimulants. Biostimulants are derived from natural or biological sources and can i) enhance plant growth and development when applied in small quantities; ii) help improve the efficiency of plant nutrients, as measured by either improved nutrient uptake or reduced nutrient losses to the environment, or both; or act as soil amendments to help improve soil structure, function, or performance and thus enhance plant response (du Jardin 2012).

Humic acid has positive effects on plant growth and nutrition (Calvo et al. 2014). In cytophotometric studies of the DNA, it was seen that humic substances increased the amount of DNA synthesis in the interphase nucleus of the meristematic cells in plants (Gorova et al. 2005). Furthermore, humic substances are accepted to be a plant growth promoter, particularly by changing the root structure and growth dynamics (Jindo et al. 2012; Canellas and Olivares 2014).

Under both normal and salt conditions, there have been many investigations related to seed germination, seedling growth (Çavuşoğlu and Ergin 2015), root development (Sivananthi and Paul 2014), plant growth and mineral nutrient uptake (Khaled and Fawy 2011), and also some metabolic changes (El-Bassiouny et al. 2014). However, there is only limited research on the effect of humic acid on cell divison (Feretti et al. 2012) and the protective role of against effects of mutagenic and genotoxic of various environmental conditions and chemicals (Gichner et al. 1990; Ferrara et al. 2000). In particular, no data have been recorded about effects of humic acid on mitotic activity and chromosomal aberrations in salinity conditions.

In the study reported here, the influence of humic acid pretreatment on mitotic activity and chromosome behaviors in root meristems of barley seeds exposed to salinity stress were investigated. So, we have aimed to clarify to some extent to what extent humic acid can alleviate salt stress, whether it stimulates cells to enter the mitosis division or not and also whether it causes any changes in the structure and behavior of chromosomes or not.

## MATERIAL AND METHODS

In the present study, barley seeds (*Hordeum vulgare* L. cv. Bülbül 89) were used. The barley seeds were subjected to surface sterilization before used. For this, seeds were kept in 1% sodium hypochlorite for ten minutes, then washed with distilled water five times and dried on filter paper at room temperature.

### *Preparation of solutions and germination of seeds*

NaCl and humic acid used in the experiments were obtained from Merck and Sigma-Aldrich firm respectively. As test solution, 28 mg/L humic acid were used due to promote germination in the best way against the inhibitory effect of salt. Concentrations of NaCl were 0.25, 0.30 and 0.35 M (molar). These salt levels and the concentration of humic acid used were determined in the result of a preliminary study. Primarily, plump-looking, robust and approximately equal-sized 20-25 barley seeds were selected. Then, sterilized seeds were soaked in test tubes filled with 28 mg/L humic acid and distilled water (control) at constant volume (50 ml) for 24 h at room temperature. At the end of this pretreatment session, the seeds from every application were arranged in 10 cm Petri dishes covered with two sheets of filter paper moistened with 7 ml of distilled water and different salt concentrations. Petri dishes were transferred in an incubator to germinate at 20 ± 1°C in continuous dark for several days.

### *Cytogenetical analyses*

When the root tips were 0.5–1 cm long, they were cut off, pretreated with a saturated solution of paradi-

chlorobenzene for 4 h at 20°C, fixed with Carnoy's Fluid I (absolute ethanol: glacial acetic acid, 3:1, v/v) for 24 h, and stored in 70% alcohol at 4°C until used. Then, the root tips were hydrolyzed in 1 N HCl at 60°C for 18 min, stained with Feulgen for 1 h, and squashed in 45% acetic acid (Sharma and Gupta 1982). After 24 h, microscopic slides were made permanent by mounting in balsam. The mitotic phases and mitotic aberrations were photographed (100×) with a digital camera (Olympus C-5060) mounted on an Olympus CX41 microscope.

### *Data analyses and statistical evaluations*

To determine the effect of humic acid and salt on the mitotic index, at least 3000 cells (approx. 1000 per slide) were scored in control and in treated groups. Chromosomal aberrations were calculated for each concentration as the percentage of 300 dividing cells counted. Statistical analysis related to all parameters was performed by using SPSS program according to Duncan's multiple range test at the level of significance $p \leq 0.05$ (Duncan 1955).

## RESULTS

### *Effects of humic acid on mitotic index and chromosome aberrations in normal conditions*

Barley seeds pretreated with 28 mg/L humic acid were germinated at 20°C in distilled water and slides were prepared with the root tips obtained. The mitotic index values calculated as a result of the cell counting procedures performed in these slides were presented in Table 1. Humic acid pretreatment caused a 30% increase in the mitotic index of barley seeds germinated in non-stress conditions as compared to those of the control group. In other words, the mitotic index of seeds treated with humic acid was even higher than seeds germinated in distilled water.

As a result of cytological analyzes, chromosomal aberrations in meristem cells of barley seeds germinated at 20°C in distilled water (control) were statistically insignificant. That is, all mitotic phases were normal (Fig. 1). However, the frequency of chromosomal aberrations in seeds germinated in distilled water after humic acid pretreatment was remarkably higher than that in the control (Table 2). For example, while the rate of chromosomal aberrations in control seeds was 0.04%, it was 0.42% in seeds pretreatmented with humic acid.

A resulting of the application of humic acid alone, chromosomal aberrations such as fragment formation, lagging chromosome, anaphase and telophase bridges, fault polarization in telophase and anaphase were frequently observed (Fig. 2).

**Table 1.** Mitotic index of barley seeds germinated in distilled water and different NaCl concentrations after humic acid pretreatment.

| Mitotic index (%) | | |
|---|---|---|
| NaCl (M, molar) | Control | Humic acid (28 mg/L) |
| 0.0 (distilled water) | *0.19 ± 0.13$^{bcd}$ | 0.27 ± 0.04$^{e}$ |
| 0.25 | 0.19 ± 0.03$^{bcd}$ | 0.17 ± 0.03$^{abcd}$ |
| 0.30 | 0.18 ± 0.01$^{abc}$ | 0.18 ± 0.02$^{bcd}$ |
| 0.35 | 0.18 ± 0.03$^{abc}$ | 0.12 ± 0.02$^{ab}$ |

The pretreatment process of seeds was performed by soaking 24 h in constant volumes of distilled water (control) or humic acid. As test solution, 28 mg/L humic acid was used. Different salt concentrations (0.25, 0.30, 0.35 M NaCl) were exogenously applied to germination medium. Data are the means of three replications ± standard deviation.

* Shows values with insignificant difference ($p \leq 0.05$) for each column shown with same letters

### *Effects of humic acid on mitotic index and chromosome aberrations in salinity conditions*

Mitotic index scores obtained from this study made to determine the activity degree of humic acid on the

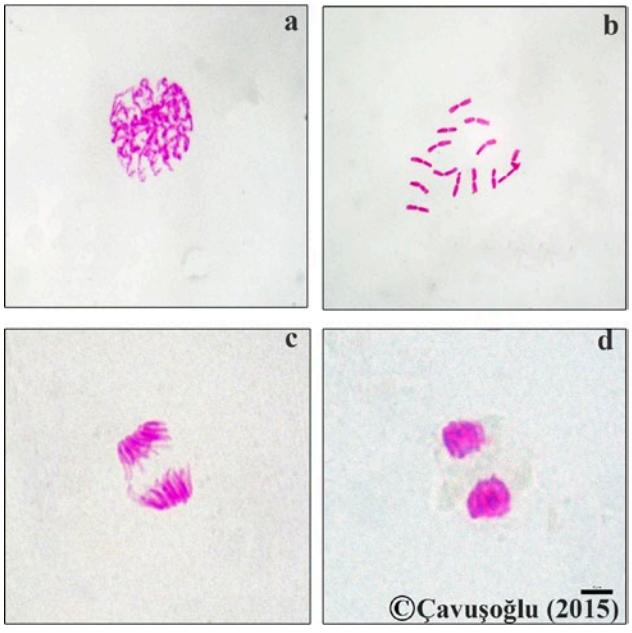


**Fig. 1.** Normal mitosis phases in root tips meristems of barley germinated in distilled water (control). (a) Prophase; (b) metaphase ($2n = 14$); (c) anaphase; (d) telophase. Scale bar = 10μm.

**Table 2.** Frequency of chromosomal aberrations of barley seeds germinated in distilled water and different NaCl concentrations after humic acid pretreatment

| Chromosomal aberrations (%) | | |
|---|---|---|
| NaCl (M, molar) | Control | Humic acid (28 mg/L) |
| 0.0 (distilled water) | $0.04 \pm 0.18^{a}$ | $0.42 \pm 0.05^{cd}$ |
| 0.25 | $0.25 \pm 0.27^{bc}$ | $0.22 \pm 0.01^{bc}$ |
| 0.30 | $0.35 \pm 0.07^{c}$ | $0.30 \pm 0.15^{bc}$ |
| 0.35 | $0.58 \pm 0.05^{d}$ | $0.04 \pm 0.04^{a}$ |

The pretreatment process of seeds was performed by soaking 24 h in constant volumes of distilled water (control) or humic acid. As test solution, 28 mg/L humic acid was used. Different salt concentrations (0.25, 0.30, 0.35 M NaCl) were exogenously applied to germination medium. Data are the means of three replications ± standard deviation.

* Shows values with insignificant difference ($p \leq 0.05$) for each column shown with same letters

mitotic index of barley seeds germinated under salt stress was summarized in Table 1.

The mitotic index value of barley seeds was statistically decreased at especially high concentrations as parallel to the increase of salt concentration as compared with control. It was found that the seeds applied alone humic acid shows a considerable increase on the mitotic index as compared with seeds germinated in distilled water. That is, the addition of 28 mg/L humic acid increased the mitotic index by 30%. However, humic acid pretreatment caused a significant decrease in the mitotic index with increasing salt concentrations. The mitotic index value (0.12) in root meristems of the seeds germinated at the highest salt concentration (0.35 M) after treated with humic acid reduced to a large extent (Table 1).

Considering all of the application groups, it was determined that humic acid pretreatment together with salinity decreased the mitotic index at 0.25 M and 0.35 M salinity, while it was at the same level with its own control at 0.30 M salinity (Table 1).

In addition to, chromosomal aberration scores were presented in Table 2. While there is a chromosomal aberration that can be ignored statistically in control seeds germinated in distilled water, screening mitotic divisions revealed the many numbers of chromosomal aberrations as parallel to increasing salt concentrations.

It was determined that chromosomal aberrations at the highest salt concentration (0.35 M) were higher approximately 60% according to those in distilled water. At the same time, alone humic acid pretreatment caused a 42% increase in chromosomal aberrations as compared to control. However, the frequency of chromosomal aberrations of seeds germinated at different salt concentrations after treatment with humic acid showed a decrease which can be considered statistically significant from those of the seeds germinated in distilled water after treatment with humic acid. Namely, humic acid has been quite successful in mitigating the detrimental effect of salt stress on chromosomal aberrations at all the salt concentrations studied here. Moreover, humic acid application at the highest salt concentration (0.35 M) significantly reduced the detrimental effect of salt stress and reached the percentage of the same abnormality (0.04) as the seeds germinated in distilled water (Table 2).

The most prominent chromosomal aberrations in seeds belonging to all application groups were the disorganizations in anaphase and telophase such as bridges,

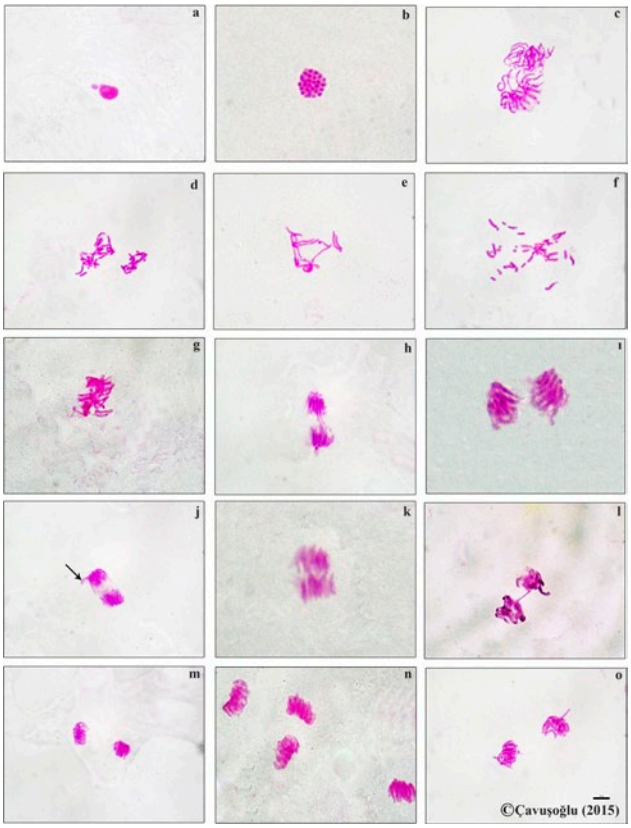


**Fig. 2.** Chromosomal aberrations in root meristems of barley seeds germinated in distilled water and different NaCl concentrations after treatment with 28 mg/L humic acid: a) micronucleus; b) granulation c) disorderly prophase; d) disrupted equatorial plate; e) uncoiling chromosomes f) fragments g) sticky chromosomes; h) bridges in anaphase and lagging chromosome; ı) fault polarization in anaphase; j) vagrant chromosome in anaphase (arrow); k) alignment anaphase; l) bridges in telophase; m) fault polarization in telophase n) distant poles in telophase; o) vagrant chromosome in telophase. Scale bar = 10 µm.

lagging chromosome, fault polarization, distant poles, vagrant chromosomes and alignment anaphase (Fig. 2h–o). In addition, other chromosomal aberrations observed were the presence of micronucleus, granulation, disorderly prophase, disrupted equatorial plate, uncoiling chromosomes, fragments, sticky chromosomes (Fig. 2a–g). The minimal common aberrations were micronucleus, granulation and disrupted equatorial plate.

## DISCUSSION

It is well known that humic substances stimulate germination in seeds of various species by increasing enzymatic activities in seed tissues during germination and decreasing mitotic activity under normal conditions (Feretti et al. 2012). However, no information has been found on effects of humic acid on mitotic activity and chromosomal aberrations especially under saline conditions. All the data on the effect of humic acid on these parameters in saline conditions are presented for the first time in this study.

In the present work, it was determined that humic acid pretreatment alone in non-stress conditions significantly increase the mitotic index of barley seeds as compared to distilled water (Table 1). Whereas, Feretti et al. (2012) have suggested in their study using various plant tests (*Allium cepa* test, *Tradescantia* and *Vicia faba* micronucleus test) that alone humic acid pretreatment decreases the mitotic index in studied two solutions. Because of the differences in the findings of these researchers may be due to the species of plant studied or the concentration of the humic acid used. However, our findings have been endorsed by the data expressed that the humic substances increase the amount of DNA synthesis in meristematic cells in plants (Gorova et al. 2005). In addition, we found that the application of alone humic acid increased the chromosome abnormality rate by 42% under normal conditions (Table 2). Our this finding is consistent with Ferretti et al. (2012)'s findings. For this reason, we can reach the result that alone humic acid pretreatment increases the mitotic index value in barley seeds germinated in distilled water but may have a genotoxic effect because of the negativity that it has shown on chromosome behavior. This reveals the fact that alone humic acid application can create mutations in various types over time.

As is known, plant growth and development are adversely affected by salinity. High salinity is an important factor negatively influencing plant growth and development even in most halophytes. At present, approximately 20% of cultivated lands in the World are affected by salinity (FAO 2015). Generally, it is suggested that salinity impairs seed germination, retards plant development and reduces productivity. In some cases, the plant dies before completing the life cycle. There have been numerous investigations conducted to explore the effects of salt on plant growth, but mechanisms of salt stress have not yet been explained precisely (Munns and Tester 2008; Zhu et al. 2016).

We determined that mitotic index in root meristems of barley seeds significantly decreased with increasing salt concentrations (Table 1). The inhibitory effects of salt stress on mitotic activity are known for a long time (Lutsenko et al. 2005). Salt induced-inhibition of cell division may relate to osmotic effect and ion uptake (Munns and Tester 2008 ), inhibition of DNA, RNA and protein synthesis (Anuradha and Rao 2001), distruption the activity of enzymes required for cells (Miller et al. 2010) and hinderance of mitosis division (Tabur and Demir 2010 a, b; Çavuşoğlu et al. 2016). It is worth mentioning again that the relation between salinity and mitotic activity was confirmed by the present work. In our study, it was also detected that there was a remarkable increase in all kinds of chromosomal aberrations at the root meristems of barley parallel to the rise of salt concentrations (Table 2). The detrimental effects of salt stress on chromosomal aberrations in plants have been studied for over the past decade. These recent studies have shown that the higher concentrations of NaCl has chromotoxic effects and increases the percentage of total aberrations (Tabur and Demir 2010 a, b). Furthermore, it was reported that these high salt concentrations delayed mitosis and caused various anaphase aberrations in barley (Tajbakhsh et al. 2006) and in onion (Çavuşoğlu et al. 2016).

There is no relevant literature data relating to effects of humic acid on either mitotic activity or/and chromosomal abnormalities in saline conditions. The present study is the first one revealing the cytogenetic responses to the salt stress of humic acid. However, there are a few studies about the effect of humic acid application against the genotoxic effects of various chemicals such as N-nitrous compounds, maleic hydrolase and some disinfectants (Gichner et al. 1990; Ferrara et al. 2000). These studies have argumented that humic acid exhibits an anticlastogenic or antimutagenic activity in different plants. Ferretti et al. (2012) determined that humic acid alone reduces the mitotic index and has genotoxic effects. However, there could not be made explanation for the effect of humic acid on these disinfectants since these investigators have not determined any evidence of the genotoxic effect of disinfectants alone.

In the present work, we analyzed that humic acid pretreatment in salt stress conditions was not sufficiently successful on the mitotic index of barley seeds, but exhibit a performance very successfully statistically on chromosome behaviors. Although humic acid application alone was caused a significant increase (42%) of chromosomal aberrations in root meristems of barley seeds germinated in distilled water, it has shown remarkable success in alleviating a large majority of these abnormalities caused by salinity in salt stress conditions. In parallel to salt concentrations rise, humic acid was reduced the detrimental effects of salinity and caused complete elimination of chromosome abnormalities at the highest salt concentration studied. That is, the application of 28 mg/L humic acid at 0.35 M salinity achieved an excellent success on the negative effect of salt stress, reaching to the same percentage (0.04) as the seeds germinated in distilled water. The important point here is that humic acid should be used at the appropriate concentrations, considering the negative effects on chromosome behaviors when it was used in non-stressed conditions. Humic acid application alone under non-stressed conditions may have functioned as a stimulator by triggering the synthesis of proteins necessary for normal cell division and by accelerating the mitotic cycle. The acceleration of the mitotic cycle may have led to a number of disruptions during the cell division and a significant increase of chromosomal abnormalities. As is known, external stimulator growth regulator applications are useless under normal conditions where there is no stress (Tabur and Demir 2010 a). Therefore, it is not surprising that the application of humic acid under stress conditions slows down mitotic activity in parallel with salt concentrations rise and eventually alleviating the negative effects of stress by regulating chromosome behaviors, and even removing (at 0.35 M salinity).

In addition, we can explain as follows the reason why various chromosomal aberrations observed during the microscopic examination of the root meristem cells of seeds belonging to all the applications: In general, accurate chromosome segregation in mitosis requires that sister kinetochores attach to microtubules emanating from opposite spindle poles. Because kinetochore attachment is a stochastic process, it is error prone and can result in chromosome malorientation (Rieder and Salmon 1998). Mitotic irregularities such as disorderly prophase, fault or distant polarization, alignment anaphase, vagrant chromosome and bridges may be mainly the result of the above mentioned reasons or spindle dysfunction. Generally, such abnormalities constitute a significant portion of chromosomal aberrations. The formations of micronuclei are likely the consequence of vagrant chromosomes and fragments. Also, some researchers reported that MNs, indicators of chromosomal genotoxicity and instability, are formed from one or more chromosomes (Bonciu et al. 2018). It is known that fragments are considered as structural changes in chromosomes and that chromosomes are affected by physical or chemical agents outside normal conditions (El-Ghamery et al. 2000). It has been reported that certain regions of chromosomes are broken by reacting with chemical substances and these regions are particularly heterochromatic regions (Rieger et al. 1973). Abnormal chromatin condensation expressed as chromatin granulation is concerned with the inhibition of enzymes and histone proteins. While laggard chromosomes could be the result of the failure of spindle apparatus to organize in normal way, sticky chromosomes may result from the improper folding of the chromatin fibres (Klášterská et al. 1976). According to some researchers, chromosomal stickiness is a marker of the toxic effect on chromatin (Fiskesjö and Levan 1993). The prophase and metaphase cells with uncoiled chromosomes may be due to disorderly chromosome contractions. The disrupted equatorial plate may result from unequal distribution of chromosomes and spindle dysfunction. Bonciu et al. (2018) asserted that nucleoplasmic bridges originate from dicentric chromosomes or occur as a result of a faulty longitudinal breakdown of sister chromatids during anaphase. It has also been reported that anaphase and telophase bridges may have been the result of inversions (Tabur and Demir 2010 b). It is thought that humic acid alone or salt concentrations used in our study may have been caused to all these abnormalities mentioned above by triggering the stimulation/ inhibition of enzymes and proteins necessary for the normal cell division, by disturbing the spindle mechanism and by accelerating mitotic cycle.

## CONCLUSION

The mechanisms by which salinity inhibits growth are complex and controversial. Moreover, these mechanisms may vary substantially according to factors, such as plant species, the developmental stage of the plant, the strength of the stress and duration of the treatment. Unfortunately, a universal mechanism about this contradiction has not been established yet. Although the causes of salinity have been characterized, our understanding of the mechanisms by which salinity prevents plant growth is still rather poor. This work may provide new conceptual tools for designing hypotheses of salt tolerance in plants. As a result, we have attempted

to serve the filling of a gap in the literature by comparing their interactions between the mitotic activity and chromosome behaviors of humic acid under normal and salt stress conditions using barley seeds, an important model plant for molecular studies. In future studies, the investigation of the effects of humic acid on fundamental metabolic events such as nucleic acid metabolism, protein synthesis, and enzyme synthesis, which may be directly or indirectly effective on mitotic activity and chromosomal abnormalities will contribute to the clarification of mentioned mechanism.

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