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Research Article

Cytogenetic and Physiologic Efficacies of Grape Seed Extract in *Allium cepa* L. Seeds Exposed to Salinity

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Abstract

In this study, effects of grape seed extract (GSE) on the seedling growth (fresh weight, radicle length, radicle number), seed germination, mitotic activity, chromosomal aberrations (CAs) and micronucleus frequency (MN) in *Allium cepa* L. seeds germinated in both saline and normal conditions investigated. In only GSE medium, althought the radicle number and radicle length of seeds partially reduced compared to the control seeds germinated in distilled water medium, the fresh weight partly ascended and germination percentage indicate statistically the same value as the control. Besides, whereas the mitotic index in root-tip meristems of *A. cepa* seeds germinated in alone GSE medium showed increase compared to the control seeds, CA and MN showed statistically the same values 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 reduced significantly the mitotic index in root-tip meristems of the seeds and escalated the number of CA and MN, which is the simplest indicator and the most effective of cytological damage. Contrariwise, inhibitive effects of salt on the mitotic activity, seedling growth, seed germination and CA significantly decreased with application of GSE but, GSE was inefficient in reducing of salt damage on MN.

Keywords: Grape seed extract, mitotic index, seed germination, chromosomal aberrations, salt stress

Tuzluluğa Maruz *Allium cepa* L. Tohumlarında Üzüm Çekirdeği Ekstresinin Sitogenetik ve Fizyolojik Etkileri

Öz

Bu çalışmada, hem tuzlu hemde normal koşullar altında çimlendirilen *Allium cepa* L. tohumlarında fide büyümesi (taze ağırlık, radikula uzunluğu ve radikula sayısı), tohum çimlenmesi, mitotik aktivite, kromozomal anormallikler ve mikronükleus sıklığı üzerine üzüm çekirdeği ekstresinin (ÜÇE) etkileri araştırılmıştır. Tek başına ÜÇE ortamındaki tohumların radikula uzunluğu ve radikula sayısı saf su ortamında çimlendirilen kontrol tohumlarına kıyasla kısmen azalmasına rağmen, taze ağırlığı kısmen artmış ve çimlenme yüzdesi ise istatistiksel olarak kontrolle aynı değeri göstermiştir. Ayrıca, tek başına ÜÇE ortamında çimlendirilen *A. cepa* tohumlarının kök ucu meristemlerindeki mitotik indeks kontrol tohumlarıyla karşılaştırıldığında artış gösterirken, kromozomal anormallikler ile mikronükleus sıklığı kontrole kıyasla istatistiksel olarak aynı değerleri göstermiştir. Diğer yandan, tuz stresi *A. cepa*'nın tohum çimlenmesi ve fide büyümesini önemli ölçüde engellemiştir. Dahası, tuz tohumların kök ucu meristemlerindeki mitotik indeksi önemli ölçüde düşürmüş ve sitolojik hasarın en basit ve en etkili göstergesi olan mikronükleus sıklığı ve kromozomal anormalliklerin sayısını arttırmıştır. Buna karşılık, mitotik aktivite, fide büyümesi, tohum çimlenmesi ve kromozomal anormallikler üzerine tuzun inhibe edici etkileri ÜÇE uygulamasıyla önemli ölçüde azalmış, fakat mikronükleus sıklığı üzerinde ÜÇE tuz hasarının azaltılmasında yetersiz kalmıştır.

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Anahtar Kelimeler: Üzüm çekirdeği ekstresi, mitotik indeks, tohum çimlenmesi, kromozomal anormallikler, tuz stresi

1. Introduction

NaCl stress is an important abiotic stress problem in irrigation areas, semi-arid and arid regions. Roughly 7% of the land area in the world, 20% of the world's cultivated areas and about half of the irrigated areas are effected by the high NaCl content. NaCl stress affects plant physiology at both whole plant and cellular levels through ionic and osmotic stress. There are two salt tolerance mechanisms; osmotic stress is associated with NaCl stress. NaCl stress contains excess sodium ion but the osmotic stress is mainly due to the lack of water, which is not directly role of sodium ions (Munns, 2002; Murphy and Durako, 2003).

In recent years, grape seed extracts have gained popularity owing to their powerful protective properties have against free radicals, oxidative stress and their antioxidant content (that protects the body from premature aging, disease and decay), predominantly proanthocyanidins and flavanols. The proanthocyanidins are comprised of a mixture of polymers, monomers and oligomers. Oligomeric proanthocyanidins are 20 times more powerful antioxidants than β-carotene and vitamins E, C. Grape seed extracts improve brain, cardiovascular, eye and skin health.

Grape seed extract (GSE) has anti-allergic, anti-bacterial, anti-microbial, anti-diabetic, anti-carcinogenic, anti-fungal, anti-inflammatory, anti-thrombotic, anti-arthritic, neuroprotective, anti-obesity, hepatoprotective, anti-cataractogenic and anti-viral effects. GSE has a preventive action on diseases such as oral/periodontal diseases, atherosclerosis and alzheimer. It also has positive activities such as chemopreventive potential, protects the circulatory system, vasodilatory properties, partially ameliorate damage induced by the chemotherapy drug, thrombosis prevention, LDL peroxidation, cholesterol reduction in serum, pharmacological properties, regulation of autonomic nerve, dilation of blood vessel, medicinal and therapeutic activities. It could be used as natural additivies in animal feeds, gastric digestive problems and dietary supplement in humans, extend the shelf life in food products (Kaur et al., 2009; Maier et al., 2009; Velmurugan et al., 2010).

Allium genus among test plants, especially *Allium cepa* L. species, possess in few number (2n=16) chromosome, very large chromosomes and rather homogenous meristematic cells (Havey, 2002). It is accepted to be one of the best biological models to studying chromosome disturbances or damage in cell cycle due to their suitable chromosomal features and a good correlation with mammalian test systems. In this study therefore, *A. cepa* was used as a test material.

No previous study has reported GSE effects on the seedling growth, seed germination, micronucleus frequency, chromosomal abnormalities and mitotic activity in salted and normal conditions yet. For these reasons, this work is designed to investigate on the

effects of GSE in decreasing of the harmful efficacies of salinity stress on the seedling growth, seed germination, micronucleus frequency, chromosomal aberrations and mitotic activity of *A. cepa* L.

2. Material and Methods

2.1. Seed, grape seed extract and salt concentrations

In the present work, seeds of *Allium cepa* L. and 0.175 M NaCl (salt) concentration were used. The concentration of GSE used in this experiment was 10 mg L^{-1}. Concentrations of GSE and salt were determined conducted in a preliminary investigation of this study.

2.2. Seed germination

The seed germination experiments were performed in set to 20°C incubator in the dark. Healthy equal-sizes of onion have used for the assay. *A. cepa* L. seeds have sterilized with the aid of NaOCl 2.5% for 10 min. and washed in distilled water for 24 hrs. 20 selected from each application group were placed in plastic containers. These bulbs have split in four groups:

 Group I (control) during 7 sequential days treated by distilled water

 Group II during 7 sequential days treated by alone 0.175 M NaCl

 Group III during 7 sequential days treated by a 10 mg L^{-1} dose of GSE

 Group IV during 7 sequential days treated by a 10 mg L^{-1} dose of GSE + 0.175 M NaCl

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 was taken, the numbers of radicle were recorded, the lengths of radicle were measured in mm, fresh weights were also determined in g / seed. All of the experiments repeated 3 times.

2.3. Statistical and cytological analysis

For cytogenetic analysis, 1-1.5 cm segment of germinated *A. cepa's* root tips was excised after a few days. *A. cepa* rootlets were pretreated using saturated para-dichlorobenzene for four hours, were fixed in 3-ethanol/1-acetic acid at room temperature for 24 hours and were stock up in 70% ethanol at 4 ^{0}C until making microscopic slides. These were hydrolysed in 5 N HCl for 45 minutes, dyed with Feulgen for 1-1.5 hrs and lysed with a drop of 45 % CH3COOH. Squashes were prepared as suggested by Sharma and Gupta (1982) to determine the presence MI, CA and MN. Microscopic preparations were made permanent by means of balsame at the end of 24 hrs, photographed mitotic phases, micronuclei and mitotic aberrations at 500 magnifications. The cell division densities of these preparations were analyzed by calculating the mitotic index (30 000 cells per sample i.e. about 10 000 per preparation). Chromosomal abnormalities calculated as the percentage of 2000 dividing cells counted for each concentration. The latter determined as a percentage between the dividing cells number (N') and the total cells number analyzed (N) according to formula: MI (%) = (N'/N) x 100 (Aslantürk and Çelik, 2005). The statistical calculations were done Duncan's multiple range test at level of significance $P \leq 0.05$ using SPSS program (Duncan, 1955).

2.4. Micronucleus (MN) assay

For micronucleus analyses, 1000 cells per slide were scored. MN was examined with the help of a binocular light microscope. For the

scoring of micronucleated cells, Fenech et al. (2003) used the protocol that they followed. These; (ı) micronucleus diameter should a tenth of main nucleus (ıı) micronucleus should separated from or marginally overlapped from the main nucleus, provided that the nucleus boundary is clearly defined (ııı) staining of micronucleus should similar to that of the main nucleus.

3. Results

3.1. Efficacies of grape seed extract on the seed growth and seedling germination

The results from Table 1 clearly demonstrate that while the germination percentage of group III displayed statistically the same value as group I (control), their radicle number and radicle length partly decreased, but the fresh weight partly elevated.

On all growth parameters examined, salt showed inhibitory effects. For instance, group I seeds germinated in distilled water medium after 7 days showed 100% germination, whereas this value was 23 % in group II seeds germinated at 0.175 M saltness. That is, salt prevented germination of *A. cepa* seeds by 77 %. Inhibitory impact of salinity stress on the seed germination was markedly mitigated by GSE application. Group IV seeds treated with GSE at said salt level showed 65 % germination (Figure 1).

Table 1. Efficacy of grape seed extract on some growth parameters of *Allium cepa* L.

	Growth parameters			
Groups	**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}	56.5 ± 1.1^{c}	44.4 ± 1.2^{c}	17.6 ± 1.2^{c}
Group IV	65 ± 5.0^{b}	14.9 ± 0.4^{b}	17.4 ± 0.7^{b}	15.8 ± 0.6^{b}

* The difference between values with the same letter in each column is not significant at the level 0.05 (±SD). Group I (control) processed by distilled water, Group II processed by alone 0.175 M NaCl, Group III processed by a 10 mg L^{-1} dose of GSE, Group IV processed by a 10 mg L^{-1} dose of GSE + 0.175 M NaCl

In addition, this extract continued its success in attenuating the negative effect of NaCl inhibition on the seedling growth parameters (radicle length, fresh weight and radicle number). The radicle length, radicle number and fresh weight of group II seeds grown in 0.175 M salted were 10.3, 12.7 and 7.0 g, while these values became 14.9,

17.4 and 15.8 g in group four, respectively (Table 1).

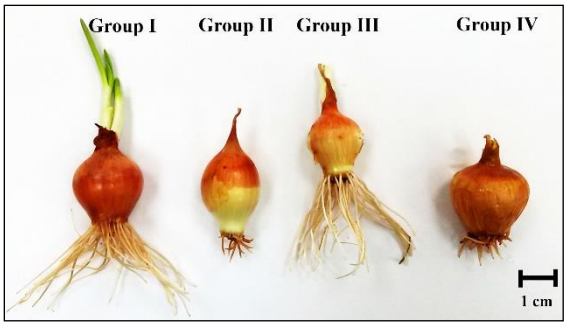

Fig. 1. Germination situations of *Allium cepa* root tip cells at the end of the seventh day. Control (Group I) seed treated by distilled water, Group II seeds treated by alone 0.175 M NaCl, Group III seeds treated by a 10 mg L^{-1} dose of grape seed extract, Group IV seeds treated by a 10 mg L^{-1} dose of grape seed extract + 0.175 M NaCl, Scale bar = 1 cm

3.2. Efficacies of grape seed extract on the micronucleus formation, chromosomal aberrations and mitotic activity

All cytogenetic parameters summarized in Table 2. Although the mitotic index of group III seeds germinated in only GSE application displayed an increase of 44% compared to the group I (distilled water) samples, the frequency of micronucleus and chromosome aberration statistically remained the same. Exposure to 0.175 M saltness resulted in significant inhibition of the mitotic index. In other words, MI of dividing root cells of the germinated seeds in this concentration of salt medium decreased by 89% compared to the group I seeds, the CAs and MN significantly increased. Simultaneous GSE+NaCl treatment (group IV) may be successful in improving unfavorable impacts of saltness on the chromosomal aberrations and mitotic activity. This performance was not successful in decreasing of harmful effects of salt on the frequency of micronucleus. All mentioned values here are statistically rather significant.

Table 2. Efficacy of grape seed extract on some cytogenetic parameters in *A. cepa* L. root tip meristems

Groups	Mitotic index (%)	Micronucleus frequency (%)	Chromosome aberration (%)
Group I	*11.6 ± 1.0^{c}	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^{c}
Group III	16.8 ± 0.7^{d}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Group IV	7.4 ± 0.3^{b}	13.6 ± 0.5^{b}	6.7 ± 0.1^{b}

* The difference between values with the same letter in each column is not significant at the level 0.05 (±SD). Group I (control) processed by distilled water, Group II processed by alone 0.175 M NaCl, Group III processed by a 10 mg L^{-1} dose of GSE and Group IV processed by a 10 mg L^{-1} dose of GSE + 0.175 M NaCl

The normal and abnormal mitotic phases showed in course of microscopic examination in *A. cepa* root tip meristematic cells show in figure 2 and figure 3. The most frequent abnormalities induced by salt were micronucleus and binucleolar. The order of chromosomal aberrations was spindle disturbance > increase in the number of nucleolus in the nucleus > polar deviation > abnormal anaphase > notched nucleus > chromosome loss > vagrant chromosome > bud > go into division of metaphase plate > ring chromosome > giant cell > irregular nucleus > bridge binucleate cell.

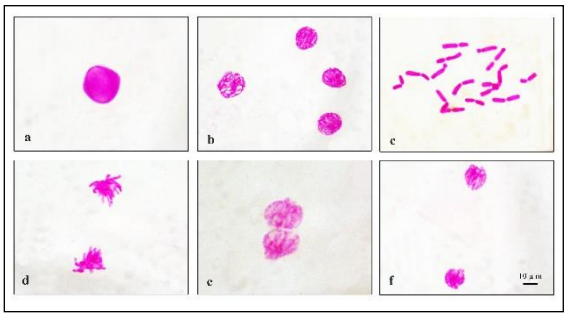

Fig. 2. The normal mitosis phases in *A. cepa* L. root tip meristems cells, scale bar = 10 µm
a) interphase b) prophases c) 2n= 16 chromosome metaphase d) anaphase e) early telophase
f) late telophase

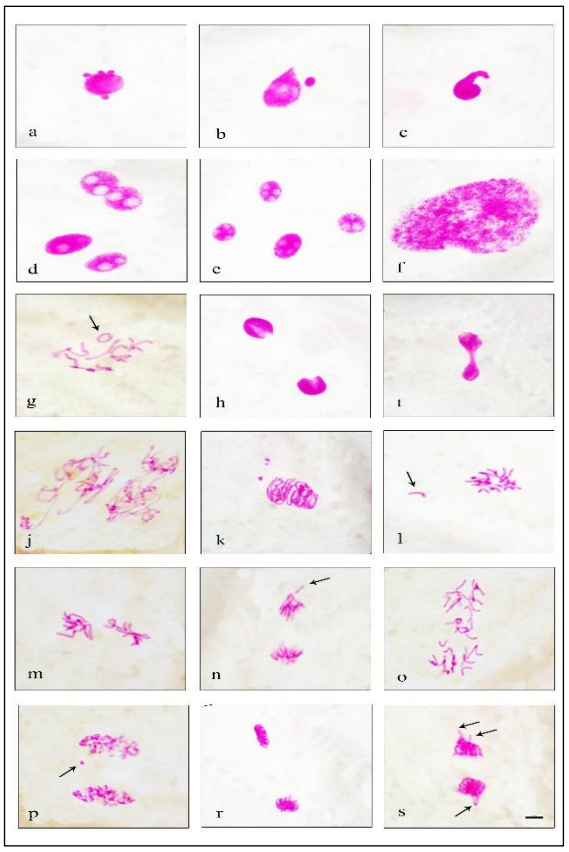

Fig. 3. Micrographs of chromosomal aberrants; a- cell bearing four nuclear buds, b- nuclear peak with micronucleus, c- irregular nucleus, d- binucleolar, e- increase in the number of nucleolus in the nucleus, f- giant cell, g- ring chromosome =arrow, h- notched nucleus, ı- bridge binucleate cell, j- spindle disturbance, k- micronuclei in prophase, l- metaphase with chromosome loss =arrow, m- go into division of metaphase plate, n- loss at anaphase =arrow, o- abnormal anaphase, p- micronucleus in telophase =arrow, r- polar deviation in telophase, s- vagrant chromosomes =arrows, Scale bar = 10 µm

4. Discussion

4.1. Cytogenetic and physiologic effects of grape seed extract 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 favorable or

unfavorable effect on seed germination and seedling growth (Çavuşoğlu et al., 2017; 2018). Unfourtunately, there is no study on the impacts of GSE on the seedling growth and seed germination. Therefore, in the study, efficacy of GSE application on the cytogenetical and physiological parameters under normal conditions requested to be tested. The study's results revealed that the germination percentage of the seeds germinated in only GSE application showed statistically the same value as the group I seeds in distilled water medium, whereas their radicle length and radicle number slightly decreased, fresh weight partly ascended as per the control (Table 1).

The efficacies of GSE application on the mitotic activity, CAs and MN under normal conditions are still unknown. But, there are a number of studies on mice that indicate that GSE prevented DNA oxidative damage through reducing total number of structural chromosomal aberrations and aberrant cells (Takahashi et al., 1999; Solorzano et al., 2001; Zern et al., 2005). Therefore, the study have firstly investigated whether GSE is effecting these parameters in normal conditions or not. Findings of this study show that the MI in *A. cepa* seeds subjected to GSE treatment under normal conditions increased by 44% as per the group I seeds in distilled water medium. Shortly, 10 mg L^{-1} GSE administration may function as a stimulator triggering the synthesis of proteins necessary for accelerate the mitotic cycle and normal cell division. MN and CAs at this dose of GSE administration showed statistically the same values compared with the control group (Table 2).

4.2. Physiologic and cytogenetic effects of grape seed extract under saline conditions

Saltness stress has a negative influences agricultural productivity throughout the world effecting production whether it is for economic gain or subsistence. The fact that the soil saltness is a factor that restricts the production of food because it limits yield of crop and the use of previously uncultivated areas. In addition, it is estimated that in the future salinity will increase its importance as a breeding objective (Flowers and Yeo, 1995). The negative impact of salt stress is possible to see at all plant levels in almost all growth stages, including seedling, germination, maturity and herbal stages. Although salinity causes ionic and osmotic stress, it causes ionic imbalances that may induce potassium deficiency and may impair the selectivity of root membranes (Muranaka et al., 2002a, b; Ranjbarfordoei et al., 2002).

As expected, cytogenetic parameters of *A.* seeds inhibited under saline conditions. Soil saltness designates the presence of high concentrations of soluble salts in soil moisture of the root zone in agriculture. Owing to their high osmotic pressures, the concentrations of these soluble salts effect plant growth restricting water uptake of the roots (Nawaz et al., 2010; Flowers and Colmer, 2015). Results of these statements are consistent with results of the present study in terms of indicating the decline in the water content and fresh weight of the seedlings in salted conditions. Inhibitory impact of NaCl on the radicle length and radicle number might stem from decline nucleic acid, cell division and protein synthesis (Mccue and Hanson, 1990; Roy et al., 2014). Only, by this extract application, the inhibitory effect of saltinity stress on all

physiological parameters was significantly eliminated (Table 1). Unfortunately, no study to date has investigated the effects of GSE on seedling growth and the seed germination in saline conditions.

That GSE alleviates salt stress on the seed germination and seedling growth can be understood from the decrease in the salt's osmotic effects. For example, at 0.175 M NaCl medium, GSE application increased the fresh weights of the seedlings compared to the control indicates this probability (Table 1). Moreover, it reduced the preventive effect of salt on the seed germination and seedling growth by stimulating mitotic activity of the embryo (Table 2).

Mitotic index measures the proportion of the cells in the mitotic phase of the cell cycle and its inhibition can be interpreted as cellular death (Rojas et al., 1993; Karaismailoglu, 2014). Cytotoxicity levels can be determined by a decrease or an increase in mitotic index. The mitotic index can be used to determine root growth rate and as a reflection of cell proliferation (Fernandes et al., 2007; Leme and Marin-Morales 2009; Urgut et al., 2016). More interestingly, this study results showed that the salt caused a reduction of mitotic index and this decrease was achieved by decreasing the number of cells entering mitotic division. Decrease in the number of divided cells suggests that the salt may have mitodepressive impacts on *A. cepa* L. cell division. Saltness might reduce the level of ribosomal biogenesis and rapidity of cell cycle.

Saltness is cytotoxic on meristematic cells of the tested plant. The results of this study clearly show that salinity adversely affects chromosome behaviors and the mitotic activity of *A. cepa* root cells. Salinity decreased MI by 89% as per the control group and showed an excessive increase in the number of CA and MN. 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, GSE+NaCl might be effective in minimize harmful effect of salt on MI and CAs. So, CAs with simultaneous GSE+NaCl application decreased by 60%. But, this application did not cause a change in the MN formation. This result shows GSE repair role against salt injuries during *A. cepa*'s mitosis.

The chromosome abnormalities are classified according to the action mechanism clastogenic and aneugenic effects. While aneugenic effect involves inactivation of a cell structure like mitotic spindles leading to chromosomal losses or malformation, clatogenic effect is characterized by induction of bridge, chromosomal break along cell division. Nuclear abnormalities are derived from various types of chromosome aberrant such as micronucleus, lobed nuclei, mini cells and nuclear buds. Chromosome bridges (Fig. 3ı) result from chromatid and/or chromosome breaks, indicating the mutagenic event in cell. Polar deviation (Fig. 3r) condition where the poles of the mitotic spindle has shifted to the corners of the cell which can interfere with the normal cell division leading to aneugenicity (Leme and Marin-Morales, 2009). Appearance of micronucleus (Fig. 3b, k, p) and budding nuclei (Fig. 3a) might be result in loss of genetic materials. Chromosomal losses (Fig. 3l, n) as well as the excess material, promoted by the DNA replication, could induce MN, which could be selected from

the cell in mini cells form (Fernandes et al., 2007). Giant cells (Fig. 3f) occur owing to incomplete cytoplasmic division but grow up with nuclear division and DNA replication before they die (Prajitha and Thoppil, 2014). Ring chromosome (Fig. 3g) is result of chromosome losses in the telomere domain (Khanna and Sharma, 2013). Vagrant chromosomes (Fig. 3s) means a deviation of mitotic spindle irregularity, an aberration which may result in delayed prophase and / or metaphase (Tkalec et al., 2009). Abnormal anaphases (Fig. 3o) might be due to spindle apparatus disturbance which allows that chromosomes to spread irregularly over cell (Amer and Alı, 1974).

5. Conclusions

There is no literature data on impacts of GSE application on the physiological and cytogenetic parameters examined in saline conditions. Therefore, the results of this study have been particularly reported for the first time in saltness conditions. As a conclusion, this study shows that GSE can significantly increase activations such as the seedling growth and seed germination under saline or alone conditions. But, mechanisms in which saltness inhibits growth are controversial and complex. They can also vary by cultivar and species. An universal mechanism has not been established yet. In spite of characterized saltine causes, the understanding of the mechanisms by which salt prevents plant growth remains weak. It is do to this reason further work is needed to learn more knowledge about the effect of GSE on the cell cycle, cell division and molecular metabolism of germination. This study suggested that GSE application at convenient doses may help to alleviate all these harmful affects on plant development in stress conditions. In summary, this study to design salt tolerance hypotheses in plants can serve to provide new conceptual tools.

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