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Potential Impact of Salt Stress on Male Reproductive Development of *Glycine Max* (L.) Merr. (Soybean)

Semra Kilic*, Arzu Seker

Department of Biology, Faculty of ArtsandScience, Süleyman Demirel University, 32260, Turkey
*Correspondingauthor: E-mail: semrakilic@sdu.edu.tr

Abstract *— Product yield and the continuity of the quality of products of plants are in parallel with their ability to tolerate or adapt to environmental factors. For this reason, it is extremely important to determine the changes in the plants under various stress conditions. Male reproductive structures are directly related to product yield and quality, and they're very sensitive to abiotic stress. Stress causes irreversible damage to plants depending on its amount and duration. The aim of this study is to determine the sensitivity of male reproductive structures of soybean seedlings and the critical salt concentration at which fertile pollen grains could be obtained in our soils whose salinity is increasing day by day. The selected soybean seedlings were exposed to increasing salt concentrations (50, 100, 150, 200, 250mM) for 6 months and they were compared with a control group in terms of flowering, pollen morphology (pollen size, exine and intine thickness, aperture structures), pollen viability, pollen germination, and pollen tube length. It was determined that, by affecting the growth process of soybean at varying grades, salt stress causes deformations in the plant's reproductive structures and decreases it's tolerance to salt stress.*

Keywords— crop, flowering, pollen germination, pollen tube growth, pollen viability.

I. INTRODUCTION

Soil salinity inhibits the growth and development of the plants' by reducing their ability to absorb water and micronutrients from the soil and it has been a threat to agriculture for nearly 3000 years [1]. Soil salinity paves the way for physiological drought by causing hyper-ionic and hyper-osmotic stress and irreversibly damages the plants [2]. While the world population is growing faster than ever abiotic stresses such as salinity, drought, heat are shrinking arable areas and significantly reducing product yield and quality [3], therefore mankind might face famine in the future.Therefore, environmental stresses negatively affect plants' vegetative and reproductive stages by hindering their morphological, anatomical and physiological parameters, and threaten the continuity of these cheap and highly cultivable sources [4]. Especially, male reproductive stage is more sensitive to stress factors such as heat, drought, salinity and low light intensity than vegetative stage[5], andthey cause anomalies in reproductive structures of plants and negatively affectsphsyiologicalproccesses of these structures such as flowering, pollination and pollen viability [6]. Reproductive stage is regulated by abiotic factors and it plays an important role in plants' ability to survive. These results indicate that reproductive stage can significantly affect product yield and quality [7].

Legumenssimbiotically balance nitrogen (N) in agricultural ecosystem. They have a wide range of use in industry and medicine, and they are best known as cheap protein source. Among the legumens, soybean (41% protein in dry matter) have the highest protein content [8]. Soybeans sensivity to salinity varies between its developmental stages. Germination rate was more sensitive than other parameters to salinity for soybeans grown in increasing salt concentrations for 45 days [9]. It's product yield decreases under salt stress and this is an indication that reproductive stage of this plant is affected more than other stages [10]. Growth, development and product yield of soybean seedlings grown under salt stress were thoroughly studied [11]. Furthermore, it has been found that salt stress delays anthesis in soybean seedlings with various genotypes and this finding is in parallel with product yield [12]. However, these studies attribute the relationship between product yield and salt stress to growth and development parameters of the seedlings. Whereas, in recent years, it has been found that male development is a lot more sensitive to stress than female development [13], andIt is noteworthy that product losses resulting from the stress effect are directly related to male fertility[14]. Because, the ecological conditions of our world is worsening day by day, and increasing the yield and quality of the foods that have a very important place in the feeding of living beings depend on the successful reproduction and development of the plants which produce them. Therefore, we tried to explain the relationship between salt stress and yield by observing the

changes in male reproductive structures and behaviour. We hypothesize that the negative impact of salt stress on male reproductive structures and behaviour directly affects the product yield. The direct relation between male reproduction structure and product yield [15] has critical importance for managing the future of agricultural production. Therefore, by investigating male reproductive structures of soybean seedlings grown under salt stress, we aim to determine the critical salt concentration at which fertile pollen grains can be obtained, and whether the pollens are suitable for fertilization after pollination.

II. MATERIAL AND METHODS

Experimental design

This study was conducted to investigate the impact of salt stress on the reproductive biology of soybean during flowering stage. *Glycine max* (L.) Merr. cv. Nova (soybean) with simillar sizes were surface sterilized with 0.1% (v/v) sodium hypochlorite and than germinated in moistened vermicutile for a week. 6 different pots, including one for control group, with a 20 l capacity (top and bottom diameters were 20 and 10 cm, respectively) filled with fine sand were prepared. 20 germinated seeds were planted in each pot at 4cm depth. After emergence, seedlings were exposed to salt stress with increasing concentrations (50, 100, 150, 200, 250 mM salt), for one time, with 100 ml. Seedlings were grown in a greenhouse at controlled temperature, humidity, light intensity, and a photoperiod of 16/8h per 24 h, for one year. Plants were irrigated daily with Hoagland's nutrient solution. Experiments were setup in completely randomized design with three replicates.

Measurements:We following exposure to salt stress (50, 100, 150, 200 mM) andcontrol (0) in growthchambers. 5-7 unbloomed flowers were randomly harvested from each pot after treatment with different salt concentrations. Collected flowers were air-dried for 1h in the vacuum furnace (25 ^{0}C) and than fixed in FAA (formaldehyde-acetic acid –alcohol-H_2O, 10-5-50-35, by vol.) fixative (about 5 min) for SEM. Mature pollen grains mounted on aluminium stubs were examined with a LEO Stereoscan 360 SEM to calculate their polar axis (P), equatorial axis (E), aperture diameters, and the P/E ratios. Pollens were prepared according to Wodehouse (1935) [16] method to determine the pollen wall thickness. The slides were observed using Olympus light microscope with X 100 objective using oil immersion.

To evaluate pollen viability, buds were randomly collected from control and treatment groups between 08:00 and 09:00. Anthers of the buds were isolated, placed on a glass slide in petri dishes, and crushed into a fine powder. Pollen grains of each group were stained with potassium iodide (I/KI) solution [17] for 1h. The numbers of fertile and sterile pollen grains were determined using a light microscope (10 X 100). Pollen grains stained in a dark color (brown) were identified as fertile pollens (viable and living pollen) and yellow or light red stained pollen grains were identified as sterile pollens.

In vitro pollen germination and pollen tube growth were determined using a pollen germination medium consisting of 15% sucrose ($C_{12}H_{22}O_{11}$), 60 mgl boric acid (H_3BO_3), and 1% agar dissolved in 100 ml of deionized water [18]. Fresh pollen grains from each pot were randomly collected from anthers of buds in the morning between 08:00 and 09:00 h. Pollen grains were dusted onto 10 ml of the germination medium and the mixtures were poured on microscope slides. Slides were placed on moistened filter paper in petri dishes. Petri dishes were covered with parafilm to maintain high humidity, and were incubated at 22 ±2 ^{0}C for 3 hours. After incubation, pollen grains were examined using a light microscope with a magnification of 10 x 100 to determine the rate of pollen germination. A pollen was considered to be germinated when the pollen tube length was either equal to or greater than the diameter of the pollen [19], and pollen germination percentage was calculated according to Luza et al.,(1987) [20].Pollen tube lengths of 50 randomly selected pollen grains from each group were measured using an ocular micrometer on a light microscope.

Cumulative stress response index (CSRI) [21] was used to reveal the reproductive responses of soybeans grown under increasing salt stress. According to this method, examined parameters of each group were calculated as the sum of the relative individual component.

Statistical analysis

Alldata of pollen parameters were statistically performed to test the significance of examined parameters by Duncan's multiple range test in analysis of variance (ANOVA) using SPSS 14.0. Differences among the mean values of the experimental data were compared with Least Significant Differences (LSD) at $P \leq 0.05$and $P \leq 0.01$. Graphs for all experimental data were constructed to determine whether the differences of the mean values between control and experiments. Data in the figures indicate mean values±standard errors (SD) based on three replicates for each application. Data represent means and the vertical bars represent the standard deviation.

III. RESULTS AND DISCUSSION

Reproductive stage in which plants' male and female structures develop and differentiate begins with the transformation of the vegetative meristem into flower meristem. The fact that the rate of deformation caused by various environmental stresses depend on the developmental stage of the plants can also mean that

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product yield and quality is related to the success of the reproductive stage. We determined the changes in all examined of soybean seedlings caused by salt stress. Salt stress altered the the process of transition between vegetative stage to reproductive stage in soybean depending on the salt concentration. At 50 mM and 100 mM salt concentrations transition was faster than the control (P≤0.05). However, at 150 and 200 salt concentrations transition was significantly delayed (P≤0.05). Even though increasing salt stress shortened the reproductive stage, flower stucturesshrinked. This may be an attempt of the plant to complete its life cycle as soon as possible to produce seeds [22]. These results may seem as positive stress at first, however, it can only be clarified by investigating the characteristics of the reproductive structures of the plants. Salinity negatively affects all metabolic functions during vegetative development stage and this in turn significantly decreases fertility during reproductive stage [23]. The number of flowers decreased by 10%, 30%, 74% and 90% at 50, 100, 150 and 200 mM salt concentrations, respectively, compared to control. 5 different salt concentrations were used in the study. However, soybean could not complete its vegetative development in 250 mM salt concentration. Therefore, there is no data from this treatment concentration (Fig 1). Stress factors such as drought and salinity can negatively affect the development of these structures due to the heterogeneous distribution of toxic ions in flower constructions [24]. While the flower numbers showed a decrease parallel to increasing salt concentration, it was determined that these structures were nearly absent especially at the highest salt concentration (P≤0.05). Excessive Na^+ accumulation in chickpea leaves grown in salt stress significantly delayed flowering and reduced reproductive structures [25].

Salinity causes anomallies during pollen formation and development processes [26]. Pollen size decreased with increasing salt concentrations, compared to control (except for 100 mM salt concentrations). Pollen diameter decreased by 1%, 4%, 4% and pollen length decreased by 2%, 4%, 4% at 50, 150, 200 mM salt concentrations, respectively, compared to control. Mean pollen size of the 100 mM salt concentration treatment group was even higher than the control with a 4% increase in pollen diameter and a 3% percent increase in pollen length (Fig 2a, Fig 5). Increasing salt concentrations caused the pollen shape to change from prolate-spheroidal (P/E=1.14-1) to spheroidal (P/E= 1.14-0.88) at the highest salt concentration (Fig. 2b, Fig. 5).

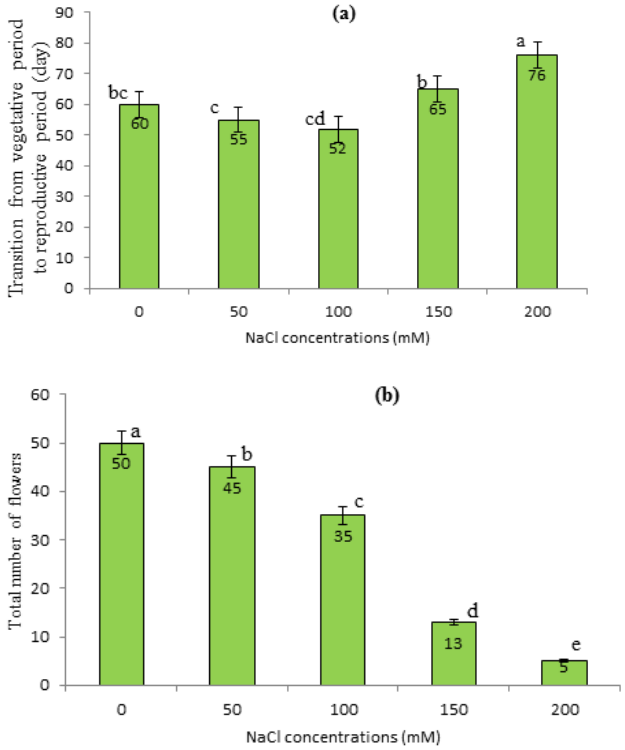

Fig. 1:The effect of increasing salt concentrations on the transition from vegative stage to reproductive stage (a) and the number of flowers of Glycine max (b)

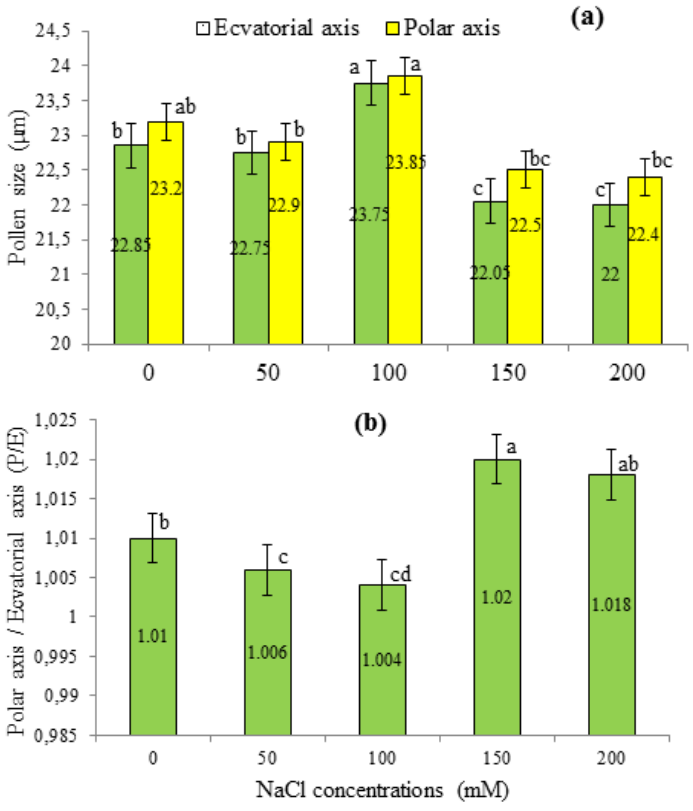

Fig. 2: Pollen size (a) and pollen shape (P/E) (b) of Glycine max exposed to salt stress

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The decrease in pollen size might be caused by the osmotic potential effect of pollen protoplast content as a result of excessive salinity [27]. On the other hand, the decrease in pollen size under the influence of stress factors also negatively affects the germination success of pollen on stigma and pollen tube length[28].

Exine and intine layers, known together as pollen wall, weren't significantly affected by increasing salt concentrations during the reproductive stage which is the most sensitive stage of plants to environmental stresses [29]. However, the fact that the highest applied salt concentration led to an increase in the thickness of both walls, even if too small, reveals the parallelism between the stress factor and the exine thickness. Thickness of the exine and intineslightly increased in parallel with increasing salt stress. Both wall structures of treatment groups were simillar to control group in width ($P \geq 0.05$). However, there was a slight increase at the highest applied salt concentration, compared to control ($P \leq 0.01$) (Fig 3). One of the conditions for becoming a fertile pollen is the presence of a well-developed pollen wall. Because any deformation in the pollen wall causes sterile pollen formation. The increase in exine width under environmental stress reveals the effort of the plant to form fertile pollen [30]. This can be a sign that pollen is protecting itself in the face of increased salt stress. The increase in exine thickness can be considered as an adaptation to the stress environment of the plant.

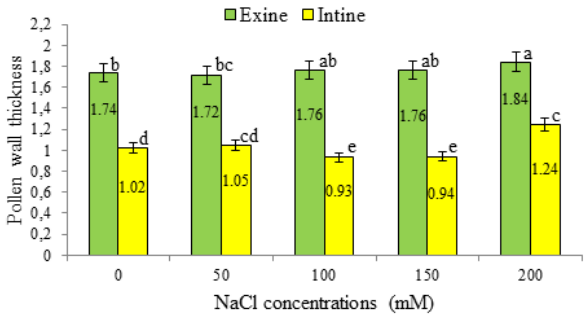

Fig. 3: Pollen wall thickness (exine and intine) of Glycine max exposed to increasing salt concentrations

Pollen germination begins with the emergence of vegetative pollen cell from the aperture. These areas are vulnerable to attack from the outside because they are openings where the pollen wall is the thinnest or non-existent. Salt stress decreased the aperture size of the pollens depending on the concentration, compared to control ($P \leq 0.05$). Aperture size decreased by 11% at 50 mM, 19% at 100 mM, 23% at 150 mM, and 29% at 200 mM salt concentrations (Fig 4 and 5). The aperture sizes in soybean seedlings exposed to salt stress decreased, and as a result pollen germination rate and pollen tube length also decreased. Any adverse effect on pollen aperture structure of plants exposed to various stress conditions also adversely affect pollen viability [31]. At the same time, these gates which provide communication between the pollen and the environment may undergo deformation due to the abnormal development of the pollen wall. This negative effect reduces the size of the apertures and significantly reduces pollen germination [32].

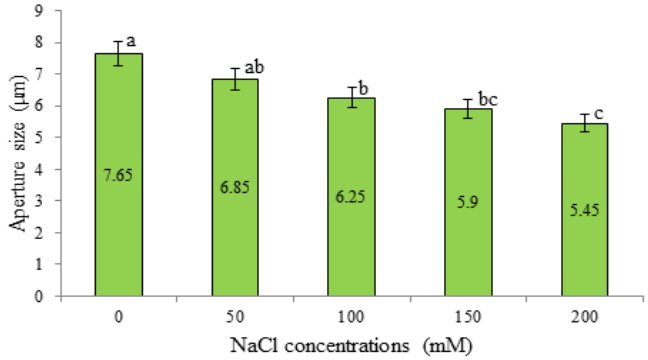

Fig. 4: The effect of salt stress on the pollen aperture size of Glycine max

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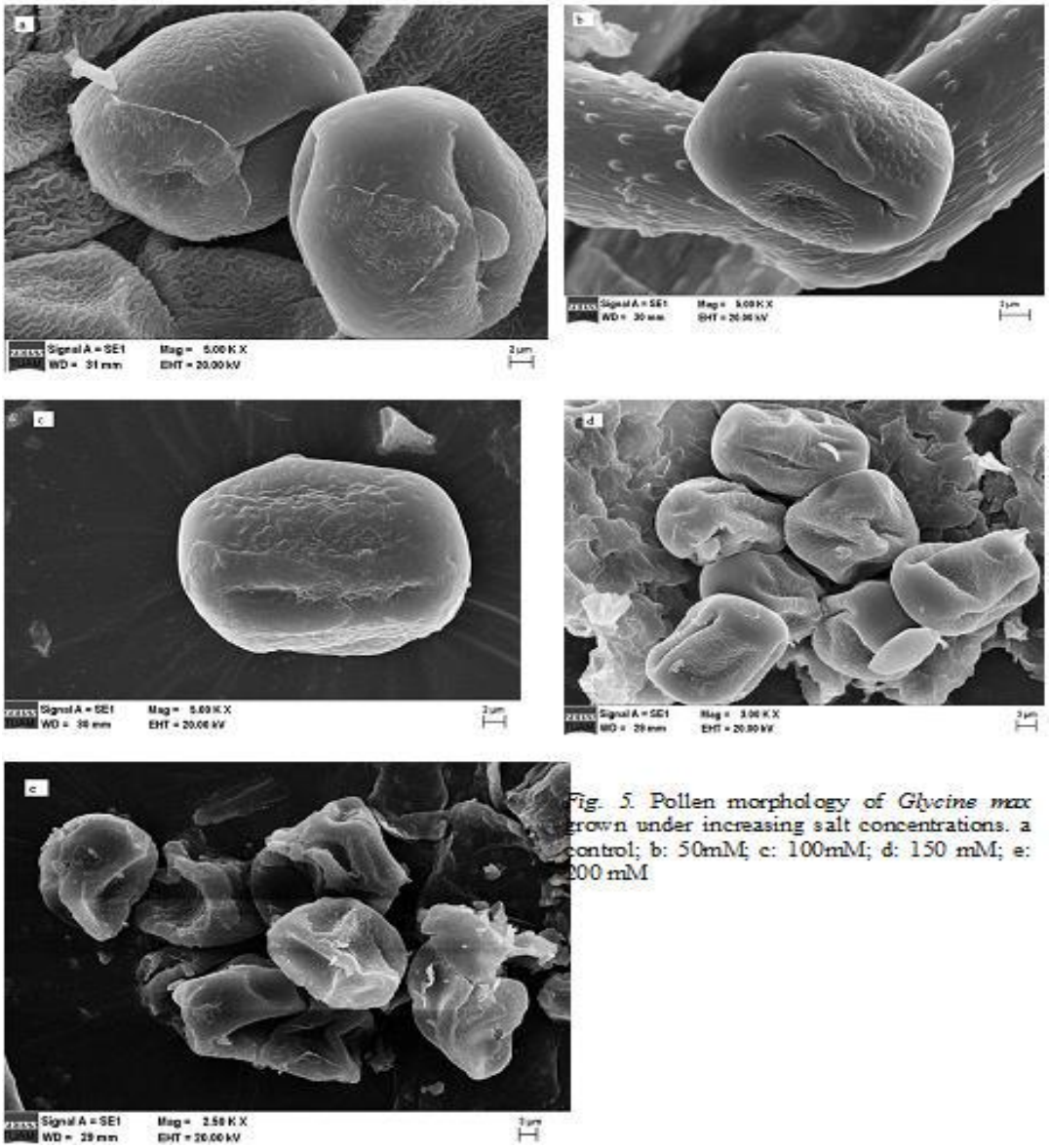

Fig. 5. Pollen morphology of *Glycine max* grown under increasing salt concentrations. a: control; b: 50mM; c: 100mM; d: 150 mM; e: 200 mM

Male reproductive stage in which microsopore main cells undergo meiosis (microsporongenesis) and microspores produce gametes (microgametogenesis) is the most sensitive stagein internal and external stresses [33] and abitoic stress during this stage causes the formation of infertile pollens [34]. Environmental stress significantly increases the recombination rate and this leads to formation of infertile pollens [35]. Pollen viability decreased with increasing salt concentrations. It decreased by 23%, 48%, 62% and 82% at increasing salt concentration, respectively, compared to control ($P \leq 0.05$). Simillar results were observed for pollen germination. However, salt stress had more negative impact on pollen germination rate. At the highest applied salt concentration, germination rate decreased by 96% ($P \leq 0.05$). The decrease in pollen viability in our study parallel to the increase in salt stress is an indication that this stress negatively affects male development in the reproductive stage. On the other hand, various stress factors impedes the passage of nutrients from leaves to anthers and this causes the formation of fragmented or infertile pollens [36]. Decrease in pollen viability causes pollination, fertilization, and product yield to decline significantly [37]. For this reason, the existence of a positive relationship between pollen viability and pollen germination can be mentioned.Because pollens can undergo germination only if they are healthy and well-developed. This is the reason why pollen germination rate (96%) decreased more than pollen viability (82%) at the highest applied salt concentration, compared to control ($P \leq 0.05$). Negative effects of salt stress on pollen viability of soybean seedlings were reflected on pollen germination at the same rate as on pollen tube length. It was observed that pollen tube lengths of successfully germinated pollens decreased as the salt concentration increased. However this negative effect was not as sever as seen on pollen vialibity and germination rate ($P \leq 0.05$). Pollen tube lengths decreased by 12%, 40%, 65%, 80% at

increasing salt concentrations, compared to control (Fig 6). A recent study on grapevines showed a positive relationship between the decrease in pollen tube growth and pollen viability [38]. When pistil structures of Arabidopsis plants exposed to salt stress is examined, no pollen germination is observed at some pistils, and in others pollen tube grows, however, it grows only a tiny amount or it is obstructed from reaching the stylus [39].

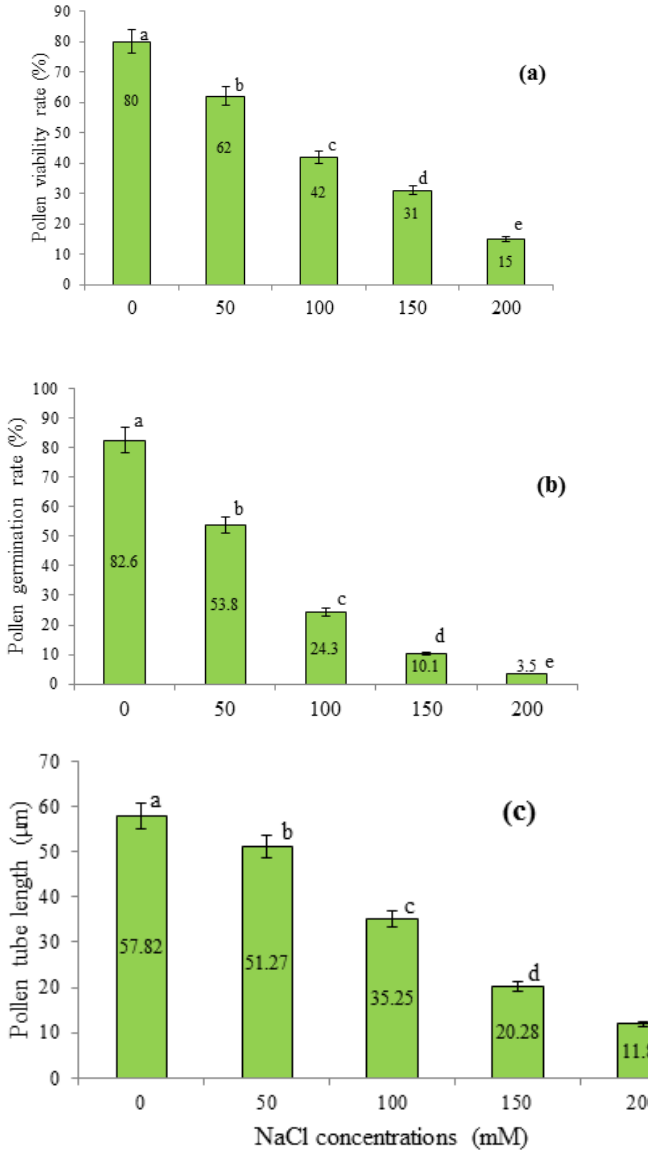

Fig. 6: Examined parameters of the reproductive structures of Glycine max grown under increasing salt concentrations. a: pollen viability; b: pollen germination rate; c: pollen tube length

IV. CONCLUSIONS

The observed decrease in all investigated parameters with increasing salt concentrations in our study was also explained by CRSI. Cumulative stress responses increased by 59%, 68%, 70% at 100, 150, 200 mMNaCI concentrations, compared to 50 mMNaCI. This increase is in inverse relationship with the tolerance level of the plant against the applied stress (Fig7). The fact that the tolerance level of the reproductive structures of *Glysine max* at the highest applied salt concentration was the lowest (-257.01) showed that the tolerance level of the plant was gradually decreasing. Our results show once again that the reproductive period is the most sensitive period to stress and as the stress level rises pollination will decrease. Therefore, it has become a necessity to speed up the work to adapt these nutrients, which have a very place in human nutrition, to our soil which becomes arid with each passing day due to excessive salinity.

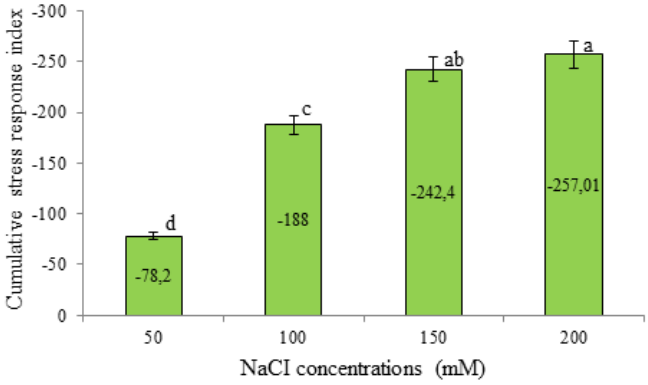

Fig. 7: Cumulative response index of all investigated parameters of Glycine max grown under increasing salt concentrations

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