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Physiological Role of Osmoregulators Proline and Glycinebetaine in Increasing Salinity Tolerance of Chickpea

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Abstract

A pot experiment was performed at greenhouse, National Research Centre, Egypt, during winter seasons 2019/2020 and 2020/2021 to investigate the physiological role of two osmoregulators proline (5 mM and 10 mM) and glycinebetaine (10 mM and 20 mM) in alleviating the deleterious effect of salinity (3000 mg/l NaCl) on quality and quantity of chickpea plant (Sakha 4 cultivar). Salinity stress significantly decreased plant dry weight at vegetative stage, all components of photosynthetic pigments, seed yield accompanied by significant increases in osmo-protectant (proline and soluble carbohydrates) in dry leaves as well as phenolic content and antioxidant activity in the yielded seeds. Regarding proline and glycinebetaine effects, results indicated that all applied treatments caused significant increases in most of the investigated parameters of chickpea plants irrigated with either tap water or saline solution relative to corresponding control. It is worthy to mention that proline treatments were more effective than glycinebetaine treatments in increasing salinity tolerance of chickpea plants that reflected in its quality and quantity. Moreover, proline treatment at 5mM was the most pronounced treatment in alleviating the deleterious effect of salinity on chickpea plants.

Keywords: Cicer arietinum L., osmoprotectant, salinity tolerance

1. Introduction

Chickpea (*Cicer arietinum* L.) is a popular legume grown in arid and semiarid regions all over the world. The chickpea crop is critical for maintaining of soil fertility, especially in arid regions. Chickpea seed includes 40- 55% carbohydrate, 13 - 33% protein, and 4-10% oil **[1]**. The chickpea is a highly sensitive crop to salinity and its production is severely affected due to salinity. According to **Dravid and Goswami [2]**, the irrigation of chickpea with saline water at 8.3 dS m^{-1} resulted in 61.6% loss in yield.

Under saline conditions, plants exhibit wide range of injurious responses, and production of reactive oxygen species (ROS)**.** The ROS are very damaged to plant tissues because they oxidize the cellular membrane and cause chlorophyll degradation and oxidation of significant molecules including lipids, proteins and DNA which causing cell damage **[3]**. Depending on the age of the plants and severity of salinity, the distribution of nutrients across the various organs of the plant is also altered. Salinity limits photosynthetic efficiency, nitrogen fixation and carbon metabolism **[4]**. Furthermore, plant growth in saline soil is influenced by a complex interaction of hormones, specific ion effects, osmotic effects, and nutritional imbalances, which all occur at the same time [**5**]. Generally, most crops tolerate salinity to a threshold level above which yields decrease as salinity increases. Plants protect themselves through a variety of mechanisms, including morphological, biochemical, and physiological changes. Different plants tolerate salinity stress by producing a variety of organic solutes known as compatible solutes (osmoprotectants) which decrease the osmotic potential and attract more water molecules into the cell, allowing the cell to maintain its turgor. These osmo-protectants are including proline, glycinebetaine, and soluble sugars, *etc.* They are characterized by high solubility in water, low molecular weight, and not hazardous to plants. In general, these osmo-protectants protect different types of plants from abiotic stress by stabilizing proteins and protecting membrane structure [**6**],

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protecting chloroplasts and cytoplasm from Na^+ damage **[7]**, maintaining the osmotic balance, and scavenging of reactive oxygen species under stressful conditions [**8, 9]**. The use of osmo-protectants has been considered as a shotgun strategy to increase plant salinity tolerance.

Proline is a proteinogenic amino acid, essential for different vital metabolic processes within the plant tissues. It is widely distributed in plants and accumulates in large amounts due to environmental stresses such as salinity, drought, and extreme of temperature. It is crucial for plant osmotic adjustment under stressful abiotic conditions. Since, exogenously administrated proline provided osmoprotection and facilitated plant development under abiotic stress conditions **[10]**.

The effect of applied proline is determined by the time of application and its concentration as well as the type of plant species and developmental stage. Its effect was dose-dependent; hence, it is important to determine the optimum level of proline treatment that can enhance economically crop plants when exposed to abiotic stress [**11]. Abbas *et al.* [12]** stated that exogenous application of proline (25 mg L^{-1}) to the *Citrus sinensis* L. increased the production of salt responsive proteins and significantly reduced the inhibitory effect of salinity stress (40 mM NaCl and 50 mM NaCl) on height and leaf number of plantlets. According to **Taie** *et al*. **[13]**, pre-sowing faba bean seed with proline at 5 mM and 10 mM enhanced salinity tolerance of faba bean by increasing photosynthetic pigments, regulating the ion accumulations, and maintaining the anatomical structure of vegetative organs. In accordance, **Dawood *et al.* [14]** reported that exogenous application of proline at 25mM partially mitigated the deleterious effect of diluted seawater (3.13 dS/m and 6.25 dS/m) on the growth of faba bean plants, whereas 50 mM proline treatment had a damaging effect similar to salinity stress.

Glycinebetaine is a quaternary ammonium compound that protects plants from the detrimental effects of abiotic stresses. Application of glycinebetaine improved growth, productivity and plant tolerance to different abiotic stress **[11]** by maintaining turgor pressure **[15]**, enhancing net CO_2 assimilation rate **[16]**, protecting the functional proteins and enzymes (e.g. Rubisco), and lipids of the photosynthetic apparatus [**17],** regulating of photosynthetic machinery and ion homeostasis **[18]**, and different types of physiological and biochemical processes **[19]**. Furthermore, **Agboma *et al*. [15]** suggested that glycinebetaine may be act as anti-transpirant, allowing the plant to access more water for a longer period of time and facilitating photosynthesis**. According to Shahbaz *et al*. [20]**, foliar-application of (50 mM) glycinebetaine on wheat plants grown under water stress conditions was the most efficient level in enhancing different growth parameters and wheat grain yield as well as the levels of some important metabolites**.** Likewise, glycinebetaine alleviated the harmful effects of salinity stress on the growth of maize seedlings by maintaining membrane permeability, enhancing antioxidant enzyme activities, and improving concentrations of K^+ and Ca^{2+} in the plants as mentioned by **Kaya *et al.* [21]**. In addition, **Dawood and Sadak [22]** mentioned that soaking canola seeds with 20 mM glycinebetaine was the most effective treatment in decreasing the harmful effect of moderate or severe drought stress on canola growth and productivity. Since, glycinebetaine treatments increased canola drought tolerance by improving the growth parameters, osmoprotectants (proline and total soluble sugars), photosynthetic pigments, seed yield, oil, protein, carbohydrate, total phenolic content, flavonoids, tannins, and antioxidant activity of the yielded seeds.

This work aimed to investigate the efficiency of proline and glycinbetain in enhancing salinity tolerance of chickpea.

2. Materials and Methods

Two pot experiments were carried out at greenhouse, National Research Centre, Egypt, during winter season 2019/2020 and 2020/2021. Chickpea seeds (Sakha 4 cultivar) were obtained from Legume Department, Agricultural Research Center, Giza, Egypt. Healthy seeds were surface sterilized with 1 % (v/v) sodium hypochlorite followed by washing with distilled water. The seeds were divided to five groups, the first group was soaked with distilled water as control, second and third were soaked with two different concentrations of proline at 5 mM and 10 mM while the fourth and fifth groups were soaked with two different concentrations of glycinebetaine at 10 mM and 20 mM for 6 hours then allowed to dry at room temperature (25 °C) for about 1h.

2.1. Experimental procedure

Pot experiment was conducted on a sandy clay soil (1:3, w/w). The pots were arranged as factorial

experiment with two factors (salinity as main factor and osmo-growth regulators as sub main factor) in a split –plot design with six replicates per treatment. Six uniform chickpea seeds were sown along a centre row in each pot at 30-mm depth. Fertilization was carried out as recommended doses. After 21 days from sowing, the seedlings were thinned to three seedlings per pot and plants were irrigated with saline solution (NaCl at 3000 mg/l).

2.2. Data recorded

At nine weeks old plants (vegetative growth stage), samples were collected to determine some growth parameters as shoot height, number of branches and leaves, fresh and dry weights of plant. Moreover, estimation of photosynthetic pigments and extraction of DNA were done in young and fresh leaves while soluble carbohydrate and proline were determined in dry leaves.

At maturity, the plants were harvested to determine number of pods /plant, seed index and weight of seeds /plant. The yielded seeds were cleaned and kept in desiccators for chemical analysis.

2.3. Chemical analysis

Photosynthetic pigments were determined according to **Moran [23]**. Total soluble carbohydrates were determined using methods described by **Smith *et al***. [24]. Proline was estimated according to **Bates *et al.* [25]**. Phenolic compounds were estimated according to **Zhang and Wang [26]**. The free radical scavenging activity was determined according to **Brand-Williams *et al.* [27]** using the 1.1-diphenyl-2-picrylhydrazil (DPPH) reagent

2.4. DNA extraction and PCR analysis

The genomic DNA was isolated from young and fresh leaves of chickpea plants using CTAB protocol [**28].** Quantitative analysis of the DNA (density of bands) was performed using 1 % agarose gels in the presence of 0.5 mg/L ethidium bromide.

PCR amplifications were carried out using 5 ISSR primers (Table 1), moreover, polymerase chain reaction (PCR) was carried out within 15 µl reaction volumes. containing 1 µl plant genomic DNA, 7.5 µL Master Mix (Gene Direx one PCRTM), 1 µL template DNA and 1 µL primer. PCR condition was an initial denaturation at 94°C for 7 min followed by 35 cycles each of 94°C for 30 s, 52°C for 45 s, 72°C for 2 min. with a final extension at 72°C for 5 min. Amplifications products were electrophoresed first on gels 1.5 % agarose in TAE buffer.

Statistical analysis

Average of two seasons was statistically analyzed by analysis of variance and differences among means were determined by least significant differences (L.S.D) according to **Silva and Azevedo [29]**.

Table 1. Inter simple sequence repeat DNA primers used in the analysis of treated chickpea.

Primers	Sequence (5\–3\)
$(AC)_8YG$	AC ACACACACACAC ACYG
$(GT)_8YG$	GT GTGTGTGTGTGT GTYG
$CGC(GATA)_4$	CGC GATA GATAGATAGATA
$(AGAC)_4GC$	AGAC AGACAGAC AGACGC
$(GATA)_4$ GC	GATA GATAGATA GATAGC

3. RESULTS and DISCUSSION

3.1. Changes in vegetative growth parameters

It was obvious that salinity stress (3000 mg/l NaCl) caused significant decreases in most of vegetative growth parameters under investigation (Table 2). Salinity stress significantly decreased plant dry weight by 13.92 % relative to untreated plant. These decrements in growth parameter under salinity stress was explained by **Hajer *et al.* [30]** who mentioned that salinity decreased plant shoot and root dry matter due to combined impacts of osmotic and Cl^- and Na^+ ions. It is well known that osmotic pressure resulted from salinity restricted plant cells to uptake water and caused defect in metabolism of plant cells **[31]**.

On the other hand, all applied treatments (proline at 5 mM and 10 mM as well as glycinebetaine at 10 mM and 20 mM) caused marked increments in all vegetative growth parameters in chickpea plants exposed to irrigation with either tap water or saline solution relative to corresponding controls (Table 2). The highest significant increase in plant dry weight was achieved by 5 mM proline followed by 20 mM glycinebetain in plants irrigated with tap water or saline solution. Application of osmolytes increased plant biomass due to the active

role of osmolytes in plant osmotic adjustment that enhanced water uptake and improved plant growth. Proline treatments increased plant growth parameters -under stress conditions - may be due to its role as a nutrient and osmoprotectant as well as its contribution in cell division and cell enlargement **[32-34]**. Regarding positive effect of glycinebetaine**,** it was mentioned by **Aldesuquy [35]** that glycinebetaine treatment counteracted the deleterious effects of stress via improving of root and shoot, leaf area, growth vigour and retention of pigments content.

Table 2: Effect of proline (Pr.) and glycinebetain (GB) on vegetative growth parameters of chickpea plants grown under salinity stress

Treatments	Shoot length (cm)	Number of branches/plant	Number of leaves/plant	Plant fresh weight (g)	Plant dry weight (g)
Control	**29.67**	**3.00**	**30.00**	**2.99**	**0.79**
Pr. (5 mM)	**33.33**	**4.33**	**34.00**	**6.17**	**1.01**
Pr. (10mM)	**31.33**	**3.33**	**31.67**	**5.89**	**0.96**
GB (10mM)	**31.00**	**3.33**	**37.33**	**4.18**	**0.90**
GB (20mM)	**32.33**	**3.67**	**39.67**	**4.29**	**0.99**
Salinity (3000 mg/l NaCl)	**25.00**	**2.67**	**19.33**	**2.69**	**0.68**
Salinity + Pr. (5 mM)	**32.00**	**3.00**	**30.67**	**4.19**	**0.90**
Salinity + Pr. (10mM)	**30.67**	**3.00**	**22.67**	**3.91**	**0.77**
Salinity + GB (10mM)	**30.67**	**3.00**	**17.00**	**3.68**	**0.78**
Salinity + GB (20mM)	**31.00**	**3.33**	**21.67**	**3.98**	**0.82**
LSD at 5%	**4.06**	**1.75**	**2.30**	**0.36**	**0.041**

3.2. Changes in seed yield and its components

Salinity stress caused significant decreases in chickpea seed yield/plant and 100 seeds weight relative to untreated plant (Table 3). The decrement in seed yield/plant was 6.79% due to salinity stress. Ashraf et al. [**36]** reported that drought decreased crop yield due to the decrease in photosynthetic pigments and diminished Calvin cycle enzymes activities**. Ali *et al.* [37]** mentioned that water stress reduced activity of numerous enzymes and leading to changes in metabolic activities and altered translocation of assimilates to seeds.

Regarding positive effect of two osmoregulators (proline and glycinebetaine), it was noted that proline treatment at 5 mM and glycinebetaine treatment at 20 mM were the highest effective treatments that significantly increased seed yield/plant and 100 seed weight in plants that irrigated with tap water or saline solution relative to corresponding controls (Table 3). The application of osmo-protectant caused increases in plant growth and yield under either normal or stress conditions due to its effect in regulating ion homeostasis and photosynthetic machinery **[18]**, improving CO_2 assimilation in plants **[38]** and enhancing biosynthesis and transport of hormones like cytokinins that may have a role in the transport of photoassimilates [**39]. Siddique *et al.* [40]** stated that proline treatment at 25mL plant^{-1} as foliar application on rice plant grown under salinity stress significantly increased the growth and development of plant and consequently increased the grain and straw yields to a significant extent. The increment in chickpea productivity (Table, 3) as the result of osmo-protectant application might be related to the increase in photosynthetic products (Table, 4) which constitute an improved supply source for sinks, leading to increase seed yield.

3.3. Changes in photosynthetic pigments

Table 4 shows that salinity stress significantly decreased all components of photosynthetic pigments. Salinity stress decreased total photosynthetic pigments by 20% relative to unstressed plants. The inhibitory effects of salinity on chlorophyll pigments (Chl a,b and Chla+b) as shown in Table (4) could be due to suppression of specific enzymes responsible for the synthesis of the green pigments. In addition, salinity stress increased the degradation of chlorophyll via increased the activity of the proteolytic enzymes such as chlorophyllase **[41]**. Furthermore, stressed plants have lower chlorophyll content, which could be attributed to damage to the photosynthetic machinery, disorder of the thylakoid membrane, and reduced CO_2 uptake and plant photosynthetic rate [**42]**.

Table 3: Effect of proline (Pr.) and glycinebetain (GB) on seed yield and yield components of chickpea plants grown under salinity stress

Treatments	Number of pods/plant	Seed yield/plant (g)	100 Seed weight (g)
Control	**3.40**	**7.07**	**33.07**
Pr. (5 mM)	**4.17**	**13.41**	**40.80**
Pr. (10mM)	**3.63**	**10.20**	**38.33**
GB (10mM)	**5.00**	**11.62**	**37.11**
GB (20mM)	**5.31**	**12.54**	**39.02**
Salinity (3000 mg/l NaCl)	**3.23**	**6.59**	**27.12**
Salinity + Pr. (5 mM)	**3.83**	**11.21**	**30.10**
Salinity + Pr. (10mM)	**3.47**	**9.72**	**29.72**
Salinity + GB (10mM)	**4.67**	**7.37**	**34.43**
Salinity + GB (20mM)	**4.67**	**10.46**	**36.33**
LSD at 5%	**0.25**	**0.05**	**0.022**

Regarding osmo-protectant effect, it was noted that the proline and glycinebetaine treatments (Table, 4) caused significant increments in all components of photosynthetic pigments relative to corresponding control plants. Proline treatments had more pronounced effect in increasing photosynthetic pigments than glycinbetain treatments. Since, proline treatment at 5 mM caused significant increases in total photosynthetic pigments of plants irrigated with tap water by 32.28%. Regarding plants irrigated with saline solution, proline treatment at 5 mM caused significant increases in total photosynthetic pigments by 50% relative to corresponding control. It is very important to mention that positive effect of 5 mM proline treatment on total photosynthetic pigments under salinity stress were more pronounced than its effect under normal conditions. The increase in total chlorophyll content due to proline application may be attributed to the increase in the rate of CO_2 diffusion and favored higher photosynthetic rate [**32]** and actual efficiency of photosystem II **[33].** Whereas, the promotive effect of glycinebetaine on photosynthetic pigments under stress could be explained basis on the efficient role of glycinebetaine in protecting various components of the photosynthetic machinery, such as rubulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) **[43],** preserving the net photosystem-II efficiency [**44]**, and enhancing the tolerance of photosynthetic apparatus of plants **[45, 46]**.

3.4. Changes in osmoprotectants (soluble sugar and proline)

Regarding estimated osmolytes- soluble carbohydrate and proline - of dry leaves at vegetative growth stage (Table 5), it was obvious that salinity significantly increased the two parameters by 32.31% and 117 % respectively relative to untreated control plant. In stressed plants, some compatible solutes (total soluble sugar and proline) accumulated, resulting in a decreased solute potential, allowing the plant cell to maintain higher water content. These solutes act as osmo-protection and/or osmotic adjustment under stress conditions. Soluble carbohydrate is a major category of osmoprotectant Because of its effectiveness in mitigating salinity stress, either by providing some desiccation resistance to plant cells or by osmotic adjustment **[47]**. The accumulation of sugars in leaves of stressed plants may be linked to its reduced growth as result of the role of salinity in the inhibition of sugar translocation or may be linked to the higher energetically cost for osmotic adjustment **[48]**. Similarly, according to **Mousavi *et al.* [49]**, and Abdallah et al. **[34]** salinity increased the quantity of soluble carbohydrates, which helped in osmotic adjustment and increased plant resistance to water stress. Regarding accumulation of proline under salinity stress, various studies have shown that abiotic stress conditions caused proline accumulation in plant tissues [**50]**. It is worthy to mention that; high ratio of proline is the sign of efficient osmotic adjustment under saline environments. A positive correlation was established between amount of proline contents and salt tolerance had been mentioned by **Abd El-Samad *et al.* [51]**. These increases in proline content under salinity stress may be attributed to reduced oxidase and proline catabolising enzymes as mentioned by **Debnath [52]**.

Table 4: Effect of proline (Pr.) and glycinebetain (GB) on photosynthetic pigments of chickpea plants grown under salinity stress

Treatments	Chlorophyll A	Chlorophyll B	Chlorophyll A + B	Carotenoids	Total photosynthetic pigments
	mg/g fresh weight				
Cont.	**2.33**	**0.94**	**3.27**	**0.42**	**3.50**
Pr. (5 mM)	**2.79**	**1.36**	**4.02**	**0.50**	**4.63**
Pr. (10 mM)	**2.67**	**1.31**	**4.09**	**0.44**	**4.49**
GB (10 mM)	**2.53**	**1.00**	**3.53**	**0.46**	**3.95**
GB (20 mM)	**2.67**	**1.13**	**3.80**	**0.48**	**4.15**
salinity	**2.04**	**0.65**	**2.70**	**0.35**	**2.80**
Salinity + Pr. (5 mM)	**2.47**	**1.11**	**3.68**	**0.44**	**4.20**
Salinity + Pr. (10 mM)	**2.58**	**0.99**	**3.46**	**0.43**	**3.84**
Salinity + GB (10 mM)	**2.30**	**0.83**	**3.28**	**0.36**	**3.71**
Salinity + GB (20 mM)	**2.45**	**0.97**	**3.27**	**0.38**	**3.81**
Salinity x GR LSD 5%	**0.015**	**0.022**	**0.017**	**0.004**	**0.004**

Under adverse environmental conditions, proline takes part in cellular osmotic adjustment **[53]**, stabilizes the membrane and proteins 3D structure [**11**] as well as takes part in the induction of stress responsive genes [**54]**. Moreover, exogenous application of proline increased its endogenous levels within plant tissues subjected to drought conditions and consequently induced plant tolerance [**11**]. Furthermore, proline may be acting as a free radical scavenger, membrane and macromolecule stabilizer, carbon and nitrogen supply thereby allowing plants to recover quickly from stress **[49]**.

Table 5: Effect of proline (Pr.) and glycinebetain (GB) on some chemical composition of chickpea plants grown under salinity stress

Treatments	Soluble carbohydrate	Proline	Phenolic content	Antioxidant activity
	(mg\g dry leaf tissues at vegetative stage)		(% in yielded seeds)	
Control	39.46	19.12	1.21	16.96
Pr. (5 mM)	57.31	44.14	1.39	25.25
Pr. (10mM)	51.19	31.50	1.29	24.41
GB (10mM)	39.04	29.28	1.29	19.15
GB (20mM)	51.09	30.31	1.31	20.23
Salinity (3000 mg/l NaCl)	52.21	41.51	1.50	19.81
Salinity + Pr. (5 mM)	63.96	56.56	1.59	30.33
Salinity + Pr. (10mM)	56.48	53.00	1.53	27.72
Salinity + GB (10mM)	51.03	43.02	1.50	22.11
Salinity + GB (20mM)	52.98	45.83	1.60	24.02
LSD at 5%	**0.51**	**0.36**	**0.009**	**0.45**

Regarding effect of osmo-protectants, it is worthy to mention that all applied treatments increased soluble carbohydrate and proline content in plants irrigated with tap water or saline solution. Proline treatments were more effective than glycinbetaine treatments. The most pronounced treatment was 5 mM proline (Table 5). Application of proline might decrease the negative effects of salinity on carbohydrate metabolism which consequently could increase the whole plant growth as pointed by **Abd El-Samad *et al.* [51]**.

Regarding glycinebetaine effect, our results are similar to those obtained by **Ibrahim [55]** who stated that sorghum plants treated with glycinebetaine under salinity stress accumulated more soluble sugars than the salinity stressed plants only.

Changes in phenolic content and antioxidant activity of the yield seeds

Regarding phenolic content and antioxidant activity of the yielded seeds (Table 5), it is clear that salinity stress significantly increased the two parameters by 23.96% and 16.80% respectively. All applied treatments caused significant increases in the antioxidant activity. The highest significant increase in the two parameters was recorded due to 5 mM

proline in plants irrigated with tap water or saline solution relative to crossponding controls.

Higher plants manifest a unique capability of the synthesis of non-enzymatic secondary metabolites as phenolics that have antioxidative role in scavenging the ROS and preventing subcellular damage during stress **[56**]. Phenolics constitute a part of cellular solutes that reduce the environmental stress on the plant. In this concern, phenolic contents protect cells from potential oxidative damage, increase stability of cell membrane **[57]** and mitigate the salinity stress injuries **[58]**. Under water deficit conditions, application of compatible solutes on maize plant increased the levels of phenolic compound as mentioned by **Ali and Ashraf [59]**.

Application of organic osmolytes, under the effect of drought condition showed a positive correlation between seed oil antioxidant activity and different antioxidant compounds **[37]**. Similarly, when plants are stressed, application of osmolytes plays an important part in their oxidative defense mechanism by enhancing the formation of antioxidant secondary metabolites. Promotive effect of osmolytes in increasing the contents of antioxidant secondary metabolites may be attributed to the role of osmolytes as a regulatory or signaling molecule to activate multiple physiological and biochemical processes as well as plant adaptation to different stress conditions [**11**]. Proline plays an important role against oxidative damages caused by ROS due to its action as singlet oxygen quencher **[60]**. The application of glycinebetaine increased rice seedlings' tolerance to salt-induced oxidative damage by up-regulating their antioxidant defense system as reported by **Hasanuzzaman *et al.* [61]**. In addition, glycinebetaine had the capacity to scavenge free radicals which is more important than their role as a mere osmolyte [**9, 11, 46]**.

Changes in DNA via PCR analysis

Data in Table (6) and Fig. (1) revealed that there are 23 reproducible bands that detected by five ISSR primers (Table 1). However, there is no any monomorphic band has been detected, but bands varied between polymorphic bands (9 bands) and unique bands (14 bands) with different molecular weight as follow:

1. IS-01: one polymorphic band has been detected by this primer in all treatments except plants treated with 20mM glycinebetaine under irrigation with both tape water and stressed plants with saline solution.

2. IS-02: two polymorphic bands with different molecular weight (456.39 and 317.49 bp) have been detected by this primer. Moreover, these polymorphic bands appeared in all treatments except control plant (irrigated with tap water) and those treated with 5mM proline and irrigated with tape water. On the other hand, another two DNA bands have been detected by this primer, they were unique bands and appeared at molecular weight **(894.40 and 876.35 bp**) **for** those treated with 5mM proline and irrigated with tape water and control plan**t** respectively.

3. IS-03: one unique band (676.99 bp) has been detected by this primer in plants treated with 5mM proline and irrigated with tape water**.** Moreover, there are three different polymorphic bands have been detected by this primer. The first one was at (942.51 bp) in plants treated with 10mM and 20mM glycinebetaine and irrigated with saline solution. Secondly, polymorphic band at (685.341bp) that appeared in plants treated with 10 mM proline and 10 mM glycinebetaine under normal condition. The third polymorphic band has been appeared at (508.20 bp) in many treatments.

4. IS-04: The type of DNA reproducible bands that detected by this primer were three polymorphic bands that varied in molecular weight. Two of them were at (712.00 and 423.00 bp) and appeared in all treatments except control plants and those treated with 20 mM glycinebetaine under normal conditions**.** Whereas, the third one has been detected at 369.14 bp and appeared in 6 treatments**.**

5. IS-05: the highest level of variation between different treatments has been detected by this primer; where six unique bands have been detected by this primer at different molecular weights. The unique bands were as follow:

- One band at molecular weight (567.58 bp) for control plants.
- Two bands at molecular weight (325.68 and 120.04 bp) for plants treated with 5mM proline and irrigated with tap water.
- One band at molecular weight (127.19 bp) for plants treated with 10 mM glycinebetaine and irrigated with tap water.
- One band at molecular weight (359.35 bp) for plants treated with 20 mM glycinebetaine and irrigated with saline solution.
- One band at molecular weight (294.32 bp) for plants treated with 10 mM glycinebetaine and irrigated with saline solution.

On the other hand, there were five polymorphic bands have been detected by primer **IS-05;** two of them appeared at different molecular weights (298.61 and 121.79 bp) in plants treated with 5 mM and 10 mM proline and irrigated with saline solution. At 960.93 bp one band has been detected in control plants and plants treated with 5 mM and 10 mM proline and irrigated with tap water**.** At 584.24 bp one band has been detected in plants treated with 5 mM and 10 mM proline and irrigated with with tap water**.** The last polymorphic band that was detected by this primer at 426.23 bp and common between all treatments except those treated with 10mM glycinebetaine under normal conditions and those treated with two levels of proline under saline conditions. Various molecular markers are available for analysis of genetic diversity, but Inter-simplesequence repeat (ISSR) is more consistent markers, as they generate a greater number of polymorphic loci per primer [**62-63**].

Table 6: Effect of proline (Pr.) and glycinebetain (GB) on molecular characteristics of chickpea plants grown under salinity stress

Primer	MW bp	Cont.	Pr.1	Pr.2	GB.1	GB.2	S	Pr.S1	Pr.S2	GB.S1	GB.S2	Polymorphism
IS- 01	**247.977**	+	+	+	+	-	-	+	+	+	+	**Polymorphic**
IS- 02	**894.40**	-	+	-	-	-	-	-	-	-	-	**Unique**
	876.35	+	-	-	-	-	-	-	-	-	-	**Unique**
	456.39	-	-	+	+	+	+	+	+	+	+	**Polymorphic**
	317.49	-	-	+	+	+	+	+	+	+	+	**Polymorphic**
IS- 03	**942.51**	-	-	-	-	-	-	-	-	+	+	**Polymorphic**
	685.34	-	-	+	+	-	-	-	-	-	-	**Polymorphic**
	676.99	-	+	-	-	-	-	-	-	-	-	**Unique**
	508.20	-	+	+	+	-	-	-	+	+	+	**Polymorphic**
IS- 04	**712.00**	-	+	+	+	-	+	+	+	+	+	**Polymorphic**
	423.00	-	+	+	+	-	+	+	+	+	+	**Polymorphic**
	369.14	-	+	+	+	-	-	-	+	+	+	**Polymorphic**
IS- 05	**960.93**	+	+	+	-	-	-	-	-	-	-	**Polymorphic**
	584.24	-	+	+	-	-	-	-	-	-	-	**Polymorphic**
	567.58	+	-	-	-	-	-	-	-	-	-	**Unique**
	426.23	+	+	+	-	+	+	-	-	+	+	**Polymorphic**
	359.35	-	-	-	-	-	-	-	-	-	+	**Unique**
	325.68	-	+	-	-	-	-	-	-	-	-	**Unique**
	298.61	-	-	-	-	-	-	+	+	-	-	**Polymorphic**
	294.32	-	-	-	-	-	-	-	-	+	-	**Unique**
	127.19	-	-	-	+	-	-	-	-	-	-	**Unique**
	121.79	-	-	-	-	-	-	+	+	-	+	**Polymorphic**
	120.04	-	+	-	-	-	-	-	-	-	-	**Unique**
Total		**5**	**12**	**10**	**9**	**3**	**5**	**7**	**9**	**10**	**11**	

Cont. = irrigated with distilled water, **Pr.1 and Pr.2=**soaked with two different concentrations of proline at 5 mM and 10mM and irrigated with distilled water, while, GB1 **and GB2**= soaked with two different concentrations of glycinebetaine at 10 mM and 20 mM and irrigated with distilled water.

S = irrigated with saline solution, **Pr.1S and Pr.2S=** soaked with two different concentrations of proline at 5 mM and 10mM and irrigated with saline solution. **GB1S and GB2S**= soaked with two different concentrations of glycinebetaine at 10 mM and 20 mM and irrigated with saline solution.

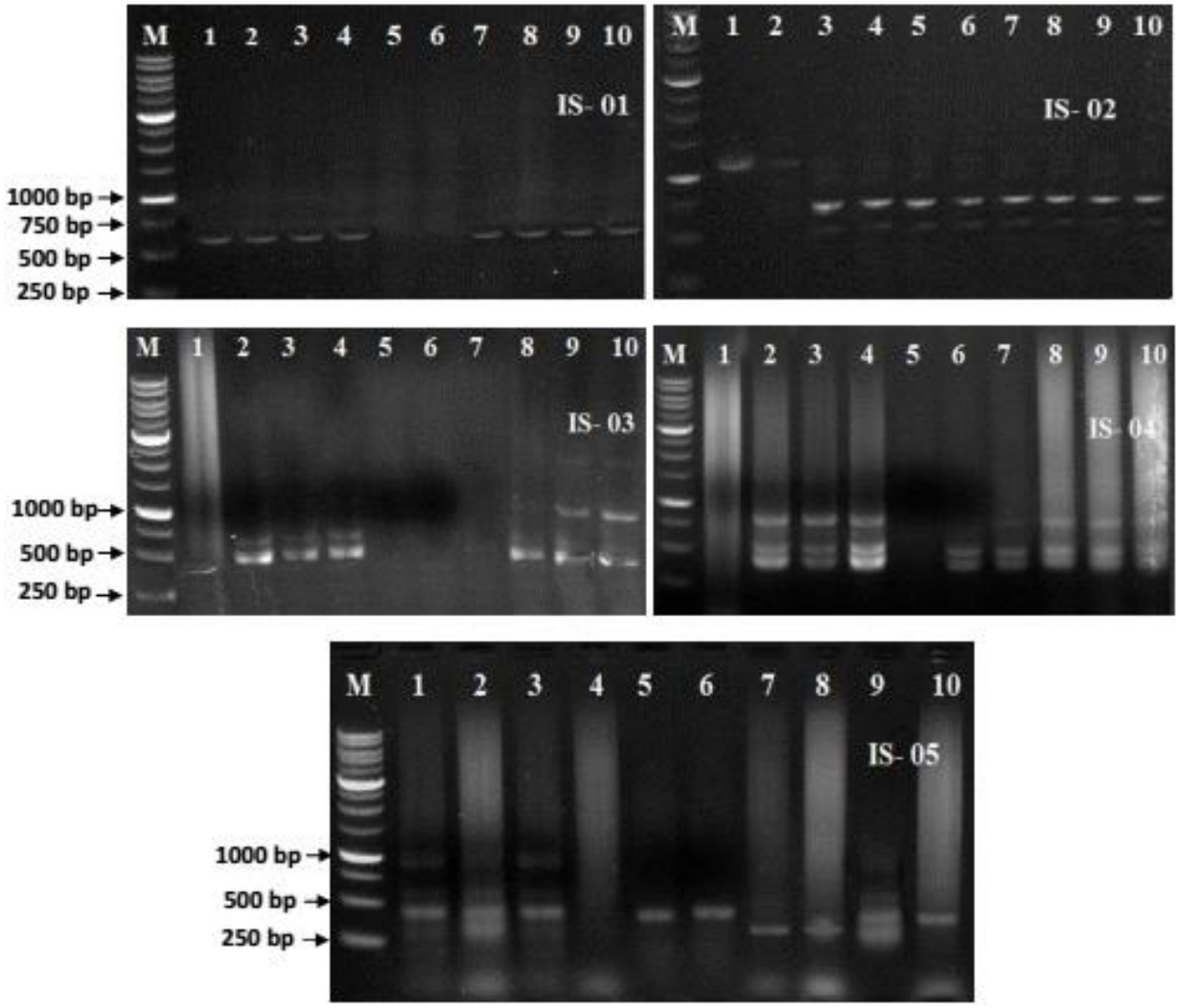

Fig. (1): Effect of proline (Pr.) and glycinebetain (GB) on ISSR generated bands of chickpea plants grown under salinity stress.

M= DNA standard marker; **1=** control (tap water); **2= Pr.** (5 mM); **3= Pr.** (10 mM);, **4=** GB(10 mM); **5=** GB(20 mM); **6=** Salinity (3000mg/l); **7= Pr.** (5 Mm)+ S; **8= Pr.** (10 mM)+S; **9=** GB (10 mM) +S; **10=** GB(20 mM) +S

4. Conclusion

It could be concluded that two osmoregulators (proline at 5 mM and 10 mM as well as glycinebetaine at 10 mM and 20mM) play an important role in alleviating the deleterious effect of salinity (3000 mg/l NaCl) on quality and quantity of chickpea plant. It is worthy to mention that proline treatments were more effective than glycinebetaine treatments in increasing salinity tolerance of ckickpea plants that reflected on its quality and quantity. Moreover, proline treatment at 5mM was the most pronounced treatment in alleviating the deleterious effect of salinity on chickpea plants.

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