Enhancing Salt Tolerance of Cucumber Using Grafting and Some Bioregulators

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ABSTRACT

Experiment was conducted in a greenhouse in El-Salehea Al-Gadeda, Sharqia Governorate during the two successive seasons of 2008/2009 and 2009/2010 to investigate the effect of grafting technique, bioregulators; salicylic acid (SA) at 0.5 & 1 mM, fulvic acid (FA) at 150 & 300 ppm and seaweed extract (SWE) at 2.5 & 5% in addition to osmoregulators (compatible solute); glycine betaine (GB) at 2.5 & 5 mM in improving salt tolerant of grafted cucumber plants (Cucumis sativus L. cv. falcon, Hybrid F1) under saline conditions. Grafting of cucumber on salt tolerance rootstock (Shintosa supreme pumpkin) significantly increased growth and fruits yield, relative water content (RWC) and antioxidant enzymes activity. Furthermore, grafting significantly increased chlorophyll, carotenoid, proline and total soluble protein concentrations. Grafting reduced membrane permeability and malondialdehyde (MDA) concentration in leaves of cucumber plants under salt stress conditions. Foliar application of SWE, SA, FA and GB significantly improved growth and yield parameters, and biochemical contents of grafted cucumber plants compared to untreated grafted plants under salt conditions. The superiority was due to SWE (5%) followed by FA (300 ppm) then GB (5 mM).

Key words: Shintosa supreme pumpkin (Cucurbita maxima x C. moschata), cucumber (Cucumis sativus), grafting, bioregulators, salicylic acid, fulvic acid, seaweed extract, compatible solute, glycine betaine, salt stress.

Introduction

Cucumber (Cucumis sativus L.) is one of the most greenhouses cultivated vegetable crops in the world because of the short cycle and its high economic value in off-season harvests, (Fonseca et al., 2003).

Soil salinity has become a serious environmental problem currently, approximately 20% of the world’s cultivated land and nearly half of all irrigated lands are affected by salinity (Zhu, 2001). Salinity imposes two constraints on plants: an osmotic effect resulting from the lower soil water potential and an ionic effect resulting from the direct toxicity of saline ions and the ion imbalance in the plants (Munns and Tester, 2008). As a consequence of these primary effects, secondary stresses, such as oxidative damage, often occur (Zhu, 2001). It is well established that the exposure of plants to salt stress can increase the production of reactive oxygen species (ROS), such as superoxide radicals (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). These ROS are so reactive that they seriously disrupt the normal metabolism of plants through oxidation of membrane lipids, proteins and nucleic acids if the plants do not have sufficient protective mechanisms (Apel and Hirt, 2004). Excessive soil salinity reduces productivity of many agricultural crops, including most vegetables which are particularly sensitive throughout the ontogeny of the plant. Plant sensitivity to salt stress is reflected in loss of turgor, growth reduction and potentially death of the plant (Cheeseman 1988; Jones, et al., 1989). Cucumber is highly sensitive to salinity (Zhu et al., 2008a). Moreover, in protected cultivation, cucumber is the primary vegetable, so the secondary salinization of protected soils has badly influenced the growth, development, and yield of cucumber (Wang, 1998).

Great efforts have been made to improve the salt tolerance of many crops by means of traditional breeding programs and, more recently, by genetic transformation (Cuartero et al., 2006). However, commercial success has been very limited owing to the complexity of salt tolerance, which is complex genetically and physiologically (Flowers, 2004).
In this regard, the role of some bioregulators to improve salt tolerance has been mentioned by many workers. Salicylic acid (SA) has been shown as an important signal molecule for modulating plant responses to environmental stress (Bergmann et al., 1994 and Breusegem et al., 2001). Humic substances (HS) and fulvic acid (FA) are relatively stable products of organic matter. They accumulate in the environmental systems to increase moisture retention and nutrient supply potentials of sandy soils (Suganaya and Sivasamy, 2006), improve soil properties, plant growth and the uptake of nutrients in case of the negative effect of salt that would inhibit plant growth and uptake of nutrient elements (Demir et al., 1999; Casierra-Posada et al., 2009; Mehanna et al., 2010). Seaweed extracts, may represent a promising strategy to reduce the use of phytochemicals in agriculture. Seaweed extract is a metabolic enhancer derived by the algae Ascophillum nodosum, it has a constant and balanced formulation containing kahydrin, phytohormones (gibberellins, auxins, cytokinines), alginic acid, macro and trace elements and betaines which synergistically contribute to the efficacy of the product. It has been proposed to increase the mineral nutrient uptake and the abiotic stress tolerance (Francesco et al., 2010).

Other attempts to improve salt tolerance have made using compatible solutes, as accumulation of these metabolites is one of the probable universal responses of plants to changes in the external osmotic potential. Metabolites with osmolytes function like sugar alcohols, complex sugars and charged metabolites are frequently observed in plants under unfavorable conditions (Hasegawa et al., 2000; Sotiropoulos, 2007). Proline and glycine betaine (GB) are known to serve as compatible osmolytes, protectants of macromolecules and also as scavengers of ROS under stressful conditions (Hellman et al., 2000; Ashraf and Foolad, 2007).

On the other hand, vegetable grafting has increased dramatically over the years to improve their adaptation to stresses. Grafting was used widely in vegetable crops focused on Cucurbitaceae (watermelon, melon, cucumber) and Solanaceae (tomato, pepper, eggplant) species (Myung and Oda, 2003; Saccardo et al., 2006). Initially, this technology has been proposed to soil borne diseases. In addition, grafted plants have showed other advantages such as the improvement of tolerance to environmental stresses (abiotic stresses); low and high temperatures (Bulder et al., 1990; Rivero et al., 2003), to improve salt tolerance of plant (Colla et al., 2006), to enhance nutrient absorption (Ruiz et al., 1997), and to improve water use (Cohen and Naor, 2002).

The present study was conducted to investigate the effect of grafting and foliar application of bioregulators (SA, FA and sea weed) and compatible solute (GB) on grafted cucumber under salt stress on several physiological parameters including growth, water status, biochemical constituents, antioxidant enzymes activity and finally yield and fruits quality under salt stress.

**Materials and Methods**

**Plant materials and cultivation**

Experiment was also conducted in a greenhouse in El-Salehea Al-Gadeda, Sharqia Governorate during the two successive seasons of 2008-2009 and 2009-2010 to investigate the effect of grafting, bioregulators [salicylic acid (SA), fulvic acid (FA) and sea weed extract (SWE)] and compatible solute [glycine betaine (GB)] on improving growth and productivity of salt sensitive cucumber plants. Salt sensitive Cucumber (Cucumis sativus, L. cv. falcon, Hybrid F1) from Sakata seed, Europe was used in this study as a scion, and Shintos Supreme pumpkin (Cucurbita maxima x C. moschata), was used as rootstock. Seeds of rootstock ‘Shintos Supreme pumpkin’ were sown on October 18th, 2008 and 2009 in 84 seedling plug trays filled with 2:1 (v:v) mixture of peat and vermiculite. Three days later, seeds of cucumber scion were sown. When seedlings of the rootstock had developed two cotyledon leaves (after 14 day from sowing), the cucumber seedlings with one true leaf were grafted onto the rootstock, using the procedure ‘Root removed single cotyledon splice grafting - RRSG’ described by Seong et al. (2003). The seedlings were incubated for 3-5 days on 25-30°C air temperature, relative humidity of 80-90% and 30-50% shading in the plastic tunnel in greenhouse. Ten days after grafting (5 days after incubation), plants were transferred to production greenhouse. Non-grafted cucumber plants were used as control with the same sowing date of cucumber plants which used as scions. The electrical conductivity (EC) of the soil and irrigation water (ground well) used for irrigation was 2.1 and 3.9 dS m⁻¹, respectively. The pH of the soil and ground
water were maintained close to 7.8 and 8, respectively. So, total salinity under study reached 3776 ppm. It could be calculated by combined soil and water salinity as follow; \((2.1 \times 640) + (3.9 \times 640) = 3776\) ppm. Fertilization was performed as recommended by Ministry of Agriculture.

**Bioregulators and osmoregulators (compatible solute) treatments**

Foliar applications of different bioregulators were carried out on seventh day after transplanting and repeated every twenty-one days intervals, the total number of foliar application reached 8 times. The concentrations of bioregulators were; 2.5, 5 mM glycine betaine (GB), 0.5, 1.0 mM salicylic acid (SA), 150, 300 ppm fulvic acid (FA) and 2.5, 5% sea weed extract (SWE) in addition to distilled water as control. Tween 20 was used as wetting agent at 0.1%. Treatments were arranged in a randomized block design with three replicates each replicate was divided into 12 plots (1 m×45 m /plot) with 100 plants per plot. Samples were taken at 70 days after planting for growth measurements, determination of leaf relative water content, membrane permeability and chemical analyses.

**Growth measurements**

Three plants from each replicate were harvested for growth measurement; plant height, shoot fresh and dry weight, and leaf area were recorded. Dry weight was determined as recommended by A.O.A.C. (2007). The leaf area (LA) was estimated using the equation

\[ LA = 0.88LW - 4.27 \]  
(Flávio and Marcos, 2005).

![Diagram of cucumber leaf showing positions of length (L) and width (W) measurements.](image)

**Fruits yield and quality measurements**

Fruits were harvested during the period of 21-165 days after sowing and total numbers of harvesting were calculated. At each harvest, the total number of fruits per plant \((N^0\text{ Plant}^{-1})\) was recorded separately. Mean fruit weight (g fruit\(^{-1}\)) and total fruit yield per plant (g Plant\(^{-1}\)) were calculated. In each treatment, 15 representatives marketable fruits were sampled (5 fruits/replicate) for determination of total soluble solid (TSS), titratable acidity, juice electric conductivity (EC) and ascorbic acid (Vit. C) concentrations.

**Determination of relative water content (RWC)**

Samples were taken from two plants per replicate (the sixth leaf from the top). Individual leaves first detached from the stem and then weighed to determine fresh weight (FW). In order to
Determine turgid weight (TW), leaves were floated in distilled water inside a closed Petri dish. Leaf samples were weighed periodically, after gently wiping the water from the leaf surface with the tissue paper until a steady state achieved. At the end of imbibition period, leaf samples were placed in a pre-heated oven at 80°C for 48 h, in order to determine dry weight (DW). Values of FW, TW and DW were used to calculate RWC using the equation below (Kaya et al., 2003):

\[ \text{RWC} \% = \left( \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \right) \times 100 \]

**Determination of membrane permeability**

Twenty leaf discs (10 mm in diameter) obtained from two plants per replicate from the young fully expanded leaves sixth leaf from the top were placed in 50 ml glass vials, rinsed with distilled water to remove electrolytes released during leaf disc excision. Vials were then filled with 30 ml of distilled water and allowed to stand in the dark for 24 h at room temperature. Electrical conductivity (EC1) of the bathing solution was determined at the end of incubation period using an electrical conductivity meter (HANNA H199301). Vials were heated in a temperature-controlled water bath at 95°C for 20 min, and then cooled to room temperature and the electrical conductivity (EC2) was measured. Electrolyte leakage was calculated as percentage of EC1/EC2 (Shi et al., 2006).

**Bio-chemical analyses**

All biochemical analyses were determined in the fully expanded sixth leaf from the top of three plants per replicate as follows:

**Determination of chlorophylls and carotenoids**

Leaf discs (0.1g) were taken from the interveinal areas of the fully opened sixth leaf from the top of three plants per replicate. Chlorophyll and carotenoids were extracted using ammoniacal acetone (10 ml). The absorbance of the solution was measured at 470, 647 and 664 nm. Formulae and extinction coefficients used for the determination of chlorophyllous pigments (total chlorophyll and carotenoids) were described by Lichtenhaler and Wellburn (1983). Carotenoids were determined using the method of Shlyk (1971).

**Determination of proline**

Proline concentration was determined according to the method of Troll and Lindsley (1955) modified by Petters et al. (1997). The reaction proceeded for 1 h in boiling water bath and the developed dye was extracted with 1 ml of toluene and measured by the spectrophotometer at 515 nm by using UV–vis spectrophotometer (CT 200 spectrophotometer). The proline concentration was determined by the standard curve of L-proline (Figure 1) and expressed as µg proline g⁻¹ fresh weight (FW).

![Fig. 1. Standard curve of L-proline](image)
Determination of malondialdehyde (MDA)

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) concentration by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). One gram of fresh leaves tissue was homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The contents were centrifuged at 10000 x g for 15 min and the absorbance of suspension was measured at 532 nm in spectrophotometer (CT 200 spectrophotometer).

Determination of antioxidant enzymes activity

Leaf tissues were homogenized in 100 mM chilled sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 15,000 x g for 15 min at 4 ºC. Supernatant was used to measure the activities of phenylalanine ammonia-lyase (PAL), peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and polyphenol oxidase (PPO). Protein concentration was determined according to the method of Bradford (1976). All enzymes activity was calculated per milligram of protein per minute. The proteins concentration were calculated by using the standard curve of bovine serum albumin (BSA) (Figure 2).

![Fig. 2. Standard curve of bovine serum albumin (BSA)](image)

Determination of phenylalanine ammonia-lyase activity (PAL)

The activity of phenylalanine ammonia-lyase (PAL; E.C 4.3.1.5) was determined by He et al., (2001). The PAL assay reaction mixture consisted of 100 µL crude enzyme extract and 900 µl of 6 µmol L-phenylalanine in 500 mM Tris-HCl buffer (pH 8.5). The absorbance was measured by spectrophotometer (CT 200 spectrophotometer) at 290 nm. Phenylalanine ammonia-lyase activity was expressed as (µmol cinnamic acid min⁻¹ mg⁻¹ protein), where one unit was determined as 1 µM of L-phenylalanine converted to trans-cinnamate and NH₃ min⁻¹.

Determination of peroxidase activity (POD)

The activity of peroxidase (POD; EC1.11.1.7) was assayed by the method of Hammerschmidt et al. (1982). The reaction mixture (2.9 ml) consisted of 0.25 % (v/v) guaiacol in 10 mM sodium phosphate buffer (pH 6 containing 10 mM hydrogen peroxide H₂O₂). One international (IU) of enzyme activity was expressed as Δ OD = 0.01 POX activity expressed as unit min⁻¹ mg⁻¹ protein.

Determination of catalase activity (CAT)

The activity of catalase (CAT; EC 1.11.1.6) was determined according to Aebi (1984). Enzyme extract (100 µl) was added to 2.9 ml of a reaction mixture containing 20 mM H₂O₂ and 50
mM sodium phosphate buffer (pH 7.0). One unit of enzyme activity was defined as the decomposition of 1 µmol of H₂O₂ min⁻¹. Catalase activity was expressed as unit min⁻¹ mg⁻¹ protein.

**Determination of superoxide dismutase activity (SOD)**

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was assayed by the method of Beauchamp and Fridovich (1971) by measuring its ability of enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The absorbance was recorded at 560 nm. One unit of SOD activity is the amount of protein required to inhibit 50% initial reduction of NBT under light. The activity of SOD was expressed at unit min⁻¹ mg⁻¹ protein.

**Determination of polyphenol oxidase activity (PPO)**

The activity of polyphenol oxidase (PPO; EC 1.14.18.1) was determined by the method of Oktay et al. (1995). One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001 min⁻¹ ml⁻¹. The enzyme activity was expressed as (unit min⁻¹ mg⁻¹ protein).

**Determination of ascorbate peroxidase (APX)**

Ascorbate peroxidase (APX; EC 1.11.1.11) was assayed by recording the decrease in absorbance at 290 nm due to a decrease in ascorbic acid concentration (Nakano and Asada, 1981). The reaction was started with the addition of H₂O₂ (ε = 2.8 mM cm⁻¹).

**Fruits analyses**

**Determination of total soluble solid (TSS)**

Ten grams of fruit samples were passed through a blander in the laboratory and homogenized using a mortar. The samples were centrifuged and the supernatant of the juice to determine the %TSS using traditional hand-held refractometer (Atago Co, Ltd., Model N1, Tokyo, Japan) (Lee et al., 1999).

**Determination of fruit juice electric conductivity (EC)**

100 grams of fruit samples were mixed through a blander in the laboratory and homogenized using a mortar. The samples were determined the EC using electrical conductivity meter (HANNA HI99301) (Lee et al., 1999).

**Determination of titratable acidity (TA)**

Titratable acidity (TA) was measured in fruits juice that was frozen at -20 °C, immediately after squeezing, to be used for further analysis. Five milliliters of juice were diluted up to 50 ml with distilled water, and then manually titrated with 0.1N NaOH using 2-3 drops of 1% phenolphthalein (Phth) as indicator. The results were calculated as a percentage citric acid (Lee et al., 1999). % TA = [(ml NaOH x 0.1) / (weight of sample titrated) x 0.064 x 100].

**Determination of L-ascorbic acid (Vit. C)**

L-ascorbic acid (Vit.C) was evaluated by the method reported by Iqbal et al., (2010). Accurately weighted 1 g of each fruit sample was placed in 25 ml conical flask, 10 ml of oxalic acid (0.05 M) solution was added and the samples were placed under shade for 24 h for extraction of Vit.C concentration. Each sample was then analysed for Vitamin C at 760 nm compared with the standard L-ascorbic acid (0.1 w/v) solution.
Statistical analysis

Growth Parameters and biochemical analysis were determined by analysis of variance using the General Linear Models procedure of Statistical Analysis system. Significance between means was tested by Tukey’s studentized range test at the 5% probability level (SAS software, Version 9.2, 2009) (SAS Institute, Inc., Cary, USA).

Results

Plant growth and yield

Plant growth

Grafting technique significantly increased cucumber growth comparing with non-grafted plants under salt stress conditions, data in Figure (3 a and b) reveal that grafting of cucumber on salt tolerance rootstock (Shintosa supreme pumpkin) gave more better growth comparing with non-grafted plants in the two successive seasons (2008/2009, 2009/2010), as indicated by fresh and dry weights, plant height and leaf area under salt condition (almost 3800 ppm). Grafted cucumber plants increased shoot fresh weight by 44%, shoot dry weigh by 41%, plant height 86% and leaf area by 102% in first season. The same trend was detected in the second one. In the same manner, foliar application with bioregulators (SWE, SA, and FA) as well osmolytes (GB) significantly improved growth parameters of grafted cucumber plants under salt conditions. The highest significant increase in growth parameters was recorded by SWE at the higher concentration (5%) followed by the lower ones (2.5%), then FA and GB at 300 ppm and 5 mM, respectively compared to other treatments and untreated plants (grafted and non-grafted) at 70 days after planting in both seasons. But SA treatments gave the lowest values comparing with other treatments in both seasons, except for SA at 0.5 mM which gave higher dry weight than other treatments at 70 days after planting in the first season. Non-grafted cucumber plants oriented to downfall after 80 days from planting, whereas grafted plants kept on doing at a later time. Therefore, the sample 70 days after planting covered life span of non-grafted cucumber and allow to compare between non-grafted and grafted cucumber.

Fruits yield

Data presented in Table (1) and Figure (4 a,b,c and d) showed that mean fruit weight significantly increased almost by 16% in grafted plants comparing to non-grafted plants at two successive seasons under saline conditions. However, foliar treatment with SWE at 5% on grafted plant significantly increased mean fruit weight (110.3 g) followed SA at 1mM (107.92 g) then FA at 150 and 300 ppm (106.69 and 106.12 g respectively) in the first season. However, other treatments did not increase mean fruit weight in the first season comparing with untreated plants. On the other hands, in the second season, the highest significant increases in mean fruit weight were recorded by SWE at 5% and GB at 2.5 mM (107.4 and 107.27 g respectively) followed by FA at 300 ppm (106.25 g) then GB at 5 mM (105.84 g) comparing with untreated grafting plant plants (100.76 g). In comparison with untreated grafted plants, all treatments significantly increased fruit number and total fruit yield in both seasons, the highest significant fruit yield were showed by SWE at 5% (13457 and 15895 g plant⁻¹) followed by FA at 300 ppm (12734 and 15194 g plant⁻¹) then GB at 5mM (12717 and 15029 g plant⁻¹) in both seasons respectively, under saline conditions.
Fig. (3 a, b): Effect of sea weed extract (SWE), salicylic acid (SA), fulvic acid (FA) and glycine betaine (GB) on the shoot growth of grafted cucumber plants (GC) onto squash shintoza under salinity stress (3800 ppm) after 70 days from planting during two seasons.
Table (1): Effect of sea weed extract (SWE), salicylic acid (SA), fulvic acid (FA) and glycine betaine (GB) on fruits yield of grafted cucumber plant (GC) onto squash shintoza under salinity stress (3800 ppm) during the two seasons.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Season 2008-2009</th>
<th></th>
<th>Season 2009-2010</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean fruit weight (g fruit⁻¹)</td>
<td>Fruit number (N Plant⁻¹)</td>
<td>Fruit yield (g Plant⁻¹)</td>
<td>Number of harvesting per plant</td>
</tr>
<tr>
<td>Cucumber</td>
<td>90.21</td>
<td>29</td>
<td>2616</td>
<td>10</td>
</tr>
<tr>
<td>GC</td>
<td>104.68</td>
<td>103</td>
<td>10718</td>
<td>28</td>
</tr>
<tr>
<td>GC+SEW 2.5 %</td>
<td>104.63</td>
<td>120</td>
<td>12552</td>
<td>32</td>
</tr>
<tr>
<td>GC+SEW 5%</td>
<td>110.33</td>
<td>122</td>
<td>13457</td>
<td>32</td>
</tr>
<tr>
<td>GC+SA 0.5 mM</td>
<td>103.52</td>
<td>117</td>
<td>12112</td>
<td>32</td>
</tr>
<tr>
<td>GC+SA 1 mM</td>
<td>107.92</td>
<td>117</td>
<td>12625</td>
<td>32</td>
</tr>
<tr>
<td>GC+FA 150 ppm</td>
<td>106.69</td>
<td>119</td>
<td>12696</td>
<td>32</td>
</tr>
<tr>
<td>GC+FA 300 ppm</td>
<td>106.12</td>
<td>120</td>
<td>12734</td>
<td>32</td>
</tr>
<tr>
<td>GC+GB 2.5 mM</td>
<td>103.66</td>
<td>119</td>
<td>12335</td>
<td>32</td>
</tr>
<tr>
<td>GC+GB 5 mM</td>
<td>105.98</td>
<td>120</td>
<td>12717</td>
<td>32</td>
</tr>
<tr>
<td>MSD</td>
<td>1.06</td>
<td>1.98</td>
<td>4.39</td>
<td>1.56</td>
</tr>
</tbody>
</table>

Leaf relative water content

Data in Figure (5) illustrated that relative water content (RWC) was significantly affected by grafting under salt condition (almost 3800 ppm), however, for grafted plants the enhancement in RWC was 12% and 13% comparing with non-grafted plants in both seasons respectively. In comparison to grafted untreated plants, all bioregulators treatments significantly increased RWC. FA at the higher concentration (300 ppm) showed the highest value followed by SWE at 5% then GB at 5 mM, but there was no significant difference between FA at 300 ppm and SWE at 5 % under salt conditions. As well as, there was no significant difference between other treatments. GB at 2.5 mM treatment gave the lowest value in both seasons.

Membrane permeability

The plasma membrane permeability (MP) significantly decreased by 20% and 22% in grafted plants compared with non-grafted plants under saline conditions in both seasons (Figure 5). However, all treatments significantly reduced the negative effect of salinity on plasma membrane permeability of grafted plants in both seasons. GB at the higher concentration (5 mM) gave the highest significant reduction (54% and 50%) of MP in both seasons followed by the lower concentration (2.5mM) then FA at 300 ppm (49% and 45%) and (45% and 39%) respectively, however, there were no significant differences between them in both seasons. As well as, there were no significant differences between FA at 300 ppm and SWE at 5% in both seasons. SWE at 2.5 % and FA at 150 ppm showed the lower values of reduction (25% and 26%) than other treatments in both seasons.
**Fig. (4 a and b ):** Effect of sea weed extract (SWE), salicylic acid (SA), fulvic acid (FA) and glycine betaine (GB) on fruits yield of grafted cucumber plant (GC) onto squash shintoza under salinity stress (3800 ppm) during the two seasons.
Fig. (4 c and d): Effect of sea weed extract (SWE), salicylic acid (SA), fulvic acid (FA) and glycine betaine (GB) on fruits yield of grafted cucumber plant (GC) onto squash shintoza under salinity stress (3800 ppm) during the two seasons.
Biochemical constituents

Biochemical constituents in leaves

Plant pigments:

(A) - Chlorophyll concentration

Data presented in Table (2) showed that, after 70 days from planting, grafted cucumber plants had higher leaf chlorophylls concentration than non-grafted plants under salt conditions in two successive seasons, but this difference was not significant. As well as, bioregulators treatments increased chlorophylls concentration compared with untreated grafted plants in both seasons. But these increments showed insignificant except for; SWE at 5%, had significantly increased chlorophyll concentration (3.54 and 4.08 mg g\(^{-1}\) FW) in both seasons comparing with untreated grafted plants (2.24 and 2.30 mg g\(^{-1}\) FW), in addition to, SWE at 2.5% and GB at 5 and 2.5 mM in the second season. However there was no significant difference between SWE at 2.5% and 5% in both seasons.

(B) - Carotenoids

In comparison to non-grafted plants, at the first sample (Table 2), carotenoids significantly increased in grafted plants, this increment was not significant in the second season. Furthermore, grafted plant (Tables 2), applied with bioregulators (SWE, SA, and FA) as well osmolytes (GB) significantly increased carotenoids concentration comparing with untreated grafted plants in both seasons under saline condition, except for SA at both concentrations in the second season. GB treatments gave the highest significant increment in carotenoids concentration followed by SWE then FA treatments in both seasons. As well as, SA at the lower concentration (0.5 mM) gave the lowest values in both seasons.
Table 2: Effect of sea weed extract (SWE), salicylic acid (SA), fulvic acid (FA) and glycine betaine (GB) on chlorophylls (Chl.), carotenoids (Carot.), proline (prol.), total soluble protein (TSP) and malondialdehyde (MDA) concentrations of grafted cucumber leaves (GC) onto squash shintoza under salinity stress (3800 ppm) after 70 days from planting during the two seasons.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Season 2008-2009</th>
<th>Season 2009-2010</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chl. (mg g(^{-1}) FW)</td>
<td>Carot. (mg g(^{-1}) FW)</td>
</tr>
<tr>
<td>Cucum.</td>
<td>1.67</td>
<td>0.15</td>
</tr>
<tr>
<td>GC</td>
<td>2.24</td>
<td>0.31</td>
</tr>
<tr>
<td>GC+SEW 2.5 %</td>
<td>3.26</td>
<td>0.58</td>
</tr>
<tr>
<td>GC+SEW 5%</td>
<td>3.54</td>
<td>0.62</td>
</tr>
<tr>
<td>GC+SA 0.5 mM</td>
<td>2.31</td>
<td>0.44</td>
</tr>
<tr>
<td>GC+SA 1 mM</td>
<td>2.33</td>
<td>0.45</td>
</tr>
<tr>
<td>GC+FA 150 ppm</td>
<td>2.45</td>
<td>0.49</td>
</tr>
<tr>
<td>GC+FA 300 ppm</td>
<td>2.48</td>
<td>0.51</td>
</tr>
<tr>
<td>GC+GB 2.5 mM</td>
<td>2.66</td>
<td>0.68</td>
</tr>
<tr>
<td>GC+GB 5 mM</td>
<td>2.68</td>
<td>0.68</td>
</tr>
<tr>
<td>MSD</td>
<td>0.68</td>
<td>0.079</td>
</tr>
</tbody>
</table>

Total soluble protein

Data presented in Table (2) showed that grafted plants recorded higher total soluble protein values than non-grafted plants at the first date in both seasons under saline condition, however, the differences between grafted and non-grafted plants in total soluble protein concentrations was significant in the second season. In addition to, all treatments significantly increased total soluble protein concentrations comparing with untreated grafted plants in both seasons, except for 0.5 mM SA in the both seasons. The highest significant values were recorded by GB at 5 mM (recorded 12.83 and 13.97 mg g\(^{-1}\) FW) and GB at 2.5 mM (recorded 12.68 and 13.73 mg g\(^{-1}\) FW) followed by SWE at 5% (recorded 11.38 and 12.83 mg g\(^{-1}\) FW) then FA at 300 ppm (recorded 11.29 and 12.45 mg g\(^{-1}\) FW) comparing with untreated grafted plants (recorded 6.76 and 7.84 mg g\(^{-1}\) FW) in both season respectively.

Proline

Proline concentration was significantly increased in grafted plants compared to non-grafted ones in both seasons under salt stress conditions (Table 2). All bioregulators treatments significantly increased proline concentration in both seasons as compared to untreated grafted plants. The highest values of proline were recorded by FA at both concentrations followed by GB and SA at both concentrations in both seasons. The differences between FA, GB and SA at 1 mM were not significant in the first season, but its reached to significant in the second one. FA at 300 ppm recorded the highest significant values (46.72 and 52.4 µg g\(^{-1}\) FW) in both seasons respectively; however SWE at the lower concentration (2.5 %) gave the lowest significant values (34.34 and 37.77 µg g\(^{-1}\) FW) in both seasons respectively.
Malondialdehyde

From Table (2) it is cleared that, non-grafted cucumber leaves under salt stress had 13.13 and 12.44 μmol g⁻¹ FW of MDA. Whereas, MDA concentration of grafted cucumber leaves was significantly reduced to 9.81 and 8.79 μmol g⁻¹ FW in both seasons respectively. Furthermore, Tables (2) also show that, all bioregulators treatments significantly reduced MDA concentration in both seasons compared with untreated grafted plants. The highest significant reduction was recorded by GB at both concentrations compared to other treatments and untreated grafted plants, in both seasons followed by FA, however, SA at the lower concentration (0.5 mM) gave the lowest reduction values (5.48 and 4.86 μg g⁻¹ FW) in both seasons respectively.

Antioxidant enzymes activity

Grafted cucumber on salt tolerance rootstock (Shintosa supreme pumpkin) significantly increased PAL, POD, CAT, SOD, PPO and APX activities as compared to non-grafted plants under saline conditions (Figure 6 a, b and c). As well as, foliar application by bioregulators (SWE, SA, and FA) as well osmolytes (GB) significantly increased PAL, POD, CAT, SOD, PPO and APX activities compared to untreated grafted plants under saline condition. The highest significant improvement was recorded by GB at 5 mM compared to other treatments and control plants at two dates.

Tabulated results in Figure (6 a, b and c) illustrate that, GB at 2.5 and 5 mM significantly increased PAL activity by 64 and 64.5% respectively, followed by FA at 300 ppm (increased by 57.5%) then SA at 0.5 and 1 mM (increased by 22 and 46 % respectively). In comparison with untreated grafted plants, SWE at 2.5% showed the same PAL activity value (15.98 unit min⁻¹ mg⁻¹ protein). GB at 5 mM recorded the highest significant of POD, CAT, SOD and PPO enzymes activity. SWE was the lowest one, but SWE exceeded SA treatments with CAT activities.

In comparison with untreated grafted plants GB at 5 mM recorded the highest significant activity of APX (increased by 41%) followed by FA at 300 ppm (almost increased by 37%) and GB at 2.5 mM (increased by 35%) then SWE at 5% (increased by 30%). However, there were no significant differences between GB at 5 mM, FA at 300 ppm and GB at 2.5 mM (70 days after planting). Also, other treatments and control did not showed any significant differences, except for 5% SWE comparing with untreated grafted plants.

Biochemical constituents in fruits

Data presented in Table (3) show that grafted plants significantly reduced titratable acidity (TA %), total soluble solids (TSS %) and electrical conductivity (EC) in fruit juice, at the first date in both seasons. There were no significant differences in TA between all treatments and untreated grafted plants under saline condition in the first season; however, these differences were significant in the second season. In comparison with untreated grafted plants, FA at 150 and 300 ppm recorded the highest reduction of TA (21 and 24% respectively) in both season followed by SA at 0.5 and 1mM (25 and 26% respectively) while GB at both concentrations showed the lowest significant reduction (28 and 29% respectively) in the second season.
Fig. (6a, b and c): Effect of sea weed extract (SWE), salicylic acid (SA), fulvic acid (FA) and glycine betaine (GB) on antioxidant enzyme activities (unit min-1 mg-1 protein) of grafted cucumber leaves (GC) onto squash shintoza under salinity stress (3800 ppm) after 70 days from planting.
Data in Table (3) indicated that, all treatments decreased TSS and EC in both seasons. There were no significant differences in TSS between FA at 300 ppm, SA at 1mM, SWE at 5% treatments and untreated grafted plants under saline conditions in the first season. Other treatments showed significant reduction in TSS, at first season. All treatments showed significant reduction in the second season comparing with non-grafted and untreated grafted plants. In comparison with non-grafted plants, Vit.C concentrations significantly increased in grafted plants in both seasons (Table 4) under saline conditions, furthermore, all treatments significantly increased Vit.C concentration in grafted plants comparing to untreated plants in both seasons. However, the highest value for Vit.C was obtained by GB at 5 mM treatment (4.99 and 5.04 mg 100g\(^{-1}\) FW) followed by SWE at 5% (4.73 and 4.71 mg 100g\(^{-1}\) FW) then SA at 1mM (4.38 and 4.50 mg 100g\(^{-1}\) FW) in both seasons respectively. FA at 150 ppm showed the lowest significant values in both seasons.

**Table 3:** Effect of sea weed extract (SWE), salicylic acid (SA), fulvic acid (FA) and glycine betaine (GB) on fruits quality of grafted cucumber plant (GC) onto squash shintoza under salinity stress (3800 ppm) after 70 days from planting during the two seasons.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TA %</th>
<th>Vit.C mg 100g(^{-1})</th>
<th>TSS %</th>
<th>EC</th>
<th>TA %</th>
<th>Vit.C mg 100g(^{-1})</th>
<th>TSS %</th>
<th>EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cucum.</td>
<td>0.4</td>
<td>3.56</td>
<td>2.2</td>
<td>2.03</td>
<td>0.44</td>
<td>3.53</td>
<td>2.4</td>
<td>2.23</td>
</tr>
<tr>
<td>GC</td>
<td>0.31</td>
<td>3.61</td>
<td>1.8</td>
<td>1.96</td>
<td>0.36</td>
<td>3.69</td>
<td>2.1</td>
<td>1.95</td>
</tr>
<tr>
<td>GC+SEW 2.5 %</td>
<td>0.29</td>
<td>4.07</td>
<td>1.9</td>
<td>1.87</td>
<td>0.26</td>
<td>4.25</td>
<td>2</td>
<td>1.86</td>
</tr>
<tr>
<td>GC+SEW 5%</td>
<td>0.28</td>
<td>4.73</td>
<td>2.1</td>
<td>1.86</td>
<td>0.27</td>
<td>4.71</td>
<td>1.9</td>
<td>1.88</td>
</tr>
<tr>
<td>GC+FA 150 ppm</td>
<td>0.28</td>
<td>3.99</td>
<td>1.8</td>
<td>1.88</td>
<td>0.25</td>
<td>3.99</td>
<td>2</td>
<td>1.84</td>
</tr>
<tr>
<td>GC+FA 300 ppm</td>
<td>0.27</td>
<td>3.86</td>
<td>1.9</td>
<td>1.76</td>
<td>0.21</td>
<td>3.79</td>
<td>2.2</td>
<td>1.82</td>
</tr>
<tr>
<td>GC+GB 2.5 mM</td>
<td>0.31</td>
<td>4.09</td>
<td>1.7</td>
<td>1.89</td>
<td>0.28</td>
<td>4.30</td>
<td>1.9</td>
<td>1.89</td>
</tr>
<tr>
<td>GC+GB 5 mM</td>
<td>0.3</td>
<td>4.99</td>
<td>1.9</td>
<td>1.85</td>
<td>0.29</td>
<td>5.04</td>
<td>1.8</td>
<td>1.87</td>
</tr>
<tr>
<td>MSD</td>
<td>0.054</td>
<td>0.0183</td>
<td>0.158</td>
<td>0.017</td>
<td>0.014</td>
<td>0.0183</td>
<td>0.118</td>
<td>0.015</td>
</tr>
</tbody>
</table>

*Titratable acidity (TA), L-ascorbic acid (Vit.C), Electric conductivity (EC), Total soluble solid (TSS).*

**Discussion**

**Effect of grafting**

Grafted cucumber on salt tolerance rootstock (Shintoza supreme pumpkin) markedly enhanced vegetative growth, fruit yield and quality comparing with non-grafted (own-rooted) cucumber under 3780 ppm of salinity (soil + irrigation water) as indicated by plant height, number of internodes / plant, root and shoot dry weights, mean fruit weight, fruit yield / plant and number of harvesting. Grafting improved fruits quality by reducing titratable acidity (TA%), total soluble solids (TSS%) and electrical conductivity (EC) in fruit juice and increasing Vit.C concentration. These results were confirmed by Canizares *et al.* (2000); Zeng *et al.* (2004); Zhong and Bie, (2007) and Huang *et al.* (2009b).

Water status in plant under salt stress is the most limiting factor allows resuming growth (Yeo *et al.*, 1985). However, the aforementioned improvement in growth and yield in grafted plants was parallel with an increase in LRWC, chlorophylls and carotenoids concentrations and reduction in
membrane permeability. The reduction in cell membrane permeability in leaves of grafted plants under salt stress has been mentioned by Chen and Wang (2008). Zhu et al. (2008b) reported that, shoot water content in the leaves of grafted plants was higher, than those of non-grafted plants at the same NaCl stress.

For better understanding salt tolerance mechanism in grafted plants which positively affected cucumber plants under salinity condition in different aspects, it was planned in this study to investigate compatible solute such as proline, antioxidant enzymes activity and malondialdehyde concentration. When plants experience unfavorable environmental conditions associated with salt, drought or low temperature, plant cell protect themselves from the stress of high concentration of intercellular salts by accumulating a variety of small metabolites that are referred to collectively compatible solutes. The accumulated osmolytes function not only restricted in osmotic adjustment, but also extended to other mechanisms for scavenging reactive oxygen species (ROS), Bohnert et al. (1995). In this concern, the present study showed significant increase in proline in grafted plants under salinity conditions.

Also, these ROS are scavenged by a variety of antioxidant defense systems that prevent ROS from reaching toxic levels, which may lead to proteins degradation, membrane damage (Chen and Murata, 2008). It was found in this study an increase in antioxidant enzymes activity (PAL, POD, CAT, SOD, PPO and APX) in grafted plants comparing with non-grafted ones. This antioxidant system was the important reason for salt tolerance of grafting plants and the protection of cell membranes from damage as noticed by decreased malondialdehyde (MDA) concentration and membrane permeability in the grafted plants, these results agree with Yang et al. (2006b); Huang et al. (2009b). Zhu et al., (2008b) reported that ROS (H$_2$O$_2$ and O$_2^-$) production rate were lower, whereas SOD, POD, CAT and APX activities in the leaves of grafted plants were higher, than those of non-grafted plants at the same NaCl stress.

Effect of bioregulators

There are no direct reports in literature concerning the effect of bioregulators on grafted cucumber under salt stress conditions. Several foliar applications (8 times) of bioregulators including seaweed extract (SWE), glycine betaine (GB), salicylic acid (SA) and fulvic acid (FA) on grafted cucumber plant increased significantly all growth parameters, number of harvesting, life span and total yield as well as fruit quality. The physiological mechanism of applied bioregulators greatly varied.

SWE exerted its affect due to its contain of several plant hormones (gibberellins, cytokinins and auxins), macro and micro nutrients and betaine (Francesco et al., 2010). The effect of previous promoter hormones in increasing plant growth, fruit setting, retardant senescence and increasing fruit yield was documented in literature (Arteca, 1996). Furthermore, the cytokinin and auxin in SWE could be improve the vascular conductivity between rootstock and scion at graft-union (Shehata and El-Shraiy, 2010) and this led to better translocation of water and nutrients. The effect of foliar spraying of SWE on non-grafted cucumber under salt stress revealed that SWE increased root/shoot ratio, dry mater content and total uptake area and active uptake of root. SWE could increase plant pigments and reduce the effect of salinity stress on membrane permeability and enhance several of antioxidant enzymes in cucumber under salt stress (Wang et al., 2005). Finally SWE could alleviate the deleterious effect of salt stress on cucumber.

As for, the promotion effect of GB foliar applications on the growth and productivity of grafted cucumber, GB exerted its effect through osmoregulation in the cells by scavenging ROS, increasing total soluble protein and proline concentrations. Accordingly, GB decreased membrane oxidation (membrane lipid peroxidation) via activation of several antioxidant enzymes. Obviously, GB markedly increased all previous physiological parameters comparing with other bioregulators treatments. Several reports were found to confirm the present results (Suriyan and Chalermpol, 2010; Zhao and Niu, 2009).

Fulvic acid is a by-product of humic acid. Humic acid is extracted from material containing well-decomposed organic matter-soil, coal, composts, etc. As humic material is decomposed by living microbes, these microbes create the most biologically complex organic compounds on earth-fulvic acid (Solange and Rezende, 2008). Fulvic Acids are more plant active than humic acids due to their
higher oxygen content, abundance of carboxyl groups and soluble in water under all pH conditions (Kulikova et al., 2005). Fulvic acid has chemical properties that allow plants to absorb more nutrients, and increases water storage capacity within a plant. Essential nutrients and vitamins, which plants may not be able to assimilate easily, will piggyback on the fulvic acid, to be transported to all cells that need them. Fulvic is so powerful that one fulvic molecule is capable of carrying 60 or more minerals and trace elements into the cells. It also prolongs the time that essential nutrients remain in the plant cells, maximizing nutritional potential. Fulvic acid increases plant metabolism, naturally increasing growth. Plants treated with a regular diet of fulvic acid have a greater resistance to fluctuations in pH. Fulvic acid act as "free-radical" scavengers, supplying vital electrolytes, enhance transport nutrients, catalyze enzyme reactions, increase assimilation, stimulate metabolism, chelate and change inorganic minerals into organically complex minerals, solubilize, energize, and transport major and trace elements to the site of need, and demonstrate amazing capacity for electrochemical balance (Nardi, 1996).

Rauthan and Schnitzer (1981) indicated that fulvic acid enhances nutrient uptake and plant growth in the soil solution, or in a foliar spray. Early in the season when plants are small, fulvic acid can be achieved in the soil solution. Once the plant expands its root system and develops a full canopy and extensive root system, the best way to promote better growth is through foliar applied fulvic acids.

Foliar application of SA was usually used to improve the adaptability of salt-sensitive cucumber under salt stress (Shang et al., 2007; Dong et al., 2009). However, the foliar application of SA on grafted cucumber increased all previous physiological parameters, but their increase ranked after SEW, GB and FA respectively.

Finally, foliar applications of SWE, SA, FA and GB significantly improved growth and yield parameters, and biochemical contents of grafted cucumber plants compared to untreated grafted plants under salt conditions. The superiority was due to SWE (5%) followed by FA (300 ppm) then GB (5 mM). From a human safety and commercially points of view, SWA at 5% application is recommended to use on grafted cucumber plants under salinity stress.

Conclusion

It was concluded in this study that the use of salt-tolerant rootstock (Shintosa supreme pumpkin) could provide a useful tool to improve plant growth, fruit yield and quality of cucumber under salt stress. As well as, application of bioregulators (SWE, FA and SA) and osmolytes (GB) improved physiological modulation in the plants to enhance salt tolerance of grafted plants. From a human healthy and commercially points of view, SWA at 5% application is recommended on grafted cucumber plants under salinity stress condition.

References


