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STOMATAL DENSITY IN THE LEAVES OF SWEET PEPPER PLANT AS AFFECTED BY CERTAIN BIO-STIMULANTS UNDER SALT STRESS CONDITIONS

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ABSTRACT

Increasing salinity levels of the two applied types decreased the number of stomata and its density on both surfaces of sweet pepper leaves as compared with control. In addition, NaCl at 4000 mg/L was more effective in this respect followed by NaCl+CaCl2 and CaCl2. On the other hand, pre-soaking sweet pepper seeds in AsA at 50 mg/L or SA at 75 mg/L increased the number of stomata and its density on both leaf surfaces as compared to control and such salinity levels. Moreover, AsA at 50 mg/L proved to be more affected than SA treatment in this respect.

INTRODUCTION

Sweet pepper (*Capsicum annuum* L.) is among the most important crops for the world human nutrition and its fruits have a good nutritional value in respect to antioxidant compounds such as vitamin C and carotenoids (Navarro *et al.,* 2006).

Salinity is a common problem for agricultural productivity as a condition where the salts in solution within the crop root zone accumulate in concentration which decrease crop yield. The plant growth is ultimately reduced by salinity stress but plant species differ in their salinity tolerance (Jamil *et al.,* 2005).

Salinity is known to affect many aspects of metabolism of plants and induces changes in their anatomy and morphology (El-Banna , 2006). These changes are often considered to be adaptive , thus increasing the chances of survival during salinity stress.

Exogenous application of antioxidants has recently gained a ground as a very promising means of mitigating the adverse effects of salt on plant growth and metabolism (Shalata and Neumann, 2001). A number of morphological changes have been observed on exogenous application of phytohormones, vitamins and yeast extract. Recently, several studies concentrated on the use of vitamins (ascorbic acid) a α-tocopherol) or phytohormones (Salicylic acid) and yeast to improve plant salt tolerance and/or reduce the harmful effect of salinity stress on plant growth (Gadalla, 2009 b; El-Banna , 2006; Ali, 2001; Elwan and El-Hamahmy, 2009).

Therefore, the present investigation was carried out to study the effect of two types of salinity on stomatal density and number on the leaves of sweet pepper plant. In addition, it was intended to investigate the ability of some bio-stimulants to alleviate the harmful of salinity stress on the tested stomatal characters.

MATEREIALS AND METHODS

The experiment was carried out in the glasshouse of the Agricultural Botany Dept., Fac. of Agriculture, Mansoura Univ. during the growing season of 2008, to study the response of stomatal density in the leaves of sweet pepper plant to different sources of salinity i.e. NaCl, $CaCl_2$ and their combination (1:1 w/w); and how to minimize its harmful effects through pre-soaking seeds in vitamin (Ascorbic acid) or bio-regulators (Salicylic acid).

Plant materials

The seeds of sweet pepper (*Capsicum annuum* L. cv. Orlando), a hybrid 'California Wonder' used in this investigation were secured from the Gohara Co. Cairo, Egypt.

Chemicals:-

1. Vitamin, ascorbic acid, Vit. C (AsA) was supplied by Sigma Chemicals Co., USA and used at the concentration of 50 mg/L.

2. Bio-regulator, salicylic acid (SA) (2-hydroxybenzoic acid) was obtained from Sigma Chemicals, Co., USA. and initially dissolved in 100 µL dimethyl sulfoxide and used at the concentrations of 75 mg/L,

3. Salts:

Sodium Chloride (NaCl) from EL-Gomhoria Co., Egypt and was used at the concentrations of 2000 and 4000 mg/L.

Calcium Chloride (CaCl2) from EL-Gomhoria Co., Egypt and was used at the concentrations of 2000 and 4000 mg/L.

Their combination, NaCl: CaCl2 1:1 (w/w) was used at the concentrations of 2000 and 4000 mg/L.

Table (1): The Molarity (Mol), Electrical Conductivity (E.C.) and pH values for different nutrient solutions.

Nutrient solution (N.S.) mg/L	N.S.	N.S.+ NaCl		N.S.+ $CaCl_2$		N.S.+ {NaCl+$CaCl_2$} (1:1) w/w			
						2000(NaCl+$CaCl_2$)		4000 (NaCl+$CaCl_2$)	
		2000 NaCl	4000 NaCl	2000 $CaCl_2$	4000 $CaCl_2$	1000 NaCl	1000 $CaCl_2$	2000 NaCl	2000 $CaCl_2$
Mol (M)	0 (Control)	3.4×10^{-2}	6.9×10^{-2}	2.0×10^{-2}	3.6×10^{-2}	1.7×10^{-2}	0.9×10^{-2}	3.4×10^{-2}	2.0×10^{-2}
Ec dSm^{-1}	2.00	5.42	8.42	4.59	7.60	5.08		8.08	
pH	5.50	5.77	5.80	5.19	5.30	5.45		5.34	

Table (2): Weights (g) of pure substances to be dissolved in 1000 liters of water to give the theoretically ideal concentrations (Cooper, 1979).

Substance	Formula	Weight
Potassium dihydrogen Phosphate	KH_2PO_4	263
Potassium Nitrate	KNO_3	583
Calcium Nitrate	$Ca(NO_3)_2$. 4H2O	1003
Magnesium Sulphate	$MgSO_4$. $7H_2O$	513
EDTA Iron	$CH_2.N(CH_2.COO)_2]_2$ Fe Na	79.0
Manganous Sulphate	$MnSO_4.H_2O$	6.10
Boric Acid	H_3BO_3	1.70
Copper Sulphate	$CuSO_4.5H_2O$	0.39
Ammonium Molybdate	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.37
Zinc Sulphate	$ZnSO_4.7H_2O$	0.44

After soaking, the sterilized seeds (25 seeds/dish) were placed in glass Petri dishes (11 cm) with a double layer of Whatman No. 1 filter paper. The dishes were left in an incubator in the dark for seed germination at 25 ± 2^0C and 90% relative humidity, and then dishes were covered with aluminum foils for darkness. In order to avoid water losses, 5 ml of the nutrient solution were added to Petri dishes, every 5 days. Thiram was added to the solution at a concentration of 2% (w/v) to control the fungi infection.

Table (3):Composition of yeast extract (according to, Nagodawithana, 1991)

Constituents		**Value (%)**	
Protein		47	
Carbohydrates		33	
Minerals		8	
Nucleic acids		8	
Lipids		4	
Approximate composition of vitamins			
Vitamines		**Value (µg/g)**	
Cholin		4000	
Niacin		300-500	
Thiamine (B_1)		60-100	
Pantorhenate (B_5)		70	
Riboflavin (B_2)		35-50	
Pyridoxine HCL (B_6)		28	
Folic acid		5-13	
Biotin		1.3	
Vit. B_{12}		0.001	
Approximate composition of minerals			
Minerals	**Value (mg/g)**	**Minerals**	**Value (µg/g)**
K	21	Cu	8.00
P	13.50	Ni	3.00
S	3.90	Sn	3.00
Mg	1.65	Cr	2.20
Ca	0.75	Mo	0.40
Zn	0.17	Se	0.10
Na	0.12	Li	0.17
Si	0.03	Va	0.04
Fe	0.02	Mn	0.02

The following experiment was carried out in the glasshouse of the Agric. Bot. Dept., Fac. of Agric., Mansoura Univ. during the spring–summer period of 2008 in a glasshouse under conditions of ambient light during winter, spring and early summer, with 10/14 light/dark period at 800–1100 µmol m^{-2s-1} PPFD, a day/night average temperature cycle of 26/15 ^{o}C and 65±5% relative humidity.

The target of the current experiment was to provide fundamental biological understanding and knowledge on sweet pepper plants growing in nutrient film technique (NFT), under different sources of salinity NaCl, $CaCl_2$ and their combinations 1:1 (w/w); and how to minimizing the harmful effects

through pre-soaking seeds in vitamin (Ascorbic acid) or bio-regulator (Salicylic acid). The seeds of sweet pepper were sown on January 13, 2008. A homogenous sweet pepper seeds were placed in 100 ml beakers and 20 ml of 1% sodium hypochlorite was added for sterilization. These were left in the solution for 5 min followed by washing under running tap water and ionized water twice then divided into 3 sets. The first set was soaked (24hours) in distilled water as control and the remaining sets (2) were separately soaked for 24 h in aqueous solution of AsA at 50 mg/L or SA at 75 mg/L. Then germinated in seedling trays (209 eye) containing peat moss and perlite (1:1) as a rooting medium moistured by nutrient cooper solution (Cooper, 1979). Trays containing the seeds were placed in a glasshouse at 28 $\pm 2^0$C to germinate.

The experimental layout consisted of 7 automatic hydroponic units (groups) (experimental plots). Each hydroponic unit comprised of two plastic channels (4 m long * 10 cm in diameter) placed on one side of the holder (4m length * 1.5 m height). Each channel had 40 pores (6 cm diameter). Every unit was provided by an electric pump representing seven groups (Table, 1) nutrient solution (2.0 dSm^{-1} as a control), 2000 mg/L NaCl (5.42 dSm^{-1}), 4000 mg/L NaCl (8.42 dSm^{-1}), 2000 mg/L $CaCl_2$ (4.59 dSm^{-1}), 4000 mg/L $CaCl_2$ (7.60 dSm^{-1}), 2000 mg/L NaCl+$CaCl_2$ (1:1) (5.08 dSm^{-1}) and 4000 mg/L NaCl+$CaCl_2$ (1:1) (8.08 dSm^{-1}).

The seedlings were transplanted to the experimental installation on Feb, 26 , 2008 (after 45 days from pre-soaking) at the stage of four/five true leaves. Two uniform seedlings were transplanted to 6 cm perforated pots (reticulated) containing peat moss and perlite (1:1) as a rooting medium.

Every two channels were divided into 3 sets, the first set was soaked in distilled water (control), AsA, at 50 mg/L and SA at 75 mg/L. Each set contained (8 replicates) 16 seedlings (two seedling/pot) spaced 10 cm representing a Nutrient Film Technique (NFT).

To keep the concentrations of sodium chloride and mineral nutrients constant, the solution was changed every 7 to 10 days and the volume of the solution was maintained by adding distilled water as required after measuring the electrical conductivity by digital conductivity meter Lutron CD-4301. A nutrient solution was pumped into the channels at a flow rate of one liter per minute from a reservoir containing 10 liters.

Sampling dates:

Samples were made at 45 days after transplanting (90 days from sowing) to study the following measurements **of** stomatal density and its number**.**

Leaf impressions were made on the adaxial (upper) and abaxial (lower) surfaces at the central portion of the 3rd upper leaf below the shoot tip. Imprints were made on the leaf using cellulose acetate in acetone (clear nail varnish) following the procedure of Rowland-Bamford *et al.* (1990). After drying for 3-5 min, each imprint was peeled off from the leaf using a transparent tape and mounted on a glass slide.

The stomatal density per unit area on the adaxial and abaxial surfaces was counted using a light microscope (Nikon, Japan at 4000 x) and the actual number of stomata was counted in a microscopical field area (0.25

mm2) with 10 random replicates per leaf from each sample (Romero-Aranda *et al.,* 2001).

Statistical analysis:

The obtained data were subjected to statistical analysis of variance according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Data presented in Tables (4 and 5) and illustrated in Figures (1-5) reveal that increasing salinity levels from 2000 to 4000 mg/L of all applied salinity types NaCl, $CaCl_2$ and their combination (1:1 w/w) decreased the number of stomata (field 400X) on adaxial and abaxial surfaces of sweet pepper as compared with control (Table 4) . Moreover, the stomatal density (stomata/mm2) was also decreased on adaxial and abaxial surfaces as compared to control (adaxial and abaxial) as shown in Table (5).

Table (4) Effect of NaCl, $CaCl_2$ and their combination or SA, AsA, as well as their combinations on Number of Stomata (field 400X) on leaf of sweet pepper adaxial and abaxial surfaces.

Salinity (A) / Treatment (C) mg/L		N.S.	N.S.+ NaCl			N.S.+ $CaCl_2$			N.S.+ (NaCl+$CaCl_2$) (1:1) w/w			Mean (C)
			Conc. (B)			Conc. (B)			Conc. (B)			
			2000	4000	Mean (A*C)	2000	4000	Mean (A*C)	2000	4000	Mean (A*C)	
No. stomata of adaxial surface												
Water		7.3	4.1	2.6	4.7	6.2	3.9	5.8	5.1	3.6	5.3	5.3
SA 75		7.7	4.1	3.4	5.1	6.5	3.9	6.0	5.4	3.7	5.6	5.6
AsA 50		10.2	4.6	3.5	6.1	6.6	3.9	6.9	5.9	3.8	6.6	6.5
Mean	A	8.4	5.3			6.2			5.9			
	B		5.4	3.6								
	A*B		4.3	3.2		6.4	3.9		5.5	3.7		
LSD at 0.05		A; 0.4	B; 0.4	C; 0.4		A*B; 0.6	A*C; 0.6		B*C; 0.6		A*B*C; 1.1	
No. stomata of abaxial surface												
Water		35.2	27.7	14.6	25.8	33.2	25.5	31.3	31.4	23.5	30.0	29.1
SA 75		37.3	28.3	21.1	28.9	33.3	25.6	32.1	32.5	25.1	31.6	30.9
AsA 50		48.7	28.4	21.6	32.9	33.3	26	36.0	32.7	25.3	35.6	34.8
Mean	A	40.4	29.2			33.1			32.4			
	B		31.2	23.1								
	A*B		28.1	19.1		33.3	25.7		32.2	24.6		
LSD at 0.05		A; 0.9	B; 0.9	C; 0.9		A*B; 1.5	A*C; 1.5		B*C; 1.5		A*B*C; 2.6	

Table (5):Effect of NaCl, $CaCl_2$ and their combination or SA, AsA, as well as their combinations on Stomatal Density (No. stomata/mm^2) on leaf of sweet pepper adaxial and abaxial surfaces.

Salinity (A) / Treatment (C) mg/L		N.S.	N.S.+ NaCl			N.S.+ $CaCl_2$			N.S.+ ($NaCl+CaCl_2$) (1:1) w/w			Mean (C)
			Conc. (B)		Mean (A*C)	Conc. (B)		Mean (A*C)	Conc. (B)		Mean (A*C)	
			2000	4000		2000	4000		2000	4000		
Stomatal Density of adaxial surface												
Water		29.2	16.4	10.4	18.7	24.8	15.6	23.2	20.4	14.4	21.3	21.1
SA 75		30.8	16.4	13.6	20.3	26.0	15.6	24.1	21.6	14.8	22.4	22.3
AsA 50		40.8	18.4	14.0	24.4	26.4	15.6	27.6	23.6	15.2	26.5	26.2
Mean	A	33.6	21.1			25.0			23.4			
	B		21.6	14.4								
	A*B		17.1	12.7		25.7	15.6		21.9	14.8		
LSD at 0.05		A; 1.5	B; 1.5		C; 1.5	A*B; 2.5		A*C; 2.5	B*C; 2.5			A*B*C; 4.4
Stomatal Density of adaxial surface												
Water		140.8	110.8	58.4	103.3	132.8	102.0	125.2	125.6	94.0	120.1	116.2
SA 75		149.2	113.2	84.4	115.6	133.2	102.4	128.3	130.0	100.4	126.5	123.5
AsA 50		194.8	113.6	86.4	131.6	133.2	104.0	144.0	130.8	101.2	142.3	139.3
Mean	A	161.6	116.8			132.5			129.6			
	B		124.8	92.6								
	A*B		112.5	76.4		133.1	102.8		128.8	98.5		
LSD at 0.05		A; 3.4	B; 3.4		C; 3.4	A*B; 5.9		A*C; 5.9	B*C; 5.9			A*B*C; 10.3

N.S.= Nutrient Solution (Control)	**SA = Salicylic acid**
AsA = Ascorbic acid	

In addition, NaCl at 4000 mg/L was more effective in this respect followed by $NaCl+CaCl_2$ and $CaCl_2$. These results are in agreement with those recorded by Cavusoglu et al. (2008) who reported that salt stress decreased radish stomata number.

Data presented in the same tables and figures reveal that, pre-soaking sweet pepper seeds in AsA at 50 mg/L and SA at 75 mg/L increased the number of stomata on adaxial surface (10.2 and 7.7) and abaxial one (48.7 and 37.3) as compared to control (7.3) on adaxial and (35.2) on abaxial. In addition, the stomatal density (stomata/mm2) was increased by (40.8 and 30.8) on adaxial surface as well as (194.8 and 149.2) on abaxial surface with treatments by AsA and SA respectively as compared to control (29.2) on adaxial and (140.8) on abaxial surfaces.

Regarding the interaction, between salinity levels and AsA or SA, the application of AsA at 50 mg/L and SA at 75 mg/L increased the number of stomata (field 400X) as well as the stomatal density (stomata/mm2) on both surfaces adaxial and abaxial as compared to untreated plants under such salinity levels in this respect, AsA at 50 mg/L was more effective than SA treatment.

The leaf structure is generally assumed to provide means of controlling water loss from plants while allowing for photosynthesis (Jones 1998). Stomata influenced in many aspects plant growth and metabolism by

controlling the exchange of gases (water vapor and CO_2) between the leaf interior and atmosphere. Gas exchange is regulated by controlling the stomatal aperture and density on the epidermis (Hetherington and Woodward 2003). In addition, the influence of stomata on these processes differs by environment. The shape of epidermal cells of the sweet pepper irregular and sinuous. The leaves are amphistomatic with more stomata on abaxial (lower) surface than the adaxial (upper) one Fig. (1 a). In this investigation, the stomatal density was highest on the abaxial surface (140.8) than adaxial surface (29.2) as recorded in Table (5).

The variation in stomatal density between the adaxial and abaxial leaf surfaces was also reported on Gerbera jamesonii (Romero-Aranda *et al.,* 1994). In addition, Ishimaru *et al.* (2001) working on rice plant revealed that on leaf surfaces, the abaxial (lower) surface had more stomata than the adaxial (upper) surface and a strong positive correlation of stomatal density between these surfaces was found. Moreover, Weng and Chen (1987) reported that the high stomatal density was associated with the high photosynthetic rate.

Rapid stomatal responses to environmental changes are important in maintaining the movement of water from soil to the leaf (Hetherington and Woodward 2003), and the role of stomatal size in this control had been widely demonstrated in plants. In addition, Muchow and Sinclair (1989) reported that the low stomatal density (larger stomata) in sorghum was associated with low epidermal conductance which was directly related to the survival of plants under severe water stress.

Small stomata which are generally associated with high densities can open and close more rapidly and provide the capacity for rapid increase in the leaf stomatal conductance, thereby maximizing CO_2 diffusion into the leaf when conditions for photosynthesis become favorable (Hetherington and Woodward 2003). As suggested by Ohsumi *et al.* (2007), one way to achieve high stomatal conductance (as exhibited by the high yielding cultivars) was to have high stomatal density.

Stomatal closure is one of the mechanisms of drought-avoidance, because it reduces transpiration water loss, thereby contributing to increased water use efficiency in water-stressed conditions (Price *et al.,* 2002). Under water-limited conditions, bigger stomata may be more advantageous because they transpire less. Some reports indicated the influence of stomatal conductance on crop growth rate during reproductive period and yield of some crops species (Fischer *et al.,* 1998) which may be associated with stomatal density. As mentioned above, high stomatal densities provide the capacity for rapid increase in the leaf stomatal conductance that maximizes CO_2 diffusion into the leaf during favorable conditions for photosynthesis. This feature is important during the ripening phase of the sweet pepper plant where current photosynthesis is the source of assimilates for fruit development.

It could be concluded that presoaking seeds of sweet pepper plant in ascorbic acid at 50 mg/L may be useful for overcoming the harmful effects of both studied types of salinity on the stomata density on both leaf surfaces .

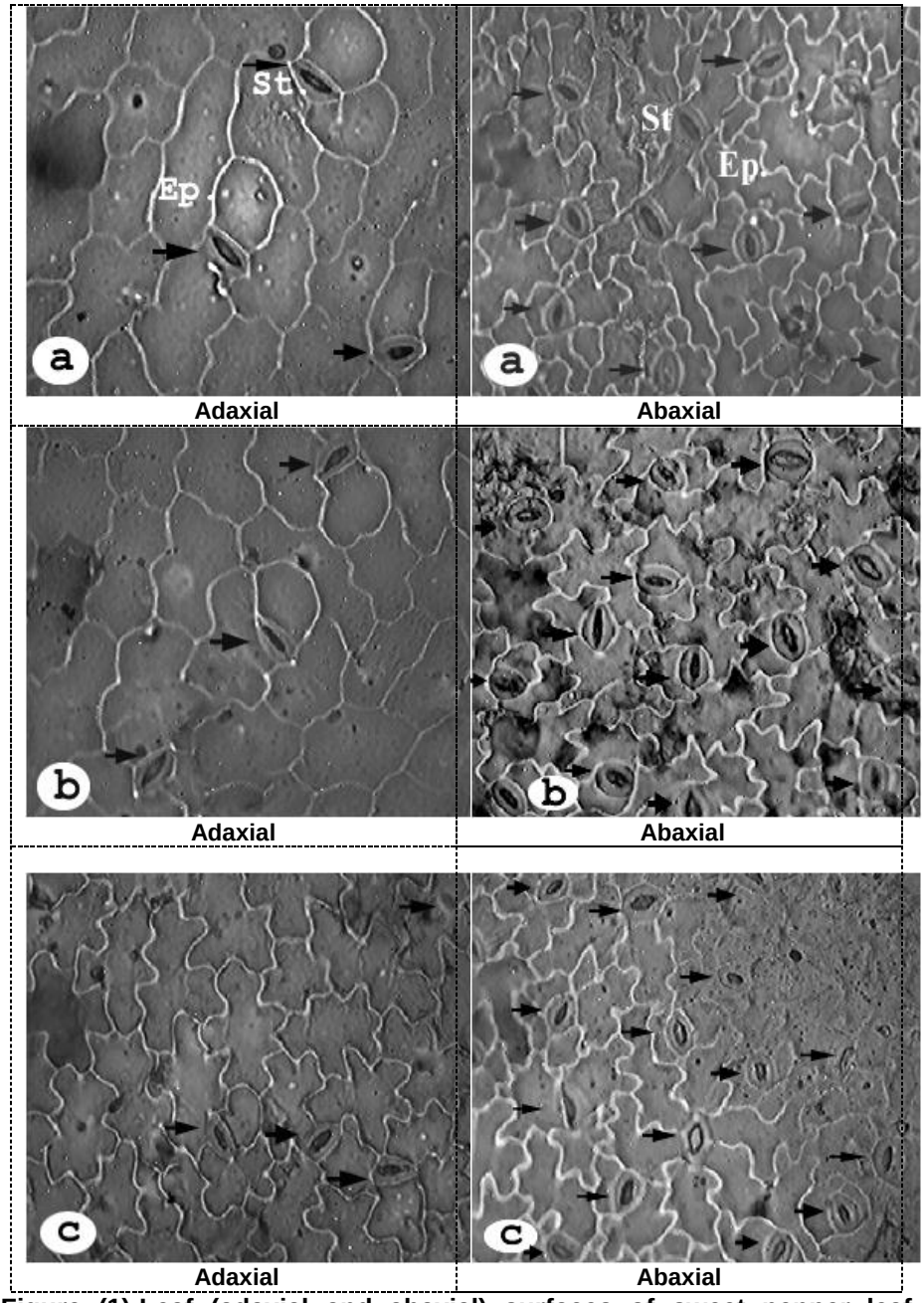


Figure (1):Leaf (adaxial and abaxial) surfaces of sweet pepper leaf showing the effect of pre-soaking seeds in SA and AsA under non-saline conditions (x400).

a = Control	
b = SA at 75 mg/L	**c = AsA at 50 100 mg/L**

Abbreviations: St.= Stomata; Ep.= Epidermis cell

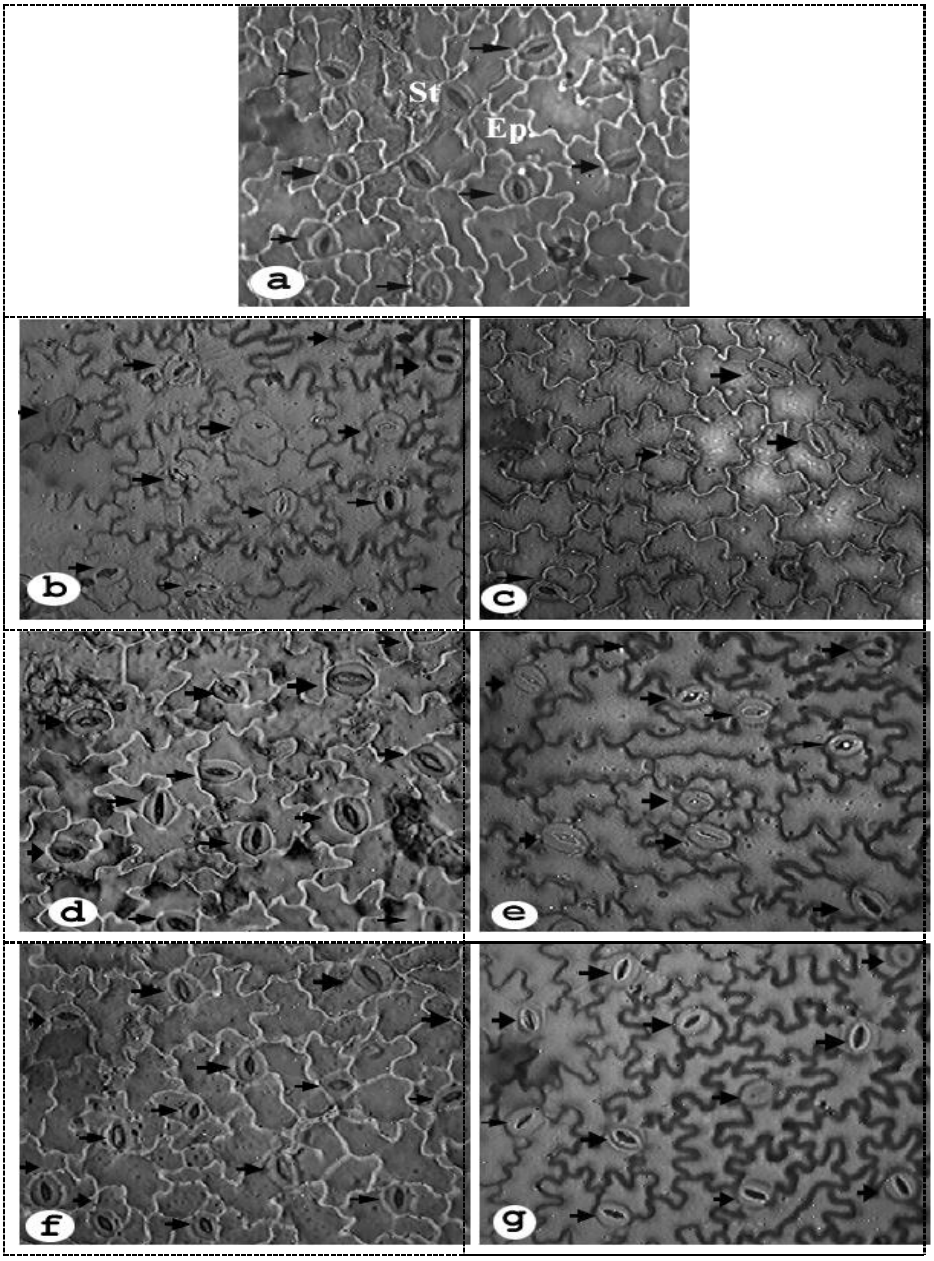
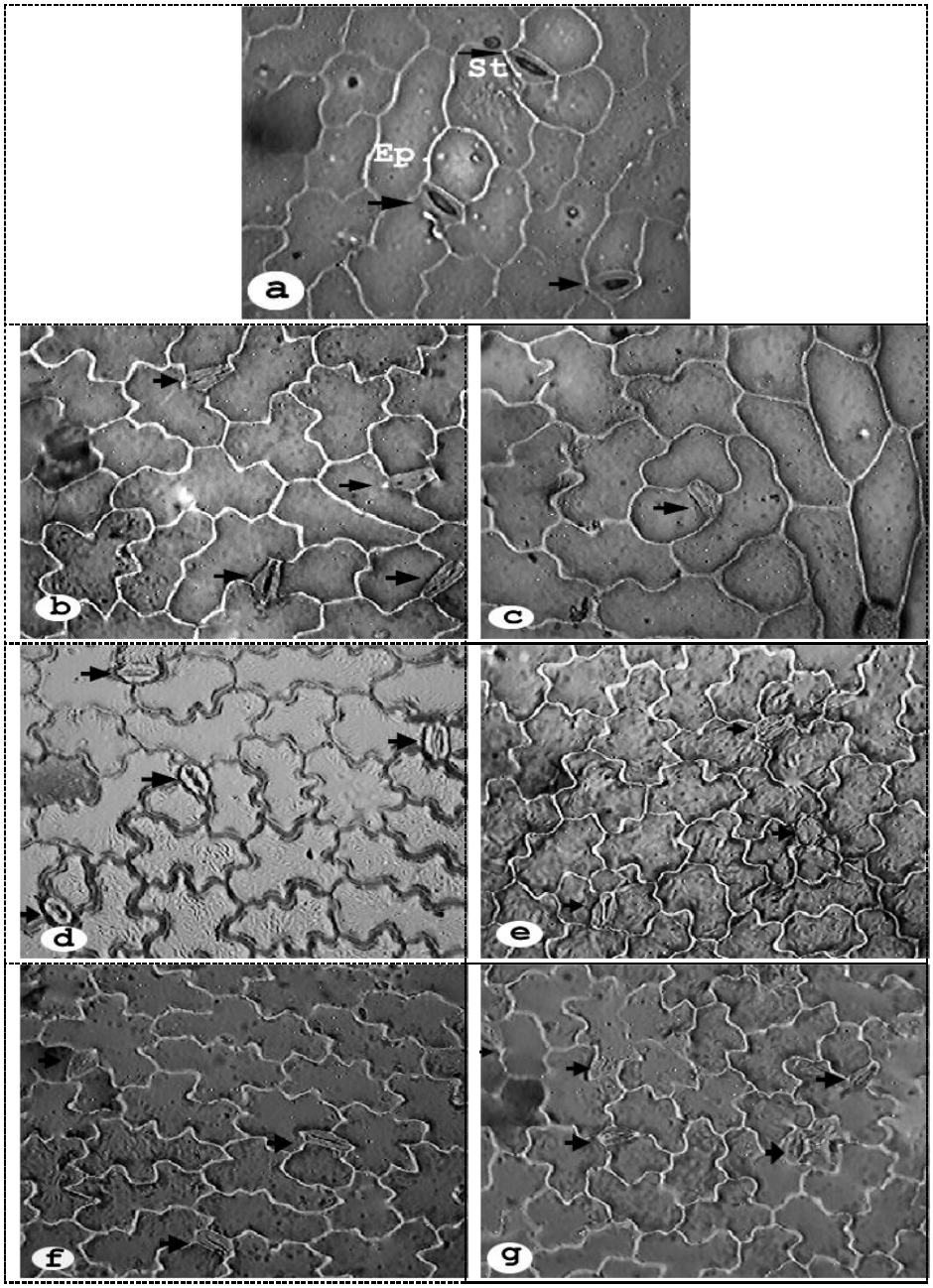


Figure (2): Leaf abaxial surface of sweet pepper leaf showing the effect of salinity applied types (NaCl, $CaCl_2$ and their combination 1:1) (x400).

a = Control	b, c = NaCl at 2000 and 4000 mg/L
d, e = $CaCl_2$ at 2000 and 4000 mg/L	f, g = NaCl+$CaCl_2$ 1:1 at 2000 and 4000 mg/L

Abbreviations: St.= Stomata; Ep.= Epidermis cell



Figure(3:Leaf adaxial surface of sweet pepper leaf showing the effect of salinity applied types(NaCl,$CaCl_2$ and their combination1:1) (x400).

a = Control	b, c = NaCl at 2000 and 4000 mg/L
d, e = $CaCl_2$ at 2000 and 4000 mg/L	f, g = NaCl+$CaCl_2$ 1:1 at 2000 and 4000 mg/L

Abbreviations: St.= Stomata; Ep.= Epidermis cell

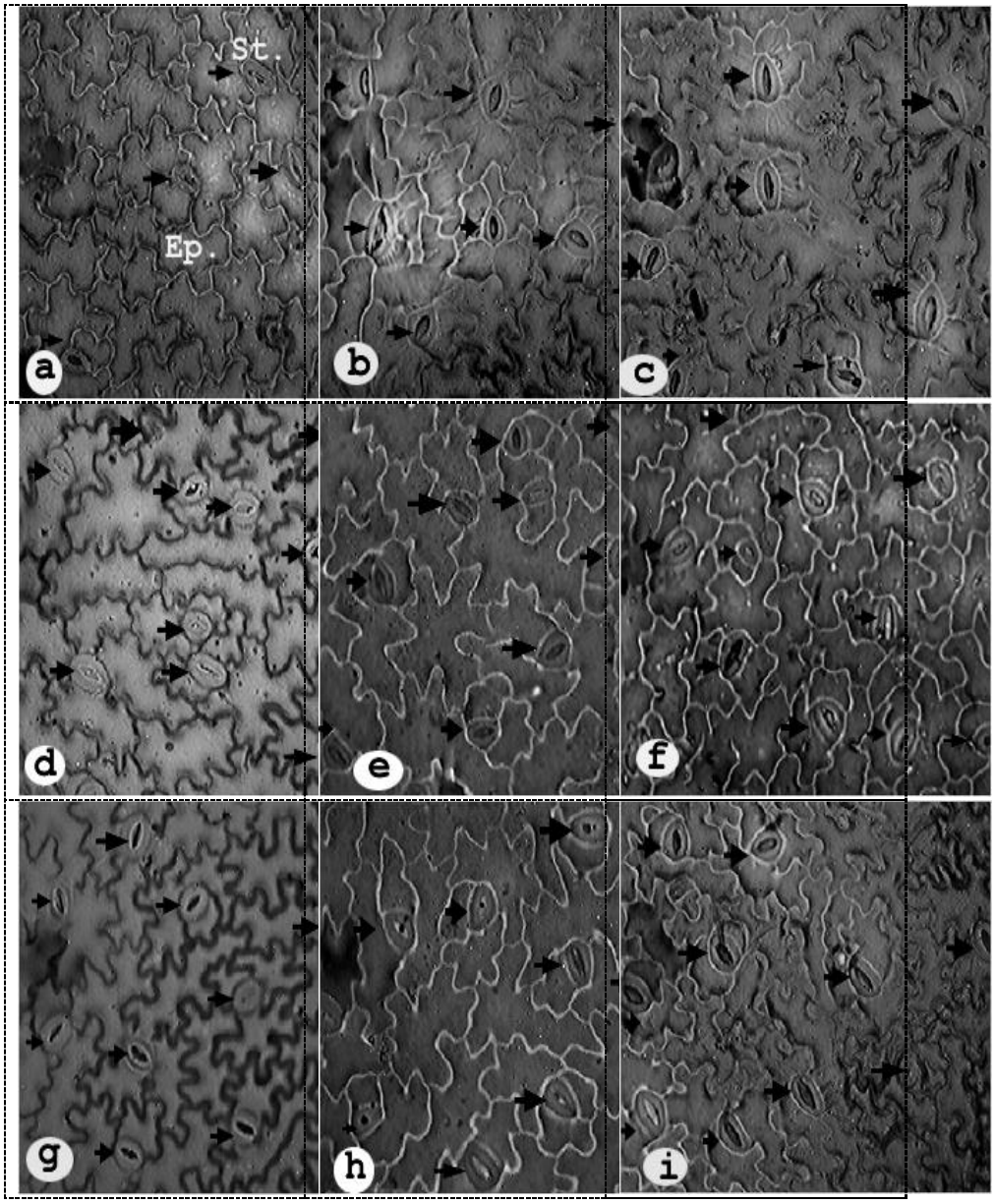


Figure (4): Leaf abaxial surface of sweet pepper leaf showing the effect of pre-soaking seeds in SA at 75 mg/L and AsA at 50 mg/L under high salinity level (4000 mg/L) NaCl, $CaCl_2$ and their combination 1:1(x400).

a = NaCl at 4000 mg/L	b = NaCl + SA at 75mg/L	c = NaCl + AsA at 50 mg/L
d = $CaCl_2$ at 4000 mg/L	e = $CaCl_2$ + SA at 75 mg/L	f = $CaCl_2$ + AsA at 50 mg/L
g=NaCl+$CaCl_2$ at 4000 mg/L	h = (NaCl+$CaCl_2$)+SA at 75 mg/L	i = (NaCl+ $CaCl_2$)+AsA at 50 mg/L

Abbreviations: St.= Stomata; Ep.= Epidermis cell

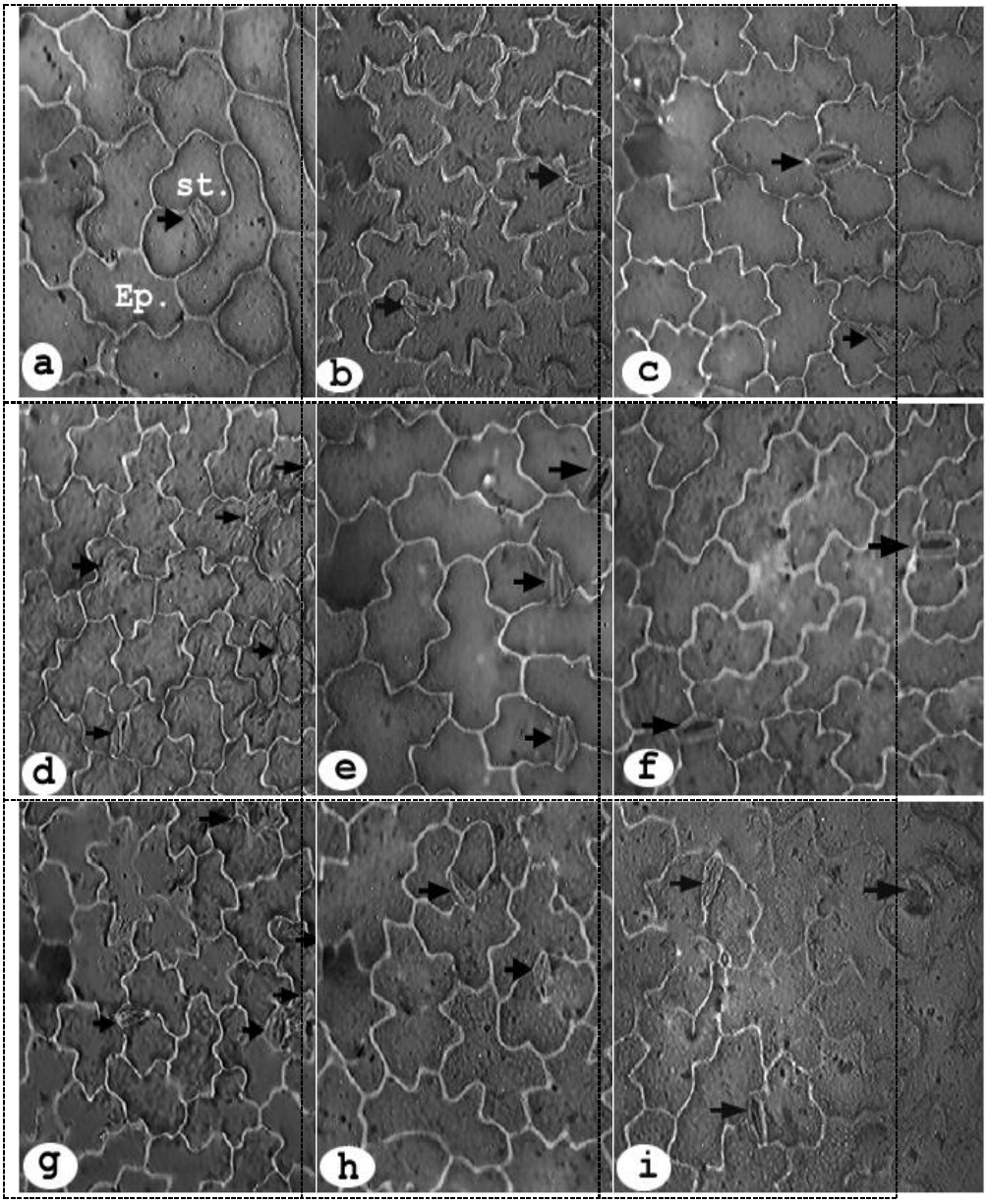


Figure (5): Leaf adaxial surface of sweet pepper leaf showing the effect of pre-soaking seeds in SA at 75 mg/L and AsA at 50 mg/L under high salinity level (4000 mg/L) NaCl, $CaCl_2$ and their combination 1:1(x400).

a = NaCl at 4000 mg/L	b = NaCl + SA at 75mg/L	c = NaCl + AsA at 50 mg/L
d = $CaCl_2$ at 4000 mg/L	e = $CaCl_2$ + SA at 75 mg/L	f = $CaCl_2$ + AsA at 50 mg/L
g = NaCl+$CaCl_2$ at 4000 mg/L	h = (NaCl+$CaCl_2$)+SA at 75 mg/L	i = (NaCl+ $CaCl_2$)+AsA at 50 mg/L

Abbreviations: St.= Stomata; Ep.= Epidermis cell

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استجابة الكثافة الثغرية فى أوراق نبات الفلفل الحلو لتأثيرات بعض المحفزات الحيوية تحت ظروف الملوحة

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أدت زيادة تركيزات الملوحة من الأملاح المستخدمة إلي حدوث نقص في عدد الثغور والكثافة الثغرية علي سطحي الورقة (العلوي والسفلي) وذلك مقارنة بالنباتات الغير معاملة. ولقد كان كلوريد الصوديوم بتركيز ٤٠٠٠ جزء في المليون الأكثر تأثيراً يليه مخلوط الملحين ثم كلوريد الكالسيوم. من جهة أخرى، أدي نقع البذور في أي من حمض الأسكوربيك بتركيز ٥٠ جزء في المليون أو حمض السالسليك بتركيز ٧٥ جزء في المليون إلي زيادة عدد الثغور والكثافة الثغرية علي سطحي الورقة وذلك مقارنة بالنباتات الغير معاملة وأي من مستويات الملوحة، وكان حمض الأسكوربيك الأكثر تأثيراً عن حمض السالسليك فى هذا الشأن.

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