STIMULATION OF HEAT STRESS TOLERANCE OF BEAN PLANTS USING SA AND CPPU

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ABSTRACT

Pot experiment was conducted in the Experimental Farm of Agricultural Botany Department, Faculty of Agriculture, Ain Shams Univ. during the two successive seasons of 2006 and 2007 to study the effect of Salicylic acid (SA at 10^{-2} , 4 x 10^{-2} µM) and N-(2-chloro-4-pyridyl)-3-phenylurea (CPPU at 20 and 40 ppm), on bean plants (*Phaseolus vulgaris*) cv. Bronco grown under high temperature conditions (at average 35-40°C /22–25 °C day/night) as the growing period conducted from May to July in both seasons. Foliar application of SA and CPPU positively affected all growth characters. The best results were noted by CPPU at 20 ppm, followed by SA treatments. Moreover, the highest chlorophyll concentration was achieved by SA at 4 x 10^{-2} µM and CPPU at 20 ppm. Furthermore, SA treatments as compared with CPPU were superior achieving the maximum number of pods per plant, pod fresh and dry weight. Meanwhile, both concentrations of SA significantly increased the concentration of total soluble protein. Most SA or CPPU or both treatments significantly improved carbohydrates, reduced and non-reduced sugars concentration in seeds and reduced fibers percentage in pods especially with SA at 4 x 10^{-2} µM. Exogenous application of SA or CPPU significantly suppressed superoxide dismutase (SOD) activities as compared to untreated plants. All the treatments caused a significant enhanced yield characters, except CPPU at 40 ppm.

Key words: *Phaseolus vulgaris*, SA, CPPU, Chlorophyll Concentration, Total Soluble Protiens, Carbohydrates, Reduced and non-reduced Sugars, Fibers, SOD.

INTRODUCTION

Bean plants (*Phaseolus vulgaris* L.) considered one of the important vegetable crops cultivated in Egypt for local market and for exportation. However, bean plants are relatively sensitive to environmental stresses that may occur in the field compared to most vegetable crops which negatively affect its growth, yield and even the quality of pods. Many investigations indicated that bean plants is very sensitive to different environmental stresses (abiotic stress) such as chilling (El-Tohamy et al., 2001), drought (Millar and Gardner, 1972; Halterlein, 1983; El-Tohamy et al, 1999) and heat stress (Dale, 1964). Freyer (1992) reported that the high incidence of temperature extremes caused by high concentration of ozone and the other pollutants subjected plants to oxidative stress which in turn negatively affects plant growth, productivity and quality. Two minutes of heat shock to the roots of *Phaseolus vulgaris* L. reduced cytokinin levels in both shoots and roots (Itai et al., 1973).

SA (Salicylic acid) belongs to plant phenolics group considered as a hormone-like endogenous regulator. SA affected several physiological processes either promoter or inhibitor depending on its concentration, plant species, development stages and environmental conditions (El-Mergawi and Abdel Wahed 2004). It plays an important role in the regulation of plant growth and development, seed germination, fruit yield, glycolysis , flowering and heat production in thermogenic plants (Raskin, 1992; Klessig and Malamy, 1994). Exogenous application of SA could regulate the activities of antioxidant enzymes and increase plant tolerance to the biotic and abiotic stress (He *et al*., 2002; Yalpani *et al.* 1994 and Szalai *et al.* 2000). Several investigators reported a positive effect of SA on photosynthesis and plant growth under stress. Treatment of bean and tomato plants with SA increased their tolerance against heat, chilling and drought stress (Senaratna at al., 2000).

CPPU (N-(2-chloro-4-pyridyl)-3-phenylurea) is a substance derived from phenylurea with cytokinin activity. It has a higher activity level than most adenine based cytokinins and metabolized even more slowly and thus last longer (Kapchina-Toteva and Stoyanova 2004). It influences cell division, morphogenesis in various species; increases fruit set in watermelon and persimmon and increases fruit size in many crops (Hayata, et al., 1995).

Cytokinins are antagonistic to many ABA mediated physiological responses such as stomatal closure, leaf senescence, leaf abscission, fruit abscission and seed germination inhibition (Kapchina-Toteva et al., 2000). However, to reduce senescence and improve retail holding, cytokinins could be applied pre-shipping to combat senescence.

Superoxide dismutase (SOD), within a cell, constitute the first line of defense against reactive O2 species (ROS) which may be due to a combination of the influences of cellular location of the enzyme and upstream sequences in the genomic sequence (Alscher et al 2002).

The aim of this study is to investigate the response of bean plants grown under heat stress to exogenous application of SA and CPPU.

MATERIALS AND METHODS

Pot experiment was conducted in the Experimental Farm of Agricultural Botany Department, Faculty of Agriculture, Ain Shams Univ. Three seeds per pot of bean (*Phaseolus vulgaris* L. cv. Bronco) were sown in 18th and 20th May in the two successive summer seasons of 2006 and 2007 respectively in PVC pots (25 cm in length and diameter) filled with 4 kg clay loamy soil (37% clay 36% silt and 27% sand), with 7.91 pH (organic matter 0.92%, Na^+ 5.4, K^+ 0.50, Ca^{++} 4.00, Mg^{++} 1.10, Cl^- 1.50, SO_4^{--} 5.80 and HCO_3^{--} 3.70 meq/1 for cations and anions). Thinning was performed after 1 weak from germination leaving one plant per pot. Basal fertilization was performed as following; Phosphorous as calcium super phosphate (15.5% P_2O_5) was mixed with soil before sowing at the rate of 3 g/pot. Nitrogen as ammonium nitrate (33.5% N) at the rate of 1.5 g/pot and potassium as potassium sulfate (48% K_2O) at the rate of 1 g/pot were added twice at 15 and 30 days after sowing. Other recommended agriculture practices for bean cultivation was followed.

Pots were arranged in complete randomized design with four replicates. Each replicate consisted of four pots with one plant per pot. Bean seeds were sown at the rates of 3 seeds to each pot.

Plants were foliar sprayed two times (30 and 45 days from sowing) with CPPU at 20 and 40 ppm, salicylic acid at 10^{-2}, $4x10^{-2}\mu M$ and control plants were sprayed with distilled water until complete covering of the plant foliage. Tween 20 at 0.05 ml/L was used as wetting agent. Temperature ranged 35-40°C /22–25 °C day/night during the course of experiment and Relative humidity was approximately 90%.Samples were taken at 55 days after sowing for growth measurements (plant height, leaves number, shoots and roots fresh and dry weight) and chlorophyll analysis from each treatment and after 70 days for yield measurements (pods number, pods fresh and dry weights) and chemical analysis.

Chemical Analysis:

Chlorophyll Determination:

Chlorophyll a (Chl a), Chlorophyll b (Chl b) and total chlorophyll (Ch) concentration were determined in the second leaf (fully expanded leaves) from shoot tip. Samples of 0.1 g leaveswas ground and extracted with 5 mL of 80% (v/v) acetone in the dark according to the methods described by Holder (1965). The mixture was filtered then the absorbance values at 645 and 663 nm were measured. Using Jenway 6105 UV/VIS Spectrophotometer, concentrations of Chl a Chl b and Ch were estimated by the equations of Arnon (1949) as follows:

- $C_{a+b} = 20.2\ D_{645} + 8.02\ D_{663}$
  C : chlorophyll
  D : absorbance

Total Soluble Protein Determination:

Total soluble protein was determined (in a dry matter basis) in seeds using the method of Bradford (1976). The soluble protein concentration was calculated from the standard curve according to Read and Northcote (1[illegible]).

Total Carbohydrates Determination:

For extraction, one gram of seeds sample was randomly taken and added to 30 ml HCL 2N. Tubes were placed in boiling water bath for 6 h. after cooling, samples transferred into calibrated flasks (100 ml). Total carbohydrates were estimated by the alkaline potassium ferricyanide method (Shales and Schales, 1945).

Total Soluble Sugars Determination:

For extraction, one gram of seeds sample was ground in a mortar with ethanol 80% for 3 times. The extracts were combined and evaporated till dryness. The dried film was dissolved in 50 ml of 10

% aqueous isopropanol. Determination of total soluble sugars, reduced and non-reduced sugars were carried out according to the method by Shales and Schales (1945).

Fiber analysis:

The method of Van Soest and Wine (1968) was used to determine fibers in samples using Dosi-fiber extractor.

Superoxide Dismutase Determination:

Activity of SOD was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) according to the methods of Giannopolitis and Ries (1977). The reaction solution (3 mL) contained 50 µmol NBT, 1.3 µmol riboflavin, 13 mmol methionine, 75 nmol EDTA, 50 mmol phosphate buffer (pH 7.8) and 20 to 50 µL enzyme extract. The reaction solution was irradiated under a blank of fluorescent lights at 75 µmol m-2 s-1 for 15 min. The absorbance at 560 nm was read against the blank (non-irradiated reaction solution) with a spectrophotometer (Spectronic Instruments, Rochester, NY). One unit of SOD activity was defined as the amount of enzyme that inhibited 50% of NBT photoreduction.

Statistical Analysis:

Data were subjected to statistical analysis of variance and were calculated according to SAS (1996).

RESULTS AND DISCUSSION

Growth parameters

Foliar application of SA and CPPU at 20 ppm significantly enhanced most of plant growth characters of bean plants. However, CPPU at 40ppm didn't give any significant increase comparing with untreated plants (Table, 1).

Maximum values of plant height, leaf numbers, and shoot fresh and dry weights were obtained by CPPU at 20 ppm followed by SA at 4 x 10^{-2} µM, and then SA at 10^{-2} µM, these effects were significant except for shoots fresh weight with SA at 10^{-2} which failed to reach the level of 5 % significance. Meanwhile, roots fresh and dry weights showed the highest value with SA treatments. The same trend was found by Martín-Mex *et al* (2005) who reported that SA affected plant size, number of leaves and flowers of African violet plant. CPPU at 40 ppm obviously inhibited plant growth. These results were in agreement with Salisbury (1985), who reported that, high concentration of cytokinins can be inhibitory to shoot and root growth. On the other hand, the stimulatory effect of CPPU at 20 ppm can be explained by the major physiological function of cytokinins, which is promoting cell division and elongation (Salisbury, 1985).

Pod Yield and yield components

As shown from Data presented in Table (2), SA at 4×10^{-2} recorded the highest values for all yield components (pod number per plant and pods fresh and dry weight) followed by SA treatment at 10^{-2} μM and CPPU at 20 ppm. Similar findings were obtained by Cleland and Ajami, (1974), who mentioned that salicylic acid (SA) when applied to herbaceous plants, affected many physiological processes such as flowering induction which in turn increased yield components. On the other hand, CPPU treatment at 40 ppm didn't reach the values of untreated plants. That result may due to the reduction in vegetative growth caused by this treatment.

Table (I): Effect of SA and CPPU on growth characters of bean plants during two seasons of 2006 &2007

Treatments	plant height (cm)	leaves No	shoot FW (gm)	shoot DW (gm)	root FW (gm)	root DW (gm)	plant height (cm)	Leaves No	shoot FW (gm)	shoot DW (gm)	root FW (gm)	root DW (gm)
			1st season						2ad season			
control	10.63	9.00	7.40	4.45	2.65	1.10	10.13	8.75	8.30	5.65	2.78	1.33
SA 10^{-2} µm	12.38	11.00	8.78	6.35	3.60	2.10	13.95	11.75	9.83	7.40	3.85	2.05
SA 4×10^{-3} µm	13.25	12.50	10.70	8.45	2.55	1.15	15.45	13.75	12.15	9.78	3.18	1.63
CPPU 20 ppm	15.75	14.50	16.28	9.25	3.28	1.60	19.25	14.50	15.48	10.00	4.08	2.15
CPPU 40 ppm	10.75	11.25	9.55	7.23	1.95	0.93	12.83	10.50	10.53	8.00	2.55	1.30
LSD	3.45	1.54	3.53	1.05	0.13	0.09	3.49	2.18	3.76	0.97	0.15	0.06

Table (2): Effect of SA and CPPU on yield components of bean plants during two seasons of 2006 &2007

Treatments	Pod No/plant	pod FW/plant (gm)	pod DW/plant (gm)	pod No/plant	pod FW/plant (gm)	pod DW/plant (gm)
		1st season			2nd season	
control	1.75	3.78	2.10	2.00	3.95	2.20
SA 10^{-2} µm	2.75	5.50	3.35	3.25	5.35	3.85
SA 4×10^{-2} µm	4.50	5.73	3.95	5.00	6.25	4.80
CPPU 20 ppm	2.00	4.28	2.75	3.25	5.00	2.85
CPPU 40 ppm	1.50	3.10	1.70	5.50	4.78	3.45
LSD	0.50	0.39	0.26	0.66	0.22	0.30

Chlorophyll concentration

The superiority of chlorophyll concentration observed with SA at 4x10-2 µ M and CPPU at 20ppm. Meanwhile, SA at 10-2 µ M and CPPU at 40 ppm didn't give any significant increase (Table 3).These results were in agreement with those of Türkyılmaz et al., (2005 on bean and Zhou et al., (1999) on corn plants. They found that, foliar spray with salicylic acid increased Chl a, Chl b and other photosynthetic pigments under normal field conditions.

It is also possible that the anti-stress substances were effective in maintaining the membrane integrity to reduce the leakage of electrolyte through its positive effect on the antioxidant enzymes system as has been demonstrated by Pinhero and Fletcher (1994) in corn seedlings. It is generally known that most of environmental stresses have in common a similar mechanism in affecting plant growth and performance. Under stress conditions, the generation of free radicals and low nutrient uptake are believed to be the main cause for damaging and dis-functioning of plant cells (Bohnert et al., 1995; Sreenivasulu et al., 2000). Regarding CPPU, it could be noticed that with low concentration (20 ppm) chlorophyll concentration [illegible] increased compared with control plants. The [illegible]

[illegible] (CPPU) [illegible] the [illegible] of [illegible] water deficit and high temperature stress. Cytokinins application (ZR) retarded the decline in photosynthetic rate for plants exposed to prolonged periods of high temperature stress, which could have been due to the effects of ZR in suppressing the decrease in photochemical efficiency and chlorophyll degradation (Liu and Huang, 2000). In contrary, Carimi et al (2004) mentioned that, the cytokinin 6-benzylaminopurine (BAP), if added at high dosage to plants and cultivated cell suspensions of Arabidopsis thaliana and Daucus carota, induce programmed cell death by accelerating senescence.

Table (3): Effect of SA and CPPU on chlorophyll (chl a, chl b and total chlorophyll) contents (mg/ml) in bean leaves during two seasons of 2006 &2007

	Chl a	Chl b	total chlorophyl	ca	cb	total chlorophyl
Treatments		1st season			2nd season	
control	0.005	0.010	6.58	0.004	0.010	6.89
SA 10^{-2} µm	0.006	0.013	9.52	0.005	0.012	8.80
SA 4×10^{-2} µm	0.006	0.013	11.19	0.006	0.013	10.96
CPPU 20 ppm	0.007	0.015	10.39	0.007	0.016	10.19
CPPU 40 ppm	0.004	0.009	9.71	0.004	0.010	9.96
LSD	0.00014	0.00047	0.99	0.00024	0.00061	1.2

Total soluble protein

It was clearly shown that CPPU at 20ppm and both concentrations of SA treatments significantly increased total soluble protein concentration as compared with untreated plants (Table 4). However, opposite trend was noticed with CPPU at 40ppm treatment which didn't give any significant increment as compared with control plants. Similar trend was obtained in both tested seasons. Such results may be attributed to proteins produced in plants in response to biotic and abiotic stress and many of these proteins are induced by phytohormones such as salicylic acid (Hoyos and Zhang 2000). Regarding plants exposure to high CPPU concentration, stressed plants recorded less of total soluble protein concentration than control plants.

Table (4): Effect of SA and CPPU on total soluble protein concentration (mg / g) in bean seeds during two seasons of 2006 &2007.

Treatments	Protein	
	1st Season	2nd season
Control	53.69	55.12
SA 10^{-2} µm	61.95	63.61
SA 4 ×10^{-2} µm	62.44	65.10
CPPU 20 PPM	57.76	59.22
CPPU 40 PPM	52.8	53.95
LSD	6.24	6.55

Total soluble sugars and carbohydrates:

Table (5) demonstrated seeds concentrations of reduced and non-reduced sugars and total carbohydrates in response to SA and CPPU treatments. It could be concluded that both SA treatments increased concentrations of reduced and non- reduced sugars and total carbohydrates followed by treatment of CPPU at 20 ppm. Khodary, (2004) suggested that SA treatment of stressed plants could stimulate their tolerance via accelerating their photosynthesis performance and carbohydrate metabolism. Concerning CPPU at 40 ppm, seeds concentration of reduced and non- reduced sugars and total carbohydrates was less than untreated plants which explained by Häggman and Haapala (2006) who found that very high amount of 6-Benzylaminopurine caused a great increase in amylase activity which was accompanied by a relatively high concentration of starch in the chloroplasts (leaf). The induction of amylase activity was thus not high enough to diminish the starch concentration. This is supposed to be due to the lack of a sink organ (seeds) since the growth of roots and shoots was greatly inhibited in concentrated solutions.

Table (5): Effect of different concentrations of SA and CPPU on total soluble carbohydrate, total soluble sugar, reduced sugar and non-reduced sugar contents (mg/g FW) in bean seeds during two seasons of 2006 & 2007.

Treatments	Total carbohydrates	Total soluble sugars	Reduced sugars	Non-reduced sugars	Total carbohydrates	Total soluble sugars	Reduced sugars	Non-reduced sugars
			1st season					
control	15.38	5.44	1.90	3.54	16.52	5.13	1.95	3.18
SA 10^{-2} µ M	28.71	8.76	3.06	5.70	30.61	10.34	3.22	7.11
SA 4 ×10^{-2} µ M	31.79	12.65	4.71	7.95	34.38	14.61	4.88	9.72
CPPU 20 ppm	24.89	8.59	2.71	5.88	27.01	9.12	2.96	6.16
CPPU 40 ppm	20.88	5.79	2.03	3.75	22.56	6.18	2.12	4.07
LSD	12.04	2.56	0.39	0.98	14.52	4.27	0.42	2.05

Fibers concentration

The percentage of fibers in bean pods is one of the most important characters indicating the quality of bean pod (Silberngel et. al., 1991). It could be observed the decrease in fiber concentration with both SA treatments and with CPPU at 20 ppm compared with control plants (Table 6). Sistrunk *et al*, (1989) pointed that high temperatures affect fiber concentration in pods of bean plants. This effect of high temperature could be reversed by SA treatments and low concentration of CPPU. However, CPPU at high concentration gave the same trend of untreated plants.

Table (6): Effect of different concentrations of SA and CPPU on % Fiber in bean pods during two seasons of 2006 & 2007.

Treatments	% Fiber	% Fiber
	1[st] season	2[nd] season
control	16.4	16.57
SA 10^{-2} μM	10.34	10.96
SA 4×10^{-2} μM	9.45	10.03
CPPU 20 ppm	**12.08**	**11.14**
CPPU 40 ppm	**15.65**	**14.12**
LSD	**2.05**	**1.98**

Superoxide Dismutase activity

Activity of SOD in all treated plants increased significantly when compared with untreated plants (Fig. 1). Applying CPPU increased SOD activity especially at 20 ppm. Meanwhile in plants treated with 4×10^{-2} μM SA, SOD activity gave the highest value followed by both concentrations of CPPU and SA at 10^{-2} μM.

Superoxide dismutase considered as key enzyme in the active- [illegible]

[illegible]

SOD activity, this activity was elevated by exogenous SA and CPPU application, especially at the higher concentration of SA. Therefore, alleviation of heat injury in bean plants by SA and CPPU could be related to the maintenance of the scavenging ability of antioxidants to super oxygen free radicals at high temperatures (Yordanova and Popova 2007).

Results in this study suggested that SA and CPPU (at low concentration) could alleviate heat stress injury in bean plants (the most effective treatment was SA at 4 x 10^{-2} μM). They improved vegetative growth, yield characters, chlorophyll concentration, total soluble protein concentration, sugar and carbohydrates concentration and SOD activity in bean plants and minimize fiber concentration. SA protecting the endogenous anti-oxidant system which is often correlated with increased resistance to oxidative stress and/or controlling the level of free radicals within plant tissues (Sreenivasulu et al., 2000). SA and CPPU may mediate the acclimation of plants to environmental stress by maintaining active antioxidants, and interact with other cellular metabolites and environmental factors in the regulation of stress responses.

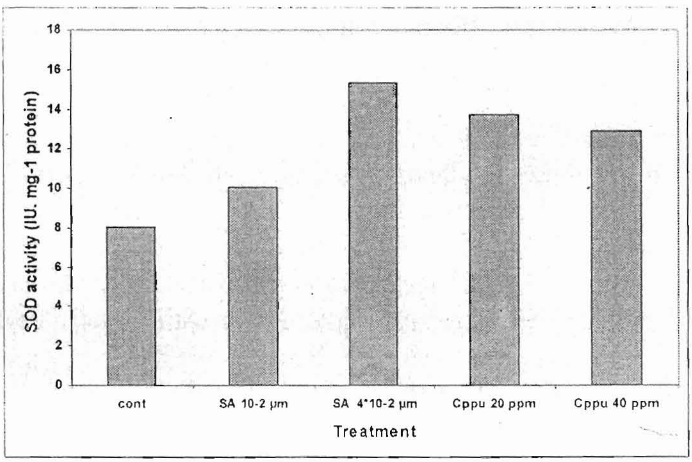

Fig.1: Superoxide dismutase (SOD) activities in bean plants as affected by foliar applications of Salicylic acid (SA) and N-(2-chloro-4-pyridyl)-3-phenylurea (CPPU)

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تشجيع نباتات الفاصوليا على تحمل الإجهاد الحراري باستخدام حامض الساليسيليك و CPPU

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أجريت التجربة في اصص بمزرعة قسم النبات ، كلية الزراعة ، جامعة عين شمس. خلال موسمى 2006 و 2007 لدراسة تأثير حامض الساليسيلك بتركيز (10^{-2} و 4×10^{-2} ميكرومول) و CPPU (N-(2-chloro-4-pyridyl)-3-phenylurea) بتركيز 20و 40 جزء في المليون ، على بعض القراءات المورفولوجية (ارتفاع النبات , عدد الاوراق، الوزن الغض و الجاف للسيقان , الوزن الغض و الجاف للجذور) و المحصول (متوسط عدد القرون للنبات و الوزن الغض والجاف للقرون) و المحتويات البيوكميائية (كلوروفيل A , B و كلوروفيل كلى و البروتينات الكلية الذائبة ، السكريات المختزلة ، و السكريات غير المختزلة ، السكريات الكلية الذائبة و الكربوهيدرات و الالياف) لنباتات الفاصوليا (*Phaseolus vulgaris*) صنف البرونكو النامية في درجة الحرارة المرتفعة خلال الفترة من مايو إلى يوليو (35-40 ° م / 22-25 ° في المتوسط) في كلا الموسمين. تمت المعاملة بالرش للنباتات بـحامض الساليسيليك و CPPU بالتركيزين .

ادت المعاملة بحامض الساليسيليك بالتركيزين (10^{-2} و 4×10^{-2}) الى تحسين صفات النمو و زيادة من المحصول يليها معاملة CPPU بتركيز 20 جزء فى المليون بينما خفضت معاملة CPPU بتركيز 40 جزء فى المليون المحصول مقارنة بالنباتات غير المعاملة .أدت معاملة SA بتركيز 4×10^{-2} و CPPU 20 جزء فى المليون الى زيادة تركيز الكلوروفيل بينما ادت معاملة SA بكلا التركيزين الى زيادة البروتينات الكلية الذائبة ، السكريات المختزلة ، و السكريات غير المختزلة ، السكريات الكلية الذائبة و الكربوهيدرات وخفضت تكوين الألياف مقارنة بمعاملة CPPU 40 جزء في المليون . بينما لوحظ نشاط انزيم سوبر اوكسيد ديس ميوتيز فى النباتات المعاملة بحمض الساليسيليك بتركيز 4×10^{-2} يلية معاملة CPPU بتركيز20 جزء فى المليون ثم 40 جزء فى المليون مقارنة بالنباتات غير المعاملة .

أوضحت نتائج الدراسة إلى أن حامض الساليسيليك وCPPU (التركيز المنخفض) ادوا الى تخفيف تاثير الإجهاد الحراري على الفاصوليا، وان افضل معاملة كانت لحامض الساليسيليك بتركيز (4×10^{-2}ميكرومتر) من حيث تحسين النمو الخضري و المحصول و زيادة تركيز الكلوروفيل و البروتينات الكلية الذائبة و السكريات و الكربوهيدرات ونشاط انزيم سوبر اوكسيد ديس ميوتيز وانخفاض نسبة الألياف. و تقترح هذة الدراسة ان مقاومة النباتات المعاملة بحامض الساليسيليك و CPPU لارتفاع درجة الحرارة يعزى الى محافظتها على نشاط مضادات الاكسدة ضد العوامل البيئية غير الملائمة.