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Evaluation of PGPR as Osmoprotective Agents for Squash (*Cucurbita pepo* L.) Growth under Drought Stress

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ABSTRACT

Evaluation of PGPR in relation with drought resistance of squash was studied *in vitro* and pot experiments. The activities of *Azotobacter chroococcum* ML1, *Bacillus circulans* ML2, *Bacillus megaterium* ML3 and *Pseudomonas fluorescence* ML4 as osmoprotective agents were estimated *in vitro*. All tested strains produced indole acetic acid (IAA) at range of (17.73-23.65 mg/ml), gibberellic acid (GA_3) (9.38-19.16 mg/ml), proline (4.69-15.06 µg/ml), exopolysaccharides (21-467 µg/ml), salicylic acid (25.98-28.35 mg/ml) and 1-aminocyclopropane-1-carboxylate (ACC-deaminase) (9.6-48.1 ml mole/ml/h). Furthermore, values of nitrogenase activity were 35.0 and 15.0 nmol C_2H_4/l/h due to using *A. chroococcum* ML1 and *Ps. fluorescence* ML4, respectively. The ability of the tested PGPR strains to enhance growth and alleviation drought stress on squash plants compared to humic acid (HA) under different irrigation levels were tested through a pot experiment. Results revealed that inoculation of squash with PGPR enhanced enzymes activities in its rhizosphere. In addition, PGPR inoculants caused significant increases in osmoregulators compounds of squash leaves and their maximum values were recorded at vegetative stage. Results also emphasized that IAA and GA_3 in squash leaves were markedly increased under drought stress when plants were inoculated with PGPR strains. Whereas, abscisic acid decreased under the same conditions. Alongside, photosynthetic pigments and oxidative enzymes were strongly affected under PGPR inoculation compared to HA or chemical fertilizers applications when squash were irrigated with 75 or 50% of field capacity. Also, growth characteristics and yield under PGPR treatments were significantly higher than those resulted from additions of HA and chemical fertilizers.

Key words: PGPR, osmoprotectants, osmoregulators, microbial activities, drought stress, squash

Introduction

Plant growth and productivity are adversely affected by various biotic and abiotic stresses. Among abiotic factors that can control plant evolution, water availability is the most important one. Water insufficiency is one of the major abiotic stresses especially in arid and semi-arid countries and it is considered as a destructive environmental stress, which is adversely decreased crop growth and productivity more than other environmental stresses (Farooq *et al*., 2012). Drought can reduce plant growth, especially vegetables such as squash, which is considered one of the most sensitive plants to deficient water and over-irrigation (Maughan *et al*., 2015). Deficiency of water has adverse effects on various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters (Hopkins, 1995). In addition, it leads to deactivate the normal balance of cells and causes increments in the production of reactive oxygen species (ROS) such as the hydrogen peroxide, superoxide radicals and hydroxyl radicals and formation of radical scavenging compounds i.e. ascorbate and glutathione (Celik and Atak, 2012). The ROS generation increases lipid peroxidation, protein degradation and nucleic acid damages. This can accumulate abscisic acid, which responsible for wilting. Moreover, drought reduces uptake and transport of nutrients and causes alongside negative effects on metabolism and then decreases the alteration in assimilate partitioning among organs (Lisar *et al*., 2012). To alleviate adverse effects of ROS, plants had antioxidant defense systems through production of enzymes like superoxide dismutase, peroxidases, polyphenol oxidase, phenylalanine ammonia lyas and catalase (Bano *et al*., 2012). Furthermore, synthesis of organic osmolytes can help plants to adjust osmotic balance without any cell impairment and encourage them to temperate ion toxicity and extract water from soil solution. Stressed plants have high ability to accumulate some important compounds such as proline (an amino acid), which

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has marked role in cell osmotic adjustment and protection of cell components during dehydration (Bhuiyan *et al*., 2016), trehalose (a non-reducing disaccharide sugar composed of two glucose units) that acts as a stress protection metabolite (Quéro *et al*., 2013), salicylic acid, which may play as a constitutive defense compound (Pacheco *et al*., 2013), phytohormones (Timmusk *et al*., 2014), oxidative enzymes (Alam *et al.*, 2013), soluble sugars and free amino acids (Bano *et al*., 2013). PGPR have various mechanisms to alleviate drought stress through stimulation of root growth by production of phytohormones, ACC-deaminase, which can hydrolyze 1-aminocyclopropane-1-carboxylic acid (ACC) which can be reducing rate of ethylene biosynthesis in plants. The presence of ACC deaminase, exopolysaccharides and osmoprotectant have roles in facilitate plant growth under stress conditions (Ahemad and Kibret, 2014). This improves leaf water potential in plants and keeps them against oxidative stress, and then increases the ability of plants to tolerate the drought. Humic substances has an important effect under abiotic stress conditions such as drought. Humic substances are well known as stimulators of plant growth by some mechanisms such as enhancing uptake and transport of nutrients, reducing uptake of toxic elements, increasing membrane permeability, respiration, photosynthesis and phosphate uptake and acting as growth hormones (Aydin *et al*., 2012). The objective of this study was to evaluate the ability PGPR strains in producing phytohormones, ACC-deaminase and osmoprotectants under laboratory conditions and to estimate their efficiency to enhance growth and alleviation drought stress on squash plants compared to applications of humic acid and chemical fertilizers.

Materials and Methods

PGPR strains:

PGPR strains, *Azotobacter chroococcum* ML1, *Bacillus circulans* ML2, *Bacillus megaterium* ML3 and *Pseudomonas fluorescence* ML4 were provided from Microbial Biotechnology and Fermentation Laboratory, Central Research Laboratories, Faculty of Agriculture, Benha University, Moshtohor, Toukh, Qalyubia, Egypt.

Evaluation of PGPR strains in vitro:

The ability of strains for phytohormones production viz. indole acetic acid (IAA), gibberellic acid (GA_3) were determined based on methods described by Patel *et al*. (2015). Nitrogenase activity was estimated as a guide for nitrogen fixing ability by strains using acetylene reduction technique given by Diloworth (1970). In addition, ACC deaminase activity was estimated using method described by Penrose and Glick (2003) through measuring the production of α-ketobutyrate generated by the cleavage of ACC by ACC deaminase. The method described by Emtiazi *et al*. (2004) was used for exopolysaccharides production by bacterial strains. Method described by Bhuiyan *et al*. (2016) was used for determination of proline production by bacterial strains. In addition, salicylic acid production in bacterial culture media was estimated according to the method described by Lukkani and Reddy (2014).

Greenhouse experiment:

A pot experiment was conducted during spring 2016 in the research greenhouse of Faculty of Agriculture, Benha University, Moshtohor, Toukh, Qalyubia, Egypt. A randomized complete block design (RCBD) with nine treatments were distributed with 3 replicates and resulted from the interact ion of three irrigation levels {100%, 75% and 50% of the field capacity (FC)} in the presence of humic acid, PGPR and chemical fertilizers as control. This experiment was carried out in plastic pots containing (10 kg soil). Soil samples were obtained from different field places and mixed well. Particle size distribution and chemical analyses were conducted according to the methods described by Faithfull (2002). Soil texture and chemical properties were presented in Table (1).

PGPR inocula preparation:

A. chroococcum ML1 was prepared in Ashby's broth medium modified by Abdel-Hafez (1966) for 4 days, while *B. circulans* ML2 was grown on Alexandrov broth medium (Zahra, 1969) for 2 days. In addition, *B. megaterium* ML3 was prepared on modified Bunt and Rovira broth medium (Abd El-Malek and Ishac, 1986) for 2 days and *Ps. fluorescence* ML4 was prepared on King's broth medium (King *et al*., 1954) for 2

days. The four inoculants were incubated at 30ºC±2 on a rotary shaker (180 rpm). Inoculants of equal volumes of the suspensions were obtained (about 10^8 cells/mL from each).

Table 1: Particle size distribution and chemical properties of the experimental soil

Particle size distribution					
Coarse sand %		4.59	Silt %		26.60
Fine sand %		25.64	Clay %		43.17
Textural class		Clay loam			
Chemical analysis					
pH		7.8	Soluble cations (meq/l)	Ca^{++}	12.1
E.C. (ds/m)		2.02		Mg^{++}	5.80
Organic matter (%)		2.12		Na^{+}	0.44
	N	1730		K^{+}	1.86
Total macronutrients (mg/l)	P	561.2	Soluble anions (meq/l)	HCO_3^-	4.40
	K	3500		$CO_3^=$	0.00
				Cl^-	9.02
				$SO_4^=$	4.60

Chemical fertilizers and humic acid:

Chemical fertilizers were applied with a full dose of inorganic nitrogen (50 kg N/fed.) as ammonium sulphate, inorganic phosphorus (25 kg P_2O_5/fed.) as super phosphate and potassium (40 kg K_2O/fed.) as potassium sulphate in two equal doses at vegetative and flowering stages of squash plants according to the recommendations of the Ministry of Agriculture, Egypt. Half dose of inorganic nitrogen was added to PGPR treatments at the beginning of the experiment. Humic acid (85%) with 56% C, 4.5% H, 31% O and 4.5% N was obtained from Sphinx for International Trade Company, Cairo, Egypt and added to soil before the cultivation process at a rate of 4 kg/fed.

Cultivation process:

Squash seeds (*Cucurbita pepo* L.) Giza 4 cultivar were obtained from Horticulture Research Institute, Agriculture Research Center, Giza, Egypt to use in this experiment. Only in PGPR treatments, seeds were soaked for 30 min. in mixture of four PGPR cell suspensions before cultivation. Arabic gum (10%) was added as an adhesive agent prior to inoculation. The PGPR inocula were added to the pots three times during growing season at a rate of 100 mL/pot.

Determinations:

Microbial enzymes in squash rhizosphere:

Soil samples of squash rhizosphere were analyzed for microbial enzymes after 30 and 60 days of sowing. Dehydrogenase (DHA) as TPF/g dry soil/h. and nitrogenase (N_2-ase) as nmol C_2H_4/g dry soil/h. activities were estimated as described by Thalmann (1968) and Diloworth (1970), respectively. Whereas alkaline phosphatase (P-ase) activity as µg P-nitrophenol/g dry soil/h. was estimated according the method of Margesin and Schinner (1994).

Osmoregulators (Compatible solutes):

Squash leaves were periodically collected after 15, 30, 45, 60 and 75 days of sowing for salicylic acid, proline and trehalose estimations as mentioned by Warrier *et al*. (2013), Marin *et al*. (2010) and Welsh *et al*. (1991), respectively.

Phytohormones, photosynthetic pigments and oxidative enzymes:

Phytohormones {indole-3-acetic acid (IAA), gibberellic acid (GA_3) and abscisic acid (ABA)} were estimated as described by Ergün *et al*. (2002); photosynthetic pigments (chlorophyll A, B and carotenoids) were determined spectrophotometrically according to Nornal (1981). Additionally, oxidative enzymes {peroxidase (PO) as absorbance at 425 nm/g fresh weight/15 min., polyphenol oxidase (PPO) as the increase in absorbance at 420 nm/g fresh weigh/30 min. and phenylalanine ammonia lyase (PAL) as µmol trans-

cinnamic acid min/ g/protein} were determined as described by Allam and Hollis (1972), Matta and Dimond (1963) and Dickerson *et al*. (1984). These estimations were conducted after 30 days of sowing.

Growth characteristics and yield:

Plant height, number of leaves and plant dry weight (shoots + roots) were determined after 30 days of sowing. Moreover, numbers of flowers, fruits and plant yield (Kg) were recorded.

Statistical analysis:

The data were statistically analyzed using Costatic (C.S.) software. For comparison between means, Duncan's multiple range test was used (Duncan, 1955).

Results and Discussion

Evaluation of PGPR in vitro:

Data in Table (2) indicated that the four PGPR stains *A. chroococcum* ML1, *B. circulans* ML2, *B. megaterium* ML3 and *Ps. fluorescence* ML4 produced considerable amounts of phytohormones viz. indole acetic acid at a range of 17.73-23.65 mg/ml and gibberellic acid at a range of 9.38-19.16 mg/ml.

Table 2: Evaluation of PGPR strains as osmoprotective agents *in vitro*

Activities	Phytohormones		Osmoprotectants			ACC-deaminase	N_2-ase
	IAA	GA_3	EPS	Pro	SA	(ml mole/ml/h.)	(nmol
PGPR strains	(mg/ml)		(µg/ml)	(µg/ml)	(mg/ml)		C_2H_4/l/h.)
A.Chroococcum ML1	17.73	17.14	363	13.4	27.08	12.8	35.0
B. circulans ML2	23.65	16.14	467	6.87	27.03	9.60	ND
B. megaterium ML3	18.18	9.38	275	4.69	25.98	48.1	ND
Ps. fluorescence ML4	19.63	19.16	21	15.06	28.35	25.4	15.0

IAA: indole acetic acid *GA_3: Gibberellic acid* *ESP: exopolysaccharides* *Pro: proline*
SA: salicylic acid *ACC: 1-aminocyclopropane-1-carboxylate* *N_2-ase: nitrogenase*

These results are agreed with Ahmad *et al*. (2011) who reported that different bacterial species and strains belonging to the genera *Azotobacter*, *Pseudomonas* and *Bacillus* able to produce IAA and different types of plant growth regulators (PGRs). Also, data indicated that the studied PGPR strains have high ability to produce osmoprotectants i.e. proline (Pro) at a range of 4.69-15.06 µg/ml, exopolysaccharides (EPS) at a range of 21-467 µg/ml and salicylic acid (SA) at a range of 25.98-28.35 mg/ml. These results were in harmony with Hoffmann *et al*. (2013) who reported that Pro is a compatible solute that can be synthesized by PGPR strains which possesses a dedicated biosynthetic pathway for it as an osmoprotectant in response to osmotic stress. In addition, (Press *et al*., 1997) proved that several bacteria can synthesize SA. Moreover, Sayyed *et al*. (2011) reported that bacterial ESP protects their producing PGPR and serves as a potential energy reserve under water deficient conditions. Moreover, the examined PGPR strains could produce ACC-deaminase at a range of (9.6-48.10 mL mole/ml/h.). These results agreed with Saraf *et al*. (2010) and Glick (2014) who mention that PGPR promote plant growth by phytohormones production and by ACC deaminase which hydrolyzes the ethylene's precursor ACC to lower ethylene level in plants as another well-reported mechanism for growth promotion. In respect of N_2-fixation ability, the tested PGPR were estimated and results showed that *A. chroococcum* ML1 and *Ps. fluorescence* ML4 gave nitrogenase activity of 35.0 and 15.0 nmol C_2H_4/l/h., respectively as agreed with Dobbelaere *et al*. (2003) who reported that the atmospheric nitrogen fixing capacity of some free-living bacteria such as *Azotobacter* and *Bacillus* could be demonstrated under *in vitro* conditions. However, results by Vermeiren *et al*. (1999) showed that different *Pseudomonas* species could fix atmospheric nitrogen. Generally, Huang *et al*. (2013) emphasized that when plants inoculated with PGPR that secreted ACC-deaminase become more resistant to wide environmental stresses such as drought. This enzyme hydrolyzed ACC into ammonia and α-ketobutyrate. Also, Verberne *et al*. (2002) reported that SA played an important role in stress alleviation in plants and as a plant hormone. Moreover, Welsh *et al*. (1991) found positive correlation between the intracellular accumulation of exogenous organic compatible solutes such as proline, trehalose and glycine betaine in bacterial cell and their tolerant to stress, add to that its role in osmotic stress decrease in plants (Bhuiyan *et al*., 2016). Along

with this, EPS producing PGPR have an important role in provide moisture and increase water holding capacity of soil under water deficient (Sayyed *et al*., 2011).

Effect of different treatments on squash grown under drought stress:

Enzymes activities in squash rhizosphere:

Regarding to the effect of different treatments on DHA, P-ase and N_2-ase activates in squash rhizosphere, the data presented in Table (3) clearly showed that DHA, P-ase and N_2-ase activates in squash rhizosphere were higher after 60 days than after 30 days from sowing. Also, results generally emphasized that values of the demonstrated enzymes were decreased with decreasing irrigation level. These results were in accordance with Baum *et al*. (2003) and Sanaullah *et al*. (2011) who indicated that the decrease of enzyme activities in soils could be exhibited to low water possibility which result in a shortage of nutrients availability that ultimately decrease all microbial activities in rhizoposition. Additionally, Williams (2007) emphasized that the microbial communities were more susceptible to the effect of water stress than other environmental stresses. Alongside, Reuben *et al*. (2013) reported that when abiotic disturbances such as drought occur, there were further changes in the bacterial community composition and abundance. This should be depended not only on the direct effect of physical stress on the microbes but also on the indirect influence of carbon availability that resulted from root exudation pattern which alleviated the stress conditions. Alongside, the lowest values of soil enzymes were observed with chemical fertilizers application under different irrigation levels. In contrast the application of HA gave high values of DHA, P-ase and N_2-ase due to its ability to reduce the negative effects of drought stress and increase the water retention capacity by soils, which could improve all vital activities, including enzymatic activities. This was in accordance with Bama *et al*. (2008) who reported that the application of HA cause increased marked increase in enzymatic activities such as dehydrogenase and phosphatase. Also, Serenella *et al*. (2002) reported that humic acid was used to enhance stress tolerance. Significantly increased activities of all estimated enzymes were observed in squash rhizosphere inoculated with PGPR under three irrigation levels compared to other treatments. This may be attributed to the synergistic effect between the four stains. Also, Sardans and Peñuelas (2005) reported that the deficient of soil water could reduce the activity of soil enzymes such as phosphatases, which were extracellularly secreted by bacteria or fungi, so these enzymes called "abiotic enzymes" because their activities reflected soil vitality.

Table 3: Effect of drought stress on microbial enzymes in squash's rhizosphere after 30 and 60 days of sowing

Activities	DHA (TPF/g dry soil/h.)		P-ase (µg P-nitrophenol/g dry soil/h.)		N_2-ase (nmol C_2H_4/g dry soil/h.)	
Treatments	After 30 days	After 60 days	After 30 days	After 60 days	After 30 days	After 60 days
100% of FC						
Chemical NPK	29.56^{f}	39.27^{f}	13.82^{g}	14.3^{g}	9.44^{g}	25.0^{e}
Humic acid	46.35^{b}	58.85^{c}	23.72^{d}	35.1^{d}	28.9^{d}	51.2^{d}
PGPR	47.32^{a}	65.74^{a}	35.54^{a}	51.6^{a}	66.1^{a}	98.3^{a}
75% of FC						
Chemical NPK	27.35^{g}	34.65^{g}	13.24^{h}	13.8^{g}	5.82^{i}	21.0^{f}
Humic acid	41.92^{e}	55.22^{d}	22.32^{e}	34.3^{e}	25.5^{e}	53.7^{c}
PGPR	46.14^{c}	63.74^{b}	33.22^{b}	46.0^{b}	38.1^{b}	81.0^{b}
50% of FC						
Chemical NPK	20.62^{h}	32.84^{h}	12.94^{i}	13.8^{g}	5.91^{h}	20.3^{g}
Humic acid	41.92^{e}	52.94^{e}	21.94^{f}	32.1^{f}	22.4^{f}	51.7^{d}
PGPR	43.91^{d}	62.93^{b}	32.74^{c}	41.5^{c}	30.4^{c}	80.9b^{c}

FC: field capacity *DHA: dehydrogenase*
P-ase: phosphatase *N_2-ase: nitrogenase*

Periodically changes in osmoregulators in squash leaves grown under water stress:

Regarding osmoregulators compounds in squash leaves viz. salicylic acid (SA) and trehalose (Tre) were gradually increased after 15 days to reach their maximum values after 45 days then decreased (Figs 1& 2). While, proline (Pro) reached their maximum after 30 days then decreased (Fig 3). In addition, osmoregulators values were higher under 50% of FC than the other irrigation levels. These results were in

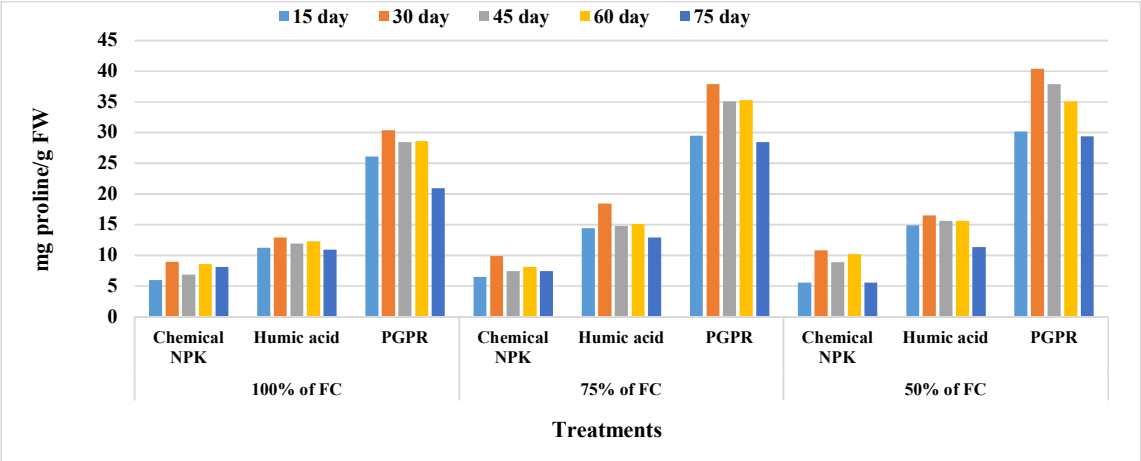

Fig. 1: Periodically changes in proline in squash leaves grown under drought stress

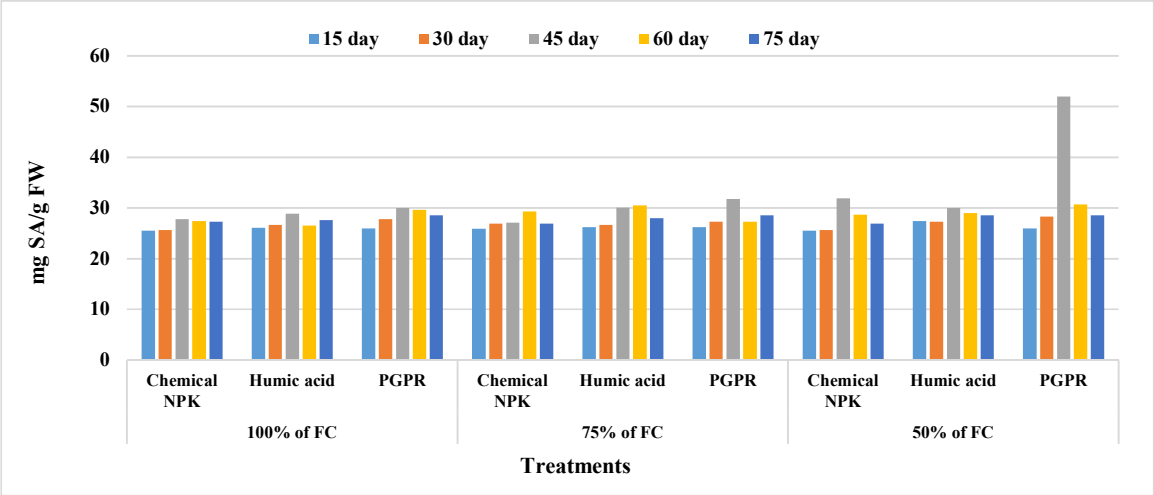

Fig. 2: Periodically changes in salicylic acid in squash leaves grown under drought stress

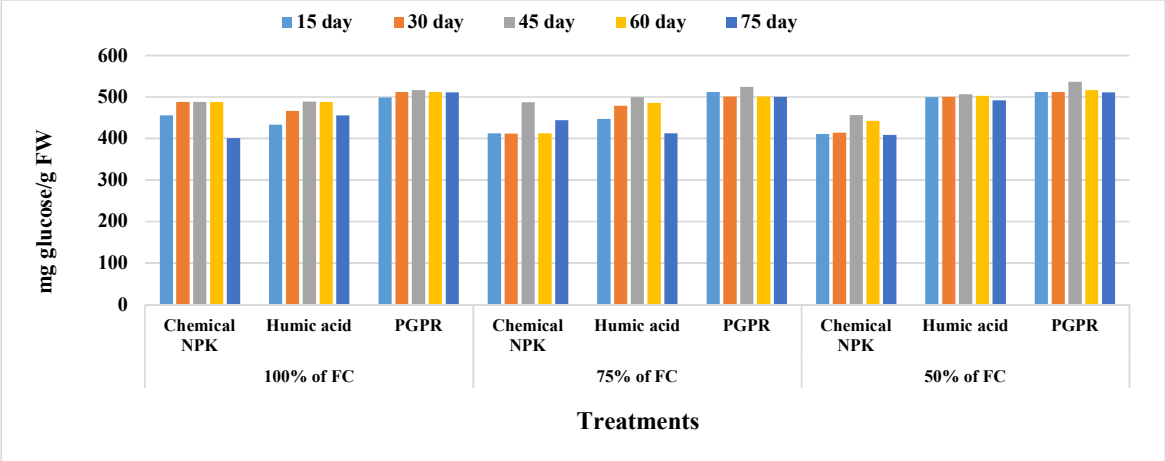

Fig. 3: Periodically changes in proline in Trehalose leaves grown under drought stress

accordance with Slama *et al.* (2014) who showed that abiotic stress conditions triggered accumulation of osmoregulators (compatible solutes) in plant tissues, and these compounds had essential roles in plant tolerance to stress conditions. Additionally, the lowest values of Pro, SA and Tre were observed in leaves of

squash that treated with chemical fertilizers (control) at all three irrigation levels. On the other hand, plants treated with HA showed more relative and significant increases in osmoregulators values than control. These results were in harmony with Kulikova *et al*. (2005) who pointed out that humic substances might show anti-stress effects under a biotic stress conditions. Also, Aydin *et al*. (2012) reported that HA application caused significant increase in Pro content in plants under abiotic stress conditions. Supplementary, squash plants inoculated with PGPR strains showed more marked enhancements in Pro, SA and Tre contents, especially under 50% of FC compared to other two irrigation levels. These results were in accordance with Pacheco *et al*. (2013) who recorded an increase in SA levels in plants grown under stress conditions after treatment with PGPR strains. The production of SA by bacteria was related to oxidative enzymes releasing in plants under stress conditions. In this respect, Shakirova (2007) emphasized that the biosynthesis of SA started from phenylalanine by one of three known paths of syntheses and the most important one was by phenylalanine ammonia lyase. In addition, Brill *et al.* (2011) proved that Pro could be considered as one of compatible solutes that PGPR synthesized in response to osmotic stress as an osmoprotectant. Indeed, Pro had high efficiency to mitigate the adverse effects of stress on plant growth through the formation of a hydration shell around cellular proteins to improve cell integrity and functions (Bhuiyan *et al*., 2016). Also, Wingler *et al*. (2012) reported that the bacterial Tre was affected by abiotic stress tolerance and might stabilized some biological structures such as proteins and membrane lipids during abiotic stresses (Duman *et al*., 2010). Furthermore, the synthesis of organic osmolytes decreased plant suffering and increased stress tolerance by triggering functions such as protection of thylakoid and plasma membrane integrity, scavenging of reactive oxygen species, as well as mitigation of other detrimental effects of biotic and abiotic stresses (Shabala *et al*., 2012). Additionally, Hayat *et al*. (2012) reported that Pro content was significantly increased under water deficit and there was a positive relationship between Pro accumulation and stress tolerance. The accumulated Pro under stress conditions participate in scavenging free radicals, buffering cellular redox potential and might also maintain $NADP^+$/NADPH ratios in plant cell. Along with this, it was also known that a low level of ROS generation might play an important role in the pre-adaptive activity of SA in seedlings with respect to extended stress situations, because ROS acted as signaling molecules that triggered the cascade of protective reactions in plants (Tarchevskii, 2002), including activation of antioxidant enzymes, favoring a decrease in the level of stress-induced generation of ROS, under subsequent conditions of stressful environment.

Phytohormones, photosynthetic pigments and oxidative enzymes:

From data presented in Table (4), it was clear that in most cases phytohormones, photosynthetic pigments and oxidative enzymes values were decreased with decreasing of irrigation level. These results were in accordance with Paul and Lade (2014) who reported that photosynthetic pigments contents (chlorophyll content and chlorophyll a/b ratio) were reduced due to the drought stress. Also, Azuma *et al*. (2010) indicated that superoxide dismutase, polyphenol oxidase and peroxidase were the main oxidative enzymes which decreased under environmental stress conditions. Data also showed that IAA, GA_3 and photosynthetic pigments values were lower in plants fertilized with chemical fertilizers under different irrigation levels compared to plants treated with HA or PGPR. But, abscisic acid (ABA) content was higher in chemical fertilizers treatment. In this respect, Man *et al*. (2011) reported that ABA increased, whereas IAA content decreased in response to drought stress. Also, Taiz and Zeiger (2010) emphasized that under drought stress conditions, ABA concentrations in leaves increased up to 50 times. Moreover, the produced ROS under drought stress were considered as a messenger in ABA transduction pathway in guard cells. ABA induced hydrogen peroxide that supposed a signal in mediating stomatal closure to reduce water loss through the activation of calcium-permeable channels in the plasma membrane (Sharma *et al*., 2012). Regarding the response of photosynthetic pigments to water stress, Celik and Atak (2012) reported that the produced ROS might cause photoinhibitory and photooxidative damage in chloroplasts. Thus, chlorophyll content could be considered as a good reflective to plant response to environmental stress because under adverse conditions chlorophyll a and b and total chlorophyll contents were decreased as well as proline concentrations. This reduction might be due to the decrease in osmotic pressure.

Regarding to the effect of HA application on all estimated parameters, results indicated that IAA and GA_3 values were significantly increased under HA application at different irrigation levels compared to chemical fertilization, whereas ABA values were decreased under the same treatments. In addition, photosynthetic pigments and oxidative enzymes values were higher in squash treated with HA than control. ABA might induce antioxidant defense systems and suppress toxicity of ROS under drought stress (Hu *et al*., 2010). These results were also in harmony with Zhang *et al*. (2008) who reported that the positive effects

Table 4: Phytohormones, photosynthetic pigments and oxidative enzymes in squash growing under drought stress

Activities	Phytohormones			Photosynthetic pigments			Oxidative enzymes		
	IAA	GA_3	ABA	Chl. *a*	Chl. $_b$	Car.	PO (Abs. at 425 nm/g FW/15 min.)	PPO (Abs. at 420 nm/g FW/ 30 min.)	PAL (µmol trans-cinnamic acid/min./g protein)
Treatments	(µg/g FW)			(mg/g FW)					
100% of FC									
Chemical NPK	4.54^{f}	6.52^{g}	0.62^{a}	2.08^{h}	1.22^{a}	0.92^{g}	53.01^{fg}	7.47^{g}	45.02^{i}
Humic acid	5.64^{d}	9.54^{b}	0.35^{d}	2.55^{f}	0.88^{f}	1.06^{f}	53.25^{f}	9.45e	70.08^{f}
PGPR	9.42^{a}	11.18^{a}	0.21^{fg}	2.84^{d}	1.14^{b}	1.55^{a}	54.78^{e}	9.45e	78.07^{e}
75% of FC									
Chemical NPK	4.24^{g}	5.32^{h}	0.53^{b}	2.65^{e}	0.99^{e}	1.28^{d}	50.13^{g}	8.73^{fg}	45.69^{g}
Humic acid	6.33^{c}	8.43^{e}	0.32^{e}	2.89^{c}	1.05^{d}	1.45^{c}	57.72^{d}	12.51^{d}	96.79^{d}
PGPR	7.54^{bc}	9.22^{c}	0.13^{g}	3.12^{b}	1.07^{cd}	1.52^{b}	79.71^{b}	13.05^{c}	105.4^{b}
50% of FC									
Chemical NPK	3.82^{h}	4.92^{i}	0.47^{c}	1.75^{i}	1.09^{c}	1.18^{e}	31.14^{h}	8.82^{f}	45.47^{h}
Humic acid	5.19^{e}	7.46^{f}	0.24^{f}	2.24^{g}	0.39^{g}	0.79^{h}	60.48^{c}	13.14^{b}	102.6^{c}
PGPR	7.96^{b}	9.10^{d}	0.11^{gh}	3.29^{a}	1.22^{a}	1.51^{b}	85.83^{a}	18.18^{a}	106.9^{a}

Abs.: Absorbance *FW: fresh weight*

of HA on plant growth and productivity due to its hormone like activities through involvement in cell respiration, photosynthesis, oxidative phosphorylation, protein synthesis, antioxidant and various enzymatic reactions. These results were also in accordance with Hrynkiewicz and Baum (2011) who reported that PGPR could promote plant growth under stress conditions by phytohormones production that promoted cell division and cell enlargement. Next to that, the use of beneficial microbes might enhance plant's tolerance to adverse environmental stresses, which including drought stress (Zahir *et al*., 2008). Also, Khaled and Fawy (2011) reported that HA could significantly increase water holding capacity of soil, reduce water evaporation and increase its use by plants under drought stress. Furthermore, HA increased cell membrane permeability, oxygen uptake, photosynthesis and improved stress tolerance (Yildirim, 2007). Also, the highest significant value of IAA was observed in plants fertilized with PGPR under normal irrigation level, whereas no significant differences were observed in IAA values in plants inoculated with PGPR and grown under 50 or 75% FC. This might be resulted from the activities of the four PGPR strains against drought stress through production of EPS and ACC-deaminase (Table 2), providing moisture and increasing water holding capacity of soil, chelating various metal ions and promoting the growth of plant. In this respect, Maheshwari *et al*. (2012) reported that the physiological adaptation and genetic potential of bacteria for drought tolerance could improve plant production. In addition, the ability of PGPR to produce SA might play an important role in decreasing ABA content in plants under drought stress. It was suggested by Man *et al.* (2011) that SA and related compounds could inhibit the abscisic acid (ABA) induced stomatal closure. Moreover, they reported that because of water deficit and consequent interruption of CO_2 and O_2 exchange an excess accumulation of ROS such as superoxide radical, these reactive oxygen species might damage membrane lipids, protein, DNA and other cell components. Though, there was correlation between stress tolerance and increasing activity of the antioxidant system in vegetable crops (Mittova *et al*., 2002). Also, Chookhampeng (2011) proved that ROS were formed in higher plants under various abiotic stresses and this could increase the efficiency of plants against the ROS by enhancing anti-oxidative enzymes including catalase, peroxidase, hydrogen peroxidase, superoxide dismutase, ascorbate peroxidase and glutathione reductase. Generally, it was clear that when squash plants grown under drought stress were inoculated with PGPR the two oxidative enzymes peroxidase and polyphenol oxidase were increased at the beginning of plant life to protect plants against ROS resulted from stress and break free and conjugated phenols which formed under this conditions. But, after that these two enzymes were decreased and the enzyme phenylalanine ammonia lyase was increased because this enzyme works on the products of the two previous enzymes. Then, binds these products with proline and produce hydroxy proline compound, which migrates from its synthesized places to the cell walls to enter the installation of lignin, which helps to strengthen cell walls to reduce transpiration process and thus reduces the stress suffered by the plant (Celik and Atak, 2012).

Growth characteristics and yield:

Results in Table (5) clearly indicated that the highest significant values of some estimated growth characteristics i.e. plant height, number of leaves/plant, number of flowers/plant and number of fruits/plant were observed in plants grown under normal irrigation level. Whereas, all investigated parameters were decreased with the decreasing of irrigation level. These results were in accordance with Hussein *et al*. (2007) who reported that water deficiency led to high decreases in plant metabolic activities and plant growth. Also, HA application caused significant increases in squash growth parameters i.e. plant height and yield/plant

compared to chemical fertilization under drought stress. In addition, plants inoculated with PGPR strains and irrigated with 50 or 75% FC had higher parameters than those grown under the same conditions and fertilized with chemical fertilization. These results might be resulted from the ability of PGPR to reduce drought stress effects on plants and then improved their growth. These results were in harmony with Nadeem *et al*. (2006) who reported that the ACC deaminase producing PGPR could promote plant growth through sequestering and cleaving plant-produced ACC and then lowering levels of ethylene. This confirmed their effective roles to improve plant growth under stress conditions. Data also showed that the highest significant records of dry weight were observed in plants treated with PGPR and grown under normal irrigation level followed by plants irrigated with 75% of FC and inoculated with PGPR. Whereas, the lowest dry weight was observed in plants received chemical fertilizers and irrigated with 50% of FC. Zhang *et al*. (2008) reported that the inoculation of vegetable seeds with PGPR containing ACC-deaminase increased the growth of seedlings by 66% under abiotic stress conditions. Regarding to the effect of different treatments on squash yield, data presented in Table (5) showed that the highest and lowest significant values of squash yield were observed in plants treated with chemical fertilizers at 100% and 50% of FC, respectively. On the other hand, plants inoculated with PGPR strains gave the highest significant yield under 50 or 75% of FC. In addition, plants treated with humic acid and grown under drought stress gave higher significant yield than those grown under same conditions and fertilized with chemical fertilizers.

Table 5: Growth characteristics and yield of squash grown under drought stress

Activities / Treatments	Plant height (cm)	Number of leaves/plant	Plant dry weight (g)	Number of flowers/plant	Number of fruits/plant	Yield/plant (Kg)
100% of FC						
Chemical NPK	47.0^{a}	18.2^{a}	7.31^{c}	50.1^{a}	20.0^{a}	2.40^{a}
Humic acid	45.6^{c}	18.0^{b}	6.88^{e}	50.0^{a}	19.7^{b}	2.20^{c}
PGPR	46.8^{b}	18.0^{b}	7.84^{a}	48.7^{b}	19.7^{b}	2.26^{b}
75% of FC						
Chemical NPK	43.0^{g}	17.2^{c}	6.23^{f}	47.4^{e}	18.8^{c}	1.90^{f}
Humic acid	44.8^{d}	16.7^{e}	6.00^{g}	48.6^{b}	16.3^{f}	1.96^{e}
PGPR	43.7^{e}	16.9^{d}	7.42^{b}	48.0^{d}	18.3^{d}	2.00^{d}
50% of FC						
Chemical NPK	35.2^{i}	14.3^{g}	5.59^{h}	47.0^{f}	12.3^{h}	1.90^{f}
Humic acid	39.1^{h}	13.8^{h}	6.01^{g}	44.0^{g}	13.7^{g}	1.95^{e}
PGPR	43.5^{f}	16.1^{f}	7.06^{d}	47.3^{e}	18.0^{e}	1.99^{de}

Conclusion

Water insufficiency is a problem that began facing the world now and one of the major abiotic stresses. As well as, it was considered the most destructive environmental stress which affects the growth and productivity in most vegetable crops all over the world. Among the sensitive vegetables to water is squash, it is very sensitive to deficient water and over-irrigation. The current study aimed to increase squash tolerance to drought stress using ACC-deaminase and osmoprotectants producing PGPR in comparison with humic acid and chemical fertilizers applications. In the first part of study, PGPR strains showed high ability to produce phytohormones, osmoprotectants and ACC-deaminase as well as, atmospheric nitrogen fixing. These mechanisms increase their ability to operate under drought stress conditions. In the second part, results emphasized that the use of these PGPR strains enhanced drought tolerance of squash and increased all activities in soil and plant including microbial enzymes activities in rhizosphere, osmoregulators, photosynthetic pigments, phytohormones and oxidative enzymes in plant. Alongside, growth characteristics and yield were enhanced under drought stress when plants fertilized with PGPR compared to other treatments.

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