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Colonization of pepper roots with salt-tolerant PGPR as an inducer for saline stress

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ABSTRACT

This study was divided into two parts, the first part was carried out *in vitro* to examine the ability of six salt-tolerant PGPR strains to colonize of two pepper cultivars roots namely Romy and Qarn Ghazal. Then, choosing one of these cultivars for greenhouse experiment. The second part was conducted in the experimental farm station of Fac. Agric. at Moshtohor during 2011 season to alleviate the ability of the selected PGPR strains (*Bacillus megaterium* D159, *Paenibacillus alvie* D139 *and Azospirillum lipoferum* D207) in combination with compost and humic acid to increase resistance of cultivated pepper under saline stress conditions and improve its productivity. The highest significant increase of macronutrients (N, P and K) uptake and photosynthetic pigments was observed in pepper inoculated with biostimulant combined with humic acid + compost. The application of humic acid combined with compost significantly decreased the proline content in pepper. On reverse, amino acids, sugars and phenols were increased with the application of PGPR combined with compost and/or humic acid.

Key words: Colonization, PGPR, saline stress, proline, humic acid and pepper.

INTRODUCTION

Salinity is one of the most critical constraints and hampers agriculture production in many areas around the world, including Egypt. The high salt content decreases the osmotic potential of soil water and consequently, this reduces the availability of soil water for plants (Aşik *et al.*, 2009). Because of the economic impact of stress and the large amount of energy required to alter the environment to suit the plant, it becomes increasingly important to utilize sustainable techniques for inducing salt tolerance in plants better adapted to stress (Smit *et al.*, 2001). PGPR have positively influence plants vitality and the ability of the plants to cope with a biotic stress conditions such as drought and salinity (Nadeem *et al.*, 2006). The interest in the consumption of pepper fruits (*Capsicum annuum* L.) is to a large extent, due to its content of bioactive nutrients and their importance as dietary antioxidants. The total area of cultivated pepper in Egypt for about 400 thousand fed. (Abrol *et al.*, 2010). Sousa *et al.* (2008) reported that the tolerance for high salinity should be criteria for many microorganisms aiming their adaptation in saline soils and aiming their capacity of colonizing the root system.

This study aimed to maximize the ability of six salt-tolerant PGPR strains to colonize pepper roots *in vitro* and then alleviate their ability to improve pepper productivity under saline stress in combination with compost and humic acid.

MATERIALS AND METHODS

Performance of PGPR strains for pepper seeds colonization *in vitro*

Petri dishes containing filter paper were autoclaved at 120ºC for15 min. Seeds of pepper varieties namely Romy(P1) and Qarn Ghazal (P2) were agitated in 70% ethanol for 5 sec for sterilizing seeds surface. The ethanol was discarded and the seeds were rinsed with sterile water. Then filter paper was moistened about 40% (Amin *et al.,* 2004). Ten seeds were put on the filter paper and 1ml of each PGPR strains suspension was added on seeds. The dishes were incubated at 25ºC for six days.

Germination rate (%), root length (cm) and colonization intensity (cfu/ml) were estimated, the most priority of pepper for PGPR strains colonization was chosen for green-house experiment.

Soil and soil mixtures

Soil of the experiment was obtained from El-Sharkia Governorate (Sahl El-Hussinia). The obtained soil was mixed with Agric. gypsum at rate of ½ ton/fed. and course sand at rate of 20 ton/fed., then subjected to mechanical and chemical analyses before using in cultivation Table (3). Soil analyses were carried out in Analysis Center and Agricultural Consultancy, Fac. Agric. at Moshtohor according to the method described by Page *et al.* (1982).

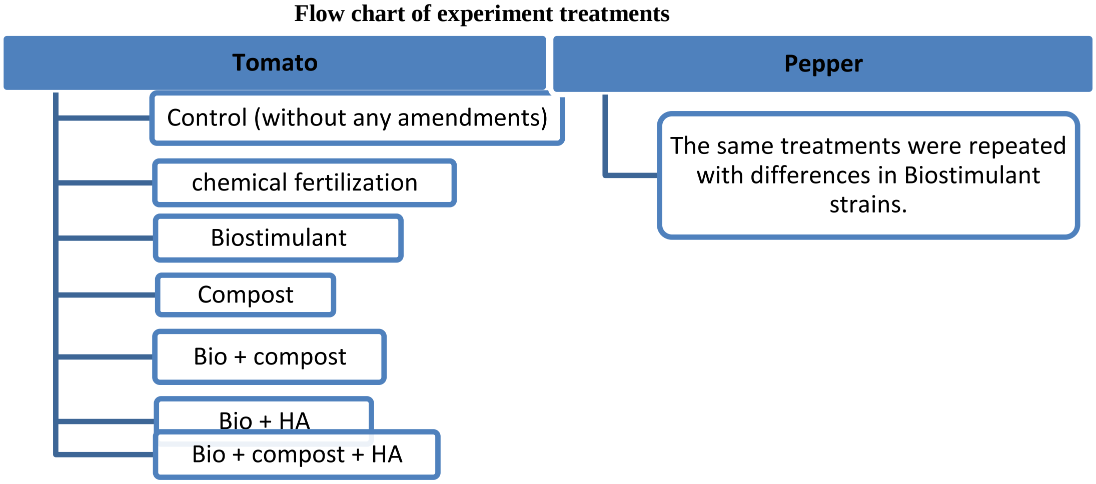
Table (1). Soil texture and chemical analyses of the experimental soil.

Parameters		Unit	Values	
	Soil texture		Soil before adjustment	Soil after adjustment
Sand		(%)	45.01	50
Silt		(%)	16.32	33
Clay		(%)	38.67	17
Textural class		(%)	Clay	Clayey silty
	Chemical analysis			
EC		dS/m	24.34	13.92
pH			8.30	8.61
Organic matter		(%)	0.62	1.12
Total nitrogen		(%)	0.010	0.114
Soluble cations	Na^+	meq./L	201	57.93
	K^+		1.34	8.27
	Ca_2^+		14.22	35.0
	Mg^{2+}		27.12	38.0
Soluble anions	$CO_3^=$	meq./L	Zero	Zero
	HCO_3^-		9.93	28.00
	Cl^-		186	81.00
	SO_4^{2-}		47.75	30.20

Experimental design

Treatments were distributed in a randomized complete block design with three replicates.

Flow chart of experiment treatments



Preparation of biostimulant inocula

The biostimulant inocula for pepper (*Bacillus megaterium D159, Paenibacillus alvie D135*and *Azospirillum lipoferum D207*) were prepared in specific broth media. Cell suspension of *A. lipoferum* contains about (10 x 10^5cfu/ml) 7 days-old on semi-solid malate medium (Dobereiner, 1978), *Bacillus megaterium* (90 x 10^6 cfu/ml) 2 days-old on modified Bunt and Rovira agar medium, modified by (Abdel-Hafez, 1966)and *P. alvie* (60 x 10^7cfu/ml) 2 days-old on King's medium (King *et al.*, 1954).

Cultivation process

Prior to cultivation, pepper seedlings were soaked by dipping the root system in a mixture of PGPR inocula (cell suspension of biostimulant) for 60 minutes before transplanting; sucrose solution (40 %) was used as an adhesive agent. The same prepared PGPR inocula were added to grown plants three times throughout the growing season at a rate of 300 ml/pot. The recommended dose of compost was 10 ton/fed for pepper. Whereas, humic acid was added to soil at rate of 4 kg/fed. Compost and humic acid were added at transplanting. A half dose of inorganic nitrogen fertilizer (50 kg N/fed) as ammonium sulphate was supplemented for treatments of biostimulant and biostimulant + humic acid. Also, a full dose of inorganic phosphorus fertilizer (25 kg P_2O_5/fed) as calcium super-phosphate and potassium fertilizer (40 kg K_2O/fed) as potassium sulphate were supplemented for all treatments in three equal doses.

Determinations

Plant samples were taken for total nitrogen, phosphorus and potassium contents according to the methods described by (A.O.A.C, 2005); A.P.H.A. (1992) and Dewis and Freitas (1970), respectively. Photosynthetic pigments (chlorophyll A & B, total chlorophyll and carotenoids) were spectrophotometrically determined according to Nornal (1982).Proline amount was determined in the plant according to the method of Bates *et al.* (1973). Total amino acids were determined according to the method described by Rosein (1957). Reducing, non-reducing and total sugars were colormetrically determined by the picric acid method described by Thomas and Dutcher (1924). Free, conjugated and total phenols were colorimetrically determined using the "Folin and Ciocalteu" reagent as described by Snell and Snell (1953). Fruits were harvested at proper maturity stage (120 days), then counted, weighed and the following data were calculated: Number of fruits/plant, individual plant yield and weight of fruit.

Statistical analysis

Statistical analysis was carried out according to Snedecor and Cochran (1989). The differences between the means value of various treatments were compared by Duncan's multiple range test (Duncan's, 1955).

RESULTS AND DISCUSSION

Colonization ability of PGPR strains for pepper roots

Data in Table (23) indicated that when pepper cultivars were inoculated with two phosphate dissolving bacteria *Pseudomonas fluorescence* D23 and *Bacillus megaterium D159*, the highest and lowest values of pepper root length were observed with P1 cultivar when inoculated with *Bacillus megaterium D159* and *Pseudomonas fluorescence* D23, respectively. These results are in agreement with Davey and O'toole (2000) who emphasized that the effective colonization of plant roots by PGPR plays an important role in growth promotion, irrespective of the mechanism of action.

Table 2. Colonization ability of PGPR strains and their effect on seed germination of pepper cultivars.

Treatments	Cultivar	Root length (cm)	Colonization intensity cfu/ml (x 10^4)
Control (without inoculation)	P1	1.8	--
	P2	1.6	--
Ps. fluorescence D23	P1	0.4	20
	P2	0.7	21

B. megaterium D159	P1	3.0	31
	P2	2.0	31
P. alvie D135	P1	3.4	290
	P2	2.9	270
A. lipoferum D178	P1	1.0	1.2
	P2	1.5	1.7
A. lipoferum D207	P1	2.4	3.5
	P2	1.5	2.8

Also, by using the nitrogen fixing bacteria namely *Azospirillum lipoferum D178* and *Azospirillum lipoferum D207* when P1 inoculated with *Azospirillum lipoferum D207* the highest root length was obvious being 2.4 cm therefore it increased with 1.6 fold comparing with P2. Whereas, unfavorably differences were observed with P1 and P2 cultivars when inoculated with *Azospirillum lipoferum D178*.

In addition, when pepper cultivars were inoculated with *Paenibacillus alvie D135* the highest root length was observed with P1. This trend of results was in concurrently with Chebotar *et al.* (2001) who refer that the colonization of roots by the introduced bacteria is very important in establishment an effective plant-bacterial interaction. The success of inoculated seeds or seedlings with beneficial bacteria is usually depends on the colonization potential of the introduced strains. The obtained results also revealed that the highest colonization intensity of pepper cultivars roots were observed when P1 was inoculated with *B. megaterium D159, Paenibacillus alvie D135* and *A. lipoferum D207*.Generally, in this experiment P1 (Romy pepper) cultivars gave the highest values

Nitrogen, phosphorus and potassium uptake in pepper shoots

It is obvious from data presented in Table (3) that the lowest records of nitrogen and phosphorus uptake were observed in moderately saline soil without any amendments (control). This may be due to the negative effect of salinity on nutrients uptake. Similar trend of results was observed by Francois and Maas (1999) who reported that the lower uptake of minerals has been observed in several plant species grown in saline conditions. The obtained results also showed that chemical fertilization gave significant higher values of N, P and K uptake than soil amended with biostimulant and higher values of P and K uptake than soil amended with compost only.

Table 3.Nitrogen, phosphorus and potassium uptake by pepper (P1) as affected by different treatments of soil amendments.

Treatments	Nitrogen (mg/plant)	Phosphorus (mg/plant)	Potassium (mg/plant)
Control	77.11^{de}	2.21^{c}	11.8^{e}
Chemical fertilization	88.13^{c}	2.92^{a}	14.8^{c}
Biostimulant	79.22^{d}	1.60^{d}	12.7^{d}
Compost	88.13^{c}	2.28^{b}	12.7^{d}
Bio. + compost	103.3^{ab}	2.18^{c}	15.2^{b}
Bio. + HA	100.4^{b}	1.02^{d}	15.6^{b}
Bio. + compost + HA	107.2^{a}	2.81^{a}	20.3^{a}

Control: without any soil amendments.
Biostimulant strains*: B. megaterium D159, P. alvie D135 and A. lipoferum D207.*
HA: Humic acid

On contrast, the highest significant records of N and K uptake were observed in soil inoculated with biostimulant combined with compost in presence of humic acid. This could be attributed to the addition of humic acid to soil which improve various soil properties and release of mineral nutrients for plant uptake. Similar results were observed by Yildirim *et al.* (2011) who reported that salt-tolerant PGPR can ameliorate the deleterious effects of salt stress by altering mineral uptake. Also, Çimrin *et al.* (2010) studied the effect of humic acid application on pepper plants cultivated in moderately saline soils. They found that humic acid can ameliorate the deleterious effects of salt stress by increasing root growth, altering mineral uptake and decreasing membrane damage, thus inducing salt tolerance in pepper.

In addition, it was obvious that when soil inoculated with biostimulant and humic acid application gave significant lower values of N and P uptake than soil amended with biostimulant combined with compost in presence or absence of humic acid. This may likely be due to the addition of compost which affected the release of nutrients to plants and increased minerals uptake (Alvarez *et al.*, 1995)

Photosynthetic pigments in pepper leaves

Data in Table (4) emphasized that the highest values of chlorophyll A& B and total chlorophyll were observed in pepper grown in soil amended with biostimulant in combination with compost and humic acid. In addition, Chemical fertilization gave higher values of chlorophyll A being 0.557 mg/g fresh leaves therefore it increased with 2.15 fold comparing to biostimulant only.

Table 4. Photosynthetic pigments of pepper's leaves (P1) as affected by different treatments of soil amendments.

.Treatments	Chlorophyll as mg / g fresh leaves			
	A	B	Total	Carotenoids
Control	0.288^{e}	0.141^{f}	0.512^{g}	0.882^{a}
Chemical fertilization	0.557^{d}	0.532^{d}	1.247^{e}	0.700^{c}
Biostimulant	0.259^{f}	0.279^{e}	0.614^{f}	0.278^{e}
Compost	0.745^{b}	0.610^{c}	1.568^{c}	0.416^{d}
Bio .+ compost	0.654^{c}	0.911^{b}	1.748^{b}	0.423^{d}
Bio. + HA	0.574^{d}	0.581^{d}	1.318^{d}	0.784^{b}
Bio. + compost + HA	1.042^{a}	1.287^{a}	2.092^{a}	0.126^{f}

Abbreviations: as those stated for Table (3)

From data presented in Table (4) it was obvious that the lowest values of chlorophyll B and total chlorophyll were observed in free amendments moderately saline soil (control).These results are in agreement with Ali *et al.* (2004) who reported that a decrease in photosynthetic pigment content of pepper plants when treated with NaCl. Reduction in chlorophyll concentrations is probably due to the inhibitory effect of the accumulated ions. In addition, results revealed that carotenoids were increased in high soil salinity. So, the highest records of carotenoids were observed in control treatment. This result is in harmony with Zahra *et al.* (2010) who reported that in high salinity levels, carotenoids content was increased. Results also emphasized that carotenoids were decreased under all soil treatments compared to control, since soil treatments which contain humic acid and biostimulant in presence of compost gave lower values of carotenoids rather than the control.

Proline accumulated in pepper leaves

The obtained results in Table (5) emphasized that when pepper plants were grown in moderately saline soil without any amendments, the highest amounts of proline were observed. These results are in harmony with Chookhampeng (2011) who reported that salinity treatments caused the increased proline content in pepper plant.

Data clearly showed that soil amended with compost in combination with biostimulant and/or humic acid significantly decreased the amounts of accumulated proline in pepper leaves being 0.25 mg/g fresh leaves therefore it decreased 8 fold comparing to control. Also, pepper inoculated with biostimulant combined with humic acid decreased proline amounts being 0.43 mg/g fresh leaves therefore it decreased with 1.8 fold comparing to biostimulant alone. This result may likely be due to the beneficial effect of boost inocula of biostimulant strains through growth period and humic acid in decreasing soil salinity. These results are in agreement with Çimrin *et al.* (2010) who studied the effect of humic acid application on pepper plants cultivated in salt-affected soils. He showed that humic acid treatment can ameliorate the deleterious effects of salt stress by increasing root growth, altering mineral uptake and decreasing membrane damage, thus inducing salt tolerance in pepper plants.Chemical fertilization gave high records of proline accumulation being 1.98 mg/g fresh leaves therefore it increased with 2.5 fold comparing with tomato inoculated with biostimulant only. This result may be attributed to the deficient of this treatment to compost, biostimulant or humic acid.

From data presented in Table (5) it is obvious that soil salinity decreased total amino acids. So, the lowest values of total amino acids were observed in moderately saline soil without any amendments. While, the highest records of total amino acids were observed in soil amended with biostimulant in combination with compost in presence of humic acid. Also, the presence of humic acid had an important role in soil salinity tolerance and so increased total amino acids. The application of biostimulant combined with humic acid gave higher values of total amino acids being 2.10 mg/g therefore it increased with 2.14 fold comparing with soil treated with chemical fertilizers or biostimulant only.

Table 5. Proline and total amino acids accumulation in pepper's leaves (P1) as affected by different treatments of soil amendments.

Treatments	Proline accumulation as mg/g fresh leaves	Total amino acids as mg/g fresh weight
Control	2.00[a]	1.09[f]
Chemical fertilization	1.98[b]	1.41[e]
Biostimulant	0.79[c]	1.44[e]
Compost	0.75[c]	2.31[d]
Bio .+ compost	0.42[d]	2.52[b]
Bio. + HA	0.43[d]	2.10[c]
Bio. + compost + HA	0.25[e]	2.79[a]

Abbreviations: as those stated for Table (3)

Estimation of phenols and sugars

Data in Table (6) revealed that the lowest values of total, free and conjugated phenols were observed in soil treated with chemical fertilizers, these results are in agreement with Rivero *et al.* (2003) who reported that the phenolic compounds were accumulated as a defense mechanism against a biotic stress. On the other hand, the highest values of total and free phenols were observed in soil inoculated with biostimulant in combination with compost in presence of humic acid. In addition, the dual inoculation of pepper with biostimulant and humic acid increased the total, free and conjugated phenols than the dual inoculation with biostimulant and compost. Data in Table (6) also emphasized that the highest significant amounts of total and reduced sugars were observed in soil amended with biostimulant and humic acid. Whereas, the lowest values of total and reduced sugars were observed in free amendments moderately-saline soil. These results are in harmony with Navarro *et al.* (2002) who observed that pepper plants exhibited slightly decrease in reducing sugar concentrations with salt application and could be explained by the increasing of fruits respiration which observed when the ionic strength of the nutrient solution increases.In addition, the highest and the lowest amounts of non-reduced sugars were observed in soil amended with biostimulant combined with compost and humic acid and soil treated with chemical fertilizers, respectively.

Table 6. Phenols, sugars and total amino acids in pepper's leaves (P1) as affected by different treatments of soil amendments.

Treatments	Phenols mg/g fresh weight			Sugars as mg glucose/g fresh weight		
	Total	Free	Con.	Total	Reduced	Non-reduced
Control	12.24[f]	7.04[e]	5.20[cd]	24.61[de]	11.84[f]	12.77[e]
Chemical fertilization	11.05[g]	6.20[f]	4.85[e]	25.16[d]	16.63[c]	8.53[f]
Biostimulant	13.77[d]	7.12[e]	6.65[b]	28.51[c]	14.40[e]	14.11[d]
Compost	15.81[b]	8.73[c]	7.08[a]	31.30[bc]	17.14[b]	14.16[d]
Bio. + compost	12.58[e]	7.60[d]	4.98[d]	32.98[b]	15.62[d]	17.36[c]
Bio .+ HA	14.62[c]	9.10[b]	5.52[c]	39.13[a]	19.66[a]	19.47[b]
Bio. + compost + HA	17.17[a]	10.5[a]	6.68[b]	38.57[ab]	17.14[b]	21.43[a]

Yield and yield components

Data in Table (7) indicated that there was significant difference between control (soil without any amendments) and other soil treatments, the control treatment gave the lowest number of fruits per plant, weight of one fruit and fruit yield/plant. This could be due to the harmful effect of salinity on pepper plants. Similar results were observed by (Navarro *et al.*, 2002) who reported that the salinity decreases the pepper yield. Data also revealed that the highest weight of one fruit was obtained from plants grown in soil amended with compost in presence of humic acid and biostimulant. This may be likely due to the beneficial effect of humic acid in decreasing soil salinity and so increasing pepper yield. These results are in agreement with Zandonadi *et al.* (2007) who reported that humic acid increased growth and yield of various crops including vegetables. Also, Ghoname and Shafeek (2005) found that adding of organic manures to sandy soil would stimulate quantitative characteristics of vegetable crops.

Table 7. Yield and yield components of pepper (P1) as affected by different treatments of soil amendments.

Treatments	Number of fruits/ plant	Weight of one fruit (g)	Fruits yield/plant (kg)
Control	4[c]	17.8[f]	0.125[g]
Chemical fertilization	6[b]	30.0[b]	0.288[b]
Biostimulant	5[b]	25.0[d]	0.130[f]
Compost	6[b]	27.3[c]	0.163[d]
Bio. + compost	8[a]	23.3[e]	0.186[c]
Bio. + HA	5[b]	28.0[c]	0.140[e]
Bio. + compost + HA	9[a]	33.5[a]	0.301[a]

Abbreviations: as those stated for Table (3)

Chemical fertilization gave significant higher fruit yield being 0.288 kg/plant therefore it increased 2.2, 1.76, 1.7 and 2.06 fold comparing to biostimulant, compost, biostimulant combined with compost and biostimulant combined with humic acid, respectively. In addition, the highest significant fruit yield of pepper was observed in soil amended with biostimulant combined with compost in presence of humic acid. This result was similar to those obtained by Abbaspoor *et al.* (2009) who emphasized that PGPR promoted the salt-tolerant PGPR *Ps. fluorescence* and *Ps. putida* can be used to overcome salinity stress and improving growth and yield of most crops under saline conditions. In addition, Grazia *et al.* (2007) who reported that the application of compost gave positive affect on most growth parameters of sweet pepper, promoting increase of precocity and yield. Also, khaled and Fawy (2011) reported that humic substances started to be given to the soil in Egypt and in other parts of the world as well to improve the crop yield.

CONCLUSION

This study could be recommended that the use of salt tolerant PGPR as biostimulant for pepper grown in moderately saline soil can improve plant defense against saline stress conditions and increase the productivity. Results also indicated that pepper inoculation with PGPR in combination with compost and humic acid enhanced plant defense to stress through the decreasing of proline accumulation and increasing of some compounds as an indicator to plant resistance for saline stress.

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إستعمار جذور الفلفل بالريزوبكتريا المنشطة لنمو النبات والمتحملة للملوحة كمحفز ضد الإجهاد الملحى

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تلعب الكائنات الحية الدقيقة دوراً حيوياً فى التربة من حيث القدرة على مساعدة النبات لتحمل ظروف الإجهاد سواء الحيوى أو البيئى مثل الملوحة، تم إجراء هذا البحث بهدف تقييم بعض سلالات الريزوبكتريا المنشطة لنمو النبات والمتحملة للملوحة من حيث قدرتها على إستعمار جذور الفلفل وبالتالى مدى إمكانية إستخدامها كمنشط حيوى للفلفل تحت ظروف الأراضى المصرية المتأثرة بالأملاح لتحسين نموه وإنتاجيته.وإتضح من تجربة المعمل أن ثلاث سلالات من الريزوبكتريا المستخدمة كانت لها القدرة على إستعمار جذور الصنف "رومى" بدرجة أكبر من الصنف "قرن غزال" ،لذلك تم إستخدام الصنف رومى فى تجربة الصوبة. ولقد اظهرت نتائج هذه التجربة أن أن أعلى معدل إمتصاص لكلاً من النيتروجين والفوسفور والبوتاسيوم فى حالة إضافة الكمبوست إلى التربة مع وجود الهيوميك والتلقيح بالسلالات المنشطة لنمو النبات، وأن أقل معدل إمتصاص للعناصر وجد فى حالة التربة الغيرمدعمة بأى إضافات (الكنترول).أيضاًكان أقل محتوى لصبغات البناء الضوئى ظهر فى حالة التربة المالحة دون تدعيمها بأى إضافات، ولكن تلقيح التربة بالسلالات المنشطة للنمو مع وجود الهيوميك أو الكمبوست أعطى قيماً أعلى من صبغات البناء الضوئى بالمقارنةبتلقيح التربة بالسلالات المنشطة للنباتات بمفردها، وقد وجد نفس الإتجاه من النتائج فى حالة كلا من الفينولات والسكريات و الأحماض الأمينية. ولكن على النقيض من ذلك وجد أن أعلى مستوى من البرولين المتراكم فى الأوراق ظهر فى حالة النباتات المنزرعة فى التربة المتأثرة بالأملاح دون تدعيمها بأى إضافات ، وأن أقل تركيز للبرولين ظهر فى حالة النباتات المنزرعة فى التربة المدعمة بالكمبوست مع وجود الهيوميك والتلقيح بالسلالات المنشطة لنمو النبات. آيضاً أظهرت النتائج أن الملوحة تؤثر تأثيراً سلبياً على محصول الفلفل حيث ظهر أقل محصول من الفلفل فى حالة التربة المالحة الغير مدعمة بأى إضافات والغير ملقحة حيوياً، ولكن وجد أن إنتاج الفلفل كان أعلى مايمكن فى حالة تدعيم التربة بالكمبوست مع وجود الهيوميك والتلقيح بالسلالات المنشطة لنمو النبات.