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EFFECT OF BIOLOGICAL AGENT *BACILLUS SUBTILIS* ON CHEMICAL COMPOSITION AND ANTIOXIDANT ENZYMES OF DATE PALM PLANTLETES (*PHOENOEX DACTYLIFERA* L.) UNDER SALT STRESS CONDITIONS

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ABSTRACT : Iraqi southern soils are facing a severe problem, which is a high accumulation of salts for many years of water irrigation; thus, it becomes necessary for search about high tolerant crops such as date palm. This current work was done at Date Palm Research Center, to investigate the inversely effects of *Bacillus subtilis* on the bad expression of salinity at three levels 10, 15 and 20mM/l on the date palm (*Phoenix dactylifera* L.) plantlets, chemical compositions as contents of total chlorophyll and carotenoid content, total soluble protein, phenols and antioxidant enzymes activity (catalase CAT and peroxidase POD). Our results proved that levels of salinity drastically severe reduction of total chlorophyll, carotenoids and increase total soluble protein and phenol compound comparable to the control treatment (without salts). Moreover, it showed that there is a close positive relation between salt stress and the antioxidant enzymes, catalase (CAT) and peroxidase (POD), which was significantly enhanced in the presence of salinity levels. Antioxidant enzymes had the defense system for salt tolerance in many plants. On the other hand, the application of *B.subtilis* had significantly ameliorated the harmful effects of salinity, which accompanied by markedly increase in all studied parameters, particularly compared to control treatment (salts only). The tolerance of date palm plantlets to soil salinity could be improved and alleviated the harmful effects of salinity by the application of *B. subtilis* to the soil.

Key words : Date palm, salt stress, chlorophyll, carotenoid, catalase, peroxidase, phenols, protein.

INTRODUCTION

One of the world's most recent agricultural problems has been soil salinity. Date palm (*Phoenix dactylifera*) is an essential tree in Iraq that it exposed to extreme conditions such as high temperatures and drought. Under these conditions, salts are dissolved and deposited in upper soil layers. The accumulation of salt in the upper layers of soil may also be due to inadequate management of irrigation. Nevertheless, plants make and accumulate a community of secondary metabolites called 'osmolytes' to relieve cell function and reduce water losses from cells. Osmolytes include proteins, amino acids, carbohydrates, and compounds of quaternary ammonium (Amal *et al*, 2007). All of these compounds engineered to mitigate the harmful effects of toxic reactive oxygen species (ROS) and enhance the functional integrity of cells (Ahmid *et al*, 2010). There are two modes of action that have alleviated the effect of oxidative damage oxidative as follows: systems remove the ROS and are restricted to allow levels and process that elevate antioxidant enzymes, *i.e.*, catalase, peroxidase and superoxide dismutase and systems that elevate oxidized antioxidants such as glutathione, glutathione reductase, ascorbate and dehydroascorbate reductase (Smirnoff, 1993). Plants may change widely in their phenolic compound contents and quantities, with both structures and their environment affecting the type, and these changes affect the quality and quantity of these compounds (DeAbreu and Mazzafera, 2005).

Plant growth-promoting rhizobacteria (PGPR) is a variety of bacteria found in the rhizosphere, root-related (Ahemad and Kibret, 2014). These species grow and contribute to the root surface area, colonize and compete with others, At the same time, different mechanisms needed to increase plant growth, shielding plants from the inverse effects (García-Cristobal *et al*, 2015). Directly, plant metabolism conversion can be identified with the involvement of rhizobacteria. Indirectly, rhizobacteria helped promote mechanisms that are important in the metabolism of plants. Examples; antibiotics, chitinases,

cellulases, 1,3-glucanases, proteases, and lipases; production of siderophores, suppressof harmfull organisms, motivated of systemic resistanceand alleviated stress impacts (Ahemad and Kibret, 2014). Armade *et al* (2014) recent studies have shown that increased photosynthesis in salt-stressed plants correlated with reduced rates of reactive oxygen species (ROS) associated with bacterial ability. Some studies have found that the application of these organisms in several plants has improved antioxidant enzymes, such as superoxide dismutase, peroxidase, catalase, polyphenol oxidase and other different compounds which related with defends (Chakraborty *et al*, 2013). In plants, activated antioxidant enzymes are related to contribute to alleviating the effect of salt pressure on plants, also by excluding hydrogen peroxide from salinity-stressed roots (Kim *et al*, 2005). There is little knowledge, however, about the mechanisms needed in defense of bacterial plant enzymes.

Bacillus is one of the main plant growth rhizobacteria that has a wide range as a research model because it has a wide various genetic and is sensitive to different environmental conditions, it also has various beneficial properties applied in agriculture, farming practices and microbiological controls (Niazi *et al*, 2014). Zhang *et al* (2008a), it is shown that the use of *B. subtilis* in Arabidopsis plants has increased photosynthesis through many modulation signals with significant regulatory roles for photosynthesis plants, including driven plants, withstand various stresses and increase the growth of plants.In other research, insulation *B. subtilis* alleviates the growth of major medicinal plants by improving the production of phytohormones such as gibberellins, auxins and cytokinins (Egamberdieva and Jabborova, 2015; Egamberdieva *et al*, 2015). Woitke *et al* (2004) find that insulation *B. subtilis* had a small number of partial products and improved vegetative plant growth. *B. subtilis* does not cause harm to humans and can also produce high rates of antibiotics in soils such as lipopeptides, polypeptides and phospholipids to kill other harmful species (Stein *et al*, 2002). Medeiros *et al* (2011) found that the bacteria *B.subtilis* had been associated withthe profilingof transcriptionalof cotton plants, which arranged biotic-stress tolerance. Zhang *et al* (2011) found that the biological agent bacteria *B. subtilis* N11 very effective in their control of banana wilt disease by strong colonization in the rhizosphere. In Arabidopsis plants found that the with bacteria *B. subtilis* (GB03) regulates the plant growth hormones, auxin, cell enlarged and increased photosynthesis by decreasing glucose sensing and levels of ABA also regulating specific tissue as well as reduces Na^+ to reduce the harmful effects of salt stress against plants also promotes salt tolerance as well as reduces total Na^+ by regulating tissue-specific expression (Zhang *et al*, 2008a). *B. subtilis* has recently found to ameliorate osmotic pressure by increasing levels of genetics for osmoprotectants (Zhang *et al*, 2010). The present study points to the reverse effects of salinity through the application of bacteria *B.subtilis* on date palm plantlets, total chlorophyll, carotenoids, antioxidant enzymes (catalase and peroxidase), phenolic compounds, and total proteins.

MATERIALS AND METHODS

This study conducted in the greenhouse and Laboratories of Date Palm Research Center, Basra University, during the period from 2018 and 2019. The experiment conducted to determine the effect of *Bacillus subtilis* on the antioxidant enzyme activity and chemical contents in date palm plantlets under salt stress conditions as a complete randomized design with three replications. Treatments were three levels of salinity stress, including 10, 15 and 20 ds/m, *B. subtilis* was at 10^8CFU/ml, the date palm plantlets that showed the same growth divided into eight groups. The identification of plant group treatments was as follows:

The treatments were: (1) uninoculated plantlets irrigated with tap water (non-saline); (2) plantlets inoculated with *B. subtilis* and irrigated with non-saline water; (3) uninoculated plantlets irrigated with saline water (10ds/m). (4) uninoculated plantlets irrigated with saline water (15ds/m). (5) uninoculated plantlets irrigated with saline water (20ds/m). (6) plantlets inoculated with *B. subtilis* and irrigated with saline water (10ds/m). (7). plantlets inoculated with *B. subtilis* and irrigated with saline water (15ds/m). (8) plantlets inoculated with *B. subtilis* and irrigated with saline water (20ds/m). Date palm plantlets were ten months old. The soil water content of experimental soil calculated when water is no longer leaving the soil sample. First, the plantlets were treated with thebacterial suspension at a concentration of 10^8 CFU/ml various above-mentioned treatments according to the soil water content of the experimental soil. Each group consists of three replicates (each replicate is one pot containing three plantlets). The levels of soil water content were controlled by weighting the pots daily. The soil water content of the control plantlets kept at 85% SWC. After completion of salt treatments, the following measurements recorded:

Determination of total chlorophyll and total carotenoid

The method of Arnon (1949) used to determine total chlorophyll, 0.5 g of fresh leaves cutting in small pieces,

then extracted in 15 mL of 80% acetone. The mixture centrifuged at 10000 x g for 5 min and then absorbance was read at 663 and 645 nm using a spectrophotometer (CECL 2021, England). The method described by Lichtenthaler and Wellburn (1983) used to calculate the total Carotenoid contents from fresh leaves at 470 nm optical density using a spectrophotometer (CECL 2021, England).

Measurement of catalase (CAT) activity

To assay the activity of catalase, the procedure of Chance and Maehly (1955) as follow. A 1.5 ml of reaction mixture containing 30 µL of distilled water, 50 µL of buffer Tris-HCl (1 M and pH = 8), five mM of EDTA, 900 µL of hydrogen peroxide (10mM) added to 20 µL supernatant. Absorption was calculated at 240 nm by spectrophotometer for 1 minute. Catalase activity measured as absorbance per minute per mg protein.

Measurement of Peroxidase (POX) activity

The procedure of MacAdam *et al* (1992) used to measure the activity of peroxidase. In this method, 3 ml of combination comprising containing 2.5 ml sodium phosphate (0.05 mM and pH = 7), 30µg leaf protein and used 20 µL of guaiacol (200 mM) as inhibitor agent . all mixture added to cuvette and before assay 10 µL of hydrogen peroxide (30%) added to reaction mixture as the electron acceptor. To calibrate the spectrophotometer to zero used control mixture without hydrogen peroxide (30%). The wave at 475 nm for 1 min. At temperature 25°C and the enzyme activity was evaluated as mg per minute to protein absorption.

Determination of soluble proteincontent (SPC)

The procedure of Lowry *et al* (1951) used to determine the total soluble protein. A 0.5 g of dry leaves was extracted with 15ml of acetone (80%). Then the mixture centrifuged (4000 rpm), 2ml of supernatants collected and added to 2ml of alkaline reagent, 50ml of Na_2CO_3 (2%) prepared in NaOH(1%) and 1ml of $CuSO_4.5H_2O$(0.5%) prepared in sodium-potassium tartrate(1%) they mixed twice then the mixture remained for 10 minute, 0.5 ml of total Folin-phenol reagent diluted 1:2 (v/v), then immediately added and mixed. After 30 min, spectrophotometer (CECL2021, England) is used to measure at 700nm. Total protein content was evaluate as $mg.g^{-1}$DW.

Determination of total phenolic content (TPC)

TPC assayed by the method reported in Alawlaqi (2014), Folin–Ciocalteu's reagent used to evaluate the TPC. 0.5 g of dry weight leaves extracted with 15ml of 80% ethanol and then the mixture was centrifuged at 10000 rpm supernatant collected and vigorous and marked to 10ml.5ml of distilled water added and shaken well, 0.5 of folin-Ciocation reagent added to the extract. After 5 minute, 2ml of sodium carbonate (20%) was added, then incubation the mixture for 30 minute. Spectrophotometer (CECL2021, England) used to measure at 660nm. for absorbance and to use pyrocatechol as standard, the content of TPC calculated in the leaf extract.

Statistical analysis

A complete randomized design employed in all experiments with four replicates, the results presented here analyzed by using the software SPSS for Windows (version 10.0). Statistical significant confirmed by ANOVA (Analysis of variance) and with revised least significant difference (RLSD) test at the probability level of 0.01, with four replicates for each parameter. All results were expressed as mean and standard deviation of the mean.

RESULTS AND DISCUSSION

Photosynthetic pigments

Results of total chlorophyll and total Carotenoid pigments (Figs. 1 and 2) indicated a significant reduction in chlorophyll and carotenoid contents under salt stress at 15 and 20 ds.m, which were 1.85 and 1.66 $mg.g^{-1}$ F.W for chlorophyll respectively and 0.013 and 0.018 $mg.g^{-1}$ F.W. for carotenoid respectively. This reduction of pigments contents might be attributed to the toxic effects of salt on the biosynthesis of pigments, increasing their degradation or maintaining damage of the chloroplast thylakoid (Rao and Rao, 1981), salinity from 10000-14000 ppm decreased chlorophyll contents in leaves on date palm (Darwesh *et al*, 2006 and Hassanein *et al*, 2009) on wheat chlorophyll contents also decreased under 200 mM NaCl (Abd -Allah *et al*, 2018 on chickpea and Shahid *et al*, 2013) on Pea*, Pisum sativum* L. Results of the pot study showed that (overall assessment) salinity significantly decreased the photosynthetic pigments (total chlorophyll and carotenoid contents) of plantlets. The maximum total pigments observed in plants inoculated with *B. subtilis* under non-salt stress and salt stress conditions. Data regarding the effect of inoculation on chlorophyll content in plants revealed that the *B. subtilis* strain caused a maximum increase of chlorophyll content in leaves of plants grown in non-salt stress conditions. This strain also increased chlorophyll and carotenoid content compared to control (plants grown in non-saline conditions). Our results showed that, relative to control plants grown in non-saline soil, salinity decreased total chlorophyll and total carotenoid content in the Date palm leaves. Similar results observed in other studies in which

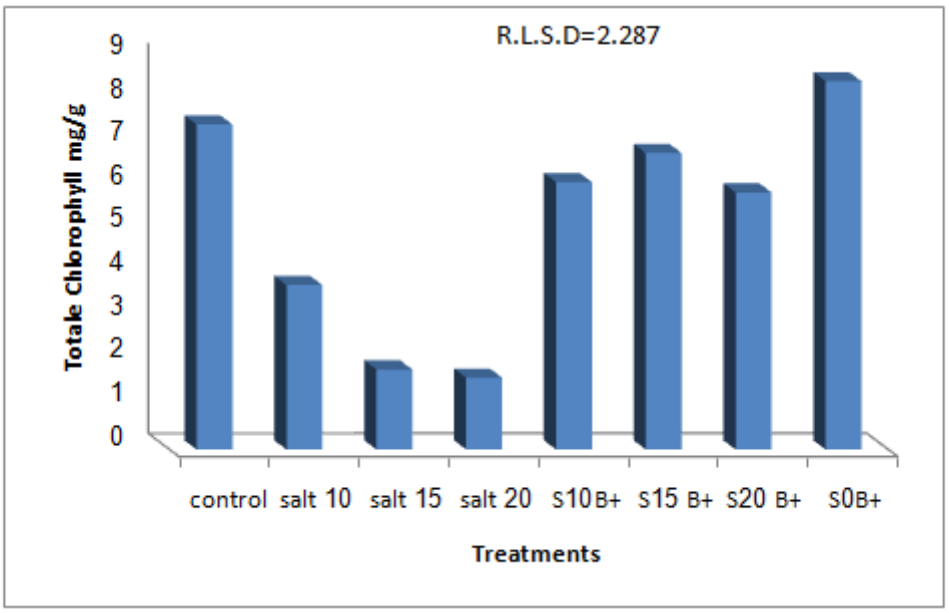

Fig. 1 : Effect of *B. subtilis* treatments on chlorophyll content at leaves of plantlets undersalt stress.

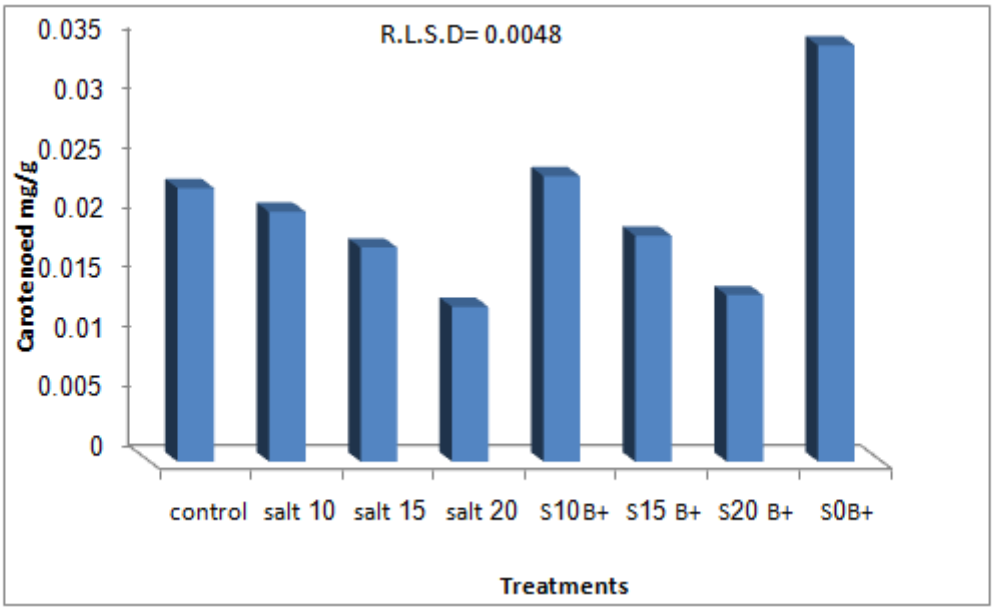

Fig. 2 : Effect of *B.subtilis* treatments on carotenoid content at leaves of plantlets under salt stress.

photosynthetic pigments significantly reduced under NaCl stress (Hashem *et al*, 2014; Abd_Allah *et al*, 2015). According to Zörb *et al* (2009), the reduction in pigment content attributed to the destructive effect of salt stress on chloroplasts in plantlets. PGPR strain *B. subtilis* increased total chlorophyll and carotenoid contents in leaves of plantlets grown under both non-saline and saline soil conditions. Mohamed and Gomaa (2012) studied the ability of PGPR *B. subtilis* to ameliorate the inhibitory effect of salt on radish (*Raphanus sativus*) in greenhouse experiments; they found that seeds inoculated with *B. subtilis* significantly increased the fresh and dry weight of roots and leaves, photosynthetic pigments, proline, total free amino acids, crude protein and N, P, K+, Ca^{2+}, and Mg^{2+} uptake compared to uninoculated control plants under saline conditions.

Antioxidant enzyme activity CAT and POD

From results on the effect of salinity levels which defined at (Figs. 3 and 4) on the activity of antioxidative enzymesCAT and POD, it shown that salinity stress at (10 and 15ds.m) increased the activities of these antioxidants, whereas lowest results found at control treatment (without salts) on the activities of enzymes CAT and POD, respectively. The plants treated with *B. subtilis* recorded the increase in the activity of CAT and POD compared with none-inoculated plants(control). The combination of treatments between salinity stress with *B. subtilis* increased the activity of CAT and POD at all

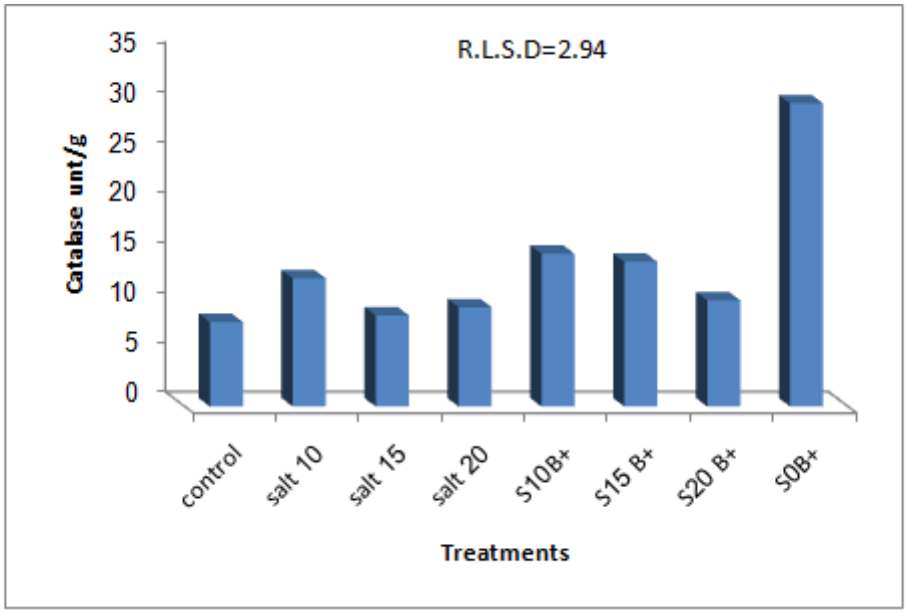

Fig. 3 : Effect *B.subtilis* treatments on catalase activity at leaves of plantlets under salt stress.

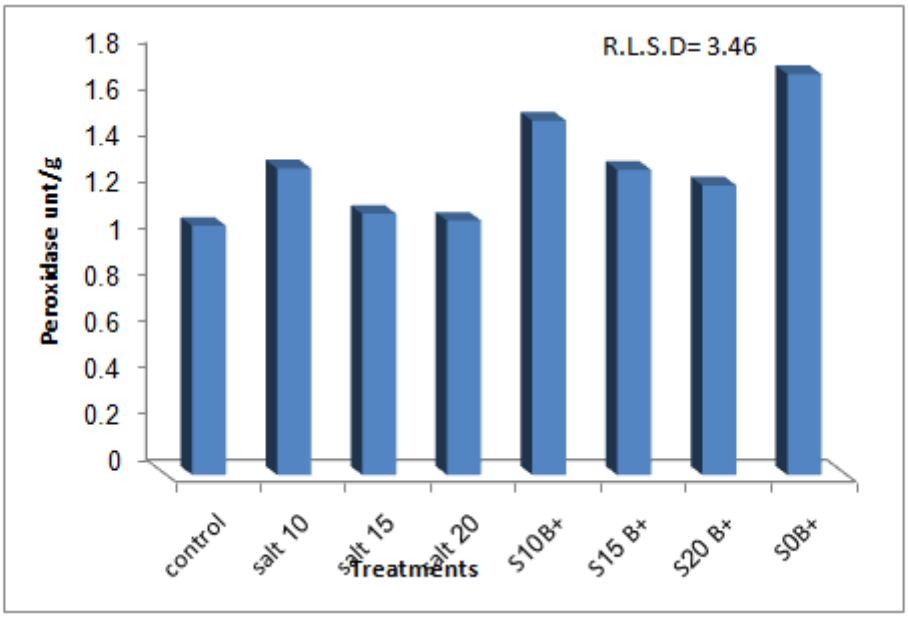

Fig. 4 : Effect of *B. subtilis* treatments on peroxidase activity at leaves of plantlets under salt stress.

salinity levels (10, 15 and 20 ds.m). In addition, the best results obtained at the combination of salinity stress treatments with *B. subtilis* significantly increased the activity of CAT and POD compared with the salinity treatments without *B. subtilis*. Abdel Latef (2011), catalase (CAT) considered to be the most critical enzyme that eliminates H_2O_2 which accumulate during salt stress and water deficit and damage the photosynthetic apparatus, also can destroy healthy metabolism through oxidative damage of lipids, proteins and nucleic acids. Pandey *et al* (2010) stated that peroxidase (POD) constituents a class of heme-containing enzymes ubiquitously present in prokaryotic and eukaryotic organisms, which regard as an antioxidant enzymes, protecting cells from the destructive influence of H_2O_2, Dasgupta *et al* (2011) on Indian mangroves, recently Abbaspour *et al* (2012) on *Pistacia vera* L. and Abdulwahid (2012) on date palm seedlings cv. Hillawi, found that NaCl from 40 to 200 mM and from 25% to 50% increased activity of CAT and POD. The activities of the antioxidative enzymes such as catalase, guaiacol peroxidase and superoxide dismutase increase under salt stress in plants and a correlation of these enzyme levels and salt tolerance exists (Mittova *et al*, 2003). The ameliorative role of *B. subtilis* against salt stress in plantlets might be due to the reduction of ROS and the up -regulation of antioxidant systems and nutrient uptake (Han *et al*, 2014; Janahiraman *et al*, 2016). Though, the

activity of antioxidant enzymes stimulated by salinity treatment, *B. subtilis* inoculation further stimulated the antioxidant system, leading to accelerated elimination of toxic ROS (Hashem *et al*, 2016). The up-regulation of CATand POD protected plantlets from free-radical-induced membrane dysfunction. The acceleration of POD activity in *B. subtilis* inoculated plantlets might be due to improved salinity stress tolerance caused by enhancement of the biosynthesis of lignins and other related protective compounds for reducing oxidative stress (Boerjan *et al*, 2003). Chen *et al* (2007) reported that higher POD activity in *Vigna unguiculata* ameliorated the effects of salt stress. Increased activity of H_2O_2 neutralizing enzymes, including CAT and POD, in *B. subtilis* inoculated plants regulates plant growth by protecting delicate organelles, such as chloroplasts, wherein key metabolic processes occur (Han and Lee 2005; Hashem *et al*, 2016). Increased CAT activity in plantlets under saline soil conditions might be due to decreased H_2O_2 production in apoplasts (Mutlu *et al*, 2009). Mittal *et al* (2012) demonstrated that higher activity of CAT in *Brassica juncea* led to tolerance against high salinity. CAT plays a crucial role during stress and eliminates H_2O_2 (which is a signaling molecule that rapidly diffuses through membranes) to prevent membrane and organelle damage (Bienert and Chaumont, 2014).

Total protein content

The results shown in Fig. 5 indicate a positive effect for salinity levels using various concentrations on the total soluble protein of date palm plantlets. It appears from the data that there was a general decrease in soluble protein content that corresponded with the increase in salt concentrations, whereas there was a general reverse in protein content, There appeared to be an inverse relationship between salt concentrations and protein content, although protein content decreasedat the higher salt concentration. Soluble proteins and proline have been shown involved in osmotic regulation in the plant, playing an essential role intolerance of the plant to salinity stress (Bartels and Sunkar, 2005) and may use as a protective strategy to alleviate Na^+ toxicity (Chen *et al,* 2002). In the current study, salinity stress caused a significant increase in soluble proteins, compared to un-salinized control plants. The reduction of protein previously recorded by Bassuony *et al* (2008) and Sadak *et al* (2010). They concluded that the reduction of protein under salinity stress suppressed by the accumulation of total amino acids and proline. Results of a study by Kapoor and Srivastava (2010) on *Vigna mungo* (L.) support the previous results. They observed a decrease in protein content when increasing salt concentration compared with control plants. The plants treated with *B. subtilis* recorded the increase in soluble protein compared with none-inoculated plants(control). The combination of treatments between salinity stress with *B. subtilis* increased total soluble protein at all salinity levels (10, 15 and 20 mM/l). The maximum increased of total soluble protein content was found at *B. subtilis* treatment alone. In addition,the best results obtained at the combination of salinity stress treatments with *B.subtilis* were significantly increased total soluble protein content compared with the salinity treatments without *B. subtilis*. *B. subtilis* protected plants from the deleterious effects of NaCl on chlorophyll pigments, which might contribute to the enhancement of the efficiency of photosynthesis bymaintaining the function of the pigment-protein complex (Rasool *et al*, 2013). Our results agree with Prathibha and Siddalingeshwara (2013) were found inoculated sorghum with *B. subtilis* was effective in seed germination and also on nutritional qualities such as total protein. Inoculation with *B. pumilus* Rs3 strain increased with 66% the total amount of seed soluble protein, probably due to stimulation of protein biosynthesis processes in soybean plants. They are considering that beans provide dietary proteins that play an essential role in human nutrition by complementing other foods (Broughton *et al*, 2003). The protein content enhancement is related to a higher relative increase in nitrogen fixation due to PGPR inoculation. Therefore, the increase of grains totals soluble protein content could be related to the enhancement of physiological activities of runner bean plants and after soybean growth (Stefan *et al*, 2010).

Total phenolic content (TPC)

The results shown in Fig. 6 indicated that the leaf phenolic compound content significantly increased at low and medium salt concentrations, 10 and 15 ds.m, respectively. At high salinity levels (20 ds.m), the accumulation of total phenols was declined. Our results agree with several reports of increasing phenolic compounds at low and moderate salinity (Navarro *et al*, 2006; Hanen *et al*, 2008). Also, the result showed that the inoculated plantlets with *B. subtilis* increased the total phenoliccomparing with control. Also, the best results were obtained at the combinationof salinity stress treatments with *B. subtilis*, which significantly increased total phenols content compared with the salinity treatments without *B. subtilis*. Also, our result showed that all inoculated treatments with *B. subtilis* were significant increased total phenols compared with non-inoculated treatments with *B. subtilis*. Phenolic acids are secondary metabolites extensively spread throughout the plant kingdom (Tomas and Espin, 2001). Phenolic compounds

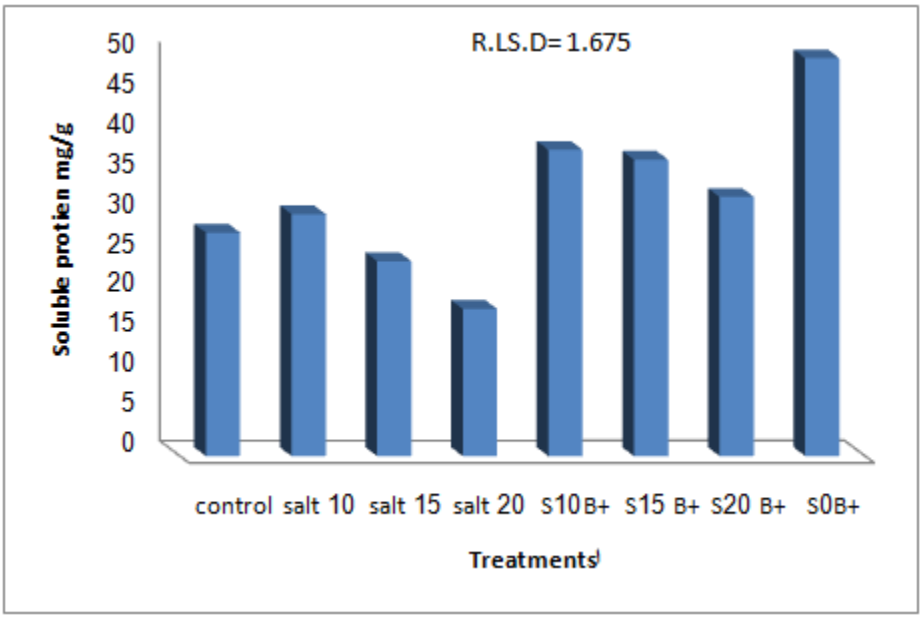

Fig. 5 : Effect of *B.subtilis* treatments on total soluble protein at leaves of plantlets under salt stress.

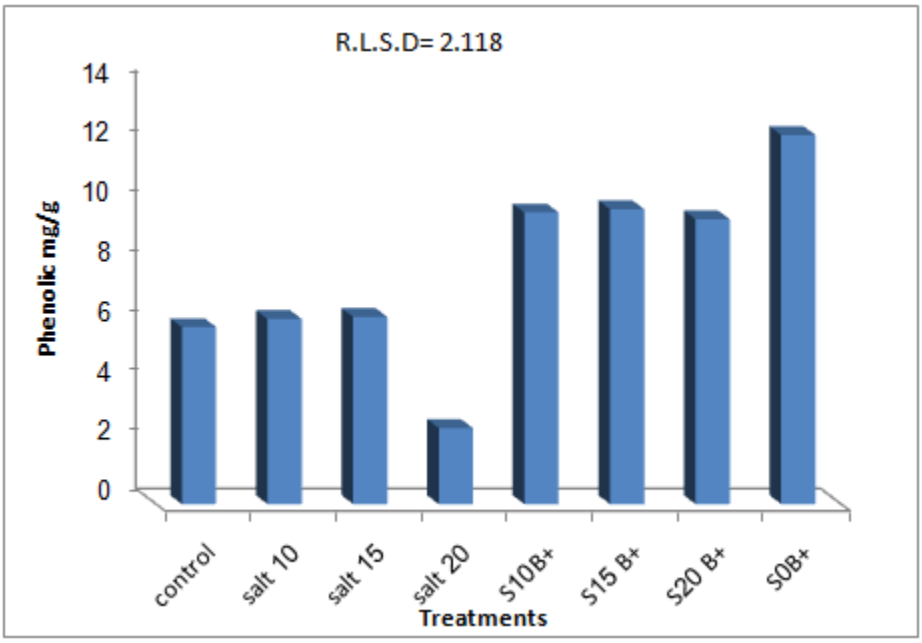

Fig. 6 : Effect of *B. subtilis* treatments on phenolic compounds at leaves of plantlets under salt stress.

are crucial for plant growth and reproduction and produced as a response to unfavorable environmental factors (light, chilling, salinity, etc.) and to defend injured plants (Valentine *et al*, 2003). The phenolic compounds increased in salinity conditions in red matured paper fruits (Navarro *et al*, 2006). Parida *et al* (2004) reported the accumulation of the phenolic content in moderate salinity in the mangrove.High accumulation of phenols in plants plays an imperative physiological role in overcoming salinity-induced oxidative stress (Hichem *et al,* 2009). Our results agreements with other others results,bd-Allah *et al* (2017) showing that the inoculation chickpea plants with *B.subtilis* increased the production of total phenols compared with control plants, also alleviated increase of total phenols content and prevented the negative effect of NaCl concentrate compared with non-inoculated plants with *B. subtilis* under NaCl concentrates. A results study of Zhou *et al* (2018) showed that the effects of salt stress on *Schizonepeta tenuifolia* Briq, the contents of antioxidants, including phenols and flavonoids, increased at low (25 mM) or moderate (50 mM) levels, but declined at severe (75 and 100 mM) levels. The accumulation of phenols in *B. subtilis*-inoculated Date palm plantlets correlated with a report by Bahadur *et al* (2007), which suggested that more considerable accumulation of phenolic compounds in pea seedlings led to higher

tolerance to fungal infection.

The results of the present study suggest that *B.subtilis* have a high potential to increase photosynthesis (total chlorophyll and carotenoid content). *B. subtilis* can indirectly enhance stress tolerance as a consequence of the increasing activity of some antioxidant enzymes during periods with intense photosynthesis. The *B. subtilis* was enhancing the increase of total soluble protein and phenolic compound content. Our study suggests that the *B.subtilis* may be used as bio fertilizers for increase production in sustainable and ecological agricultural systems. However, further studies are necessary in order to evaluate the impact of beneficial bacteria introduction into soilecosystems.

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