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The Role of Trehalose-producing *Bradyrhizobium japonicum* and *Azotobacter chroococcum* in Enhancing Salinity Tolerance of *Zea mays* Plants

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Salinity is one of the most potent abiotic elements in nature and is damaging to both plants and microbes. Osmostress response in bacteria involves the accumulation of small organic compounds called compatible solutes as trehalose. In this work the synthesis and accumulation of trehalose by *Bradyrhizobium japonicum* strain (ARC 517) were investigated. Different sources of carbon, nitrogen, initial pH, and inoculum level were studied in order to increase trehalose productivity. An optimal production medium containing glucose and yeast extract was found suitable for trehalose production. The results showed that keeping the pH of the culture broth at 6.0 is important for trehalose production. Moreover the optimal level of inoculum was 4.0%. The optimized parameters gave a maximum trehalose production of 22.65 mg ml^{-1}. Scanning electron microscope showed that

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cells of *B. japonicum* that enhanced for trehalose was aggregated and became the longest with length about 3 times higher than that of control. The results of field experiment revealed that, the *Zea mays* plants treated with *B. japonicum* strain (ARC 517) that enhanced for trehalose + *Azotobacter chroococcum* retained higher relative water content (RWC), chlorophyll content, K^+/Na^+ ratio as compared to other treatments. Positive correlation between trehalose overproduction and high-yield parameters were observed under saline conditions. These findings suggest that trehalose overproduction could be a beneficial characteristic for biofertilizers.

Keywords: Azotobacter chroococcum; Bradyrhizobium japonicum; carbon sources; salinity; trehalose; Zea mays.

1. INTRODUCTION

Salinity stress is a global issue in agriculture that has a detrimental impact on plant growth and productivity [1]. Biofertilizer is a substance that contains living microorganisms, which, when applied to seed, plant surfaces, or soil, mobilizes the availability of nutrients particularly by their biological activity, and promotes plant growth [2]. High osmolarity conditions cause bacteria to lose their cytoplasmic water and turgor. The term most usually used to describe this is osmotic stress. The only known way by which bacteria can restore and maintain cytoplasmic volume and turgor within the limits which allow development is to raise the osmolarity of the cytoplasm by accumulation of low molecular weight organic osmolytes (compatible solutes or osmoprotectants) [3]. These include sugars (such as trehalose and sugar alcohols), amines (such as glycinebetaine and polyamines), and amino acids (primarily proline), all of which are easily soluble in water and harmless at high doses. One of these osmolytes (compatible solutes) is trehalose, a non-reducing disaccharide that consists of two units of D-glucose linked by a glycosidic linkage, plays an important physiological role as an abiotic stress protectant for a large number of organisms, including bacteria, yeasts and plants. Several recent studies have demonstrated the involvement of trehalose in numerous signaling and metabolic pathways in plants, as well as its vital role in plant growth and development and the achievement of stress (e.g. drought, salinity and temperature) tolerance [4]. Trehalose is a substance that is present in many different types of life, including plants, invertebrates, and microorganisms [5,6] demonstrated that trehalose can protect organisms from external challenges such as heat, alcohol, osmotic, and oxidative stress. Trehalose can shield a variety of biological structures from abiotic stressors, according to [7]. Trehalose is a storage carbohydrate that builds up as cells transition into their stationary growth phase, as demonstrated by [8]. The importance of trehalose in the osmostress response has been well established in several rhizobia [9].The production of trehalose by rhizobia grown aerobically in liquid culture is variable; some strains produce negligible amounts, while others accumulate significant quantities and overproduction of trehalose can be occured in the presence of a variety of osmotic-stress agents (hexose sugars, inorganic salts, and pyruvate) as indicated by [10]. Trehalose's significance in bacterial survival both inside and outside of a host may be explained by its capacity to protect bacterial cells against a variety of stressors. For instance, *Bradyrhizobium japonicum* [11], S*inorhizobium meliloti* [12], and *Rhizobium leguminosarum* [13]. [14] proved that increasing the concentration of trehalose *in Bradyrhizobium japonicum* increases survival of bacteria on soya bean seeds.

Various researches demonstrated that rhizobia can act as plant growth promoting bacteria (PGPB) for non-legumes, such as sunflower [15] , wheat [16], rice [17] and maize [18] .The ability of *Rhizobium* to colonize roots of non-legumes was confirmed previously by [19] and *Rhizobium* that well known as a symbiotic N fixer is reported as asymbiotic (associative & endophytic) microorganisms [17], where they benefit plant growth and act as phytohormone producer, phosphate solubilizer and to some extent, as nitrogen fixer as indicated by [20].

Research on *Azotobacter chroococcum* in agricultural production has demonstrated its significance in enhancing plant nutrition and soil fertility [21,22] confirmed that inoculation with *A. chroococcum* improves crop resistance to salinity. Moreover, [23] proved that the combined application of *Bradyrhizobium* sp*.* and *Azotobacter* sp. showed a significant increase in overall performance of mungbean plants compared to other single (fertilizers) treatments. One of the most important crops grown

worldwide is maize, which has industrial use in addition to being important for human and animal food. This crop is extremely vulnerable to abiotic stress brought on by drought, excessive salinity, and extreme temperatures, which can result in yield reductions of up to 15% and an estimated loss of 16 million tonnes of grain [24]. It is therefore necessary to put strategies in place to reduce these losses as [25] who proved that the exogenously application of soaking rice seeds with 25 mM of trehalose could alleviate the harmful effects of salinity stress [26] proved that foliar application of trehalose had significant and positive effect on most growth parameters of wheat plants [27] confirmed that trehalose can efectively alleviate salt stress and enhance salt tolerance of tomato [28] concluded that trehalose treatments (100 µM or 500 µM) had pronounce effect in alleviating the harmful effect of moderate and severe drought stress on cowpea plant and enhanced its drought tolerance [29] demonstrated that trehalose was involved in the process of mitigating salt stress toxicity in tomato plants and provided specific insights into the effectiveness of trehalose in mediating salt tolerance.

The aim of this study was to investigate the ability of bradyrhizobial strain for trehalose accumulation and effect of different nutritional and environmental factors on trehalose production. Scanning electron microscope for bacterial morphological observation. Effect of application of *Bradyrhizobium japonicum* strain (ARC 517) that enhanced for trehalose and *Azotobacter chroococcum* (singly or co-inoculated) on various growth parameters of *Zea mays* plants grown under salinity stress.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Bradyrhizobium japonicum strain (ARC 517) and *Azotobacter chroococcum* were used in the present study. Strains were kindly provided by the Biofertilizers Production Unit, Agricultural Microbiol. Res Dept., Soils, Water and Environ. Res. Instit., Agric. Res. Center (ARC), Giza, Egypt.

2.2 Trehalose Determination

50 mL of the bacterial cultures were extracted for trehalose analysis by centrifuging them at 12,000 g for 15 min. at 4°C after incubating them at 28 ± 2°C for 3 days. After being cleaned with distilled water, the cells were extracted in 16 mL of 0.5 M cupric acetate for 3 hours at 4°C. After the supernatant was collected, 2 mL of the supernatant was incubated at 100°C for 5 min with 4 mL of a buffer containing 0.2% (w/v) anthrone-sulfuric acid. Trehalose was then measured at 590 nm as previously mentioned in accordance with [30].

2.3 *In vitro*, Factors Affecting Trehalose Production in Culture Media

Carbon source: Different carbon sources as glucose, fructose, sucrose, galactose, maltose, and mannitol were used as carbon source.

Nitrogen source: Nitrogen sources as yeast extract, peptone and ammonium sulphate were used as nitrogen source.

Initial pH: Different initial pH values (4, 5, 6, 7, 8, 9) were adjusted to determine the optimal initial pH for trehalose production.

Level of inoculum: Various inoculum levels 1%, 2%, 3%, 4%, 5% (v/v) were studied for their effects on trehalose production.

2.4 Ultrastucture of Trehalose Producing *Bradyrhizobium japonicum*

The tested strain was subjected to scanning electron microscopy observations, as described by [31]. The cells were first fixed with glutaraldehyde for sample preparation, rinsed with 0.2 M phosphate buffer (pH 7.4), and then dehydrated with gradient ethanol. The sample was dehydrated and dried using a CO_2 critical point desiccator. The dry material was then cut into pieces of the appropriate length, adhered to the table, and sprayed with gold. The morphology of strain cells was then examined using a scanning electron microscope at the Faculty of Agriculture, El-Mansura University, in the presence of salt concentration.

2.5 Soil Analysis

According to [32], the physical and chemical characteristics of the experimental soil were identified and are displayed in Table (1). Electrical conductivity (EC) and soil pH were measured in accordance with [33]. Utilizing the modified Kjeldahel approach, available N was calculated [32]. Olsen's approach [33] was used to colorimetrically determine available P. The

Flame-Photometer was used to calculate the available K [34].

Table 1. Some physico-chemical properties of used soil

Property	Values
Mechanical analysis	
Coarse sand (%)	4.20
Fine sand (%)	15.20
Silt (%)	35.30
Clay (%)	45.30
Texture grand	Clay
Physical analysis	
pH (1:2.5)	8.12
E.C.(dSm^{-1})	12.82
$CaCO_3$ %	11.50
Organic Matter %	0.47
Chemical analysis	
Macronutrients (ppm)	
N	107.1
P	2.95
K	171.16
Micronutrients (ppm)	
Fe	1.85
Mn	2.16
Zn	0.868
Cu	0.61
Soluble Cations ($meql^{-l}$):	
Ca^{++}	22.50
Mg^{++}	35.50
Na^{+}	68.50
K^{+}	1.48
Soluble Anions ($meql^{-l}$):	
CO_3^{--}	0.00
HCO_3^{-}	4.50
Cl^{-}	95.50
SO_4^{--}	27.98

2.6 Field Experiment

A Field experiment was carried out at El-Rowad village, Sahl El-Hussinia, El-Sharkia Governorate, Egypt, during summer season 2018 using a randomized complete block design to study the effect of enhanced *B. japonicum* strain (ARC 517) for trehalose, *A.chroococcum* and their Co-inoculation on maize growth and yield under saline soil conditions. The treatments were applied as follows:

1. Control (Recommended dose of NPK)
2. *A. chroococcum* + half dose of NPK
3. *B. japonicum* strain (ARC 517) on YEM + half dose of NPK.
4. *B. japonicum* strain (ARC 517) on optimized media+ half dose of NPK.
5. *B. japonicum* strain (ARC 517) on optimized media+ *A. chroococcum* + half dose of NPK.

Grains of *Zea mays* plants (C.V Single cross173) were inoculated with gamma irradiated vermiculite-based inoculants. All treatments received the half recommended dose of phosphorus, potassium and nitrogen (except control) according to Stander agriculture practices recommended by Ministry of Agriculture.

2.7 Relative Water Content

Relative water content (RWC) percentage was determined as described by [35]. Randomly selected young, completely formed leaves were taken from the plants. The fresh weight of the leaves was determined and they were immersed in distilled water in a test tube before being incubated in a refrigerator for 24 hours. With the help of tissue paper, the leaves were dried off, and the weight of the fully turgid leaves was calculated. The leaves were dried in an oven for 24 hours at 72°C. Finally, the dry weight was measured, and the relative water content was calculated using the equation below:

$$RWC\ (\%) = [(FW- DW)/(TW-DW)]x100$$

2.8 Pigment and Cation content

Chlorophyll-a, chlorophyll-b and total chlorophyll content were performed according to [36]. Absorbances were determined at 645, 652, 663 and 470 nm respectively. Calculations were estimated by using equations of [37]. K^{+} and Na^{+} contents in plant were determined according to [38].

2.9 Yield Components of Maize Plants

At harvesting stage the plants of three replicates in each treatment were harvested to calculate yield ($ton.fed^{-1}$), straw and weight of 100 grains (g) after drying at 70°C for 48 hr.

2.10 Statistical Analysis

The obtained results were statistically analyzed using the general linear models procedure of [39]. The differences were statistically tested using Duncan's multiple range tests.

3. RESULTS AND DISCUSSION

3.1 Trehalose Production as Affected by Carbon Source

The ability of *B. japonicum* strain (ARC 517) for trehalose biosynthesis was investigated in presence of different carbon sources. The results in Fig. (1) indicated that, the bacterial strain has the ability to produce intracellular trehalose and glucose was the best carbon source for trehalose production (19.43 mg ml^{-1}) followed by sucrose and mannitol (13.56 and 13.45 mg ml^{-1}).These results agree with [40] who reported that Rhizobium isolates from root nodules of *Phaseolus vulgaris* were able to grow well in the presence of glucose, galactose, mannitol, sucrose, and mannose. Moreover [41] proved that, rhizobia accumulate osmolytes products and the accumulation of these osmolytes is dependening on the carbon source in the growth medium. Several microbes have the capacity to accumulate large amounts of extracellular trehalose when supplied with glucose, according to [42,43] established that a strain of *Micrococcus varians* produced trehalose extracellularly from glucose.

3.2 Trehalose Production as Affected by Nitrogen Source

Regarding nitrogen sources that are essential for trehalose biosynthesis, it was discovered that yeast extract and peptone were both good for trehalose accumulation in culture broth, whereas trehalose was limited in ammonium sulfate-containing media, demonstrating the need for essential organic factors for trehalose biosynthesis (Fig. 2). These findings are consistent with [42], who demonstrated that media containing organic nitrogen sources produced more trehalose than those containing inorganic nitrogen sources.

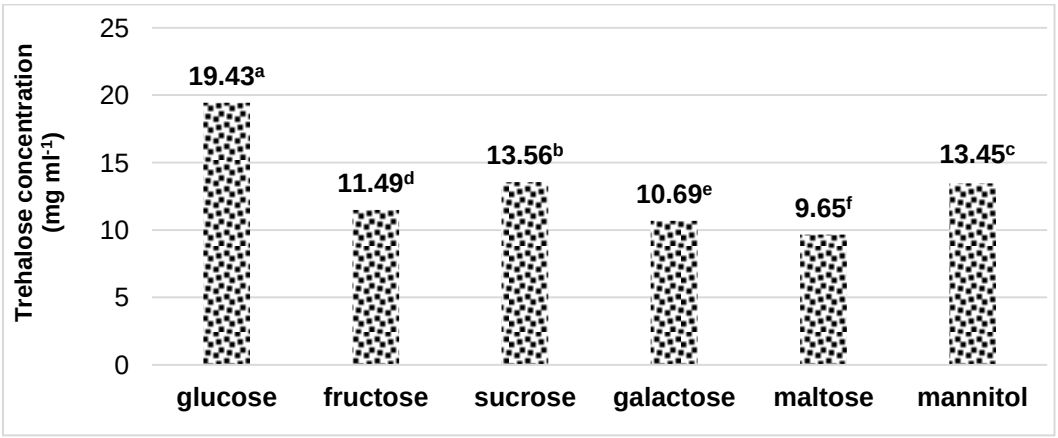

Fig. 1. Effect of different carbon sources on trehalose production by *B. japonicum* strain (ARC 517)

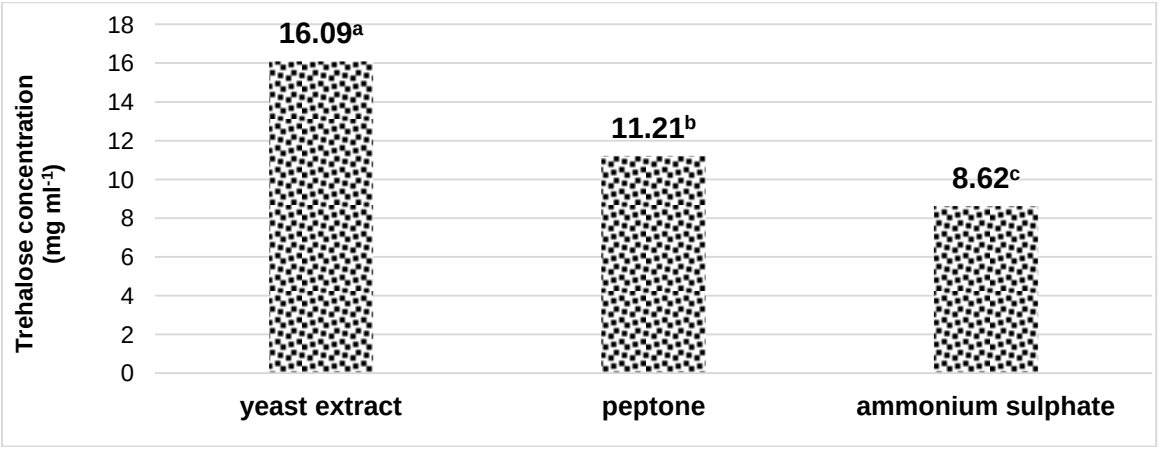

Fig. 2. Effect of nitrogen sources on trehalose production by *B. japonicum* strain (ARC 517)

3.3 Effect of Initial pH

According to [44], the pH adjustment increased the accumulation of trehalose; therefore, several initial pH values were utilised to evaluate their impacts on trehalose production, and the findings are given in Fig. (3). The most effective pH for the synthesis of trehalose was determined to be pH6.0 (17.24 mg ml^{-1}), followed by pH 7 (16.09 mg ml^{-1}). Lower or higher pH inhibited trehalose yield. This finding is consistent with [43] who demonstrated that the pH of the culture broth at 6.0 allowed *Micrococcus varians* strain to produce trehalose at its highest rate [44] demonstrated that maintaining the pH at 7 resulted in the best rate and yield of trehalose synthesis in *Propionibacterium* sp.

3.4 Effect of Level of Inoculum

The effects of different inoculum levels on the production of trehalose were investigated, and the results are shown in Fig. (4). The highest possible trehalose yield (17.24 &12.41mg ml^{-1}) was obtained at 4.0% and 3% inoculum level respectively [42] proved that 4.0% of inoculum increased trehalose productivity, this may be because a low inoculum density may give insufficient biomass causing reduced product formation. The above optimized parameters gave a maximum trehalose production of 22.65 mg ml^{-1}.

3.5 Scanning Electron Microscopy for Bacterial Morphology Evolution

Salt stress resulted in changes to the cell shape of the *B. japonicum* strain (ARC 517), as seen in Fig. (5). The cells were short and uniform in shape when there was no NaCl present. Furthermore, the bacteria grew slightly longer in the presence of 3% NaCl, about two times longer than in the control. Additionally, *B. japonicum* strain (ARC 517) cells that were improved for trehalose aggregated and grew to be the longest with lengths that were around three times greater than the control. These results agree with [45] who indicated that Bradyrhizobia are short rod shaped (0.5-0.9 µm by 1.2- 3.0 µm). Previous studies have shown that when exposed to high salinity, halophilic bacteria must alter their morphology to overcome salt stress [46]. The findings of the current study are in line with earlier findings [47] that indicated that PGPR cells grew longer in response to abiotic stress. According to [48], mother cells can increase their cell surface area in response to harmful environmental elements, allowing them to store more nutrients needed for the life activities of future generations. These findings are also similar to [49], who demonstrated that the salt-tolerant rhizobial strain's capacity to collect osmoprotectants may promote bacterial growth by lowering the osmotic stress brought on by salts.

3.6 Relative Water Content (RWC) %

Reduced osmotic potential is related to decreases in Relative Water Content (RWC) under stressful conditions [50]. Under osmotic stressors, increased accumulation of soluble sugars such as trehalose may act as an osmoprotectant to stop water loss from plant cells [51]. Determination of RWC % indicated that, the plants treated with *B. japonicum* strain (ARC 517) enhanced for trehalose +*A. chroococcum* retained higher water contents (59.8%) followed by single inoculation of the *B. japonicum* strain (ARC 517) enhanced for trehalose and control plants (50.8% and 49.3%) respectively. On the other hands *A. chroococcum* and *B. japonicum* strain (ARC 517) grown on YEM media gave lower results.This might be due to the fact that trehalose reduced the growth-inhibiting effects of salinity stress, improving the water status of plant tissues and increasing relative water content as indicated in Fig. (6). [52] indicated that trehalose treatment on maize plants improves water retention and plant tolerance through osmoregulation and stomatal closing at stress. Previous studies have shown that trehalose addition in saline medium protects *Catharanthus roseus* from the salt's negative effects on growth, RWC, and photosynthesis [53].

3.7 Photosynthetic Pigments

Salinity affects the survival and growth of rhizobia in soil [54], alters the protein and lipopolysaccharide content of cells, reduces the number of rhizobia in plant inoculants, reduces plant growth and photosynthesis [55]. Table (2) showed that treatment of *B. japonicum* strain (ARC 517) that optimized for trehalose + *A.chroococcum* significantly increased photosynthetic pigments (chlorophyll a, chlorophyll b and total chlorophylls) followed by single inoculation of *B. japonicum* strain (ARC 517) optimized for trehalose and *A. chroococcum* respectively as compared to that of *B. japonicum* strain (ARC 517) based on YEM media or untreated control plants. This action of trehalose

is supported by the findings of earlier research with rice plants, where exogenous trehalose enhanced photosynthetic pigment levels under stress [56]. In addition, [57] confirmed the promotive role of trehalose on photosynthetic pigments of wheat plant under drought stress. This stimulatory effect might be due to the role of trehalose in maintaining stability of chlorophyll envelope and maintaining chloroplast osmotic potential [58,59] confirmed that trehalose acts as a positive regulator of stress tolerance in plants.

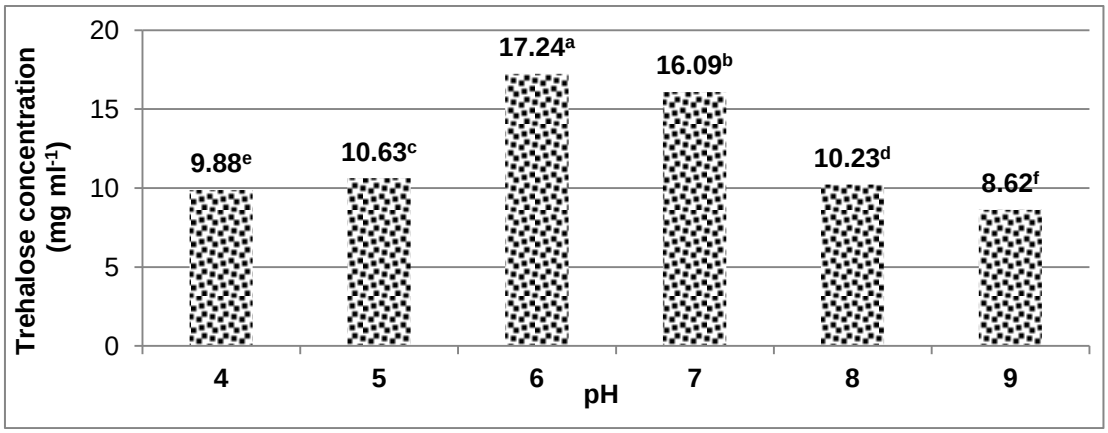

Fig. 3. Trehalose production by *B. japonicum* strain (ARC 517) as affected by pH

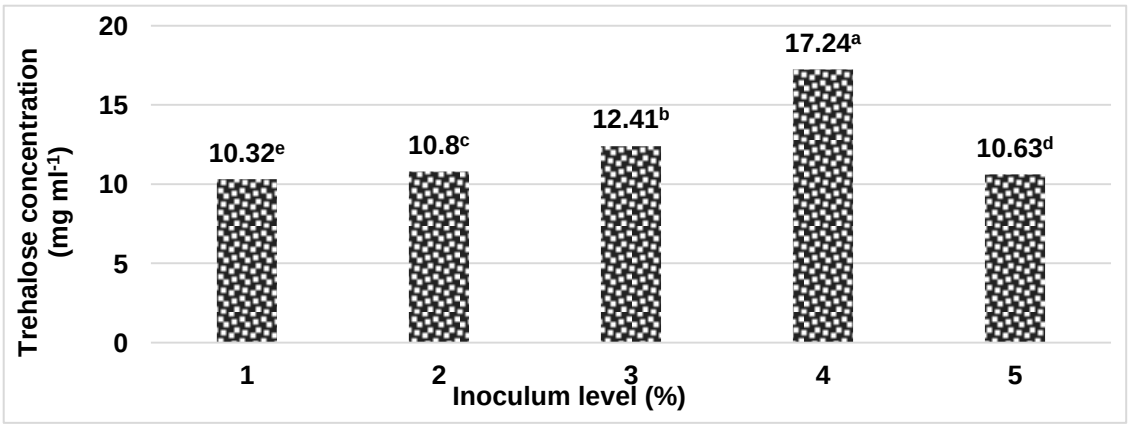

Fig. 4. Trehalose production by *B. japonicum* strain (ARC 517) as affected by inoculum level

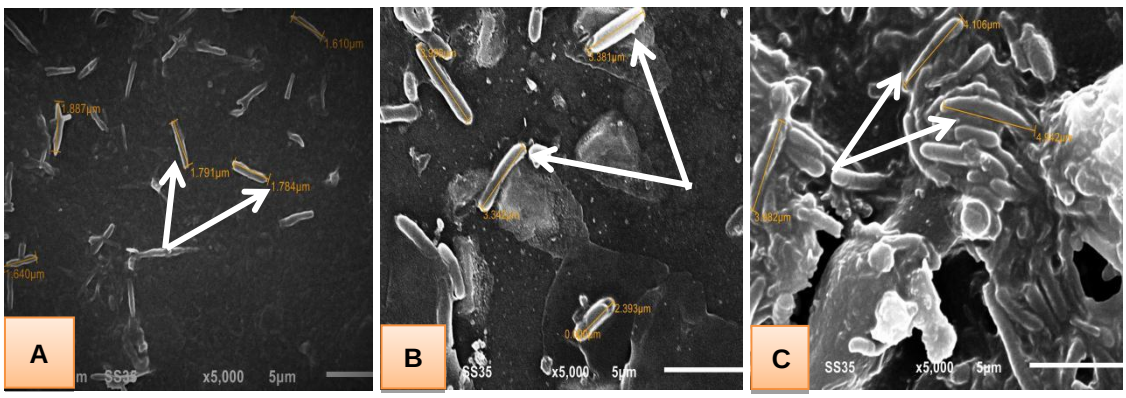

Fig. 5. Scanning electron microscopy images of *B. japonicum* strain (ARC 517) that enhanced for trehalose in presence of 0 and 3% NaCl

(A) B. japonicum in zero NaCl. (B) B. japonicum in presence of 3%. (C) .B. japonicum that enhanced for trehalose in presence of 3%

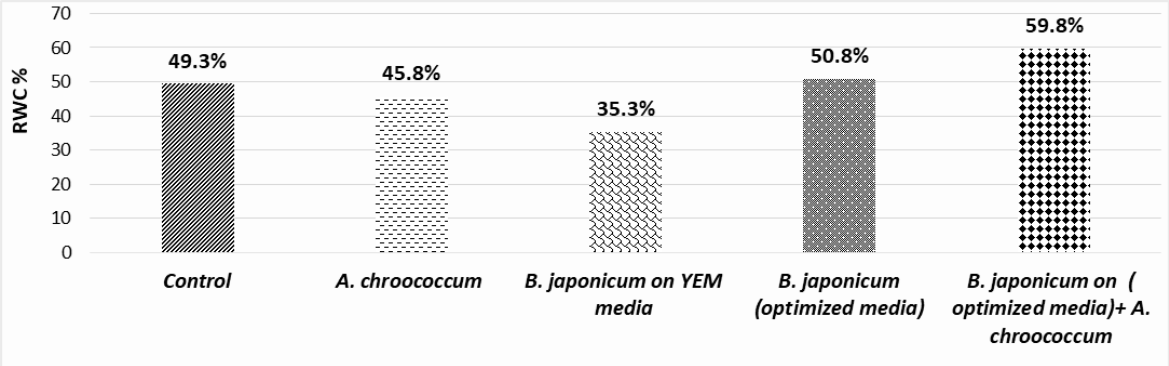

Fig. 6. Relative Water Content as affected by trehalose producing *B. japonicum* strain (ARC 517)

Table 2. Effect of *B. japonicum* strain (ARC 517) enhanced for trehalose on photosynthetic pigments in *Zea mays* plants

Treatments	ch-a	ch-b	total chl-
Control (Recommended dose of NPK)	1.2^{c}	1.53^{d}	3.39^{d}
A. chroococcum .+ half dose of NPK	1.02^{d}	1.77^{c}	3.55^{c}
B. japonicum strain(ARC 517) on YEM media	0.87^{e}	1.45^{e}	2.46^{e}
B. japonicum strain (ARC 517) based on optimized media	1.45^{b}	3.05^{b}	3.89^{b}
B. japonicum strain (ARC 517) based on optimized media + *A. chroococcum*	2.15^{a}	3.22^{a}	5.53^{a}

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test.

Table 3. Effect of *B. japonicum* strain (ARC 517) enhanced for trehalose on %K, %Na and K^{+}/Na^{+} ratio of *Zea mays* plants grown in saline soil

Treatments	% K	% Na	K / Na Ratio
Control (Recommended dose of NPK)	3.01^{e}	2.49^{b}	1.20
A. chroococcum + half dose of NPK	3.10^{c}	2.31^{d}	1.30
B. japonicum strain(ARC 517) on YEM media + half dose of NPK	3.08^{d}	2.6^{a}	1.18
B. japonicum strain(ARC 517) on optimized media + half dose of NPK	3.41^{b}	2.49^{b}	1.37
B. japonicum strain(ARC 517) based on optimized media + *A. chroococcum* + half dose of NPK	3.49^{a}	2.44^{c}	1.40

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test

Table 4. Effect of *B. japonicum* strain (ARC 517) enhanced for trehalose on yield components of *Zea mays* plants

Treatments	Grains (ton fed^{-1})	Straw (ton fed^{-1})	Weight of 100 grains (g)
Control (Recommended dose of NPK)	1.639^{a}	1.775^{a}	26.3^{c}
A. chroococcum. + half dose of NPK	1.572^{b}	1.275^{d}	26.5^{b}
B. japonicum strain(ARC 517) on YEM media + half dose of NPK	1.330^{d}	1.075^{e}	21.2^{e}
B. japonicum strain(ARC 517) on optimized media + half dose of NPK	1.400^{c}	1.325^{c}	23.5^{d}
B. japonicum based on optimized media + *A. chroococcum* + half dose of NPK	1.573^{b}	1.600^{b}	31.6^{a}

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test

3.8 Plant Cation Uptake

In trehalose treatments, shoot of inoculated plants maintained a higher K^+/Na^+ ratio as compared to control plants especially *B. japonicum* strain(ARC 517) enhanced for trehalose production + *A.chroococcum* (Table 3). Similar results were reported by [60] who showed that exogenous trehalose treatment significantly reduce the accumulation of Na^+ in the leaves, indicating that It might influence the cellular exclusion of Na^+ in a direct or indirect way to affect ion selectivity. K^+ is considered as a helpful ion that benefits plants under stress and salty conditions [61]. Further, [62] emphasized that salinity increased Na^+ and decreased K^+ concentration, thus decreasing K^+/Na^+ ratio. However, bacterial inoculation resulted in significant decrease of Na^+ and increasing K^+ concentration, and consequently K^+/Na^+ ratio could be increased.

3.9 Yield and Components of *Zea mays* Plants

The data in Table (4) revealed that yield, straw and weight 100 grains of *Zea mays* plants increased by co-inoculation of *B. japonicum* that enhanced for trehalose + *A. chroococcum* as compared to control and *B. japonicum* strain (ARC 517) based on YEM media. These results agree with [63] who proved that inoculation of maize plant with *Azospirillum brasilense* containing higher levels of trehalose confers drought tolerance and a significant increase in leaf and biomass. Regarding the stimulatory effect of trehalose on seed yield, [64] discovered that osmoregulators reduced fruit abscission by decreasing ethylene production, increasing the quantity of fruits and seeds and, as a result, the amount of seeds produced per plant. Additionally, the use of osmoregulators may improve photosynthetic pigments, which would enhance dry matter accumulation and seed yield [65].

4. CONCLUSION

One of the key mechanisms for bacterial tolerance to stress conditions like salinity is the buildup of suitable solutes such as trehalose. This work clearly showed that the optimized parameters of media growth gave a maximum trehalose of 22.65 mg ml^{-1} that leads to the improving in plant growth under salinity stress. Co-inoculation of *B. japonicum* strain (ARC 517) enhanced for trehalose and *A. chroococcum* can improve some of the growth indices *Zea mays* plant under saline stress conditions. These results suggest that trehalose overproduction might be a desirable property for biofertilizers.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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