Original Research Article

The Role of trehalose-producing *Bradyrhizobium japonicum* and *Azotobacter chroococcum* in enhancing salinity tolerance of *Zea mays* plants

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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) ~~was~~ wree 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 cells of *B. japonicum* that enhanced for trehalose was aggregated and became the longest with length about 3 fold 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 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: Salinity, trehalose, *Bradyrhizobium japonicum*, carbon sources, *Azotobacter chroococcum* ,*Zea mays*.

1. INTRODUCTION

Salinity stress is a worldwide agricultural problem that negatively affects plant growth and production [1]. Several beneficial microorganisms are inoculated into the soil or on the seed surface to improve agricultural yield [2].When bacteria are exposed to environments of elevated osmolarity, they lose cytoplasmic water and turgor. This is commonly referred to as osmotic stress. The only known method by which bacteria can restore and maintain cytoplasmic volume and turgor within the limits which allow growth, is to increase the osmolarity of the cytoplasm by accumulation of low molecular weight organic osmolytes (compatible solutes or osmoprotectants) [3]. These include amino acids (mainly proline), amines (such as glycinebetaine and polyamines), and sugars (such as trehalose and sugar alcohols), all of which are readily soluble in water and nontoxic at high concentrations.

Trehalose is one of these osmolytes (compatible solutes) which is a disaccharide of two D-glucose molecules linked by a glycosidic linkage[4].Trehalose is found in various organisms, including microorganisms, invertebrates and plants [5]. It has been demonstrated in the past that the production of trehalose plays a significant role in protecting bacteria against stress and osmoregulation. [6] proved that, trehalose is capable of protecting the organisms against environmental stresses, such as thermal, alcohol, osmotic and oxidative stress. [7] confirmed that, trehalose could protect numerous biological structures against abiotic stresses. [8] proved that trehalose acts as a storage carbohydrate and accumulates as the cells enter the stationary growth phase.

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].The ability of trehalose to protect bacterial cells from a range of stresses might explain the role of this sugar in the survival of bacteria both inside and outside of a host. For example, in *Rhizobium leguminosarum* [11], *Sinorhizobium meliloti* [12] and *Bradyrhizobium japonicum* [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 crop production has shown its importance in improving plant nutrition and amelioration of 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.

Maize is one of the main crops around the world, not only for its importance in both human and animal consumption but also for its industrial uses. This crop is highly sensitive to abiotic stress caused by drought, excessive salinity, and extreme temperature, reducing yield up to 15%, reaching an estimated loss of 16 million tons of grain, which makes necessary to implement strategies that minimize the losses caused by stress in this crop [24]. [25] 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 exogenous trehalose can increase the salt tolerance of plants.

The aim of this study was to investigate the ability of bradyrhizobial strain for trehalose accumulation and effect of different nutritional and cultural conditions on trehalose production to determine optimal conditions for maximum trehalose production, scanning electron microscope for bacterial morphological observation and investigate the 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.

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2. MATERIAL 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

For trehalose determination, 50 mL of the bacterial cultures incubated at 28 ± 2°C for 3 days was harvested by centrifuging at 12,000× g for 15 min at 4 °C. The cells were washed with distilled water and then extracted in 16 mL of 0.5 M cupric acetate at 4° C for 3 h. After the supernatant was collected, 2 mL of the supernatant was incubated with 4 mL of 0.2% (w/v) anthrone-sulfuric acid buffer at 100°C for 5 min. Subsequently, trehalose ~~was~~ were determined at 590 nm as previously described according to [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*

Scanning electron microscopy observations of the tested strain was obtained as described by [31]. For sample preparation, the cells were first fixed with glutaraldehyde, washed with 0.2 M phosphate buffer (pH 7.4) and dehydrated with gradient ethanol. The dehydrated sample was dried with a CO2 critical point desiccator, after which the dried sample was divided into fragments of suitable length, glued to the table and sprayed with gold. Subsequently, the morphology of strain cells in the presence of salt concentration was done with a scanning electron microscope at Faculty of Agriculture ,El-Mansura University.

2.5 Soil analysis

Physical and chemical properties of the experimental soil were determined as shown in Table (1) as described by [32].Soil pH and electrical conductivity (EC) were measured according to [33].Available N was determined according to the modified Kjeldahel method [32]. Available P was determined colorimetrically according to Olsen's method [33]. Available K was determined using the Flame-Photometer [34].

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.

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 ($meql^{-1}$):	
Ca^{++}	22.50
Mg^{++}	35.50
Na^{+}	68.50
K^{+}	1.48
Soluble Anions ($meql^{-1}$):	
CO_3^{--}	0.00
HCO_3^{-}	4.50
Cl^{-}	95.50
SO_4^{--}	27.98

2.8 Relative Water Content

Relative water content (RWC) percentage was determined as described by [35]. Fresh fully-developed leaves were collected randomly from the plants. The fresh weight was measured and the leaves were submerged in a test tube filled with distilled water and incubated in a refrigerator for 24 h. The leaves were blotted dry using tissue paper and the fully turgid weight was measured. The leaves were oven-dried for 24 h at 72 °C. Eventually, the dry weight was recorded and the relative water content was determined using the equation below:

RWC (%) = (fresh weight − dry weight) / (fully turgid weight − dry weight) × 100

2.9 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 the equations of [37]. K^{+} and Na^{+} contents in plant were determined according to [38].

2.10 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.

2.11 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. [42] stated that, several microorganisms had the ability to accumulate abundant extracellular trehalose when supplied with glucose. [43] confirmed that–,the extracellular production of trehalose from glucose by a strain of *Micrococcus varians*.

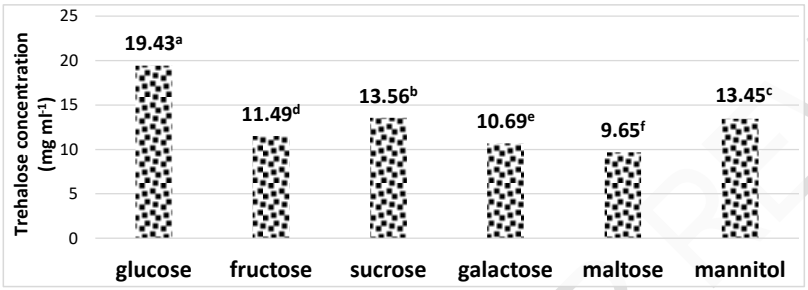

Fig.1. Effect of different carbon sources on trehalose production by *B. japonicum* strain (ARC 517).

3.2 Trehalose production as affected by nitrogen source

Concerning nitrogen sources which play an important role in trehalose biosynthesis, yeast extract was found to be beneficial for trehalose accumulation in the culture broth followed by peptone while trehalose was limited in the medium containing ammonium sulfate indicating the necessity for essential organic factors for trehalose biosynthesis (Fig. 2).These results agree with [42] who confirmed that higher production of trehalose occurred in the media containing organic nitrogen sources as compared to the media containing inorganic nitrogen sources.

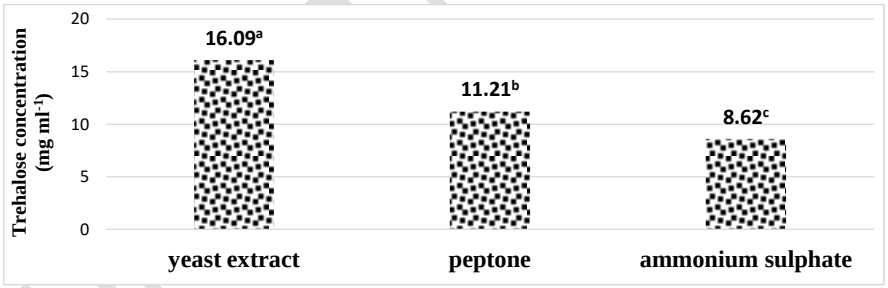

Fig.2. Effect of nitrogen sources on trehalose production by *B. japonicum* strain (ARC 517).

3.3 Effect of initial pH

The pH adjustment increased the accumulation of trehalose as indicated by [44] hence different initial pH values were used to study their effects on trehalose production and the results are shown in Fig.(3). pH 6.0 was found to be most suitable for trehalose production (17.24 mg.ml^{-1}) followed by pH 7 (16.09 mg.ml^{-1}). Lower or higher pH repressed trehalose yield. This result agree with [43] who showed that, Keeping the pH of the culture broth at 6.0 facilitated the

maximum trehalose production rate of *Micrococcus varians* strain. [44] showed that the highest production rate and yield of trehalose in *Propionibacterium* sp. was found when controlling the pH at 7.

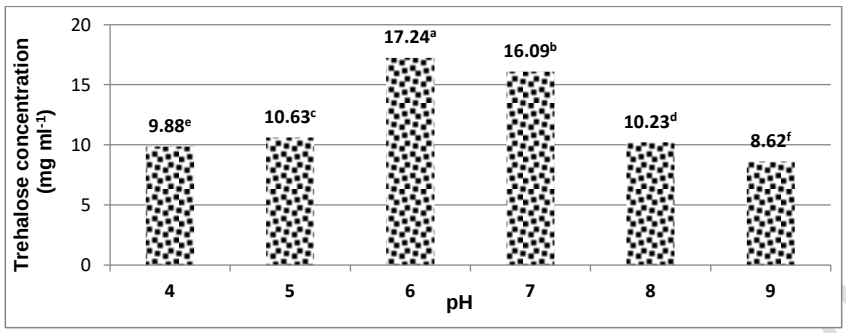

Fig.3. Trehalose production by by *B. japonicum* strain (ARC 517) as affected by pH

3.4 Effect of level of inoculum

Various inoculum levels were implemented to study their effects on trehalose production and the results are presented in Fig. (4). The maximum 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} (not tabled)

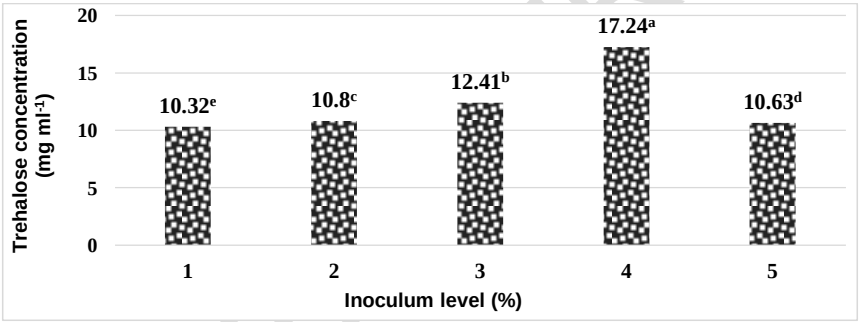

Fig. 4. Trehalose production by by *B. japonicum* strain (ARC 517) as affected by inoculum level

3.5 Scanning electron microscopy for bacterial morphology evolution

As shown in Fig. (5), the cell morphology of *B. japonicum* strain (ARC 517) was altered under salt stress. In the presence of 0% NaCl, the cells were relatively short and regular in shape. Moreover in the presence of 3% NaCl, the bacteria became slightly longer with length about 2 folds as compared to control. Moreover, cells of *B. japonicum* strain (ARC 517) that enhanced for trehalose aggregated and became the longest with length about 3 folds higher than the control. Previous research has demonstrated that halophilic bacteria must modify their morphology to cope with salt stress when exposed to high salinity [45]. The results of the present study are consistent with previously results that showed that PGPR cells lengthened in response to abiotic stress [46]. [47] confirmed that mother cells can store more nutrients needed for the life activities of future generations by increasing the cell surface area when they meet dangerous substances in the environment. These results are also consistent with[48] who proved that, the ability to accumulate osmoprotectants in the salt-tolerant rhizobial strain may be beneficial to bacterial growth through reducing osmotic stress caused by salts.

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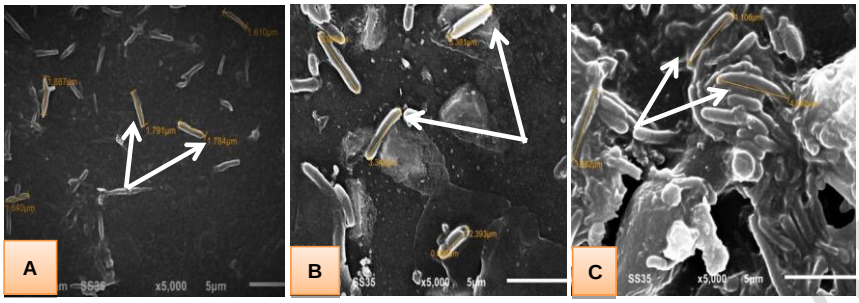

Fig.5. Scanning electron microscopy images of *B. japonicum* strain (ARC 517) that enhanced for trehalose in presence of 0 and 3% NaCl

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

3.6 Relative Water Content (RWC) %

Decreases in Relative Water Content (RWC) under stress conditions are associated with reduced osmotic potential [49]. Higher accumulation of soluble sugars as trehalose may function as an osmoprotectant to prevent water loss from plant cells under osmotic stresses [50]. 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 may be because of trehalose reduced inhibitory effects of salinity stress on growth thus improving water status of plant tissues and relative water content increased as shown in Fig. (6). [51] 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 supplementation of trehalose in saline medium protects *Catharanthus roseus* from the inhibitory effects of salt on growth, RWC, and photosynthesis [52].

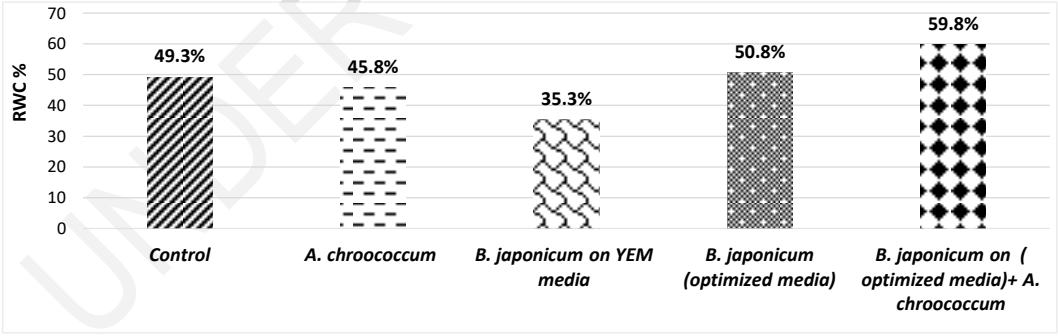

Fig .6. Relative Water Content as affected by trehalose producing *B.japonicum* strain (ARC 517).

3.7 Photosynthetic pigments

Salinity affects the survival and growth of rhizobia in soil [53], alters the protein and lipopolysaccharide content of cells, reduces the number of rhizobia in plant inoculants, reduces plant growth and photosynthesis[54]. 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 effect of trehalose is corroborated with the results of previous studies with rice plant, where exogenous trehalose improved photosynthetic pigment contents under stress [55]. In addition, [56] 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 [57]. [58] confirmed that trehalose acts as a positive regulator of stress tolerance in plants.

Table 2. Co-inoculation of *B. japonicum* strain (ARC 517) higher in trehalose+ *A. chroococcum* and photosynthetic pigments in *Zea mays* plants.

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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.

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 [59] who showed that exogenous trehalose treatment significantly reduce the accumulation of Na^+ in the leaves, indicating that it might play a direct or indirect role in determining ion selectivity by modulating cellular exclusion of Na^+. K^+ is considered as a beneficial ion that confers an advantage to plant under saline and stress [60]. Further, [61] 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.

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**
Azotobacter sp. + 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 + *Azotobacter* sp.+ 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.

3.9 Yield and components of *Zea mays* plants.

The data in Table (4) revealed that yield, straw and wt.100 Grains of *Zea mays* plants increased by co-inoculation of Bradyrhizobial strain that enhanced for trehalose + *A. chroococcum* as compared to control and *B. japonicum* strain (ARC 517) based on YEM media. These results agree with [62] 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,[63] found that osmoregulators decreased fruit abscission due to its role in reducing ethylene production, leading to increase fruits and seeds number and consequently increased seed yield per plant. Moreover, application of osmoregulators may be enhancing photosynthetic pigments, leading to enhanced dry matter accumulation and increased seed yield [64].

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

Treatments	Grain (ton.fed^{-1})	straw (ton.fed^{-1})	wt. 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.

4. CONCLUSION

The accumulation of compatible solutes as trehalose is referred as one of the main mechanisms of bacterial tolerance to stress conditions such as salinity. 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.

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