Screening efficiency of diazotrophic bacteria by assessing photochemical and biochemical attributes of maize genotype against combined stress environment.

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Abstract

Due to climate change, salinity and low moisture becomes a foremost abiotic stress that curtails the plant productivity worldwide. As a curative approach, the role of diazotrophic bacteria to mitigate combined stress (salinity + moisture) in maize plants by modulating the photochemical physiological and growth traits were investigated. The diazotrophic free nitrogen fixer bacteria exhibited several plant growths promoting activities. In this research, maize seeds were coated with two different units of diazotrophic bacteria (Azotobacter specie) i.e. B1; 4.8 x 10^5 CFU mL^{-1} from dilution 10^{-6} and B2; 4.8 x 10^{11} CFU mL^{-1} from dilution 10^{-2}. The maize plants were grown in pots (size; 15 × 18 cm) a complete randomized design having 3 sets with four replicates of each treatment (B0, B1and B2) later to facsimile the natural environment, two weeks old treated and untreated (B0) maize plant were exposed to steady additions of combined; salt and moisture stress (55% moisture +100mM NaCl and 35% moisture +150mM NaCl). To patterned the activity of treatments, the fluorescence and chlorophyll content of maize plants were observed before, during and after stress by non destructive fluorometer and chlorophyll meter. Under combined condition, the maize plants expressed lower biomass due to aberrant physiological functions. However, the presence of diazotrophic bacteria favoured the better photosystem II activity, lower photoinhibition, unhindered electron flow in thylakoid membrane, and boosted protein contents in maize plants. The untreated plants data represents higher red-yellow color in IR thermal imaging which shows increased temperature (low water content) whereas treated plants show blue color which stated decreased temperature (higher water content) in maize leaf. It was observed that bacterial coating decreases the heat dissipation, absorption per reaction center by increasing the efficiency of light reaction (φPo / (1- φPo), performance index, maximum quantum yield of photochemistry and biochemical reaction (ψo / (1- ψo). Moreover, the results revealed lower reactive oxygen species (ROS) production in the maize leaves, improved the photosynthetic performance, carbohydrate content which ultimately increased the plant length under higher stress. This study concluded that coating of diazotrophic bacteria potentially improved the photochemistry and biochemical attributes by regulating electron flow which enabling maize plants to bear severe salt and moisture stress. It is suggested that the use of diazotrophic bacteria advance the sustainability and minimize the yield coasts even in harsh environment.

Keywords: Maize, Diazotrophic Bacteria, Seed coating, Photochemistry, Combined stress Measuring stress phases.

Highlights

- The seed coating with diazotrophic Bacteria balanced the photochemistry and chlorophyll content in maize plants under different stress phases (during and after stress).
- Lower reactive oxygen species (ROS) production in the maize treated leaves, improved the photosynthetic performance.
- IR images reflected less heat dissipation in treated plants.

Introduction

Maize (*Zea mays* L.) is a useful food crop which offer nutrition and used as fodder in animal farms. Maize is ranked third in Pakistan in terms of production after wheat and rice. Around sixty-six percent of Pakistan's maize is irrigated; the balance is grown solely from rainwater. Pakistan produced 3.037 million tonnes of maize in 2007, up from 0.38 million tonnes in 1947-50. (Tariq *et al*., 2010). Recently the demand of maize crop is increasing due to its usage in milling industry as well as food for poultry. In this regard there is need to improve agricultural tactics and exploring new sustainable techniques to enhance the maize production in hypersaline environments. According to previous reports, maize plant is tolerant to mild abiotic stress, however, severe, or prolonged stress causes adverse effects on growth and production (Munns and Tester., 2008, Abdelgawad *et al*., 2019, Siddiqui *et al*., 2022).

With the global population expected to reach 9.6 billion by 2050, agricultural productivity must be enhanced to feed an additional 2 billion people by that time (Diacono and Montemurro., 2015). Salinisation of arable land may hinder the world's ability to provide a consistent food supply for its expanding population (Parihar *et al*., 2015). Annually, salinity stress reduces production of vital cereal crops like maize, rice, and wheat (Kaya *et al*., 2013, Siddiqui *et al*., 2017 and Razzaq *et al*., 2020). Salinisation in Pakistan's dry to semiarid climate has rendered 8.6 million hectares of agricultural land unsuitable (Syed *et al*., 2021). Because of inadequate rainfall and high evapotranspiration petition, combined water and salt stress frequently occurs simultaneously or sequentially in heavily irrigated fields in arid and semi-arid areas, posing a serious risk to crop construction (Farooq *et al*., 2015; Zelm *et al*., 2020).

Consequently, strong anthropogenic pressures and overexploitation, oasis ecosystems are becoming increasingly fragile. The rhizopheric bacteria is the well-known factor contributing plant growth promoting properties typical of rhizospheres in non-arid ecosystems. Moreover, rhizosphere bacteria contribute to neutralizing the negative consequences of salinity and drought stresses in a sub-optimal environment. It is great source to provide accessibility of essential mineral and maintain pH in dry soil particles inducing plant physiological responses against water losses, supporting the metabolites productions and contributing plant hormone maintenance and nutrient homeostasis (De *et al*., 2013). Plant growth promoting bacteria, naturally associated with plants, have been shown to be essential partners for improving plant tolerance to stressful conditions (Marasco *et al*., 2012). The multifarious plant growth-promoting potentials of bacterial endophytes under both ordinary and stressful atmosphere are thought to be responsible for the development of growth and yield of the plants (Walitang *et al.,* 2023). Inorganic availability, root establishment, symbiotic connotation and nitrogen fixation are the few traits exhibit by free living bacteria.

To defend from gigantic yield misfortune a few endeavours have been made for plant change in accordance with endure abiotic stress, which draw in complex physiological and photochemical efficiencies (Javid *et al*., 2011). Plant biomarkers have gained a lot of interest over the last decade as pre-stress indicators for a variety of economically significant crop yields. Moreover, the effective use of light energy to drive photosynthesis in the key to photosynthetic performance under combined stress environment. Chlorophyll fluorescence and infrared heat sensing are non-destructive, sophisticated, accurate, and time-saving phenotyping techniques in plant physiology that are used to test for stress tolerance in abiotic stress conditions. Chlorophyll fluorescence is utilized to analyses PSII and PSI activities, which reveal plant photosynthetic capacity during drought stress (Siddiqui *et al*., 2014). The photosynthetic performance and biochemical activities express the tolerance of plants under combined stress. Photochemical efficiencies of a plant are very important factor which increases the development of additional metabolic sinks to increase the consumption of photosynthetic electrons. The efficiency of thylakoid membrane is linked with photosystem II (PSII) because it is not only responsible for capturing and absorbing light energy but also generating ATP, NADPH, and splitting of water molecule during light reaction of photosynthesis. In saline environment photosystem II is greatly altered due to many reasons which lead to elevation of reactive oxygen species (ROS) concentrations in chloroplast (Umar *et al*., 2019).

Seed coating with microbial fertilisers can reduce the need for inoculants, leading to cost savings and enhanced efficiency (Oliveira *et al*., 2016). According to Rouphael *et al*. (2017), seed coating can improve agricultural sustainability. These strategies, the use of microbes is shown to be successful and eco-friendly approach to enhance plant's health under salinized conditions (Vejan *et al*. 2016). It is assumed that free living nitrogen fixer diazotrophs can make nitrogen and other mineral accessible to the plants in saline habitat due to its ability to solubilizes the soil mineral and fixes gaseous nitrogen. Azotobacter is able to produce growth hormones like auxins and gibberellins which enhancing root growth and leads to increased nitrogen fixation which ultimately improve crop yield (Verma *et al*., 2014). Therefore, present study is designed to investigate the sustainable role of azotobacter in photochemical efficiency and physiological tolerance in maize plants under combined salt and moisture stress environment.

Material And Methods

***Isolation and Purification of Bacteria from Soil Specimen*:**

The soil specimen was collected from the rhizosphere of a palm tree that is situated next to Ibrahim Masjid at the University of Karachi in Pakistan at a depth of 8 to 40 cm. The soil was then assessed to monitor pH and EC. Several serial dilutions, reaching a final fraction of 10^{-6} were made with distilled water utilizing 10 gm of soil samples. Among the total fraction (10^{-1}

to 10^{-6}), 1 ml of the suspension from each fraction 10^{-3} and 10^{-6} was poured into an Ashby's mannitol agar petri plates (mannitol 20000; dipotassium phosphate 200; magnesium sulphate 200 sodium chloride 200; potassium sulphate 100; calcium carbonate 5000; agar 15000 mg L^{-1}; pH 7.8) and was incubated at 28 °C for 48 hours. The bacterial associations appearing on the medium (10^{-6}) were selected and re-streaked three to four times on fresh plate of Ashby's mannitol agar medium for the purity of culture. Morphological characteristics of the isolated colony appearances (i.e. shape, diameter, opacity, size, texture and color of the bacterial colony and their growth rate) were identified by using the criteria of Bergey's Manual of Systematic Bacteriology. Microscopic study of the colonies was complete by using Gram staining method (Gram, 1884) and Exopolysaccharide (EPS) production was evaluated by streak the strains on Congo Red Agar (CRA) via modified method (Freeman *et al*, 1989).

Stress tolerance activity of microbial isolates:

The bacterial isolates were screened for their stress tolerance activity by streak on Ashby's medium containing 15% PEG +100Mm NaCl, 25% PEG+150MmNaCl respectively and pH were adjusted at 7.4 + 2. Later modified media poured into 3 petri dishes for each set and were permitted to solidify. Subsequently, with the aid of sterile wire loop newly identified colony were selected and streaked on to the modified media petri dishes. The inoculated petri dishes were kept for six days at 28°C, afterwards the growth was evaluated.

Preparation of inoculum:

The bacterial isolates from 25% PEG+150MmNaCl medium was now added into Ashby's broth (100 ml) to the flask for 24 hours. Subsequently, when the bacterial colony grown, the flasks were shaken mechanically intended for 4 hours per day for one week at 28°C at a speed of 80 rpm (Fig 1). Afterwards the bacterial broth centrifuging for 20 minutes at 9000 rpm and the pallets were collected. Later, the pallets of bacteria were systematically shaken using a vortex machine by re-suspended into distilled water. For the count of bacterial colonies, the resuspended culture diluted ten times in a 0.85% physiological saline solution (10^{-1} to 10^{-10}) and thoroughly shaken. 1 ml of the suspension from each dilution, spread out on Ashby's medium and incubated for 24 hours. Later the colony forming unit (CFU) was 4.8 x 10^{5} CFU mL^{-1} from dilution 10^{-6} and 4.8 x 10^{11} CFU mL^{-1} from dilution 10^{-2} calculated using the plate count method.

Seed collection & Seed coating:

The maize seeds (*Zea mays* L.) cv. P1429 were collected from the Department of Seed Certification, Government of Pakistan. After collection the seeds underwent for two different treatments from dilution 10^{-2} plate and dilution 10^{-6}.1% carboxymethyl cellulose (CMC) at the percentage of 1:1 to make a slurry with the final bacterial concentration. Later, the maize seeds were soaked in the particular bacterial CMC solution for 2 hours. After being soaked for 24 hours, the seeds were dried in a laminar flow by air and sowed in the respective treatment pots.

Experimental maneuver:

The research experiment was conducted in the Stress Physiology Phenomics center, Department of Botany, University of Karachi, Pakistan. The maize seeds were grown in the germination trays. Later, after one week of sprouting, maize seeds were transplanted into clay pots (size 15 × 18 cm) filled with air-dried autoclaved sandy loam (Fig 1). The pots were arranged into 3 sets i.e., Control, 55% moisture content+100mM NaCl and 35% moisture content+150mM NaCl, for each treatment and every set has four replicates. Subsequently the combined stress was applied after two weeks of transplantation, which lasted for about 14 days and the stress was applied in steady augmentations by sub irrigation method. The parameters of soil were alternatively scrutinized for soil moisture content, pH and EC. Control plants were maintained at more than 70% of moisture content throughout the research. The plants were imperilled to the different invasive and non-invasive measurements after the completion of stress.

Morphological Parameters:

Parameters like plant length and plant biomass were assed through inch tape and weighing machine.

Chlorophyll fluorescence:

The chlorophyll a' fluorescence was measured by fluorometer (OS-30p+, Opti-science, USA). The readings were taken before, during and after stress between 8:30 AM and 10:30 AM on the newest and fully opened leaves with particular consideration to leaf angle and shadowing. For dark adapted data measurements, leaf clips were used for 30 minutes. The methodology was carried out using pulses of saturating light lasting 1.6 s (6.8 mol m^{-1} s^{-1} PAR) and weakly modulated red light (0.5 mol $m^{-1}s^{-1}$). The protocols of Maxwell & Johnson; 2000, Strasser *et al*., 2010 and Stirbet & Govindjee., 2011 were used for JIP test. Critical parameters including performance indices, quantum efficiencies, the density of reaction centers, energy fluxes (ABS/RC, TRo/RC, ETo/RC, DIo/RC) and various technical fluorescence parameters were measured to monitor the photochemical efficiency of PSII.

Formula:

$ABS/RC = (Mo/VJ)/\varphi Po$
$TRo/RC = Mo/VJ$
$ETo/RC = (Mo/VJ) \times \psi Eo$
$DIo/RC=(ABS/RC) – (TRo/RC)$
$PI_{ABS} = (RC/ABS) \times [\varphi Po/(1 – \varphi Po)] \times [\psi Eo/(1 – \psi Eo)]$
$Fv/Fm =(Fm-Fo)/Fm$

Chlorophyll content index (CCI):

By using a chlorophyll meter, CCI was measured in the youngest completely expanded leaves before, during and after stress between 8:30AM and 10:30AM (Model CL-01, Hansatech, UK).

Malondialdehyde (MDA) content:

For the extraction, the frozen plant sample (0.1g) were crushed with 4ml reagent 1 (0.5% trichloro-acetic acid). Later the homogenate sample were centrifuged at 5000 rpm form about 15 minutes. After the centrifugation, the supernatant was placed in a distinct tube and residue were discarded. For estimation, 1ml of the supernatant were mixed with the 3 ml of reagent 2 (20g of trichloro-acetic acid and 0.5g of thiobarbituric acid dissolved into 100ml distilled water). Afterwards incubated the test tube content into boiling water bath at 100°C for about half an hour. After incubation, cool the contents to room temperature and measure the optical density (O.D.) at 532 and 600 nm. MDA-TBA's extinction coefficient is 155 mM^{-1} cm^{-1}. (Dhindsa *et al.*, 1981).

$$\text{Concentration of TBAR } (\mu M) = \frac{\text{Absorbance at 532 nm} - \text{Absorbance at 600 nm}}{155} \times 1000$$

Hydrogen peroxide (H_2O_2) content:

For the extraction, the frozen plant sample (0.1g) were crushed in a prechilled pestle and mortar with 4ml of 0.1% trichloro-acetic acid. Afterwards, homogenate content was centrifuged at12000 rpm for up to 15 minutes, the residue was discarded and the supernatant was taken in a separate test tube. For estimation, 0.5 ml of 10mM potassium phosphate buffer having pH: 7 and 1 ml of 1 M potassium iodide were added in 0.5 ml of supernatant. The optical density (O.D.) of the whole content were measured against a TCA blank at 390 nm. (Velikova *et al*., 2000). The H_2O_2 content was assessed by referring to a standard curve and stated as mmol g^{-1} per FW.

$$\text{Total of } H_2O_2 \text{ in } \mu\text{mol } g^{-1} = \frac{(conc\ of\ regression \times Volume\ of\ extract \times Dilution\ Factor)}{(Weight\ of\ sample) \times 1000}$$

Phenols:

For extraction, the frozen plant sample (0.5g) were crushed in a prechilled pestle and mortar with 10ml of 1N HCL. Afterwards, homogenate content was centrifuged at 3000 rpm for 10 minutes and the supernatant was then dried in an oven at 80°C. Ater one day 10 ml of 85% ethanol were added in dried sample and shake well to dissolve phenols. For estimation, 1 ml supernatant was taken in test tubes and add 5 ml of folin's reagent then incubate the whole setup for 5 minutes in dark. After the incubation, add 4 ml of saturated sodium carbonate and kept in dark for another 30 minutes. The optical density was recorded after incubation at 760 nm and the total phenol expressed in mg/g $^{-1}$ using standard curve (Swain & Hillis.,1959).

$$\text{Phenols content (mg/g FW)} = \frac{(Volume\ of\ extract \times Value\ from\ Standard\ curve \times 100)}{(Weight\ of\ sample) \times 1000}$$

Total proteins:

Bradford, (1976) protocol was used for total protein quantification. 1.0 gm of leaves were crushed in 10 ml of Tris- HCl buffer and centrifuged at 5000 revolutions per minute for 15 minutes. The supernatant 1.0 ml was added up in 5 ml of Bradford assay mixture (1 ratio of the dye stock with 4 ratio of distilled water). The dye stock is made by the ratio of 0.5g per mL coomassie brilliant blue dye, 25% methanol and 42.5% H_3PO_4.The optical density was recorded at 595nm. The total proteins were enumerated using a standard curve and stated in mg g^{-1} of fresh mass/weight.

$$\text{Protein content (mg/g FW)} = \frac{(Volume\ of\ extract \times Value\ from\ Standard\ curve \times 100)}{(Weight\ of\ sample) \times 1000}$$

Carbohydrate content:

Total soluble carbohydrate can be estimated by the method of Yemm and Willis (1954). For extraction 0.5g of fresh leaf sample was crushed in 5ml of 80% ethanol and centrifuged at 3000rpm for 15mins. For estimation, add 4 ml anthrone reagent (0.4 g anthrone in 100 ml ice cold freshly prepared 95% sulphuric acid) and heated in a water bath at 100ºC for 10 minutes. Later it was cooled down and optical density was measured at 620nm. Standard curve was generated using the graded concentration (10, 25, 50, 75 and 100) µg/ml of glucose.

Carbohydrate content (mg/g FW) = $\frac{(Volume\ of\ extract \times Value\ from\ Standard\ curve \times 100)}{(Weight\ of\ sample) \times 1000}$

Infrared (IR) Thermal Imaging to Degree Plant Temperature:

The thermal images were taken before harvesting with a FLIR-E5 (FLIR Systems, USA). IR images were capture from a distance of 1 m from the plant pots and concurrently, graphic color images were also saved automatically in a machine. A steel sheet was used to minimize the plant background temperature. Before the measurement, the system was optimized for half an hour and then images were taken. The images were extracted directly from the IR camera into the computer and generated using FLIR Software 2.10.

Statistical analysis:

Statistical analysis of respective treatment and control data were managed in Excel 2016 and SPSS ver 20. Significant variances amid the mean values of altered treatments were assessed by means of a post hoc test (Bonferroni) and characterized as alphabets on the bar's graphs ($p \leq 0.05$). Principal component analysis for biochemical parameters were calculated by using R studio ver 4.3.1 software.

Results

Morphological characterization of diazotrophic bacteria:

The isolated colonies were identified as Azotobacter by using different cell test and morphological characters. The colonies were moderately large and irregular in size, rod shaped, glistening creamy opaque in colour and the diameter of the colony is 14mm. Isolated bacteria exhibited pink or red staining and other Gram-negative traits. It was proposed that the gram-negative bacteria had a thin peptidoglycan cell wall encircled by a lipopolysaccharide-containing outer membrane. Moreover, the results showed positive exopolysaccharide producing capacity. The colonies appeared with dark brown to black colonies in congo red agar (CRA).

Soil parameters:

The effects of diazotrophic bacteria on soil traits were studied under combined stress conditions. The results showed that the treated plants maintain soil pH, organic and moisture content of the maize plants as compared to untreated plants (Table no 1).

Plant morphological traits and Biochemical analysis:

It was noted that the plant traits were reduced under stress conditions, however due to the seed coating of diazotrophic bacteria maize plants were showed substantial improvements in plant length and biomass. Moreover, the maize plants which are subjected to B2 (4.8 x 10^{11} CFU/ml) concentration tremendously increased plant length (40cm) and biomass (2.3g) as compared to B0 (untreated plants) which is 29 cm and 01g under higher combine stress (Table no. 2). The biochemical attributes like carbohydrates content, protein contents were reduced where as hydrogen peroxide, malonaldehyde and phenol contents were increase under stress condition (Fig 2a). It was revealed from the results that diazotrophic bacteria treatments (B1and B2) mitigate the deleterious effects of moisture and salt combine stress by reducing the oxidative stress markers in maize plants as compared to B0. Moreover, it was observed from the data set that B2 maintained significant energy production (carbohydrate content) and B1 increased more protein content under higher stress conditions. Furthermore, PCA−biplots were created to demonstrate the important data variations of biochemical and morphological traits of treated and untreated maize plants. The distribution in diverse ordinates based on their variances under control and stress environments. The PC1 and PC2 described 97.0% and 2.8%, correspondingly, of the total changeability across treatments in the control and stress condition, whereas the dimensions (dim1 and dim2) showed 84.8% and 7.9% respectively, of the entire variation in the traits and stress environments (Fig 2a). The PC1 was strongly connected with diazotrophic applications (B1 and B2) and PC2 was strongly associated with untreated (B0) plants (Fig 2c). In comparison, treated plants showed the negative correlation between the oxidative stress markers and morphological traits whereas, untreated plants showed positive correlation and make an acute angle under higher and moderate stress.

Chlorophyll content and Photochemical assessments:

Chlorophyll content index (CCI) and Photochemical traits were measured before, during and after stress to evaluate the effects of diazotrophic bacteria applications in maize plants under combine stress. The results showed that combine stress effect the photosynthetic performance of plants, however treated plants maintained significant photochemical efficiencies by mitigate the deleterious effects of salt and moisture stress (Fig 3). It was observed from the data set that maximum quantum yield of primary photochemistry (Fv/Fm) and Performance index per absorption (PI_{ABS}) exposed significant difference during and after stress. However, the treated plants had comparatively higher quantum efficiencies in comparison to untreated plants under stress conditions. The chlorophyll content index of B1 and B2 was significantly improved after stress phase as compared to B0 might be due to the sustainability in quantum efficiencies.

Energy Fluxes:

The effect of diazotrophic bacteria on Absorption per reaction centre (ABS/RC), Trapping per reaction centre (TRo/RC), Electron transport per reaction centre (ETo/RC) and Dissipation per reaction centre (DIo/RC) was observed under combine stress condition before, during and after stress phases in maize plants (Fig 4). It was observed that ETo/RC were reduced whereas, DIo/RC were increased under stress condition however, treated plants improves the flow of electron and maintain the heat loss dissipation during and after stress as compared to untreated plants. The trapping and absorption per active reaction centre were increased in untreated plants might be due to the disrupt in the flow of mobile electron carriers. Moreover, diazotrophic Bacteria was better to overcome the negative effects of combined stress on maize plants and maintain the better performance of photosynthetic apparatus by sustain the TRo/RC and ABS/RC.

Chlorophyll a fluorescence Transient Curve:

The JIP test is a widely used technique in plant physiology that measures the fluorescence emitted by chlorophyll molecules to assess the efficiency of the plant's photosynthetic energy conversion and response to environmental stressors. It was confirmed by the OJIP measurements that in combined stress B1 and B2 primed maize plants showed better fluorescence intensity and were enhanced from 215 to 715 µmol (photon) m^{-2} s^{-1} from initial to final stress phases. In B2 subjected plants indicating effective augmentation in photosynthetic performance by increasing the flow of electron 215 to 465 µmol (photon) m^{-2} s^{-1} as compared to B0 treated maize plants (Fig 5). The untreated plants showed the flat curve from O-P step under high stress conditions.

Radar Plot:

The effect of diazotrophic bacteria on Physiological state of the photosynthetic apparatus (Fo/Fm), The ratio of photochemical to nonphotochemical quantum efficiencies (Fv/Fo), Electron transport per maximal trapping rate of absorbed photons (ETR), Probability that trapped electron was transferred to ETC beyond QA (Yo), The efficiency of light reaction (φPo /(1- φPo)), The efficiency of biochemical reaction (ψo /(1- ψo)), Relative variable fluorescence at J step (Vj) and The number of active PSII per excited cross section (RC/CSo) was observed under combine stress condition before, during and after stress phases in maize plants (Fig 6). The data set presented the variations between the stress phases, however B0 disastrous to maintain Fo/Fm, Fv/Fo and ETR activities under stress conditions. Moreover, it was exhibited from the results that B2 subjected plants showed higher physiological state of the photosynthetic apparatus and the number of active PSII per excited cross section from initial to final stage of stress. Fv/Fo was relatively low in untreated plants as compared to treated plants however, B1 exhibited higher efficiency of light reaction and biochemical reaction under stress conditions during and after stress in maize plants. The fluorescence at J step was relatively increase in B2 as compared to B1 treated plants which indicated the better flow of electron.

Infra Red Thermography:

IR thermography of combined stress exposed to maize plants were measured (Fig. 7). Red color in images displayed higher temperature (low Water Content) while blue color expressed lower temperature (higher Water Content) in leaf. B0 Plants under stress exhibited the greater temperature compared to B1 and B2. Results obtained from thermal images have shown that untreated plants exhibited higher average temperature (45.7 to 43.8^{O}C) as compared to treated

B1 and B2 plants (36.9 to 32.3 and 29.5 to 36.4) under low (S1) and high (S2) stress conditions compared to treated respectively.

Discussion

The climate change creates abiotic stress like salinity and low moisture which becomes the foremost stress that curtails the plant productivity worldwide. As a curative approach, diazotrophic bacteria isolated from the harsh environment and use as an eco friendly method to improve agricultural output by mitigating combined stress. In this research, maize seeds were coated with two different units of diazotrophic bacteria (Azotobacter specie) i.e. 4.8 x 10^5 CFU mL^{-1} from dilution 10^{-6} and 4.8 x 10^{11} CFU mL^{-1} from dilution 10^{-2} and exposed to two different combine stress conditions (55% moisture +100mM NaCl and 35% moisture +150mM NaCl) and photochemical, biochemical and growth traits were investigated. It was observed from the fig 8 that B1 and B2 mitigate the stress by improving plant growth were as compared to B0 under 35% moisture +150mM NaCl (S2).

According to Qurashi and Sabri (2011) exopolysaccharide not only protect bacterial cells, but also aid them minimise sodium toxicity in soil, which promotes plant growth under salinity. Based on the findings, it is possible that the strains form extracellular EPS in order to deal with stress. At high stress, bacterial cells accumulate more intracellular osmolytes, which shields them from the negative effects of soil salinity and moisture stress (Fig 9). Bacterial cells under combined stress in the rhizosphere use the same method to ensure their survival however, more molecular research into osmo-adaptation is required to fully comprehend the mechanism. The study suggested that inoculating selected EPS-producing bacteria could serve as a useful tool for alleviating salinity stress in sensitive plants (Ashraf *etal*., 2004). The EPS binds with the toxin ions that balance the osmotic activities and ion homeostasis which leads to improves the soil structure.

The plant growth was related to the smooth photosynthetic performance. PI_{abs} in plant leaves is an important tool to indicate the effects of stress on photosynthetic performance. In present study the PI_{abs} was decreased under stress without Diazotrophic Bacteria. Moreover, the values of PI_{abs} were increased in the maize plants treated with diazotrophic bacteria. The better performance index might be due to the higher CCI values in maize plants primed with diazotrophic bacteria. Umar *et al*. (2019) reported that the stomatal closure under osmotic stress is linked with lower PI_{abs} values. Intrinsic quantum efficiency of PSII photochemistry (Fv/Fm) decreased under severe salt stress indicate that the proportion of the PSII reaction centre was damaged or inactivated (Kalaji *et al*., 2016). Maize plants primed with B2 diazotrophic bacteria expressed highest Fv/Fm under stressed and unstressed maize plants. From before to after stress phases, Fv/Fm values were not much affected in maize plants with diazotrophic bacteria due to greater values of Fm under combined stress.

The OJIP transient curve becomes vital photosynthetic trait to quantify the impact of stress on plants. The downward OJIP curve under salt stress represented the hurdle in electron transport due to the shrinkage of plastoquinone pool size and inefficiency of PSI acceptor side (Stirbet *et al*. 2018; Umar *et al*., 2019). OJIP However, the higher values of OJIP curve of maize leaves indicated the lesser inactive reaction centres in PSII under stress environments with diazotrophic bacteria. The decline in OJIP curves were observed under combined stress (Fig 5). The higher OJIP curves in maize plants primed with diazotrophic bacteria exhibited more active PSII reaction centres as compared to un-primed plants (Kalaji *et al*,. 2011). From before to after stress phases B1 and B2 electron flow was maintain as compare to B0 plants which shows flat cure O-P step under high stress condition. It is suggested that diazotrophic bacteria alleviated the restrictions imposed on electron flow in thylakoid membrane and improved the production of ATP and NADPH even under stress. These results indicated greater tolerance and efficient photochemical activities in primed maize plants under salt stress (Tsimilli-Michael and Strasser 2008; Stirbet and Govindjee., 2011).

Combined stress suppressed the photosynthetic efficiency and triggered the dissipation mechanism to remove excessive light energy. A small part of PSII absorbed photon and re-emit as chlorophyll a fluorescence. Under combined stress, better Fv/Fo values in Diazotrophic Bacteria plants reflecting unfluctuating electron flow in thylakoid membrane, less photodamage and higher vibrant RCs as compared to un-primed plants. The better PSI and PSII functions in maize plants treated with Diazotrophic Bacteria may be due to unhindered electron transport through plastoquinone and plastocyanin. The values of Vj viz relative variable fluorescence after illumination of a dark-adapted sample in maize plant increased under combined stress (Fig 6). The complex Vj values expressed more damage in the water splitting complex at PSII donor side. Underneath combined stress, the Fm/Fo which is the indicator of electron transport rate through PSII was increased in diazotrophic bacteria primed plants as compared to un-primed plants. Umar *et al*. (2019) reported that greater Fm/Fo values due to the Q_A reduction rate was greater as compared to oxidation rate of Q_B, this situation reflected a lower hindrance in electron flow at donor side of PSII. Fv/Fo which explain the performance of oxygen evolving complex (OEC) on the donor side of photosystem II is the most sensitive trait for smooth electron flow in photosynthetic light reaction. The decline in the Fv/Fo indicates the impairment in electron flow (Pereira *et al*., 2000). In this research, Fv/Fo decreased under saline environment. However, maize plants primed with diazotrophic bacteria exhibited the higher values of this ratio as compared to un-primed plants. It is suggested that smooth electron flow in primed maize plants was due to the efficient working of OEC under combined stress.

In this experiment, Maize Plants exposed to combined stress expressed the changes in cell membrane permeability and membrane integrity (Fig 2). Cell membrane integrity is the characteristic of the regulation of ionic movements and used as the tool to compute the damage intensity (Siddiqui *et al*., 2022). Our results have shown that the concentration of MDA is increased that cause membrane impairment under combined stress. The coating of maize seeds with Diazotrophic Bacteria curtailed the breakdown of aldehydic lipid production, causing reduction in MDA contents under combined stress. The application of Diazotrophic Bacteria in maize plants maintained the membrane integrity and reduced the electrolyte leakage under combined stress as compared to unstressed plants. These results exhibited that Diazotrophic Bacteria had defensive role to increase membrane stability by reducing peroxidation of lipids. Umar and Siddiqui (2018) suggested that damaged cellular membrane was due to the higher

H_2O_2 concentration in plant cell. PCA was used in this study to identify the most important selection traits for stress tolerance, by using the first and second principal components (Fig 2b, c). PCA–biplot is a type of multivariate analysis that combines traits and objects in two dimensions together and minimizes overlapping variations, facilitating the determination of main characters for selection. The PCA revealed that the B1 and B2 treated plants morphological traits were negatively correlated with the oxidative stress markers whereas positive related with carbohydrate and protein contents. However untreated plants showed the positive relation between oxidative stress and growth traits.

The surplus MDA ad H_2O_2 under combined tress damages the membrane, disturb the photosynthetic activities, and hinder the enzymatic activities. Results revealed that the application of diazotrophic Bacteria caused decline in the concentration of H_2O_2 in maize plants under saline environments. Silveira *et al*. (2003) reported that osmotic substances i.e., sugar and sugar-containing alcohol are accumulated in plant cells to mitigate osmotic stress caused by combined stress. The lower quantity of H_2O_2 in maize plants primed with Diazotrophic Bacteria uttered the relief of stress related glitches (Tahjib-Ul-Arif *et al*., 2018).

The diazotrophic bacteria primed maize plants expressed lowest ABS/RC and DIo/RC due to elevated active reaction centres as compared to un-primed plants. These results expressed that presence of diazotrophic bacteria minimize the inactivation of RC and increased the quinone pool size in maize plants which ultimately increased the rate of Q_A reduction under combined stress. The relatively higher TRo/RC in diazotrophic bacteria primed maize plants enhanced the electron transport per RC (ETo/RC) which reflected the ability of RC to re-oxidize Q_A^- (Grieco *et al.,* 2015). It is suggested that un-primed maize plants had higher absorption under stress and more light is reflected back to the environment in the form of heat or as light energy. Energy flux model expressed stress tolerance persuaded by diazotrophic bacteria primed maize plants under combined stress.

Heat dissipation under coupled stress was exhibited in IR pictures. IR pictures of leaf temperature are directly proportional to stress progression. In this study, B1 and B2 plants had lower leaf temperature (blue colour) than B0 plants in stress conditions. When exposed to combined, sensitive cultivars' leaf temperatures rise. The lower leaf temperature in B2 plants could be attributed to improved water usage efficiency and increased RWC. The sensitivity of the IR thermal camera not only identified stress-tolerant cultivars, but it was also non-invasive and time-saving. It was assumed that combined stress increased thermal dissipation (DIo/RC), resulting in elevated leaf temperature. Furthermore, stomata play an important function in supporting leaf moisture, resulting in reduced leaf temperature (Siddiqui *et al*., 2014; Kwon *et al*., 2015).

Plants acquire some physiological and biochemical tactics to thwart stressed condition. The higher PI_{abs}, Fv/Fm, and CCI were due to less aggregation of electron in the apparatus of photosynthetic light reaction and reduced the dissipated energy in Maize plants treated with Diazotrophic Bacteria. The traits of light reaction expressed the fortification of D1 and D2 protein of PSII in primed plants under combined stress. This investigation corroborated that JIP test is highly useful to comprehend the impact of the Diazotrophic Bacteria in maize plants under combined stress environments. This research suggested that Diazotrophic Bacteria in maize plants could acquire a better symbiotic relationship in combined stressed conditions.

Conclusions

Diazotrophic bacteria is well known for its diverse range of secondary metabolites. These endosymbionts suppress the negative effects of combined stress in maize plants. Diazotrophic bacteria was collected form saline environments to study its potential to alleviate the effects of stress in maize plants. In accordance with current finding, it is suggested that diazotrophic bacteria enabled plants to survive under unfavourable environmental conditions. Diazotrophic bacteria able to induce tolerance by increasing photochemical and biochemical efficiencies to mitigate the effects of combined stress and improve overall plant growth. The enhancement in photosynthetic performance in diazotrophic bacteria treated maize plants represented minimum risk of PSII damage under combined stress. Therefore, the nomination of relevant chlorophyll *a* fluorescence parameter is important for sensing stress in maize plants with and without diazotrophic bacteria. These substantial eco-physiological advantages suggested that diazotrophic bacteria could be better plant bio-intoxicant for improving maize plants under harsh environment.

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Fig. 1: Experimental setup in vitro and vivo of diazotrophic bacteria of maize genotype against combined stress environment.

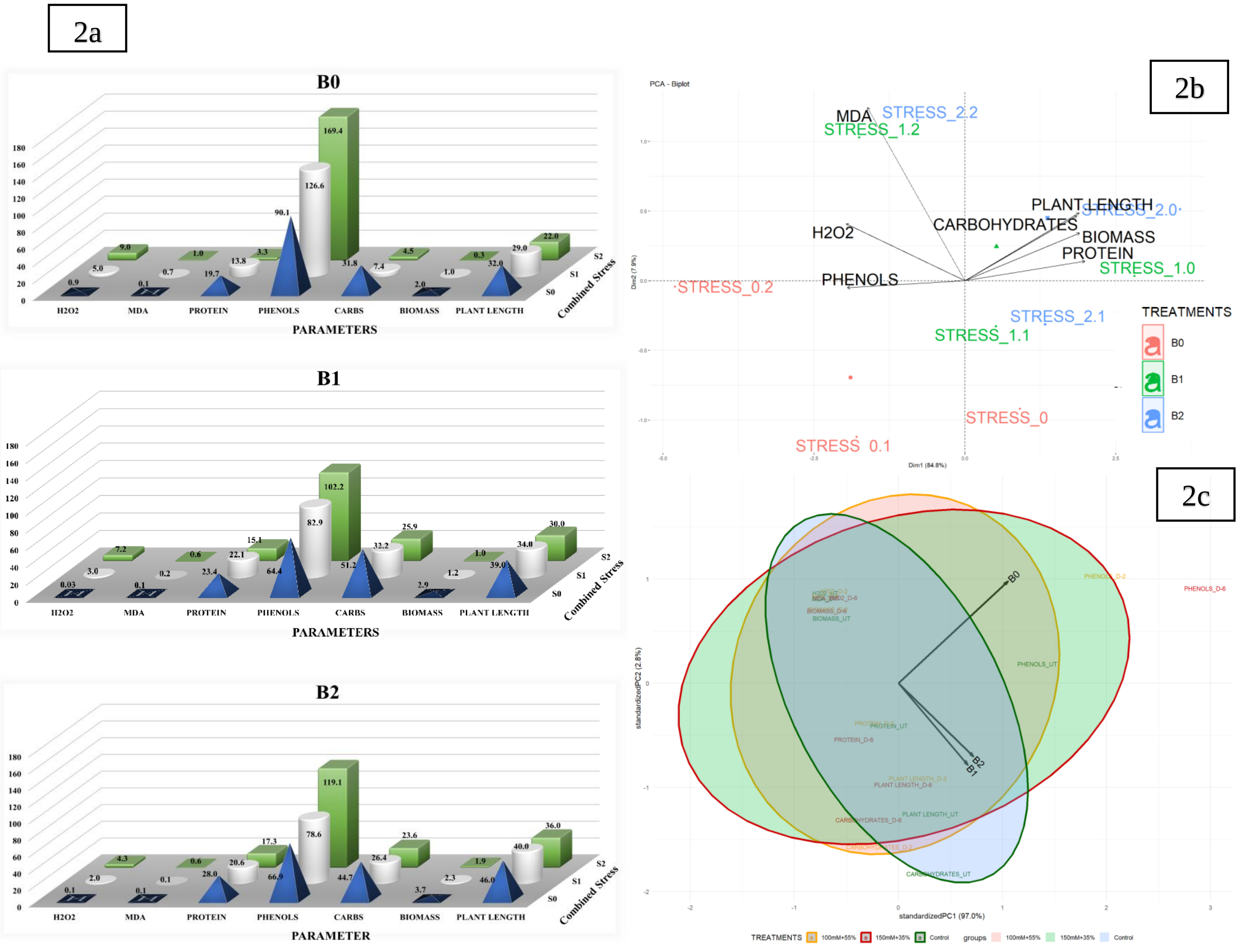

Fig. 2 (a) Effect of *diazotrophic bacteria* on biochemical and morphological traits i.e Hydrogen peroxide (H_2O_2), Malonaldehyde (MDA), Proteins contents, Phenols contents, Carbohydrates contents, Plant biomass and Plant length. **(b)** PCA-Biplot dimension (Dim) analysis between the stress and traits with respect to treatments where C represents 0, 1.0 and 2.0; 55%ms +100mM NaCl represents 0.1, 1.1 and 2.1; and 35%ms +150mM NaCl represents 0.2, 1.2 and 2.2. **(c)** PCA-Biplot (PC1 and PC2) analysis between the parameters under control and stress condition (55%ms +100mM NaCl and 35%ms +150mM NaCl) and with respect to treatments (B0; without Bacteria, B1; 4.8 x 10^5 CFU mL^{-1}, B2; 4.8 x 10^{11} CFU mL^{-1}).

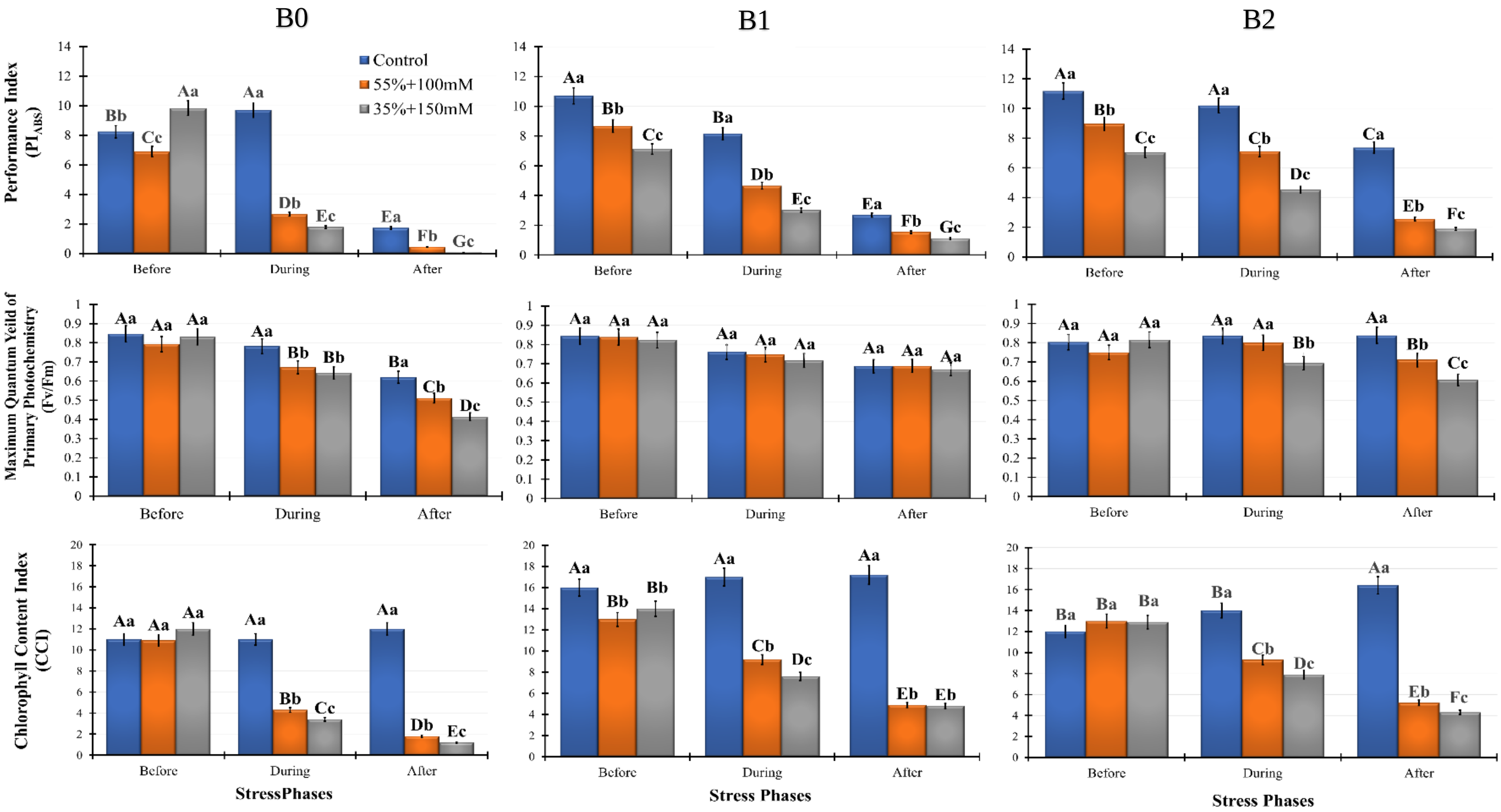

Fig. 3 Effect of *diazotrophic bacteria* on Performance index (PI_{ABS}), Maximum Quantum Yield of Primary Photochemistry (Fv/Fm) and Chlorophyll Content Index (CCI) before, during and after stress phases under control and different combine stress conditions in maize plants. B0: without bacteria, B1: 4.8 x 10^5 CFU $mL^{-1}$and B2; 4.8 x 10^{11} CFU mL^{-1}.Vertical line on the bar represents mean standard error (S.E). Small similar alphabet shows non-significant difference at $p \leq 0.05$ (Bonferroni Post-Hoc test) between treatments whereas large alphabets showed difference among the treatments.

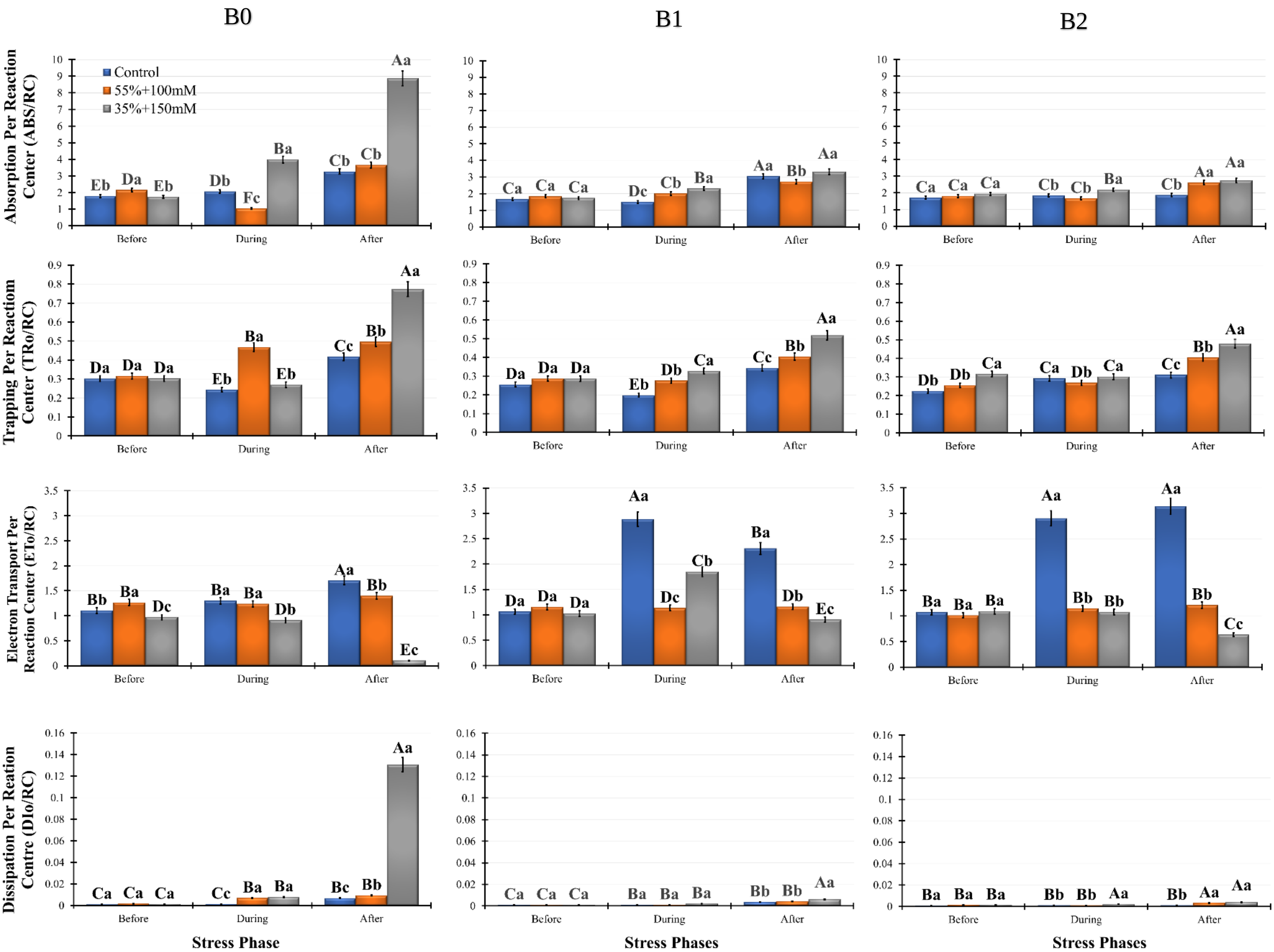

Fig. 4 Effect of *diazotrophic bacteria* on energy fluxes (ABS/RC, TRo/RC, Eto/RC and Dio/RC) before, during and after stress phases under control and different combine stress conditions in maize plants. B0: without bacteria, B1: 4.8 x 10^5 CFU $mL^{-1}$and B2; 4.8 x 10^{11} CFU mL^{-1}.Vertical line on the bar represents mean standard error (S.E). Small similar alphabet shows non-significant difference at $p \leq 0.05$ (Bonferroni Post-Hoc test) between treatments whereas large alphabets showed difference among the treatments.

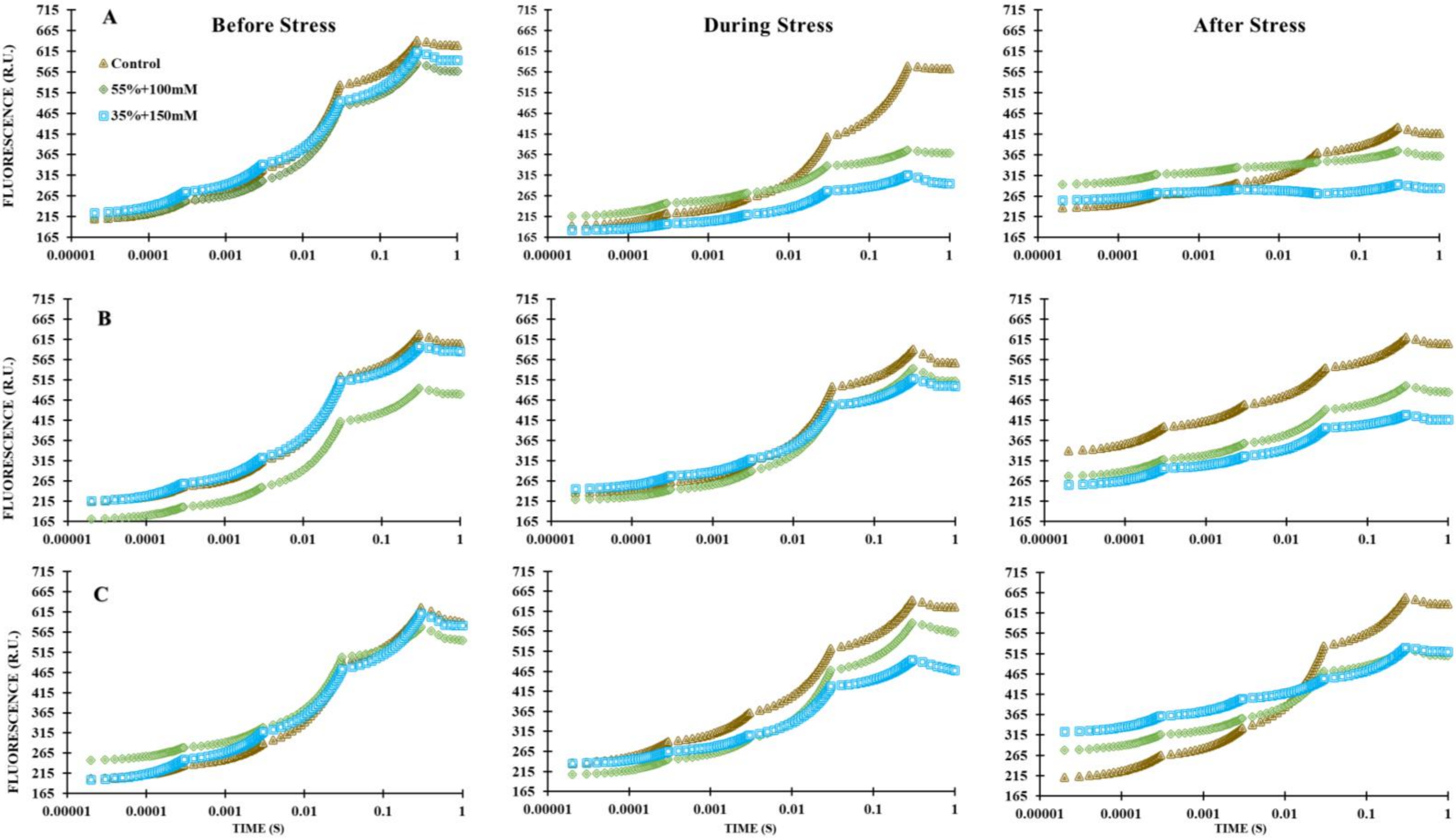

Fig. 5 Effect of *diazotrophic bacteria* on fluorescence transient curve before, during and after stress phases under control and different combine stress conditions in maize plants. A= B0: without bacteria, B=B1:4.8 x 10^5 CFU $mL^{-1}$and C=B2; 4.8 x 10^{11} CFU mL^{-1}.

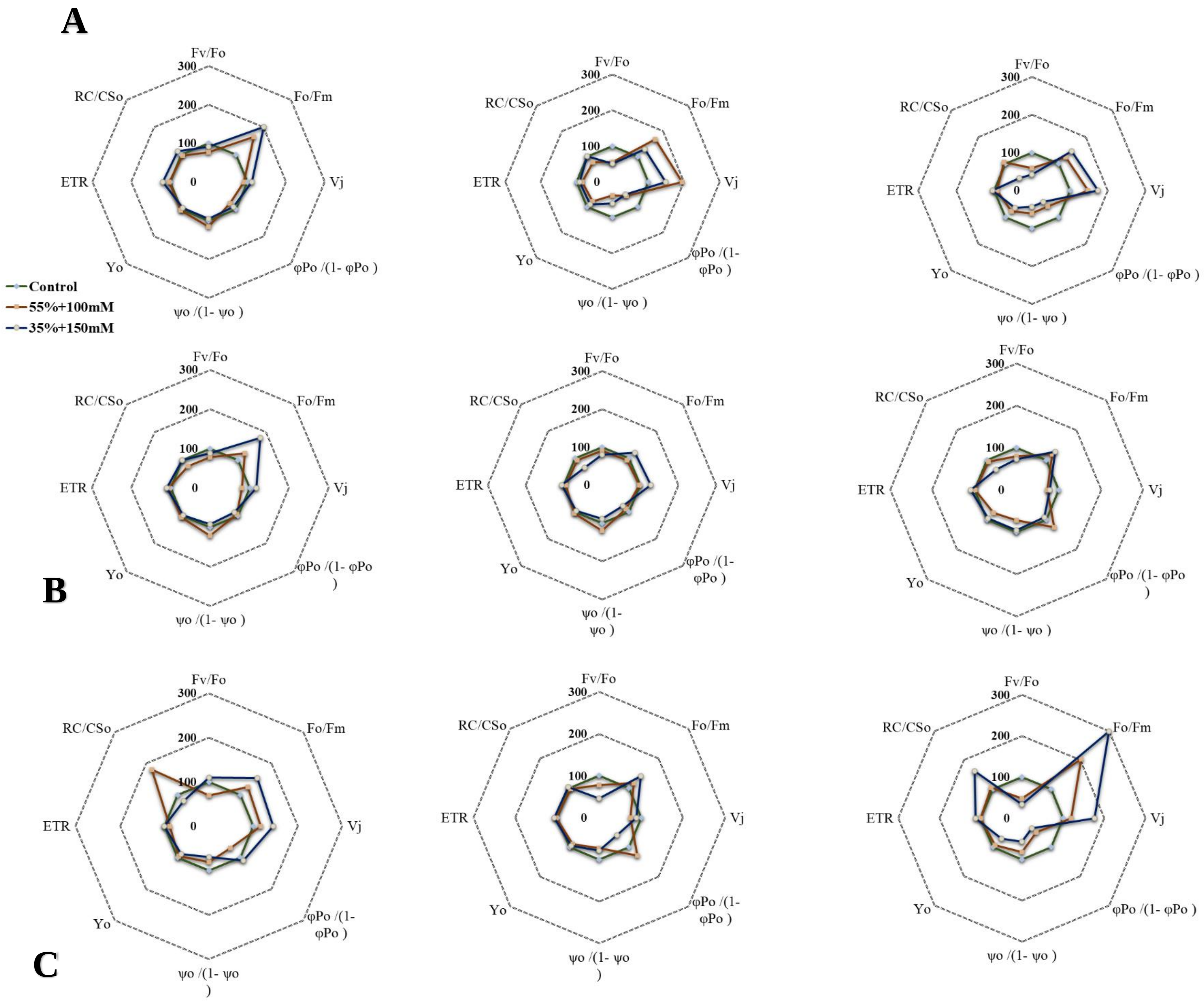

Fig. 6 Effect of *diazotrophic bacteria* on Physiological state of the photosynthetic apparatus (Fo/Fm), The ratio of photochemical to nonphotochemical quantum efficiencies (Fv/Fo), Electron transport per maximal trapping rate of absorbed photons (ETR), Probability that trapped electron was transferred to ETC beyond QA (Yo), The efficiency of light reaction (φPo /(1- φPo)), The efficiency of biochemical reaction (ψo /(1- ψo)), Relative variable fluorescence at J step (Vj) and The number of active PSII per excited cross section (RC/CSo) before, during and after stress phases under control and different combine stress conditions in maize plants. A= B0: without bacteria, B=B1:4.8 x 10^5 CFU $mL^{-1}$and C=B2; 4.8 x 10^{11} CFU mL^{-1}.

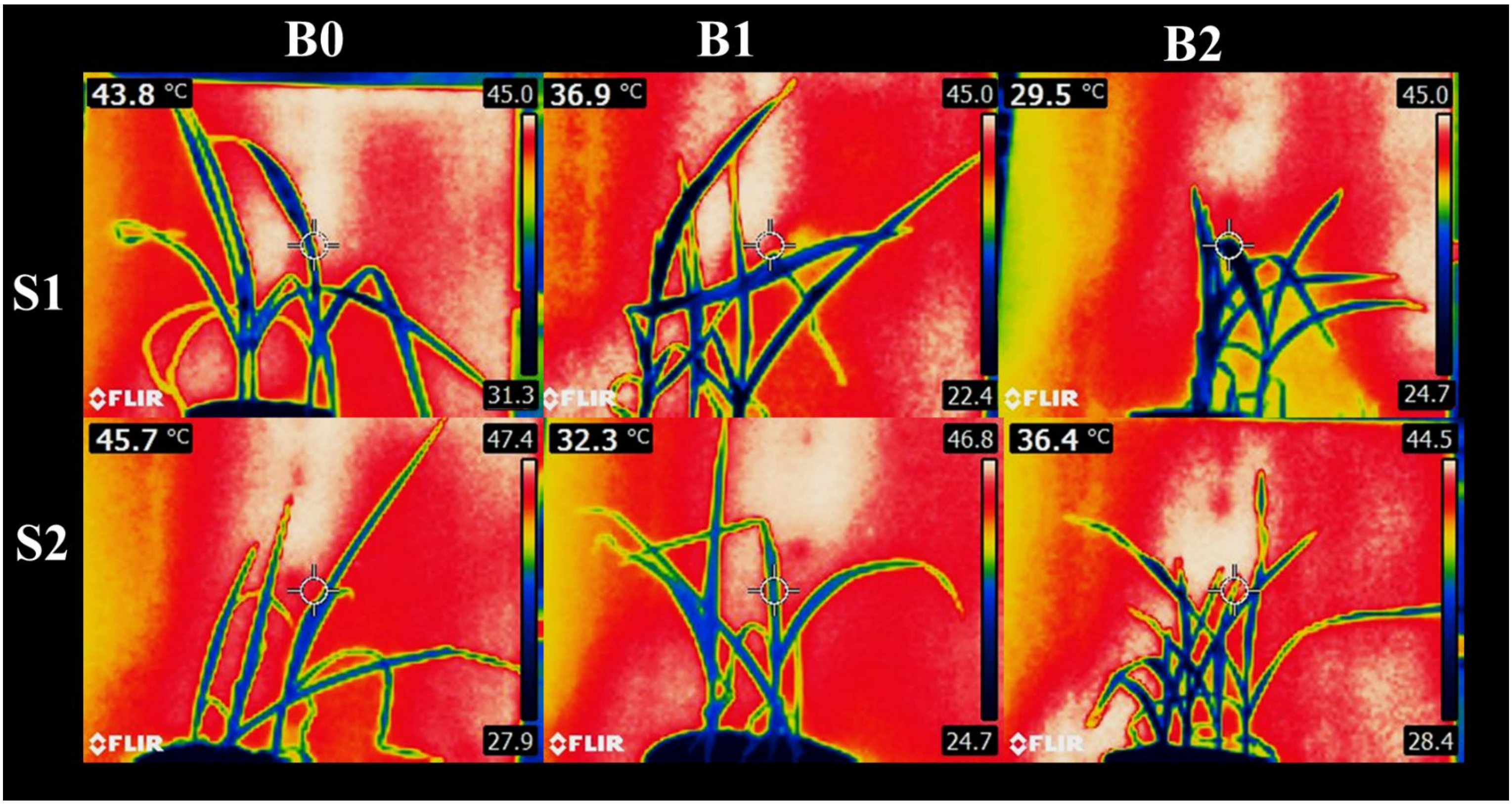

Fig. 7 Infra Red Thermography of maize plants after stress. Legends represents; B0: without bacteria, B1:4.8 x 10^5 CFU $mL^{-1}$and B2; 4.8 x 10^{11} CFU mL^{-1} , Where S1= 55%ms +100mM NaCl and S2=35%ms +150mM NaCl.

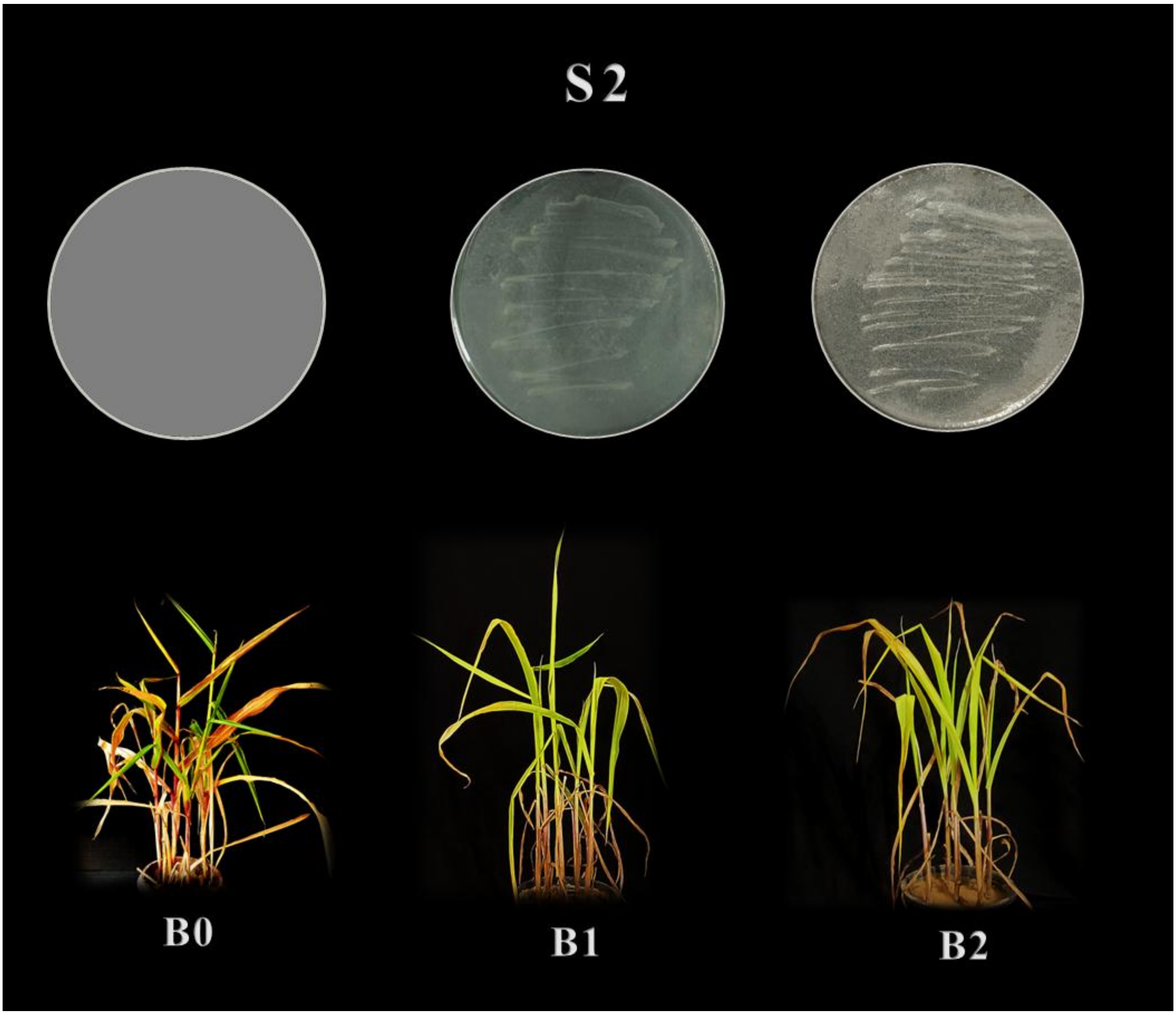

Fig. 8 *Bacterial and* Maize plant morphology under combined stress. In vivo stress measurements; 35%ms +150mM NaCl where as in vitro; 25% PEG+150MmNaCl (S2). Legends represents; B0: without bacteria, B1:4.8 x 10^5 CFU $mL^{-1}$and B2; 4.8 x 10^{11} CFU mL^{-1}.

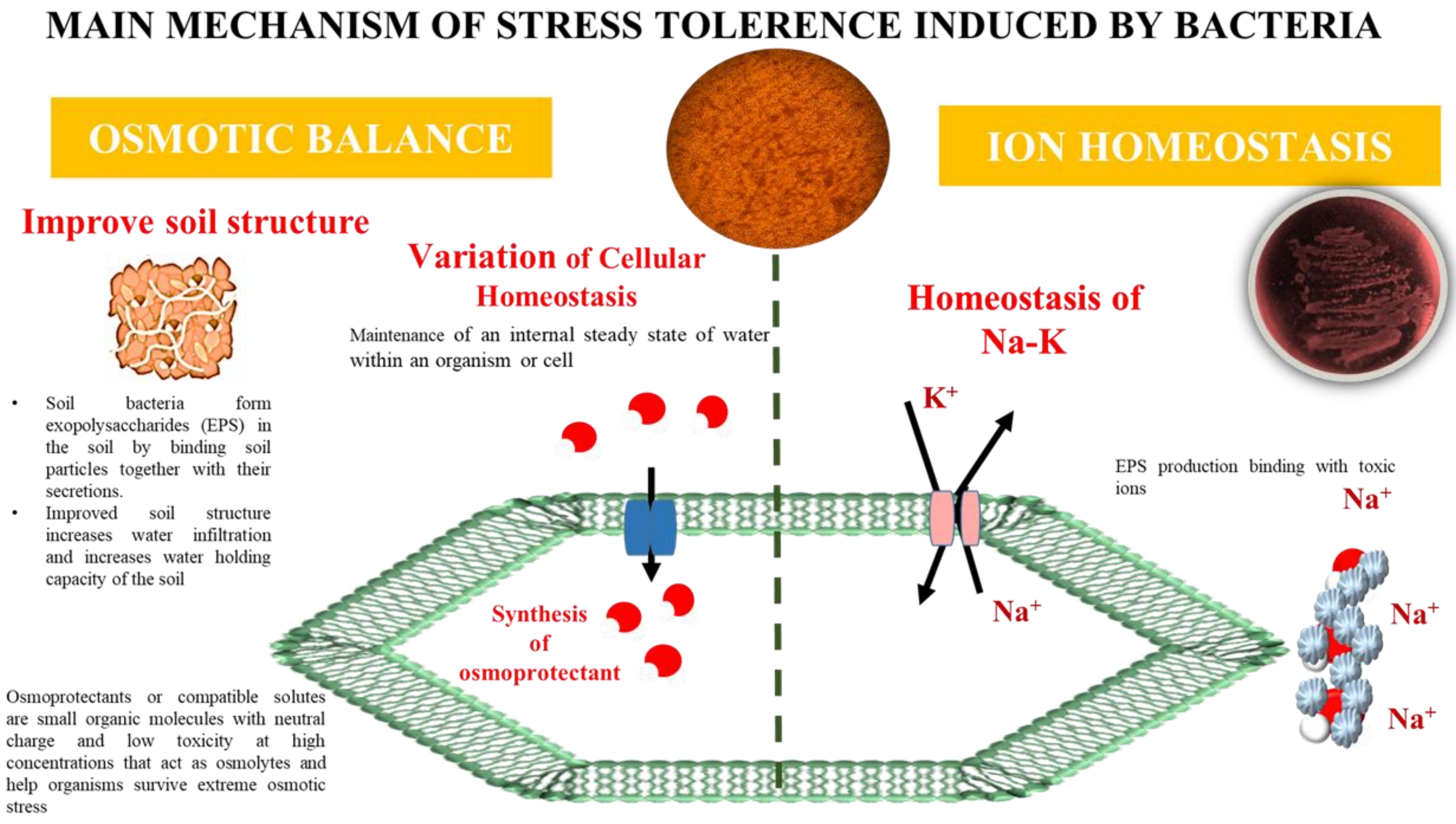

Fig. 9 General mechanism of diazotrophic bacteria under stress conditions.

Table. 1: Showing different soil parameters such as soil moisture content (SMC), organic content, electrical conductivity (EC) and potential of hydrogen (pH) monitored after stress application with and without *Diazotrophic bacteria*. B0: without bacteria B1: 4.8 x 10^5 CFU/ml and B2: 4.8 x 10^{11} CFU/ml

	B_0				**B_1**				**B_2**			
NaCl (mM)	**SMC (%)**	**Organic content**	**EC dS/m**	**pH**	**SMC (%)**	**Organic content**	**EC dS/m**	**pH**	**SMC (%)**	**Organic content**	**EC dS/m**	**pH**
Control	72	1.42	0.19	7.09	75	1.68	0.16	7.05	74	1.98	0.16	7.06
55%Ms+100mM	54	0.34	9.71	7.39	55	1.02	9.41	7.28	54	1.11	9.40	7.31
35%Ms+150mM	33	0.33	14.55	8.01	35	0.62	14.11	7.92	35	0.71	14.07	7.87

Table. 2: Showing different morphological and biochemical parameters such as plant total length, plant biomass, carbohydrate contents, protein contents, hydrogen peroxide content, malondialdehyde contents and phenol contents monitored after stress application with and without *Diazotrophic bacteria*. B0: without bacteria B1: 4.8 x 10^5 CFU/ml and B2: 4.8 x 10^{11} CFU/ml.

Treatments	**Combined Stress conc.**	**Plant length (cm)**	**Plant Biomass (g)**	**Carbohydrate (mg/g^{-1}FW)**	**Protein (mg/g^{-1}FW)**	**Hydrogen peroxide (µmolg^{-1}FW)**	**Malondialdehyde (µg^{-1}FW)**	**Phenols (mg/g^{-1}FW)**
B_0	Control	32.00 ± 0.02	2.00 ± 0.02	31.85 ± 1.20	19.72 ± 0.11	0.90 ± 0.001	0.10 ± 0.0002	90.07 ± 1.01
	55%Ms+100mM	29.00 ± 0.03	1.00 ± 0.06	07.41 ± 0.33	13.79 ± 0.03	5.00 ± 0.013	0.70 ± 0.0001	126.57 ± 2.03
	35%Ms+150mM	22.00 ± 0.03	0.30 ± 0.02	04.45 ± 0.02	3.29 ± 0.01	9.00 ± 0.002	1.00 ± 0.0001	169.40 ± 5.10
B_1	Control	39.00 ± 0.12	2.90 ± 0.02	51.24 ± 2.01	23.39 ± 0.30	0.03 ± 0.001	0.08 ± 0.0001	64.40 ± 1.20
	55%Ms+100mM	34.00 ± 0.11	1.20 ± 0.08	32.17 ± 1.02	22.12 ± 0.41	3.00 ± 0.003	0.20 ± 0.0002	82.90 ± 1.02
	35%Ms+150mM	30.00 ± 0.03	1.00 ± 0.04	25.89 ± 0.95	15.09 ± 0.03	7.20 ± 0.003	0.60 ± 0.0001	102.23 ± 3.14
B_2	Control	46.00 ± 0.14	3.70 ± 0.03	44.68 ± 2.01	27.95 ± 0.32	0.08 ± 0.001	0.09 ± 0.0002	66.90 ± 1.03
	55%Ms+100mM	40.00 ± 0.03	2.30 ± 0.04	26.38 ± 1.02	20.55 ± 0.04	2.00 ± 0.001	0.12 ± 0.0004	78.57 ± 1.02
	35%Ms+150mM	36.00 ± 0.11	1.90 ± 0.05	23.62 ± 0.83	17.25 ± 0.04	4.30 ± 0.003	0.58 ± 0.0032	119.07 ± 2.03