



Research article

Ascorbic acid formulation for survivability and diazotrophic efficacy of *Azotobacter chroococcum* Avi2 (MCC 3432) under hydrogen peroxide stress and its role in plant-growth promotion in rice (*Oryza sativa* L.)

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ABSTRACT

Oxidative stress generates reactive oxygen species which causes cell damage of living organisms and are normally detoxified by antioxidants. Indirect reports signify the damages caused by reactive oxygen species and neutralized by antioxidant, but the direct evidence to confirm this hypothesis is still unclear. To validate our hypothesis, an attempt was made in a diazotrophic bacterium (*Azotobacter chroococcum* Avi2) as a biological system, and hydrogen peroxide (H₂O₂) and ascorbic acid were used as oxidative stress and antioxidant supplement, respectively. Additionally, rice plant-growth attributes by Avi2 was also assessed under H₂O₂ and ascorbic acid. Results indicated that higher concentration of H₂O₂ (2.5 mM–4.5 mM) showed the complete mortality of Avi2, whereas one ppm ascorbic acid neutralized the effect of H₂O₂. Turbidity, colony forming unit, DNA quantity, *nifH* gene abundance, indole acetic acid and ammonia productions were significantly ($p < 0.5$) increased by 11.93%, 17.29%, 19.80%, 74.77%, 71.89%, and 42.53%, respectively in Avi2-treated with 1.5 mM H₂O₂ plus ascorbic acid compared to 1.5 mM H₂O₂ alone. Superoxide dismutase was significantly ($p < 0.5$) increased by 60.85%, whereas catalase and ascorbate peroxidase activities were significantly ($p < 0.05$) decreased by 64.28% and 68.88% in Avi2-treated with 1.5 mM H₂O₂ plus ascorbic acid compared to 1.5 mM H₂O₂ alone. Germination percentage of three rice cultivars (FR13a, Naveen and Sahbhagi dhan) were significantly ($p < 0.5$) increased by 20%, 13.33%, and 4%, respectively in Avi2-treated with 0.6 mM H₂O₂ plus ascorbic acid compared with uninoculated control. Overall, this study indicated that ascorbic acid formulation neutralizes the H₂O₂-oxidative stress and enhances the survivability and plant growth-promoting efficacy of *A. chroococcum* Avi2 and therefore, it may be used as an effective formulation of bio-inoculants in rice under oxidative stress.

1. Introduction

Oxidative stress (OS) is one of the major environmental stresses which cause an adverse effect on bacterial cell physiology that leads to either its growth rate reduction or complete mortality under extreme circumstances (Imlay, 2019; Kashmiri and Mankar, 2014; Tong et al., 2019). The OS theory of ageing postulates that reactive oxygen species (ROS) formed exogenously or endogenously under normal metabolic

processes, play a direct role in the ageing process (Chacar et al., 2019; Son et al., 2019; Zhang et al., 2017). Denham Harman proposed a ‘free radical theory’ of ageing which states that generation of endogenous oxygen radicals in cells causes a cumulative damage (Carocho et al., 2019). The excess ROS attacks on cell membrane, damages the DNA, oxidize amino acids, proteins and lipids (Asada, 1999; Richardson et al., 2003; Son et al., 2019). Finkel and Holbrook (2000) also reported that generation of ROS due to oxidative stress had a significant role on

Abbreviations: OS, oxidative stress; ROS, reactive oxygen species; H₂O₂, hydrogen peroxide; Avi2, *Azotobacter chroococcum* Avi2; AA, ascorbic acid; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; RDA, recommended daily allowance; LB, Luria Bertani broth medium; CFU, colony forming unit; IAA, indole acetic acid; NH₃, ammonia; RT-PCR, real-time polymerase chain reaction; GP, percentage of seed germination; SVI, seedling vigor index; Lr, mean root length; Ls, mean shoot length; RWC, Relative water content; FWt, fresh weight of shoots; TWt, weight at full turgid; DWt, dry weight; ANOVA, analysis of variance; PCoA, principal coordinate analysis; dbrDA, distance-based redundancy analysis; DHA, dehydroascorbic acid; PGP, plant growth promotion

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ageing. Free radicals which are generated under OS cause damage to bacterial cells and this would mainly happen because of asymmetry between production of ROS and a biological system's ability to detoxify the reactive intermediates which leads to imbalance in the ROS production and antioxidant defense system (Birben et al., 2012).

Antioxidant defense enzymes such as superoxide dismutase, glutathione, glutathione peroxidase, glutathione reductase, thioredoxin, vitamin C, vitamin E are possessed by most of the living organisms to protect against oxygen intermediates (superoxide anion, hydroxyl and hydrogen peroxide radicals) (Gill and Tuteja, 2010; Mishra et al., 2015). However, these native antioxidants were not enough to prevent organisms' severe damage or death due to the formation of higher ROS (Zhang et al., 2017). Therefore, exogenous antioxidant additives may be required to reduce the oxidative stress. Though several reports indicated the exposure of hydrogen peroxide (H_2O_2) as an oxidative stress to human pathogenic bacteria such as *Salmonella typhimurium* (Christman et al., 1989); *Streptococcus pneumonia* (Pericone et al., 2003); *Escherichia coli* (Imlay, 2019; Kohen and Shalhoub, 1994; Li and Imlay, 2018) and its scavenging mechanism, but its effect on beneficial bacteria was very meager and insufficient (Dimkpa et al., 2009). Many indirect reports signify the impact of antioxidant as anti-ageing agents as it has ability to detoxify the effect of ROS (Carocho et al., 2019; Imlay, 2019; Son et al., 2019), but there is no direct confirmation to showcase this hypothesis. Therefore, to prove this hypothesis, we used a bacterium (*Azotobacter chroococcum* Avi2, a diazotroph) as a model biological system, H_2O_2 as an external source of oxidative stress and ascorbic acid as an antioxidant source.

Diazotrophs play a significant role in the rhizosphere in enhancing plant growth-promotion by supplying fixed nitrogen to the plants (Kumar et al., 2017a, b; Kumar et al., 2018; Kumar et al., 2019a). Present study showed how the various concentration of H_2O_2 affected the *A. chroococcum* (Avi2) alone and in combination with ascorbic acid to neutralize such an ample amount of ROS. Based on the extensive survey of scientific literature, there is limited information on the effects of antioxidant on the diazotrophic bacteria (Karthikeyan et al., 2007; Kelman et al., 2009). Perhaps, no reports were found on the survival and functional variations of *A. chroococcum* (Avi2) under H_2O_2 stress with or without the presence of ascorbic acid. Earlier many researchers recommended the use of derivative forms of ascorbic acid for the removal of hydrogen peroxide residues from H_2O_2 -treated fruits and vegetables (Sapers and Simmons, 1998; Sapers et al., 1999) which supported the idea that ascorbic acid might have beneficial effects as a free radical scavenger under excessive OS for bacterial health and also protects their functional traits.

Therefore, the present study was performed to assess i) the survival and growth of *A. chroococcum* (Avi2) under various concentrations of H_2O_2 alone and then in combination with standardized ascorbic acid dose as per recommended daily allowance for a male or female human body i.e. 60 mg day^{-1} (Ozkan et al., 2004), ii) the variation of functional traits (ammonia, indole acetic acid production, defense enzyme production, and nitrogen-fixing efficiency) were observed in *A. chroococcum* (Avi2) in response to H_2O_2 and ascorbic acid. In addition, seed germination, seedling vigor index and relative water content were also observed in *A. chroococcum* (Avi2)-inoculated rice plants under H_2O_2 and ascorbic acid.

2. Materials and methods

2.1. Survival and mortality of *A. chroococcum* Avi2 in different concentration of H_2O_2 and ascorbic acid

In the present study, *A. chroococcum* strain Avi2 (MCC no. 3432; NCBI accession no. KP099933) (Banik et al., 2019), H_2O_2 (HiMedia, India) and ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) were used as experimental materials for biological marker, oxidative stress and antioxidant, respectively. Treatments for this study were control (only

Avi2), 0.6 mM H_2O_2 , 1.5 mM H_2O_2 , 2.5 mM H_2O_2 , 3.5 mM H_2O_2 , 4.5 mM H_2O_2 , 1.5 mM H_2O_2 + ascorbic acid (AA), 2.5 mM H_2O_2 + AA, 3.5 mM H_2O_2 + AA, 4.5 mM H_2O_2 + AA. Three replications for each treatment were maintained. Firstly, overnight grown Avi2 strain (100 μL) was inoculated in test tubes containing 10 mL sterilized Luria Bertani (LB) broth medium (Bertani, 1951) with different concentration of H_2O_2 (0 mM–4.5 mM). Then the required dose of ascorbic acid (1 ppm) was added in test tubes containing a different concentration of H_2O_2 (0 mM–4.5 mM) in LB broth media and incubated for 48 h at $30 \pm 1^\circ\text{C}$ in shaker (150 rpm). The dose of ascorbic acid was calculated as per recommended daily allowance for a male or female human body weight (60 mg d^{-1}) (Ozkan et al., 2004). Growth of Avi2 was measured at 660 nm using the UV spectrophotometer (Specord, 2000; Analytik Jena, Germany). Viable cells of Avi2 under different treatments were measured by adopting the serial dilution method and represented in colony forming unit (CFU) per mL (Kumar et al., 2017a, c). Briefly, 1 mL of bacterial culture was added to 9 mL of sterile diluents. From this suspension, the higher dilutions were made and 0.1 mL was plated onto Jensen's agar media and incubated for 72 h at $30 \pm 1^\circ\text{C}$. Quantification of DNA was measured using NanoDrop[®] ND-1000 spectrophotometer.

2.2. Biochemical characterization of *A. chroococcum* Avi2 under H_2O_2 and ascorbic acid

Carbohydrate utilization of *A. chroococcum* Avi2 under different treatments was analyzed by using KB009 HiCarbohydrate TM kit (Hi-Media). Briefly, the Avi2 was grown in 5 mL LB broth under different concentration of H_2O_2 and ascorbic acid and allowed to incubate for one day ($\text{OD} \leq 0.5$ at 660 nm). 50 μL broth of each treatment was dispensed in KB009 HiCarbohydrate TM kit and incubated at $35 \pm 2^\circ\text{C}$ for 48 h and the result was analyzed based on change in colour of each well containing different carbon sources (Garland and Mills, 1991).

2.3. Assay for indole acetic acid production

Indole acetic acid (IAA) production of *A. chroococcum* Avi2 under different treatments was assessed (Bric et al., 1991; Kumar et al., 2017a). Briefly, for quantitative estimation of IAA, Avi2 strain was inoculated in 5 mL LB broth (containing 5 mM tryptophan) and incubated at $30 \pm 2^\circ\text{C}$ for 4 days under dark and then 2 mL of Salkowski reagent (2% of 0.5 M FeCl_3 in 35% HClO_4) was added to the tubes. Pink colour development was observed and measured at 530 nm. IAA content was expressed as $\mu\text{g mL}^{-1}$ using a standard curve of IAA.

2.4. Ammonia (NH_3) production

Overnight grown *A. chroococcum* Avi2 (100 μL) culture was inoculated in 10 mL peptone broth and nitrogen-free respective media, incubated at $30 \pm 0.1^\circ\text{C}$ for 48 h in an incubator shaker (120 rpm). After growth, 0.5 mL broth, 1 mL sodium borate buffer (pH 8.5; 0.1 M) and 0.5 mL L-asparagine solution (0.04 M) were mixed and incubated at $30 \pm 2^\circ\text{C}$ for 10 min. The reaction was stopped with 0.5 mL trichloroacetic acid (0.1 N), centrifuged at 12,000 rpm at 4°C and the released NH_3 was determined spectrophotometrically at 450 nm by Nesslerization following Cappuccino and Sherman (1992).

2.5. Estimation of superoxide dismutase activity

Superoxide dismutase (SOD) (EC 1.15.1.1) activity in *A. chroococcum* Avi2 treatments was analyzed as per methodology of Chakraborty et al. (2015). Briefly, 3 mL reaction mixture was prepared by adding 13.33 mM methionine, 75 μM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate, 0.1 mL enzyme extract in 2 mL distilled water. The reaction was initiated by adding 2 mM riboflavin (0.1 mL) and tubes were placed

under illuminating chamber (two 15 W fluorescent lamps) for 15 min. Illuminated and non-illuminated reaction mixtures were used for calibration and the absorbance of these were recorded at 560 nm. The SOD activity was calibrated using tubes lacking enzyme per unit time and expressed as unit mg^{-1} protein min^{-1} .

2.6. Estimation of catalase activity

The catalase (CAT) (EC 1.11.1.6) activity in *A. chroococcum* Avi2 treatments was assayed by measuring the loss of H_2O_2 (Aebi, 1984) in a reaction mixture (3 mL) consisting of 0.5 mL of 75 mM H_2O_2 and 1.5 mL of 0.1 M phosphate buffer (pH 7) along with the addition of 50 μL of chilled bacterial enzyme extract. The decrease in absorbance at 240 nm was observed for 1 min in an UV-visible spectrophotometer. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed. The initial and final contents of H_2O_2 were calculated by comparing with a standard curve drawn with known concentrations of H_2O_2 and expressed as μmol H_2O_2 reduced mg^{-1} protein min^{-1} .

2.7. Estimation of ascorbate peroxidase activity

The ascorbate peroxidase (APX) (EC 1.11.1.11) activity was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm (Nakano and Asada, 1981). The final 3 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2 , 0.1 mL enzyme in which 0.1 mL of H_2O_2 was added to initiate the reaction. Decrease in absorbance was measured spectrophotometrically and the APX activity was measured by calculating the decreased ascorbic acid content with a known concentration of ascorbic acid using standard curve and expressed as μmol ascorbate oxidized mg^{-1} protein min^{-1} .

2.8. Quantification of *nifH* gene efficacy under H_2O_2 stress by real-time polymerase chain reaction (RT-PCR)

The abundance of *nifH* genes was measured using Eppendorf iCycler with fluorescent dye iQ SYBR[®]Green qPCR Kit (CloneTech Takara, Japan) as per manufacturer's instruction. qPCR protocols were optimized for PCR efficiency using the universal diazotrophic primer pairs *nifH* (Poly et al., 2001), two replicates of each sample were used for detection. Quantitative PCR of *nifH* was performed in a 25 μL reaction mixture containing: 5 μL of template DNA ($\sim 3\text{--}15$ ng μL^{-1}), 12.5 μL of iQ SYBR[®], 0.25 μL of each primer stock solution (10 μM), and nuclease-free water. The initial denaturation step was at 95 °C for 15 min prior to reaction step, followed by 40 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. Melting curve analyses were performed at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. To determine the absolute quantities of the *nifH* gene existence, a standard was prepared for PCR. *nifH* genes were amplified by conventional PCR with the *nifH* primer set. The PCR product was extracted from agarose gel, purified with Wizard[®] SV Gel and PCR Clean-Up System (Promega, USA) and cloned into a TOPO-TA plasmid vector (Invitrogen USA) in *Escherichia coli*. Plasmid DNA was extracted from *E. coli* colonies with inserts using a QIAprep Spin Miniprep Kit (Qiagen, USA). Plasmid DNA was assessed for quantity and quality using a NanoDrop[®] ND-1000 spectrophotometer and sequenced. A 10-fold dilution series of plasmid DNA was prepared, ranging from 3×10^1 to 3×10^8 copies. The *nifH* copies were calculated from a standard constructed by plotting plasmid DNA concentrations versus quantification cycles which produced linear ($R^2 > 0.95$) standard curve.

2.9. Effect of oxidative stress on seed germination bioassay with *A. chroococcum* Avi2

Seed germination test was performed as per methodology of Shende et al. (1977). This experiment was conducted at Microbiology

laboratory-National Rice Research Institute, Cuttack (20°25' N, 85°55' E, 24 m above mean sea level) during November–December 2017. During the study period, mean temperatures, relative humidity, and precipitation were 22.5 °C, 78.5% and ~ 0 mm, respectively. Briefly, Rice seeds were surface sterilized with 0.1% of HgCl_2 for 3 min followed by successive washings (at least ten times) with sterile distilled water to remove traces of HgCl_2 . Seeds were soaked in 72 h ($\geq 10^7$ cells mL^{-1}) older Avi2 culture broth for about one hour. The excess culture broth was drained off from the seeds. Then 0.8% sterile agar was poured into sterilized plates and allowed to solidify. Rice seeds (25 per plate) were placed on soft agar plates using a sterile forceps. Different concentration of H_2O_2 (0.6 mM, 1.5 mM, 2.5 mM, 3.5 mM, 4.5 mM and 5.5 mM) (Supplementary Fig. 2D) added onto the plate and incubated for 5 days at 30 °C.

2.10. Effect of ascorbic acid treated *A. chroococcum* Avi2 culture on seedling vigor under oxidative stress

One ppm of ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) was dropped on the soft agar plates to examine the germination as well as the plant growth promoting (PGP) traits such as germination percentage, shoot length, root length, seedling vigor index, fresh weight, dry weight and relative water content of three rice cultivars (FR13a, Naveen and Sahbhagi dhan) under different treatments of *A. chroococcum* Avi2 and H_2O_2 (uninoculated control; Avi2 + ascorbic acid; Avi2 + 0.6 mM H_2O_2 ; Avi2 + 0.6 mM H_2O_2 + ascorbic acid) and each treatment was replicated thrice. The plates containing seeds were incubated at 30 °C for 5 days. After fifth day, the seed germination percentage and on the eighth day, root and shoot length in mm, fresh and dry seedlings weight in mg seedling⁻¹, seedling vigor index (SVI), and relative water content (RWC) were measured and calculated as per methodology of Kaydan and Yagmur (2008) (Supplementary Fig. 2A, B, C). Germination percentage (GP) = (Total number of seeds germinated/Total number of seeds in all replicates) $\times 100$. Seedling vigor index (SVI) = [Mean root length (L_r) + Mean shoot length (L_s)] \times Percentage of seed germination (GP). Relative water content (RWC) = [(FWt - DWt)/(TWt - DWt)] $\times 100$. Where FWt is the fresh weight of shoots, TWt is the weight at full turgid i.e. measured after floating the shoots for 24 h in sterile distilled water in the light at 37 °C and dry weight (DWt) was estimated at 70 °C until a constant weight was gained.

2.11. Statistical analysis

Data were statistically analyzed by using online server of statistical computing for NARS, Indian Agricultural Statistics Research Institute, New Delhi, India (<http://www.iasri.res.in/sscnars>). The mean difference comparison between the treatments was analyzed by analysis of variance (ANOVA) and subsequently by Tukey's HSD at 5%. Based on principal coordinate analysis (PCoA), two biplots were constructed, one by using the relevance of plant-growth promoting activity, enzyme assay and *nifH* data, explaining the effect of antioxidant under H_2O_2 stress in different treatments of bacterial culture and another one by using the responses of plant-growth promoting traits (germination percentage, shoot length, root length, seedling vigor index, fresh weight, dry weight, and relative water content) in three rice cultivars (FR13a, Naveen and Sahbhagi dhan) and these were grouped into three clusters, analyzed by Bray-Curtis distance-based redundancy analysis (dbRDA) using the Vegan package in R software. Heatmap and correlation plot were also generated using the Vegan package in R software version 3.5.0.

Table 1

Survivability of *Azotobacter chroococcum* (Avi2) under the influence of H₂O₂ and ascorbic acid. T: turbidity (optical density at 660 nm); NT: not turbid; ND: not detected; HP: hydrogen peroxide (H₂O₂) doses in milli molar (mM); AA: ascorbic acid (1 ppm); CFU: colony forming unit of Avi2 (X10⁸) mL⁻¹ on Jensen medium; QD: quantification of total DNA concentration of Avi2 (ng μL⁻¹). Values within a column followed by the same letters are not significantly different at Tukey HSD (*p* < 0.05).

Treatment	Growth of Avi2	Turbidity	CFU of Avi2	QD of Avi2
Control	T	0.86e	0.63e	17.20f
0.6HP + Avi2	T	1.01c	0.98d	20.13e
1.5HP + Avi2	T	1.55b	1.10c	29.73b
2.5HP + Avi2	NT	ND	ND	ND
3.5HP + Avi2	NT	ND	ND	ND
4.5HP + Avi2	NT	ND	ND	ND
1.5HP + Avi2 + AA	T	1.76a	1.33a	37.07a
2.5HP + Avi2 + AA	T	1.63b	1.20 b	25.30c
3.5HP + Avi2 + AA	T	1.61b	1.10c	22.37d
4.5HP + Avi2 + AA	T	0.94d	1.03d	20.03e

3. Result

3.1. Effect of hydrogen peroxide (H₂O₂) on *A. chroococcum* Avi2

Survivability of *A. chroococcum* Avi2 was investigated under influence of different concentrations of H₂O₂ (0–4.5 mM). Higher concentration (2.5–4.5 mM) of H₂O₂ showed the complete mortality of *A. chroococcum* Avi2 (Supplementary Fig. 1 and Table 1).

3.2. Effects of ascorbic acid on survivability and growth of *A. chroococcum* Avi2 under influence of H₂O₂

Addition of ascorbic acid (1 ppm) detoxified the effect of H₂O₂ (2.5–4.5 mM)-treated *A. chroococcum* Avi2 and interestingly, it revived the bacterial growth under H₂O₂ stress (Supplementary Fig. 1 and Table 1). Significantly (*p* > 0.05) higher turbidity (1.76) was found in Avi2 treated with 1.5 mM H₂O₂ and ascorbic acid (1 ppm) compared to Avi2 treated with 1.5 mM H₂O₂ alone. However, a gradual decrease of turbidity was recorded in treatments having higher concentration of H₂O₂ (2.5–4.5 mM) (Table 1). Significantly (*p* > 0.05) higher colony forming unit (CFU) and DNA quantity were recorded in Avi2 treated with combination of 1.5 mM H₂O₂ and ascorbic acid (1 ppm) (Table 1).

3.3. Carbon source utilization of *A. chroococcum* Avi2 under oxidative stress

The heatmap result showed that out of 36 carbohydrate sources, 3 carbon sources such as malonate, citrate, and mannitol were utilized by the *A. chroococcum* Avi2 under controlled condition (without addition of H₂O₂) (Fig. 1). Frequency of carbon-source utilization by the *A. chroococcum* Avi2 was increased under H₂O₂-induced treatments. Results also showed that the highest number (6) of carbohydrate (fructose, dextrose, sucrose, inulin, sorbitol and esculin) was utilized by *A. chroococcum* Avi2 exposed with 4.5 mM H₂O₂ along with 1 ppm of ascorbic acid compared to the control.

3.4. In vitro plant-growth promoting traits of *A. chroococcum* Avi2 under influence of H₂O₂ and ascorbic acid

Abundance of *nifH* gene varied in the range of 0.98 × 10⁶–4.36 × 10⁶ copies mL⁻¹ in Avi2 treatments (Fig. 2), wherein the maximum copies of *nifH* gene were recorded in treatment 1.5 mM of H₂O₂ + 1 ppm of ascorbic acid (4.36 × 10⁶ copies mL⁻¹) compared to treatment 1.5 mM of H₂O₂ (1.10 × 10⁶ copies mL⁻¹). Among all treatments, indole acetic acid (IAA) and ammonia (NH₃) productions were recorded in the range of 10.75–38.25 μg mL⁻¹ and

13.58–50.83 mM mL⁻¹, respectively. Treatment 1.5 H₂O₂ + Avi2 + AA showed the highest IAA (38.25 μg mL⁻¹) and NH₃ (50.83 mM mL⁻¹) productions compared to other treatments (Table 2).

3.5. Antioxidant enzyme activities of *A. chroococcum* Avi2 under influence of H₂O₂ and ascorbic acid

The highest superoxide dismutase (SOD) activity was observed in *A. chroococcum* Avi2 treated with 1.5 mM of H₂O₂ + 1 ppm of ascorbic acid (2.35 unit mg⁻¹ protein min⁻¹) and the lowest activity was recorded in 4.5 mM of H₂O₂ + 1 ppm of ascorbic acid (0.04 unit mg protein⁻¹ min⁻¹). The highest activity of catalase (CAT) was recorded in *A. chroococcum* Avi2 treated with 4.5 mM of H₂O₂ + 1 ppm of ascorbic acid (0.32 μmol H₂O₂ reduced mg⁻¹ protein min⁻¹), whereas ascorbate peroxidase (APX) activity was recorded higher in 3.5 mM of H₂O₂ + 1 ppm of ascorbic acid (0.64 μmol ascorbate oxidized mg⁻¹ protein min⁻¹). However, lower CAT and APX activities (0.05 μmol H₂O₂ reduced mg⁻¹ protein min⁻¹ and 0.13 μmol ascorbate oxidized mg⁻¹ protein min⁻¹, respectively) were observed in the treatment of 1.5 mM H₂O₂ + 1 ppm ascorbic acid (Table 2).

3.6. Principal coordinates analysis of growth parameters, plant-growth promoting traits and antioxidant enzyme activities of *A. chroococcum* Avi2 under influence of H₂O₂ and ascorbic acid (AA)

Six treatments (control, 1.5 mM H₂O₂, 1.5 mM H₂O₂ + AA, 2.5 mM H₂O₂ + AA, 3.5 mM H₂O₂ + AA, 4.5 mM H₂O₂ + AA) and nine parameters (turbidity, CFU, total DNA, *nifH* gene, IAA, NH₃, SOD, CAT and APX) were analyzed using principal coordinate analysis (PCoA) with the help of two principal factors (PC1 and PC2). The PCoA explained total variance of 83%, in which principal component 1 (PC1) and principal component 2 (PC2) showed 64.8% and 18.2% variances, respectively (Fig. 3). In the plot, the arrows indicated the different variables which demarcated the different distance from the origin. Correlation level among the quantified variables indicated that lines in the same direction were more closely correlated. In PC1 maximum contributed parameters viz. *nifH* gene quantification (15.49%), superoxide dismutase activity (15.24%), total DNA (14.05%) and indole acetic acid production (11.89%) had high correlation (*p* < 0.05) with the treatment 1.5 mM of H₂O₂ + 1 ppm ascorbic acid. In PC2, the following parameters had maximum contribution viz. colony forming unit (CFU) of viable Avi2 cells (30.12%), turbidity (27.19%), ammonia (15.63%), catalase (12.12%) (Fig. 3). Most of the parameters namely (turbidity, CFU, total DNA, *nifH* gene, IAA, NH₃ and SOD) were positively correlated with 1.5 mM H₂O₂ + AA, whereas catalase and ascorbate peroxidase were found in the opposite direction of 1.5 mM H₂O₂ + AA, depicting a negative correlation with 1.5 mM H₂O₂ + AA. However, catalase and ascorbate peroxidase had higher positive correlation with 1.5 mM H₂O₂ as compared with other treatments. In the biplot ordination plane, control was found to farthest, showing less variability.

3.7. Effect of *A. chroococcum* Avi2 in rice plant growth promotion under influence of H₂O₂ and ascorbic acid

Statistically analyzed data of Avi2 treated rice seedlings of three cultivars (FR13a, Naveen and Sahbhagi dhan) under influence of H₂O₂ and ascorbic acid are presented in Supplementary Fig. 3 and Fig. 4. It was seen that more than 0.6 mM concentration of H₂O₂ inhibited the seed germination (Supplementary Fig. 2D). Germination percentage of three cultivars in different treatments (control or uninoculated; Avi2 only; Avi2 + ascorbic acid; Avi2 + 0.6 mM H₂O₂; Avi2 + 0.6 mM H₂O₂ + ascorbic acid) showed in the range of 82.67–98.67% (Sahbhagi dhan), 86.67–100% (Naveen), 56–80% (FR13a), whereas the highest germination percentage was recorded in Naveen (100%) followed by Sahbhagi dhan (98.67%) and FR13a (80%) under treatment Avi2 + 0.6 mM of H₂O₂ + 1 ppm ascorbic acid compared to control. After 15

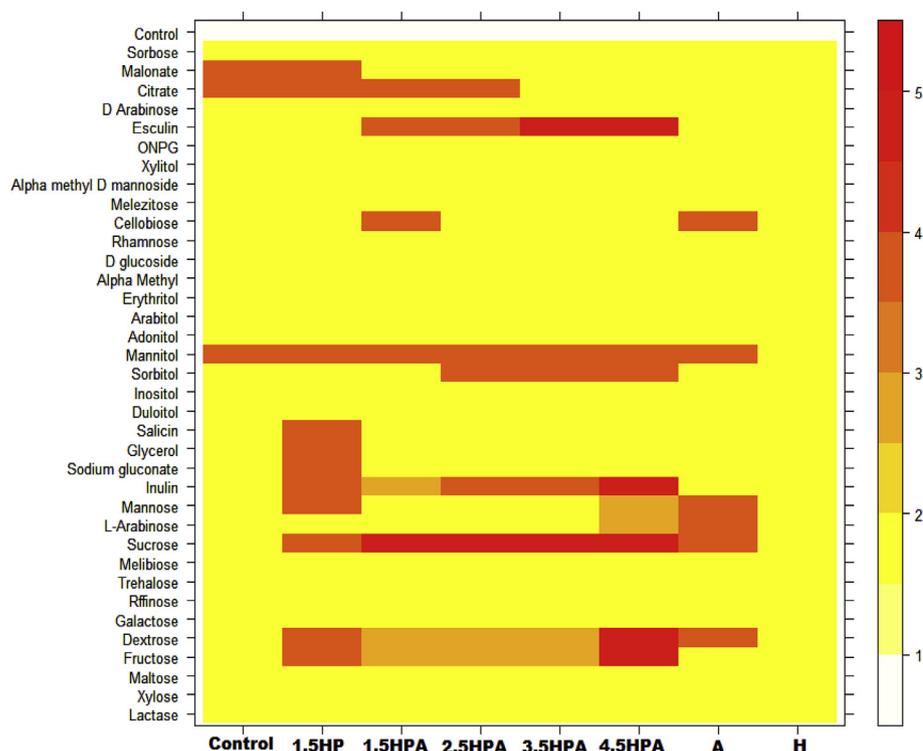


Fig. 1. Heat map of carbon source utilization of *A. chroococcum* Avi2 under different treatments. Control: Avi2 in LB broth medium without H₂O₂; H: only H₂O₂ (without Avi2 inoculant); A: only ascorbic acid (without Avi2 inoculant); 1.5 HP: 1.5 mM H₂O₂ + Avi2 inoculant; 1.5HPA: 1.5 mM H₂O₂ + Avi2 inoculant + ascorbic acid; 2.5 HPA: 2.5 mM H₂O₂ + Avi2 inoculant + ascorbic acid; 3.5 HPA: 3.5 mM H₂O₂ + Avi2 inoculant + ascorbic acid; 4.5 HPA: 4.5 mM H₂O₂ + Avi2 inoculant + ascorbic acid. Light yellow colour shows lower and dark red colour shows higher carbon utilization by subsequent treatment of Avi2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

days of treatments, varied plant growth parameters were observed viz., shoot length (Sahbhagi dhan: 17.5–28.33 mm; Naveen: 19.67–39.50 mm; FR13a: 15.50–37 mm); root length (Sahbhagi dhan: 22.83–63.33 mm; Naveen: 41.50–54.33 mm; FR13a: 42.50–74.17 mm); seed vigour index (SVI) (Sahbhagi dhan: 33.31–90.47; Naveen: 52.99–93.83; FR13a: 37.91–88.99); fresh weight of seedlings (Sahbhagi dhan: 57.33–76.67 mg; Naveen: 33.33–59.17 mg; FR13a: 61.50–89.67 mg); dry weight of seedlings (Sahbhagi dhan: 20.67–24.50 mg; Naveen: 17.00–24.67 mg; FR13a: 22.00–31.33 mg) and relative water content (RWC) (Sahbhagi dhan: 58.57–71.48%; Naveen: 48.97–66.46%; FR13a: 62.96–65.87%) (Fig. 4 and Supplementary Fig. 3). Highest SVI was recorded in Naveen variety (93.83) followed by Sahbhagi dhan (90.47), FR13a (88.99), whereas highest RWC was observed in Sahbhagi dhan (71.48%) followed by FR13a (65.87%) and Naveen (66.46%) under treatment Avi2 + 0.6 mM of H₂O₂ + 1 ppm ascorbic acid (Fig. 4 and Supplementary Fig. 3).

3.8. Principal coordinates analysis (PCoA) of rice plant-growth promoting traits of *A. chroococcum* Avi2 under influence of H₂O₂ and ascorbic acid

PCoA biplot analysis of the parameters obtained from rice varieties

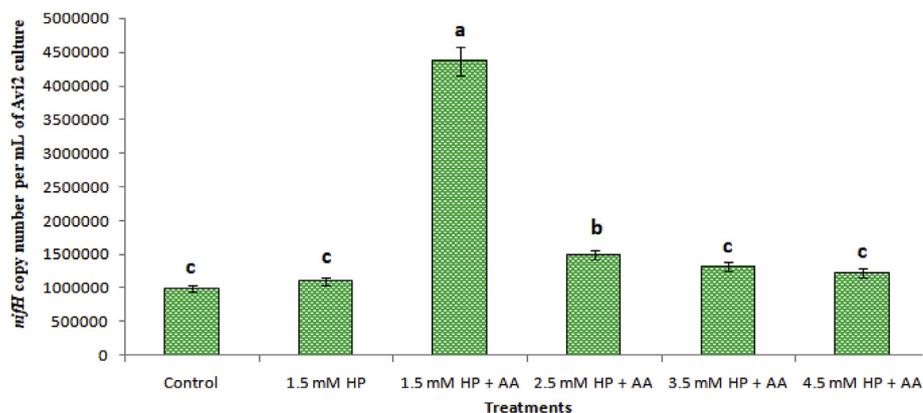


Table 2

Variation of plant-growth promoting and antioxidant enzyme activities of *Azotobacter chroococcum* (Avi2) under influence of H₂O₂ and ascorbic acid. HP: hydrogen peroxide (H₂O₂) doses as mM; AA: ascorbic acid (1 ppm); *nifH*: quantification by real time PCR (*nifH* gene X10⁶ copies mL⁻¹ culture); IAA: indole acetic acid (µg mL⁻¹); NH₃: ammonia production (mM mL⁻¹); SOD: superoxide dismutase (unit mg protein⁻¹ min⁻¹); CAT: catalase (µmol H₂O₂ reduced mg⁻¹ protein min⁻¹); APX: ascorbate peroxidase (µmol ascorbate oxidized mg⁻¹ protein min⁻¹). Values within a column followed by the same letters are not significantly different at Tukey HSD (p ≤ 0.05).

Treatment	<i>nifH</i>	IAA	NH ₃	SOD	CAT	APX
Control (Only Avi2)	0.98f	18.17d	46.71b	0.64c	0.13c	0.37c
1.5HP + Avi2	1.10e	10.75e	29.21e	0.92b	0.14c	0.36c
1.5HP + Avi2 + AA	4.36a	38.25a	50.83a	2.35a	0.05d	0.13e
2.5HP + Avi2 + AA	1.49b	25.42b	32.38c	0.51d	0.13c	0.25d
3.5HP + Avi2 + AA	1.32c	20.94c	30.67d	0.25e	0.21b	0.64a
4.5HP + Avi2 + AA	1.22d	18.58d	13.58f	0.04f	0.32a	0.49b

seedling (5 days after sowing) was shown in the ordination plane (Fig. 5), in which the PCoA could explain total variance of 75.9%, with principal component 1 (PC1) representing 44.9% and principal

Fig. 2. Quantification of *nifH* gene in Avi2 treated with H₂O₂ and ascorbic acid. Control: Avi2 in LB broth medium without H₂O₂; 1.5 mM HP: 1.5 mM H₂O₂ + Avi2 inoculant; 1.5 mM HP + AA: 1.5 mM H₂O₂ + Avi2 inoculant + ascorbic acid; 2.5 mM HP + AA: 2.5 mM H₂O₂ + Avi2 inoculant + ascorbic acid; 3.5 mM HP + AA: 3.5 mM H₂O₂ + Avi2 inoculant + ascorbic acid; 4.5 mM HP + AA: 4.5 mM H₂O₂ + Avi2 inoculant + ascorbic acid. Line above the bars represent standard errors (n = 3). Different letters above the bars indicate significant difference among the treatments at Tukey HSD (p < 0.05).

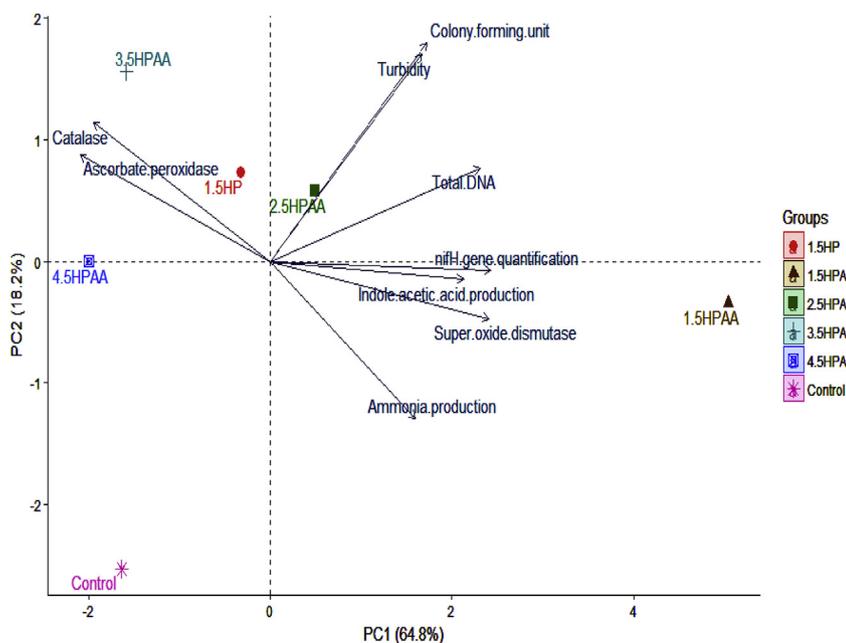


Fig. 3. PCoA biplot-based correlation between parameters (super oxide dismutase, ascorbate peroxidase, catalase, turbidity, colony forming unit, ammonia production, total DNA, indole acetic acid production, *nifH* gene quantification) and treatments (control; 1.5HP- 1.5 mM H₂O₂; 1.5 HPAA- 1.5 mM H₂O₂ + ascorbic acid; 2.5 HPAA- 2.5 mM H₂O₂ + ascorbic acid; 3.5 HPAA- 3.5 mM H₂O₂ + ascorbic acid; 4.5 HPAA- 4.5 mM H₂O₂ + ascorbic acid). HP: hydrogen peroxide (H₂O₂) doses as mM; AA: ascorbic acid (1 ppm); *nifH*: real time PCR (*nifH* gene X10⁶ copies mL⁻¹ culture); IAA: indole acetic acid (μg mL⁻¹); NH₃: ammonia production (mM mL⁻¹); SOD: superoxide dismutase (unit mg protein⁻¹ min⁻¹); CAT: catalase (μmol H₂O₂ reduced mg⁻¹ protein min⁻¹); APX: ascorbate peroxidase (μmol ascorbate oxidized mg⁻¹ protein min⁻¹).

component 2 (PC2) representing 31.0% variances, respectively. The PCoA was able to cluster the responses in three different rice varieties into three clusters, shown as elliptical circles. The parameters germination percentage, shoot length, root length, seedling vigor index, fresh weight, dry weight, and relative water content were represented as vector lines from which it was found that root length usually has a highly positive correlation with relative water content with high associations particularly associated with FR13a. Germination percentage and shoot length were founded to be higher in Naveen variety, whereas root length was higher in FR13a. Treatments were also presented (control or uninoculated, Avi2, Avi2 + ascorbic acid, Avi2 + 0.6 mM H₂O₂, Avi2 + 0.6 mM H₂O₂ + ascorbic acid) which were represented as points in the ordination plane. Treatment with Avi2 + 0.6 mM H₂O₂ + ascorbic acid showed the highest variance across all the three cultivars, whereas control or uninoculated showed least variances in all three cultivars (Fig. 5).

Overall, results conclude that higher concentration of H₂O₂ (2.5 mM–4.5 mM) showed the complete mortality of *Avi2*, whereas one ppm ascorbic acid neutralized the effect of H₂O₂. Ascorbic acid significantly ($p < 0.5$) increased the turbidity, CFU, DNA quantity, *nifH* gene abundance, IAA, NH₃, SOD in *Avi2*-treated with 1.5 mM H₂O₂ plus ascorbic acid compared to 1.5 mM H₂O₂ alone. Germination percentage of three rice cultivars (FR13a, Naveen and Sahbhagi dhan) were significantly ($p < 0.5$) increased by 20%, 13.33%, and 4%, respectively in *Avi2*-treated with 0.6 mM H₂O₂ plus ascorbic acid compared with uninoculated control.

4. Discussion

Our result indicated that the treatments containing more than or equal to 2.5 mM H₂O₂ showed the complete mortality of *A. chroococcum* *Avi2*. The most probable reason of bacterial mortality might be due to oxidation of their cell walls (Lee et al., 2004). Another reason might be the permeability of H₂O₂ through diffusion into cell membranes where it oxidizes the OxyR transcription factor which inhibits to encode the defensive enzyme (Imlay, 2015; Li and Imlay, 2018). Interestingly, *A. chroococcum* *Avi2* was found to survive at higher concentration of H₂O₂ after addition of 1 ppm of ascorbic acid. Here, ascorbic acid acts as a strong antioxidant (Arrigoni and De Tullio, 2002; Orsavová et al., 2019) which detoxifies the effect of H₂O₂ leading to bacterial survival. Previous reports also indicated that ascorbic acid acts as both the anti- and

pro-oxidant in the cell (Ozkan et al., 2004). Antioxidant is a useful biomarker of oxidative stress as it tends to react with the excess ROS during severe stress condition (Orhan et al., 2018). Several oxidative stress-responsive enzymes such as SOD, CAT, and APX have been reported as an innate defense under response of oxidative stress (Mishra et al., 2015). However, the innate native defense system is not enough to cope up with this issue; therefore, exogenous antioxidant (ascorbic acid, tocopherol, uric acid, and glutathione) might play a pivotal role in these aspects, which corroborates with our study.

The ROS mediated oxidative stress is normalized by two ways, one is by enzymatic antioxidants (SOD, CAT, and APX) and another one is by non-enzymatic antioxidants (ascorbic acid, glutathione, α -tocopherol, carotenoids etc.) (Das and Roychoudhury, 2014; Orsavová et al., 2019; Waśkiewicz et al., 2014). Under oxidative stress environment, SOD acts as a first line of defense. The present study showed that SOD was increased by 30.43% in *Avi2*-treated with 1.5 mM H₂O₂ compared with *Avi2* without treatment of H₂O₂ (control) and its activity was increased by 72.76% and 60.85% once *Avi2*-treated with ascorbic acid compared to control and 1.5 mM H₂O₂ alone, respectively, which indicated that SOD efficiency was increased almost double in presence of ascorbic acid. However, SOD activity decreased with increased concentration of H₂O₂ as ascorbic acid oxidized H₂O₂ and formed dehydroascorbate which effluxes outside the cell by glucose transporter channel (Guaiquil et al., 2001). Contrastingly, CAT and APX activities were decreased by 64.28% and 68.88%, respectively in *Avi2*-treated with 1.5 mM H₂O₂ and 1 ppm of ascorbic acid compared to 1.5 mM H₂O₂ alone. Reports also revealed that CAT and APX reduced H₂O₂ to H₂O and dehydroascorbate using ascorbic acid as a reducing agent (Das and Roychoudhury, 2014).

The present study also demonstrates a concurrent mechanism (increased of SOD concentration with decreased concentration of CAT and APX) of bacterial protection against H₂O₂ stress in presence of ascorbic acid which showed a good model of balanced antioxidant defense enzyme systems inside the cells. Reports also revealed that CAT never saturated by H₂O₂ at any concentration, might give the defense protection under the higher concentration of H₂O₂ (Lledies et al., 1998). PCoA analysis data revealed that CAT and APX activities were higher at increasing concentration of H₂O₂ as these were positively correlated (12% and 7% contribution, respectively) to each other, whereas their activities normalize when exogenous antioxidant (ascorbic acid) was added in the culture. Thus, bacterial growth occurred by balancing the

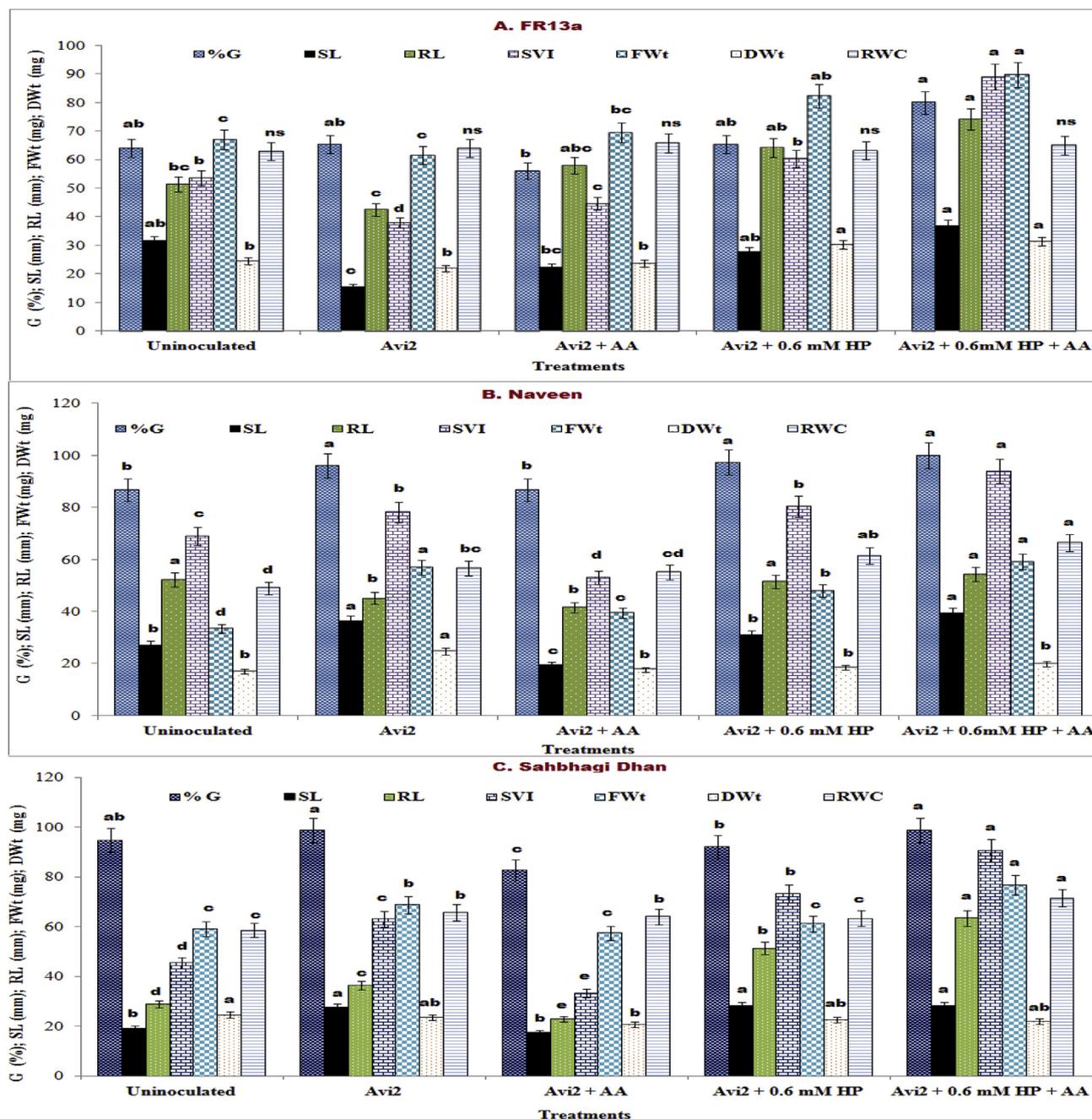


Fig. 4. Correlation of various treatments (uninoculated, Avi2, Avi2 + ascorbic acid, Avi2 + 0.6 mM H₂O₂, Avi2+0.6 mM H₂O₂ + ascorbic acid) and plant growth parameters. Germination percentage (%G), shoot length (SL in mm), root length (RL in mm), seed vigor index (SVI), fresh weight of seedlings (FWt in mg seedling⁻¹), dry weight of seedlings (DWt in mg seedling⁻¹) and relative water content (RWC) of three rice cultivars: A- FR13a, B- Naveen, C- Sahbhagi Dhan. Line above the bars represent standard errors (n = 25). Different letters above the bars indicate significant difference among the treatments at Tukey HSD (p ≤ 0.05).

defense system with exogenous supplemented ascorbic acid at a certain concentration of H₂O₂.

According to Ortiz et al. (2015), bacterial strains modulate the plant antioxidant responses by decreasing oxidative stress; which helps to improve plant growth promotion (PGP) under stress condition. The present study showed that IAA and NH₃ productions were increased by 71.89% and 42.53%, respectively in treatment 1.5 mM H₂O₂ with 1 ppm of ascorbic acid compared to the same treatment without ascorbic acid. This improvement helps plants for better growth under adverse condition. Researchers have already reported that ROS attack macromolecules like lipids, proteins, and DNA and converted them into oxidized form (Orhan et al., 2018). These radicals may cause oxidation of nucleotides and subsequent DNA breakage. Literature also suggested

that H₂O₂ oxidizes the ascorbic acid and forms dehydroascorbic acid (DHA). DHA, an active form of ascorbic acid, is imported into the bacterial cellular system via glucose transporters and trapped therein by reduction back to ascorbate by glutathione and other thiols (Welch et al., 1995). Thus, by means of this mechanism, in our study, ascorbic acid in one way detoxified the effect of H₂O₂ oxidative stress and another way enhanced the PGP efficacy of bacteria which ultimately showed the greater potential to retrieve bacteria under OS through enzymatic activity and DNA repairing system. Similarly, the present investigation proved that *nifH* gene efficiency was increased by 74.77% in Avi2-treated with 1.5 mM of H₂O₂ in combination with 1 ppm ascorbic acid compared to 1.5 mM of H₂O₂ alone and simultaneously decreased along with the increased concentrations of H₂O₂.

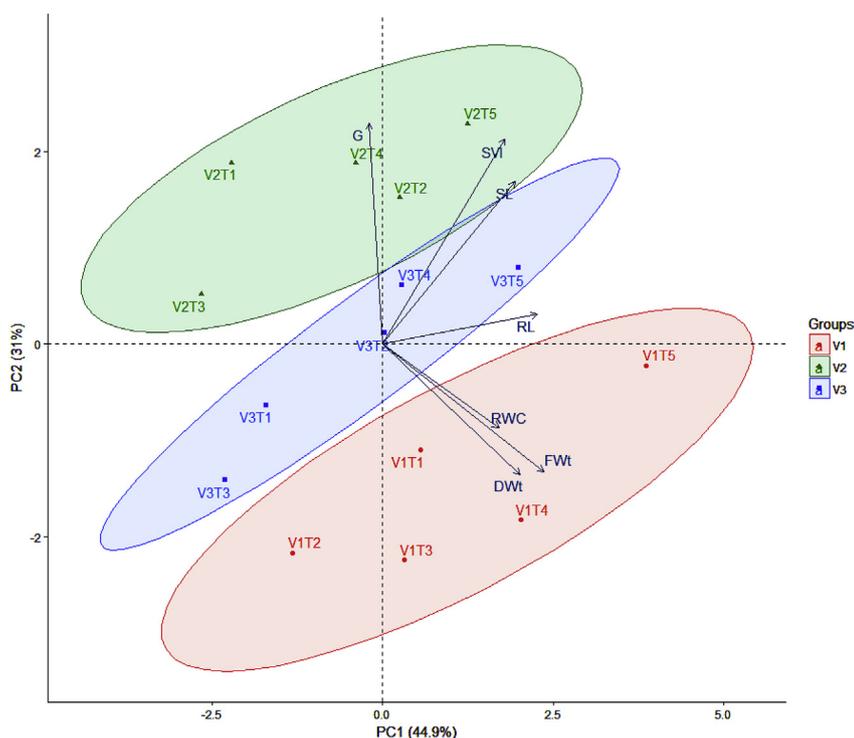


Fig. 5. PCoA biplot correlations between treatments (T1- uninoculated, T2- *Avi2*, T3- *Avi2* + ascorbic acid, T4- *Avi2* + 0.6 mM H_2O_2 , T5- *Avi2* + 0.6 mM H_2O_2 + ascorbic acid) and plant growth parameters. Germination percentage (%G), shoot length (SL in mm), root length (RL in mm), seed vigor index (SVI), fresh weight of seedlings (FWt in mg seedling⁻¹), dry weight of seedlings (DWT in mg seedling⁻¹) and relative water content (RWC) of three rice cultivars: FR13a (V1), Naveen (V2) and Sahbhagi Dhan (V3).

The result of seed germination of three rice cultivars (FR13a, Naveen, Sahbhagi dhan) showed the highest percentage (20%, 13.33%, and 4%, respectively) when treated with *Avi2* and ascorbic acid under 0.6 mM H_2O_2 compared to 0.6 mM H_2O_2 alone. A similar trend of results had been shown in seedling vigor index (SVI) and relative water content (RWC). Rice is one of the essential foods for growing Asian population and its production mostly depends on usage of chemical fertilizers and pesticides which deteriorates the sustainability of soil health (Chatterjee et al., 2018; Kumar et al., 2017c, 2019b; Munda et al., 2016; Sharma et al., 2019). Biofertilizers are one of the best alternative solutions to supplement the reduced doses of chemical fertilizers without compromising the crop yield (Abhilash et al., 2016; Kaviya and Anbarasi, 2018). Even though the uses of biofertilizers are well-known practice but the reliable and consistent effect under field condition is still a bottleneck for a wider use (Malusa et al., 2012) due to the incomplete understanding of the relationships between the plants and microorganisms and the soil environmental conditions (Artursson et al., 2006). For the sustainability of crop production, diazotrophs provide growth promotion, crop protection, heavy metal stress tolerance and even mitigate abiotic stresses (Singh, 2016; Vejan et al., 2016). Researchers also used the potential microbes for seed priming which improved plant growth and health beyond the germination and seedling emergence stage (Singh, 2016). But there are numerous biotic and abiotic factors that severely damaged such microbes; only a few had the tolerance ability against abiotic stresses such as drought, salinity, and metal toxicity (Dimpka et al., 2009). That is why, we used ascorbic acid formulations as antioxidant in the present study which may serve as one of the vital constituents in biofertilizers formulations to enhance the sustainable plant-growth promotion of rice under oxidative stress.

5. Conclusion

The present study shows the role of ascorbic acid as an antioxidant in *A. chroococcum Avi2*, a diazotrophic bacterium under H_2O_2 oxidative stress and gave direct evidence of H_2O_2 mediated oxidative stress as an ageing agent for *A. chroococcum Avi2*, because it causes complete mortality of bacterium at higher concentration. Interestingly, our study

also reported that antioxidant acts as an anti-aging agent, because the growth of *A. chroococcum Avi2* was regained after addition of 1 ppm ascorbic acid. Besides, it enhanced the seed germination and promotes better growth of rice seedling. Overall, the findings suggest that by using antioxidant as one of the ingredients in biofertilizers formulations, may overcome the drawback of microbial inoculation in agricultural crops and also improve the performance of beneficial microorganisms under oxidative stress.

Authors contribution

UK, SG, and MK conceived of and designed the study. UK and MK analyzed and interpreted the data. UK drafted the paper and PP, HP, KC, PS, SNC, PKN, and AKN critically revised it for important intellectual content. All authors gave final approval of the version to be published.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.04.003>.

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