Saudi Journal of Biological Sciences 30 (2023) 103664

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Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Synergistic effects of biofilm-producing PGPR strains on wheat plant colonization, growth and soil resilience under drought stress

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ARTICLE INFO

Article history:
Received 20 February 2023
Revised 3 April 2023
Accepted 21 April 2023
Available online 28 April 2023

Keywords:
Drought stress
Mixed biofilms
PGPR
Plant growth
Rhizoplane colonization
Synergistic interaction
Soil enzymes

ABSTRACT

Drought stress substantially impedes crop productivity throughout the world. Microbial based approaches have been considered a potential possibility and are under study. Based on our prior screening examination, two distinct and novel biofilm-forming PGPR strains namely *Bacillus subtilis*-FAB1 and *Pseudomonas azotoformans*-FAP3 are encompassed in this research. Bacterial biofilm development on glass surface, microtiter plate and seedling roots were assessed and characterized quantitatively and qualitatively by light and scanning electron microscopy. Above two isolates were further evaluated for their consistent performance by inoculating on wheat plants in a pot-soil system under water stresses. Bacterial moderate tolerance to ten-day drought was recorded on the application of individual strains with wheat plants; however, the FAB1 + FAP3 consortium expressively improved wheat survival during drought. The strains FAB1 and FAP3 displayed distinct and multifunctional plant growth stimulating attributes as well as effective roots and rhizosphere colonization in combination which could provide sustained wheat growth during drought. FAB1 and FAP3-induced alterations cooperatively conferred improved plant drought tolerance by controlling physiological traits (gs, Ci, E, iWUE and P_N), stress indicators (SOD, CAT, GR, proline and MDA content) and also maintained physico-chemical attributes and hydrolytic enzymes including DHA, urease, ALP, protease, ACP and β glucosidase in the soil. Our findings could support future efforts to enhance plant drought tolerance by engineering the rhizobacterial biofilms and associated attributes which requires in-depth exploration and exploiting potential native strains for local agricultural application.

1. Introduction

Agriculture is currently facing many challenges, including global population growth that is expected to exceed 8 billion by 2030. At the same time, climate change, primarily in terms of global warming and fluctuations in rainfall frequency, may adversely affect crop production in many parts of the globe (Smol, 2012; Joshi et al., 2020; Banerjee et al., 2021). In the last five years, India has witnessed an increase in annual temperature by 0.7 °C, which has affected about 36 percent of rain-fed agriculture production via drought (Joshi et al., 2020). Drought and irregular rainfall events are compelling farming communities to adopt stress-tolerant and short-duration crop varieties. Although short-duration crop varieties are suitable in drought-proofing contingency plans, productivity is low in comparison to long-duration varieties (Joshi et al., 2020) which results in lower yield production and affects subsequent crop planting times.

Drought stress threatens wheat growth and productivity, and risks possible crop failures (Joshi et al., 2020; Ansari et al., 2021). As a result, there is increased interest in identifying methods to improve crop drought tolerance and enhance growth in water-stressed environments. Current approaches involve promoting either drought-tolerant cultivars or making crops more resilient to environmental stresses.

Development of drought-tolerant crops requires complicated genetic engineering techniques due to the enormous number of genes involved and the fact that the plant may be subjected to several stresses, further complicating the process of identifying

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2023.103664
1319-562X/

acceptable phenotypes (Price et al., 2002; Wang et al., 2003; Khan et al., 2019). Genetic modifications may not only result in the loss of important and useful attributes, but also has the potential to asset a single crop species (Eckerstorfer et al., 2019; Esse et al., 2020). Furthermore, in addition to being a time-consuming and labor-intensive operation, transgenic crops may pose environmental hazards and not be accepted by farmers or the public. An alternative to traditional crop breeding and genetic engineering, i.e., microbially-based support, could be a more economically applicable strategy for alleviating drought stress in crops in an eco-friendly manner. Plant growth promoting rhizobacteria (PGPR) have attracted substantial attention for imparting abiotic stress tolerance and mitigation of challenging environmental conditions (Chauhan et al., 2015; Gontia-Mishra et al., 2016; Joshi et al., 2020; Ansari et al., 2021). The use of native plant-beneficial bacteria has recently came under research scrutiny as a promising approach to combat drought-inflicted damage. However, performance of PGPR is not always consistent due to various abiotic and biotic constraints occurring in the soil–plant system. The biofilm state of growth offers bacteria with the capability to survive, compete, colonize and perform with consistency under stressful conditions in the soil system which, in turn, supports mitigation of crop stress in a sustainable manner. Few researches have demonstrated that biofilm-producing PGPR help plants in ameliorating water deficiency stress by changing the root framework, consequently leading to improved nutrient consumption and water removal capability (Joshi et al., 2020; Ansari et al., 2021).

Wheat (*Triticum aestivum*) is susceptible to accumulation of ROS (reactive oxygen species) that are synthesized during drought stress (Abid et al., 2018; Hasanuzzaman et al., 2020). Excessive levels of ROS cause oxidative stress which triggers synthesis of antioxidants including superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and glutathione reductase (GR) and proline to alleviate water stress. Water limiting conditions are additionally associated with adverse impacts on photosynthetic attributes of crops (Ansari et al., 2019; Ansari and Ahmad, 2019a; Hasanuzzaman et al., 2020). Water stress also causes deleterious impact on soil nutrient cycling and accessibility to crops (Homyak et al., 2017; Siebert et al., 2019).

PGPRs have been extensively explored over the past decade (Glick, 2014; Vurukonda et al., 2016; Vasseur-Coronado et al., 2021). However little attention have been made on new emergent traits such as biofilms, root/rhizosphere colonization, stress mitigation of plant growth promoting rhizobacteria. Such emergent features may result from synergistic interactions among PGPR species. As a result of these beneficial interaction, the functional capabilities of the bacterial consortium may be substantially greater than the combined totals of individual isolates (Seneviratne et al., 2011; Ren et al., 2015; Yang et al., 2021). Bacteria frequently form multispecies biofilms and work as a community (Stoodley et al., 2004; Hansen et al., 2007; Yang et al., 2021). In our previous study, we determined compatible and synergistic interaction among rhizospheric bacterial isolates on biofilm production, i.e., the two isolates formed more biofilm biomass when cultured together in comparison to the sum of single species biofilms (Ansari and Ahmad, 2019a). A study conducted by Yang et al. (2021) showed that a combination of four bacterial species (*Paenibacillus amylolyticus*, *Microbacterium oxydans*, *Stenotrophomonas rhizophila* and *Xanthomonas retroflexus*) displayed enhanced biofilm formation and supported greater drought tolerance in crops in comparison to the effects of individual strains. Whether such compatible and synergistic impacts of multispecies biofilms results in emergent traits on drought tolerance of plants is generally unexplored. It is established that biofilms contain approximately 97% water; therefore, they have the potential to maintain water requirement for plants during drought conditions (Flemming et al., 2016; Yang et al., 2021). We hypothesized that PGPR strains with multiple PGP properties and capable of developing mixed strong biofilms may synergistically more stable and perform with consistency under drought stress condition. In this study, wheat plants were grown in a soil-pot system and inoculated with a dual species consortium from *Bacillus subtilis*-FAB1 and *Pseudomonas azotoformans*-FAP3 to assess their effects on drought tolerance of wheat. In addition to these, consortium-induced differences in wheat physiology, antioxidative systems, yield production, and root and rhizosphere colonization, as well as soil physico-chemical assets which may mitigate the deleterious consequences of drought on crop performance were investigated.

2. Materials and methods

2.1. Bacterial selection and screening based on multifunctional PGP traits

Two drought tolerant bacterial isolates namely *Bacillus subtilis* strain FAB1 and *Pseudomonas azotoformans* strain FAP3 were selected from our previous study (Ansari and Ahmad, 2019). These bacterial strains were further screened for multifarious plant growth promoting (PGP) attributes including synthesis of IAA (indole acetic acid), siderophore synthesis, phosphate solubilization, and HCN (hydrogen cyanide) and NH_3 (ammonia) generation via standard methods as stated elsewhere (Ahmad et al., 2008; Ansari et al., 2019, Ansari and Ahmad, 2019).

Synthesis of ACC deaminase was estimated using standard method (Penrose and Glick, 2003). Briefly, test plates were prepared by adding 3 mM of ACC in DF culture medium as a nitrogen source instead of $(NH_4)_2SO_4$. Freshly-grown bacterial cultures were spot inoculated and incubated to determine ACC deaminase production qualitatively in the test isolates (Dworkin and Foster, 1958). A quantitative evaluation of 1-aminocyclopropane-1-carbox ylate deaminase (ACC deaminase) synthesis was conducted on the basis of microbial ability to employ ACC as the key nitrogen element. The α-KB (α-ketobutyrate) synthesized due to breakdown of ACC by the action of ACC-deaminase and the enzymatic activity was determined quantitatively (Penrose and Glick, 2003). The unit of ACC-deaminase activity was measured in μmol α-KB/mg protein/hour.

Bacterial tolerance to water stress was tested by including polyethylene glycol (PEG-6000) in the growth medium. Briefly, aliquot of 100 μl overnight-grown test isolates was spot inoculated on their respective growth media containing 40% PEG and assay plates were incubated at 28 ± 2 °C for 24 to 48 h. Bacterial growth on plates was examined in terms of minimum inhibitory concentration (MIC). Successful growth of bacterial isolates was designated as drought stress-tolerant (Ansari et al., 2021).

2.2. Interaction studies between FAB1 and FAP3 under planktonic growth

Both the isolates were tested for interactions using co-culture plate, overlay and cross-streak methods to observe positive and negative relations. The bacterial combination was prepared for both the isolates and evaluated for interaction in the planktonic mode. For co-culture plate and cross streak plate methods, NA plates were prepared and freshly grown bacterial cultures were spot inoculated adjacent to each other in combination. Cultures were cross-streaked on plates using a nichrome loop in combination and assay plates were kept under incubation at 28 ± 2 °C for 48 h following which the plates were examined and observations designated as positive (compatible) or negative interaction (Ansari and Ahmad, 2019).

Bacterial interaction was also assessed by the over-lay technique using soft agar plates. In this assay, an equal amount of test bacterium was mixed in soft agar and moderately poured on the surface of the primary layer previously inoculated with the other test bacterium. Test plates were kept for incubation at 28 ± 2 °C for 48 h; after the incubation time, estimation in the terms of growth inhibition between isolates was conducted to determine compatibility (Ren et al., 2015, Ansari and Ahmad, 2019).

2.3. Interaction of FAB1 and FAP3 in biofilm mode on glass coverslips

Interaction in biofilm mode by selected isolates (FAB1 and FAP3) was conducted on glass surfaces using crystal violet assay with slight modification as elaborated by O'Toole and Kolter (1998). One ml of freshly-grown FAB1 and one ml of FAP3 isolate (1:1) were infused into each well of a 12-well plate (flat bottom) containing fresh medium and cover slips (20 mm). Afterwards, experimental 12-well plate were kept under incubation for 24–48 h at 28 °C. At the completion of incubation, coverslips were removed from each well using sterile forceps, cleaned with PBS (phosphate buffer saline) and stained with 0.1% crystal violet solution for 20 min. Following staining process, coverslips were gently cleaned with PBS and observed under a light microscope (Olympus BX60, Japan) at 100x. For biofilm development of a single isolate, two ml was placed into the 12-well tissue culture plate. The remainder of the procedure is identical to that provided above.

2.4. Interaction of FAB1 and FAP3 in biofilm mode under SEM

Interaction of the selected isolates in biofilm development was assessed on glass surfaces. Freshly-grown bacterial cultures were co-inoculated in 1:1 ratio (FAB1 and FAP3) in 12-well plates containing fresh medium and a glass cover slip in trplicate. Plate was incubated for 24 to 48 h at 28 ± 2 °C. Following incubation, cover slips were gently removed and washed thrice with 1x PBS (pH 7.2) to eliminate softly adhered bacteria. The formed biofilm on the surface of cover slip was then fixed at room temperature for 4 h using 2.5 % glutaraldehyde (v/v). The fixed biofilms were processed for dehydration using a gradient of absolute ethanol from 30% – 100% (v/v) and the test samples were dehydrated with critical point dryer (CPD; Quorum Technologie-K850WM) in CO_2. Samples were coated with gold for observation under SEM (scanning electron microscope) (JEOL, 76510LV, Japan). For single-species biofilm development, two ml of freshly-grown bacterial culture was collected and prepared for SEM as elaborated above (Ansari and Ahmad, 2019).

2.5. Interaction of FAB1 and FAP3 in biofilm state on root surfaces

Ten day-old seedlings of wheat plants were uprooted aseptically and cleaned using sterile DDW to eliminate all adhering soil particles and surface sterilized. The seedlings were soaked in over-night grown mixed culture (1:1 ratio) of FAB1 and FAP3 for 6 h at 28 ± 2 °C. Seedlings were removed after dipping and cleaned thrice with PBS to eliminate loosely adhered bacteria. The seedlings were processed for fixation in 2.5% glutaraldehyde (v/v) in freshly prepared 0.1 M PBS (pH 7.2) for 4 h. Fixed root samples were desiccated using a gradient of ethanol (20–100 %) and desiccated via critical point dryer in CO_2 (Ansari et al., 2019). To avoid tissue destruction, pressure was gradually released. The samples were coated with gold particle (22 nm) before observing under SEM. For single-species biofilm development on root surfaces, cultures were grown separately and root samples were immersed. Root samples were processed for SEM examination as elaborated above.

2.6. Microtiter plate assay for biofilm development under drought stress

Quantification of biofilm development by the FAB1 and FAP3 isolates, both single and mixed-species was executed using 96-well ELISA microtiter plates by adopting the method of O'Toole (2011). For single-species biofilm formation, test isolates were grown at 28 °C for 24–48 h in their appropriate growth media. Each well of the microtiter plate received 160 µl of culture ($\sim 10^7$ CFU/ml) and the plate was incubated for 24–48 h at 28 °C. Following incubation, each well was washed thrice with PBS and subsequently stained using 0.1% crystal violet for 20 min. All the experimental wells were then cleaned thrice with PBS to eliminate unbound dye. The bound crystal violet with biofilm ring was extracted by adding 95% ethanol (Sigma, India) and absorbance of the extracted CV was recorded at 590 nm under an ELISA plate reader (Thermo Scientific Multiskan EX, UK). The range of biofilm development (weak, moderate, strong) was calculated using Christensen's formula as stated by Stepanović et al. (2000). For dual species biofilm production, 80 µl of each isolate was inoculated in each well of 96 well plates in triplicate. All subsequent procedures were same as described above. In the drought stress experiment, the growth medium containing 40% of PEG-6000 was included to assess its impact on biofilm formation (Ansari et al., 2021).

2.7. Quantitative estimation of exopolysachharides (EPS) under drought stress

Quantitative estimation of EPS production by the isolates in both single-species and mixed form was performed according to Mody et al. (1989). Freshly grown pre-inoculum of test isolates, both single and in mixed form, were grown overnight in their appropriate liquid media (Hi-media) for 24 h at 28 ± 2 °C. Pre-inocula (500 µl) was supplemented to freshly prepared culture medium (50 ml volume) and permitted to grow for five days at 120 rpm in a rotatory shaking incubator (Remi, Pvt. Ltd. India) at 28 ± 2 °C. Following incubation, 200 ml of culture was centrifuged for 20 min at 11500 rpm at 4 °C. The culture supernatant was collected and filtered using a nitrocellulose filter having 0.45 µm pore size. EPS was precipitated from the final filtrate following addition of chilled concentrated ethanol (3 volumes) and solution was stored overnight at 4 °C for EPS precipitation. After EPS precipitation, EPSs were dehydrated for 48 h at 80 °C and weight was calculated for single-species and mixed bacterial cultures. For the drought stress experiment, the growth medium contained 40% of PEG-6000 ((Ansari et al., 2021).

2.8. Alginate quantification assay under drought stress

Quantitative extraction of alginate from overnight-grown cultures of FAB1 and FAP3 in single and dual culture was carried out under normal and drought-stressed environments. Bacterial cultures were centrifuged at full speed (10,000 rpm) for 10 min and the cell-free supernatant was removed. An equal volume of absolute isopropanol was added to culture supernatant to isolate deacetylated alginate and extracted alginate was stored for 1 day at 4 °C. Followed this, precipitate was collected using centrifuge for 10 min at 10,000 rpm; afterwards, the extracted pellet was then gradually washed with 1 ml of 70 and 96% ethanol, dehydrated at 37 °C for 15 min and blended in 1 ml of sterile DDW. To measure the amount of alginate, 100 µl of the suspension was removed and prepared with Milli-Q water up to 1 ml volume. Subsequently, an aliquot 1 ml borate sulfuric acid (BH_5O_7S) solution was applied, followed by addition of 30 µl of freshly made carbazole reagent and solution was thoroughly mixed. After 15 min at room temperature, the mixture was tested against a reagent blank for absorbance at

500 nm. Production of alginate was expressed in μg/mg wet biomass (Wozniak et al., 2003). In the case of alginate extraction under drought stress, the growth medium contained 40% of PEG-6000 and the above-described procedures were carried out.

2.9. Cell surface hydrophobicity assay under drought stress

Bacterial attachment to hydrocarbons was calculated using cell surface hydrophobicity (CSH) in isolates FAB1 and FAP3 in single-species and mixed form using standard methods (Rosenberg et al., 1980). Test isolates were incubated in their respective liquid culture media and hydrophobicity determined after 4 d incubation. To assess percentage hydrophobicity, freshly grown culture (5 ml) was centrifuged for 10 min at 8,000 rpm and cell pellets were re-dissolved at pH 7.4 in phosphate-magnesium buffer. The absorbance was recorded with a UV–visible spectrophotometer (Shimadzu-8500 II) at 400 nm and denoted as the initial bacterial cell suspension. Aliquot of 5 ml culture was applied to 0.2 ml $C_{16}H_{34}$ and then mixed robustly with a vortex mixer (Borosil, bhrmi 1.0). Following separation of the aqueous phase, absorbance was measured at 400 nm and the observed value was designated as the final concentration in the aqueous phase. For the drought stress experiment, 40% of PEG-6000 was present in the media and the above-stated procedures were applied. Calculation of percent hydrophobicity was as follows:

$$\textit{Percent hydrophobicity}\ (\%) = 1\ --\ (A_1/A_0) \times 100$$

Where A_1 denotes initial bacterial cell suspension absorbance and A_0 denote aqueous phase absorbance.

2.10. Bacterial motility assays under drought stress

Bacterial motility (swimming and swarming) in the isolates FAB1 and FAP3, both individually and in mixed culture, was assessed according to Adler (1966). Briefly, soft agar plates were prepared by adding 0.3 and 0.5% agar (w/v) to assess swimming and swarming motility, respectively. A total of 3 μl of bacterial cultures (10^{-7} cells/ml) were spot inoculated on plates. To prevent dehydration, all plates were tightly wrapped with parafilm tapes and kept for incubation at 28 ± 2 °C for 48 h. Bacterial motilities were consequently carried out by recording swarm and swim diameter following the incubation for 24 h and results were expressed in millimeter (mm). In the water stress experiment, bacterial cultures were grown under 40% PEG in the media and procedures were followed as elaborated above.

2.11. Measurement of plant physiological parameters

Assessment of physiological attributes such as photosynthetic rate (P_N), stomatal conductance (gs), transpiration rate (E), vapor pressure deficit (VpDL), intercellular CO_2 concentration (Ci), respiration ($\mu molm^{-2}\ s^{-1}$) and intrinsic water use efficiency (iWUE) were assessed on 3 replicates of each treatment using portable photosynthesis system (LI-6400; USA) furnished with blue and red LED light source on four mature leaves at similar physiological age per treatment. At the time of measurement, temperature, average light intensity, relative humidity and CO_2 concentration were set at 500–502 μmol $m^{-2}s^{-1}$, 29.83–32.69 °C, 446–669 μmol^{-1}, 36.02–48.95%, respectively. All measurements were conducted between 09:30–12:00 pm (0930–1200).The ratio of (A) to (E) was considered as the intrinsic water use efficiency (iWUE) in the leaf (Ansari et al., 2021).

2.12. Estimation of stress markers (CAT, SOD, GR, MD Aand proline content)

Thirty days old leaves (0.5 g) were amalgamated in 1 ml of extraction buffer containing 100 mM potassium phosphate at pH 7.0 with 1% polyvinylpyrrolidone [w/v] and 0.1 mM EDTA at 4.0 °C). The prepared homogenate was subjected for centrifugation for 15 min at 4.0 °C at 15,000 × *g*. Aliquots were stored at −80 °C until analysis. In the collected supernatant, the amount of protein was estimated according to Bradford (1976). The supernatant was also utilized for CAT, SOD and GR assays.

Estimation of CAT (catalase) enzyme was conducted in 3 ml of reaction mixture by measuring reduction of absorbance by induced breakdown of H_2O_2 in the presence of enzyme (Duerig et al., 2009). In a reaction mixture carrying 100 μl enzyme extract, 20 mM H_2O_2 and 50 mM sodium phosphate buffer (pH 7.0), the rate of reduction in absorbance was monitored over 3 min at 240 nm. For the control, 100 μl of protein mixture and 50 mM sodium phosphate buffer (pH 7.0) was used, whereas H_2O_2 (20 mM) and sodium phosphate buffer (50 mM) used for blank. The catalase activity was expressed in H_2O_2 (μmoles) separated in per min of mg^{-1} of protein.

Measurement of SOD (superoxide dismutase) in leaves was performed according to Beauchamp and Fridovich (1971). An order of 100 μl enzyme extract, 13 mM methionine, 75 mM nitrobluetetrazolium (NBT), 40 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, and 2 mM riboflavin were added to a 3 ml mixture. For the blank, reaction mixture with enzyme extract was held in the dark, while the mixtures containing enzyme (treatments) and without protein (control) were kept in the light. To initiate the reaction, light was turned on which remained on for 30 min and absorbance was recorded at 560 nm. SOD activity is calculated as the difference between NBT reduction by a protein-free mixture in the light and NBT reduction by a protein-containing mixture. The expression of the enzyme quantity required to prevent 50% of the initial NBT decrease in the light is considered as one unit of activity.

Glutathione reductase (GR) enzyme was measured in accordance with Smith et al. (1989). When DTNB (5,5′-dithiobis-2-nitro benzoic acid) was converted to TNB by GSH during the reaction process, an increase in absorbance was recorded at 412 nm under spectrophotometer. A total 1 ml of assay volume was made up of ten μl of leaf extract was included with DTNB (0.75 mM), GSSG (1 mM) and NADPH (0.1 mM). The reaction was sparked by the addition of GSSG (oxidized glutathione) in the final step, and the rise in absorbance was recorded for 3 min. Using the extinction coefficient of TNB (14.15 $M^{-1}\ cm^{-1}$), which was reported as mmole TNB per minute per gram of fresh weight, the enzyme activity of GR was estimated.

The quantity of lipid peroxidation was performed by estimating MDA content and the result was expressed as μmol MDA per gram of fresh weight in reaction mixture (Heath and Packer, 1968),

To quantify proline content, 0.5 g of leaves were collected from 3 plants from each treatment and the method of Bates et al. (1973) was used. Concisely, leaf samples were amalgamated in 5 ml of sulfosalycylic acid, 2 ml glacial acetic acid and 2 ml ninhydrin reagent in a mixture. After 30 min of heating and cooling the reaction mixture, toluene (6 ml) was applied to the reaction mixture. The chromophore containing toluene was considered for proline estimation against the standard curve of proline using UV–visible spectrophotometer at 520 nm. Proline content was expressed as μmoles/g of fresh weight.

2.13. Measurement of plant growth parameters under drought stress

Growth attributes of wheat plants, inoculated with isolates FAB1 and FAP3 both individually and in mixed formulation were

measured under both normal as well as ten-day drought stress in soil microcosms. Drought stress was created by modifying the ten-day irrigation gap to the plants. Bacterial inoculation was performed by the seed soaking method. Healthy wheat (*Triticum aestivum* L.) var. PBW 343 seeds were processed for surface-sterilization using 1% NaClO for 1 min and washed thrice with sterile DDW. Surface sterilized wheat seeds were then soaked in freshly-grown cultures of test isolates for 6 h. Following soaking, seeds were sown in earthen pots containing 5 kg soil. All experiments pertaining to plant growth under water stress were conducted in a climate-controlled greenhouse.

Three replicate wheat plants from each treatment were harvested and data documented for length (cm) of shoot and root, root number, fresh and dry weight (mg) of shoot and root, RWC (seedling) % and RAS/RT (mg/mg). Samples of each treatment were then air-dried for dry weight calculation. Measurement of root and shoot length was measured as described by Ansari et al. (2021).

2.14. Quantification of seed attributes and straw yield

A total eight treatments were designed (T1- no drought; T2- drought; T3- FAB1 inoculated; T4- FAP3 inoculated; T5- FAB1 + FAP3 inoculated; T6- FAB1 + drought; T7- FAP3 + drought and T8- FAB1 + FAP3 + drought). Earthen pots of each treatments were maintained up to harvesting time in green house condition. Wheat plants from 3 pots of each treatments were harvested at 120 days after sowing (DAS). Estimation of seed attributes and straw yields such as grain yield (g/1000 seeds), grains per spike, straw yield (g/plant) and grain protein (mg/g FW) were performed in plants inoculated with FAB1 and FAP3 individually and in dual culture under ten-day drought stress. All the above parameters were measured as described previously ((Ansari et al., 2021).

2.15. Root and rhizosphere colonization under drought stress

Bacterial root and rhizosphere colonization by the isolates FAB1 and FAP3 individually and mixed was executed under ten day water stress. The standard method was adopted for the colonization study as elaborated (Simons et al., 1996) with slight modification. Uniform-size healthy wheat seeds (cv. 343) were processed for surface-sterilization as explained in the earlier section. Surface sterilized wheat seeds were dipped with overnight freshly grown FAB1 and FAP3 both individually and mixed liquid culture for at least 6 h using gum guar powder (1%) for appropriate adhesion. For the control, seeds were soaked in sterile water. Both culture-soaked and non-soaked seeds were sown in earthen pots as control (i.e., without water stress) and maintained in a climate-controlled greenhouse. To moisten the soil, 10% plant nutrient solution (PNS) (v/w) was applied regularly. For bacterial root colonization assay, 1 g of root was taken from uprooted seedling and cleaned gradually in DDW (sterile) to eliminate softly attached bacteria from the surface of root. One gram of root was placed aseptically in an eppendrof tube, diluted in sterile NSS, and cyclomixed at full speed. Colony forming units (CFU) were estimated by plating root sample (100 µl) on their respective growth plates contained 30 µg/ml rifampicin for selective isolation. Plates were left for appropriate incubation at 28 ± 2 °C for 48 h. The isolates were resistant to 30 µg/ml rifampicin (Zhang et al., 2014; Ansari and Ahmad, 2018) and results were expressed as CFU/g root. The CFU of root colonization assay were recorded at 10, 20, 30, 60 and 90 days after seeding. At the same time intervals, rhizospheric soil (i.e., that which was strongly attached to the surface of root) was collected and prepared serial dilution into the NSS. A total of 100 µl of each dilution was spread on KB and Nutrient agar media supplemented with rifampicin at 30 µg/ml for discriminatory selection. The assay plates were kept for incubation at 28 ± 2 °C for 48 h and observations were calculated in CFU/gm of soil.

2.16. Assessment of soil physico-chemical attributes

The physico-chemical attributes of rhizospheric soil were determined under both normal as well as ten-day water-stressed conditions from each treatment. Soil pH and EC (electrical conductivity) (1:5 soil:water ratio) were calculated using digital pH meter (digital-7310P, WTW, Germany) and conductivity meter (EdgeEC; Romania), respectively. Determination of soil organic carbon (OC) was carried out using potassium dichromate (1 N) and back-titrated using 0.5 N $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ (Spertus, 2021). Soil nitrogen (total and available; NH_4-N) were estimated by Kjeldahl method using Kelplus KES12L; Pelican Equipments, Chennai, India (Moore and Diehl, 1962), with the help of an automatic nitrogen estimation equipment. A total P was determined by the colorimetric method using vanadomolybdophosphoric acid (Muir, 1952) and extractable P by 1 M acidic NH_4F (Recena et al., 2017) using a spectrophotometer (EVOLUTION201, Thermo Scientific, USA). Total plus water-soluble exchangeable K was estimated by the NH_4CH_3-CO_2 method (Wang and Scott, 2001) using a flame photometer (Model No. 128, Systronics, India).

2.17. Determination of soil hydrolytic enzyme activities

Activities of hydrolytic enzymes were assayed in both normal and ten-day water-stressed rhizospheric soil from each treatment. The activity of dehydrogenase was estimated spectrophotometrically using TTC reduction (2,3,5-triphenyltetrazolium chloride) to TPF (triphenylformazan) as stated elsewhere (Benefield et al., 1977). Concisely, 5 g of soil from each treatment was blended with 5 ml prepared solution of TTC in 100 mM of tris- HCl (pH 7.7) and kept for incubation in the dark for 24 h at 28 °C at 100 rpm on a shaker. For the control, five ml of tris HCl without TTC was used. Following incubation, each tube was filled with 40 ml of acetone and incubated again for 2 h with shaking every 10 min in the dark. Absorbance was recorded at 540 nm after three ml of supernatant was collected and centrifuged. To prepare a blank, added 5 ml tris buffer to the soil sample without addition of TTC. Measurement of dehydrogenase enzyme activity was calculated in μg of formazan/g sample (mg TPF/g dry wt/h).

Acid and alkaline phosphatase activity were estimated by spectrophotometric method based on the quantification of *p*-nitrophenol ($C_6H_5NO_3$) released following incubation of soil with $C_6H_6NO_6P$ (*p*-nitrophenyl phosphate) at 37 °C for 1 h. Concisely, 1 ml of *p*-nitrophenyl phosphate solution (15 mM), 0.25 ml of toluene, and 4 ml of MUB (pH 6.5 for acid phosphatase and pH 11.0 for alkaline phosphatase assay) were added to the 1 g of soil in the same buffer. 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added to the mixture after the mixture was incubated at 37 °C for 1 h. Before filtering the blank preparation, immediately added 1 ml of $C_6H_6NO_6P$, 4 ml of 0.5 M NaOH and 1 ml of 0.5 M $CaCl_2$. Afterwards, the soil suspension was carefully mixed and sieved with filter paper (Whatman no. 2). At 400 nm, the absorbance was measured in triplicates, and the enzymatic activity was estimated in g of PNP/g dry wt/h.

Activity of β glucosidase was evaluated spectrophotometrically using the substrate PNG (*p*-nitrophenyl-β-D-glucoside) following the standard method (Tabatabai, 2018). 1 g of soil, 0.25 ml of toluene, 4 ml of MUB (pH 6.0) and 1 ml of PNG (25 mM) were applied to the same buffer and mixed with solution. In the reaction mixture, 14 ml of tris buffer (pH 12) and ml of 0.5 M $CaCl_2$ were applied after appropriate incubation at 37 °C for 1 h. Subsequently, Whatmann filter paper was used to sieve the suspension of the soil.

At a wavelength of 400 nm, the absorbance was measured, and enzymatic activity was estimated as g of PNP/g dry weight/h.

Quantification of urease was determined using the standard protocol of Kandeler and Gerber (1988) which is relies on spectrophotometric estimation of ammonium following incubation of the sample with urea. In Erlenmeyer flask, five g of wet soil and 2.5 M urea solution was placed and left for appropriate incubation at 37 °C for 2 h. Following the appropriate incubation, KCl solution (50 ml) was added and the suspension placed for 30 min on a shaker. For the blank preparation, urea was replaced with equal volume of MQ water. The suspension was sieved through filter paper and the filtrate tested for NH_4^+ content at 690 nm using UV visible spectrophotometer. Activity of urease enzyme was calculated as μg NH_4/g dry wt/h.

Protease activity was measured via the protocol of Ladd and Butler (1972). The concentration of Tyrosine (Tyr) was estimated in soil samples following the incubation with ($C_{20}H_{27}FN_2$) sodium caseinate. One g wet soil was suspended with 5 ml sodium caseinate and 5 ml tris buffer solution. Soil suspension was incubated for 2 h at 50 °C in a shaking water bath. Following incubation, an aliquot of TCA solution (5 ml of) was amended. The resulting suspension was centrifuged at 8000 rpm and 5 ml of supernatant was removed in fresh tubes. The collected supernatant was blended in 7.5 ml of the alkaline reagent. For blank, solution was prepared with sodium caseinate (5 ml) at the end of the incubation and instantly prior to addition of TCA. Followed the incubation for 15 min at room temperature, absorbance was recorded at 700 nm and enzymatic activity of protease was calculated as μg Tyr g^{-1} soil h^{-1}.

2.18. Statistical analysis

All the generated data in this study were analyzed statistically using ANOVA (analysis of variance) on SPSS statistics 28. At the 5% probability level, variations among the mean values of treatments were compared in accordance with Duncan's Multiple Range Test (DMRT). All the graphs were prepared on SigmaPlot™ version 12.0.

3. Results

3.1. Selection and screening of rhizobacteria from wheat rhizoplane

Two well characterized and identified PGPR strains *Bacillus subtilis*-FAB1 and *Pseudomonas azotoformans*-FAP3 were selected in this study from previous published research. These strains were isolated from the wheat rhizoplane. Characteristics of some of these isolates have been reported earlier (Ansari and Ahmad, 2019). All isolates exhibited varying degrees of IAA synthesis. Maximum IAA production was recorded in FAB1 (135.6 ± 2.3) and FAP3 (126.5 ± 1.60 μg/ml). Varied levels of phosphate solubilization were also observed in the isolates; maximum phosphate solubilization was in FAB1 (181.7 ± 1.95) and FAP3 (149.8 ± 2.05 μg/ml). The isolates varied in terms of siderophore production; FAB1 and FAP3 exhibited greatest production (26.3 ± 1.95 and 29.5 μg/ml) *in vitro*. Production of HCN and NH_3 in FAB1 and FAP3 was also positive as depicted in Table 1.

Relatively, maximum synthesis of ACC deaminase was detected in FAB1 (71.8 ± 1.90) and (58.9 ± 2.05 μmol α-KB/mg Pr^h) in comparison to other tested isolates. All isolates except FAB1 and FAP3 grew poorly and did not tolerate this level of water stress and the FAB1 and FAP3 isolates revealed better growth even in 40% PEG-amended media (Table 1).

3.2. Rhizobacterial interaction in planktonic mode of growth

Interactions among these two isolates were examined under the planktonic mode of growth by using co-culture plate, overlay and cross streak technique *in vitro*. The bacterial combination displayed positive interaction and no negative interaction was recorded in any of the set. The FAB1 and FAP3 interacted positively with each other (**Table S1**).

3.3. Molecular identification of FAB1 and FAP3

The two most promising isolates (FAB1 and FAP3) were identified based on their sequence matched through NCBI-BLAST. Isolate FAB1 showed 99.99 percent sequence similarity to *Bacillus subtilis* and FAP3 displayed 99.99 percent sequence similarity to *Pseudomonas azotoformans*. Trimmed and aligned sequences of 16S rRNA were deposited to NCBI (National Center for Biotechnology Information), USA with accession number MG192145 for FAB1 and KY110951 for the FAP3 isolate. The ancestral tree was built up to designate the isolate identification and molecular based characterization (**Figure S1**).

3.4. Rhizobacterial interaction in biofilm mode on glass and root surface

Based on interaction of rhizobacteria in the planktonic mode of growth, these two most promising isolates (FAB1 and FAP3) were selected for further interaction in the biofilm state on glass and root surfaces. This combination was analyzed for its ability to form a compatible biofilm on a glass surface and visualized by both light microscopy and SEM. These two isolates displayed high compatibility and formed a strong biofilm. No negative interaction was observed in the FAB1 + FAP3 mixed biofilm (Figs. 1 and 2).

Interaction of FAB1 + FAP3 in biofilm mode on wheat root was positive; cells formed a dense and compact biofilm. The isolates displayed high compatibility as evident under SEM (Fig. 3).

3.5. Influence of water stress on PGP traits and ACC deaminase

Synthesis of IAA under 40% water stress decreased by 9.2 in FAB1 and 12.2% in FAP3. Only 4.2% reduction ($p > 0.05$) was observed in the mixed FAB1 + FAP3 inoculation in comparison to the non-stress control (Table 2). Phosphate solubilization was reduced by 7.6% (FAB1) and 27.1% (FAP3) while only 6.3% reduction was recorded in the mixed FAB1 + FAP3 culture under drought stress when compared to non-stressed conditions. Siderophore production was also impeded in both among the individual isolates and in mixed culture under drought stress and siderophore production declined in FAB1 (16.7%) and FAP3 (35.3%), while 20.4% reduction was observed in mixed culture (FAB1 + FAP3). All the above PGP traits declined in single and mixed culture under drought stress; however, inhibition was not complete. Overall, the least reduction in all PGP attributes was recorded by mixed the FAB1 + FAP3 combination at high water stress (Table 2**)**.

Synthesis of ACC deaminase declined by 1.3, 2.4 and 2.6% in FAB1, FAP3 and FAB1 + FAP3, respectively, under drought stress in comparison to control (Table 2). All reductions in ACC deaminase were non-significant.

3.6. Determination of biofilm and its linked traits under drought stress

Drought stress at 40% PEG resulted in inhibitory effect to biofilms and their associated traits. The FAB1 isolate showed 7.0% reduction in biofilm formation followed by EPS (12.0%) alginate (17.8%) and CSH (12.3%). Similarly, all traits were reduced in FAP3 (9.2% biofilm, 17.4% EPS, 19.4% alginate, and 23.2% CSH) under

Table 1
In vitro screening of FAB1 and FAP3 strains based on multifunctional plant growth promoting traits and elevated water stress tolerance abilities.

Isolates	IAA (μg/ml)	P-solubilization (μg/ml)	ACC deaminase (μmol α-KB/mg Pr^{-h})	Siderophore		HCN	NH_3	Water stress tolerance (40% PEG)
				CAS agar	$FeCl_3$ test			
Bacillus subtilis-FAB1	135.6 ± 2.30[a]	181.7 ± 1.95[a]	71.8 ± 1.90[a]	26.3 ± 1.95[ab]	+	–	+	+
Pseudomonas azotoformans-FAP3	126.5 ± 1.60[b]	149.8 ± 2.05[b]	58.9 ± 2.05[b]	29.5 ± 2.15[a]	+	+	+	+

The values in the data represented mean ± S.E. Means followed by the same superscript letter are not significantly different ($P = 0.05$) using Duncan's multiple range test. '+' indicates positive and '–' represent negative results.

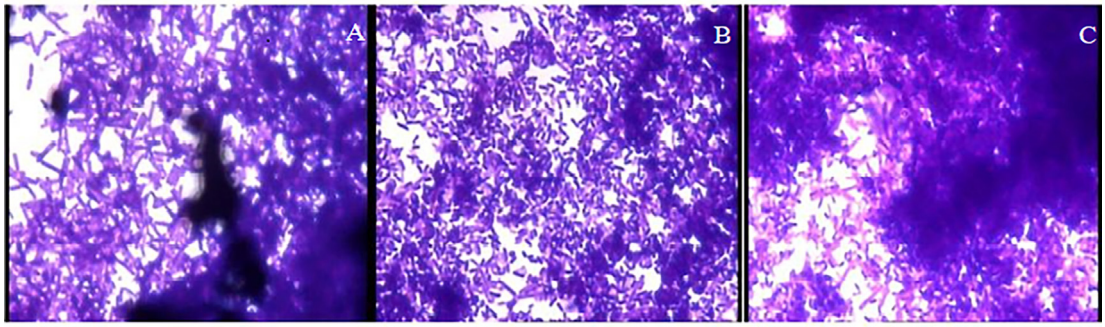

Fig. 1. Light micrograph of biofilm development on glass surface by FAB1 (A) and FAP3 (B) individually and in mixed form of FAB1 + FAP3 (C) on glass surface stained with crystal violet (1 %).

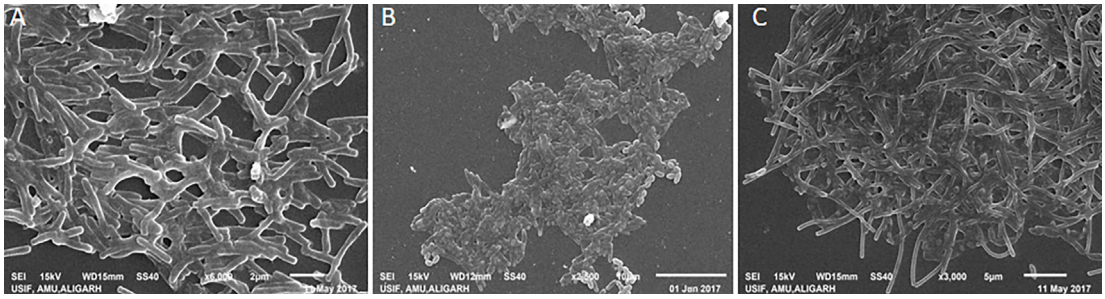

Fig. 2. Scanning electron micrograph of biofilm development on glass surface by FAB1 (A) and FAP3 (B) individually and in mixed form of FAB1 + FAP3 (C).

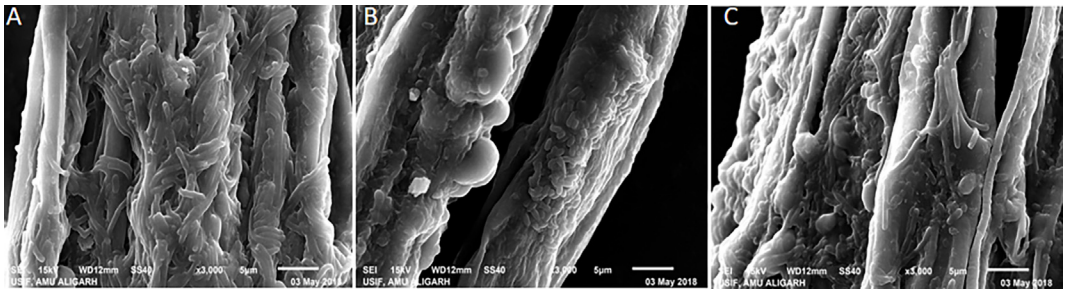

Fig. 3. Scanning electron micrograph of biofilm development on wheat root surface by FAB1 (A) and FAP3 (B) individually and in mixed form of FAB1 + FAP3 (C) by the root dip method.

drought stress compared to control. The reduction in attributes was significantly decreased in mixed cultures (FAB1 + FAP3) under same stress condition In contrast, the motility (i.e., swarming and swimming) of the isolates was relatively enhanced in both single and mixed cultures under water stress (Table 3).

3.7. Impact of drought stress on seedling germination

Under non-stressed conditions, seed germination was enhanced by 10.0, 5.0 and 16.0% treated with FAB1, FAP3 and mixed cultures, respectively compared to control. Drought stress resulted in inhibitory effect on seed germination 2.3% to 3.6% in single and mixed treatment. Decline in percent seed germination was not significant ($p > 0.05$) in the individual isolates. The lowest decline under drought stress was in the mixed culture (**Fig. S3**).

3.8. Influence of ten-day drought stress on photosynthetic attributes

The ten-day water stress period resulted in adverse impacts on photosynthetic attributes; reductions were 75.0% (gs), 16.0% (Ci),

Table 2
Influence of 40 % drought stress (DS) on the synthesis of plant growth promoting attributes in FAB1 and FAP3 individually and in mixed culture.

Treatments	IAA (μg/ml)	P-solubilization (μg/ml)	Siderophore (μg/ml)	ACC deaminase (μmol α-KB/mg Pr^{-h})
	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
FAB1	135.50 ± 1.18^{c}	181.53 ± 1.13^{c}	26.43 ± 1.16^{d}	71.73 ± 1.04^{c}
FAP3	126.43 ± 1.16^{d}	149.53 ± 1.11^{e}	29.40 ± 1.18^{c}	59.20 ± 0.91^{e}
FAB1 + FAP3	178.87 ± 1.18^{a}	213.43 ± 1.18^{a}	41.67 ± 1.07^{a}	86.77 ± 3.45^{a}
FAB1 (40% DS)	123.40 ± 1.02^{d}	170.67 ± 3.32^{d}	21.70 ± 1.27^{e}	70.67 ± 1.13^{d}
FAP3 (40% DS)	111.33 ± 1.13^{e}	109.37 ± 1.19^{f}	19.40 ± 1.14^{e}	57.60 ± 1.10^{e}
FAB1 + FAP3 (40% DS)	171.47 ± 1.01^{b}	199.80 ± 1.24^{b}	33.47 ± 1.05^{b}	81.30 ± 1.27^{b}

The values in the data represented mean ± S.E. Means followed by the same superscript letter are not significantly different (P = 0.05) using Duncan's multiple range test.

Table 3
In vitro evaluation of biofilm and its associated traits in FAB1 and FAP3 alone and in mixed culture under 40% drought stress (DS).

Treatments	Biofilm (OD)	EPS production (μg ml^{-1})	Alginate production (μg ml^{-1})	Swarming motility (mm)	Swimming motility (mm)	CSH (%)
	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
FAB1	1.73 ± 0.10^{abc}	910.33 ± 1.10^{b}	123.60 ± 1.14^{c}	30.00 ± 1.15^{bc}	37.00 ± 1.15^{abc}	73.00 ± 1.15^{b}
FAP3	1.53 ± 0.11^{bc}	619.67 ± 1.33^{d}	103.47 ± 1.10^{d}	34.00 ± 1.15^{a}	39.00 ± 1.15^{ab}	69.00 ± 1.15^{c}
FAB1 + FAP3	1.97 ± 0.10^{a}	989.60 ± 1.30^{a}	236.70 ± 1.18^{a}	28.00 ± 1.15^{c}	36.00 ± 1.15^{bc}	81.00 ± 1.15^{a}
FAB1 (40% DS)	1.59 ± 0.12^{abc}	801.17 ± 0.87^{c}	101.87 ± 1.18^{d}	32.00 ± 1.15^{ab}	40.00 ± 1.15^{a}	64.00 ± 1.15^{d}
FAP3 (40% DS)	1.38 ± 0.12^{c}	512.33 ± 1.07^{e}	83.43 ± 1.10^{e}	35.00 ± 1.15^{a}	37.00 ± 1.15^{abc}	53.00 ± 1.15^{e}
FAB1 + FAP3 (40% DS)	1.86 ± 0.15^{ab}	911.60 ± 1.27^{b}	202.33 ± 1.07^{b}	30.00 ± 1.15^{bc}	35.00 ± 1.15^{c}	71.00 ± 1.15^{bc}

The values in the data represented mean ± S.E. Means followed by the same superscript letter are not significantly different (P = 0.05) using Duncan's multiple range test. EPS; exopolysachharides, CSH; cell surface hydrophobicity.

48.1% (E), 32.9 (iWUE) and 56.3% (P_N) in non-inoculated wheat plants. Application of the isolates individually and in combination enhanced all attributes at ten-day water stress. Enhancement by inoculation of FAB1 was 100% (gs), 9.9% (Ci), 50.0% (E), 29.2% (iWUE) and 77.1% (P_N). Plants inoculated with FAP3 showed increases of 200% (gs), 13.1% (Ci), 64.3%, (E), 34.4%, (iWUE) and 96.2% (P_N) when compared to non-inoculated drought stress control (Table 4**)**. The mixed FAB1 + FAP3 plants displayed maximum enhancement in all photosynthetic traits: 200% (gs), 17.2% (Ci), 78.6% (E), 40.8% (iWUE) and 117.1% in P_N under ten days drought stress in comparison to control.

3.9. Influence of ten-day drought stress on plant stress markers

Levels of stress markers (antioxidants) CAT, SOD, GR activity, MDA and proline content increased significantly ($p < 0.05$) in plants exposed to ten-day drought stress. Percent enhancement in catalase was 65.1%, SOD activity 82.7%, GR activity 85.3%, MDA 28.7% and proline content 76.1% (Table 5**)**.

Levels of stress markers were significantly ($p < 0.05$) decreased in the presence of the isolates; maximum reduction was recorded in plants inoculated with the FAB1 + FAP3 combination. Reduction in catalase activity was 45.1%, SOD activity 31.5%, GR activity 34.4%, MDA 25.7%, and proline content 34.9% under ten-day drought stress when compared to the non-inoculated control (Table 5). Generally, the inoculation of rhizobacteria reduced the adverse effect of water stress and lowered the level of antioxidants.

3.10. Assessment of ten-day drought stress on plant growth attributes

Root length, shoot length, and root number, shoot fresh weigh, shoot dry weight, root fresh weight, root dry weight, RWC and RAS/RT ratio under non-drought-stressed conditions significantly ($p < 0.05$) increased in the presence of the tested isolates; greatest increases were recorded in plants inoculated with the combination of FAB1 + FAP3. The respective enhancement in shoot length was 5.8%, root length 7.4%, root number 30.8%, shoot fresh weight 44.4%, shoot dry weight 23.3%, root fresh weight 24.1%, root dry weight 21.8%, RWC 1.7% and RAS/RT 33.3% over the control **(Table S2)**.

Values of plant growth attributes were significantly ($p < 0.05$) reduced under ten days drought stress in non-inoculated plants. Percent reduction in shoot length was 12.4%, root length 29.9%, root number 23.1%, shoot fresh weight 21.2%, shoot dry weight

Table 4
Influence of ten days drought stress (DS) on photosynthetic parameters [stomatal conductance (gs); intercellular CO_2 concentration (Ci); transpiration rate (E); intrinsic water use efficiency (iWUE); photosynthetic rate (PN)] in the presence and or absence of FAB1 and FAP3 individually and in mixed inoculation.

Treatment	gs (mmol m^{-2} s^{-1})	Ci (μmol CO_2 mol air^{-1})	E; (mmol m^{-2} s^{-1})	iWUE (μmol CO_2 /mmol H_2O)	P_N (μmol m^{-2} s^{-1})
	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
Control (WD)	0.43 ± 0.01^{b}	30.79 ± 0.01^{e}	30.89 ± 0.01^{e}	205.26 ± 1.20^{b}	16.10 ± 0.05^{f}
Control (DS)	0.71 ± 0.01^{a}	56.25 ± 0.01^{a}	57.25 ± 0.01^{a}	264.13 ± 1.16^{a}	28.25 ± 0.01^{a}
FAB1	0.43 ± 0.01^{b}	29.23 ± 0.01^{f}	26.23 ± 0.01^{f}	207.24 ± 1.09^{b}	17.05 ± 0.01^{e}
FAP3	0.42 ± 0.01^{b}	29.12 ± 0.01 ^{g}	25.68 ± 0.01 ^{g}	206.26 ± 1.11^{b}	15.25 ± 0.01 ^{g}
FAB1 + FAP3	0.42 ± 0.01^{b}	28.01 ± 0.00 ^{h}	25.01 ± 0.00 ^{h}	207.53 ± 1.10^{b}	15.21 ± 0.01 ^{g}
FAB1 (DS)	0.38 ± 0.01^{b}	39.54 ± 0.01^{c}	41.54 ± 0.01^{c}	191.41 ± 1.24^{d}	21.35 ± 0.01^{b}
FAP3(DS)	0.46 ± 0.11^{b}	40.12 ± 0.01^{b}	40.12 ± 0.01^{b}	194.49 ± 1.16 ^{cd}	20.21 ± 0.01^{c}
FAB1 + FAP3(DS)	0.39 ± 0.01^{b}	38.53 ± 0.01^{d}	37.53 ± 0.01^{d}	196.26 ± 1.18^{c}	18.38 ± 0.01^{d}

The values in the data represented mean ± S.E. Means followed by the same superscript letter are not significantly different (P = 0.05) using Duncan's multiple range test. WD indicate without drought in the experiments.

Table 5
Impact of ten days drought stress (DS) on stress markers in the presence and or absence of FAB1 and FAP3 individually and in mixed inoculation on wheat plant.

Treatment	CAT activity (m moles min^{-1} g^{-1}FW)	SOD activity (% increase)	GR activity (μmol min^{-1} g^{-1} FW)	MDA content (moles g^{-1} FW)	Proline (μg.g^{-1}. FW)
	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
Control (WD)	0.43 ± 0.01^{b}	30.79 ± 0.01^{e}	30.89 ± 0.01^{e}	205.26 ± 1.20^{b}	16.10 ± 0.05^{f}
Control (DS)	0.71 ± 0.01^{a}	56.25 ± 0.01^{a}	57.25 ± 0.01^{a}	264.13 ± 1.16^{a}	28.25 ± 0.01^{a}
FAB1	0.43 ± 0.01^{b}	29.23 ± 0.01^{f}	26.23 ± 0.01^{f}	207.24 ± 1.09^{b}	17.05 ± 0.01^{e}
FAP3	0.42 ± 0.01^{b}	29.12 ± 0.01 ^{g}	25.68 ± 0.01 ^{g}	206.26 ± 1.11^{b}	15.25 ± 0.01 ^{g}
FAB1 + FAP3	0.42 ± 0.01^{b}	28.01 ± 0.00 ^{h}	25.01 ± 0.00 ^{h}	207.53 ± 1.10^{b}	15.21 ± 0.01 ^{g}
FAB1 (DS)	0.38 ± 0.01^{b}	39.54 ± 0.01^{c}	41.54 ± 0.01^{c}	191.41 ± 1.24^{d}	21.35 ± 0.01^{b}
FAP3 (DS)	0.46 ± 0.11^{b}	40.12 ± 0.01^{b}	40.12 ± 0.01^{b}	194.49 ± 1.16 ^{cd}	20.21 ± 0.01^{c}
FAB1 + FAP3 (DS)	0.39 ± 0.01^{b}	38.53 ± 0.01^{d}	37.53 ± 0.01^{d}	196.26 ± 1.18^{c}	18.38 ± 0.01^{d}

The values in the data represented mean ± S.E. Means followed by the same superscript letter are not significantly different ($P = 0.05$) using Duncan's multiple range test. CAT; catalase, SOD; superoxide dismutase, GR; glutathione reductase, MDA; Malondialdehyde. WD indicates without drought.

19.3%, root fresh weight 46.1%, root dry weight 31.2%, RWC 15.4 % and RAS/RT 25.1% compared to plants grown without water stress. In general, inoculation of rhizobacteria reduced the adverse effect of drought. The respective enhancement in shoot length was 8.4%, root length 28.6%, root number 20.3%, shoot fresh weight 21.2%, shoot dry weight 24.3%, root fresh weight 71.3%, root dry weight 41.6%, RWC 16.2% and RAS/RT 26.2% under drought stress when compared to non-inoculated control (**Table S2**).

3.11. Grain attributes and straw yield under water stress

Under non-drought stressed conditions, the number of grains per spike, grain yield, grain protein and straw yield were significantly ($p < 0.05$) improved in the presence of the isolates. Maximum enhancement was recorded in plants inoculated with the combination of FAB1 + FAP3. The respective increase in grain number per spike was 4.9%, grain yield 26.0%, grain protein 5.0%, and straw yield 22.2%, respectively, over control.

Values of all the attributes of seeds and yield of straw were reduced significantly at ten days water stress in non-inoculated plants. Percent reduction in grain per spike was 9.8%, grain yield 17.6%, grain protein 26.5%, and straw yield 37.0% in drought-affected and non-inoculated treated plants. Grain attributes and straw yield were significantly ($p < 0.05$) enhanced in the presence of the tested isolates. Greatest improvements under drought stress were recorded in plants inoculated with the FAB1 + FAP3 mixed. The respective increases in grain per spike were 8.1%, grain yield 14.1%, grain protein 32.7%, and straw yield 49.0% in comparison to the non-inoculated and drought-stress control (Table 6).

3.12. Rhizoplane and rhizosphere colonization under water stress

Bacterial viable counts on the wheat rhizoplane at 30 DAS were Log 7.3 (FAB1), 7.1 (FAP3) and 7.5 CFU/g root (FAB1 + FAP3), and at 60 DAS were 6.4 (FAB1), 6.2 (FAP3) and 6.7 CFU/g in FAB1 + FAP3 under non-drought-stressed conditions. Bacterial viability was reduced but not completely inhibited in the rhizoplane under drought stress. Bacterial viability at 30 DAS was 7.0 (FAB1), 6.8 (FAP3) and 7.2 (FAB1 + FAP3) while at 30 DAS was 6.1 (FAB1), 5.9 (FAP3) and 6.3 log CFU (FAB1 + FAP3). The mixed inoculation resulted in better colonization on the rhizoplane under ten days water stress up to 60 DAS (**Fig. S4a**).

Bacterial viable counts in the rhizosphere were 7.4 (FAB1), 7.3 (FAP3) and 7.7 CFU/g of rhizospheric soil (FAB1 + FAP3) at 30 DAS, while at 60 DAS values were 7.0 (FAB1), 6.9 (FAP3) and 7.2 (FAB1 + FAP3) under non-drought-stressed conditions. The ten-day drought stress altered the structure of rhizosphere colonization at 30 and 60 DAS. Bacterial viability at 30 DAS was 7.1 (FAB1), 6.9 (FAP3) and 7.3 (FAB1 + FAP3) while at 30 DAS was 6.8 (FAB1), 6.5 (FAP3) and 6.9 Log CFU/g of rhizospheric soil (FAB1 + FAP3) (**Fig. S4b**). Generally, the isolates in the single inoculation revealed comparable patterns in terms of rhizoplane and rhizosphere colonization. The isolates in the mixed inoculation displayed better overall rhizoplane and rhizosphere colonization and survived for the long-term even under drought-stressed conditions.

3.13. Influence of water stress on physico-chemical properties of wheat rhizospheric soil

The ten-day drought stress resulted in adverse effects on physico-chemical properties of non-inoculated rhizospheric soil. The pH increased from 7.65 to 8.52 and EC increased from 870.23 to 890.12 μS/cm. Soil TOC decreased from 0.704 to 0.501%, and concentrations of total and available P declined somewhat (**Table S3**).

Rhizospheric soil under drought stress and inoculated with bacterial isolates experienced improvement in soil physico-chemical attributes and reduced adverse impacts of drought stress as compared to drought stress-alone plants. The FAB1 + FAP3 treatment resulted in better recovery in soil physico-chemical parameters compared to the isolates individually. Soil pH declined from 8.52 to 7.71, and EC from 890.12 to 728.19 μS/cm. Soil TOC increased from 0.50 to 0.70% (**Table S3**).

3.14. Impact of drought stress on soil hydrolytic enzymes of wheat rhizospheric soil

Drought stress at ten days imparted adverse impacts on soil hydrolytic enzymes; significant ($p < 0.05$) declines in enzyme synthesis were noted: 28.1% (DHA), 9.5 % (urease), 18.2% (ALP), 25.6% (protease), 21.8% (ACP) and 25.7% (β-glucosidase), respectively, over the non-inoculated control.

Enzyme synthesis increased upon application of the isolates, and maximum enhancement was recorded where rhizospheric soil was inoculated with the FAB1 + FAP3 mixture. The respective improvements were as follows: DHA (38.5%), urease (9.9%), ALP (10.3%), protease (28.1%), ACP (24.3) and in β-glucosidase (31.4%) under drought stress when compared to water stress alone-treated rhizosphere (Table 7). Generally, inoculation of rhizobacteria reduced the deleterious impact of water stress. The most effective treatment was the FAB1 + FAP3 mix, which significantly reduced the adverse effects generated by drought stress for all soil enzymes.

Table 6
Effect of ten days drought stress (DS) on grain attributes and straw yield in the presence and or absence of FAB1 and FAP3 alone and in consortium on wheat plants.

Treatments	No. of grains per spike	Grain yield (g/1000 seeds)	Grain protein (mg g^{-1} FW)	Straw yield (g/plant)
	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
Control (WD)	41.00 ± 1.11^{a}	31.90 ± 0.12^{c}	88.30 ± 0.13^{d}	8.10 ± 0.12^{c}
Control (DS)	37.00 ± 1.15^{ab}	26.30 ± 0.14^{e}	64.90 ± 0.12 ^{h}	5.10 ± 0.12^{f}
FAB1	43.00 ± 1.15^{a}	38.20 ± 0.12^{b}	91.20 ± 0.14^{c}	9.20 ± 0.13^{b}
FAP3	43.00 ± 1.14^{a}	39.37 ± 0.37^{ab}	91.80 ± 0.12^{b}	9.50 ± 0.14^{b}
FAB1 + FAP3	43.00 ± 1.13^{a}	40.20 ± 0.12^{a}	92.70 ± 0.11^{a}	9.90 ± 0.11^{a}
FAB1(DS)	42.50 ± 0.21^{a}	28.90 ± 0.11^{d}	82.70 ± 0.12 ^{g}	6.90 ± 0.11^{e}
FAP3(DS)	42.40 ± 0.12^{a}	29.10 ± 0.13^{d}	84.70 ± 0.11^{f}	7.20 ± 0.12^{e}
FAB1 + FAP3(DS)	40.00 ± 1.15^{ab}	30.00 ± 1.15^{d}	86.10 ± 0.12^{e}	7.60 ± 0.13^{d}

The values in the data represented mean ± S.E. Means followed by the same superscript letter are not significantly different (P = 0.05) using Duncan's multiple range test.

4. Discussion

Rhizobacterial communities that perform together are commonly regarded as beneficial to their hosts and to soil fertility. PGPR have been investigated extensively among microbial residents of the rhizosphere for useful attributes (Ansari and Ahmad, 2019b, Murali et al., 2021). These microbes cooperate in mixed communities and produce biofilms for effective root and rhizosphere colonization (Ansari and Ahmad, 2018, Wu et al., 2019). Aside from biotic stress resistance, PGPR have also been documented for efficiently activating abiotic stress resistance in plants (Chandra et al., 2020, Ansari et al., 2021, Yang et al., 2021). Sufficient numbers of these bacteria either on the root or in the rhizosphere synthesize varied biomolecules for promoting plant growth and drought-response enzymes (e.g., ACC deaminase) to mitigate environmental stress (Ansari et al., 2021, Murali et al., 2021). Wheat is considered a drought-sensitive crop; therefore, water scarcity creates severe constraints for cultivation and productivity (Vurukonda et al., 2016, Ansari et al., 2021). Considering these collectively, we conducted this study to assess the efficacy of two novel and promising multifunctional PGPR with strong biofilm-forming ability in both single and mixed treatments on wheat to mitigate drought stress and improve soil resilience.

Two most desired bacterial isolates were included in this study, which were isolated from rhizoplane of wheat crop. These isolates belonging to Bacillus and Pseudomonas spp. displayed varied ranges of multifunctional PGP traits, ACC deaminase activity, and biofilm production, both qualitatively and quantitatively *in vitro*. The FAB1 and FAP3 isolates exhibited maximal values of the above traits and displayed optimal growth at 40% drought. All isolates were evaluated for possible interactions in their respective combination. The FAB1 and FAP3 isolates in combination revealed positive interaction and did not restrict growth of their partner isolates and therefore these isolates displayed strong positive interaction and revealed compatible behaviour in the planktonic mode of growth, and were selected for further investigation. Isolates FAB1 and FAP3 were identified as *Bacillus subtilis* and *Pseudomonas azotoformans*, respectively, based on 16S rRNA gene sequencing.

Research has been published on bacterial interactions in soil under 'normal' conditions; however, interaction between root-adhered beneficial bacteria under drought stress and their interactive functionalities is as poorly explored (Ren et al., 2015, Herschend et al., 2018, Ansari and Ahmad, 2019a, Bhat et al., 2020, Peng et al., 2021;). It is presumed that bacterial interaction in the biofilm mode is a prerequisite for enhanced rhizosphere and rhizoplane colonization in natural soil systems. Multispecies biofilms maintain microbial community diversity and sustain soil ecosystems; as a result, plant growth is sustained even under harsh environmental conditions (Ren et al., 2015, Wu et al., 2019, Ansari et al., 2021;). The selected rhizoplane bacteria (FAB1 and FAP3) displayed a strong positive interaction in biofilm mode on glass and wheat root surfaces as visualized by light microscopy and SEM. These isolates revealed dense and strong biofilm production individually as well as in combination, and colonized root surfaces effectively.

Drought stress hampers metabolic activities of beneficial soil bacteria and therefore directly hinders plant growth (Ren et al., 2015, Peng et al., 2021). Drought stress (40%) resulted in reduction in bacterial PGP traits including IAA synthesis, P solubilization, siderophore production, and ACC deaminase activity in FAB1 and FAP3 in single culture. However, combination of FAB1 + FAP3 displayed non-significant reduction in PGP attributes even at higher drought stress. The combination of these drought-tolerant isolates resisted water stress and maintained all growth attributes at stable levels. Such activities of a dual bacterial system may stimulate plant growth and mitigate water stress in natural soil systems.

Drought stress generally alters the structure of rhizobacterial biofilms, whether mono- or mixed species biofilms. This disorganization is mainly due to negative interference in biofilm-associated

Table 7
Influence of 10 days drought stress on soil hydrolytic enzymes of wheat rhizospheric soil in the presence and or absence of FAB1 and FAP3 individually and in mixed inoculation after 30 days.

Treatments	DHA (µg/gdwt/h)	Urease (µg/gdwt/h)	ALP (µg/gdwt/h)	Protease (µg/gdwt/h)	ACP (µg/gdwt/h)	ß glucosidase (µg/gdwt/h)
	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
Control (WD)	135.33 ± 1.13^{d}	301.73 ± 0.07^{d}	14.30 ± 0.12^{a}	4.30 ± 0.12^{bc}	14.20 ± 0.12^{d}	98.50 ± 0.12^{d}
Control (DS)	97.30 ± 1.15 ^{g}	273.20 ± 0.12 ^{h}	11.70 ± 0.12^{e}	3.20 ± 0.12^{e}	11.10 ± 0.12 ^{h}	73.20 ± 0.12 ^{h}
FAB1	159.50 ± 0.12^{b}	378.20 ± 0.12^{c}	13.40 ± 0.12^{b}	4.60 ± 0.12^{b}	15.30 ± 0.12^{b}	142.30 ± 0.12^{b}
FAP3	149.90 ± 0.12^{c}	386.70 ± 0.12^{b}	13.50 ± 0.12^{b}	4.40 ± 0.12^{bc}	14.70 ± 0.12^{c}	133.70 ± 0.12^{c}
FAB1 + FAP3	201.60 ± 0.12^{a}	488.90 ± 0.12^{a}	9.50 ± 0.12^{f}	5.30 ± 0.12^{a}	23.90 ± 0.12^{a}	178.83 ± 0.07^{a}
FAB1(DS)	129.50 ± 0.12^{e}	298.40 ± 0.85^{f}	11.50 ± 0.12^{e}	3.90 ± 0.12^{d}	13.10 ± 0.12^{f}	91.30 ± 0.12^{f}
FAP3 (DS)	123.37 ± 1.18^{f}	288.30 ± 0.12 ^{g}	12.10 ± 0.12^{d}	3.50 ± 0.12^{e}	12.70 ± 0.12 ^{g}	89.70 ± 0.12 ^{g}
FAB1 + FAP3 (DS)	134.77 ± 1.18^{d}	300.13 ± 0.03^{e}	12.90 ± 0.12^{c}	4.10 ± 0.12 ^{cd}	13.80 ± 0.00^{e}	96.20 ± 0.12^{e}

The values in the data represented mean ± S.E. Means followed by the same superscript letter are not significantly different (P = 0.05) using Duncan's multiple range test. DHA; dehydrogenase, ALP; alkaline phosphatase, ACP; acid phosphatase, WD; without drought, DS; drought stress.

functions such as EPS production, microbial motility, alginate and CSH production in the soil matrix. Therefore, drought stress is considered a major environmental constraint to bacterial colonization in the rhizosphere and on the root surface (Bogino et al., 2013, Ansari and Ahmad, 2019b, Haque et al., 2020). Hence, we tested the selected bacterial isolates for biofilm production and related functions under 40% water stress. All tested traits declined in non-significant range in individual FAB1 and FAP3 cultures at high water stress, and a non-significant decline in biofilm-associated functions was noted in the dual culture system (FAB1 + FAP3). The dual culture system survived well and displayed maximum production of all traits under drought stress. The FAB1 + FAP3 mix also demonstrated greater synthesis of all the above attributes during drought stress.

Drought management has become increasingly important in recent years, limiting the variety of crops available for agricultural production. Several novel methods for mitigating the deleterious effects of abiotic stress have been attempted in order to achieve higher yields; however, they have not yet generated useful results in wheat crops (Singh et al., 2017, Wang et al., 2019, Ansari et al., 2021). Therefore, the selected isolates were subjected for seedling germination enhancement and drought mitigation ability using mono- and dual-culture inoculation. Our findings indicate that these isolates, both singly and in combination, performed well and enhanced percent seedling germination even under 40% water stress. Both isolates revealed comparably similar enhancement in seedling germination; however, the dual culture displayed greatest enhancement in seedling germination under drought conditions compared to single-culture inoculation.

Photosynthetic attributes (leaf gas exchange) and chlorophyll content have been widely accepted as indicators of plant stress (Ansari and Ahmad, 2019a, Qaseem et al., 2019, Ansari et al., 2021). These attributes are negatively influenced by abiotic stress, ultimately resulting in stunted growth of plants. Our study, corroborated with previous studies, revealed that photosynthetic attributes (gs, Ci, E, iWUE and P_N) in leaves of non-inoculated plants declined significantly during ten-day drought conditions. Application of bioinoculants FAB1 and FAP3 in monoculture restored all physiological attributes and chlorophyll pigments even under drought conditions. Improvement was observed in FAB1 and FAP3 with comparably similar restoration in photosynthetic traits; however, dual culture (FAB1 + FAP3) inoculation displayed greatest restoration in physiological traits to normal levels even under drought stress.

Under drought stress, plants trigger essential antioxidant systems to reduce cell damage and prevent cell death. Antioxidants including CAT, SOD, GR, MDA and proline are well-documented stress markers as they tend to accumulate and increase by repressed catabolic pathways during oxidative stress. Variations in enhanced levels of these antioxidants under drought may be due to the fact that they do not follow a static model, and fluctuate with stress level (Khaleghi et al., 2019, Ansari et al., 2021). No previous studies were identified that discuss differences in stress marker levels in plant-bacterial interactions under drought conditions. In the current study, levels of all stress markers significantly increased in non-inoculated wheat plants under ten days drought. Application of bioinoculants in monoculture (FAB1 and FAP3) and dual culture (FAB1 + FAP3) lowered the levels of stress markers. Reductions observed in FAB1 and FAP3 were comparable, but dual culture (FAB1 + FAP3) inoculation exhibited maximum reestablishment in all antioxidants to their normal state even under drought stress.

It is recognized that PGPR inoculation of single and mixed cultures improve plant growth attributes in both normal and stressed environments (Li et al., 2019, Joshi et al., 2020, Ansari et al., 2021). Despite these findings, little is documented regarding the impact of biofilm-forming beneficial bacteria on wheat growth attributes under high drought stress. Our study demonstrated that FAB1 and FAP3 improved growth attributes including root length, shoot length, root number, root fresh weight, root dry weight, shoot fresh weigh, and shoot dry weight, RWC and RAS/RT ratio, even under high drought stress. Growth stimulation was observed in FAB1 and FAP3, and both isolates exhibited comparable enhancement in plant attributes. Bioinoculants in dual culture (FAB1 + FAP3) resulted in the greatest improvement in all parameters and ameliorated the deleterious impacts of drought to the plants. No similar results have been published in this context. The above bacterial combination could be applied in field systems to enhance crop growth and mitigate drought issues in natural soil ecosystems.

Bioinoculants in dual culture (FAB1 + FAP3) improved all attributes (grains per spike, grain yield, grain protein and straw yield) to a greater extent at high drought and normalized the severe impacts of stress compared to non-inoculated plants. Single culture inoculation resulted in enhancement in all grain attributes, with comparable results between the two inoculants; however, bioinoculants in dual culture (FAB1 + FAP3) resulted in the greatest improvement in all attributes. This effect may be a result of intrinsic properties of bacterial compatibility in the dual culture system and effective colonization, as well as stress-alleviating abilities in the rhizosphere (Ansari et al., 2021, Yang et al., 2021).

Rhizoplane and rhizosphere colonization by single species are infrequent in natural ecosystems, including agricultural soil where micro-communities have the potential to form multispecies biofilms of great density and megadiversity (Burmølle et al., 2010, Burmolle et al., 2014, Ansari and Ahmad, 2019a, Yang et al., 2021). Such situations are believed to expedite the formation of complex associations among various species. Numerous studies have focused on interspecies interaction in human microbial populations in healthy conditions (Ren et al., 2015, Kommerein et al., 2017), but research on multispecies biofilms in the rhizosphere and rhizoplane among soil beneficial bacteria under abiotic stress is yet to be explored. In this study, for the first time, we evaluated root and rhizosphere colonization by mono and dual cultures and their existence under drought. Isolates FAB1 and FAP3 as single species colonized the rhizoplane and rhizosphere comparably up to 30 and 60 days under drought; dual culture displayed even more effective colonization of the rhizosphere and root surface of wheat under drought as evidenced by bacterial counts. Our study clearly indicates that these bioinoculants in dual species exist as robust colonizers and could be applied for field trials for commercialization.

Rhizospheric beneficial bacterial communities perform key roles in soil fertility by maintaining and increasing nutrient mobilization and acquisition, soil aggregation and other functional attributes (Alimi et al., 2021, Lazcano et al., 2021). However, the role of multispecies bacterial biofilms in maintaining and enhancing soil physico-chemical attributes under stress conditions is relatively unknown. In our study, the ten-day drought resulted in marked fluctuations in pH, EC, %TOC, and levels of total and available N, P and K. Upon application of FAB1 and FAP3 in both single and dual culture inoculation, all fertility parameters improved significantly and were restored to their normal state even under drought. Therefore, these novel isolates could be used to enhance and maintain soil health against drought situations.

Soil hydrolytic enzymes such as DHA, urease, ALP, protease, ACP and β-glucosidase are widely known as key players for restricting pathogens and enhancing soil fertility (Jog et al., 2012, Raklami et al., 2019, Wu et al., 2019). The role of mixed bacterial biofilms in the regulation of soil enzymes is under exploration for improvement of soil health in an eco-friendly manner. Our study is the first to report on the influence of mixed bacterial biofilm in dynamics of soil hydrolytic enzymes. Activities of all enzymes declined signifi-

cantly under drought conditions in the absence of bioinoculants. Use of the isolates resulted in maximum recovery for all soil enzymes even under drought conditions. Single-species inoculation resulted in comparable recovery of all soil enzymes; however, dual culture inoculation demonstrated the highest recovery of soil hydrolytic enzymes. This phenomenon may be due to inherent bacterial drought tolerance and efficient biofilm production over longer periods. To date, no research has been published to compare data for further investigation and/or establishment. The combination of these novel bacterial strains could be further exploited for their performance in field conditions to improve wheat productivity and drought mitigation.

5. Conclusions

Inoculation of mixed biofilm-forming strains *Bacillus subtilis* (FAB1) and *Pseudomonas azotoformans* (FAP3) resulted in improved growth and productivity of wheat in comparison to inoculation of individual cultures. All treatments enhanced resistance of wheat to drought. The mixed species biofilm-forming strains displayed effective colonization in the rhizosphere and rhizoplane for long periods and performed consistently even during drought. The mechanism of drought stress tolerance demonstrates that FAB1 and FAP3 in single and combined forms initiate various signals in the wheat plant that are transferred from roots to leaves which balance and maintain physiological (photosynthesis) and biochemical (antioxidative) systems to protect the integrity of cellular dynamics of plants. These strains also acted as key players to maintain and improve soil health and fertility by sustaining physicochemical attributes and hydrolytic enzymes of rhizospheric soil even under elevated drought conditions. This study concludes that *B. subtilis* and *P. azotoformans* in dual culture could be applied to mitigate elevated drought conditions in wheat and maintain soil enzymes as indicator of soil health. Further field based evaluation is recommended for practical applications.

6. Authors' contributions

FAA and IA conceived the idea and designed the study. FAA conducted all experiments and generated research data under the supervision of IA. All generated data were statistically analyzed by FAA and critically checked and verified by IA. FAA wrote the manuscript and IA and JP edited the same. All contributing authors gave their consent for publication in the present form.

Ethical approval

In this original research, the authors did not used an animal or human participants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Author FAA thankfully acknowledges the UGC-MHRD, India for granting the fellowship (Maulana Azad National Fellowship: MANF) to support this study. The University Sophisticated Instrumentation Facility (USIF), AMU, Aligarh is gratefully acknowledged by the authors for providing SEM analysis. The authors are also thankful to Macrogen Inc. Seoul, South Korea for bacterial identification at molecular level. Author FAA is also highly grateful to ICAR-NBAIM India for kind support to analyze soil enzymatic activities and physico-chemical traits.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103664.

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