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Biocontrol potential of native isolates of *Beauveria bassiana* against cotton leafworm *Spodoptera litura* (Fabricius)

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The entomopathogenic fungus (EPF), *Beauveria bassiana*, is reported as the most potent biological control agent against a wide range of insect families. This study aimed to isolate and characterize the native *B. bassiana* from various soil habitats in Bangladesh and to evaluate the bio-efficacy of these isolates against an important vegetable insect pest, *Spodoptera litura*. Seven isolates from Bangladeshi soils were characterized as *B. bassiana* using genomic analysis. Among the isolates, TGS2.3 showed the highest mortality rate (82%) against the 2nd instar larvae of *S. litura* at 7 days after treatment (DAT). This isolate was further bioassayed against different stages of *S. litura* and found that TGS2.3 induced 81, 57, 94, 84, 75, 65, and 57% overall mortality at egg, neonatal 1st, 2nd, 3rd, 4th, and 5th instar larvae, respectively, over 7 DAT. Interestingly, treatment with *B. bassiana* isolate TGS2.3 resulted in pupal and adult deformities as well as decreased adult emergence of *S. litura*. Taken together, our results suggest that a native isolate of *B. bassiana* TGS2.3 is a potential biocontrol agent against the destructive insect pest *S. litura*. However, further studies are needed to evaluate the bio-efficacy of this promising native isolate *in planta* and field conditions.

The reduction of crop losses due to insects is becoming a bigger challenge for the world's food production. Due to concerns about their impact on human health, the environment, and the food chain, many of the older, less expensive chemical insecticides are no longer being registered[1]. New technologies like expensive, more selective chemicals and genetic modification are being used, but this increased selection pressure accelerates the evolution of resistance in insect pests. Global agriculture urgently needs more environmentally friendly pest management techniques.

The tobacco caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), is one of the most devastating pests of 120 crop plants, including cauliflower, groundnuts, cotton, onions, tomatoes, brinjal, turnips, and cabbage[2]. Each year, it goes through five to six overlapping generations, and if it is not promptly treated, it might cause significant crop losses up to complete destruction[3]. Insecticides are the most often used method for controlling this problem. Although this is effective in reducing pest populations in the short term, long-term exposure to insecticides may cause S. *litura* to develop the 3 R's issues, viz., resistance, resurgence of insects, and residues on crops, like other Noctuidae members[4]. In addition, the use of pesticides leads to ecological imbalances by destroying non-target organisms and their natural enemies, parasites, and predators. The public's growing concern over the potential ecological and health risks of synthetic pesticides has shifted the focus of research toward more environmentally benign methods for controlling insect pests[5].

Insect-pathogenic or entomopathogenic fungi (EFP) (Fungi: Ascomycota, Order: Hypocreales) cause disease in insects. These entomopathogens are used as biocontrol agents, or "biopesticides," for the management of insect pests[6]. They provide an alternative to chemical insecticides for protecting crops[7] and reducing the harmful environmental impacts of chemical insecticides[8]. An increasing number of products based on EPF are being registered as insecticides and used in developed and developing countries like the United States of America, the United Kingdom, Australia, Canada, China, India, etc.[8].

Among the members of the genus Hypocreales, *Lecanicillium* sp., *Beauveria* sp., and *Metarhizium* sp. have been effectively used to control aphids, lepidopteron larvae, and other pests[9]. Among them, *Beauveria bassiana*

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(Balsamo) Vuillemin is responsible for causing white muscardine disease in a variety of insects. *Beauveria* infects the insect by degrading the host cuticle using mechanical and chemical forces, which are particularly advantageous in pest control because direct ingestion of fungal propagules is not needed by insects, thus also becoming active against the non-feeding stages of insects[10]. In addition, among the cyclic hexadepsipeptide mycotoxins produced by the different EPF, beauvericin, produced by *B. bassiana,* has shown the most effective larvicidal properties[11].

Like other Hypocreales, the species of *Beauveria* show pleomorphic life stages. They are often described as cryptic fungi, i.e., morphological characteristics are changed in response to the environment, and thus morphological description fails to clarify their systematics at species level[12]. Nowadays, researchers are using polymerase chain reaction (PCR) based molecular techniques to reconstruct the *Beauveria* phylogeny for accurate identification of *Beauveria* species. Among the molecular markers, the internal transcribed spacer (ITS) region of rDNA is considered a universal bar code for fungus identification[13]. But in case of Hypocreales, ITS analysis produced low resolution in many cases and failed to resolve the phylogeny of *Beauveria*[14]. Additional genomic markers like translation elongation factor-1α (TEF) are needed for the species-level determination of *Beauveria* to be made correctly[14].

Although *B. bassiana* showed a broad spectrum of pathogenicity against a wide range of insects, its bio-efficacy depends on the isolation source and life stages of the target stages. Insecticide resistance and resurgence issues can be effectively addressed by controlling insect pests with local isolates of fungus[15]. These native isolates also have higher survival and persistence abilities under local environmental conditions[16]. In conservation agriculture guidelines, it is also important to isolate potential native bioagents to prevent contamination from imported biopesticides. In addition, the pathogenicity of the biocontrol agent differs according to the different life stages of the target insect[17]. Identification of the more suspectable stage of insects against fungal inoculum increases the bio-efficacy of biological control strategies in field conditions. Therefore, the present investigation was carried out to isolate and molecularly characterize native *Beauveria* isolates and test their bio-efficacy against different life stages of *S. litura.*

Results

Isolates of *Beauveria*. Among the isolated fungal isolates on selective medium, seven isolates showed characteristics of the morphology of *Beuaveria* species. The single fungal colonies of the isolates were white in color, round, lightly elevated with a powdery appearance, and lightly downy with circular rings. Conidia were hyaline and round. Single cell conidiophores were short, densely clustered, and hyaline (Fig. 1).

Molecular identification and phylogeny of *Beauveria* isolates. The partial sequence datasets of ITS and TEF were processed and analyzed individually through Geneious V.11 software, and accession numbers were obtained from NCBI (Table 1). The genomic ITS and TEF data of seven isolated *Beauveria* isolates showed BLAST similarity, with many references to *B. bassiana* in BLAST search results in the NCBI database. The reconstructed maximum likelihood phylogenetic tree of the ITS data set showed that the seven morphologically characteristic isolates were clustered with the reference *B. bassiana* isolate with a moderate bootstrap support value (60%) (Fig. 2). Furthermore, a tree constructed with the TEF data set showed the maximum support (100%) for the clade containing isolated *Beauveria* isolates and references to *B. bassiana* (Fig. 3). Thus, both the ITS and TEF data sets confirmed the isolated strains as *B. bassiana*.

Biomass production of the fungal isolates. Overall mean mycelial growth revealed that the fungal isolate TGS2.3 (388.27 ± 10.29 mg/100 mL) exhibited the highest biomass production, and the lowest growth was observed in TGS1.2 (208.8 ± 8.03 mg/100 mL) (Fig. 4).

Insect bioassay. Seven days following infection of the 2nd larval instar by seven *B. bassiana* isolates revealed that TGS2.3 had the highest mortality rates (81.72 ± 2.15%) followed by TGS2.1 (72.40 ± 3.46%),

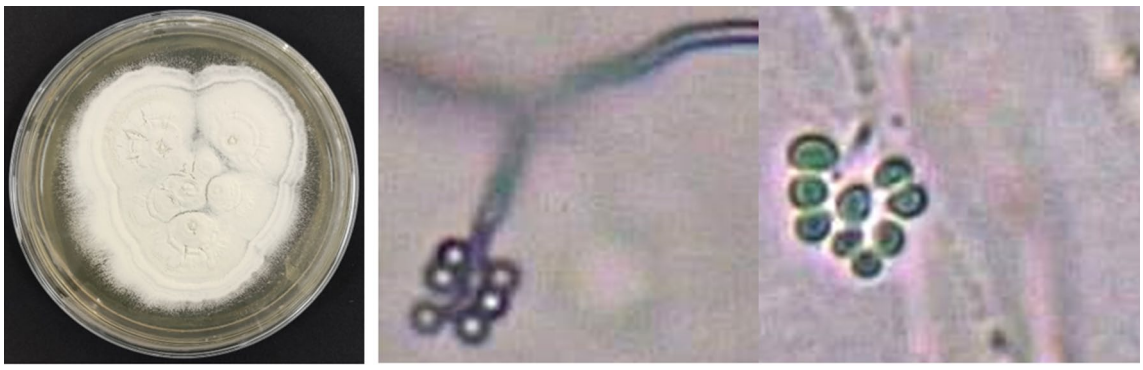

Figure 1. Morphological features of a representative *Beauveria* isolate TGS2.3. (**a**) 15 days old culture, (**b**) Conidiophore with conidia.

Serial no.	Isolate's name	ITS accession	TEF accession
1	KSS1.1	OP784778	OP785280
2	KSS2.2	OP784779	OP785281
3	TGS1.2	OP784780	OP785282
4	TGS2.1	OP784781	OP785283
5	TGS2.3	OP784782	OP785284
6	BeauA1	OP784783	OP785285
7	BeauD1	OP784784	OP785286

Table 1. NCBI accession numbers of isolated *B. bassiana* isolates.

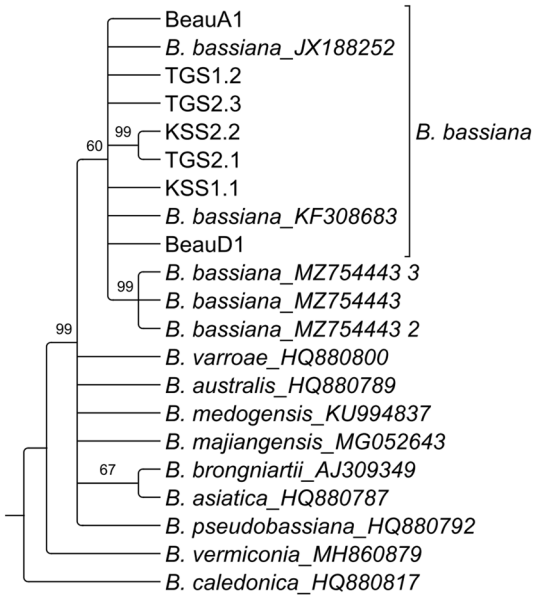

Figure 2. Maximum likelihood phylogenetic tree of the ITS data set of 1000 bootstrap replications in GTR-GAMMA model.

BeauD1 (61.29 ± 1.08%), BeauA1 (51.61 ± 2.15%), KSS1.1 (49.46 ± 4.69%), TGS1.2 (46.59 ± 2.71%), and KSS2.2 (43.73 ± 3.78%) (Fig. 5).

The findings implied that the death of 2nd instar larvae of *S. litura* treated with TGS2.3 and TGS2.1 occurred mostly during the first two days of infection, especially on the first day for TGS2.3. The mortality was caused more gradually from day-one to day-seven by the other *Beauveria* isolates, viz. BeauA1, BeauD1, KSS1.1, KSS1.2, KSS2.2, and TGS1.2 (Fig. 6).

As the first day was when the most deaths occurred, results were statistically analyzed to ascertain which isolates induced the highest day-one mortality (causing high mortality within 24 h of infection). Samples infected with TGS2.3 (56.67 ± 7.02%) had the highest day-one mortality, followed by TGS2.1 (43.33 ± 3.51%) (Fig. 7).

Hatchability and neonate larval mortality after TGS2.3 treatment. Egg hatchability was drastically reduced in the TGS2.3-treated eggs compared to the control. The isolate TGS2.3 induced 81.25 ± 2.75% egg mortality, whereas in control it was 18.5 ± 2.65% (Fig. 8). The 7-days post treatment data also revealed that TGS2.3 induced 57.25 ± 6.34% neonatal larval mortality, whereas in control it was 8.25 ± 2.63% (Fig. 9).

Bioassay against different larval stages of *S. litura* by *B. bassiana* isolate TGS2.3. The larvae treated with the TGS2.3 isolate succumbed to fungal infection and showed different mortality rates in various larval stages. The highest mortality was recorded in 1st instar larvae (94.45 ± 4.60%) and the lowest was in 5th instar larvae (56.56 ± 2.07%). The mortality rates in 3rd and 4th instar larvae were statistically similar (Fig. 10).

Cumulative mortality over 7 days demonstrates that 1st instar larvae had the highest day-one mortality, which progressively rose until the 4th day. The death of 2nd instar larvae began on day-one and subsequently increased until day-five. The 3rd instar larvae did not die until the 3rd day, and the death rate progressed until the 6th day. The death of larvae in the 4th and 5th instars occurred on the 4th day and subsequently increased

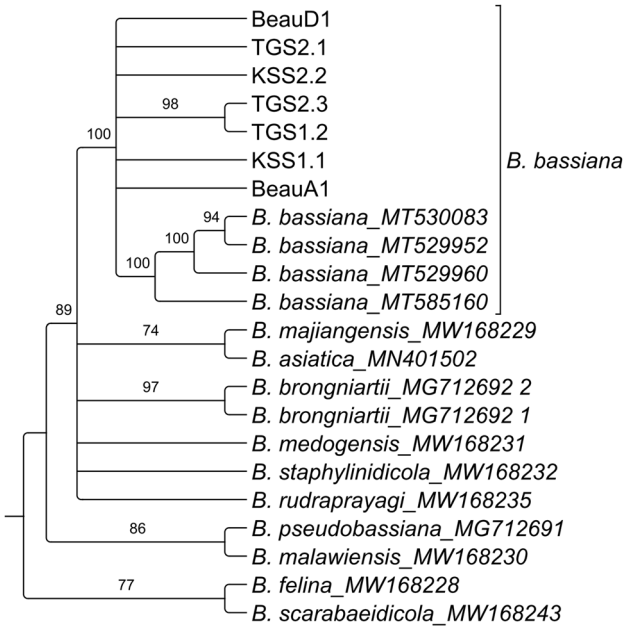

Figure 3. Maximum likelihood phylogenetic tree of the TEF data set of 1000 bootstrap replicates in GTR-GAMMA model.

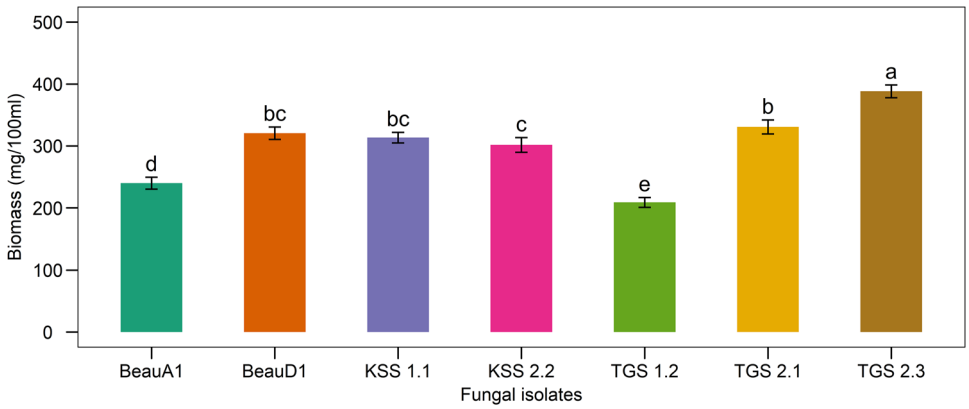

Figure 4. Biomass production of the *B. bassiana* isolates in SDA liquid broth. Values (means ± SEs) with different alphabetical letter(s) show statistically significant differences (lsd, $p < 0.05$).

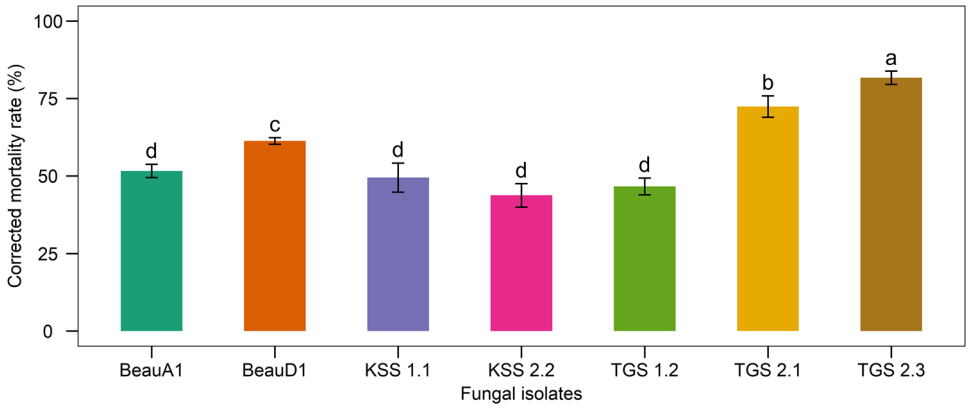

Figure 5. Mortality rates of 2nd instar larvae of *S. litura* treated with *Beauveria* isolates. Values (means ± SEs) with different alphabetical letter show statistically significant differences (lsd, $p < 0.05$).

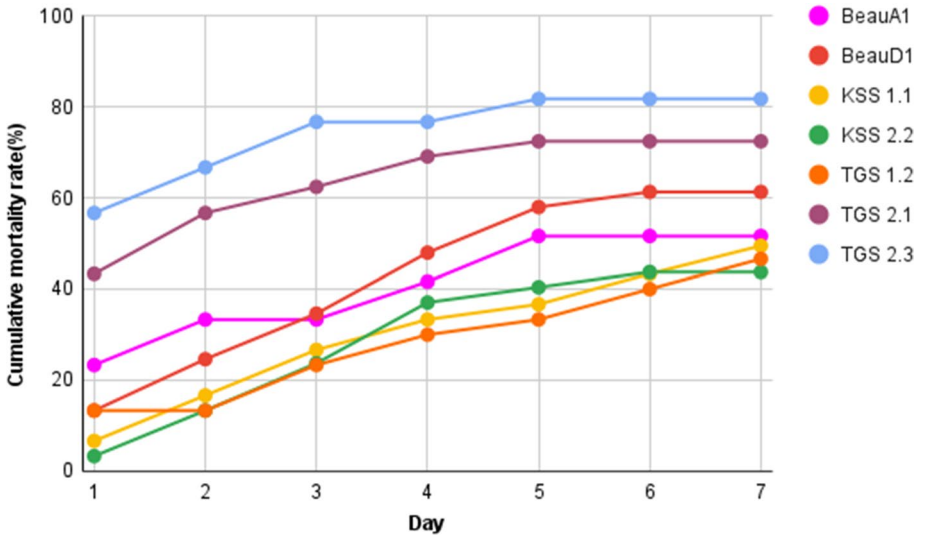

Figure 6. Cumulative mortality rates of *S. litura* treated with *B. bassiana* isolates over 7 days.

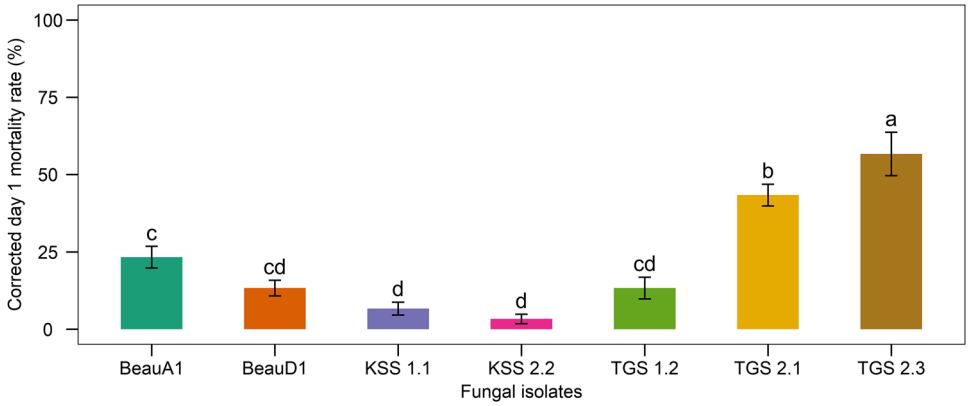

Figure 7. Day-one mortality of 2nd instar larvae of *S. litura* treated with *B. bassiana* isolates. Values (means ± SEs) with different alphabetical letter(s) show statistically significant differences (lsd, $p < 0.05$).

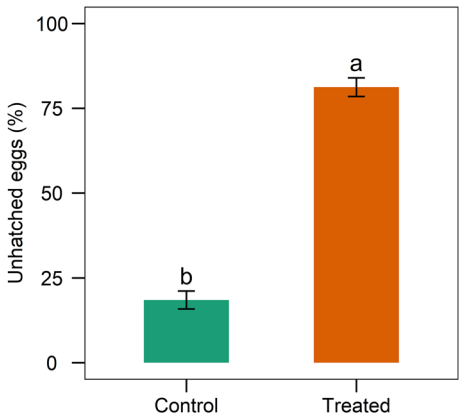

Figure 8. Mortality of *S. litura* eggs infected with *B. bassiana* isolate TGS2.3. Values (means ± SEs) with different alphabetical letter show statistically significant differences (lsd, $p < 0.05$).

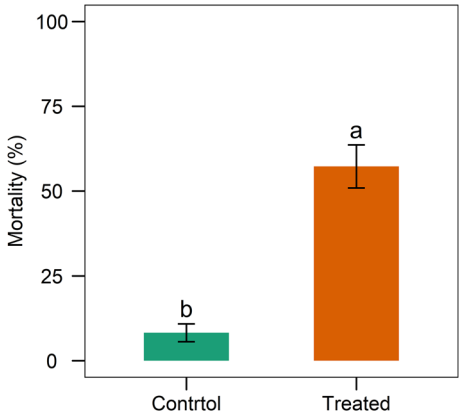

Figure 9. Mortality of neonate stage larvae treated with TGS2.3 at egg stage. Values (means ± SEs) with different alphabetical letter show statistically significant differences (lsd, $p < 0.05$).

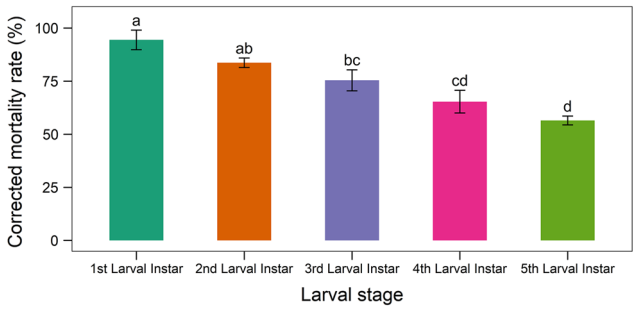

Figure 10. Mortality rates of different larval stages of *S. litura* treated with *B. bassiana* isolate TGS2.3. Values (means ± SEs) with different alphabetical letter(s) show statistically significant differences (lsd, $p < 0.05$).

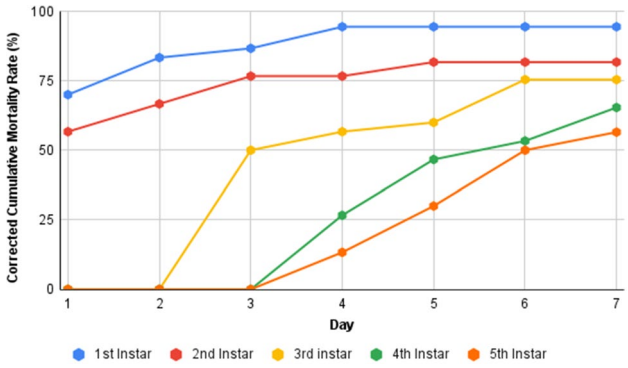

Figure 11. Cumulative mortality of different larval instar of *S. litura* treated with *B. bassiana* isolate TGS2.3.

until the 7th day (Fig. 11). Overall, the mortality across various time points revealed all larval instars of *S. litura* to be susceptible to the fungus TGS2.3.

Mycosis and sub-lethal effects. The mobility of the infected larvae was reduced. The larvae were stiff and rigid after dying. Within two days of death, the deceased larvae began to produce mycelium. (Fig. 12). The *B. bassiana* infection was verified by the slides prepared from this fungus' growth. When compared to control larvae, *B. bassiana* negatively impacted the emergence of adults from the 2nd, 3rd, 4th, and 5th instars. A smaller number of adults (7.11–37.94%) emerged from fungus treated larvae than from control larvae (94%) (Fig. 13).

Deformities. The fungal infection caused a wide range of abnormalities. When some of the treated larvae molted into pupae, they did not entirely detach from the exuvium (Fig. 14). Some pupae lacked a completely

Figure 12. Larval cadaver of *S. litura* mycosed by *B. bassiana* isolate TGS2.3.

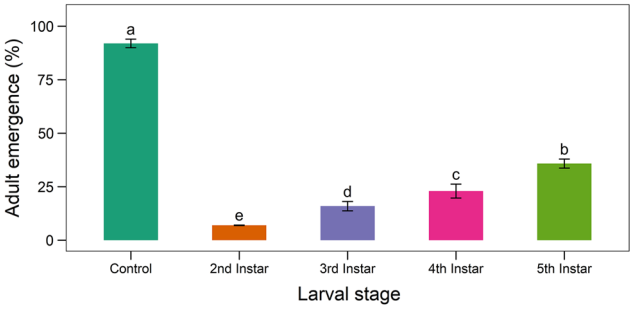

Figure 13. Adult emergence of larvae of *S. litura* due to treatment with *B. bassiana* isolate TGS2.3. Values (means ± SEs) with different alphabetical letter show statistically significant differences (lsd, $p < 0.05$).

Figure 14. Undetached adult from exuvium.

developed cuticle. When 2nd instar larvae were treated with *B. bassiana*, they had 9.33 ± 2.08 percent pupal deformities. Similarly, the pupal deformity was 7.67 ± 1.53, 10 ± 2 and 6.67 ± 1.53 percent owing to the treatment of 3rd, 4th, and 5th instar larvae, respectively (Fig. 15). Adults developed from fungus infected larvae had 3.67–10% deformities (Fig. 16) with folded, undeveloped wings (Fig. 17). The control group, however, showed no deformation.

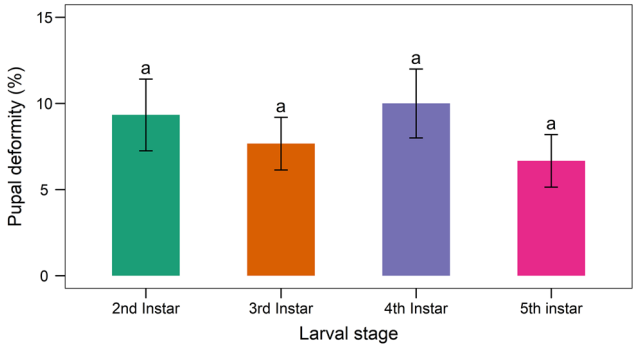

Figure 15. Pupal deformity of larvae of *S. litura* due to treatment with *B. bassiana* isolate TGS2.3. No statistically significant difference was observed among the values (means ± SEs) (lsd, $p < 0.05$).

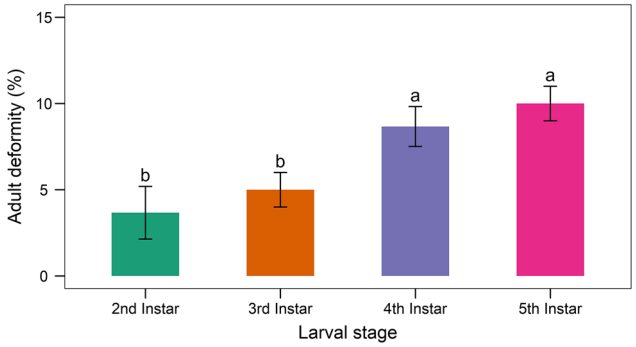

Figure 16. Adult deformity of larvae of *S. litura* due to treatment with *B. bassiana* isolate TGS2.3. Values (means ± SEs) with different alphabetical letter show statistically significant differences (lsd, $p < 0.05$).

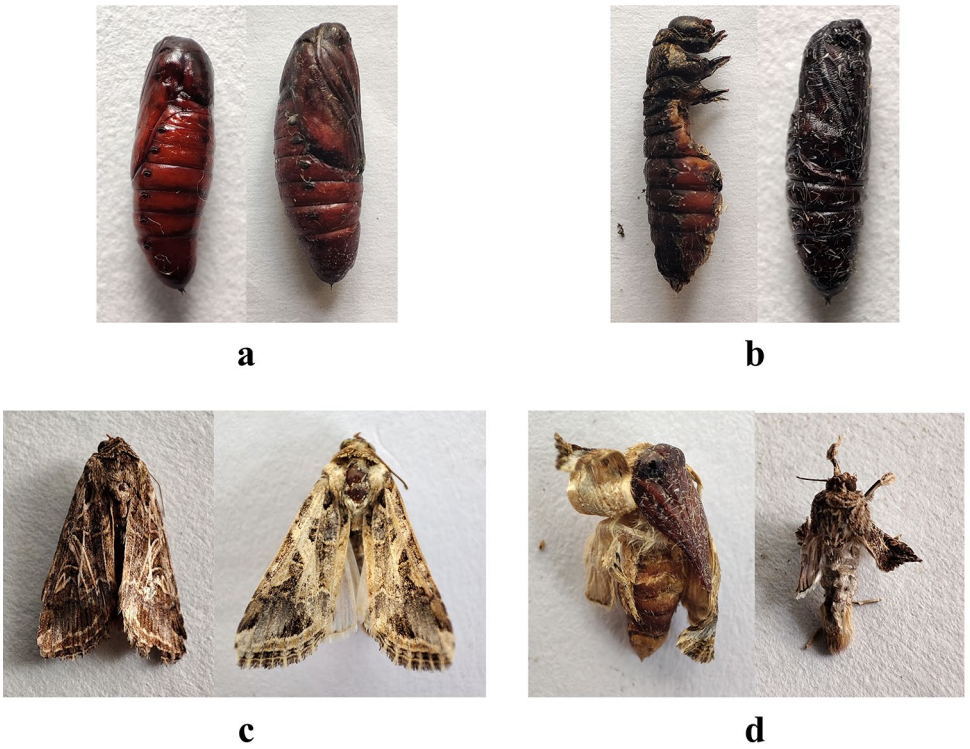

Figure 17. (**a**) Normal pupae, (**b**) Deformed pupae, (**c**) Normal adult, (**d**) Deformed adult.

Discussion

The tobacco caterpillar (*S. litura*) is one of the most devastating pests of various crops. Insecticides are the most commonly used method for controlling this problem. The use of pesticides leads to ecological imbalances by destroying non-target organisms and their natural enemies, parasites, and predators. The public's growing concern over the potential ecological and health risks of synthetic pesticides has shifted the focus of research toward more environmentally benign methods. Among them, *B. bassiana* causes white muscardine disease in a wide range of insects. Insecticide resistance and resurgence issues can be effectively addressed by controlling insect pests with local isolates of fungus and targeting more suspectable stages of insects.

In this study, seven *B. bassiana* isolates were isolated from soil samples and reported for the first time in Bangladesh as local isolates. The morphology described by previous studies[5,18].was similar to that of our seven isolates. The ITS phylogeny produced a moderate support value for these seven isolates, which confirmed the inadequacy of the ITS analysis that had been previously reported[1,14,19]. However, ITS could be used for quick screening of a wide range of field isolates because of its PCR amplification efficiency[20–22] and the availability of reference data[23]. Further molecular analysis with TEF data supported the phylogenetic position of seven isolates in the *B. bassiana* clade very strongly and proved its efficiency in resolving phylogenies for Hypocreales fungi[1,14,19].

To find the best insect pathogenic *B. bassiana* isolate, the overall and daily mortality of 2nd instar larvae was investigated to determine the mortality induced by each fungal isolate. The highest mortality rate was induced by *B. bassiana* isolate TGS2.3 and could be because of higher insect pathogenic properties like conidial adhesion, germination rate, growth condition, or the production of enzymes or secondary metabolites. The very first stage of fungal infection is conidial attachment, and the strength of conidial attachment is a crucial indicator of the virulence of an entomopathogenic fungus. Fungal cell attachment to the cuticle may involve specific receptor-ligand and/or nonspecific hydrophobic and electrostatic mechanisms[24–26]. If the adhesion strength of EPF is weakened, then it could result in the washing off of the conidia from the host, thus preventing the infection[27]. The fluctuation of virulence among different isolates of *B. bassiana* may be due to their different levels of hydrophobic nature or their biochemistry.

Secondary metabolite synthesis might let EPF get past the immunological defenses of the insects and hasten mycosis[6]. According to some research, EPF *B. bassiana* creates host-specific secondary metabolites that, at low quantities, may result in 50% mortality[11,28]. The strain TGS2.3, which showed the highest insect mortality rate, may produce biologically active compounds with insecticidal activity against *S. liturta*. Further investigation is required to determine the bioactive compounds produced by *B. bassiana* isolate TGS2.3. The investigation and production of these compounds may open up new arrays of possibilities for controlling invasive crop pests.

The parameters, such as conidial germination and the production of hydrolytic enzymes are associated with the virulence of EPF[21,29–31]. A faster germination rate was found to exhibit higher virulence in *B. bassiana*[29]. The day-one mortality of TGS2.3 was the highest among all the isolates, which suggested that TGS2.3 has a higher germination rate. Hydrolytic enzymes such as protease, chitinase, and lipase are secreted by EPF to degrade the cuticle of host species to infect them[32]. Higher enzyme activity may be one of the reasons for the higher virulence of TGS2.3. Further investigation is needed to verify these hypotheses for our high-performing *Beauveria* candidate, TGS2.3.

The immobility of eggs is the main reason for insect vulnerability to microbial infections[33]. The nutrient requirement of an egg to develop into a hatchling is excessive, and they are highly targeted by pathogenic microbes at this stage[34]. This study showed that the eggs of *S. litura* were highly susceptible to TGS2.3. Similar results were found in previous studies where *B. bassiana* induced egg mortality in *S. frugiperda*[35,36] and *Phthorimaea operculella*[37]. The isolate TGS2.3 also induced mortality in neonate larvae, which is similar to another study conducted by Idrees et al.[17].

The mortality of larvae was highest in the 1st instar, and it gradually decreased with the advancement of each stage. The 1st instar larvae experienced 38% higher mortality than the 5th instar larvae. This indicates the decreased susceptibility of larvae with age. Shweta and Simon[38] used *B. bassiana* against *S. litura* Fab. (Tobacco Caterpillar) in which the 1st and 2nd instar larvae showed higher mortality than the later stages. These variations in mortality between various instars might be attributed to enzymatic activity. According to different studies, detoxification enzyme activity changes significantly across and within developmental stages. The activity is modest in the egg stage, rises with each larval or nymphal instar, and ultimately decreases to zero during pupation[39,40].

The EPF isolate TGS2.3 demonstrated sub-lethality over the life stages of *S. litura*. Pupal and adult deformities were produced as a consequence of the fungal infection in the larval stage. The larvae were unable to adequately transition into pupae. Insect molting has reportedly been hampered by *B. bassiana*[41]. Since the development of new cuticles during the molting process heavily depends on nutrients, any stage in the process might be affected if there is a nutritional imbalance in the hemolymph caused by a fungal infection. This sub-lethality of *B. bassiana* isolate TGS2.3 suggests a relatively prolonged sub-lethal influence of the fungi on *S.litura*, which may reduce *S.litura* populations more effectively in addition to direct mortality.

In summary, this study found that, the most potent isolate, TGS2.3, was effective against egg hatching and all stages of *S. litura* caterpillars and suggested that this fungal isolate could be utilized to target both the hatching and feeding stages of this target insect. Alongside, early stages of larval development of *S. litura* were more susceptible to fungal infection. The sub-lethal effects also demonstrated that once exposed to an entomopathogen, *B. bassiana* isolate TGS2.3 has the capability to kill insects at any descendant life stage of insect and reduce adult emergence. This study warrants further *in planta* evaluation in both laboratory and field conditions to evaluate the bio-efficacy of native *B. bassiana* isolates precisely. However, the findings of this research provided the potential for developing alternative *S. litura* pest control techniques as well as for limiting the use of synthetic pesticides, thereby minimizing their detrimental effects on the ecosystem.

Methods

Collection of soil samples. Soil samples were obtained from the Bhawal Sal Forest and agricultural fields in Gazipur, Bangladesh. To construct the composite sample, five different soil samples weighing a total of 250–300 g were mixed from a depth of 10–15 cm using a soil sampler. Until they were all studied, the soil samples were kept in distinct zip-lock bags with labels and maintained at 4 °C in a cold room.

Isolation of fungus. A soil suspension containing five grams of soil and 50 mL of 0.1% Tween 80 was made in a screw-cap plastic tube and incubated at room temperature for 3 h after being sieved through a 5 mm screen. Five inversions of each tube were performed at intervals of 30 min. The tubes were retained for 20 s after incubation to allow for sedimentation, and 100 µL of supernatant from each tube was plated on a Petri plate with Sabouraud dextrose agar (SDA) medium (peptone 10 g/L, agar 10 g/L, dextrose 40 g/L, and CTAB 60 mg/L). To avoid bacterial contamination, streptomycin (30 mg/L) was also added. Following inoculation, all plates underwent a two-week incubation period at 22 °C. Every 2–3 days, plates were checked for recognizable, thick, compact white mycelium development. Hypocreales-like isolates were isolated and sub-cultured.

Morphological study. Both the vegetative and reproductive structures of fungal colonies on SDA were examined using microscopy immediately after sporulation. From the outermost part of the fungal colony, little plaques were transferred to glass slides and inspected under a compound light microscope.

Sub-culture, DNA isolation, and molecular characterization. On SDA agar plates without antibiotics, the fungal isolates were sub-cultured for DNA isolation and sequencing. The procedure described by Islam[1] was used to extract the DNA.

Briefly, a little lump of fungal mycelium from a 7-day-old culture was placed in an Eppendorf tube, mashed with a sterile plastic pestle, and then suspended in 1 mL of lysis buffer (400 mM Tris–HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl, and 1% SDS), which was then incubated at 50 °C for 1 h in a heat block. A volume of 150 µL of precipitation buffer (5 M potassium acetate, 60.0 mL; glacial acetic acid, 11.5 mL; distilled water, 28.5 mL) was added in the tube and vortexed shortly, then incubated on ice for 5 min. The supernatant from the centrifugation was transferred to a fresh tube along with 500 mL of isopropanol to precipitate the DNA. After centrifugation at 18,000 rcf for 20 min, the DNA pellet was recovered and washed with 1 mL of 70% ethanol. After being air dried for ten minutes, the DNA pellet was dissolved in 100 µL of Tris–EDTA (TE) buffer. In a nanodrop, the DNA's purity was examined. Polymerase chain reaction (PCR) was used to amplify the ITS region using the primers ITS1F: CTTGGTCATTTAGAGGAAGTAA and ITS4R: TCCTCCGCTTATTGATATGC in accordance with the profile: denaturation at 90 °C for 2 min, then 35 cycles of denaturation at 95 °C for 30 secs, annealing at 55 °C for 30 secs, extension at 72 °C for 1 min, and finally extension at 72 °C for 15 min[1]. The 5′-TEF region was amplified using EF1TF (5′-ATGGGTAAGGARGACAAGAC) and EF2TR (5′-GGAAGTACCAGTGATCATGTT) after the profile underwent an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 40 s, 65 °C for 40 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min[19]. The PCR product was electrophoresed in 1% agarose in 1 × TBE buffer at 120 V with GelRed nucleic acid stain and photographed with a molecular imager under UV light. For sequencing, the PCR products were sent to Macrogen, Korea.

Sequence analysis and phylogenetic tree preparation. The Sanger sequencing data of the fungal isolates were produced, and a BLAST search on the National Center for Biotechnology Information (NCBI) database was completed. The partial sequence datasets of ITS and TEF were submitted to NCBI for getting accession number. The sequences matched reference genome sequences obtained from NCBI. The Geneious V.11's MAFT plug-in was used for multiple alignments, and the final alignment was fixed manually. Phylogenetic trees were developed by maximum likelihood analysis for the data sets using the Geneious V.11 RAxML plug-in using rapid bootstrapping and searching for the best scoring ML tree from 1000 bootstrap replicates in the GTR-GAMMA model.

Insect rearing. Eggs of *S. litura* were obtained from the existing culture at the Entomology Division, Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh. Homogenous larvae were obtained from eggs hatched on the same day. The larvae were grown in sterile plastic boxes containing pieces of okra disinfected with 0.5% (v/v) sodium hypochlorite for 10 min, maintained at 25 ± 2 °C and 65 ± 5% RH[42].

Production and Collection of *Beauveria* conidia. Sabouraud's Dextrose Agar (SDA) medium was used in this study. A 10 mm actively grown culture of *B. bassiana* was placed individually at the center of the 60 mm petri dish containing 10 mL of solid SDA media[43]. The inoculated plates were incubated at 28 °C for 7 days. The conidial suspension of the isolates was then prepared by flooding the dishes with 10 mL of sterile Tween 80 (0.05%), the agar surface was gently scraped with sterile glass rods, and the suspension was collected in sterile 250 mL beakers. The suspension was then adjusted to 50 mL and mixed using a hand mixer to separate and disperse the conidia, and finally the conidial density was adjusted to 1.5×10^8 conidia per ml using a hemocytometer[44]. Before the bioassay experiment, conidial germination was tested on SDA agar medium.

Growth in liquid medium. A volume of 250 mL Sabouraud's Dextrose Broth (SDB) was prepared in a 500 mL Schott bottle, and the final pH was adjusted to 6.5. The liquid broths were then inoculated with a 10 mm culture disc of the fungus. Three replications were maintained for all the *B. bassiana* isolates. The entire setup was kept in a shaker incubator at 25 °C temperature at 120 rpm for 10 days. White cotton ball-type growth was

observed after 7 days. The mycelia were then filtered through a pre-weighed filter paper and dried in a hot air oven at 70 °C until a constant weight was obtained. This revealed the biomass production capability of all the fungal isolates [43].

Virulence of *B. bassiana* isolates against eggs and hatched larvae. Freshly laid egg masses that were 1–2 days old were collected and counted under a dissecting microscope. A batch of 50 eggs was separated using a hairbrush and transferred into a petri dish. A volume of 10 mL of conidial suspension (1.5×10^8 conidia/mL) was made using 0.05% Tween 80. The suspension was then sprayed over the egg masses. For control, only Tween 80 was used. Each treatment was repeated four times. 7 days after the treatment (DAT), the number of hatched and unhatched eggs was counted. The newly hatched larvae were then fed, incubated at 25 ± 2 °C, and monitored for the next 7 days. The mortality of each treatment was carefully recorded[17].

Insect bioassay. Freshly laid eggs were collected and hatched to obtain homogenous larvae. The assay was conducted on 2nd instar larvae of *S. litura*. A set of 10 larvae in triplicate were dipped individually into a 10-mL conidial suspension of *Beauveria* isolates (1.5×10^8 conidia/mL) for 5 s. After treatment, transferred each set of larvae to a separate, sterile plastic box. To each box, added moist blotting paper and a piece of disinfected okra as feed. Changed the paper and feed on alternate days. At 7 DAT, the mortality of larvae was recorded according to the isolates[42].

Evaluation of sublethal effects. Larvae that survived the fungal treatment were further reared until pupation at 25 ± 2 °C and 60–70% relative humidity to see the sublethal activity, such as variation in development, any kind of deformity, and longevity compared to the control. Observations were made on larval and pupal deformity, adult emergence, and any morphological deformity in various developmental stages[3].

Statistical analysis. Mortality was corrected by Abbott's formula[45]. The percent data were transformed by the arcsine transformation. The data were subjected to an analysis of variance (ANOVA), followed by a comparison of the means of different treatments using the least significant difference (LSD). Analyses were performed using R version 3.4.2.

Data availability

The partial sequence data of ITS and TEF genomic regions of fungal isolates during the current study are available in the NCBI repository under the Accession Numbers OP784778–OP784784 and OP785280–OP785286 (will be available on December 4 2022), respectively. The statistical datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

S.M.N.I. conceptualized the idea, supervised experiments, wrote and edited the manuscript. M.Z.H.C. designed experiments, analyzed data and wrote the manuscript. M.F.M. performed fungal and molecular study, M.B.M. conducted insect bioassay, T.I. contributed in interpretation, reviewed and edited the manuscript. Correspondence and requests for materials should be addressed to S.M.N.I. or T.I.

Competing interests

The authors declare no competing interests.

Additional information

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Seed priming with *Beauveria bassiana* improves growth and salt stress response in rice

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ABSTRACT

One of the most important abiotic factors that hinder plant development, growth, and production is salt stress. In recent years, there has been a lot of interest in the biological treatment of salt stress in plants using beneficial microbes. The fungal endophyte *Beauveria bassiana* provides a wide variety of ecosystem services, like suppressing insect pests and pathogens and enhancing plant growth. However, the role of *B. bassiana* in reducing salt stress in plants has not yet been clarified. This study was undertaken to evaluate the performance of *B. bassiana* isolate BeauA1 primed rice under salt stress by estimating rice growth, stress parameters, and mitigator characteristics. Primarily, rice seeds were primed with BeauA1 and placed in an agar medium with 120 mM NaCl (≈12 dS m^{-1} salt solution) to observe the role of BeauA1 in the early establishment of rice seedlings in salt conditions. Seed priming with BeauA1 resulted in an enhancement of rice growth attributes under both control and NaCl stress conditions. In the pot experiment, the BeauA1 primed rice seedlings were planted in soil with different concentrations of salt, viz. 8, 10, and 12 dS m^{-1}. The BeauA1 primed rice plants showed improvement in leaf succulence, leaf area, photosynthetic pigments, and shoot relative water content (RWC), leading to enhanced growth under both salt stress and control conditions. The biochemical study found that BeauA1 considerably increased proline content, total soluble sugars, total carbohydrates, and K^+/Na^+ in leaves. The antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione *S*-transferase (GST), and nonenzymatic antioxidants phenol and flavonoid were upregulated in BeauA1-primed plants under both control and stressed conditions. Further significant reductions of the lipid peroxidation products malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) by BeauA1 under salt stress were consistent with higher antioxidant activities in salt stress conditions. Principal component analysis (PCA) further validated BeauA1-primed plants' modulation of growth, antioxidant defense, and reduction of MDA and H_2O_2 in rice under salt-stress conditions. Our findings indicated that utilizing BeauA1 to reduce salt stress would be a useful strategy to increase rice yield in salt-affected regions.

1. Introduction

The world's most productive regions for sustainable agriculture are now being affected by soil salinization in many countries (Clarke et al., 2015). An increase in soil salinity is perceived as an obvious constraint for plant populations locally and globally (Munns and Tester, 2008; Minhas and Dagar, 2016). Globally, 1.5 million hectares (Mha) of irrigated land are expelled from production each year owing to soil salinity, and more than 45 Mha of irrigated land have been affected by salt. By 2050, it is predicted that more than 50% of the agricultural land will have started to become salinized (Jamil et al., 2011; Kumar and Sharma, 2020). To ensure sustained agricultural productivity, effective strategies should be established with local and global implications (Pereira et al., 2020).

Rice (*Oryza sativa* L.), one of the most important cereal crops, is a staple food for more than 3.5 billion people globally, mainly in

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Bangladesh, China, and India. The cultivation of rice in these areas is hindered by salt, the second most prevalent problem after drought. Due to its great sensitivity to salt, especially during the seedling and reproductive phases, salinity has a profound effect on rice's development and productivity (Lutts et al., 1995; Khan and Abdullah, 2003; Lou et al., 2012; Mishra et al., 2013). According to the International Rice Research Institute (IRRI) (2006), soil salinity above EC 4 dS m^{-1} is moderate for rice, and soil salinity above 8 dS m^{-1} is severe. Salinity may cause a number of morphological, physiological, or biochemical alterations and symptoms in rice plants, as well as death in severe circumstances (Gupta et al., 2021). Efforts to increase rice resistance to salt stress through classical breeding have produced limited success, and most of the current varieties are unable to tolerate excessive salinity (Rabbani et al., 2013; Solis et al., 2020).

The detrimental consequences of soil salinity on plants often take the form of osmotic stress, ion imbalance, disruption of nutritional balances, oxidative damage from reactive oxygen species (ROS), metabolic abnormalities, and decreased cell division (Ali et al., 2004; Parida and Das, 2005; Tahjib-Ul-Arif et al., 2018; Hafez et al., 2021a). Together, these effects reduce plant development, growth, and, eventually, crop production (Gharib et al., 2016). Osmotic stress caused by highly salinized soil prevented nutrient absorption and decreased water absorption, which resulted in physiological dehydration (Hafez et al., 2015). The sodium ion alone damages plants when they are exposed to soil salinity, and a larger concentration of Na^+ in the root zone decreases K^+ absorption due to their antagonistic actions. (James et al., 2011; Hussain et al., 2017; Saad Kheir et al., 2019; Hafez et al., 2021b). The source-sink connection and photosynthesis are disturbed because of the excess Na^+ concentration in the cytoplasm brought on by salt salinity, which interferes with cellular activities like protein and enzyme synthesis (Ahmad and Prasad, 2011; Hirasawa et al., 2017; Chourasia et al., 2021). In response to salt stress, there is an increase in the production of ROS in plants, including singlet oxygen, superoxide, hydroxyl radicals, and hydrogen peroxide. Numerous cellular components, including proteins, lipids, and DNA, can be affected by salinity-induced ROS production, interfering with vital cellular processes in plants (Gupta and Huang, 2014; Kumar et al., 2017; Khedia et al., 2019). Reactive oxygen species (ROS) can cause oxidative damage to plant cells, but plant cells also have a variety of defense and repair mechanisms that can reduce the possibility of this occurring. The induction of ROS-scavenging enzymes is the most common method of detoxifying reactive oxygen species (ROS) that are produced during the stress response. These ROS-scavenging enzymes include both enzymatic antioxidants, like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), and ascorbate peroxidase (APX), as well as non-enzymatic antioxidants, like glutathione (GSH), ascorbate (AsA), total phenolics, total flavonoids, tocopherols, carotenoids, etc. (Tanveer and Shabala, 2018; Mostofa et al., 2020; Tewari et al., 2021; Bhat et al., 2022). Cellular osmolytes such as proline and soluble sugars also play a crucial role in osmotic adjustment while acclimating to osmotic stresses (Ozturk et al., 2021; Ghassemi-Golezani and Abdoli, 2022).

Plant-microbe interactions lead to a variety of rhizospheric and systemic responses that improve plants' capacity to respond metabolically to salt stress (Nguyen et al., 2016; Jamil et al., 2022). Root colonization by arbuscular mycorrhiza alleviated salt stress by activating the ROS-scavenging system in wheat (Talaat et al., 2014). The species of *Trichoderma* mitigate salt stress by stimulating root growth (Mastouri et al., 2010) and the antioxidative defense system (Ahmad et al., 2015). Thus, the use of plant growth-promoting microorganisms in crop cultivation has become a viable strategy to reduce crop loss under challenging environmental conditions, such as salinity (Calvo et al., 2014; Chauhan et al., 2022). *Beauveria bassiana* (Bals.-Criv.) Vuill (Hypocreales: Cordycipitaceae) is an endophytic fungus that dwells in plant tissues and provides protection against herbivorous pests and pathogens. It also increases nutrient uptake and water use efficiency under stress conditions (Dara et al., 2017; Eid et al., 2019). However, the role of *Beauveria* in improving plant growth under salt stress conditions is still not studied. Considering the above facts, the present study evaluated *Beauveria* primed rice performance under salt stress conditions by estimating rice physiological traits and elucidating the role of *B. bassiana* in mitigating rice salt stress-induced oxidative damage by estimating stress parameters, enzymatic antioxidants, and non-enzymatic osmoprotectants.

2. Materials and Methods

2.1. Plant and fungal materials

Rice seeds of BRRI Dhan 89 were collected from the Bangladesh Rice Research Institute (BRRI), Bangladesh. The *Beauveria bassiana* isolate BeauA1 (GenBank: OP784783) from our culture collection was used as experimental material. The fungus was previously isolated from Bhawal Sal Forest and characterized by morphology and genomic analyses (Islam et al., 2023).

2.2. Experiment 1: in vitro evaluation of BeauA1 colonized rice performance under salt stress conditions

This *in vitro* experiment was carried out to examine the colonization capacity of BeauA1 in rice roots and evaluate its effect on rice seedlings under both normal and salt-stress conditions.

2.3. Fungal inoculum preparation

Spores of BeauA1 were collected from a 14-day-old fungal culture, diluted in Tween-80 (0.05%) solution to achieve 1×10^8 conidia/ml, and stored at 4 °C.

2.3.1. in vitro evaluation

Sterilized, healthy rice seeds were soaked in distilled water and kept in the dark for 48 h at room temperature for faster germination. Half of the germinated rice seeds were soaked in the BeauA1 spore suspension for 24 h. Another half of the germinated rice seeds were soaked in a Tween-80 solution for 24 h. Seven germinated rice seeds were placed in each transparent jar (height × diameter = 13 cm × 6 cm) containing 1.0% agar medium under four treatment conditions, i) seed without BeauA1 and salt solution (Control), ii) seed only treated with BeauA1, iii) seed only treated with 120 mM salt NaCl (≈12 dS m^{-1} salt solution), and iv) seed treated with both BeauA1 and 120 mM NaCl. There were three replications per treatment. Plants were grown in an incubator (Thermostable GC-450, DAIHAN Scientific, Korea) for a day/night cycle of 16/8 h with 65% relative humidity and 600 mol m^{-2} s^{-1} light intensity. After 15 days of NaCl treatment, rice seedlings were collected, to estimate plant characteristics.

2.3.2. Confirmation of endophytic colonization by BeauA1

A small piece of root (approximately 3 mm) cut carefully during harvest and preserved in zipper bags was used immediately after harvesting. Each sample was surface sterilized using 70% ethanol, followed by rinsing three times with sterile distilled water and drying on sterile paper towels. Each clipped root was then cut into six pieces with an average length of 0.5 mm. All the pieces were put on petri dishes (6 pieces per 60-mm petri dish) with SDAY medium with 2 mg/L of each antibiotic penicillin, streptomycin, and tetracycline. Then, petri dishes were sealed with parafilm and incubated at 25 °C in the incubator for one week. Plates were checked every 2–3 days to observe fungal development. *B. bassiana* growth in treated plant roots was recorded by observing its traits visually in petri dishes (Parsa et al., 2013).

2.3.3. Experiment 2: Evaluation of BeauA1 colonized rice performance under salt stress conditions in the pot experiment

The purpose of the pot experiment was to assess BeauA1's impact on rice grown on soil with varying salt concentrations.

2.3.3.1. Soil and pot preparation. Plastic pots (height × diameter = 20 cm × 11 cm) were filled with a 4 kg soil mixture (cow dung: sand: soil: 1:0.5:2 (in weight basis). Ten days after sowing, urea (@ 4.0 g per liter of water) was applied to ensure an adequate supply of nitrogen fertilizer.

2.3.3.2. Fungal treatment of rice seeds. Rice seeds were primed with or without BeauA1 and planted in pots with eight treatment levels: T1) Rice plant without BeauA1, no salinity condition (Control); T2) Rice plant with BeauA1, no salt condition (BeauA1); T3) Rice plant in 8 dS m^{-1} salt without BeauA1 (8 dS m^{-1}); T4) Rice plant in 8 dS m^{-1} salt with BeauA1 (8 dS m^{-1} + BeauA1); T5) Rice plant in 10 dS m^{-1} salt without BeauA1 (10 dS m^{-1}); T6) Rice plant in 10 dS m^{-1} salt with BeauA1 (10 dS m^{-1} + BeauA1); T7) Rice plant in 12 dS m^{-1} salt without BeauA1 (12 dS m^{-1}); and T8) Rice plant in 12 dS m^{-1} salt with BeauA1 (12 dS m^{-1} + BeauA1). Each treatment consisted of six plants and had four replications. In a net house, the pots were arranged randomly during the rice-growing season in Bangladesh (January to April).

After 30 days, the soil salinity at the targeted level in the pot was achieved by the method described by Kusvuran et al. (2013) and Saleque et al. (2015). Thirty-day-old seedlings of T1 and T2 were irrigated with tap water. Others were irrigated with 8 dS m^{-1}, 10 dS m^{-1}, and 12 dS m^{-1} of saline water (600 ml to each pot) at a one-day interval to induce moderate and high stress for 14 days (7 times in total). During the night, the pots were covered with temporary shade made with polythene sheets to prevent any precipitation. 14 days after salinity treatment, the plants were harvested when visual symptoms of salinity stress appeared to assess various morphological, physiological, and biochemical characters.

2.3.3.3. Observation of growth parameters. Growth parameters, viz., shoot length, root number, and fresh and dry weight of shoot and root, were recorded 14 days after treatment imposition at the final harvest.

2.3.3.3.1. SPAD value and chlorophyll content determination. Leaf greenness was estimated using the SPAD (Soil Plant Analysis Development) value at final harvest using the SPAD meter (Model 502 Plus, Konica-Minolta, Japan). At each evaluation, the content was measured three times from leaf to base, and the average was used for analysis. For estimating photosynthetic pigments, 0.1 g of fresh leaf sample was homogenized in 2 ml of 80% acetone, and chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were estimated from the leaf samples using the method described by Arnon (1949) and Lichtenthaler and Wellburn (1983).

2.3.3.3.2. Measurement of leaf area, leaf succulence, and relative water content (RWC). The upper three leaves were taken from each plant for the determination of the total leaf area, according to Carleton and Foote (1965). Leaf succulence was estimated using 9 leaves collected from three plants of each treatment (3 leaves/plant). Leaf succulence was calculated for each sample using leaf fresh weight and leaf area according to Silveira et al. (2009). The relative water content (RWC) was estimated according to Nishiyama et al. (2011) by using turgid weight (TW) and dry weight (DW).

2.3.4. Potassium (K^+) and sodium (Na^+) determination

The K^+ and Na^+ contents in rice shoots and roots were estimated by the method of Mendes et al. (2006) using a flame emission spectrophotometer (Spectrolab Analytical, UK).

2.3.4.1. Proline content, total soluble sugar, and total carbohydrate contents determination. Proline extractions were done using the method outlined by Bates et al. (1973). Total soluble sugar and total carbohydrates were assessed at 1.5 ml of 80% ethanol by homogenizing 0.1 g of fresh leaf samples following Dubois et al. (1956) using glucose as a carbohydrate standard.

2.3.4.2. Analysis of membrane Lipid Peroxidation and hydrogen peroxide (H_2O_2) contents. Malondialdehyde (MDA), a degraded byproduct of the membrane lipid's peroxidized polyunsaturated fatty acid content, was measured to calculate the degree of membrane lipid peroxidation using thiobarbituric acid (TBA) as the reactive material described by Heath and Packer (1968). Hydrogen peroxide (H_2O_2) content was estimated following the methods of Yu et al. (2003) by homogenizing fresh leaf samples (0.1 g) with 1.5 ml of 0.1% trichloroacetic acid (TCA).

2.3.4.3. Total phenolics and flavonoid contents determination. The leaf sample (0.1 g) was homogenized with 1.5 ml of methanol (100%) followed by centrifugation at 11,500g for 15 min at 4 °C (TOMY MX-307 high-speed refrigerated microcentrifuge, Japan). The same supernatant was then used to quantify total phenolics and total flavonoid contents following the protocols of Ainsworth and Gillespie (2007) and Zhishen et al. (1999), respectively.

2.3.4.4. Protein and enzyme activity determination. Using pre-cooled mortars and pestles, the leaf samples (0.5 g) were homogenized in 1 ml of extraction buffer containing 1 mM ascorbic acid, 1 M KCl, 0.5 M K-P buffer (pH 7.0), β-mercaptoethanol (5 mM), and 10% (v/v) glycerol. The homogenates were centrifuged at 11,500g for 15 min to extract the supernatants, which were then utilized to measure the protein and antioxidant enzyme activity. The extraction process was done under cold conditions. The protein concentration of each sample was determined following the method of Bradford (Bradford, 1976), using BSA as a protein standard.

The catalase (CAT, EC: 1.11.1.6) activity was determined using a spectrophotometer (Genesys 10 S UV/ Vis, Thermo Fisher Scientific, UK) by the method of Hasanuzzaman et al. (2014). The peroxidase (POD, EC: 1.11.1.7) activity was assessed according to the method of Hemeda and Klein (1990). The glutathione S-transferase (GST, EC: 2.5.1.18) and ascorbate peroxidase (APX, EC: 1.11.1.11) activities were measured by the protocols of Hasanuzzaman et al. (2014).

2.3.5. Statistical analysis

One-way analysis of variance (ANOVA) and post-hoc analyses were conducted to determine significant variations among the treatments (LSD, $P < 0.05$) using Statistix v.10 software. Hierarchical clustering and principal component analysis (PCA) for the first two components (PC1 and PC2) were carried out using the "pheatmap" and "devtools" packages in R, respectively.

3. Results

3.1. BeauA1 improved the phenotypic appearance of rice plants under in vitro salt stress conditions

Under *in vitro* non-stressed conditions, rice seed primed with BeauA1 enhanced the visual appearance of the "BeauA1″ plants compared with the "Control" plants (Fig. 1). The rice plants under 120 mM NaCl stress for 15 days exhibited considerable phenotypic disruption, including stunted growth, early senescence, leaf decolorization (changed to pale and yellow), a decrease in root length, and wilting (Fig. 1). In contrast, plants treated with BeauA1 significantly decreased salinity-induced toxic effects in "120 mM NaCl + BeauA1″ plants, and as compared to the corresponding salt-stressed plants, their improved phenotypes, such as reduced wilting and yellowing of leaves, delayed leaf senescence, and increased root length, were visible (Fig. 1).

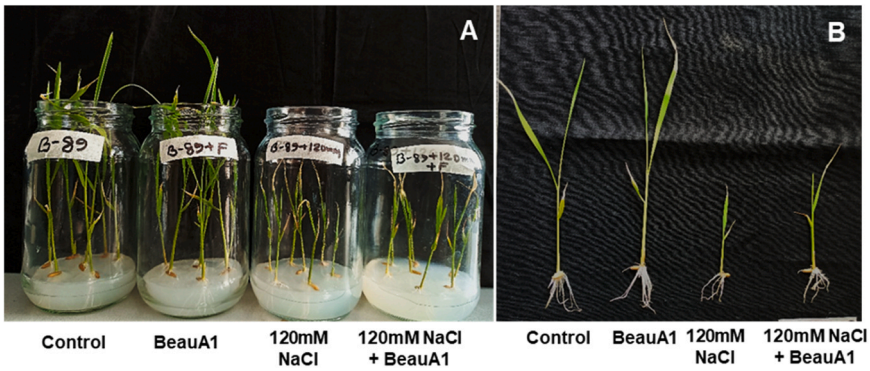

Fig. 1. Effects of BeauA1 on the phenotype of rice plants under salt stress conditions. (A) Representative photographs of rice plants with or without salinity in 1.0% agar medium at 15 days. (B) View of representative leaves and roots showing a positive effect of *Beauveria* on salt-stressed rice plants.

3.2. BeauA1 improved rice growth under in vitro salinity

BeauA1-primed only plants exhibited improved shoot and root length, shoot and root fresh weight, shoot and root dry weight, and root number by 13.69%, 6.71%, 7.58%, 7.98%, 8.97%, 22.63%, and 21.43%, respectively, over the untreated control (Fig. 2).

Fifteen days after NaCl treatment, the 120 mM of NaCl stress decreased rice seedling growth. The shoot height, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, and root number decreased in salt-treated plants by 50.37%, 24.39%, 61.42%, 57.42%, 60.30%, 61.32%, and 35.71%, respectively, compared to the control (Fig. 2). However, compared to NaCl-stressed plants, BeauA1-primed plants showed improved shoot height, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, and root number by 15.43%, 9.68%, 20.13%, 24.28%, 23.89%, 56.38%, and 21.43%, respectively, under the 120 mM NaCl condition (Fig. 2).

4. BeauA1 colonized rice roots under saline condition

BeauA1 colonization was confirmed by visual observation of a white fungal colony on root sections (supplementary data, Fig. S1). The average colonization was 72.22% in only BeauA1-primed plants and 66.67% in the "BeauA1 and 120 mM NaCl" treatment. No fungal colonization was found in "control" and only "120 mM NaCl" treated plants.

4.1. BeauA1 improved the phenotypes of rice plants under soil salinity conditions

The toxic effects of soil salinity on rice plants were examined at three levels of salt stress (8, 10, and 12 dS m^{-1}) in pots. Rice plants exposed to 8, 10, and 12 dS m^{-1} salt stress for 14 days showed substantial phenotypic disruption, including early senescence, chlorosis, and even burning of leaves, stunted growth, and wilting, as compared with the "Control" plants (Fig. 3). On the other hand, the rice seeds primed with the endophytic fungus BeauA1 notably reduced the salt-induced detrimental effects and improved plant growth compared to the plants treated with saline water (Fig. 3).

4.2. BeauA1 improved the growth parameters of rice plant under soil salinity stress

BeauA1-treated only plants showed enhancements of the shoot and root length, root and shoot fresh weight, and root and shoot dry weight by 13.29%, 37.25%, 77.17%, 39.81%, 61.53%, and 41.86%, respectively (Fig. 4). The leaf area, leaf fresh weight, and leaf succulence were improved by 29.21%, 100%, and 52.25%, respectively, in BeauA1-treated plants compared with the corresponding data obtained from the "Control" plants (Fig. 5).

Inversely, in comparison with the untreated control, 8, 10, and 12 dS m^{-1} salinity levels noticeably decreased shoot height (by 3.45%, 5.55%, and 10.27%, respectively), root length (by 12.18%, 17.66%, and 23.14%, respectively), shoot fresh weight (by 14.93%, 25.79%, and 35.74%, respectively), root fresh weight (by 10.86%, 32.60%, and 45.65%, respectively), shoot dry weight (by 23.25%, 30.23%, and 39.53%, respectively), and root dry weight (by 23.07%, 38.46%, and 46.15%, respectively) (Fig. 4). The 8, 10, and 12 dS m^{-1} salinity levels also reduced leaf area (by 18.80%, 27.33%, and 35.81%, respectively), fresh leaf weight (by 25%, 37.5%, and 56.25%, respectively), and leaf succulence (by 10.12%, 14.13%, and 31.26%, respectively) (Fig. 5).

Rice plants with BeauA1 exhibited significant enhancement of plant growth under salt-stress conditions. Remarkable improvements in shoot height (by 14.42%, 13.81%, and 13.67%), root length (by 42.43%, 40.32%, and 38.17%), shoot FW (by 45.74%, 37.19%, and 28.16%), root FW (by 75.94%, 74.19%, and 70.37%), shoot DW (by 57.57%, 50%, and 46.15%), root DW (by 90%, 88.88%, and 85.71%) (Fig. 4), leaf area (39.52%, 43.12%, and 47.35%), leaf succulence (48.92%, 21.62%, and 37.34%), and leaf fresh weight (108.33%, 80%, and 53.33%) (Fig. 5) were improved in "8 dS m^{-1} + BeauA1″, "10 dS m^{-1} + BeauA1″ and "12 dS m^{-1} + BeauA1″ plants, respectively, when contrasted with the corresponding "8 dS m^{-1}", "10 dS m^{-1}", and "12 dS m^{-1}" plants.

4.3. BeauA1 improved SPAD value and photosynthetic pigments of rice plants under soil salinity stress

Salinity stress caused a profound decrease in SPAD chlorophyll value in rice leaves. Upon exposure to 8, 10, and 12 dS m^{-1} salt stress, the SPAD value decreased (by 10.75%, 17.58%, and 20%, respectively) compared to the untreated control (Table 1). Application of BeauA1 reduced these effects of salt stress. Under salt stress conditions (8, 10, and 12 dS m^{-1}), treated rice plants with BeauA1 showed increased SPAD values (10.75%, 10.48%, and 3.24%, respectively) compared to the salt-stressed plant alone. The value found with the "8 dS m^{-1} + BeauA1″ treatment combination was almost similar to the control. In addition, BeauA1-primed only plants also enhanced the SPAD value by 10.69% compared to the untreated control plants (Table 1). Although SPAD value profoundly decreased under salt stress conditions, BeauA1, "8 dS m^{-1} + BeauA1″, "10 dS m^{-1} + BeauA1″, "12 dS m^{-1} + BeauA1″ treatments significantly increased the value (Table 1).

To assess the role of the endophytic fungus *B. bassiana* on the photosynthetic parameters under salt stress, the quantities of photosynthetic pigments like Chl a, Chl b, total Chls, and carotenoids in salt-exposed rice leaves were determined (Table 1). In comparison with control, a sharp decline in the content of Chl a (by 21.98%, 53.40%, and 61.78%), Chl b (by 40.29%, 55.22%, and 59.70%), total Chls (by 29.75%, 54.29%, and 61.34%), and carotenoids (by 26.16%, 44.85%, and 58.87%) was recorded in the leaves of 8, 10, and 12 dS m^{-1} salinity stresses, respectively (Table 1). In contrast, the application of BeauA1 protected photosynthetic pigments from salinity-induced toxic effects

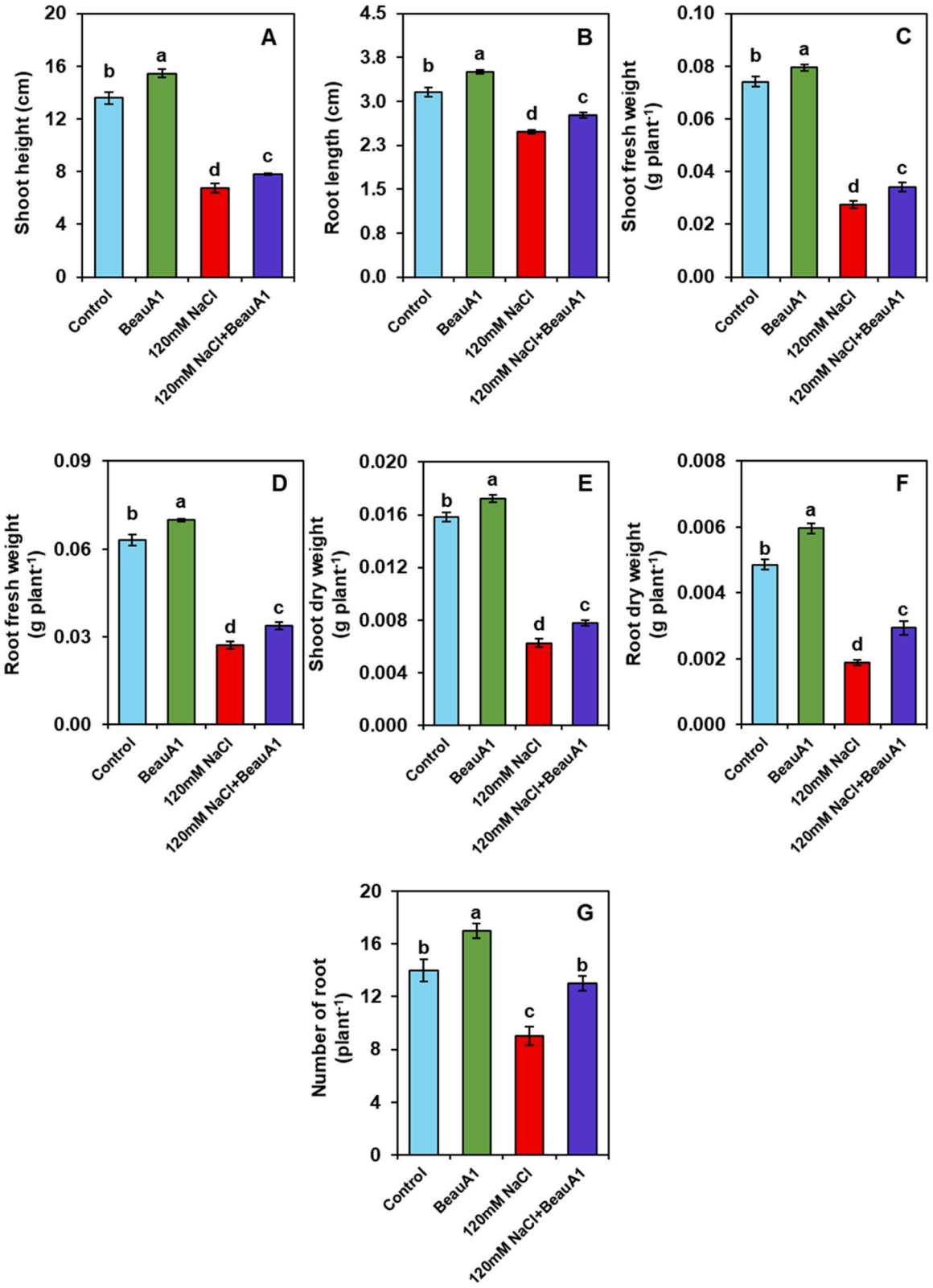

Fig. 2. Effects of BeauA1 on the overall growth of salt-stressed rice plants. (A) shoot height, (B) root length per plant, (C) shoot fresh weight per plant, (D) root fresh weight per plant, (E) shoot dry weight per plant, and (F) root dry weight per plant in leaves of rice plants (G) number of roots per plant, subjected to 120 mM salt stress for 15 days. Values (means±SEs) with different alphabetical letter(s) above the bars show statistically significant differences (lsd, $P < 0.05$) among the treatments. BeauA1, *Beauveria bassiana* isolate BeauA1.

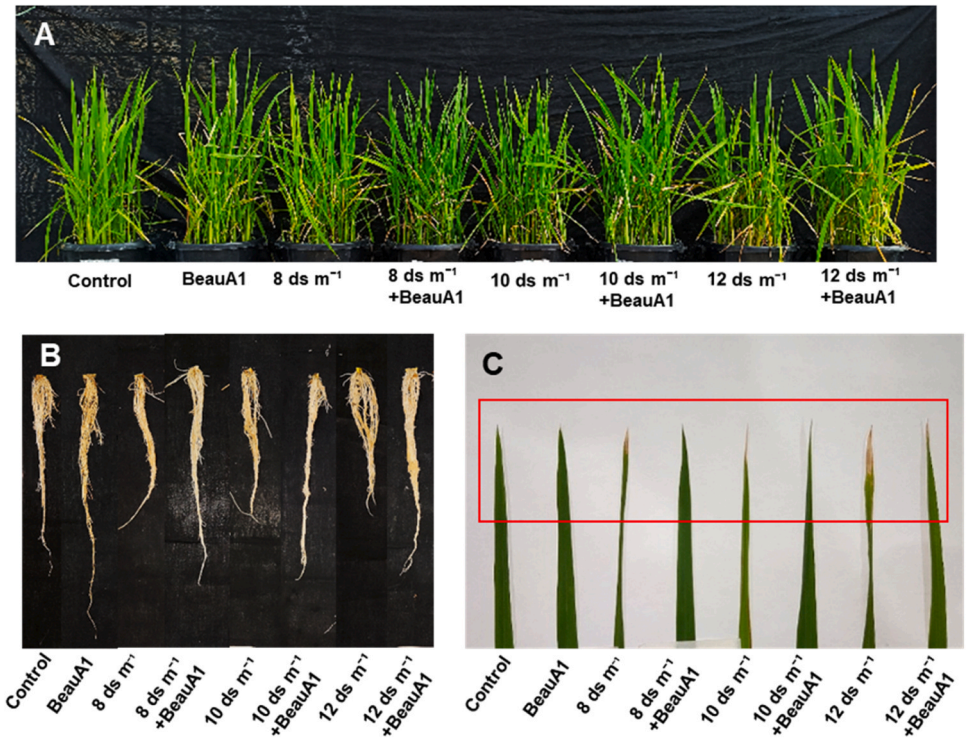

Fig. 3. Effects of BeauA1 on the phenotype of rice plants under salt stress conditions. (A) Representative photographs of rice plants after exposure to a gradient of salinity for 14 days. (B) Roots showing a positive effect of BeauA1 on salt-stressed rice plants. (C) The symptomatic appearance of leaves of rice plants under salt stress.

and showed remarkable increases in the levels of Chl a (24.16%, 23.59%, and 20.54%), Chl b (28.75%, 26.66%, and 7.40%), total Chls (25.76%, 24.83%, and 15.87%), and carotenoids (32.91%, 28.81%, and 27.27%) in responses to 8, 10, and 12 dS m^{-1} salt doses, respectively, when compared with salt-treated only plants. Nonetheless, non-stressed rice plants treated with BeauA1 also showed increased contents of Chl a (20.94%), Chl b (16.41%), total Chls (18.71%), and carotenoids (9.34%) when compared with untreated controls (Table 1).

4.4. BeauA1 elevated relative water and osmoprotectants contents in salt-stressed rice plants

To evaluate whether salt tolerance induced by BeauA1 was dependent on the water status and osmoprotectant levels in rice plants, relative water content (RWC), total soluble sugars (TSS), total carbohydrates (CHO), and proline content (Table 2) in rice leaves were determined. Shoot RWC of rice plants markedly decreased by 10.82%, 12.80%, and 14.77% at 8, 10, and 12 dS m^{-1} of salinity levels, respectively, as compared with untreated control. In contrast, under saline conditions, plants treated with BeauA1 greatly rescued RWC by increasing shoot RWC by 6.64%, 6.50%, and 7.29% at 8, 10, and 12 dS m^{-1} salinity levels, respectively, compared with salt-stressed plants (Table 2).

Imposition of 8, 10, and 12 dS m^{-1} salt stress led to an enhancement in the contents of proline (126.96%, 200.56%, and 265.16%), total soluble sugars (142.2%, 224.77%, and 311.47%), and total carbohydrates (150.71%, 206.07%, and 271.43%), respectively, as compared with those of untreated control plants (Table 2). In contrast, "8 dS m^{-1} + BeauA1," "10 dS m^{-1} + BeauA1," and "12 dS m^{-1} + BeauA1″ plants, respectively, displayed significantly increased levels of proline (by 59.40%, 46.35%, and 91.44%), total soluble sugars (by 67.99%, 61.15%, and 53.73%), and total carbohydrates (by 48.29%, 40.60%, and 36.53%), respectively, than the corresponding salt-stressed plants only (Table 2). BeauA1-primed non-stressed plants increased the contents of proline, total soluble sugars, and total carbohydrates by 79.21%, 67.43%, and 63.92%, respectively, compared with untreated control plants (Table 2).

4.5. BeauA1 improved the K^+/Na^+ ratio in salt-stressed plants

Rice plants exposed to 8, 10, and 12 dS m^{-1} salt stress showed a marked increase in the levels of Na^+ (by 348%, 464.99%, and 701.80% in shoots, and by 258.66%, 345.87%, and 555.67% in roots, respectively), as well as a notable decrease in K^+ contents (by 74.83%, 80.43%, and 82.51% in shoots, and by 41.98%, 45.42%, and 48.59% in roots, respectively) in comparison to untreated control (Fig. 6). Consequently, significant reductions in the K^+/Na^+ ratios (by 94.44%, 96.57%, and 97.86% in shoots and by 83.88%, 87.80%, and 92.35% in roots, respectively) were observed in 8, 10, and 12 dS m^{-1} salt stress, compared to the Control (Fig. 6).

In contrast, "8 dS m^{-1} + BeauA1″ and "10 dS m^{-1} + BeauA1″ salt stress showed elevated levels of Na^+ (by 51.47, 9.47% in shoots, and by 41.50, 24.30% in roots, respectively), and "12 dS m^{-1} + BeauA1″ exhibited a decreased level of Na^+ (9.10% in shoot and 5.35% in root) and an increased level of K^+ (by 201.01%, 120.13% and 71.45% in shoots, and by 193.17%, 50.83% and 78.88% in roots, respectively), resulting in a higher K^+/Na^+ ratio (by 101.28%, 102.08% and 90% in shoots, and by 106.41%, 20.33% and 91.89% in roots, respectively) when compared with the shoots and roots of 8, 10 and 12 dS m^{-1} salt stress (Fig. 6).

Similarly, in comparison with the Control, BeauA1 showed an increased accumulation of both Na^+ (by 5.92% in shoot and by 24.61% in root) and K^+ (by 29.08% in shoot and by 76.73% in root) and thus maintained a higher K^+/Na^+ ratio (by 22.66% in shoot and by 47.93% in root) (Fig. 6).

4.6. BeauA1 reduced oxidative stress in rice plants

One of the most significant damages in plants subjected to

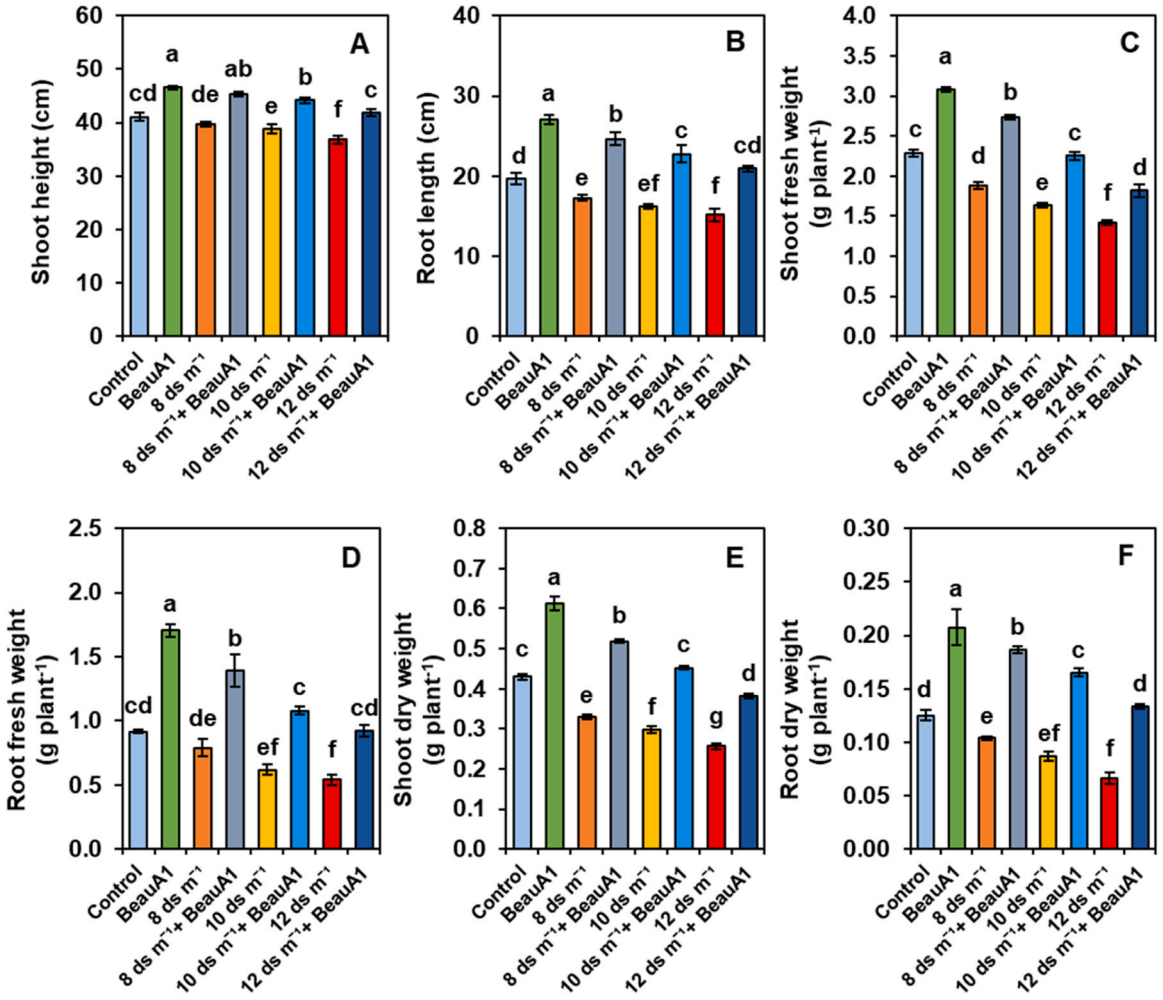

Fig. 4. Effects of BeauA1 on growth-associated attributes of rice plants under salt stress conditions. (A) shoot height, (B) root length per plant, (C) fresh shoot weight per plant, (D) root fresh weight per plant, (E) shoot dry weight per plant, and (F) root dry weight per plant in leaves of rice plants subjected to 8,10 and 12 dS m^{-1} salt stress. Values (means±SEs) with different alphabetical letter(s) above the bars show statistically significant differences (lsd, $P < 0.05$) among the treatments. BeauA1, *B. bassiana* isolate BeauA1.

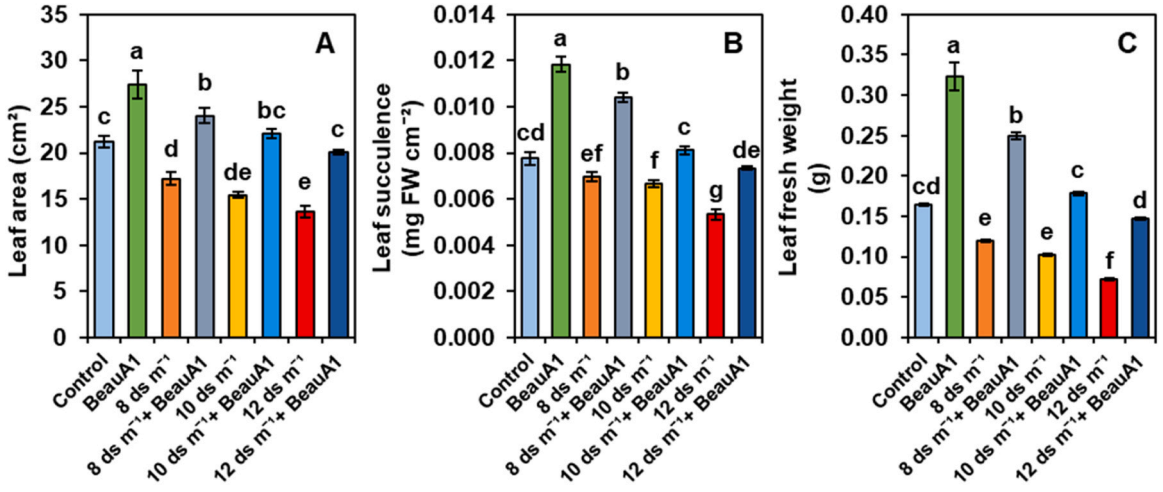

Fig. 5. Effects of BeauA1 on growth-associated attributes of rice plants under salt stress conditions. (A) leaf area, (B) leaf succulence, (C) leaf fresh weight in leaves of rice plants subjected to 8,10 and 12 dS m^{-1} salt stress. Values (means±SEs) with different alphabetical letter(s) above the bars show statistically significant differences (lsd, $P < 0.05$) among the treatments. BeauA1, *B. bassiana* isolate BeauA1; FW, fresh weight.

Table 1
Effects of *Beauveria* on SPAD Value and chlorophyll (Chl) a, Chl b, total Chls, and carotenoids contents in leaves of rice plants subjected to 8, 10 and 12 dS m^{-1} salt stress. Different alphabetical letter(s) with values (means±SEs) show statistically significant differences (lsd, $P < 0.05$). Chl, Chlorophyll; FW, fresh weight; BeauA1, *B. bassiana* isolate BeauA1.

Treatments	SPAD Value	Chl a content (mg g^{-1} FW)	Chl b content (mg g^{-1} FW)	Total Chls content (mg g^{-1} FW)	Carotenoid content (mg g^{-1} FW)
Control	32.08 ± 0.99^{b}	1.91 ± 0.01^{b}	1.34 ± 0.02^{b}	3.26 ± 0.01^{b}	1.07 ± 0.04^{b}
BeauA1	35.51 ± 0.93^{a}	2.31 ± 0.00^{a}	1.56 ± 0.01^{a}	3.87 ± 0.01^{a}	1.17 ± 0.01^{a}
8 dS m^{-1}	28.54 ± 0.41^{de}	1.49 ± 0.00^{d}	0.80 ± 0.02^{d}	2.29 ± 0.02^{d}	0.79 ± 0.00^{d}
8 dS m^{-1}+ BeauA1	31.61 ± 0.86^{bc}	1.85 ± 0.02^{c}	1.03 ± 0.02^{c}	2.88 ± 0.02^{c}	1.00 ± 0.01^{c}
10 dS m^{-1}	26.61 ± 1.03^{ef}	0.89 ± 0.01^{f}	0.60 ± 0.01^{e}	1.49 ± 0.02^{f}	0.59 ± 0.00^{e}
10 dS m^{-1}+ BeauA1	29.40 ± 0.14^{cd}	1.10 ± 0.01^{e}	0.76 ± 0.01^{d}	1.86 ± 0.01^{e}	0.76 ± 0.02^{d}
12 dS m^{-1}	25.29 ± 0.97^{f}	0.73 ± 0.01^{g}	0.54 ± 0.01^{f}	1.26 ± 0.02^{g}	0.43 ± 0.02^{f}
12 dS m^{-1}+ BeauA1	26.11 ± 1.00^{ef}	0.88 ± 0.01^{f}	0.58 ± 0.02^{ef}	1.46 ± 0.02^{f}	0.56 ± 0.01^{e}

Table 2
Effects of *Beauveria* on shoot relative water content, proline, total soluble sugars, and total carbohydrates rice leaves exposed to 8, 10 and 12 dS m^{-1} salt stress. Different alphabetical letter(s) with values (means±SEs) show statistically significant differences (lsd, $P < 0.05$). FW, fresh weight; BeauA1, *B. bassiana* isolate BeauA1.

Treatments	Shoot relative water content (%)	Proline content (μmole g^{-1} FW)	Total Carbohydrates (mg g^{-1} FW)	Total Soluble Sugars (mg g^{-1} FW)
Control	94.13 ± 1.03^{b}	1.78 ± 0.26^{g}	2.80 ± 0.31^{g}	2.18 ± 0.10^{g}
BeauA1	97.41 ± 0.53^{a}	3.19 ± 0.10^{f}	4.59 ± 0.23^{f}	3.65 ± 0.17^{f}
8 dS m^{-1}	83.94 ± 0.34^{ef}	4.04 ± 0.27^{e}	7.02 ± 0.24^{e}	5.28 ± 0.26^{e}
8 dS m^{-1}+ BeauA1	89.52 ± 0.22^{c}	6.44 ± 0.12^{c}	10.41 ± 0.20^{c}	8.87 ± 0.20^{c}
10 dS m^{-1}	82.08 ± 0.37^{fg}	5.35 ± 0.16^{d}	8.57 ± 0.19^{d}	7.08 ± 0.23^{d}
10 dS m^{-1}+ BeauA1	87.42 ± 0.37^{d}	7.83 ± 0.13^{b}	12.05 ± 0.12^{b}	11.41 ± 0.19^{b}
12 dS m^{-1}	80.22 ± 0.26^{g}	6.50 ± 0.17^{c}	10.40 ± 0.23^{c}	8.97 ± 0.18^{c}
12 dS m^{-1}+ BeauA1	86.07 ± 0.19^{de}	9.41 ± 0.18^{a}	14.20 ± 0.29^{a}	13.79 ± 0.36^{a}

environmental stresses is oxidative stress induced by ROS (Chao et al., 2010). Given the role of ROS in salt stress tolerance, we investigated the effects of BeauA1 on ROS scavenging. Reactive oxygen species, such as H_2O_2, and lipid peroxidation products, such as MDA, are recognized as cellular indicators of oxidative stress. The leaves of 8, 10, and 12 dS m^{-1} plants exhibited significant enhancements in the levels of H_2O_2 by (145.73%, 204.03%, and 291.46%), and malondialdehyde (MDA) by (160%, 260%, and 379%), respectively, as compared to the untreated control plant leaves (Fig. 7). The addition of BeauA1 to salt-stressed plants played a decisive role in reducing the levels of MDA (by 41.95%, 30.73%, and 25.90%, respectively) and H_2O_2 (by 53.37%, 34.29%, and 33.21%, respectively) compared with the plants exposed to 8, 10, and 12 dS m^{-1} salt levels only (Fig. 7). BeauA1 application to plants grown under normal conditions also reduced the levels of MDA and H_2O_2 by 30.45% and 15.69%, respectively, in the leaves compared to those of the untreated control (Fig. 7).

4.7. BeauA1 elevated antioxidant enzyme activities in salt-stressed rice plants

To evaluate whether the enzymatic system plays a role in BeauA1-induced tolerance to salt, we examined the activities of some antioxidant enzymes in rice leaves, including CAT, POD, APX, GST, and the contents of total phenolics and total flavonoids. Salt stress at 8, 10, and 12 dS m^{-1} increased the activities of POD by (139.24%, 298.73%, and 513.92%), APX by (71.42%, 161.90%, and 366.67%), GST by (62.51%, 100.56%, and 260.34%), and CAT by (105.12%, 143.66%, and 208.37%), respectively, as compared with corresponding values in control plants (Fig. 8). In contrast, notably improved activities of CAT (by 47.32%, 38.63%, and 22.42%), APX (66.66%, 38.18%, and 18.36%), POD (74.07%, 58.41%, and 49.27%), and GST (48.41%, 31%, and 20.72%) were observed in "8 dS m^{-1} + BeauA1," "10 dS m^{-1} + BeauA1," and "12 dS m^{-1} + BeauA1″ plants, respectively, in contrast to those observed in 8, 10, and 12 dS m^{-1} plants (Fig. 8). In addition, BeauA1-primed plants showed significant improvements in the activities of CAT, APX, POD, and GST by 49.65%, 157.14%, 48.10%, and 94.56%, respectively, compared to the "Control" plants (Fig. 8).

On the other hand, plants treated with 8, 10, and 12 dS m^{-1} salinity levels showed an increased amount of total phenolics (30.55%, 47.22%, and 61.11%) and total flavonoids (by 40.47%, 76.19%, and 92.85%), respectively, over that of untreated control plants (Fig. 8). By comparison, further enhancements in the content of total phenolics (by 23.40%, 22.64%, and 20.68%) and total flavonoids (by 49.15%, 36.48%, and 30.86%) were found in "8 dS m^{-1} + BeauA1," "10 dS m^{-1} + BeauA1," and "12 dS m^{-1} + BeauA1″) plants, respectively, compared to the 8, 10, and 12 dS m-1 salt-stressed plants (Fig. 8). In the absence of salt treatment, BeauA1 seed priming increased total phenolics and flavonoids by 11.11% and 90.47%, respectively, relative to control plants (Fig. 8).

4.8. Visualization of data with a clustered heatmap and unveiling the treatment-variable relationship by PCA

The parameters were subsequently sorted into four distinct groups using the hierarchical clustering approach, and a heatmap was created to display the performance of various parameters under various treatment conditions (Fig. 9). Cluster-A included all plant growth parameters, viz., shoot height, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, fresh leaf weight, leaf area, leaf succulence, RWC, Chl a, Chl b, total chls, carotenoids, and shoot and root K^+/Na^+. Cluster-B included salt stress indicators, viz., MDA and H_2O_2. Cluster-C included the salt stress mitigators, antioxidants, and osmoprotectants (phenol, flavonoid, total soluble sugars, total carbohydrates, CAT, POD, APX, GST, and proline). Compared with the "Control" plants, the parameters of cluster-A revealed a declining trend in 8, 10, and 12 dS m-1 salt-stressed plants, except for Chl a in 8 dS m^{-1} stress. Interestingly, "8 dS m^{-1} + BeauA1," "10 dS m^{-1} + BeauA1," "12 dS m^{-1} + BeauA1," and "BeauA1″ treated plants displayed a reverse trend than those of 8, 10, and 12 dS m^{-1} salt-stressed plants for cluster-A parameters, with the exception of Chl a, Chl b, total chls, and carotenoids in "12 dS m^{-1} + BeauA1″ and "10 dS m^{-1} + BeauA1″-treated plants (Fig. 9). Compared to the "Control" plants, the variables in Cluster-B showed an upward trend under 8, 10, and 12 dS m^{-1} of salt stress. There was a decline in the levels of these parameters in "8 dS m^{-1} + BeauA1," "10 dS m^{-1} + BeauA1," and "12 dS m^{-1} + BeauA1″ treated plants in relation to salt-stressed plants only (Fig. 9). BeauA1-primed plants showed a decreasing tendency in the variables of Cluster-B.

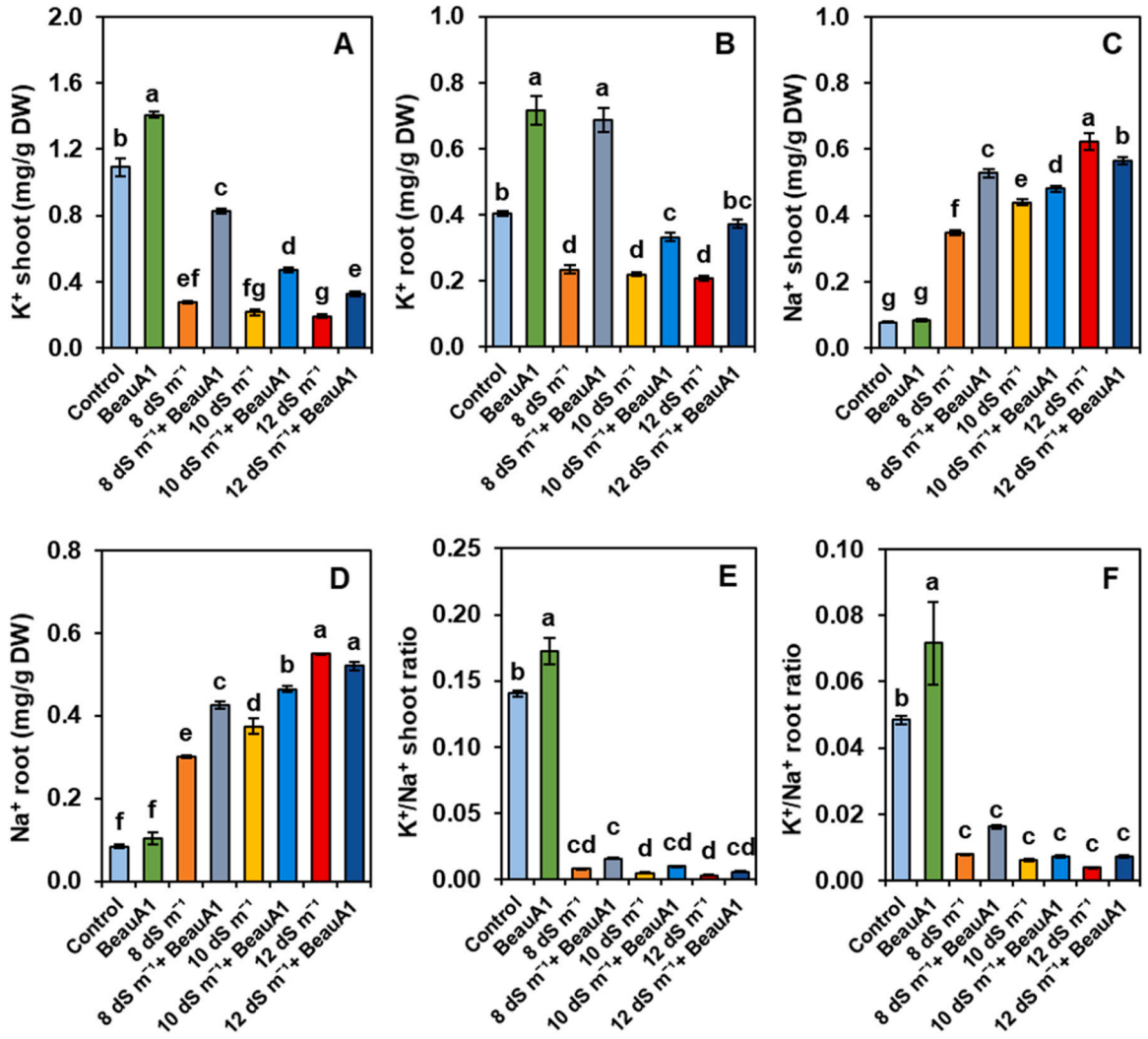

Fig. 6. Effects of BeauA1 on K^+ and Na^+ contents in shoot and root of rice plants subjected to 8, 10, and 12 dS m^{-1} salt stress. Different alphabetical letter(s) with values (means±SEs) show statistically significant differences (lsd, $P < 0.05$). DW, dry weight; BeauA1, *B. bassiana* isolate BeauA1.

Compared to "Control" plants, parameters of Cluster-C exhibited an increasing tendency under 8, 10, and 12 dS m^{-1} stress conditions. However, there were further increases in the levels of these parameters in "8 dS m^{-1} + BeauA1," "10 dS m^{-1} + BeauA1," and "12 dS m^{-1} + BeauA1″ treated plants (Fig. 9). BeauA1 application decreases these parameters compared to salt-stressed and BeauA1-treated salt-stress plants, except for flavonoid.

Principal component analysis (PCA) was then used to ascertain how various treatments and variables were related (Fig. 9). PC1 (67.0%) and PC2 (29.1%) accounted for most of the variability. Notably, parameters of Cluster-A were found to have a close association with BeauA1 and "8 dS m^{-1} + BeauA1″ treatments, while Cluster-B variables were found to be closely related to "12 dS m^{-1}" treatments (Fig. 9). Parameters of Cluster-C were found to have a close association with BeauA1 and the "12 dS m^{-1} + BeauA1″ and "10 dS m^{-1} + BeauA1″ treatments (Fig. 9). Nonetheless, BeauA1-treated plants showed a close relationship with Cluster-A and -C variables instead of Cluster-B variables (Fig. 9).

5. Discussion

Historically, *B. bassiana* has been considered a potent entomopathogenic agent that is commonly utilized to control insect pests in crop cultures and forest stands (Sandhu et al., 2012; Rai et al., 2014; McKinnon et al., 2017). Recently, its endophytic colonization of many different plant species has been shown to enhance plant growth and protection (Parsa et al., 2013; Vidal and Jaber, 2015). In this study, we elucidated the role of *B. bassiana* isolate BeauA1 in salt stress mitigation in rice, which is discussed as follows:

Salinity significantly retards plant growth by reducing cell size and cell production rate, causing ion toxicity and soil/plant osmotic imbalance (Abdel Latef, 2010). Our study revealed that BeauA1 treatment of rice seeds reduced the detrimental effects of salt and enhanced plant phenotypic appearance and growth by raising root and shoot biomass under *in vitro* saline stress (Figs. 1 and 2). Similar outcomes were found when *Trichoderma longibrachiatum* isolate T6 was used in wheat to reduce the effects of salt stress on an agar medium (Zhang et al., 2016). The pot experiment also showed that under both control and salt stress environments, BeauA1-primed plants had significantly improved phenotypes and increased root, shoot, and leaf features (Figs. 3, 4, and 5). Improvement of plant growth by various isolates of *Trichoderma* sp. under salt stress was reported in wheat (Rawat et al., 2011), mustard (Ahmad et al., 2015), *Ochradenus* (Hashem et al., 2014), maize, and rice (Rawat et al., 2012). The PCA results confirmed our findings by demonstrating that, as compared to rice plants that were only treated with salt, BeauA1-treated plants showed a greater degree of the positive relationship with plant growth traits under salt-stressed circumstances (Fig. 9). The possible explanation for the improvement of plant development under abiotic stress conditions is the release of plant

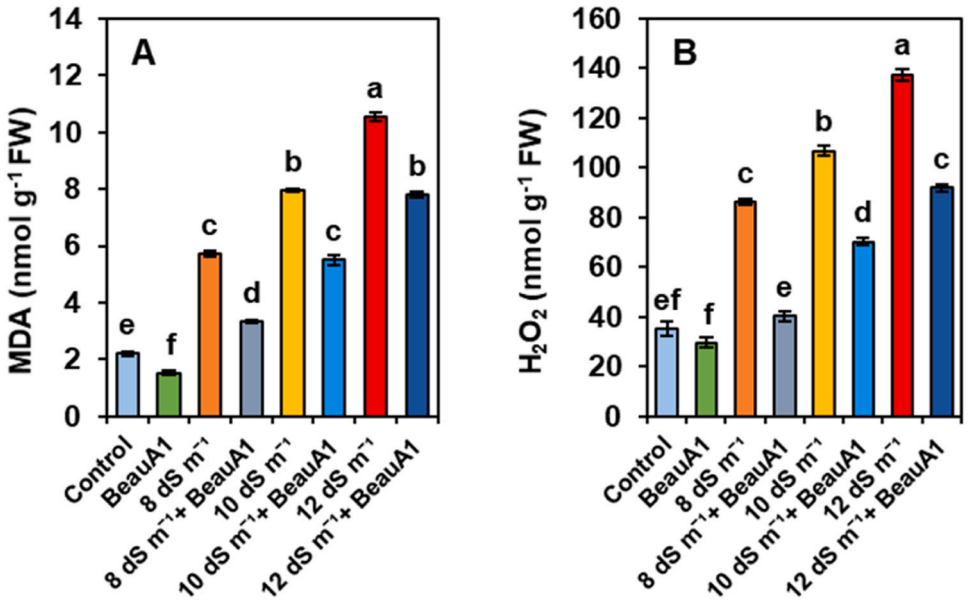

Fig. 7. Effects of BeauA1 on (A) MDA content and (B) H_2O_2 content of rice plants under salt stress. Values (means±SEs) of each treatment were attained from four biological replications (n = 4). Values (means±SEs) with different alphabetical letter(s) above the bars show statistically significant differences (lsd, $P < 0.05$) among the treatments. BeauA1, *Beauveria bassiana* isolate BeauA1; FW, fresh weight.

growth-promoting substances such as gibberellic acids (GAs) and indole acetic acid (IAA) by endophytic fungi (Khan et al., 2011; Khan et al., 2012). The growth and salt tolerance in BeauA1-primed plants are likely to be caused by the enhanced activity of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) and accelerated production of IAA, which were explained in attaining salt tolerance in wheat by *T. longibrachiatum* isolate T6 (Zhang et al., 2019).

The amount of chlorophyll in a plant is often used as a measure of its abiotic tolerance. The current study also found that salt stress dramatically reduced the amount of chlorophyll in rice plants; however, BeauA1 priming considerably raised the amount of chlorophyll in both normal and stress situations (Table 1). The increased chlorophyll content in lettuce after *B. bassiana* treatment is also reported (Macuphe et al., 2021). In agreement with our findings, Soliman et al. (2020), Kumar et al. (2017), Yusnawan et al. (2021), and Zhang et al. (2016) all showed that *Trichoderma* inoculation led to an increase in chlorophyll production under salt stress. It was observed that RWC was reduced by different levels of salt stress in rice plants but increased by applying BeauA1 (Table 2). Leaf RWC was reduced by salt treatment, which indicated a loss of turgor that resulted in limited water availability for the cell extension process. Maintaining a substantial amount of RWC in the leaf is the main tactic for maintaining sufficient plant growth under salinity (Siddiqui et al., 2014). It was reported that, in response to salt stress, the application of *Trichoderma* showed an improved ability to maintain additional intracellular water relations, resulting in better biomass production (Hashem et al., 2014). According to our results, the PCA also showed a positive relationship between the salt-stressed plants treated with BeauA1 and the elevated levels of chlorophyll and leaf RWC (Fig. 9).

Proline, as a normal osmoprotectant, accumulates when plants suffer from abiotic stress, which stabilizes the membranes and stops the breakdown of proteins and enzymes (Farooq et al., 2009). Our result shows that the proline content was increased under different levels of salt stress compared with the control plants (Table 2). On the other hand, rice treated with BeauA1 had significantly higher proline levels than the untreated or salt-stressed plants, which was supported by PCA analysis (Table 2 and Fig. 9). Ali et al. (2022) reported that salt application increased proline accumulation in maize. The association of endophytes significantly enhanced proline content in maize, barrel clover, rice, and wheat under salt stress conditions (Bagheri et al., 2013; Li et al., 2017; Dief et al., 2021; Ali et al., 2022; Khomari et al., 2018).

Our findings demonstrated that after treating salt stress alone, the amount of total soluble sugars and total carbohydrates in rice plants significantly decreased (Table 2). Application of BeauA1 increased the total soluble sugar and total carbohydrates in the rice plants grown under salt stress or non-saline stress, compared to the control. Significant variations in the total carbohydrate levels of the stressed experimental plants were noted by Ouhaddach et al. (2018). Abiotically stressed soybean plants with the aspersions *Aspergillus niger* and *Aspergillus violaceofuscus* were shown to accumulate large levels of total sugars in seedlings (Ismail et al., 2020a; Ismail et al., 2020b). Ghabooli and Kaboosi (2022) found that the endophytic fungus *Serendipita indica*--treated plants showed higher levels of carbohydrates and soluble sugars than untreated plants under drought stress.

Since regulation of K^+ homeostasis by maintaining a higher K^+/Na^+ ratio is the big challenge for crop plants under salinity stress, we estimated K^+ and Na^+ accumulation in leaves and roots of rice plants under salt either treated or not treated with BeauA1. We found that the content of Na^+ was increased and K^+ was decreased, respectively, in rice plants under salt stress alone (Fig. 6). Our result showed conformity to the findings of Zhang et al. (2018), where Na^+ and K^+ were sufficiently increased and decreased, respectively, in rice genotypes under salt stress. This might be due to the Na^+-induced K^+ efflux from the cell (Demidchik et al., 2014) either utilizing the activation of K^+ outward rectifier channels through membrane depolarization or by the inhibition of the Arabidopsis K transporter (AKT1), which is an important factor for K^+ uptake and transport (Véry et al., 2014). Application of BeauA1 increased Na^+ and K^+ in the rice plants grown under salt or non-saline stress compared to the control (Fig. 6). Similar findings were reported by Abd El-Baki and Mostafa (2014), who showed that treatment with *Trichoderma harzianum* increased the accumulation of both Na^+ and K^+ ions under salinity stress. Because K^+ regulates a myriad of physiological processes, including stomatal regulation and photosynthesis (Mostofa et al., 2022), cytosolic K^+/Na^+ ratios are a crucial issue for salt tolerance (Shabala and Pottosin, 2014). We found that the K^+/Na^+ ratio significantly decreased under salt stress (Fig. 6). The K^+/Na^+ ratio was, however, positively correlated with BeauA1-primed plants and increased in BeauA1-primed salt-stressed rice plants compared to

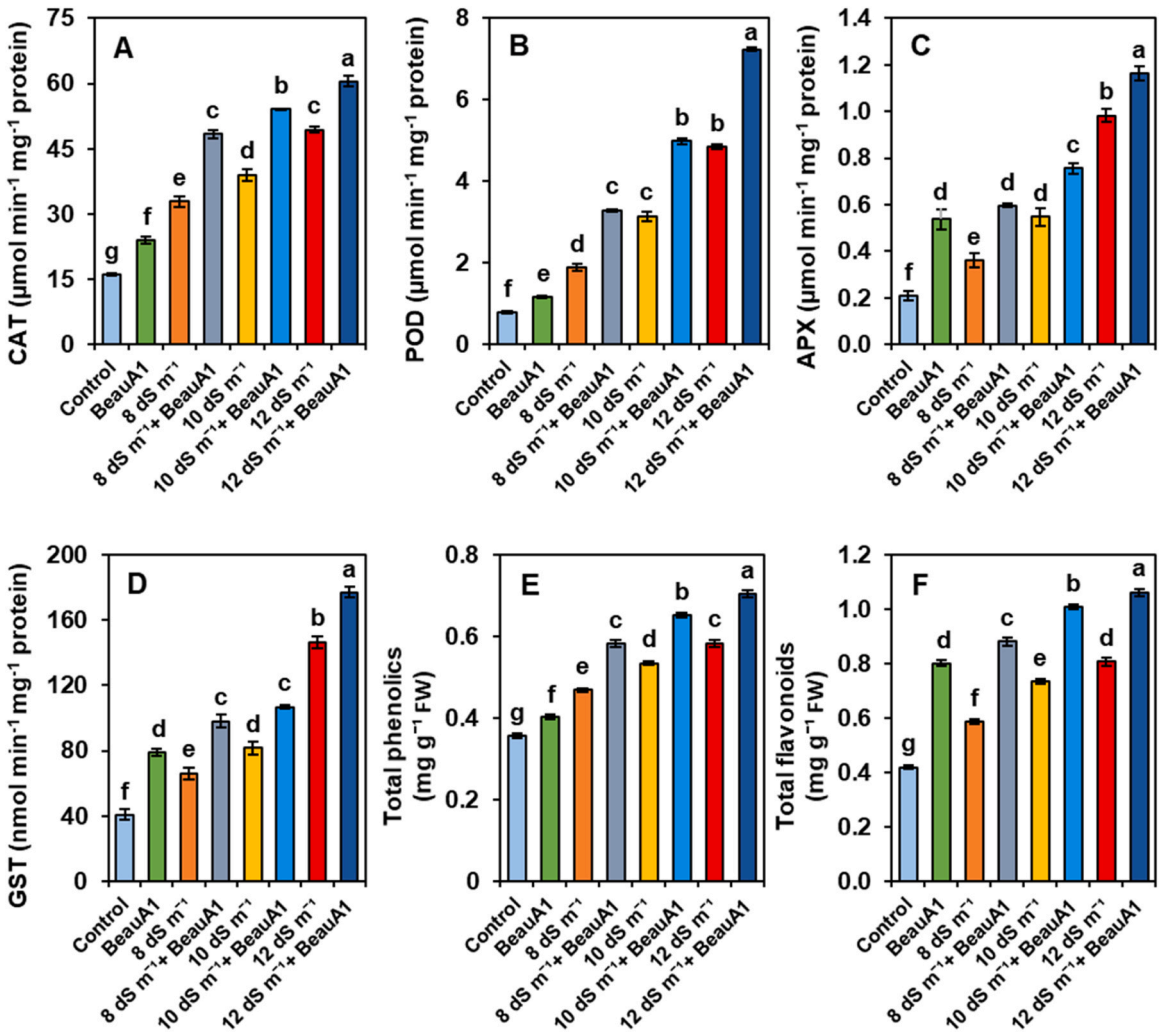

Fig. 8. Effects of BeauA1 on the activity of (A) CAT, (B) POD, (C) APX, (D) GST, (E) Total phenolics, and (F) Total flavonoids in the leaves of rice plants under salt stress. Values (means±SEs) with different alphabetical letter(s) above the bars show statistically significant differences (lsd, $P < 0.05$) among the treatments. BeauA1, *Beauveria bassiana* isolate BeauA1; FW, fresh weight.

salt-stressed only plants (Figs. 9 and 6). Our findings were in agreement with those of Zhang et al. (2019), who demonstrated that cucumber seedling treatment with *T. harzianum* under salt stress maintained cellular homeostasis by preserving a greater K^+/Na^+ ratio. During salt stress, enhanced compartmentalization of Na^+ in the vacuoles is crucial to managing lower water potential in the cell (Fan et al., 2015). Thus, the higher K^+/Na^+ ratio in BeauA1-primed salt-stressed plants could be managed by higher root biomass (Figs. 3b and 4) to accelerate compartmentalization of more Na^+ in the vacuoles of enlarged root biomass as compared to salt-stressed plants only. A similar pattern of salinity acclimation was found in the halophytic plant *Suaeda salsa* (L.), which followed a higher root biomass to absorb more Na^+ under salt stress (Wang et al., 2021). Along with that, the maintenance of a higher K^+/Na^+ ratio in both shoot and root is likely to be caused by the enhanced expression of the Na^+/H^+ antiporter gene in BeauA1-primed salt-stressed plants, which was explained by the *T. longibrachiatum* T6-led salt stress acclimation process in wheat (Zhang et al., 2019).

Oxidative stress mediated by ROS has been a common issue for plants whenever exposed to abiotic stresses (Mostofa et al., 2015; Ghosh et al., 2022; Arora et al., 2020). The matter is consistent with the present study, where induction ROS, H_2O_2, and the lipid peroxidation product MDA were found to be higher in salt-stressed plants (Fig. 7). However, BeauA1-inoculated plants produced a lower accumulation of H_2O_2 and MDA, suggesting the essential role of BeauA1 in the salt stress acclimation process (Fig. 7). Priming wheat seed with isolates of *T. harzianum* Th-13, Th-14, and Th-19 decreased the MDA accumulation under both saline and non-saline conditions (Rawat et al., 2011), which agrees with us. Similar findings were observed by many investigations, such as the inoculation of *Trichoderma* spp. in plants reduced the accumulation of H_2O_2 and MDA significantly (Hajiboland et al., 2010; Hashem et al., 2014a). According to PCA results, BeauA1-primed salt-exposed rice plants had a negative relationship with ROS products as well as MDA levels when compared to salt-stressed non-treated plants (Fig. 9). Along with those, our findings suggest plants' acclimation to salt tolerance is aided by endophytic fungi, including *B. bassiana*.

To counteract ROS and maintain membrane integrity under salinity stress, modulation of antioxidant metabolisms is crucial for improving abiotic stress tolerance in land plants (Huchzermeyer et al., 2022; Singh, 2022). In enzymatic defense systems, SOD acts as the first-line scavenger for protecting cellular damage against ROS (Bowler et al., 1992). Further, POD, CAT, and APX are involved in decomposing H_2O_2 to H_2O while acclimating to oxidative stress (Mittler, 2002). We found that dose-dependent salt treatments alone significantly increased the activities of APX, POD, GST, and CAT (Fig. 8). As opposed to salt-stressed plants alone, BeauA1 treatment boosted the activities of APX, POD, GST, and CAT in salt-stressed rice plants (Fig. 8). *T. harzianum* colonized mustard (*Brassica juncea*) plants and mitigated NaCl stress by inducing an antioxidative defense system (Ahmad et al., 2015), consistent with

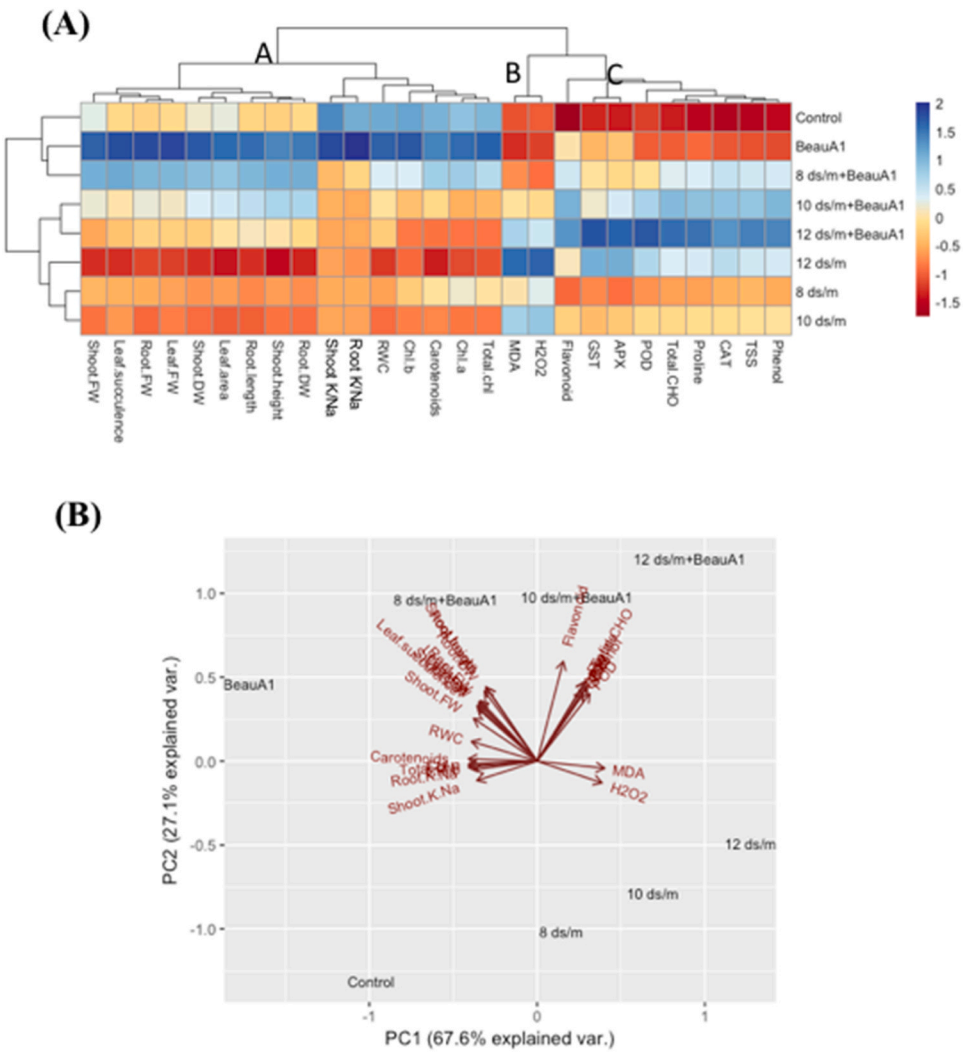

Fig. 9. (a) Clustering heatmap visualizing relationships of different parameters under different treatments at a glance. Normalized mean values of different parameters were used to prepare the heatmap. The parameters were grouped into three (A-C) distinct clusters. The color scale indicates the changing trend of the normalized mean values of different parameters under different treatments. (b) Principal component analysis (PCA) was conducted to unveil the direction of variation towards the treatments.

our findings. Besides, the effective colonization of endophytes elevated the antioxidant enzymes CAT, SOD, POD, and APX and elevated phytohormones, mainly salicylic acid (SA) and gibberellic acid (GA), that are directly involved in stress tolerance (White Jr. and Torres, 2010; Jha and Subramanian, 2016; Lata et al., 2018; Metwally and Soliman, 2023). The application of arbuscular mycorrhizae also elevated salt stress in cucumber by increasing defense enzymes SOD, CAT, and APX (Santander et al., 2020). The PCA, which demonstrated a strong positive correlation between the activities of enzymatic antioxidants and the salt-stressed plants treated with BeauA1, provided further support for our findings (Fig. 9). Our observations of the increased POD, CAT, APX, and GST activity in rice by BeauA1 under salt stress demonstrate the effectiveness of this endophyte in the salt acclimation process of rice plants.

Along with enzymatic and non-enzymatic antioxidants, plants also play a crucial role in acclimating to abiotic stresses, including rice (Dionisio-Sese and Tobita, 1998; Wang et al., 2019; Narayanasamy et al., 2020). By quenching singlet oxygen, absorbing and neutralizing free radicals, decreasing peroxides, and relieving the salinity impact, phenolic substances, especially flavonoids, decrease the oxidative process (Chen et al., 2019; Kumar et al., 2020; Shah and Smith, 2020). In our study, we found that total phenolics and flavonoids were significantly increased under various salt concentrations, and BeauA1 further boosted their activity in both non-saline and saline-stress environments compared to their controls (Fig. 8). The positive associations of BeauA1-primed plants with total phenolics and flavonoids were evident from PCA results (Fig. 9). Pearl millet plants subjected to extreme salt stress had higher phenolic content, according to Khushdil et al. (2019), whereas endophyte-inoculated plants showed far lower levels of stress. Similar results were also found in Ali et al. (2022), where the endophyte association exhibited improvement in the phenolic and flavonoid contents of the maize plants under salt stress. Similar increases in phenolics and flavonoids have been reported in groundnut (Yusnawan et al., 2021), buckwheat, barley (Yang et al., 2018), and maize (Kumar et al., 2017), which are cultivated in saline soil. Along with those, our results showed that the endophyte *B. bassiana* has a function in triggering the host plants' stress-tolerance defense mechanisms.

6. Conclusion

Our study presented the first-ever evidence that rice seeds primed with BeauA1 increased root and shoot growth, chlorophyll content, K^+/Na^+ accumulation, relative water content, proline, and carbohydrates content in rice plants under saline stress and suggested that BeauA1 effectively regulated osmoprotectants for plants under salinity stress. Moreover, endophytic fungus BeauA1 increased enzymatic antioxidants CAT, APX, POD, GST, and nonenzymatic antioxidants phenol and flavonoid metabolisms in rice plants and reduced MDA and H_2O_2 in salt-stressed conditions, suggesting its role in enzymatic salt stress acclimation. Therefore, the use of BeauA1 in rice would be an effective technique for both growth promotion and salt stress alleviation strategies.

CRediT authorship contribution statement

T.A. conducted the experiment, conducted data curation, and wrote the first draft. A.A.M. and N.Z. contributed to enzyme determination, recording data, and analysis. M.A.H. and M.M.H. contributed to supervision, interpretation, and review. T.K.G. contributed to biochemical analysis and interpretation, and M.Z.H.C. contributed to laboratory assays and editing. S.M.N.I. conceptualized the idea, supervised experiments, and reviewed and edited the manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Shah Mohammad Naimul Islam reports administrative support and equipment, drugs, or supplies were provided by Bangabandhu Sheikh Mujibur Rahman Agricultural University. Shah Mohammad Naimul Islam reports a relationship with Bangabandhu Sheikh Mujibur Rahman Agricultural University that includes: employment. There is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2023.105427.

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