Plant Physiology and Biochemistry 144 (2019) 127–134

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Salt stress alleviation in *Pennisetum glaucum* through secondary metabolites modulation by *Aspergillus terreus*

Faiza Khushdil[a], Farzana Gul Jan[a], Gul Jan[a], Muhammad Hamayun[a,**], Amjad Iqbal[b,*], Anwar Hussain[a], Nusrat Bibi[a]

[a] Department of Botany, Garden Campus, Abdul Wali Khan University Mardan, Pakistan
[b] Department of Agriculture, Garden Campus, Abdul Wali Khan University Mardan, Pakistan

ARTICLE INFO

Keywords:
Salinity tolerance
Endophytic fungi
Aspergillus terreus
Pennisetum glaucum
Physicochemical attributes

ABSTRACT

The growth promoting activities of the isolated endophyte *Aspergillus terreus* from *Aloe barbendsis* was studied in the salt stressed *Pennisetum glaucum* (pearl millet). A significant (P = 0.05) increase in the root-shoot lengths, fresh and dry weights and chlorophyll content of pearl millet seedlings was noticed after colonization by *A. terreus* under normal conditions. At 100 mM NaCl stress and *A. terreus* inoculation, the growth rate of pearl millet seedlings were significantly (P = 0.05) inhibited. Furthermore, the IAA production, relative water content (RWC), chlorophyll, soluble sugar, phenol and flavonoid contents were significantly decreased, whereas proline content and lipid peroxidation were increased. On the contrary, pearl millet seedlings inoculated with *A. terreus* retained significantly (P = 0.05) higher amounts of RWC, chlorophyll, soluble sugar, phenol and flavonoid contents under 100 mM salt stress. The higher IAA production in *A. terreus* associated seedlings rescued the plant growth and development under salt stress. Moreover, the LC MS/MS analysis of *A. terreus* cultural filtrate revealed the presence of quinic acid, ellagic acid, calycosin, wogonin, feruloylquinic acid, caffeic acid phenylethyl ester, D-glucoside, myricetin, propoxyphene and aminoflunitrazepam. The results of the study conclude that innoculation of *A. terreus* improves the NaCl tolerance in pearl millet by ameliorating the physicochemical attributes of the host plants.

1. Introduction

Plant microbe interactions are vital for the survival of both the partners under normal as well as stress conditions. Among the plant interacting microbes, endophytic fungi have gained remarkable attention during last few decades. Endophytes play an important ecological and physiological role in plant symbiosis (Ismail et al., 2019; Nusrat et al., 2019; Jan et al., 2019; Ikram et al., 2018). Endophytic fungi can promote plant growth and yield through assimilation of nitrogen, phosphate solubalization, hydrogen cyanide (HCN) and ammonia production (Hamayun et al., 2017; Hussain et al., 2018; Ismail et al., 2018; Mehmood et al., 2018, 2019). Fungal endophytes can also secrete secondary metabolites and/or phytohormones that can control plant growth under stressful conditions (Ikram et al., 2018; Hamayun et al., 2017; Mehmood et al., 2018; Hussain et al., 2015; Khan et al., 2018; Bilal et al., 2018). For the better metabolic activities, plants are dependent on endophytes in stressed environment (Kavamura et al., 2013). Various crops cultivated in arid or semi-arid regions are frequently exposed to wide range of environmental stresses. Among these, salinity stress severely affects plant growth and metabolism and hence results in reduced biomass production. Studies have shown that salt stress can provoke several morphological, physiological, and metabolic responses in plants leading to increased lipid peroxidation and inactivation of antioxidant enzyme (Nusrat et al., 2019). In fact, plants consist of well-known biological process (production of osmolytes, scavenging ROS and altering water movement) in response to stress (Nusrat et al., 2019). Besides, plants accumulate several metabolites in the cytoplasm, such as proline and soluble sugars to help themselves against stress (Nusrat et al., 2019). However, severe salt stress can affect the nutrient availability, uptake, partitioning and transportation within the plant organs due to ionic imbalance and water deficiency, alters the physiological activities of the plants (Farooq et al., 2015).

The development of salt stress cultivars are expensive and time consuming, therefore, a cheap and quick solution is required. It is assumed that the use of fungal endophytes could be a cost effective and sustainable approach to enhance the salt tolerance of economically

* Corresponding author.
** Corresponding author.
E-mail addresses: hamayun@awkum.edu.pk (M. Hamayun), amjadiqbal@awkum.edu.pk (A. Iqbal).

https://doi.org/10.1016/j.plaphy.2019.09.038
Received 15 July 2019; Received in revised form 22 September 2019; Accepted 23 September 2019
Available online 23 September 2019
0981-9428/

important crops, such as pearl millet. Pearl millet has greater nutritional value in terms of dietary fiber, high levels of energy and proteins. These play a vital role in protecting human ailments, such as cancer and diabetes (Jukanti et al., 2016). Though pearl millet has the ability to thrive in less fertile soil, high temperature and low rainfall (Onyango et al., 2013), but salinity stress can drastically effect the crop growth and yield. To compensate the effect of salt stress, the isolated novel endophytic fungal strain(s) were aimed to be used in order to help the pearl millet seedlings under salinity stress.

2. Materials and methods

2.1. Collection and sterilization of plant materials

In the present study, *Aloe barbadensis* miller was collected from the different areas of Tehsil Takht bhai. The plants were collected, labeled, sealed in sterile bags and shifted to the Plant Microbe Interaction (PMI) laboratory at Abdul Wali Khan University, Garden Campus, Mardan. The plants were stored at 4 °C till further processing. The leaves were detached and washed with running tap water to remove dust particles. The leaf samples were then surface sterilized with 70% ethanol for 30 s (Tran et al., 2010). Following sterilization, the samples were washed three times with autoclaved distilled water. The leaves samples were cut into small pieces (5 mm/segment) and were placed with the help of forceps in petri plates having Hagem medium. The Hagem medium consisted of KH_2PO_4 (0.05%), NH_4Cl (0.05%), $MgSO_4.7H_20$ (0.05%), glucose (0.5%), $FeCl_3$ (0.1%), agar (1.5%) and 80 ppm streptomycin. The pH (5.6 ± 0.1) of the medium was adjusted. After placing the leave samples on Hagem media plates, the plates were shifted to an incubator pre set at 30 °C and kept for 7-days. During the incubation of 7-days fungal hyphae were developed that were transferred to Potato Dextrose Agar (PDA), supplemented with 100 μg/ml of streptomycin to inhibit bacterial growth. The plates were then incubated at 30 °C for 7-days to allow the growth of mycelium (Redman et al., 2011).

2.2. Collection of culture filtrates and mycelia

The isolated strains were inoculated in 50 mL of Czapek broth (1% glucose, 0.05% KCl, 1% peptone, 0.05% $MgSO_4.7H_2O$, 0.001% $FeSO_4.7H_2O$; pH = 7.3 ± 0.2) for 7-days in a shaking incubator at 30 °C and 120 rpm (Khan et al., 2008). The culture filtrated was separated from the fungal mycelia by passing through the Whatman filter paper. The separated fungal culture filtrate (50 mL) and mycelium (3–5 g) of each fungus were immediately shifted to −80 °C freezer and then freeze dried before screening.

2.3. Screening of fungal stains

2.3.1. Determination of IAA in fungal culture filtrate

Previously collected culture filtrate (1.5 mL) of each strain was taken in a seperate eppendorf tubes and centrifuged at 10000 rpm for 2 min. After centrifugation, 1 mL of the supernatant was mixed with 2 mL of Salkowski's reagent. The development of a pink colour indicated IAA production. The absorbance was measured at 530 nm through UV–vis spectrophotometer (Perkin Elmer) and IAA was quantified by using standard curve (Ahmad et al., 2005).

2.3.2. Estimation of flavonoids in fungal culture filtrate

Total flavonoids were estimated by using aluminium chloride colorimetric method (Saravanan and Parimelazhagan, 2014). In a test tube, 4.3 mL of methanol (80%) was added to 0.5 mL of each culture filtrates. Then, 0.1 mL of aluminium chloride (10%) and 0.1 mL of potassium acetate (10%) was added to the tubes. The samples were incubated for 30 min at room temperature. After incubation, the samples were shaken and the absorbance were recorded at 510 nm.

2.3.3. Quantification of total phenolics

Total phenolics were calculated using Folin Ciocalteau reagent. To 0.5 mL of the culture filtrates in test tubes, 0.5 mL of ethanol were added and the tubes were centrifuged at 10000 rpm for 20 min. Distilled water (4 mL) and Folin Ciocalteau reagent (0.5 mL) were then added to each tube, followed by the addition of Na_2CO_3 (2 mL) after 3 min. The mixtures were heated on water bath till the appearance of dark blue colour. The tubes were cooled and the absorbance was measured at 650 nm by UV–vis spectrophotometer (Jayanthi et al., 2011).

2.3.4. Production of ammonia

The capacity of endophytic fungal isolates to produce ammonia was checked qualitatively by the method as described by Singh et al. (Singh et al., 2014). Nessler's reagent (0.1 mL) was added to the 2 mL of culture filtrate. The conversion of deep yellowish colour to brownish indicated the maximum ammonia production, whereas faint yellow colour indicated the minimum ammonia production by the tested isolates.

2.3.5. Hydrogen cyanide (HCN) production

For the production of HCN all the isolates were screened in PDA medium plates amended with 4.4 g/l glycine as described by Chadha et al. (Chadha et al., 2015). A Na_2CO_3 (2%) and picric acid (0.5%) soaked filter paper were then placed in a PDA medium plates streaked with fungal isolates. The Plates were then covered with parafilm and kept in incubator for 7-days at 28 °C for the production of HCN. A colour change of filter paper from yellow to orange brown indicated a positive result.

2.3.6. Phosphate solubilization (PSM)

Pikovskaya's agar medium [10 g Glucose, 0.2 g KCl, 2.5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.5 g Yeast extract, 0.002 g $FeSO_4.7H_2O$, 0.002 g $MnSO_4.H_2O$, and 15 g Agar in 1 L of distilled water] was used to detect solubilization of tri-calcium phosphate. Fungal isolates were inoculated in Petri plates containing Pikovskaya's agar medium and the plates were incubated at 28 °C for 3-days. Phosphate solubalization was confirmed by the production of halos around the fungal hyphae (Wahyudi et al., 2011).

2.3.7. Salt tolernt fungal strains

Fungal isolates were checked for salt tolerance by following the method of Al Tamie (Al Tamie, 2014). To, 50 mL of Czapek broth (liquid media) in 250 mL conical flasks, different concentrations of NaCl (0, 50, 100 and 150 mM) were added. The inoculum of fungl isolate was then added to the each flask. The flasks were shifted to the shaking incubator operated at 27 °C and 120 rpm and kept there for 7-days. After 7-days, the fresh and dry weights, IAA, flavonoids and phenolics of the cultures were measured.

2.3.8. Genomic DNA extraction and fungal identification of the potent strain

Genomic DNA extraction and PCR of the most promising strain were performed according to method of Khan et al. (Khan et al., 2008). The potent fungal isolate was identified by sequencing internal transcribed region (ITS) of 18S rDNA, using universal primers ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). A Big Dye terminator cycle sequencing kit v.3.1 was used for that purpose. Both PCR sequencing and amplification was analyzed by an automated DNA sequencing system (Applied Biosystems, Foster City, USA) at the Macrogen, Inc., Seoul, Korea. The obtained PCR product was initially sequenced and then subjected to a homology search by using online tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast).

2.3.9. LC MS/MS analysis of compounds in the culture filtrate of potent strain

The fungal culture having the ability to exhibit antioxidant activity, were further processed for documentation of bioactive compounds by LC MS/MS (LTQ XL, Thermo Electron Corporation, USA) analysis (Khan et al., 2017). The detection was performed through direct injection mode with Electron Spray Ionization (ESI) probe, at positive-mode. The capillary temperature was kept at 280 °C, while the sample flow rate was set at 8 µl/min. The mass range was selected from 50 to 1000 m/z. The collision induced dissociation energy (CID) during MS/MS was kept in the range of 10–45, depending upon the nature of the parent molecular ion. As a mobile phase, the ratio of methanol and acetonitrile was 80:20 (*v*/v) for the HPLC fractions. The MS parameters for each compound were optimized to ensure the most favorable ionization, ion transfer conditions and attained optimum signal of both the precursor and fragment ions by infusing the analytes and manually tuning the parameters. The source parameters were identical for all of the analytes.

2.3.10. Plant growth promotion assay of the selected endophytic strain under saline conditions

Pearl millet seeds were surface sterilized with 70% ethanol for 30 s. The seeds were washed thrice with sterilized distilled water and allowed to germinate in autoclaved petri plates. The seeds were placed at uniform distance in Petri plates having double layer of wet filter paper. The plates were then transferred to the incubator operated at 25 °C and kept till the seeds germinated (Khan et al., 2008). Equally germinated seedlings were shifted to autoclaved soil in sterile pots. The spore suspension was applied to each pot after the establishment of seedlings. These seedlings were kept for one week under normal conditions for plant fungal symbiotic association. The experiment was designed in such way that it had four sets of plants:

(i) Control plants without endophytes and salt stress
(ii) Salinity (100 mM NaCl) stressed plants
(iii) Plants inoculated with endophytes
(iv) Plants inoculated with endophytes under salinity stress (100 mM NaCl).

After a week, when the pearl millet seedlings got established, 10 mL of NaCl (100 mM) was given to plants of group (ii) and (iv). The application of 10 mL of NaCl was repeated after every third day till 20th day. The plants in group (i) and (iii) were given 10 mL of tap water only after every three days. Afetr the 20th day, root and shoot fresh weight (g), root and shoot lengths (cm) of all the seedlings were measured. The dry weight of the seedlings was recorded after drying the seedlings in an oven at 70 °C for 48 h. The pearl millet seedlings from various groups were also tested for biochemical analysis.

2.3.11. Estimation of pearl millet growth parameters

Root and shoot length was measured manually with the help of a scale. Plant fresh weight was measured directly using analytical balance, while plant dry weight was estimated after drying the samples in an oven for 48 h at 70 °C.

2.3.12. Analysis of chlorophyll contents

Chlorophyll content of pearl millet seedlings was calculated by the method of Maclachlan and Zalik (1963). Approximately, 0.3 g of fresh sample was macerated in 5 mL of acetone and the sample was transferred in a 15 mL falcon tube. The tube was centrifuged at 1000 rpm for 5 min. The supernatant were collected and the final volume was adjusted to 7 mL with acetone. The optical densities were recorded at 663 nm, 646 nm and 470 nm by using UV–vis spectrophotometer.

2.3.13. Determination of relative water content (RWC)

The method of Khan et al. (Khan et al., 2013) was adopted for the analysis of RWC.

$$\% RWC = \frac{FW - DW}{TW - DW} \times 100$$

2.3.14. Estimation of proline contents

Proline accumulation was determined using the method of Bates et al. (Bates et al., 1973). Fresh or frozen leaf sample (0.3 g) was crushed in 4 mL of 3% sulfosalicylic acid and the solution was transferred in to falcon tube. The falcon tube was centrifuged for 15 min at 3000 rpm and the supernatant was collected in tube. The collected supernatant (2 mL) was then mixed with 2 mL of acid ninhydrin in a test tube. The mixture was placed in a water bath for 1 h at 100 °C. After cooling the reaction mixture were extracted with 4 mL of toluene and the absorbance was measured at 520 nm using spectrophotometer.

2.3.15. Measurement of lipid peroxidation

Lipid peroxidation was determined by following the method of Hernández et al. (Hernández et al., 2001). Fresh leaf sample (0.5 g) was homogenized in 2 mL of 0.1% (TCA) trichloroacetic acid. The mixture was centrifuged at 10,000 rpm and 4 °C for 10 min. The supernatant (0.5 mL) was collected and mixed with 1.5 mL thiobarbituric acid (0.5%) in trichloroacetic acid (20%). The contents were incubated (90 °C for 2 min) and then centrifuged at 10,000 rpm for 5 min after cooling on an ice bath. The optical density of the supernatant was checked at 532 and 600 nm.

2.3.16. Estimation of total soluble sugars

The total soluble sugars were estimated according to the method of Dubois et al. (Dubois et al., 1956). Fresh leaves sample (0.5 g) was homogenized in 10 mL of distilled water. The homogenate was then centrifuged at 3000 rpm for 5 min. The collected supernatant (0.5 mL) was mixed with 10 µL of phenol (80%), followed by the addition of 1 mL H_2SO_4. The solution was carefully vortexed and allowed to stand on a bench at room temperature for 10 min. The optical density was finally checked at 520 nm.

2.4. Statistical analysis

For statistical analysis, One-way analysis of variance (ANOVA) at P = 0.05 was used, followed by Duncan's Multiple Range Test (DMRT) by using SPSS for windows.

3. Results

3.1. Selection of best endophytic fungal strain

A total of 14 strains were initially isolated and screened for plant growth promoting ability. However, the endophytic fungal strain marked as AL4 showed phosphate solubilization, HCN and ammonia production (Table 1).

The fungal isolate AL4 produced IAA, flavonoids and phenolics in appreciable quantities. The culture filterate of AL4 contained 8.14 µg/mL of IAA, 44.0 µg/mL of flavonoids and 65.21 µg/mL of phenolics (Table 1).

The fungal strain AL4 was also screened for halotolerance in Czapek

Table 1
Plant growth promoting activity and bioactive compounds in culture filtrate of *A. terreus*.

Fungal strain	IAA µg/mL	Flavonoids µg/mL	Phenols µg/mL	Ammonia	HCN	Phosphate solubilization
AL4	8.14	44.0	65.21	+	+	+

+ = show maximum potential.

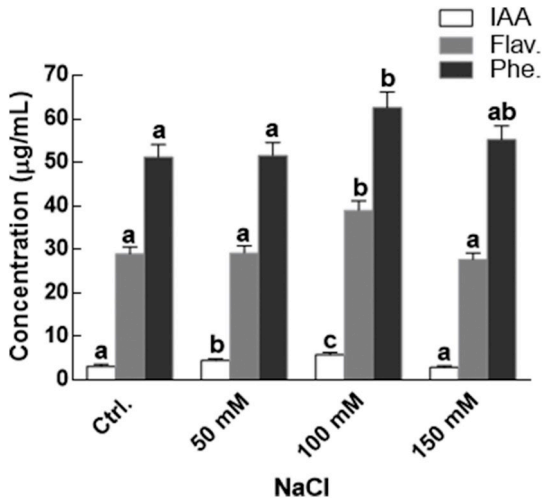

Fig. 1. Effects of salt stress on IAA, flavonoids and phenols (µg/mL) secretion by AL4. Ctrl. = without salt stress; IAA = indole accetic acid; Flav. = flavonoids; Phe. = phenolics. Each bar represents the mean of triplicated data with ± SE. Means followed by different letters are significantly different from their respective bars (Duncan's multiple-range test; $p < 0.05$).

broth medium amended with varying concentration of NaCl (50, 100 and 150 mM). The result showed that the production of IAA, phenolics and flavonoids by the isolated fungal strain AL4 was unaffected in 50 mM and 100 mM NaCl. However, when NaCl concentration was increased to 150 mM, the secretion of IAA, phenolics and flavonoids by fungal strain AL4 were significantly decreased (Fig. 1).

3.2. Identification of fungal isolate AL4

The phylogenetic analysis of the fungal isolate AL4 was carried by using the neighbor joining (NJ) method and the tree was constructed from 12 taxa and aligned ITS sequences with 100 bootstrap replications. The strain was selected through the BLAST search showing the maximum sequence homology similarity. Results of BLAST search showed highest sequence similarity (70%) between the fungal isolate AL4 and *A. terreus* strains (*A. terreus* PF26, *A. terreus* AN4, *A. terreus* ATE1 and *A. terreus* wlp.R). The tree indicated that the isolated strain AL4 belongs to *A. terreus* family (Fig. 2).

3.3. Effect of A. terreus on growth of pearl millet under salinity stress

NaCl stress caused decline in shoot and root length, fresh and dry weight of pearl millet plants (Fig. 3A & B). Inoculation of the NaCl stressed pearl millet plants with *Aspergillus terreus* mitigated the salt stress by restoring all growth parameters to the level of control. The pearl millet plants, when inoculated with fungal strain *A. terreus*, a longer root (78 cm) and shoots (20 cm) were observed as compared to the control plants root (52 cm) and shoots (18 cm). Moreover, when salinity stressed peal millet were inoculated with *A. terreus*, the plant fresh and dry weight was recorded as 10.6 g and 2.4 g, which were higher than the non-inoculated NaCl stressed plants (Fig. 3A & B).

3.4. A. terreus enhanced proline, total chlorophyll and relative water content (RWC) in NaCl treated pearl millet

The change in relative water content, total chlorophyll and proline content related to the effect of NaCl and *A. terreus* are shown in Fig. 4A-C. In comparison to control, RWC were decreased at 100 mM concentration, however, the co-inoculation of *A. terreus* has recovered the losses in relative water content (Fig. 4A). Application of *A. terreus* to NaCl supplemented pearl millet plants showed an increase in chlorophyll content (44.2 µg/mL), while great losses in chlorophyll contents were observed in non-inoculated pearl millet plants under NaCl stress (Fig. 4B). Furthermore, the proline content was high in salt treated non-inoculated pearl millet plants as compared to inoculated plants (Fig. 4C).

3.5. A. terreus decreases lipid peroxidation in NaCl-treated plants

A decline in malondialdehyde (MDA) content was perceived in pearl millet plants treated with *A. terreus* (Fig. 5A). Maximum increase in MDA content (3.87 µg/mL) was observed in NaCl supplemented pearl millet plants as compared to control. However, when the plants were treated with *A. terreus*, a reduction in MDA content was noticed (Fig. 5A).

3.6. A. terreus enhanced soluble sugar in pearl millet plants under salt stress

A. terreus enhanced the soluble sugar in pearl millet plants under salinity stress relative to the control. Present results indicated that soluble sugar increased (44.75 µg/mL) in plants that were inoculated with fungal strains *A. terreus* (Fig. 5B). Similarly, the concentration of soluble sugar increased (47.03 µg/mL) in plants that were inoculated with fungal strains *A. terreus* and stressed with 100 mM NaCl. On the other hand, a significant reduction was noticed in soluble sugar when pearl millet plants were subjected to 100 mM salt stress alone (Fig. 5B).

3.7. A. terreus enhanced IAA, flavonoids and phenols in inoculated plants under salt stress

Higher amounts of phenols and flavonoids were recorded in *A. terreus* associated pearl millet plants that were further increased, when NaCl treated pearl millet plants were inoculated with *A. terreus* (Tables 2–4). On the contrary, IAA production was observed to reduce when the non-inoculated plants were exposed to 100 mM salt stress as compared to the control plants and *A. terreus* inoculated plants. Infact, salt stress resulted in the reduced IAA content in non-associated pearl millet leaves, stem and root at 100 mM NaCl stress, whereas plants inoculated with *A. terreus* indicated maximum increase in leaves, stem and root IAA (Tables 2–4).

3.8. Identification of compound by LC-MS/MS method

The LC-MS/MS spectra of the analyzed samples were compared with standards and the compounds were identified by comparing our data with standard data of literature. The LC-MS/MS deduced the presence of quinic acid, ellagic acid, calycosin, wogonin, feruloylquinic acid, caffeic acid phenylethyl ester, D-glucoside, myricetin, propoxyphene and aminoflunitrazepam in the culture filtrate of *A. terreus* (Table S1 & Figs. S1–S10).

4. Discussion

In the present study, a total of 14 endophytic fungi were isolated from *Aloe barbadensis* for their salt resistance and growth promoting potential. A variation in the production of IAA by the isolated fungal endophytes from *A. barbadensis* were noticed under salinity stress. However, the fungal endophytes *A. terreus* has produced IAA in significantly higher amounts under saline contditions. This might be due to the fact that *A. terreus* has the ability to adopt the salt stress, where the other fungal species were either failed to survive under saline conditions or their growth ere significantly inhibited. Nusrat et al. (Nusrat et al., 2019) and Mehmood et al. (Mehmood et al., 2018) has also demonstrated that the endophytes has the ability to release IAA under saline conditions. To see whether an endophyte has the ability to prmote growth, it is necessary to test it for phosphate solubalization, IAA, hydrogen cyanide (HCN), ammonia and secondary metabolites

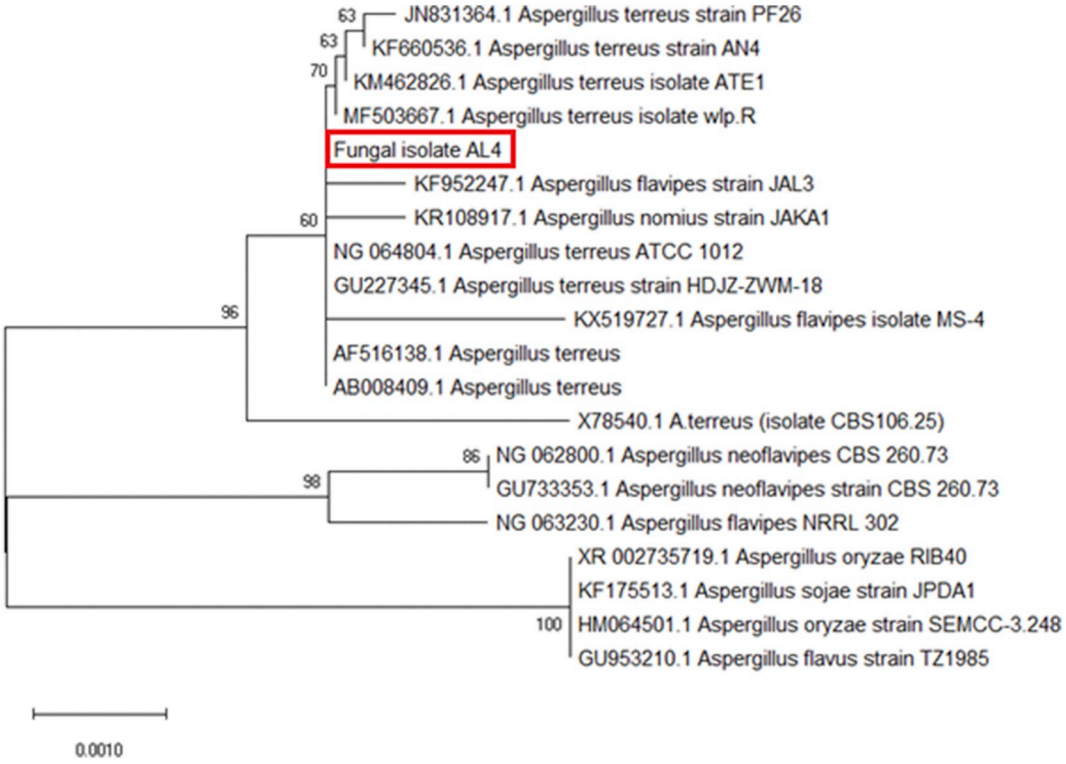

Fig. 2. Phylogenetic analysis of fungal isolate AL4.

production (Nusrat et al., 2019). In fact, nitrogen and phosphate are the essential elements for the production of biomolecules, like amino acids, nucleic acid, NADH and ATP that are imperative for the survival of an organism (Bartwal et al., 2013). Furthermore, it will be right to say that ammonia production and phosphate solubilization is as important as nitrogen fixation. Therefore, the strain *A. terreus* was selected as a best endophytic strain that has survived the saline conditions and revealed plant growth promoting traits.

The utilization of plant symbiotic microbes, especially fungal endophytes had proven to be beneficial in developing strategies to promote plant growth under salinity stress. The salt stress severely reduces the growth parameters due to the non-availability of nutrients (Farooq et al., 2015). In the present study, all the growth parameters of pearl millet were decreased with increasing salinity; however the adverse effects of salinity stress were reduced after the inoculation of stressed plants with *A. terreus*. The profound impact of *A. terreus* on pearl millet under salt stress was judged interms of root and shoots growth, root and shoot weight, secretion of phytohormones and stress markers. The percent decrease in shoot and root length were 36% and 40% with NaCl stress. However an increase of 97% and 63% was noticed in A. terreus inoculated pearl millet plants. Fresh and dry weights of pearl millet were significantly enhanced by fungal isolates in the absence of NaCl. Moreover, under saline condition (100 mM NaCl), the chlorophyll *a* and *b* contents of pearl millet were reduced (39.3% and 45%, respectively), but a non-significant difference were noted in fungal inoculated plants under 100 mM NaCl stress and non-inoculated plants under control conditions. A 72 and 52% percent increase in Chlorophyll *a* and *b* were noted when plants were inoculated with A. terreus under salinity. Similar results have been shown in previous studies, where plant growth parameters were supported by the endophytes under stressful conditions (Ismail et al., 2019; Nusrat et al., 2019; Jan et al., 2019; Ikram et al., 2018).

Plant metabolites, such as proline act as an osmolite or antioxidant under salt stress, which is a source of organic nitrogen (Hayat et al., 2012). In the current study, significant accumulation of proline in salt stressed pearl millet plants were noticed that were kept increasing with an increase in NaCl. The application of *A. terreus* has further increased the accumulation of proline by 2.4 folds. This result was supported by the outcomes of Nusrat et al. (Nusrat et al., 2019). The increased accumulation of proline might be due to enhanced enzymatic activities (Ahmad et al., 2010). The accumulation of soluble sugar is also an important indicator of stressful conditions (Mohammadkhani and

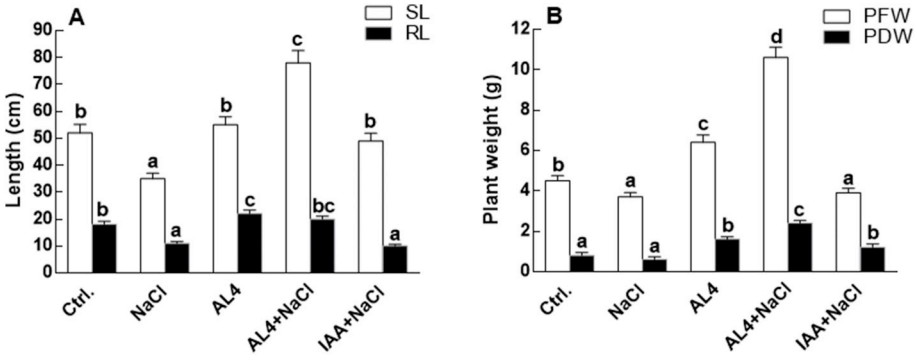

Fig. 3. Effect of NaCl on shoot and root length (A) and weight (B) of *P. glaucum* inoculated with or without *A. terreus* AL4. SL = shoot length; RL = root length; PFW = plant fresh weight; PDW = plant dry weight. Each bar represents the mean of triplicated data with ± SE. Means followed by different letters are significantly different from their respective bars (Duncan's multiple-range test; $p < 0.05$).

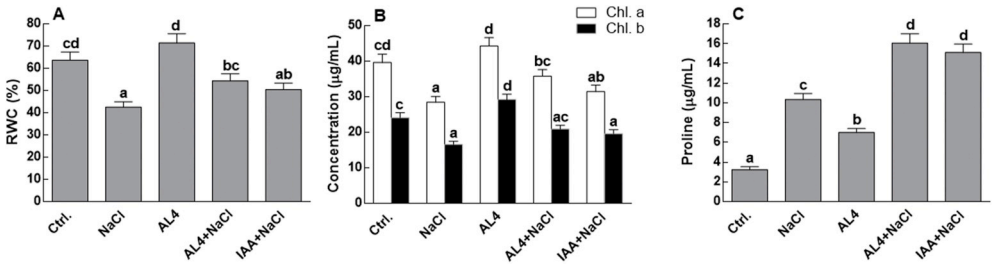

Fig. 4. Effect of NaCl on RWC (A), chlorophyll (B) and proline (C) of *P. glaucum* inoculated with or without *A. terreus* AL4. RWC = relative water contents; Chl.a = chlorophyll *a*; Chl. B = chlorophyll *b*. Each bar represents the mean of triplicated data with ± SE. Means followed by different letters are significantly different from their respective bars (Duncan's multiple-range test; $p < 0.05$).

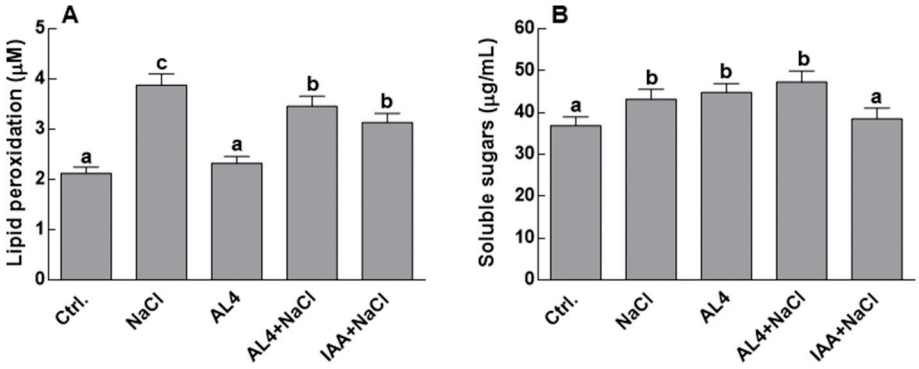

Fig. 5. Effect of NaCl on lipid peroxidation (A) and soluble sugar (B) of *P. glaucum* inoculated with or without *A. terreus* AL4. Each bar represents the mean of triplicated data with ± SE. Means followed by different letters are significantly different from each other (Duncan's multiple-range test; $p < 0.05$).

Table 2
Effect of NaCl on leaves, stem and root IAA of *P. glaucum* with or without *A. terreus* association.

Treatment	Leaves	Stem	Root
Control	1.05 ± 0.3b	1.01 ± 0.3b	0.89 ± 0.30b
NaCl	0.58 ± 0.2a	0.41 ± 0.14a	0.54 ± 0.21a
AL4	1.89 ± 1.47d	1.74 ± 1.32d	1.54 ± 1.12d
AL4 + NaCl	1.67 ± 0.69c	1.51 ± 0.71c	1.24 ± 0.34c
IAA + NaCl	1.82 ± 0.90c	1.65 ± 0.89c	1.54 ± 0.4c

Each data point represents the mean of triplicated data with ± SE. Means followed by different letters are significantly different from each other (Duncan's multiple-range test; $p < 0.05$).

Table 3
Effects of NaCl on leaves, stem and root flavonoids of *P. glaucum* with or without *A. terreus* association.

Treatment	Leaves	Stem	Root
Control	28.7 ± 1.61a	28.1 ± 1.5a	26.9 ± 1.5a
NaCl	42.3 ± 2.5c	40.3 ± 2.1c	38.6 ± 1.9c
AL4	38.7 ± 2.1b	38.2 ± 2.2b	36.1 ± 2.04b
AL4 + NaCl	44.9 ± 2.6d	43.8 ± 2.4d	41.3 ± 2.2d
IAA + NaCl	45.3 ± 2.6d	42.1 ± 2.3d	40.1 ± 2.1d

Each data point represents the mean of triplicated data with ± SE. Means followed by different letters are significantly different from each other (Duncan's multiple-range test; $p < 0.05$).

Heidari, 2008), therefore, an increase was noticed in soluble sugar after treating pearl millet plants with 100 mM NaCl. Correspondingly, the oxidative damages that plant undergoes under salt stress is the result of change in lipid peroxidation (Parvin et al., 2019). In the current study, lipid peroxidation was enhanced to 82.5% in the pearl millet plants treated with NaCl (100 mM). However, lipid peroxidation in pearl millet plants inoculated with *A. terreus* was reduced to 20%. Under NaCl stress, the relative water content decreased by 49% in the non-associated pearl millet plants, whereas the application of *A. terreus* has retained the relative water contents to the level of control. The present

Table 4
Effect of NaCl on leaves, stem and root phenolics of *P. glaucum* with or without *A. terreus* association.

Treatment	Leaves	Stem	Root
Control	26.8 ± 1.3a	24.8 ± 1.2a	22.5 ± 1.1a
NaCl	36.5 ± 1.7c	34.5 ± 1.7c	32.1 ± 1.6c
AL4	31.8 ± 1.6b	30.2 ± 1.4b	29.8 ± 1.2b
AL4 + NaCl	40.1 ± 2.0d	38.3 ± 1.9d	36.7 ± 1.5d
IAA + NaCl	39.9 ± 1.9d	37.4 ± 1.7d	36.8 ± 1.5d

Each data point represents the mean of triplicated data with ± SE. Means followed by different letters are significantly different from each other (Duncan's multiple-range test; $p < 0.05$).

data is also supported by the Nusrat et al. (Nusrat et al., 2019) and Sarwat et al. (Sarwat et al., 2016). Plant under stress conditions also requires flavonoids and phenolics to defend itself and to scavenge the toxic radicals (Bartwal et al., 2013). In the present case, pearl millet plants inoculated with *A. terreus* had produced higher amounts of flavonoids and pheolics under 100 mM NaCl stress. The higher production of flavonoids and pheolics by *A. terreus* associated pearl millet plants posed great tolerance to 100 mM NaCl stress. The results are in accordance with that of Ali and Ismail (2014), who showed an increase in flavonoid contents of tomato fruit under salt stress. Nusrat et al. (Nusrat et al., 2019) also reported the higher production of phenolics in okra plants treated with elevated salt solutions.

To identify the comounds of interest in *A. terreus* inoculated pearl millet plants, one of the promising analytical tools, i.e. LC-MS/MS was used. MS not only allows excellent sensitivity to low amount of samples within short time, but also determines natural compounds of known and unknown structures (Saldanha et al., 2013). According to the retention time and MS spectra, the unknown compounds were identified by comparing it with reference standards. Quinic acid, calycosin, ellagic acid, wogonin, myricetin, propoxyphene, 7-Aminoflunitrazepam, feruloylquinic acid, caffeic acid and D glucoside were identified as bioactive molecules in the extract of *A. terreus* inoculated pearl millet plants. These compounds can act as antioxidants (Li et al., 2017), regulates radical scavenging activities and Malondialdeyde (MDA) (Guo

et al., 2012), and exhibits anticancer and anti-inflammatory activities (Tai et al., 2005).

Funding statement

Abdul Wali Khan University Mardan has financially supported the project.

Ethics approval and consent to participate

Our study doesn't involve any human, animal or endangered species.

Consent for publication

No consent/approval at the national or international level or appropriate permissions and/or licenses for the study was required.

Availability of data and material

All the data are included in the manuscript.

Conflicts of interest

The authors declare that there is no competing interest of any nature related to this manuscript.

Author's contribution

FK, NB, GJ and FGJ designed and performed all the experiments. AI and AH analyzed the data and wrote the manuscript. AI and MH edited the manuscript and arranged the resources for the work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.038.

References

Ahmad, F., Ahmad, I., KHAN, M.S., 2005. Indole acetic acid production by the indigenous isolates of Azotobacter and fluorescent Pseudomonas in the presence and absence of tryptophan. Turkish J. Biol. 29, 29–34.

Ahmad, P., Jaleel, C., Sharma, S., 2010. Antioxidant defense system, lipid peroxidation, proline-metabolizing enzymes, and biochemical activities in two Morus alba genotypes subjected to NaCl stress. Russ. J. Plant Physiol. 57, 509–517.

Al Tamie, M.S., 2014. Effect of salinity on the fungal occurance in Al-Shega area at Al-Qassim, Saudi Arabia. Res. J. Microbiol. 9, 287.

Ali, H.E.M., Ismail, G.S.M., 2014. Tomato fruit quality as influenced by salinity and nitric oxide. Turk. J. Bot. 38, 122–129.

Bartwal, A., Mall, R., Lohani, P., Guru, S., Arora, S., 2013. Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. J. Plant Growth Regul. 32, 216–232.

Bates, L.S., Waldren, R.P., Teare, I., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205–207.

Bilal, L., Asaf, S., Hamayun, M., Gul, H., Iqbal, A., Ullah, I., Lee, I.-J., Hussain, A., 2018. Plant growth promoting endophytic fungi *Asprgillus fumigatus* TS1 and *Fusarium proliferatum* BRL1 produce gibberellins and regulates plant endogenous hormones. Symbiosis 76, 117–127.

Chadha, N., Prasad, R., Varma, A., 2015. Plant promoting activities of fungal endophytes associated with tomato roots from central Himalaya, India and their interaction with Piriformospora Indica. IJPBS 6, 333–343.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.t., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Farooq, M., Hussain, M., Wakeel, A., Siddique, K.H., 2015. Salt stress in maize: effects, resistance mechanisms, and management. A review. Agron. Sustain. Dev. 35, 461–481.

Guo, C., Tong, L., Xi, M., Yang, H., Dong, H., Wen, A., 2012. Neuroprotective effect of calycosin on cerebral ischemia and reperfusion injury in rats. J. Ethnopharmacol. 144, 768–774.

Hamayun, M., Hussain, A., Khan, S.A., Kim, H.-Y., Khan, A.L., Waqas, M., Irshad, M., Iqbal, A., Rehman, G., Jan, S., 2017. Gibberellins producing endophytic fungus *Porostereum spadiceum* AGH786 rescues growth of salt affected soybean. Front. Microbiol. 8, 686.

Hayat, S., Hayat, Q., Alyemeni, M., Wani, A., Pichtel, J., Ahmad, A., 2012. Role of proline under changing environments: a review. Plant Signal. Behav. 7, 1456–1466.

Hernández, J.A., Ferrer, M.A., Jiménez, A., Barceló, A.R., Sevilla, F., 2001. Antioxidant Systems and O2. − /H2O2 production in the apoplast of pea leaves. Its relation with salt-induced necrotic lesions in minor veins. Plant Physiol. 127, 817–831.

Hussain, A., Shah, S.T., Rahman, H., Irshad, M., Iqbal, A., 2015. Effect of IAA on in vitro growth and colonization of Nostoc in plant roots. Front. Plant Sci. 6, 46.

Hussain, A., Hamayun, M., Rahman, H., Iqbal, A., Shah, M., Irshad, M., Qasim, M., Islam, B., 2018. Bioremediation of hexavalent chromium by endophytic fungi; safe and improved production of *Lactuca sativa* L. Chemosphere 211, 653–663.

Ikram, M., Ali, N., Jan, G., Jan, F.G., Rahman, I.U., Iqbal, A., Hamayun, M., 2018. IAA producing fungal endophyte *Penicillium roqueforti* Thom., enhances stress tolerance and nutrients uptake in wheat plants grown on heavy metal contaminated soils. PLoS One 13, e0208150.

Ismail, Hamayun, M., Hussain, A., Iqbal, A., Khan, S.A., Lee, I.-J., 2018. Endophytic fungus *Aspergillus japonicus* mediates host plant growth under normal and heat stress conditions. BioMed Res. Int. 2018.

Ismail, Hamayun, M., Hussain, A., Afzal Khan, S., Iqbal, A., Lee, I.-J., 2019. *Aspergillus flavus* promoted the growth of soybean and sunflower seedlings at elevated temperature. BioMed Res. Int. 2019, 1–13.

Jan, F.G., Hamayun, M., Hussain, A., Jan, G., Iqbal, A., Khan, A., Lee, I.-J., 2019. An endophytic isolate of the fungus *Yarrowia lipolytica* produces metabolites that ameliorate the negative impact of salt stress on the physiology of maize. BMC Microbiol. 19, 3.

Jayanthi, G., Kamalraj, S., Karthikeyan, K., Muthumary, J., 2011. Antimicrobial and antioxidant activity of the endophytic fungus Phomopsis sp. GJJM07 isolated from Mesua ferrea. Int. J. Curr. Sci. 1, 85–90.

Jukanti, A., Gowda, C.L., Rai, K., Manga, V., Bhatt, R., 2016. Crops that feed the world 11. Pearl Millet (Pennisetum glaucum L.): an important source of food security, nutrition and health in the arid and semi-arid tropics. Food Security 8, 307–329.

Kavamura, V.N., Santos, S.N., da Silva, J.L., Parma, M.M., Ávila, L.A., Visconti, A., Zucchi, T.D., Taketani, R.G., Andreote, F.D., de Melo, I.S., 2013. Screening of Brazilian cacti rhizobacteria for plant growth promotion under drought. Microbiol. Res. 168, 183–191.

Khan, S.A., Hamayun, M., Yoon, H., Kim, H.-Y., Suh, S.-J., Hwang, S.-K., Kim, J.-M., Lee, I.-J., Choo, Y.-S., Yoon, U.-H., 2008. Plant growth promotion and Penicillium citrinum. BMC Microbiol. 8, 231.

Khan, A.L., Waqas, M., Hamayun, M., Al-Harrasi, A., Al-Rawahi, A., Lee, I.-J., 2013. Co-synergism of endophyte Penicillium resedanum LK6 with salicylic acid helped Capsicum annuum in biomass recovery and osmotic stress mitigation. BMC Microbiol. 13, 51.

Khan, I., Rahman, H., El-Salam, N.M.A., Tawab, A., Hussain, A., Khan, T.A., Khan, U.A., Qasim, M., Adnan, M., Azizullah, A., 2017. Punica granatum peel extracts: HPLC fractionation and LC MS analysis to quest compounds having activity against multidrug resistant bacteria. BMC Complement Altern. Med. 17, 247.

Khan, M.A., Hamayun, M., Iqbal, A., Khan, S.A., Hussain, A., Asaf, S., Khan, A.L., Yun, B.-W., Lee, I.-J., 2018. Gibberellin application ameliorates the adverse impact of short-term flooding on *Glycine max* L. Biochem. J. 475, 2893–2905.

Li, S., Wang, Y., Feng, C., Wu, G., Ye, Y., Tian, J., 2017. Calycosin inhibits the migration and invasion of human breast cancer cells by down-regulation of Foxp3 expression. Cell. Physiol. Biochem. 44, 1775–1784.

Maclachlan, S., Zalik, S., 1963. Plastid structure, chlorophyll concentration, and free amino acid composition of a chlorophyll mutant of barley. Can. J. Bot. 41, 1053–1062.

Mehmood, A., Hussain, A., Irshad, M., Hamayun, M., Iqbal, A., Khan, N., 2018. In vitro production of IAA by endophytic fungus *Aspergillus awamori* and its growth promoting activities in *Zea mays*. Symbiosis 1–11.

Mehmood, A., Hussain, A., Irshad, M., Hamayun, M., Iqbal, A., Rahman, H., Tawab, A., Ahmad, A., Ayaz, S., 2019. Cinnamic acid as an inhibitor of growth, flavonoids exudation and endophytic fungus colonization in maize root. Plant Physiol. Biochem. 135, 61–68.

Mohammadkhani, N., Heidari, R., 2008. Drought-induced accumulation of soluble sugars and proline in two maize varieties. World Appl. Sci. J. 3, 448–453.

Nusrat, B., Gul, J., Farzana, G.J., Muhammad, H., Amjad, I., Anwar, H., Hazir, R., Abdul, T., Faiza, K., 2019. *Cochliobolus sp.* acts as a biochemical modulator to alleviate salinity stress in okra plants. Plant Physiol. Biochem. 139, 459–469.

Onyango, C., Ochanda, S., Mwasaru, M., Ochieng, J., Mathooko, F.M., Kinyuru, J., 2013. Effects of malting and fermentation on anti-nutrient reduction and protein digestibility of red sorghum, white sorghum and pearl millet. J. Food Res. 2, 41.

Parvin, K., Hasanuzzaman, M., Bhuyan, M., Nahar, K., Mohsin, S.M., Fujita, M., 2019. Comparative physiological and biochemical changes in tomato (Solanum lycopersicum L.) under salt stress and recovery: role of antioxidant defense and Glyoxalase systems. Antioxidants 8, 350.

Redman, R.S., Kim, Y.O., Woodward, C.J., Greer, C., Espino, L., Doty, S.L., Rodriguez, R.J., 2011. Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS One 6, e14823.

Saldanha, L.L., Vilegas, W., Dokkedal, A.L., 2013. Characterization of flavonoids and phenolic acids in Myrcia bella cambess. Using FIA-ESI-IT-MSn and HPLC-PAD-ESI-IT-MS combined with NMR. Molecules 18, 8402–8416.

Saravanan, S., Parimelazhagan, T., 2014. In vitro antioxidant, antimicrobial and anti-diabetic properties of polyphenols of Passiflora ligularis Juss. fruit pulp. Food Sci. Human Wellness 3, 56–64.

Sarwat, M., Hashem, A., Ahanger, M.A., Abd_Allah, E.F., Alqarawi, A., Alyemeni, M.N., Ahmad, P., Gucel, S., 2016. Mitigation of NaCl stress by arbuscular mycorrhizal fungi through the modulation of osmolytes, antioxidants and secondary metabolites in

mustard (Brassica juncea L.) plants. Front. Plant Sci. 7.
Singh, P., Kumar, V., Agrawal, S., 2014. Evaluation of phytase producing bacteria for their plant growth promoting activities. Int. J. Microbiol. 2014.
Tai, M.C., Tsang, S.Y., Chang, L.Y., Xue, H., 2005. Therapeutic potential of wogonin: a naturally occurring flavonoid. CNS Drug Rev. 11, 141–150.
Tran, L.-S.P., Shinozaki, K., Yamaguchi-Shinozaki, K., 2010. Role of cytokinin responsive two-component system in ABA and osmotic stress signalings. Plant Signal. Behav. 5, 148–150.
Wahyudi, A.T., Astuti, R.P., Widyawati, A., Mery, A., Nawangsih, A.A., 2011. Characterization of Bacillus sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. J. Microbiol. Antimicrob. 3, 34–40.